Mechanisms of pathogenesis of African pneumococcal serotype 1 isolates during nasopharyngeal carriage and invasive disease

Thesis submitted in accordance with the requirements of the University of Liverpool for the degree of Doctor in Philosophy by

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Abstract

Background: *Streptococcus pneumoniae* is a human pathogen responsible for serious diseases such as pneumonia, septicaemia and meningitis. There are over 90 pneumococcal serotypes, with serotype 1 being a major cause of invasive disease worldwide. Despite its high invasiveness, serotype 1 is rarely isolated during nasopharyngeal carriage. The aim of this study is to understand the various mechanisms that determine the pathogenicity of serotype 1.

Methods: *In vitro* techniques were used to determine adhesion to and invasion of epithelial cells by serotype 1, and to assess its ability to avoid phagocytosis as well as to uncover the mechanisms involved. Furthermore, two *in vivo* models of infection in mice were used to assess serotype 1 virulence and ability to colonise and carry in the nasopharynx. Immunological techniques including flow cytometry and enzyme-linked immunosorbent assays were applied to determine the host immune responses to serotype 1. Furthermore, the gene expression of key virulence factors and metabolic pathways was studied in serotype 1 and compared to the less virulent serotype 2 laboratory reference strain D39.

Results: Serotype 1 is highly virulent, causing the death of 80-100% of infected mice by 48h post-infection when using an invasive pneumonia model. In a nasopharyngeal carriage model in mice, serotype 1 is able to establish colonisation, although at a lower density and for a shorter period of time than other serotypes. Serotype 1 is not able to induce an early recruitment of T regulatory cells or early production of the cytokines TGF-β1 and IL-10. The induction of these modulatory cells and cytokines is associated with maintenance of carriage and provides protection to the host during pneumococcal pneumonia. Therefore, the failure to induce immune-modulatory cytokines and regulatory T cells may lead to the clearance of serotype 1 in the nasopharynx and reduced protection against pneumococcal dissemination. Moreover, the gene expression analysis of key virulence factors and metabolic pathways showed that serotype 1 is less adapted to be a successful coloniser but is, in contrast, very successful at becoming invasive.

Conclusions: The unique characteristics of the type 1 capsule are likely to influence the immune responses against this serotype; however, differences in the expression of non-capsular determinants may also have a key role in the invasiveness of serotype 1. Although serotype 1 is not successful at maintaining nasopharyngeal carriage, it is highly successful at becoming invasive.

Acknowledgements

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I would like to thank my family who have worked very hard to give me the opportunity to get to this stage in my career, even if that meant living in a different country. They have all given me great moral support, and at times, I even got some "scientific advice" from my father.

I would like to thank all my friends who have been there for me, especially when I needed a break, or even a few formatting lessons. You know who you are.

Last but not least, I would like to thank all the members in the Kadioglu lab for helping me during all the stages of the PhD. I am very grateful for their technical support and for the great discussions we have had throughout the years, some of which have led to important experiments included in this thesis. However, I am the most grateful for their time and moral support during the many frustrating times. Again, you know who you are.

I would like to finish by mentioning a Spanish saying that I have had to remember many times during the past four years:

"La paciencia es la madre de la ciencia"

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Abbreviations

APC = Allophycocyanin

ATP = adenosine triphosphate

BAB = Blood agar base

BCA = Bicinchoninic acid assay

BEAS-2B = Human bronchial-epithelial cell line

BHI = Brain heart infusion

Bp = Base pairs

CAP = Community acquired pneumonia

CBP = Choline-binding protein

CD = Cluster of differentiation

CDC = Cholesterol-dependent cytolysin

CFU = Colony forming units

CLN = Cervical lymph nodes

CP = Capsular polysaccharides

CPS = Capsule locus

CSF = Cerebrospinal fluid

DMEM = Dulbecco’s Modified Eagle’s Medium

DMF = Dimethylformamide

DMSO = Dimethyl sulfoxide

DNA = Deoxyribonucleic acid

DPBS = Dulbecco's phosphate-buffered saline

ECM = Extracellular matrix

EDTA = Ethylenediaminetetraacetic acid

EEP = Early exponential phase

ELISA = Enzyme-linked immunosorbent assay

EMBL-EBI = European Bioinformatics Institute

ESP = Early stationary phase

Fc = Fragment crystallizable

fcR = Fc receptor

FCS = Foetal Calf Serum

FI = Fluorescent intensity

FITC = Fluorescein isothiocyanate

HBSS = Hanks' Balanced Salt Solution

HIV = Human immunodeficiency virus

Hyl = Hyaluronidase lyate

Ig = Immunoglobulin

IL = Interleukin

INF = Interferon

IPD = Invasive pneumococcal disease

IVIG = Intravenous immunoglobulin

KC = Keratinocyte chemoattractant

KEGG = Kyoto Encyclopedia of Genes and Genomes

LEP = Late exponential phase

LSP = Late stationary phase

Lyt = Autolysin

MEM = Minimum Essential Medium

MEP = Mid-exponential phase

MIP = Macrophage Inflammatory Protein

MLST = Multi locus sequence type

MHC = Major histocompatibility complex

MRC = Medical Research Council

NADH = Nicotinamide adenine dinucleotide

NALT = Nasal associated lymphoid tissue

Nan = Neuraminidase

NCTC = National collection of type cultures

NLRP3 = NOD-like receptor family pyrin domain containing 3 inflammasome

OD = Optical density

OPKA = Opsonophagocytic killing assay

OR = Odds ratio

PAGe1 = Pneumococcal African Genomic Consortium 1

PavA = Pneumococcal adherence and virulence factor A

PBS = Phosphate-buffered saline

PCho = Phosphorylcholine

PCR = Polymerase chain reaction

PCV = Pneumococcal conjugate vaccine

PE = Phycoerythrin

PE/Cy7 = Phycoerythrin/Cyanine dye7

pIgR = Polymeric immunoglobulin receptor

PLNA = Pneumolysin deficient pneumococcus

Ply = Pneumolysin

PPV = Pneumococcal polysaccharide vaccine

PsaA = Pneumococcal surface adhesin A

PspA = Pneumococcal surface protein A

PTS = Phosphotransferase system

RNA = Ribonucleic acid

RPMI = Roswell Park Memorial Institute medium

Sec = Secretion systems

SNP = Single nucleotide polymorphism

SP = *Streptococcus pneumoniae*

SpxB = Pyruvate oxidase

Srt = Sortase

SSA = Sub-Saharan Africa

ST = Sequence type

TGF = Transforming growth factor

TLR = Toll-like receptor

TNF = Tumour necrosis factor

TOD = Time of death

TPM = Transcripts per million

UK = United Kingdom

USA = United States of America

WHO = World Health Organisation

Xpt = Xanthine phosphoribosyltransferase

ZPS = Zwitterionic polysaccharides

Introduction

1. Introduction

1.1. *Streptococcus pneumoniae*

Streptococcus pneumoniae, or the pneumococcus, is a Gram-positive, facultative anaerobic bacterium that is usually found in pairs (diplococcus), but can also be found singularly or forming short chains. As it is diploid in nature, it was for that reason that in 1920 Winslow and Broadhurst gave to it the original name of *Diplococcus pneumoniae*, which was later changed by Kauffman to *Streptococcus pneumoniae* in 1974 [1-3].

The pneumococcus is often found as part of the "normal" nasopharyngeal flora. However it can also become pathogenic and, as such, it is a major pathogen for humans. The pneumococcus is the causative agent of severe infections such as pneumonia, meningitis and sepsis, but it is also responsible for relatively minor infections such as otitis media, bronchitis and sinusitis.

S. pneumoniae causes invasive pneumococcal disease (IPD) mainly in children, the elderly and immune-suppressed people [4]. The pneumococcus is a leading cause of mortality and morbidity globally resulting in a huge burden to the already fragile health systems of developing countries and as such remains a major contributor to high childhood death rates in these countries, in particular in sub-Saharan Africa, where the incidence of pneumococcal disease is considerably higher (Figure 1) [5]. Furthermore, in year 2000 the number of deaths attributed to the pneumococcus in children under 5 was approximately 826,000, 11% of those belonging to HIV positive children, with the highest mortality rates and case-fatality ratios found in sub-Saharan Africa and South East Asia [5].

In developed countries the pneumococcus is the most common cause of hospitalisation due to community acquired pneumonia (CAP), such as in the United Kingdom, where there is an average mortality rate of approximately 20%, but can reach 60% in the elderly [6].

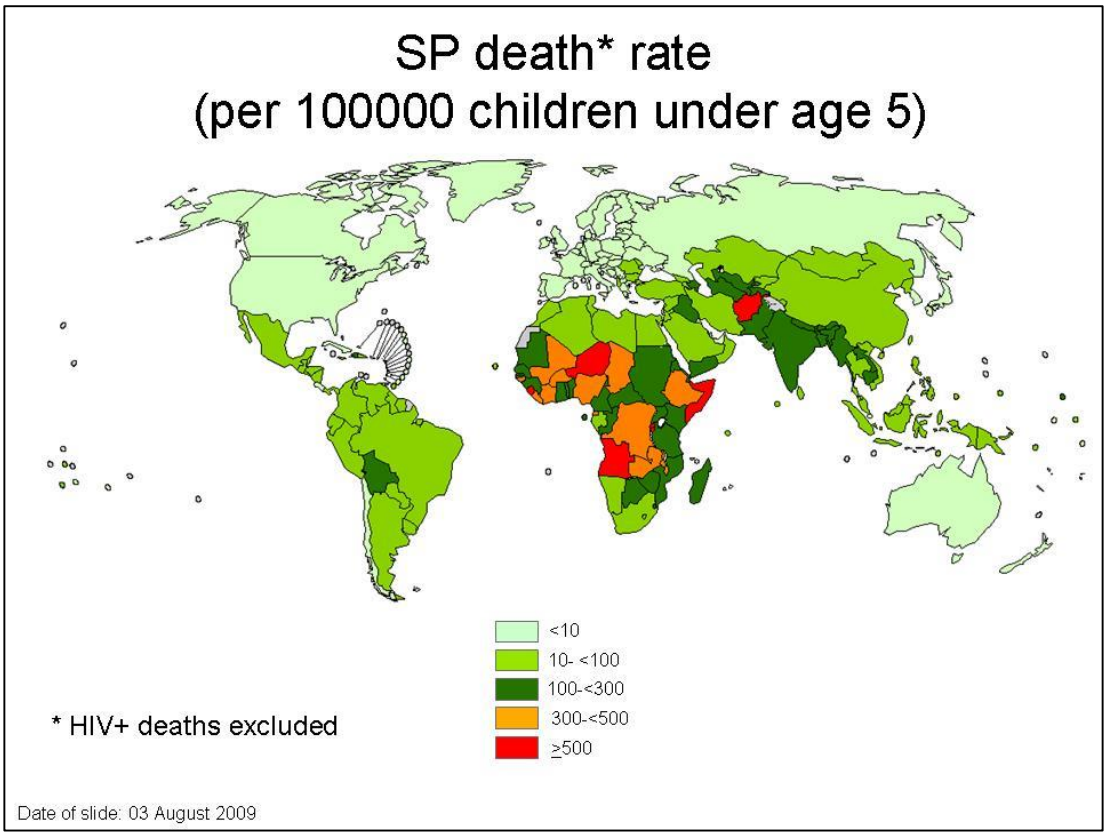

Figure 1. Rate of deaths caused by *S. pneumoniae* in children under the age of 5 [7].

Even though the pneumococcus can cause severe invasive diseases, it is commonly carried asymptomatically in the nasopharynx, which acts as the main reservoir for this pathogen. Carriage rates vary depending on age, geographic location, HIV status and serotypes that are being carried [8, 9]. Globally, between 30% and 70% of children under the age of five carry *S. pneumoniae* asymptomatically in their nasopharynx depending on geographical location [9-11], but the carriage rate is dramatically reduced in adults to less than 10% [10-12]. Not only does pneumococcal carriage occur

more often in children than in adults, but they are also more likely to have more new serotype acquisitions during the same period of time when compared to adults [8].

The first important contact between the pneumococcus and the host is through nasopharyngeal colonisation, which is considered to be a prerequisite for invasiveness [13]. The pneumococcus is commonly carried asymptomatically in the nasopharynx of healthy adults; however, it can progress into serious diseases such as pneumonia, sepsis and meningitis (Figure 2) [13].

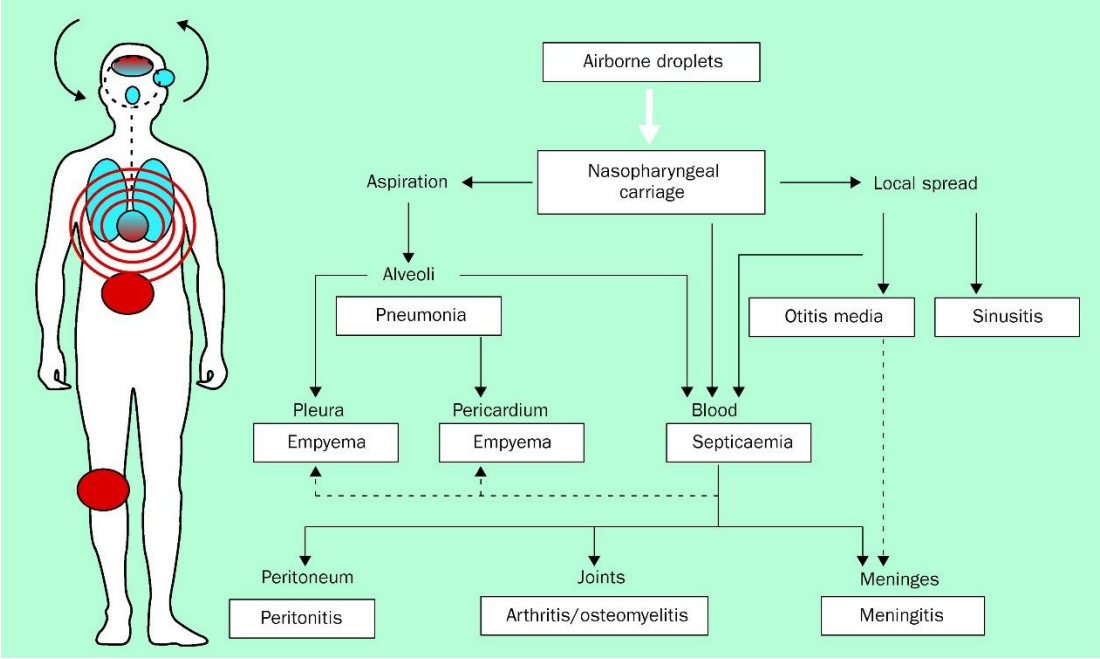

Figure 2. Pathogenic route for *Streptococcus pneumoniae* infections [13].

According to previous studies, pneumococcal infection usually occurs after the acquisition of a new strain, and pneumococcal disease does not occur without prior nasopharyngeal carriage by the same strain [11, 13, 14]. Moreover, the nasopharynx acts as a reservoir for the pneumococcus, allowing the pathogen to easily spread within communities [12, 13, 15, 16]. Since nasopharyngeal carriage seems to be an inevitable step prior to invasiveness it is an area of high importance to study and understand the

interactions between the host and the pathogen and the mechanisms by which asymptomatic nasopharyngeal carriage can progress into invasive pneumococcal disease.

1.2. Epidemiology

There are more than 90 different pneumococcal serotypes which are characterised based on the chemical structure of their polysaccharide capsule. Even though most pneumococcal serotypes are capable of colonising the nasopharynx of healthy humans, there are some serotypes that are rarely found in carriage. The serotype distribution in carriage varies depending on age and geographical location.

Europe	USA	Asia	Africa
6B, 6A, 19F, 23F, 14	6B, 6A, 19F, 23F, 14	6, 14, 15, 19, 23	6A, 6B, 13, 14, 15, 19F, 23F

Table 1. Common serotypes causing nasopharyngeal carriage in different geographic locations.

As observed in Table 1, the serotype distribution in Europe and USA is quite similar, with serotypes 6A, 6B, 19F, 23F and 14 being the most common serotypes [13, 17-20]; serotypes 6, 14, 15, 19, and 23 the most common serotypes in Asia [13, 21-24]; and serotypes 6A, 6B, 13, 14, 15, 19F and 23F the most common serotypes in Africa [9, 13, 25-28]. Even though there is some variability, there are some serotypes, such as 6, 14, 19 and 23, that are consistently found among all geographical regions in high carriage rates, but also as the most common causes of invasive disease. However, there are other serotypes such as 1 and 5 that are rarely found in carriage, although they are common causes of invasive disease [29].

Some studies have shown that there is a relationship between capsular type and ability to cause disease, with some serotypes highly associated with invasive disease and other serotypes highly associated with carriage [27]. Therefore, the virulence of an isolate is strongly associated with its capsule.

The "invasive odds ratio" is used to quantify the virulence of a specific serotype. The ratio for serotype X is calculated using the formula $OR_x = (ad)/(bc)$ where *a* is the number of invasive isolates of a serotype X, *b* is the number of invasive isolates of other serotypes, *c* is the number of carried isolates of a serotype X, and *d* is the number of carried isolates from other serotypes [30].

	Brueggemann et al. [27]	**Hanage et al. [31]**	**Sa-Leao et al. [32]**	**Shouval et al. [33]**	**Kronenberg et al. [34]**	**Rivera-Olivero et al. [35]**
Study period (yrs)	1994-2001	1995-1999	2001-2003	2000-2004	2002-2004	2006-2008
Study area (country)	UK	Finland	Portugal	Israel	Switzerland	Venezuela
Highly invasive serotypes	**1, 4, 14, 18C**, 7F, 9V, 19A	**6B, 14, 18C, 19A**, 4, 7F, 9V, 38	**1, 3, 4, 5, 7F, 8, 9N, 9L, 12B, 14, 18C, 20**	**1, 5, 12F**, 9V, 18C, 19A, 19F	**1, 4, 5, 7F, 8, 9V, 14**, 19A	**1, 5, 7F, 18**, 3, 14, 19F
Less invasive serotypes	**23F**, 3, 6B, 15B/C, 19F	**6A, 11A, 35F**, 3, 9N, 10, 15, 19F, 22, 23F	**6A, 6B, 11A, 15B/C, 16F, 19F, 23F, 34, 35F, 37**	3, 6A, 6B, 11A, 14, 15A, 15B/C, 21, 23F, 35B	**3, 7, 10, 11, 15, 19F, 23**, 6B, 9, 22, 23F	6A, 6B, 19A, 23F

Table 2. Invasive disease potential of different pneumococcal serotypes in different geographical locations based on the invasive odds ratio [29]. The serotypes in bold show the ones significantly different.

As shown in Table 2, the serotypes that are highly invasive or less invasive can vary slightly depending on the geographical location, but some of the serotypes are consistently high or less invasive across countries [29]. The highly invasive serotypes, and therefore more related to invasiveness than carriage, are serotypes 1, 4, 5, 14, 18C

and 7F. On the other hand, the main serotypes that are more associated to nasopharyngeal carriage are types 23F, 6A, 6B and 19F [29].

Considering that certain serotypes are more invasive than others, it is clear that the polysaccharide capsule is an important factor in the virulence of the pneumococcus; however, there are other pneumococcal factors that have been shown to play key roles during invasive disease and also during nasopharyngeal carriage.

1.3. Pneumococcal virulence factors

The pneumococcus is an encapsulated bacterium; surrounded by a polysaccharide capsule, with a cell wall and cell membrane beneath. Even though the capsule is an important virulence factor there are proteins and enzymes located in and on the surface of the pneumococcus that also contribute to the virulence of this pathogen by either providing host immune evasion mechanisms or by actively interacting with the host [36].

The pneumococcal genome is on average 2,088,534 bp [37]. Around 74% of the pneumococcal genome consists of genomic content that is present in all pneumococcal isolates (core genome), whilst the rest of the genome consists of genes present in a subset of isolates belonging to specific clonal lineages (accessory genome) [37, 38]. Many of the accessory genes are clustered in regions of three or more genes which are distributed along the pneumococcal genome; these regions are called islets or accessory regions [38].

Through comparative genomic analysis it has been found that highly invasive isolates may lack some of the known bacterial virulence factors, suggesting a significant redundancy among virulence genes [39]. Having a better understanding of the different

virulence factors, their role during carriage and invasive disease and their expression during various stages of infection, can lead to the development of potential new vaccine candidates. The main pneumococcal virulence factors are shown in Figure 3 and each factor will be discussed in turn.

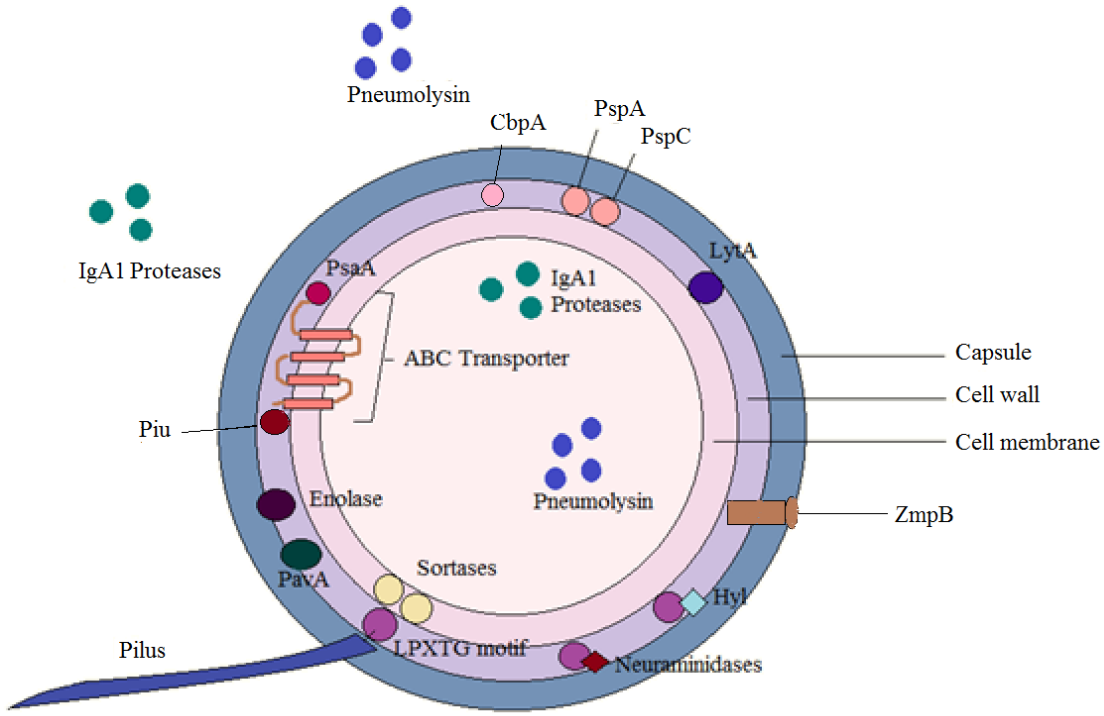

Figure 3. Selection of the main pneumococcal virulence factors: choline binding protein A (CbpA), pneumococcal surface proteins (PspA and PspC), autolysin (LytA), pneumococcal iron uptake ABC transporter (Piu), hyaluronidase lyase (Hyl), neuraminidases, sortases, pilus, enolases, pneumococcal surface adhesion (PsaA), pneumolysin, IgA1 proteases, and zinc metalloprotease B (ZmpB) [12].

1.3.1. Polysaccharide capsule

The pneumococcal capsule is the outer layer of the pathogen and is usually formed by neutral or negatively charged chains of polysaccharides. In most serotypes, the capsular polysaccharides (CPs) are covalently attached to the peptidoglycan layer of the cell wall, as in other Gram positive bacteria [40, 41].

The capsule is a key virulence factor that allows the bacteria to evade the host immune system by protecting the bacteria against opsonophagocytosis [42], even though it also provides highly immunogenic antigens that can facilitate phagocytosis through the adaptive immune system, therefore giving protection against the pneumococcus [43]. The high immunogenicity in humans has led to the development of serotype specific vaccines. There are over 90 different structurally and serotypically distinct serotypes, and it is believed that the high diversity in pneumococcal serotypes is an evolutionary adaptation for an increased antigenic diversity imposed by the human immune system [44]. The geographical distribution of different serotypes is a result of the natural and vaccine-mediated immunity against the pneumococcus [45-51].

Pneumococcal strains that belong to the same serotypes can be further divided in subgroups or clonal groups by differences in the sequences of 7 house-keeping genes; this method is known as Multi Locus Sequence Typing (MLST) [28]. The sequence typing of pneumococci has enabled researchers to study the evolution and expansion of clones within the same serotype. It is well known that recombination is the main source of genetic exchange in the pneumococcus [37], which can also include alleles from the seven house-keeping genes used for MLST [52].

The majority of pneumococcal isolates produce a capsule, and this capsule can be detected using capsular type-specific antisera. Whilst most isolates can be classified according to their positive results during type-specific serological tests, a small proportion of isolates (0.5-2.2% recovered from sterile sites, and 10% recovered from non-sterile sites) show negative results during these tests, reflecting either a lack of capsule or the presence of a capsule that cannot be typed to any known serotype [53]. Those strains are known as non-typeables [53, 56]. Strains that do not produce a

polysaccharide capsule have been shown to be less virulent [56, 57], however some unencapsulated strains have been found to cause conjunctivitis [58, 59].

The presence or absence of capsule in a pneumococcal strain can be preliminarily determined by the morphology of the colonies grown on a 5% blood agar plate: whilst an encapsulated strain has a mucoid and opaque appearance, an unencapsulated strain is flat and transparent (Figure 4) [42].

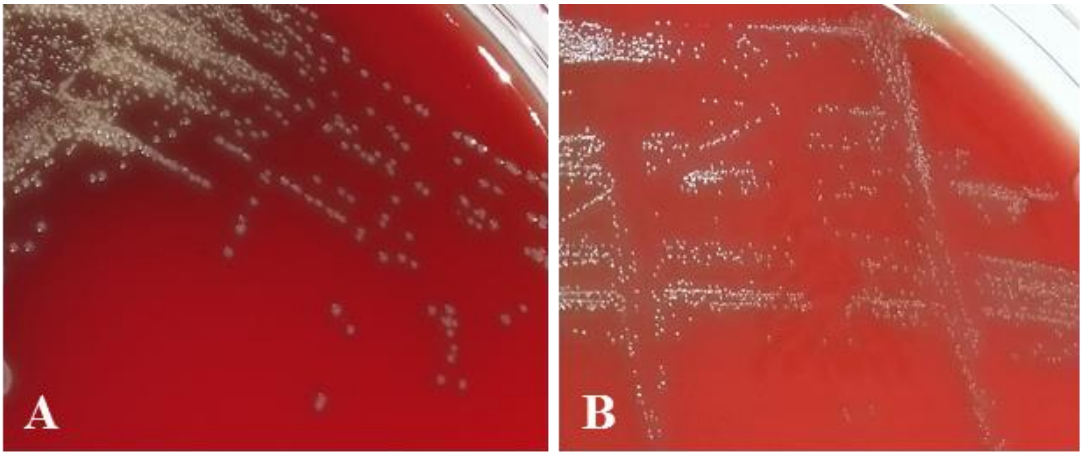

Figure 4. Colony morphology of the encapsulated serotype 2 strain D39 (A) and the un-encapsulated serotype 2 strain R6 (B).

The colony morphology differs in encapsulated and unencapsulated strains, however a flat and transparent colony is not always an indication of unencapsulation. Some pneumococcal serotypes have the ability to change the opacity of their colonies, being able to show at least three different phenotypes (Figure 5) [60]. Whilst the opaque colonies seem to be larger, mucoid and raised/domed, the transparent colonies appear smaller and more transparent in the centre, which is known as a bull's eye. This ability of the pneumococcus to change the opacity of its colonies is reversible and it is known as phase variation [60].

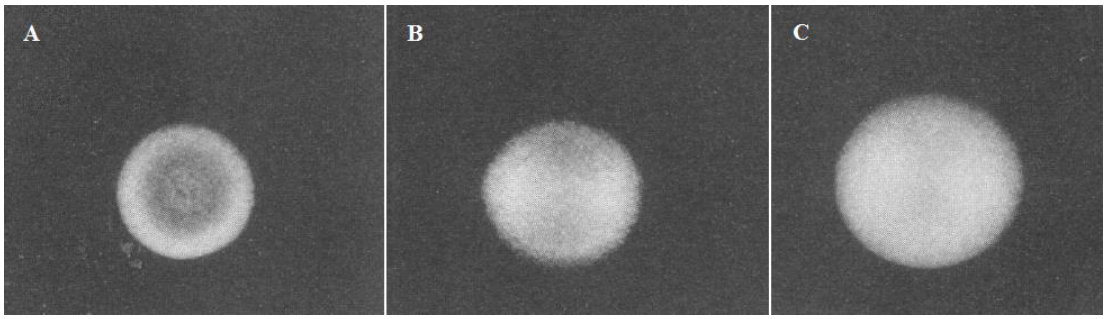

Figure 5. Different opacity phenotypes derived from a single isolate: A) Transparent, B) Intermediate and C) opaque [60].

The transparent colonies have a similar morphology to the unencapsulated strains; however, these transparent colonies produce less capsular polysaccharide but more teichoic acid than the opaque colonies [57]. Furthermore, the increased production of teichoic acid affects the expression of choline-binding proteins, which are surface proteins that bind to the choline-containing teichoic acids. Choline binding proteins are associated with increased adherence; therefore, transparent variants have been shown to be better colonisers whilst opaque variants have been shown to be more virulent [57, 60, 61]. Since phase variation depends on production of capsular polysaccharides, a thicker capsule is associated with virulence whilst a thinner capsule is associated with adhesion and colonisation [60, 61].

The genes needed for the production of the pneumococcal capsule are clustered together in the *cps locus* [54], and it has been shown that this locus is in all serotypes, located in the same chromosomal location [62]. The *cps locus* consists of serotype-specific genes that are surrounded by genes that are common to all serotypes [63]. Recombination during nasopharyngeal colonisation is the main source of genetic exchange in the pneumococcus; this genetic exchange can occur between different pneumococcal strains or between the pneumococcus and other bacterial species, such as other streptococcal species [64]. When recombination occurs between different

pneumococcal strains there can be exchange of genes within the *cps locus*, which can lead to the acquisition or loss of serotype-specific capsular genes leading to a change of the capsular serotype. As a result, these bacteria have a genetic background encoding a specific serotype, but a capsule locus of a different serotype [65]. This phenomenon is known as capsular switching. Capsular switching was first observed in 1928 by Griffith [66], but has since been observed on repeated occasions [62, 65, 67-69]. It is believed that capsular switching occurs regularly in the pneumococcus and it can alter the virulence potential of the bacteria [69-71]. Different studies have shown that although capsular type has an important effect on virulence, the complex interaction of capsular and non-capsular determinants have a synergistic effect on the final virulence potential of the pneumococcus [70, 71]. These observations suggest that the capsule has an effect on the accessibility and functionality of surface proteins; however, an altered expression of other genetic determinants may overcome the effect of the capsule on the accessibility and functionality of those surface proteins [70-72].

In summary, the presence or absence of capsule, its thickness and its type can determine the virulence of a specific strain. However, even though the pneumococcal polysaccharide capsule is an important virulence factor there are other factors involved in the virulence of the pneumococcus.

1.3.2. Pneumolysin

Pneumolysin is a 53kDa protein that is composed of 471 amino acid residues and is synthesised by most pneumococcal isolates [73]. It is a member of the family of cholesterol-dependent cytolysins (CDC) that are synthesised by twenty different Gram positive bacteria [74]. These CDCs are pore-forming toxins that, at high

concentrations, lead to the disruption of cholesterol-containing membranes resulting in the lysis of the cell [75].

Pneumolysin consists of four domains, of mainly β sheet structure. Domain 4 contains a conserved tryptophan-rich motif that is thought to interact with cholesterol, being therefore essential for cytolytic activity [76, 77, 78]. Whilst domain 4 is responsible for interaction with mammalian membranes, domain 3 is responsible for pore formation by insertion in the mammalian membrane (Figure 6) [79-81].

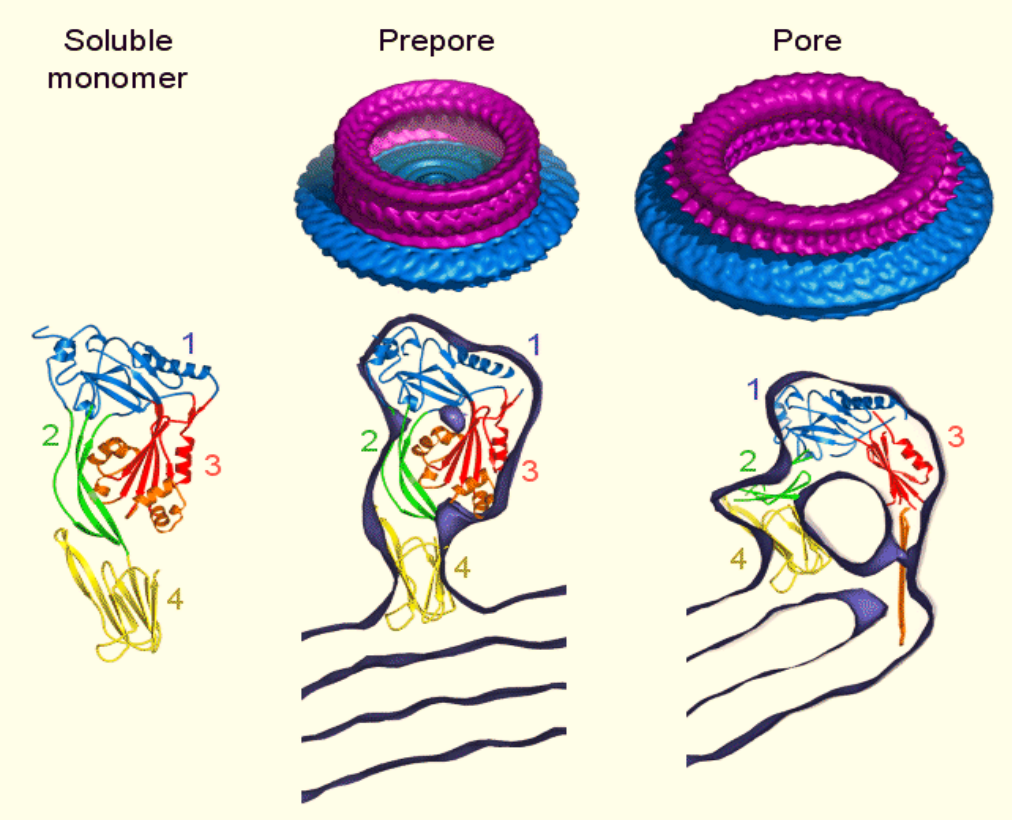

Figure 6. Pore formation on eukaryotic membranes by pneumolysin. Soluble monomer consists of 4 domains: domain 1 (blue), domain 2 (green), domain 3 (red) and domain 4 (yellow). The prepore is formed by the interaction of domain 4 with mammalian cells, whilst the pore forming activity relies on domain 3 [78].

As shown in Figure 6, pneumolysin is produced as a soluble monomer which binds to cholesterol-containing membranes and then oligomerises into a large ring-shaped assembly of around 40 subunits and 30nm diameter; this ring-shaped assembly, known

as a pre-pore assembly, can then punch a large hole in the target membrane leading to the lysis of the cell [82].

Pneumolysin is a negatively charged toxin that is produced by the pneumococcus and stored in the cytoplasm instead of the membrane of the bacteria [74, 83]. In contrast to all other CDCs, pneumolysin lacks a signal peptide that allows the secretion of the protein via the type II pathway [84].

Pneumolysin is released during the exponential growth phases of the pneumococcus, but its release is significantly increased during the stationary phase suggesting that pneumolysin is secreted due to the activity of autolysin [74, 83, 85]. Nonetheless, some observations suggest that there might be other secretion pathways that are autolysin-independent and domain 2-dependent [86]. The two main observations that support the hypothesis of an autolysin-independent secretion pathway are: a) pneumolysin is detected in cultures during early stationary phase, when autolysis has yet not occurred [87], and most important b) the secretion of pneumolysin is not altered in the absence of autolysin [88]. Therefore, there may be an alternative unknown mechanism through which pneumolysin is released independently of bacterial lysis. Previous studies have shown that pneumolysin can be found bound to the cell wall during growth stages where autolysin is not occurring [89]. Moreover, pneumolysin released upon cell lysis is not able to reassociate with the cell wall of intact cells indicating that pneumolysin is release through an export system which is dependent on the domain 2 of pneumolysin [86].

Since pneumolysin can punch holes in red blood cells the haemolytic activity levels of pneumolysin can be determined using an haemolytic activity assay where pneumolysin or bacterial lysates are incubated with red blood cells for a short period of time to

assess the lytic activity of different types of pneumolysin or different pneumococcal strains (Figure 7) [73].

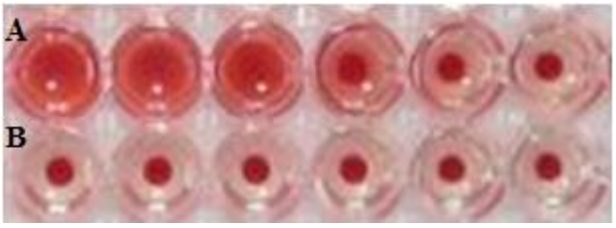

Figure 7. Example of haemolytic assay showing the different levels of lysis (A) and the absence of lysis (B) of red blood cells after incubation with 1:2 serially diluted pneumolysin and a negative control, respectively.

Even though pneumolysin is present in virtually all pneumococcal isolates, some sequence types from serotypes 1, 7F and 8 have been found to produce pneumolysin expressing either low or undetectable levels of haemolytic activity [90]. Despite producing a low or non-haemolytic version of pneumolysin, these isolates are highly invasive, which suggests that the immunomodulatory properties of pneumolysin are independent of its cytolytic activity [90].

At high concentrations pneumolysin is able to cause cell and tissue damage due to its ability to lyse cholesterol-containing membranes, but it also contributes to the pathogenesis of the pneumococcus at the early stages of the infection where the concentration of pneumolysin is low (Figure 8) [91].

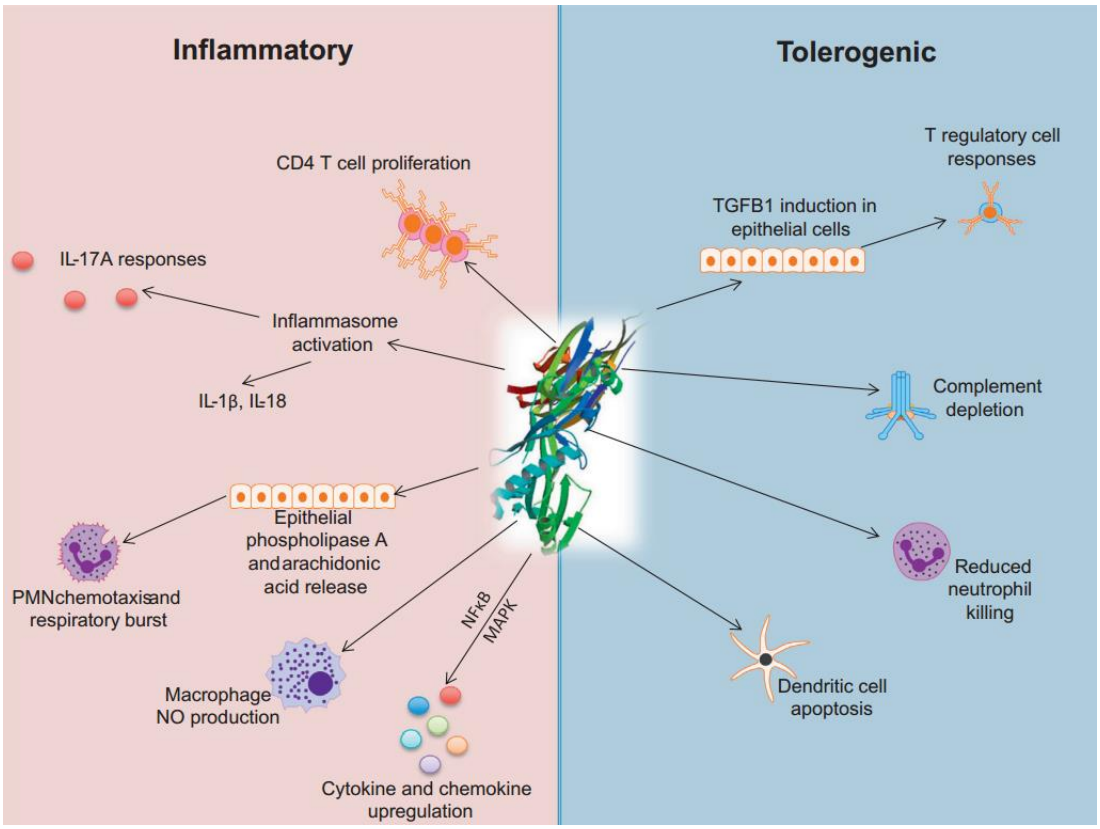

Figure 8. Effect of pneumolysin at sub-lytic concentration in the host [92].

At low concentrations pneumolysin can induce apoptosis [93], antibody-independent activation of the classical complement pathway by binding to the Fc region of IgG [94, 95], induction of pro-inflammatory reactions in immune cells [96], and TLR4-independent activation of the NLRP3 inflammasome [97]. Previous studies have suggested that pneumolysin is able to activate TLR4 receptors [98, 99], mediating inflammatory responses in the host; however, more recent studies have cast doubt on this and have shown that pneumolysin is a potent activator of inflammatory responses in the absence of TLR4 activation [97, 100]. It is possible that bacterial lipopolysaccharide (LPS) contamination during the pneumolysin purification process from recombinant *E.coli* may be responsible for the activation of TLR4 [92].

A number of publications have demonstrated the role of pneumolysin *in vitro* and *in vivo* by using pneumolysin deficient mutants, such as PLN-A (pneumolysin deficient

D39, serotype 2), in murine models of infection [12, 101-103]. Pneumolysin-deficient pneumococci have significantly lower ability to adhere to respiratory epithelial cells and, even though it is able to colonise the nasopharynx of mice for a short period of time, it is found at a lower density and is rapidly cleared by the host [101, 102]. A high dose intra-nasal challenge usually leads to high numbers of pneumococci in the lung and subsequently blood and therefore death of the mouse; however, in the absence of pneumolysin bacterial clearance is increased in the lung and this leads to a prolonged survival of the mice [103].

Pneumolysin has pro-inflammatory effects that lead to the release of IL-8, IL-1α, IL-1β, TGF-β1, TNF-α and the production of IL-6 [92, 97, 104]. Moreover, pneumolysin has been shown to be a potent activator of the inflammasome, which plays a key role in anti-pneumococcal immunity by promotion of IL-1β and IL-17A responses [97, 92, 100, 105]. Although cytokine activation by pneumolysin should be detrimental to the pneumococcus, it has been shown otherwise: pneumolysin enhances bacterial dissemination by increasing tissue permeability due to tissue damage caused by uncontrolled production of pro-inflammatory cytokines [102, 106, 107].

Therefore, pneumolysin is an important virulence factor in the invasiveness of the pneumococcus and in its ability to colonise the nasopharynx.

1.3.3. Choline-binding proteins (CBP)

The pneumococcal cell wall is composed of peptidoglycan, teichoic acid, lipoteichoic acid and phosphorylcholine (PCho) [108]. There is a series of surface-exposed proteins that are anchored to the cell wall through the PCho [36, 109].

As observed in Figure 9, there are a large number of choline binding proteins, but not all of them are produced by all of the pneumococcal strains [109]. The number of CBPs can range between 10 (serotype 2, strain R6; the unencapsulated derivative of D39) and 15 (serotype 4 strain TIGR4) [108]. The function of the more abundant and important choline-binding proteins is discussed further.

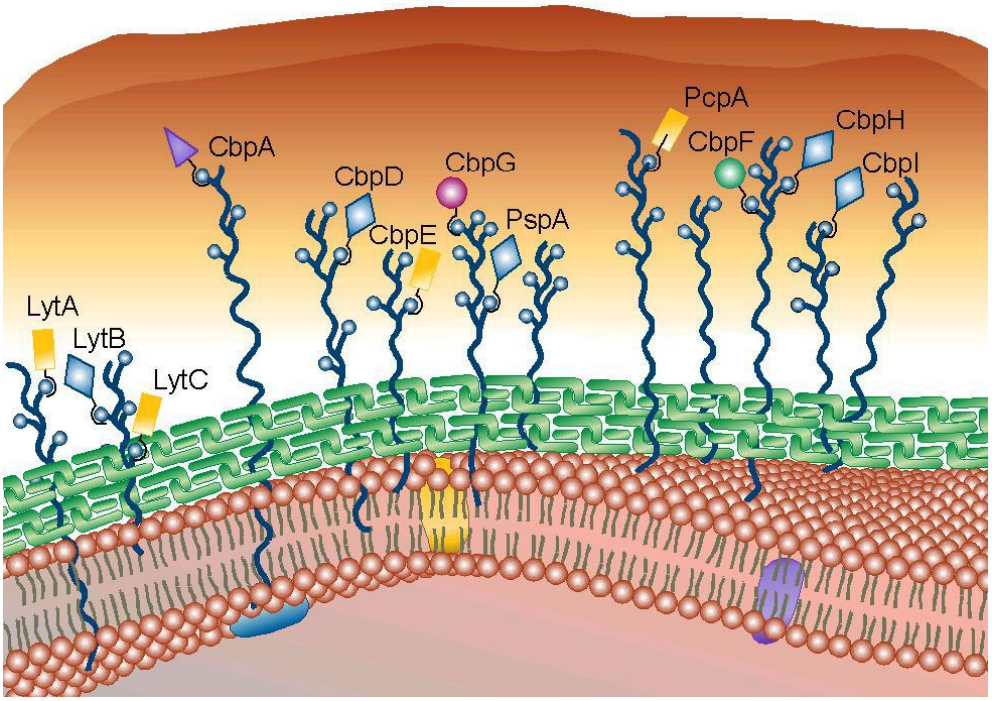

Figure 9. Choline Binding Proteins anchored to the cell wall of the pneumococcus: LytA, LytB, LytC, CbpA, CbpD, CbpE, CbpG, PspA, PcpA, CbpF, CbpH and CbpI [109].

1.3.3.1. Pneumococcal surface protein A (PspA)

The pneumococcal surface protein A is a CBP that is located in the cell wall of the pneumococcus and has a molecular weight that can vary from 67 to 99 kDa [110]. PspA is a highly variable protein and this variability is determined by the N-terminal module, whilst the C-terminal module enables the protein to anchor to the pneumococcal cell wall [36]. It is believed that the exposed negatively charged extreme of the protein prevents the binding of C3 to the pneumococcus, therefore

inhibiting complement-mediated opsonisation which leads to a reduction in phagocytosis of the pneumococcus by the host [111].

1.3.3.2. Choline –binding protein A (CbpA)

Also known as pneumococcal surface protein C (PspC), SpaA and PbcA, the CbpA consists of 665 amino acid residues and is an adhesion protein that connects the teichoic and lipoteichoic acids from the pneumococcal cell wall to glycoconjugates from human cells [12]. This protein is connected to the cell wall though the C-terminal module, whilst the N-terminal module provides the binding site to the human cells. It is considered to be the most important adhesion molecule in the pneumococcus because it is responsible for the adhesion and invasion of the bacteria into the epithelial cells of the nasopharynx, lungs and endothelial cells [112]. CbpA adheres to epithelial cells by binding to an extracellular domain of the polymeric immunoglobulin receptor (pIgR) which is responsible for the transport of secretory IgA to the surface of nasopharyngeal epithelial cells. Through this mechanism, pneumococci can use the human cellular machinery to be translocated across epithelial cells into the bloodstream [113].

1.3.3.3 Autolysin

Autolysins are a group of enzymes responsible for the degradation of the peptidoglycan layer of the cell wall of bacterial organisms [12]. There is a variety of autolysins, but the best characterised member of this group is the pneumococcal LytA amidase, which cleaves the N-acetylmuramoyl-L-alanine bond of the peptidoglycan leading to the lysis of the bacteria [114]. The molecular structure of LytA is composed of two different domains: the C-terminal domain which binds the enzyme to the cell

wall, and the N-terminal domain which is the functional part [36]. The activity of LytA leads to cell lysis and therefore cell death, but it is also involved in cell wall growth and turnover [115, 116]. LytA is an important virulence factor because, by lysing the cell wall, it facilitates the release of the cytoplasmatic contents of the bacteria, which includes the important cytolysin pneumolysin [12, 83, 117]. Different experiments with mice have shown that LytA deficient pneumococcal strains are less virulent than their parent wild type strains in both intraperitoneal and intranasal challenge models [118-121].

1.3.4. Hyaluronate lyase (Hyl)

Hyaluronate lyase is a surface protein found in a variety of pathogens and its function is to breakdown mammalian hyaluronic acid by cleaving the 1,4-glycosidic linkage between N-acetyl-β-D-glucosamine and D-glucuronic acid residues [122]. Hyaluronic acid is found in mammalian connective tissue and extracellular matrix, and is involved in different biological processes such as cell migration and differentiation, wound healing and inflammation [36, 85]. The activity of hyaluronidases increases the permeability of mammalian tissues therefore increasing the accessibility of the pneumococcus to those tissues [36]. As a result of this, the pneumococcus has an increased ability to colonise mammalian tissues and to translocate between different tissue niches [85].

1.3.5. Neuraminidases

Neuraminidases, also known as sialidases, cleave terminal sialic acid from host glycans located in body fluids and cell surfaces, such as glycolipids, glycoproteins and cell-surface oligosaccharides [36]. By doing this, neuraminidases alter the host cell

surface, which can lead to an increased susceptibility to pneumococcal colonisation, and also releases sugars as carbon and energy sources that are beneficial for the growth of bacteria [123]. Three different neuraminidases have been identified in the pneumococcus: NanA, NanB and NanC [124]. The NanA gene (*nanA*) is found in the core genome and is therefore found in all pneumococcal strains [125]. While the genes encoding NanB and NanC are found in the accessory genome which means that they and are not found in all pneumococcal strains [38]. Both NanA and NanB, the most well characterised neuraminidases, are secreted proteins, but NanA possesses an LPxTG motif present in the C-terminal module, which allows it to bind to the cell wall [38, 124, 126]. The different types of neuraminidases have different biological properties and different molecular sizes, suggesting variations in their efficacy depending on what tissue they are colonising [119]. Supporting this idea is evidence that NanC is more commonly detected in strains isolated from cerebrospinal fluid (CSF) than in those isolated from nasopharyngeal carriage [125]. Several studies have shown that NanA contributes to all the processes that contribute to pneumococcal infection: colonisation, dissemination into the lungs [107], sepsis [127] and passage across the blood-brain barrier [128]; but there are contradictory studies that have found no significant role for NanA, although these studies have come from one laboratory and not from several groups as is the case for studies that support an important *in vivo* role for neuraminidases [129, 130]. Experiments performed using NanA and NanB mutants, *nanA*Δ and *nanB*Δ respectively, show that these proteins are key factors for adherence to cultured epithelial cells, which suggests an important role during colonisation [123, 127]. In a similar way to pneumolysin, purified neuraminidase is toxic for mice but can also confer some degree of protection against pneumococcal infection [131].

1.3.6. PavA

The pneumococcal adhesion and virulence A (PavA) protein is a surface adhesin that binds to one of the components of the extracellular matrix (ECM); fibronectin, through heparin binding domains [132]. Fibronectin acts as a link between pathogens and integrins from host epithelial cells [133]. It has been shown that in other Gram-positive bacteria, like *Streptococcus pyogenes*, the presence of proteins binding to fibronectin plays an important role in adherence to epithelial cells and invasion of host cells [132-135]. The use of pneumococcal PavA deficient mutants in a murine sepsis model has shown a reduction in virulence in the absence of PavA [132]. During a long term nasopharyngeal colonisation model it was observed that in the absence of PavA, the pneumococcus is cleared from the nasopharynx over time, suggesting that PavA has an important role during colonisation [136]. In an invasive pneumonia model, where infection develops into pneumococcal pneumonia and subsequently progresses into septicaemia and death of mice in 80-100% of the cases, the PavA mutant was unable to seed into blood and therefore progress into septicaemia and host death. Instead, the PavA deficient pneumococcus was able to remain in the lung causing a chronic pneumococcal infection without being cleared [136]. In a meningitis model, where the infection is introduced through an intracisternal injection, the number of bacteria recovered from brain and blood after an infection with PavA deficient pneumococcus was consistently lower than in an infection with PavA-producing pneumococcus and there was decreased mortality in infections with the PavA mutant [133, 136]. These studies suggest that PavA is a key factor during nasopharyngeal colonisation and translocation of the pneumococcus between compartments due to its role in adherence and invasion to host cells [136]. Even though PavA is known to bind to fibronectin, it does not so exclusively, as PavA mutants still retain 50% fibronectin-binding activity

[133]. One of the other proteins able to bind to fibronectin is PavB [137]. During pneumonia, mice infected with PavB deficient pneumococci exhibit longer survival times and a delay in transmigration to the lungs when compared to wildtype pneumococcus infected mice [137]. Whilst PavB deficient pneumococci show reduced adherence to epithelial cells *in vitro*, which correlates to the observed decrease in bacterial numbers during a nasopharyngeal carriage model, no differences were observed in virulence during a meningitis model. These observations suggest that PavB is an important virulence factor only in upper respiratory tract infections [137].

1.3.7. Enolase

Enolase is a pneumococcal surface protein of 47kDa which binds to human plasmin and plasminogen, has α-enolase activity and is needed for viability [138]. The serine protease plasmin, the activated form of the glycoprotein plasminogen, plays a crucial role in fribrinolysis (dissolving blood clots), homeostasis and degradation of the extracellular matrix (ECM) [139-141]. It is thought that enolases open the plasminogen molecule conformation leading to an enhanced plasmin generation which gives the bacteria an efficient proteolytic activity that targets plasmin substrates in the host (fibrin, fibronectin, thrombospondin, laminin, and von Willebrand factor) [142]. Binding of the pneumococcus to circulating plasminogen also enhances bacterial attachment to host cell surfaces which has been shown to increase virulence in a mouse model [143, 144].

1.3.8. PsaA

PsaA is a metal ion-binding lipoprotein that, alongside PsaB and PsaC (an ATP binding protein that releases ions into the cytoplasm and an integral membrane protein

that transfers the ions in and out, respectively), composes the ABC tranporter for Mn^{2+} or Zn^{2+} uptake in *S. pneumoniae* [145]. Manganese is an element that has been shown to be essential for the growth and survival of the pneumococcus by acting as a cofactor for a large number of enzymes [146, 147]. Whilst the pneumococcus needs manganese to maintain cellular functions, large concentrations of this element can be toxic, which results in the need to have mechanisms, such as the ABC transporter, to carefully regulate intracellular manganese homeostasis [145, 148]. Zinc competes with manganese for the binding site on the ABC transporter inhibiting its acquisition [149].

PsaA is located on the surface of all known pneumococcal serotypes, and not only is part of the ABC transporter, but can also act as an adhesin [150, 151]. Some studies performed with PsaA deficient pneumococcus have shown that in the absence of PsaA there is a reduction in virulence and nasopharyngeal colonisation *in vivo* and a reduction in adherence to epithelial cells *in vitro* [152].

1.3.9. Immunoglobulin A1 Protease

Streptococcus pneumoniae produces a protease that specifically cleaves human serum IgA1 and secretory IgA. IgA is an immunoglobulin that plays an important role in mucosal immunity by inhibiting adhesins and toxins, and enhancing pathogen clearance [153, 154]. The immunoglobulin A1 protease is an enzyme that cleaves a proline-threonine bond in the hinge region of the immunoglobulin, which results in the formation of intact Fc and Fab fragments [153, 155]. Since the Fab fragments alone are not able to bind to antigen, the activity of IgA1 protease leads to antibody inactivation eliminating the protective role of IgA, making the presence of IgA1 protease an important factor in the pathogenesis of *S. pneumoniae* [153, 155].

1.3.10. Pili and sortases

Pili or fimbriae are long structures composed of associated protein subunits called pilins or fimbrins that are assembled by sortases [156, 157]. Pili were firstly thought to be characteristic of Gram-negative bacteria until they were first observed in Gram-positive bacteria in 1968 [158]. Since then, a wide range of Gram-positive bacteria have been found to express pili including *S. pneumoniae* [159].

In the pneumococcus the pili are approximately 3nm in diameter with a length that can vary between 0.1 and 5 μm, and can be visualised only using immunoelectron microscopy (Figure 10) [156].

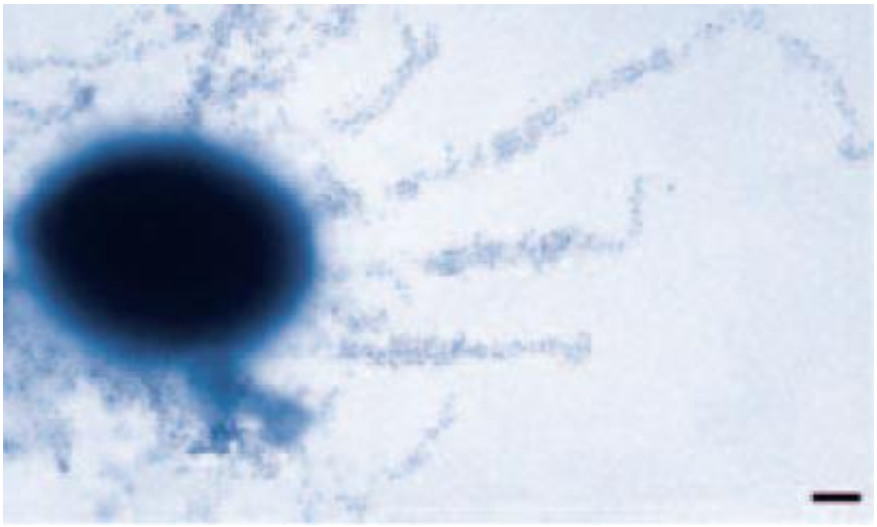

Figure 10. Immunoelectron microscopy of the main pilus component in *S. pneumoniae* (RrgB) [160].

In all Gram-positive bacteria, the genes encoding pilus proteins (i.e. RrgB) and sortases are clustered in the same genetic locus and they are transcribed in the same direction, indicating that they are part of an operon [160].

There are two important sortases in Gram-positives: sortase D (SrtD) and sortase A (SrtA). SrtD is responsible for the polymerisation of pili, whilst SrtA is responsible for the anchoring of the pili to the cell wall (Figure 11) [157].

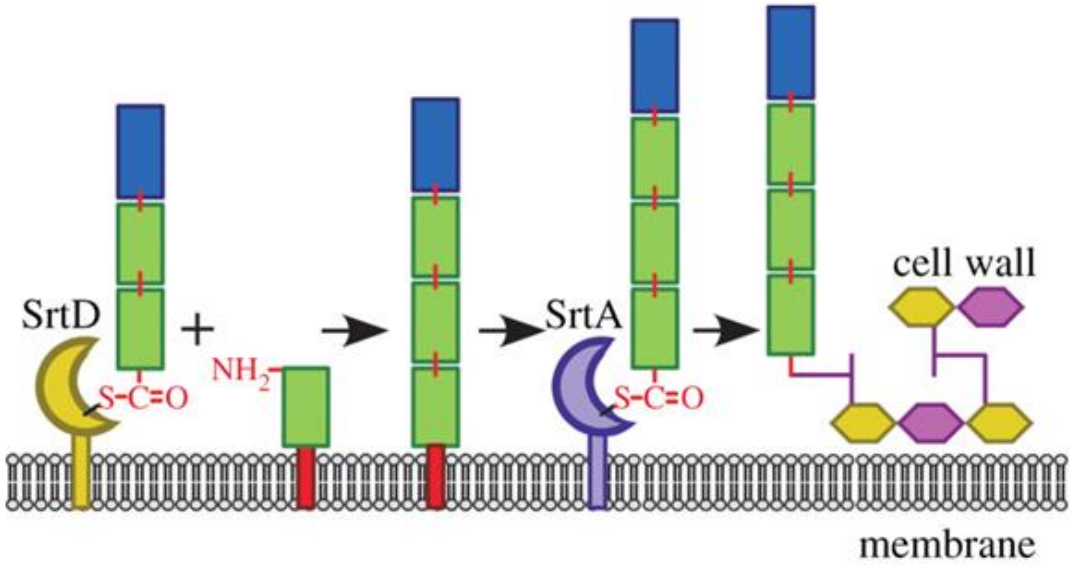

Figure 11. Formation of pili via the sortase-assembly mechanism: sortase D is responsible for the binding of different pilins (green and blue boxes), whilst sortase A is responsible for the anchoring of the pilus to the cell wall [157].

In *S. pneumoniae* two different pathogenicity islands have been found to contain pili genes, the widely known PI-1 (*rlrA* islet) and the PI-2 [38]. Not all pneumococcal isolates express pili however. The *rlrA* pilus islet is present in genetically related pneumococcal strains preferentially associated, but not only, with the carriage associated serotypes 6B, 9V, 19F and 35B [38, 161]. On the other hand, PI-2 is associated with serotypes 1, 2, 7F, 19A and 19F [38]. Since these pathogenicity islands are flanked by inverted repeats, which are characteristic of mobile genetic elements, isolates containing pili can be found globally; however, pili are present in approximately 30% of invasive pneumococcal isolates [38, 160, 162, 163].

Pili appear to be very important in the interaction of the pneumococcus with the host epithelial cells. The pilin subunits RrgA encoded by the pathogenicity islet *rlrA* acts

as an adhesin which binds to respiratory cells: RrgA deficient pneumococcus show reduced adherence to epithelial cells, whilst an overexpression of RrgA shows increase adherence [156].

Non-piliated isolates have been shown to be less virulent than piliated isolates in pneumonia and bacteraemia models of infection in mice. Moreover, it was observed that pilus expression enhances adherence to lung epithelial cells and piliated isolates were able to out-compete non-piliated isolates in nasopharyngeal colonisation models in mice. In addition, it was also shown that pili can stimulate host inflammatory responses by inducing IL-6 and TNF production [159].

1.3.11. Gene expression of virulence factors

The expression of many pneumococcal genes is influenced by external stimuli [164]. Furthermore, it has been shown that the gene expression of virulence factors change during the different *in vitro* growth stages of the pneumococcus [164]. Genes encoding proteins responsible for the biosynthesis of the polysaccharide capsule, fatty acid biosynthesis and cell division have been shown to be down-regulated during the stationary phase when compared to the exponential phase [164], while genes encoding for neuraminidases, proteases and heat shock proteins were found to be up-regulated during the stationary phase [164]. These observations suggest that the expression of specific genes change during different growth phases of the pneumococcus, where genes involved in growth and division are up-regulated during the exponential growth phase, when the pneumococcus is growing quickly.

In vivo studies of pneumococcal gene expression have been extensively done using microarrays, although the gene expression of only a small range of serotypes has been

studied, with the serotype 2 strain D39 being the most commonly used [165-169]. Studies of *in vivo* pneumococcal gene expression were mainly focused on the expression of genes encoding Ply, CbpA, PspA, PsaA, Cps2A, NanA and SpxB [167, 168]. Those studies showed that the expression of different genes vary during an intra-peritoneal infection where *psaA* and *cps2A* were highly up-regulated during the early hours of infection, whilst the up-regulation of *ply* and *pspA* occurred in later stages of the infection. In contrast, no differences were observed in the expression of *cbpA* throughout the course of infection [168]. Although gene expression *in vitro* varies when compared to *in vivo* infections, it was shown that genes up-regulated during *in vivo* infection, were also more expressed *in vitro* when compared to other virulence factors [168].

Other studies have investigated the differential expression of specific virulence factors in different sites of infection (i.e. nasopharynx, lungs and blood); these studies showed that although some genes are up-regulated in all infection sites, some are mainly expressed in the nasopharynx [167]. These observations emphasise the idea that certain proteins are important during nasopharyngeal carriage, others are important for invasiveness and others have an important role during both nasopharyngeal carriage and invasive disease.

Gene expression is an important tool to understand the pathogenesis of *S. pneumoniae*; however, little is known about the effect of differential gene expression in the differences observed in pathogenesis of different serotypes. Therefore, the study of differential gene expression between different serotypes may help elucidate the mechanisms through which some serotypes are more invasive than others.

Differential gene expression of key pneumococcal virulence factors will be discussed further in Chapter 6.

1.4. Pneumococcal vaccines

In 1880, *S. pneumoniae* was independently isolated in New Orleans and Paris by George M. Sternberg and Louis Pasteur respectively, and it was later recognised in 1881 in two separate publications, to be the principal cause of pneumonia, meningitis and bacteraemia [3, 170-172]. The first person to demonstrate humoral immunity in rabbits following pneumococcal infection was Albert Fränkel, who in 1886, observed that rabbits that had recovered from a pneumococcal infection were resistant to reinfection [3]. Between 1895 and the 1940s, anti-pneumococcal serum was commercially available through the H.K. Mulford catalogue [3, 173]. During the early 1900s efforts were made to purify and concentrate type-specific antiserum, which was then used to treat patients resulting in a reduction in mortality cases [173- 176].

Also during the early 1900s a different type of approach was taken in the war against pneumococcal infections: immunisation with whole-cell killed pneumococcal vaccines [3]. Sir Almroth E. Wright, a British physician, performed a series of clinical trials during the 1910s to test the efficacy of whole-cell killed pneumococcal vaccines, in which they were using circulating strains in their whole cell preparations, but not considering serotype-specificity [177]. In 1916-17, Oswald T. Avery and Alphonse R. Dochez isolated the pneumococcal polysaccharide capsule which allowed the pneumococcus to be classified by serotypes.

Development of the ‘quellung reaction’ by Franz Neufeld, a method in which antibodies from sera exposed to previous pneumococcal infection bind specifically to

subsets of pneumococcal capsules, along with the realisation of serotype-specificity (Figure 12), led to the development of serotype-specific whole-cell killed pneumococcal vaccine [3, 178, 179].

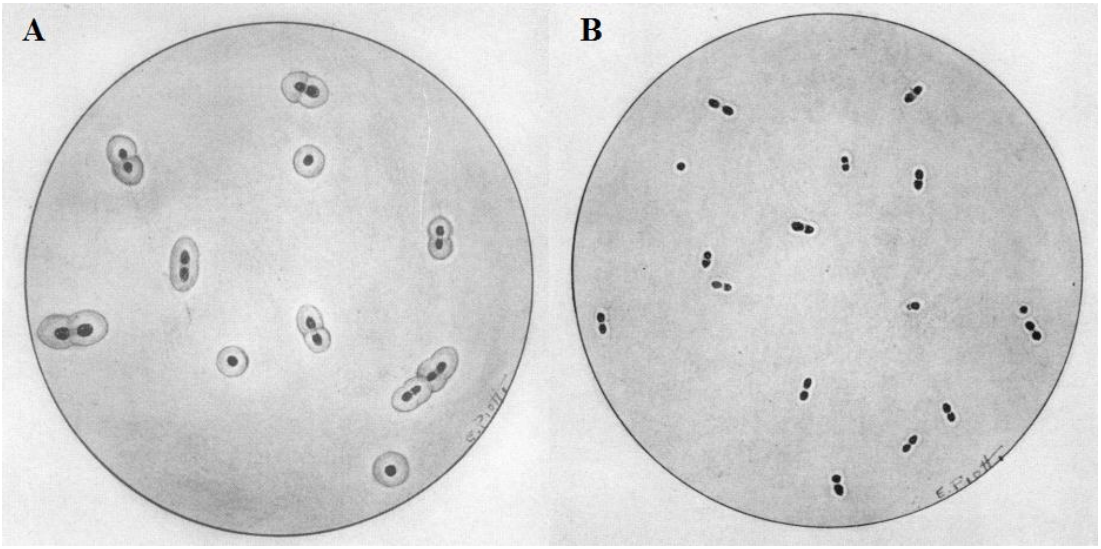

Figure 12. Pneumococcus positive (A) and negative (B) for the 'quellung reaction'. Serotype-specific anti-capsular antibodies bind to the pneumococcal capsule allowing its visualisation; the capsule appears to swell due to the increased surface tension [180].

In 1927, Oscar Schiemann and Wolfgang Casper discovered the immunogenicity of the polysaccharide capsule, whilst in 1929, Avery conjugated pneumococcal polysaccharides to proteins to improve immunogenicity [3, 181, 182].

Between the 1930s and the 1970s, various pneumococcal polysaccharide vaccines (PPVs) were developed and tested, until 1977, when a 14-valent PPV license was approved [3]. PPV14 targeted 70-80% of invasive pneumococcal disease in the US and was indicated for people above 50 and for anyone older than 2 with certain underlying health conditions [3, 183, 184]. The need for protection against a wider range of serotypes resulted in the development of the PPV23 in 1983, which covered approximately 87% of pneumococcal bacteraemic disease in the US [185].

Polysaccharide vaccines have been shown to protect more efficiently against bacteraemia than pneumonia, but they protect more against pneumonia than nasopharyngeal carriage [186-188].

1.4.1 Pneumococcal polysaccharide vaccines

Pneumococcal polysaccharide vaccines, such as the PPV23, have been shown to be highly immunogenic in adults, unfortunately children under five years of age are unable to produce a significant increase in antibody after vaccination [186, 189, 190]. The capsular polysaccharide is poorly immunogenic in children because it produces antibodies through T-cell-independent mechanisms [12]. T cell independent mechanisms produce an immune response that is not long-lasting, and boosters do not increase its duration of protection [190, 191]. Children under the age of 2 have an immature immune system which makes the polysaccharide vaccines not effective in that age group. Moreover, it was observed that PPV23 was not able to enhance herd protection due to its reduced effect in nasopharyngeal carriage [190]. The inefficiency of polysaccharide vaccines in children, a group at high risk of invasive pneumococcal disease, and its inability to provide "herd immunity" led to the development of vaccines in which the capsular polysaccharides were conjugated to a protein (diphtheria carrier protein) to increase the immunogenic effect of the vaccine in children; conjugated vaccines activate a T-cell dependent antibody response [3, 192]. The first pneumococcal conjugated vaccine to be licensed in the US was PCV7, which came in 2000, thirteen years after the first protein-conjugated vaccine to be licensed: the vaccine against *Haemophilus influenzae* type b [3].

In summary, the limitations of polysaccharide vaccines are: lack of immunogenicity in small children, transient efficiency in the healthy elderly, lack of efficiency in the

frail elderly, failure to induce mucosal immunity and low impact on nasopharyngeal carriage which results in low herd protection [192]. Nonetheless, PPV23 is, together with PCV13, currently recommended to all adults over the age of 65 in the US [190, 193].

1.4.2. Conjugate pneumococcal vaccines

In the US, before the introduction of the first pneumococcal conjugate vaccine (PCV7), there were around 65,000 cases of invasive pneumococcal disease (IPD) annually. Of these cases, 25% occurred in children under five, and 80% of those cases were caused by serotypes covered by PCV7 [194, 195]. Shortly after the introduction of PCV7 into the US, there was a reduction in cases of IPD; even though there was a higher reduction in the incidence of cases covered by PCV7-types (94%), there was also an overall reduction of cases by all serotypes (45%) [194]. Interestingly, it was observed that this reduction in IPD cases was not only seen amongst vaccinated children, but also amongst unvaccinated children and adults, which suggests that vaccination with conjugate vaccines provides herd protection [194, 196, 197].

Pneumococcal conjugate vaccines have been shown to reduce nasopharyngeal carriage and therefore transmission [198]. However, an increase in IPD cases caused by serotypes not included in the 7-valent vaccine (i.e. serotype 19A) highlighted the need of developing new vaccines with broader serotype coverage [3, 194]. Since PCV7 was first developed, two more pneumococcal conjugate vaccines have been licensed, PCV10 and PCV13, whilst PCV15 is currently being developed for licensure [199]. Serotypes 1 and 5 were included in PCV10 and PCV13 because they cause approximately 10% of IPD cases globally, a number that goes up to a third of the cases

in some developing countries [200]. Nonetheless, the efficacy of PCV10 and PCV13 against those serotypes has been shown to be limited [192, 193].

Serotype distribution varies between countries, and even though PCV7 is highly effective in some countries, in some developing countries it only provides 25% coverage against IPD-causing strains [201]. Therefore, the development of PCV10 and PCV13 should further reduce the paediatric IPD mortality rates in developing countries, where the incidence of pneumococcal disease is higher [202-204]. Recent studies have been evaluating the effect of PCV10 and PCV 13 showing a reduction in invasive disease caused by serotype 19A since the introduction of PCV13 [205]. Furthermore, there was a reduction in invasive disease in developing countries, such as Brazil and Peru, after the introduction of PCV10 and PCV13 [206-208].

A summary of the serotype coverage and the year of introduction of the main pneumococcal vaccines can be found in Table 3.

Vaccine	Serotype coverage	Year/place of introduction
PPV14	1, 2, 3, 4, 5, 6A, 7F, 8, 9N, 12F, 18C, 19F, 23F, 25F	1977 USA
PPV23	1, 2, 3, 4, 5, 6B, 7F, 8, 9N, 9V, 10A, 11A, 12F, 14, 15B, 17F, 18C, 19F, 19A, 20, 22F, 23F, 33F	1983 USA
PCV7	4, 6B, 9V, 14, 18C, 19F, 23F	2000 (infants) USA and Europe
PCV10	1, 4, 5, 6B, 7F, 9V, 14, 18C, 19F, 23F	2009 (infants) Europe
PCV13	1, 3, 4, 5, 6A, 6B, 7F, 9V, 14, 18C, 19A, 19F, 23F	2010 (infants) in USA and Europe 2011 (adults) in USA and Europe

Table 3. Main pneumococcal vaccines, serotype coverage and years of introduction (3).

1.4.3. Serotype replacement

Conjugate vaccines effectively reduce the risk of carriage and transmission of serotypes covered by the vaccines. In contrast, the risk of carriage and transmission of serotypes not covered by the vaccines has increased, resulting in a higher incidence of IPD caused by non-vaccine-covered serotypes [198]. Globally, the main serotypes responsible for IPD are 1, 5, 6A, 6B, 14, 19F and 23F, with serotypes 1, 5 and 6A not included in the first PCV, as previously mentioned [200, 209].

The serotype distribution profile of the pneumococcus has fluctuated over time due to factors such as changes in socio-economic conditions, demographic changes, introduction of antibiotics, adaptations by the pneumococcus and most importantly: the introduction of vaccines [210, 211]. After the introduction of PCV7 an increase of IPD cases by non-PCV7-covered serotypes was observed in different countries, most of this increase being attributable to serotype 19A which is now included in PCV13 [49, 200, 212- 217]. The fact that IPD cases by non-vaccine-covered serotypes increase after the introduction of a vaccination program suggests that developing vaccines with higher serotype coverage will constantly be needed. Unfortunately, the development of conjugated vaccines that cover all or most of the serotypes is not feasible due to the reduced cost-effectiveness [192, 218]. For that reason, the current research towards new vaccines is focusing on the development of vaccines based on broadly representative, serotype-independent and highly-conserved pneumococcal antigens [192].

1.4.4. Potential vaccine candidates

The current approach for new-generation vaccines is the development of non-polysaccharide antigens that are common in most pneumococcal serotypes [201].

These new vaccines are: protein vaccines, DNA vaccines, and whole-cell vaccines with low-level expression of capsular polysaccharides [192, 218]. Currently, the more developed potential vaccines are protein vaccines.

Many of the pneumococcal virulence factors have been suggested as potential vaccine candidates (Table 4); however, PspA, PsaA and pneumolysin are the leading candidates [219]: recent publications show that PspA conjugated to a flagella protein is able to enhance immunity against pneumococcal infections in mice [220]; immunisation studies with PsaA showed increased protection against carriage; pneumonia and bacteraemia in mice [221-223]; and previous immunisation studies in mice have shown increase protection against invasive disease after immunisation with pneumolysin [131, 73].

Even though these proteins can individually enhance immunity against the pneumococcus, it has been shown that in combination, this immunity is increased [224-226].

Protein	Ligand
Pneumococcal surface adhesin A (PspA)	E-cadherin
Pneumococcal surface protein C (PspC)	Polymeric Ig receptor FH domain of complement factor H Vitronectin
Choline-binding protein E (CbpE)	Plasminogen
α-Enolase	Plasminogen
Fibronectin-binding protein A (PfbA)	Fibronectin and plasminogen
Pneumococcal adherence and virulence factors A and B (Pav A/B)	Fibronectin and plasminogen
Polyhistidine triad (Pht) proteins	Unknown
Pneumococcal serine-rich repeat protein (Psrp)	The intermediary filament protein
PcpA	Uncertain
Pneumococcal pili PI-1 and PI-2	Collagen, fibronectin and laminin
Type IV pilus	DNA
Neuraminidases A and B (Nan A/B)	Expose cryptic adhesion sites on target cells
Pneumococcus-specific glycosyl hydrolase 25 (GHIP)	Exposes cryptic adhesion sites on target cells
Hyaluronate lyase (SpnHL)	Hydrolyses extracellular matrix
NADH oxidase	Contactin 4, chondroitin 4, sulphotransferase, laminin
Pneumococcal protein endopeptidase O (PepO)	Plasminogen and fibronectin
Zinc metalloproteinase B (ZmpB)	Collagen IV
Cell wall-associated serine protease (PtrA)	Collagen IV
Autolysin A (LytA)	Promotes autolysis and release of Ply
Pneumolysin (Ply)	Facilitates invasion via pore-forming activity and activates NLRP3 inflammasome

Table 4. Pneumococcal proteins with vaccine potential [192].

1.5. Host immune response to pneumococcal infection

As previously described, the pneumococcus is a common commensal of the nasopharynx of healthy humans, however asymptomatic colonisation can progress into invasive disease depending on different factors such as viral infections, age, pneumococcal serotype or most importantly, host susceptibility [227]. The main risk groups for IPD are children, the elderly and the immunocompromised, which suggests that an impaired host immune system leads to increased progression of pneumococcal nasopharyngeal carriage into invasive disease [12, 13]. In children, the immaturity of the immune system leads to poor immunogenesis and therefore reduced protection against the pneumococcus [186, 189, 190]. In the elderly, the increased susceptibility to pneumococcal disease is due to a variety of age-related changes in the immune system [92]: reduced cough reflex [228], reduced responsiveness by macrophages and monocytes to TLR ligands [229], defects in innate immune chemotaxis [230], impaired dendritic cell function [231], reduced T cell responses [232], and reduced B cell antibody production [233].

Due to the high incidence of pneumococcal carriage in the population, there is high exposure of the respiratory mucosa to the pneumococcus [92]. For that reason, humans have developed a plethora of innate and acquired, humoral and cellular immune responses to the pneumococcus [92].

1.5.1. Immune responses during nasopharyngeal colonisation

The nasopharynx is usually where the first encounter with the pneumococcus occurs; for that reason, the host immune responses taking place in the nasopharynx influence the clearance or persistence of carriage and its potential progression into invasive disease [92]. Asymptomatic carriage is not only beneficial to the pathogen, but also to

the host where it provides a natural boosting mechanism to sustain protective immunity against disease in adults [92, 234]. However, a dysregulation in the balance between immune tolerance and inflammatory responses could lead to the development of invasive disease [92, 104, 235].

As observed in Figure 13, the host has resistance mechanisms to detect and eliminate invading pathogens; however, some of these resistance mechanisms can also cause damage in the host through inflammation. For that reason, the optimal immune response is determined by the fine balance between efficient pathogen clearance and acceptable levels of inflammation [235].

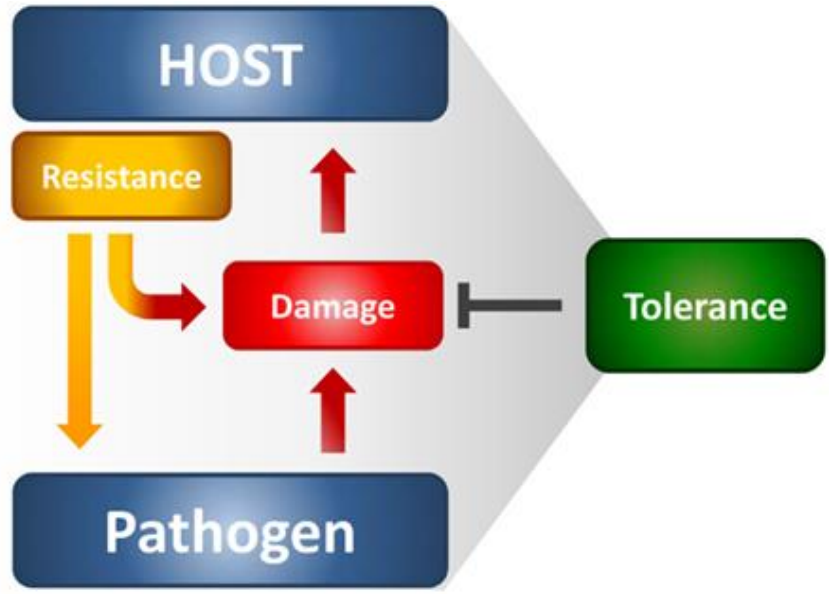

Figure 13. Host resistance and tolerance defence strategies during infection [235].

Previous studies have shown that *S. pneumoniae* induces active transforming growth factor (TGF)-β1 and T regulatory cells in the nasopharynx of mice during the early stages of colonisation, an induction that is crucial for prolonged pneumococcal carriage [104]. Moreover, elevated levels of T regulatory cells and Interleukin-10 (IL-

10) in the nasal associated lymphoid tissue (NALT) has been associated with persistence of pneumococcal carriage [236]. In summary, pneumococcal induction of TGF-β1, IL-10 and T regulatory cells in the nasopharynx limits the production of pro-inflammatory cytokines reducing tissue damage and providing an immune-tolerance mechanism that allows prolonged pneumococcal carriage [92, 104, 236]. In the absence of TGF-β1, IL-10 and T regulatory cells induction the pneumococcus is cleared and nasopharyngeal carriage is prevented [104]. Since nasopharyngeal carriage is a pre-requisite for invasiveness it would be acceptable to consider nasopharyngeal carriage as unfavourable for the host; however, there is a production of anti-pneumococcal antibodies during carriage that protects the host from invasive disease [92, 237].

1.5.2. Immune responses during invasive pneumococcal disease

Anti-pneumococcal antibodies are produced during asymptomatic nasopharyngeal carriage, with IgM and IgG being the most common types produced in serum, and IgA the most common type produced in mucosal surfaces [92, 234, 238-242]. These antibodies act as a protective mechanism against invasive disease; however, other host immune mechanisms are also involved in the clearance of the pneumococcus during invasive disease [92].

Pneumococci can descend from the upper respiratory tract (nasopharynx) to the lower respiratory tract (lungs) by aspiration of respiratory secretions; however, the lungs have been adapted to restrict bacterial growth by producing enzymes, such as lysozyme and surfactant proteins, capable of killing external pathogens [92]. Normally, alveolar macrophages are able to phagocytose pathogens and to maintain a

non-inflammatory state by producing anti-inflammatory mediators such as TGF-β and IL-10 [92, 104].

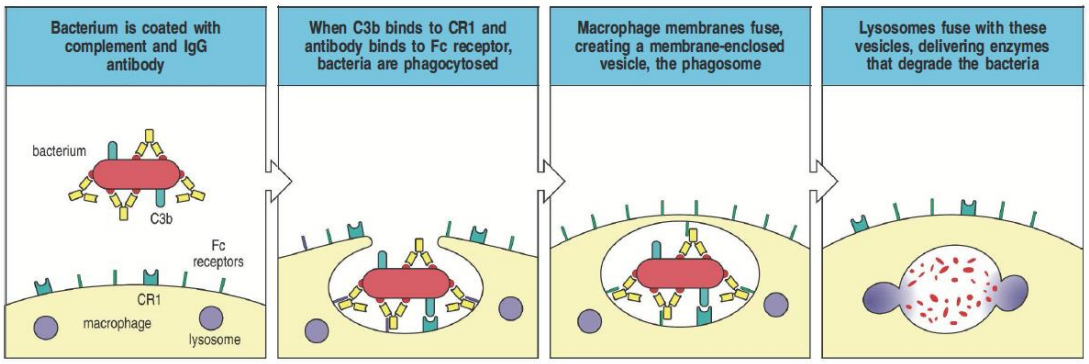

Figure 14. Phagocytosis process [243].

As observed in Figure 14, phagocytes such as macrophages and neutrophils, ingest pneumococci through an opsonin-dependent mechanism in which pneumococci have been opsonised by immunoglobulin or complement and are detected by the phagocytes through the Fc receptor (FcR) or the complement receptor (CR) respectively [13, 92]. The complement system is a collection of soluble proteins present in blood and other body fluids that are responsible for opsonisation of invading organisms, acting as early protection during infection [92, 243]. It is organised into three different pathways depending on how the activation is initiated: the lectin pathway (initiated by mannose-binding lectin and ficolins which bind to carbohydrate structures on microbial surfaces), the classical pathway (initiated by the recognition by C1q of microbial surfaces or antibodies already bound to the surface of the pathogen) and the alternative pathway (initiated by spontaneous hydrolisis of C3 which can then bind to bacterial surfaces) [243]. When activated, these pathways cleave the complement protein C3 into its active form C3b, which acts as an active opsonin stimulating phagocytosis by neutrophils and macrophages through the complement receptor (CR) [92, 243]. The complement system also leads to the lysis of pathogens via formation of a membrane-

attack complex (MAC) which disrupts the bacterial cell membrane causing lysis. However, the pneumococcus produces a phosphoglycerate kinase that acts as a complement inhibitor by inhibiting the formation of the membrane attack complex (MAC) [244].

Mouse models of infection have shown the importance of complement in the immunity against the pneumococcus; mice with genetic deficiencies in all three complement pathways have shown increased susceptibility to pneumococcal infections [92, 245-247].

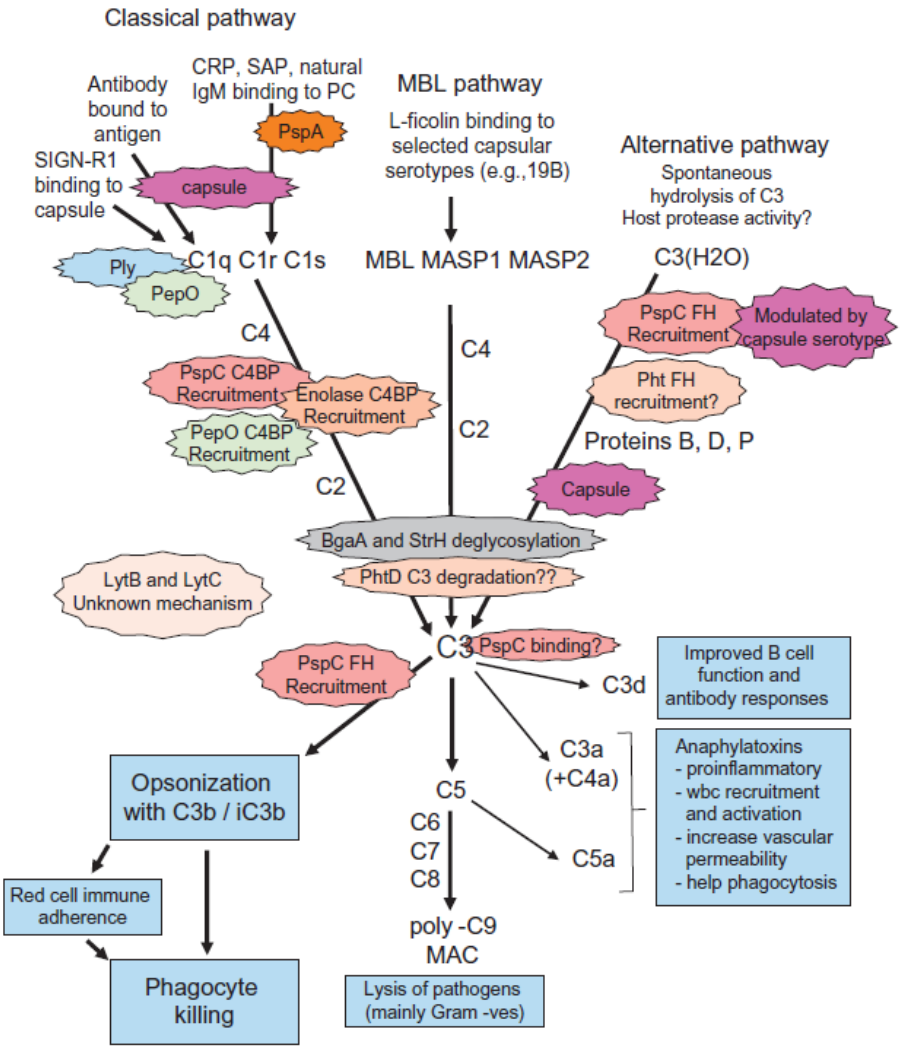

Figure 15. Mechanisms of complement identification of *S. pneumoniae* and the bacterial factors that prevent complement activation. The mechanisms of initiation by *S. pneumoniae* are indicated at the top and major immune effects of complement activation are shown in the light blue boxes. The *S. pneumoniae* factors interfering with complement activity are shown in coloured balloons at their recognized sites of action [92].

As shown in Figure 15, the complement system is able to recognise the pneumococcus through recognition of: phosphocholine from the cell wall, antibody bound to surface antigens, capsular polysaccharide and components of selected serotypes [92]. However, the pneumococcus has developed mechanisms to inhibit complement activation. Some of the inhibition mechanisms found in the pneumococcus are: capsule, which although it can be recognised by some complement proteins it can also reduce susceptibility to complement by blocking access to surface protein antigens [248-250]; the pneumococcal surface protein PspA, which inhibits C1q binding and therefore inhibits the classical pathway [329, 330]; and the pneumococcal PspC, which is able to inhibit both the alternative and the classical pathways by binding to factor H and C4BP respectively [251, 252].

Both macrophages and neutrophils are phagocytes involved in the clearance of the pneumococcus during infection; whilst macrophages are present in healthy lungs aiding to control bacterial growth, neutrophils are recruited during pneumonia via inflammatory cytokine recruitment [92, 253]. During the early stages of pneumonia the host needs to rely on innate immune responses and memory immune responses [92]. In mice, neutrophils start to accumulate in the lungs between 12 and 16 hours post-infection which coincides with a period of rapid bacterial growth occurring after a decline in bacterial numbers [254]. The interaction of the pneumococcal surface with Toll-like receptor 2 (TLR2), an innate immune receptor present on the surface of mammalian cells, leads to cytokine secretion (TNF-α, IL-1, IL-6, IL-8, IL-10, IL-12 and IL-17) which is responsible for pneumococcal clearance in the lung [92, 255, 256]. At this stage, if the infection is not contained the pneumococcus can cause IPD by translocating into the blood and causing bacteraemia [92].

1.6. The pneumococcus in animal models of infection

The pneumococcus is a human-adapted commensal pathogen; however, many vertebrates like mice, ferrets and even Zebra fish can be infected with pneumococci, and reproduce many aspects of invasive human disease [38].

The most common animal used to study the pathogenesis of the pneumococcus is the mouse due to: a) the genomes of many mouse strains have been sequenced and therefore their genome can be manipulated to create knock-out or transgenic animals [257], b) there is a variety of well-characterised strains with varying susceptibilities to pneumococcal disease [258, 259], c) there is a variety of anti-mouse antibodies and other immunological resources available for immunological studies in mice, and d) there is a number of well-defined models that mimic human pneumococcal disease [260].

There are two types of mouse strains: inbred and outbred. Inbred mice, such as BALB/c and CBA mice, offer reproducibility of responses, which provides a more uniform phenotype during colonisation and infection [260]. Outbred mice on the other hand, offer phenotypic diversity which mimics the natural variation in the human population of immune responses during nasopharyngeal colonisation and infection [260]; for that reason outbred mice were used in this study.

In animal models of infection there is a large variation in the outcome of disease depending on the dose, the administration route, the mouse strain, the pneumococcal strain and even the sex of the mice [261]. For that reason, it is important to use the same variables throughout one specific study to allow comparability between experiments. Two well characterised mouse models of infection were used during this study: the invasive pneumonia and nasopharyngeal carriage models [254, 238].

Pneumococcal serotype 2 (strain D39) is a commonly used laboratory strain, with a large amount of mechanisms of pathogenesis and host immune responses to pneumococcal infection based on studies done using D39 [107, 121, 238, 254, 258, 260, 262-265]. Published data using the invasive pneumonia model used in this study, showed that all mice intra-nasally infected with 10^6 bacteria in a 50µl volume of PBS showed signs of disease by 24h post-infection, and had become moribund by 48h post-infection [254]. Furthermore, in published data which established the nasopharyngeal model used in this study, D39 was able to sustain stable colonisation in the nasopharynx of mice intra-nasally infected with 10^5 bacteria for a minimum of 28 days (Figure 16) [238].

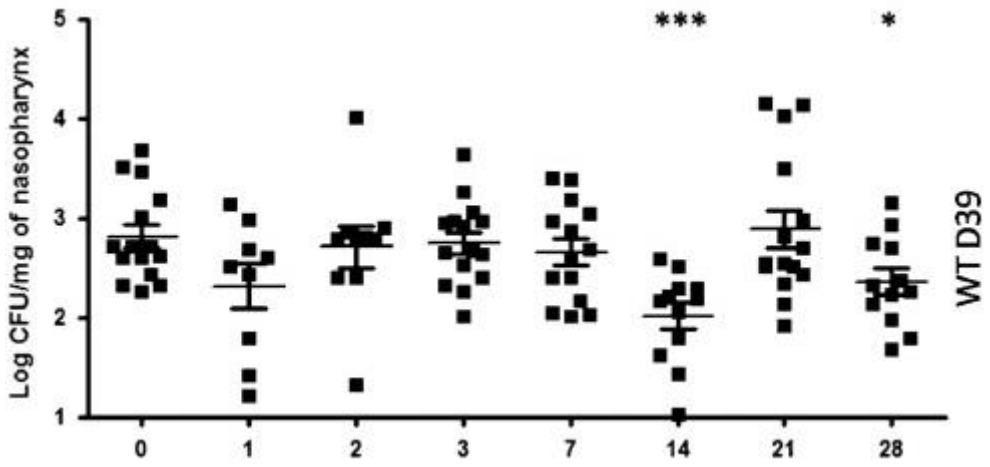

Figure 16. Density of nasopharyngeal colonisation by D39 expressed as Log CFU/mg of total homogenised nasopharynx [238].

Since these models have been well-characterised, the virulence as well as the density and duration of nasopharyngeal colonisation of D39 can be used as a reference to compare and study the virulence and nasopharyngeal colonisation patterns of other strains and serotypes.

1.7. Pneumococcal Serotype 1

Serotype-1 was one of the first serotypes to be identified [266]. It has a zwitterionic capsular polysaccharide (ZPS), which differs from other pneumococcal serotypes [267]. Whilst most polysaccharide capsules are either neutral or negatively charged, the zwitteronic capsule is charged both negatively and positively. The serotype-1 capsule has a trisaccharide repeating unit with one positive charge and two negative charges (Figure 17) [268].

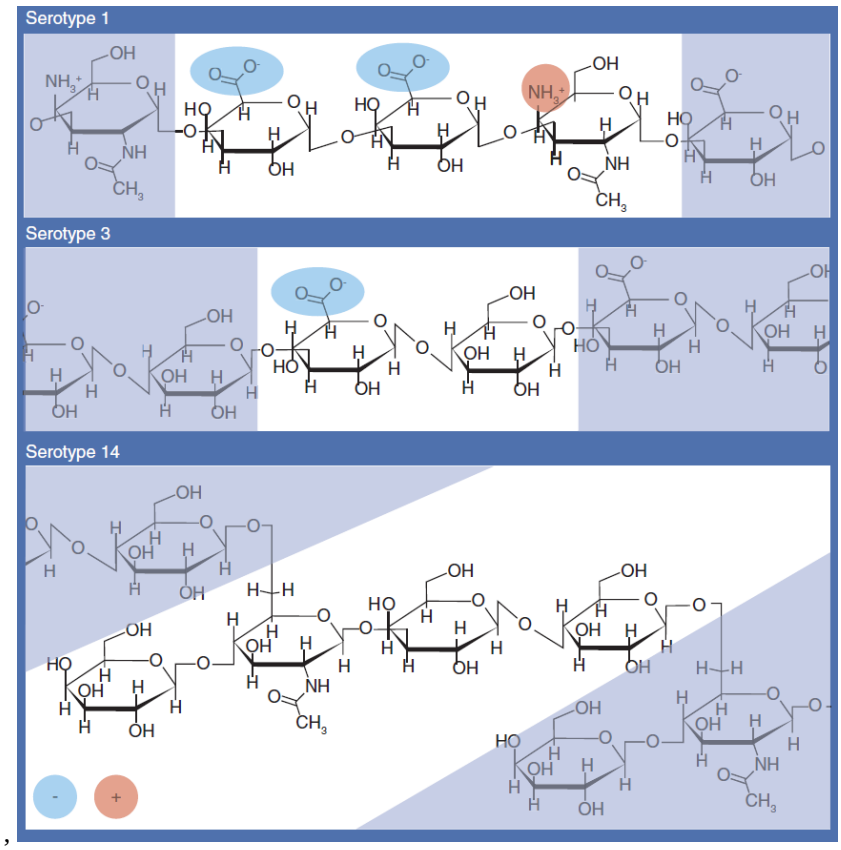

Figure 17. Differences in the structure of the polysaccharide repeating unit of different pneumococcal serotypes [269].

1.7.1. Epidemiology of serotype 1

Pneumococcal serotype 1 is known for being a major cause of invasive disease, especially in developing countries, and a high proportion of the population affected by

it are young adults under the age of 30, in contrast to other serotypes that mainly affect children and the elderly [270-272].

Serotype 1 causes common pneumococcus-associated diseases such as pneumonia, bacteraemia, meningitis and otitis media; but it is also found causing other uncommon diseases like empyema and peritonitis [269]. Empyema is an accumulation of pus and bacteria in the pleural space (space between the lung and the inner surface of the chest wall) that can occur after bacterial pneumonia, trauma, lung abscesses or surgery [273]. Approximately half of the patients with pneumonia develop fluid in the pleural space, with 5-10% of these developing empyema [274]. Empyema is a serious complication of pneumonia that can develop into necrosis, cavitation, or fistulas in the thoracic cavity [275]. Pneumococcal serotype 1 is the main cause of empyema in children and it is a common cause in adults. The incidence rates of empyema have increased in recent decades, which is thought to be a result of the introduction of pneumococcal vaccines and consequent serotype replacement [275].

What makes serotype 1 special is that, unlike other serotypes, it is rarely found in nasopharyngeal carriage [269, 270]. In a study dating from 1937, it was observed that around 22% of pneumococcal pneumonia cases in children, and 33% in adults, were caused by serotype 1 isolates, whilst only 2% of the isolates found in carriage were serotype 1 [276]. Since then, many studies have observed the same pattern [22, 277-280]. The fact that nasopharyngeal carriage with serotype 1 seems to be much lower than invasive disease suggests that serotype 1 has a high attack rate, which means that the probability of carrying it is low, but once it is carried, there is a high probability of developing invasive disease [281].

Even though nasopharyngeal carriage with serotype-1 is rare, there are reports where serotype-1 carriage has been observed [30, 282]. Moreover, in closed communities and during outbreaks when serotype-1 isolates are found causing nasopharyngeal carriage, the probability of finding other individuals carrying serotype 1 is higher [283-205]. This suggests that serotype-1 is able to colonise the nasopharynx and spread amongst close contacts, but the duration of colonisation is short when compared to other serotypes [269].

1.7.2. Consequences of low carriage rates in serotype 1 distribution

The low duration and density of carriage by serotype 1 results in less genetic diversity due to low chances of horizontal gene transfer, which occurs mainly during nasopharyngeal carriage [269, 270]. Furthermore, low genetic diversity results in distinctive geographic differences in serotype-1 clones [281]. A clone (or Sequence Type) is a group of isolates with identical multilocus sequence type (MLST) allelic profile, which is determined by 7 house-keeping genes (*ddl*, *spi*, *recP*, *aroE*, *gdh*, *gki* and *xpt*) [286].

A study of the clonal diversity of 488 serotype-1 isolates from 27 different countries (including 11 in Africa) showed clear differences in the geographic distribution of each clone (Figure 18) [270]. The serotype 1 isolates studied clustered into four distinct lineages: a) lineage A containing isolates form Europe, South America and Oceania; b) lineage B containing isolates from Africa; c) lineage C containing isolates from South America; and d) lineage D containing isolates from Asia [270].

Figure 18. Phylogeography of the 488 pneumococcal serotype 1 isolates. Maximum-likelihood phylogenetic tree based on the whole genome SNPs of serotype 1 isolates annotated with country of origin. The colour of each isolate indicates the continent of origin: red, Africa; orange, Asia; green, Europe; blue, South America (the Brazilian group is highlighted by a circle, the Argentinian and Peruvian isolates are highlighted by a star); purple, Oceania. Specific lineages referred to in the text are labelled: A (lineage A, Europe, South America & Oceania), B (lineage B, Africa), C, (lineage C, South America), D (lineage D, Asia). Note that Asian isolates are present in all of the lineages [270].

In the same study, the phylogeography of serotype 1 within Africa was analysed, showing that the African isolates clustered into 6 different clades with a high level of geographical structure [270]. Within the African isolates, 2305 orthologous genes were identified, with 59% of these genes found in all isolates (therefore belonging to

the core genome) and 37% found in at least two isolates (therefore belonging to the accessory genome). The remaining genes were unique to single isolates [270]. The high percentage of genes not shared between all African serotype 1 isolates suggest that the accessory genome may reflect host-specific selective pressures between regions and may therefore explain differences in disease severity between regions [270]. Moreover, European serotype 1 ST306 isolates have been shown to produce non-haemolytic pneumolysin due to mutations found in the pore-forming region of the protein [287]. These observations suggest that the pathogenesis of serotype 1 isolates from different geographic regions should be studied differently due to the genetic differences observed between isolates from different origins which may lead to differences in virulence and pathogenicity.

1.7.3. Epidemiology of serotype 1 in Africa

The incidence of invasive disease caused by serotype 1 can vary in time; there are periods of low incidence of serotype-1 invasive disease followed by periods of high incidence, or outbreaks [269]. In most serotypes, different sequence types can coexist at the same time, while in serotype 1 pre-existing clones are constantly being replaced by new clones [269, 270, 288, 289].

Serotype 1 is the most common cause of pneumococcal meningitis in the African meningitis belt [290, 291, 292]. The African meningitis belt region is an area in Africa with a high incidence of meningitis disease which include a total of 26 nations (Figure 19) [292, 293].

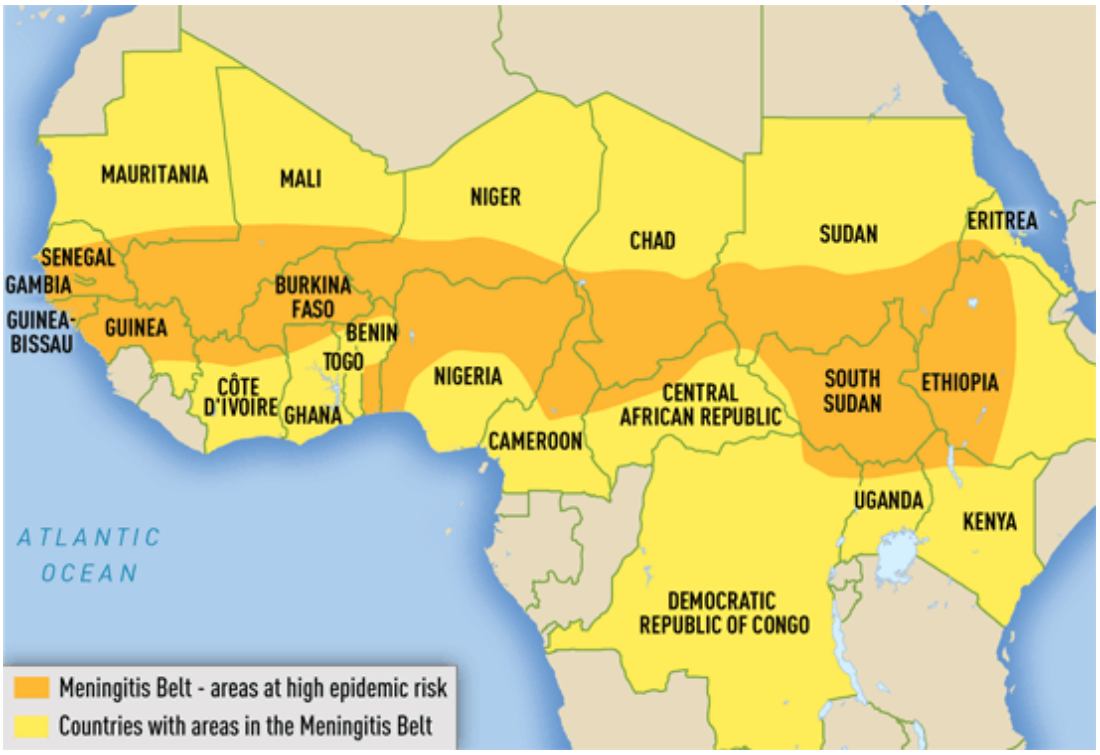

Figure 19. African meningitis belt [293].

As previously mentioned, serotype 1 is one of the capsular types included in the conjugated vaccines currently being used (PCV10 and PCV13); however, the efficacy of these vaccines against serotype 1 has been shown to be limited [192, 294]. Hence, the study of the pathogenic mechanisms of African serotype 1 is of key importance to understand the factors responsible for the mortality and morbidity caused by serotype 1 in the developing countries of Sub-Saharan Africa.

1.7.4. Immune response to serotype 1

Pneumococcal polysaccharide capsule is presented to B cells on the cell surface of antigen presenting cells, probably through complement receptors, which leads to a T-cell-independent response leading to the production of antibodies in individuals over the age of 2 [269]. In contrast, the type 1 capsular polysaccharide is able to associate to the major histocompatibility complex II (MHC II) allowing recognition by $CD4^+$ T

cells and differentiation into Th1 (producing INF-γ) and Th17 cells (producing IL-17A) (Figure 20) [269, 295-297].

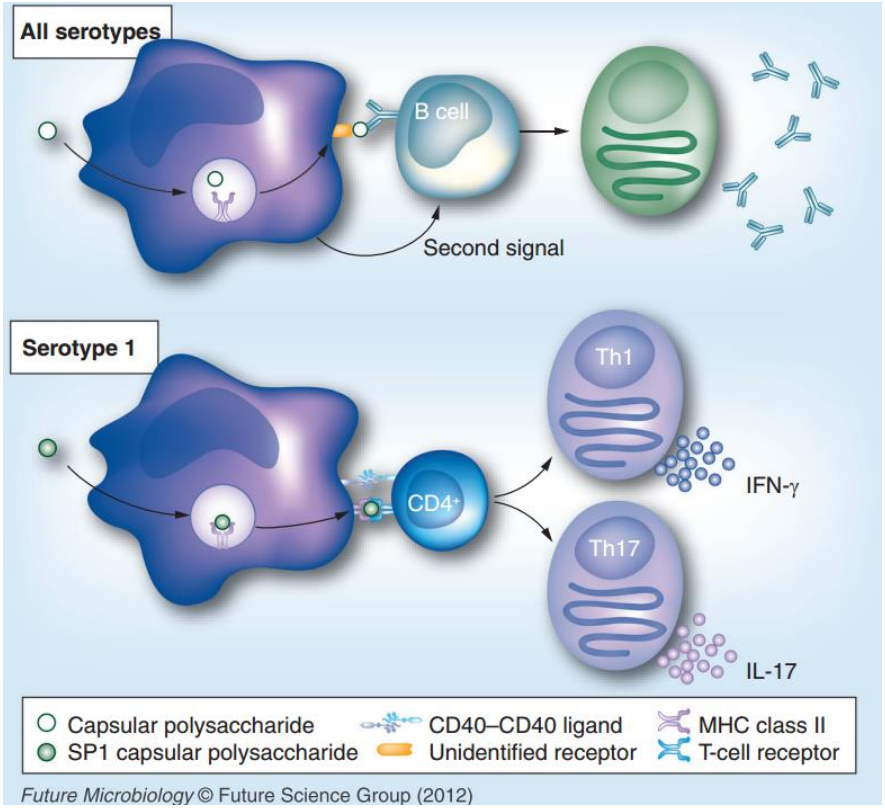

Figure 20. Immune response to pneumococcal serotype 1 capsule compared to other serotypes[269].

However, capsular type 1 is particularly resistant to opsonisation and complement deposition and moreover, $CD4^+$-dependent immunity has been shown to be reduced in mice when using protein-conjugated immunisation and hence could be the reason why PCV10 and PCV13 are not as efficient against serotype 1 as against other capsular types [269, 192, 294].

1.7.5. Serotype 1 in animal models of infection

Previous studies have shown serotype 1 to induce a low immune response and be less virulent than other serotypes in mouse models of infection. However, the isolates used in these studies were ST228, ST306, ST227 and ST304 isolates, all of which belong

to lineage A, which includes isolates from Europe, North America and Australia (Table 5) [270, 281, 295, 298-301].

Europe, North America and Australia	Africa and Israel	Chile
ST306, ST617, ST228, ST227, ST304, ST305	ST217, ST303, ST614, ST612, ST613, ST618, ST3081	ST615, ST611, ST616, ST300

Table 5. Geographic location of the most common sequence types from serotype 1 [281].

In one study, the isolate used belonged to ST217 (the most common sequence type from lineage B, which includes isolates form Africa), although the partially resistant inbred mouse strain C57BL/6 was used. [295, 300, 301]. More recently, a study showed that a serotype 1 ST618 isolate from the Gambia, was highly virulent in a mouse model of bacteraemia where the mice were infected intra-venously [302].

1.8. Research aims

The incidence of invasive disease caused by serotype 1 isolates ranges from 10% in developed countries up to 30% in some developing countries. These observations together with the reduced efficacy of the pneumococcal conjugate vaccine against serotype 1 highlights the importance of understanding the various mechanisms that determine the pathogenicity of this serotype.

Furthermore, the high genetic diversity observed between serotype 1 isolates across the world suggests that isolates from different geographic locations may be phenotypically quite different and hence observations based on one lineage may not be applicable to other lineages. To this end, as most of the studies to determine virulence and host immune responses during serotype 1 infections have been done

using isolates from lineage A (Europe, North America and Australia) the aim of this thesis was to study isolates from lineage B (Africa), which includes major disease causing sequence types, of which ST217 is the most common. As Sub-Saharan Africa bears the heaviest burden of pneumococcal disease across the world and serotype-1 is the major invasive disease causing serotype, the overall aim of this study was to determine the mechanism of pathogenesis and host immune interactions of African serotype 1 isolates belonging to ST217.

Materials and methods

2. Materials and Methods

All the reagents used were from Sigma Aldrich® (St. Louis, USA) unless stated otherwise.

2.1. Pneumococcal bacterial strains

The *Streptococcus pneumoniae* isolates used in this project were representative isolates from the blood of patients with sepsis, from the cerebrospinal fluid of patients with meningitis, from lung aspirates of patients with pneumonia or from the nasopharynx of asymptomatic individuals. The isolates from Malawi were collected for ongoing studies at two different locations: the Queens Elizabeth Central Hospital in Blantyre (the leading state-run hospital in Malawi based in the economic capital, Blantyre), and the Karonga district of Malawi (located in the rural Northern Region of Malawi). The isolates from The Gambia were provided by the MRC Unit which store isolates collected from different areas in the country. The data available for these isolates can be found in Table 6. In some experiments, the strain D39 serotype 2 of *S. pneumoniae* (NCTC 7466) and its unencapsulated R6 isotype were used as a control. Two non-serotype 1 isolates were used for competition experiments alongside serotype 1 isolates; the serotypes chosen were 6B and 19F, which are commonly found in asymptomatic carriage.

For long-term storage the isolates were maintained in bead stocks (Microbank^{TM}, Pro-Lab, Bromborough, UK) at -80°C, and streaked out onto 5% Horse Blood agar plates (Oxoid, Basingstoke, UK) when needed. After incubating the plates overnight at 37°C, alpha-haemolytic and optochin resistant colonies were observed. During most of the experiments, liquid bacterial stocks were used. The stocks were obtained by incubating

a streak of colonies from a blood plate in 20% BHI serum (Oxoid) at 37°C for 10-12h, and were then kept at -80°C for long-term storage. The stocks were ready to be used in the experiments after checking the purity and concentration of the stocks using the Miles and Misra method [303].

Sample ID	Carriage / Invasive	Blood/ pneumonia/ CSF/Naso	ST	Location	Year	Age	Serotype	Child / Adult
A41626	Invasive	Blood	217	Blantyre	2006	54	1	adult
A42174	Invasive	Blood	217	Blantyre	2006	30	1	adult
A47864	Invasive	Blood		Blantyre	2007	37	1	adult
B11510	Invasive	CSF	217	Blantyre	2005	42	1	adult
B13969	Invasive	CSF	217	Blantyre	2006	38	1	adult
B17099	Invasive	CSF	217	Blantyre	2007		1	adult
C9471	Invasive	CSF	217	Blantyre	2004	3	1	child
C10305	Invasive	CSF		Blantyre	2004	3	1	child
C10827	Invasive	CSF		Blantyre	2004	9	1	child
D25796	Invasive	Blood	217	Blantyre	2004	8	1	child
D48309	Invasive	Blood	217	Blantyre	2008	4.8	1	child
D42785	Invasive	Blood		Blantyre	2007	10	1	child
MLAB7062	Carriage	Naso		Blantyre	2006		NT	adult
W000168	Carriage	Naso		Karonga	2008	26	1	adult
W001477	Carriage	Naso		Karonga	2009	16	1	child
W001640	Carriage	Naso		Karonga	2009	6w	1	child
W001641	Carriage	Naso		Karonga	2009	4	1	child
W002818	Carriage	Naso		Karonga	2009	2	1	child
W004030	Carriage	Naso		Karonga	2010	2	1	child
W004737	Carriage	Naso		Karonga	2010	10w	1	child
L001564	Invasive	Blood		Karonga	2010	29	1	adult
PNI 830	Invasive	Pneumonia	217	The Gambia	2009	45	1	adult
SVT 23946	Carriage	Naso	217	The Gambia	2006	12	1	child
D33275	Carriage	Naso	9533	Malawi			6B	
19F	Carriage	Naso	177	Europe			19F	

Table 6. List of pneumococcal isolates used in this study.

2.2. Confirmation of serotype 1 isolates by PCR

The Malawian isolates were confirmed to be serotype-1 by amplification of the *WchC* gene by colony PCR. Individual colonies grown overnight in 5% Blood Agar plates were picked and placed in DNase free water to be lysed. PCRs were performed using a Techne Flexigene thermal cycler (Therem, Stone, UK) and using ReadyMix™ Taq PCR Reaction Mix according to the manufacturer's instructions.

The sequence of the forward primer used for this PCR was 5'-CTAATGCTAAATTAATCTTAGG-3', whilst the sequence of the reverse primer was 5'-ATTTCATCTCCCTTGATAAACG-3'. This primer amplifies a 401bp fragment. The thermocycling conditions for this PCR are shown in Table 7.

The presence or absence of the *WchC* gene was determined by separation of the DNA fragments using a 1% agarose gel electrophoresis.

Step	Temperature	Time
Initial Denaturation	95°C	5 min
35 cycles	95°C	30 sec
	50°C	30 sec
	72°C	1 min
Final extension	72°C	10 min
Hold	15°C	

Table 7. Thermocycling conditions for the serotype-1 capsule PCR.

2.3. Determination of presence or absence of the pneumolysin gene

The presence of the pneumolysin gene in the Malawian isolates was also determined by colony PCR by amplifying the *ply* gene. PCRs were performed using a Techne Flexigene thermal cycler and using ReadyMix™ Taq PCR Reaction Mix according to

the manufacturer's instructions. The sequence of the forward primer used for this PCR was 5'- GTCGCAAGCATTCTCCTCTC-3', whilst the sequence of the reverse primer was 5'- GGCTGATTTCGCTGAACAAG-3'. This primer leads to the amplification of a 1501bp fragment. The thermocycling conditions for this PCR are shown in Table 8.

The presence or absence of the *ply* gene was determined by separation of the DNA fragments using a 1% agarose gel electrophoresis.

Step	Temperature	Time
Initial Denaturation	95°C	5 min
35 cycles	95°C	30 sec
	50°C	30 sec
	72°C	1 min
Final extension	72°C	10 min
Hold	15°C	

Table 8. Thermocycling conditions for the pneumolysin PCR.

2.4. Haemolytic assays

The haemolytic activity of the different Malawian isolates was determined using a modified version of a method published by James C. Paton in 1983 [73, 90]. The bacterial isolates were grown in BHI to late exponential phase ($OD_{500} < 1$) and then lysed by treatment with 0.1% sodium deoxycholate. The total protein of the lysates was determined using the BCA Protein Assay Kit (Thermo Scientific, Loughborough, UK). Approximately 85µg of total protein was used for the determination of the haemolytic activity of each isolate by making serial dilutions of the lysates and incubating them with a 4% suspension of sheep red blood cells (Oxoid). After a 30min

incubation at 37°C the suspension was centrifuged at 3000g for 5min and the OD_{540} of the supernatants was measured to determine the haemolytic activity. This experiment was performed in triplicate.

2.5. Growth curves

The OD_{550} of 200µl of a suspension of 10^3CFU/ml bacteria in 20% BHI serum was determined every 30min for a total of 15h using a BMG Labtech FLUOstar OMEGA microplate reader (BMG Labtech, Aylesbury, UK)..

2.6. Tissue culture

The cell lines used for this study were the human bronchial-epithelial cells BEAS-2B (ATCC® CRL-9609™), the human nasopharyngeal epithelial cells Detroit-562 (ATCC® CCL-138™) and the promyelocytic leukaemia human cell line HL-60 (ATCC® CCL-240™).

The BEAS-2B is an adherent cell line that was maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 5% inactivated Foetal Calf Serum (FCS) and 1% Penicillin-Streptomycin solution. The cells were kept in horizontal 75cm^2 flasks at 37°C and 5% CO_2 until confluent. Once confluent, approximately every 2-3 days, the cells were split into a new flask after being detached using 5ml of 1xTripsin/EDTA. The Detroit-562 is also an adherent cell line and was maintained in Minimum Essential Medium Eagle (MEM) supplemented with 10% FCS and 1% Penicillin-Streptomycin solution. This cell line was kept under the same conditions as the BEAS-2B and the cells were split after incubation with 5ml 1xTrypsin to induce the detachment of the cells from the flask.

The HL-60 cell line was maintained in suspension in RPMI-1640 Phenol Red free medium (Invitrogen, Thermo Scientific, Loughborough, UK) supplemented with 20% FCS. The cells were kept in vertical 75cm^2 flasks at 37°C and 5% CO_2, and the media was changed every day. This cell line was used for opsonophagocytic killing assays, for which the cells had to be differentiated into polymorphonuclear lymphocytes (neutrophils) by incubating 2x10^{5}cells/ml in 100ml of medium with 750µl of dimethylformamide (DMF) for 4 days. For opsonophagocytic killing assay the viability of the cells after differentiation needed to be above 90%, which was determined with trypan blue.

2.7. Adhesion and invasion assays

The adhesion and invasion assays were performed using the lung epithelial cells BEAS-2B and the nasopharyngeal epithelial cells Detroit-562 following a modified version of a protocol published by Paton in 1996 [152].

Approximately 10^5 cells were seeded in 24 well plates and grown in their appropriate media (DMEM for BEAS-2B, and MEM for Detroit-562) in the presence of FCS and antibiotics. The cells were grown at 37°C and 5%CO_2 until confluent, approximately 48h after seeding. Frozen stocks of known concentrations of bacteria were used for this assay. The bacteria were pelleted down and re-suspended in the appropriate medium (with FCS but without antibiotics to a final concentration of 10^6CFU/ml. One ml of the bacterial suspension was added to the seeded cells after those had been washed 3 times with PBS (Oxoid). The 24 well plates were then incubated for 2h at 37°C and 5% CO_2. For each experiment two 24 well plates were used, one of them to assess the adherence of the bacteria to the surface of the cells, the other one to assess the invasion of the bacteria into the cells.

After 2h of incubation both plates were washed 5 times with PBS to remove the bacteria that were not adhered or invading. In the plate designated to determine the invasion of the bacteria 1ml of medium with FCS and 5ug of penicillin was added to each well and incubated at 37°C and 5%CO_2 for a further 2h. For the plate designated to assess adhesion, 100µl of 1xTrypsin/EDTA were added to each well and the plate was incubated for 10min at 37°C and 5% CO_2. Once the cells were detached they were lysed by incubating them for 10min in 1ml of 0.05% Triton X-100 media with FCS. Finally, the viable bacterial number was determined using the Miles and Misra method, by serially diluting the sample and incubating the dilutions overnight on 5% BAB plates at 37ºC [303].

After the extra 2 hours of incubation with penicillin, the cells in the plate used to determine the invasion were washed 3 times with PBS to remove the penicillin. After the washes with PBS the cells were detached and lysed as with the plate designated to determine bacterial adhesion. The viable bacterial numbers were determined using the Miles and Misra method [303]. The number of adhered bacteria was determined by subtracting the number of invading bacteria (invasion plate) to the total number of bacteria (adhesion plate).

These experiments were performed in triplicate on three independent days and controls without bacteria and without cells were used every time.

2.8. Capsule thickness

The bacterial capsule thickness was determined using a modified version of the FITC-dextran exclusion method originally published by Gates in 2004 [304, 305]. This

method consists of measuring the zone of exclusion of FITC-dextran which correlates with capsule size.

Briefly, a single colony from an overnight culture on 5% BAB plates was incubated for approximately 8-12 hours at 37°C in 10ml of BHI to mid-log phase. The bacteria were then sub-cultured to again, a mid-log phase. Finally, 2µl of 200kDa FITC-dextran (Thermo Scientific) was added to 10µl of the culture and pipetted onto a microscope slide. The slides were observed using a Nikon Eclipse 80i fluorescence microscope (Nikon, Kingston Upon Thames, UK). At least 100 individual bacteria were analysed for each sample using the ImageJ software. These experiments were kindly done by Laura Jacques, a PhD student in the lab.

2.9. Opsonophagocytic killing assays

The opsonophagocytic killing assay with HL-60 cells is used to determine the ability of different bacterial strains to avoid phagocytosis by human neutrophils, and is also used to determine the opsonising ability of different serums against different bacteria. The assay used in this study is a modification of the protocol published by Romero-Steiner in 2003 [306].

Frozen bacterial stocks of known concentration were washed in PBS and re-suspended to a final concentration of 10^5CFU/ml in HBSS (Invitrogen). 10µl of the bacterial suspension (10^3 bacteria) were incubated with 20µl of opsonin for 30min at 37°C and shaking at 175rpm. The opsonin used differed in each assay, being either serum obtained from naïve or infected mice, or pooled human IgG (IVIG, Grifols Therapeutics, New York, USA). During the opsonisation time the neutrophils were prepared by centrifugation at 250g for 10min and re-suspending them in 1ml $DPBS^{-/-}$

(Invitrogen). The concentration of the cells was determined using a Neubauer chamber and trypan blue, being adjusted to a final concentration of $1.125x10^7$cells/ml. After opsonisation 40µl of the neutrophils suspension ($4x10^5$ neutrophils) were added to the bacteria, followed by 10µl of baby rabbit complement (Invitrogen). The cells were then incubated for 45min at 37°C and shaking at 175rpm. Finally, 10µl of the suspension were plated out onto 5% BAB plates and incubated overnight to determine the number of viable bacteria.

The controls used in this assay include bacteria only, bacteria with neutrophils, bacteria with opsonin, bacteria with complement, bacteria with neutrophils and opsonin, bacteria with opsonin and complement, and bacteria with neutrophils and complement. The experiments were done in triplicate on three independent days.

2.10. Complement deposition

The complement deposition assay is an assay where human serum is added to the bacteria and the deposition of C3 on the surface of the bacteria is determined by flow cytometry. The human serum used in this experiment was a pool from different healthy individuals. Thiazol orange was used as a bacterial stain to be able to gate and select live bacterial cells during the flow cytometry acquisition. The C3 deposition on the bacteria was determined using a mouse-anti-human C3 antibody (Abcam, Cambridge, UK) and an anti-mouse-APC antibody (Clone m2a-15F8) (eBiosciences, Hatfield, UK).

Briefly, a 5ml suspension of 10^5CFU/ml in BHI was made using bacterial frozen aliquots of known concentration. The bacterial suspension was centrifuged at 3350g for 10min after allowing the bacteria to re-activate for 15min at 37°C. The bacterial

pellet was re-suspended in 5ml PBS and the suspension was equally distributed in 3 eppendorf tubes. All tubes were centrifuged and the bacterial pellet was re-suspended with 100µl of 20% serum diluted in PBS and 1% Gelatine Veronal Buffer. After an incubation of 30min at 37°C and 5% CO_2 the bacteria were washed with 900µl of PBS and centrifuged at 17,000g for 3min. The pellet of the first two tubes was re-suspended in 100µl of PBS whilst in the third tube it was re-suspended with 100µl of mouse-anti-human C3 (1:300 dilution in PBS). The bacteria were again incubated for 30min at 37°C and 5% CO_2 followed by another wash with 900µl of PBS. After centrifugation at 17,000 for 3min the pellets of the first and second tubes were re-suspended again in 100µl of PBS whilst in the third tube the pellet was re-suspended with 100µl of anti-mouse-APC (1:400 dilution in PBS). The bacteria were then incubated at 4°C for 30min in the absence of light. After the incubation the bacteria were washed twice with 900µl and a further 100µl of PBS. Finally, 5µl of Thiazol orange (1:1000 dilution in PBS) was added to tubes two and three before the samples were analysed using the BD Accuri™ C6 flow cytometer (BD Biosciences, Oxford, UK). The first two tubes were used as controls whilst the third tube was used to determine the mean fluorescence intensity, used to determine C3 deposition. These experiments were performed in triplicate on three independent days.

2.11. Invasive pneumococcal disease model in mice

The induction of IPD in mice was carried out following the protocol previously described by Kadioglu in 2000 and 2002 [102, 254]. The experiments were performed at the University of Liverpool following the Home Office guidelines for animal procedures.

Seven to ten week old female MF1 outbred mice (Charles River, Margate, UK) were temporally anaesthetised with 2.5% (vol/vol) isoflurane (Abbott, Chicago, USA) over oxygen delivered at a rate of 1L/min. The bacteria were administered intra-nasally in a 50µl volume of sterile PBS at a concentration of approximately 10^6CFU/ml. The bacterial dose was prepared using frozen aliquots of known concentration.

The mice were monitored and signs of disease recorded. The illness chart classifies mice as normal, hunched, piloerect, lethargic, moribund or dead. Each level of illness was scored as not present, present or strongly present. Mice were culled by CO_2 asphyxiation and cervical dislocation at pre-established specific time points or when showing signs of lethargy, as established by the Home office licence.

Blood was collected in heparinised tubes by tail-bleeding at different time points and by cardiac puncture at time of death. The bacterial load in blood was determined using the Miles and Misra method [303]. For some experiments the collection of serum was required. In these cases a minimum of 500µl of blood were collected by cardiac puncture in the absence of heparin and the tube was left to rest for either 30min at room temperature or overnight at 4°C. The blood was then centrifuged for 10min at 2,000g and the serum was collected and stored at -20°C for future analysis.

Lung, nasopharyngeal tissue and brain were obtained for bacterial load determination. The tissues were collected in sterile PBS, weighted and homogenised by mechanical disruption using a T10 ULTRA-TURRAX® homogeniser (IKA®, Staufen, Germany). After homogenisation 20µl of the samples were serially diluted in PBS, plated onto 5% BAB plates and incubated overnight at 37°C. When necessary, the remaining sample was centrifuged at 300g for 5min to pellet down the cells and the supernatants were stored at -20°C for future cytokine analysis.

When the tissues were required for cellular profiling using flow cytometry they were mashed and sieved using 40µm cell strainers (Corning, New York, USA) instead of homogenised by mechanical disruption. The cells were then ready to be stained or ready to be re-suspended in freezing media (75% RPMI, 15% FCS and 10% DMSO) and stored at -80°C for future analysis.

2.12. Nasopharyngeal carriage model in mice

The induction of pneumococcal nasopharyngeal carriage in mice was performed following the method previously described by Richards in 2010 [238]. The experiments were done at the University of Liverpool following the Home Office guidelines for animal procedures.

Seven to ten week old female MF1 outbred mice (Charles River) were temporally anaesthetised with 2.5% (vol/vol) isoflurane (Abbott) over oxygen delivered at a rate of 1L/min. The mice were infected intra-nasally with 10µl of sterile PBS containing approximately 10^5CFU/ml of bacteria. The bacterial dose was made using frozen stocks of known concentration.

The mice very rarely developed any sign of disease, therefore they were culled at pre-established time points by CO_2 asphyxiation followed by cervical dislocation.

For bacterial load determination and cytokine analysis blood, lung, brain and nasopharyngeal tissue were collected at time of death and processed following the same guidelines as for the invasive mouse model. Serum was obtained as previously described and used for antibody and cytokine analysis. The cervical lymph nodes (CLN) and the nasal-associated lymphoid tissue (NALT) were collected in PBS, mashed and sieved through a 40µm cell strainer for flow cytometry analysis.

2.13. Assessment of long-term protection acquired during carriage

In order to assess the long term protection induced during nasopharyngeal carriage with serotype 1 isolates, twenty 7-10 week old female MF1 outbred mice (Charles River) were infected with the bacteraemia isolate and then re-infected 21 days later with the same isolate. The mice were infected with 10^5 bacteria in 10 µl of sterile PBS and let to rest for 21 days before infecting them again with another 10^5 bacteria in 10µl of sterile PBS. The doses, as for the nasopharyngeal carriage model, were done using frozen stocks of known bacterial concentration. The re-infection was performed 21 days after the first infection to ensure that most of the bacteria from the first infection had been cleared. The mice were then culled 0, 1, 3 and 7 days post re-infection.

The nasopharynx was collected for bacterial load determination and for cytokine analysis. The cervical lymph nodes and the nasal-associated lymphoid tissue were collected in PBS, mashed and sieved for flow cytometry analysis.

2.14. Nasopharyngeal competition experiments in mice

Competition experiments were performed in mice to assess the ability of different serotypes to out-compete one another during nasopharyngeal carriage. In order to do this, 7-10 week old female MF1 outbred mice (Charles River) were infected with 10^5 bacteria in 10µl of sterile PBS and were left to rest for 7 days until the second infection with a different serotype. The time between infections was 7 days in order to allow the first introduced serotype to establish colonisation before the addition of the second serotype.

For bacterial number determination blood, lung, brain and nasopharyngeal tissue were collected at different time points and processed as previously described. After bacterial

number determination, the nasopharyngeal tissue was harvested and the supernatant was used for cytokine analysis. Serum was collected as previously described and used for antibody and cytokine analysis. The cervical lymph nodes and the nasal-associated lymphoid tissue were collected in PBS, mashed and sieved for flow cytometry analysis.

2.15. Flow cytometry

Lungs, cervical lymph nodes, brain, and nasal-associated lymphoid tissue were collected in PBS, mashed through cell strainers and centrifuged for 5min at 300g, as previously described. After centrifugation the pellets were re-suspended in freezing media and stored at -80ºC for future staining and acquisition. For the lung cells, the pellet was treated with 10% red blood cell lysis (BD Biosciences) for 5min in order to remove the red blood cells from the cell suspension. The blood was also treated with red blood cell lysis and stored at -80ºC for future staining and acquisition.

For staining and acquisition the samples were thawed and washed twice with PBS before being incubated with purified anti CD16/CD32 Fc blocking antibody (eBiosciences) for 10min at 4ºC. Following the incubation with Fc blocking antibody, cell surface markers were stained using a combination of monoclonal antibodies conjugated with FITC, PE, PECy7 and APC fluorochromes (Biolegend, London, UK). An intracellular monoclonal antibody against Fox P3 was also used for staining T regulatory cells (Biolegend). The panels used in this study can be seen in Table 9. The samples were acquired using a BD FACSCaliburTM flow cytometer (BD Biosciences) and analysed using FlowJo vX.0.6.

Panel 1	Panel 2
APC - CD45 (immune cells) Clone I3/2.3	APC – F4/80 (macrophages) Clone BM8
PE – FoxP3 (T regulatory cells) Clone FJK-16S	PE – CD19 (B cells) Clone eBio1D3
PE/Cy7 - CD4 (T cells) Clone GK1.5	PE/Cy7 – Gr1 (neutrophils) Clone RB6-8C5
FITC – CD3 (T cells) Clone 17A2	FITC – CD45 (immune cells) Clone 30-F11

Table 9. Staining panels used for flow cytometry.

2.16. Cytokine analysis

Levels of cytokines from the nasopharynx, lungs, brains and serum of mice were determined using enzyme-linked immunosorbent assays (ELISA). Commercially available kits from eBiosciences were used for all cytokines except for MIP-2 and KC, which were purchased from Sigma and Invitrogen respectively. The levels of KC, MIP-2, IL-1β, IL-10, IL-6, IL-17A, TGFβ, TNFα, and INFγ in the nasopharynx of mice carrying pneumococcus were determined following manufacturer instructions. The levels of IL-6, IL-17A, MIP-2, INFγ and TNFα were determined from lungs, brain and serum of mice with invasive pneumococcal disease.

2.17. Determination of antibody levels in serum and nasal mucosa

Enzyme-linked immunosorbent assays (ELISAs) were used to determine the levels of total IgG from serum and IgA from the nasal mucosa following manufacturer instructions (eBiosciences).

2.18. Capsule switch detection

The determination of capsule switching events during nasopharyngeal co-colonisation was studied during a competition experiment in which serotype 6B was introduced 3 days after a carriage infection with the serotype 1 bacteraemia isolate (A42174). Ten

isolates were recovered as controls from the nasopharynx of mice on day 3 post-infection with serotype 1, but before the introduction of the 6B isolate. A further 300 single isolates were obtained from the nasopharynx of co-colonised mice at days 1, 3 and 7 post 6B introduction. The phenotypic determination of the capsule type of 46 of the 300 isolates was performed using serotype 1 specific latex antisera (Immulex™, Statens Serum Insitut, Copenhagen, Denmark). The genotypic determination of the capsule type of those 46 isolates was done by sequencing the xanthine phosphoribosyltransferase (*xpt*) housekeeping gene (accession number: Q8RJS3), which has different alleles for the serotypes 1 and 6B, allowing differentiation.

2.19. RNA extraction

Growth curves of the serotype 2 strain D39 and the serotype 1 strain A42174 from bacteraemia were performed by inoculating 250ml of BHI with $2.5x10^6$ bacteria and incubating them at 37ºC in a water bath. Samples were taken every hour to determine the optical density (OD_{500}). This experiment was done in triplicates and plotted to determine the five phases of growth: early exponential phase (EEP), mid-exponential phase (MEP), late exponential phase (LEP), early stationary phase (ESP) and late stationary phase (LSP).

Ribonucleic acid (RNA) from each growth phase was stabilised to avoid sacrificing the integrity of the RNA by incubating the bacteria with a solution of 5% phenol (Invitrogen) and 95% ethanol for a minimum of 30min in ice. Following stabilisation, the RNA was extracted using a phase separation method using TRIzol® (Invitrogen) [307]. Briefly, the cells were centrifuged and re-suspended in TRIzol® to allow bacterial lysis. The lysed bacteria were mixed with chloroform to separate the RNA from DNA, lipids and proteins. Once the aqueous phase was collected (RNA), the

sample was washed and the RNA was precipitated using ethanol. Finally, the RNA was re-suspended in DNase/RNase free distilled water (Invitrogen). The RNA extraction was done in an RNase-free area and using RNase-free materials to avoid RNA degradation.

The quantity of RNA was determined using the Qubit® Fluorometer (Thermo Scientific). A 2100 Bioanalyzer (Agilent Technologies, Stanta Clara, USA) was used to assess the quality of the RNA: if the RNA is not degraded two bands should be observed, if the RNA is degraded the two bands disappear into a smear. Once confirmed that the RNA was not degraded the samples were sent to Vertis Biotechnologie AG (Freising, Germany) for Illumina NextSeq 500 sequencing (Illumina).

Different methods were used to optimise the RNA extraction from bacteria infecting different tissues of mice, but no method was found to provide RNA of good enough quantity and quality.

The growth curves and RNA extraction for the serotype 2 strain D39 were kindly done by Reham Yahya, a PhD student in the lab.

2.20. Transcriptomic analysis

The raw sequencing data was sent from Vertis Biotechnologie AG in FastQ format. The genomes were annotated by another PhD student in the lab, Chrispin Chaguza. Assembly of the sequence reads was performed using Velvet and SPADes assembly programs. The resulting contigs of the genomes were then ordered by ABACAS and annotated using a combination of the RATT and PROKKA prokaryotic annotation pipelines. Orthologous genes between the A42174 (EMBL-EBI Accession number: ERS353646) and the D39 strains (Genebank Accession number: NC_008533) were

identified using CD-HIT. The mapping of the RNA-Seq reads against the annotated genomes to infer transcripts was done by Dr. Karsten Hokamp at Trinity College, Dublin, as previously described [308]. Finally, the expression analysis of the transcriptome was done in collaboration with Prof. Jay Hinton (University of Liverpool) using the GeneSpring 7.3 software (Agilent Technologies).

2.21. Statistical analysis

The GraphPad Prism 5® software was used to create all the graphs shown in this thesis and to do the statistical analysis of the results. The individual tests used are explained in the figure legends.

In vitro characterisation of African serotype-1 pneumococcal isolates

3. *In vitro* characterisation of African serotype-1 pneumococcal isolates

3.1. Introduction

Phylogeographic analysis based on whole genome sequencing of *Streptococcus pneumoniae* serotype 1 isolates has led to the conclusion that serotype 1 clusters into four distinct lineages, each of which is associated with a single continent [270]. Different isolates from different continents have been shown to be phenotypically different during infection [270, 287]. This difference in pathogenicity highlights the importance of analysing isolates forming different lineages separately. Across sub-Saharan Africa, serotype 1 remains one of the most common causes of invasive pneumococcal disease, causing approximately 20% of invasive pneumococcal disease cases in Sub-Saharan Africa and the African meningitis belt [290-292, 309, 310]. The high propensity for this serotype to cause invasive disease across the continent highlights the need for understanding how it causes disease and its genotypic and phenotypic characteristics which contribute to its pathogenesis.

The Pneumococcal African Genomic Consortium 1 (PAGe1) is a consortium funded by the Bill and Melinda Gates Foundation that focuses on the genomic analysis of pneumococcal serotype 1 isolates across Africa. This consortium is currently analysing more than 900 sub-Saharan serotype 1 genomes to understand the molecular mechanisms of pathogenesis of this highly invasive pneumococcal serotype. ST217 is one of the most prevalent serotype 1 sequence types found in Africa [295, 300, 301]. For that reason, a group of 20 different Malawian serotype 1 ST217 isolates were selected as representatives of different infection sites (nasopharynx, blood and CSF)

to link the genomic analysis of the 900 sub-Saharan serotype 1 isolates to their *in vitro* and *in vivo* phenotypic characteristics.

In vitro experiments were used to determine the phenotype of serotype 1 pneumococci using 3 experimental designs: a) by determining the role of pneumolysin on the virulence of serotype 1 ST217, b) by assessing the ability of ST217 isolates to adhere and invade human cells and c) by assessing the ability of ST217 to evade the host immune system.

3.2. Serotype 1 confirmation by PCR

To ensure all the isolates selected for this study were indeed serotype 1, all the isolates were analysed for the presence or absence of the *wchC* gene. The product of the *wchC* gene is a putative acetyltransferase responsible for the acetylation of the type 1 capsule [311]. All the isolates were positive for the presence of this gene (Figure 21).

To subsequently validate the PCR results, with one exception, all isolates were confirmed to be serotype 1 *in silico* through whole genome sequencing. The only exception was the MLAB7602 isolate which was later confirmed as non-typeable by full genome sequencing. Non-typeable isolates are considered to be encapsulated bacteria that have lost their capsule through the loss of either the whole capsule operon or a selection of genes inside the operon [53]. Since this isolate was positive for the *wchC* gene by PCR we hypothesised that this isolate could have been a serotype 1 isolate before losing the capsule or that it had acquired the *whcC* gene by recombination [65].

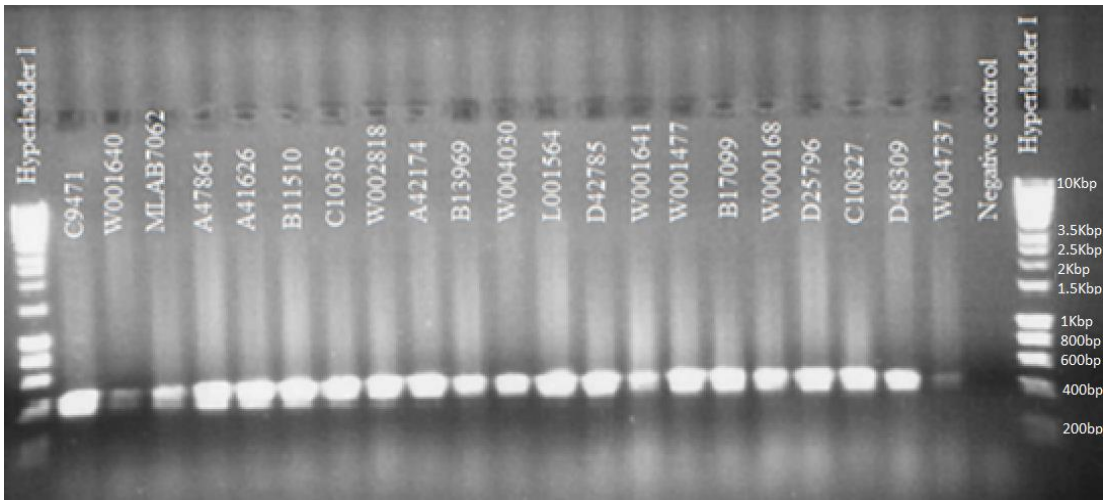

Figure 21. PCR for the *wchC* gene, representative of a serotype 1 capsule, shown as presence or absence of the 401bp gene product.

3.3. Determination of presence or absence of the pneumolysin gene by PCR.

Pneumolysin has been found to be produced by approximately 99% of all pneumococcal serotypes, including serotype 1 [312]. Across Europe, ST306 is the most common sequence type associated with serotype 1, while the ST217 strain is commonly found in sub-Saharan Africa. The type and levels of production of pneumolysin has been shown to be different between clones, with ST306 producing a non-haemolytic form of pnumolysin [287]. Since pneumolysin is a major virulence factor for pneumococcal pathogenesis, a PCR was performed to detect the presence or absence of the pneumolysin gene (*ply*) in all 20 ST217 isolates used in this thesis. All the Malawian isolates had the pneumolysin gene present (Figure 22).

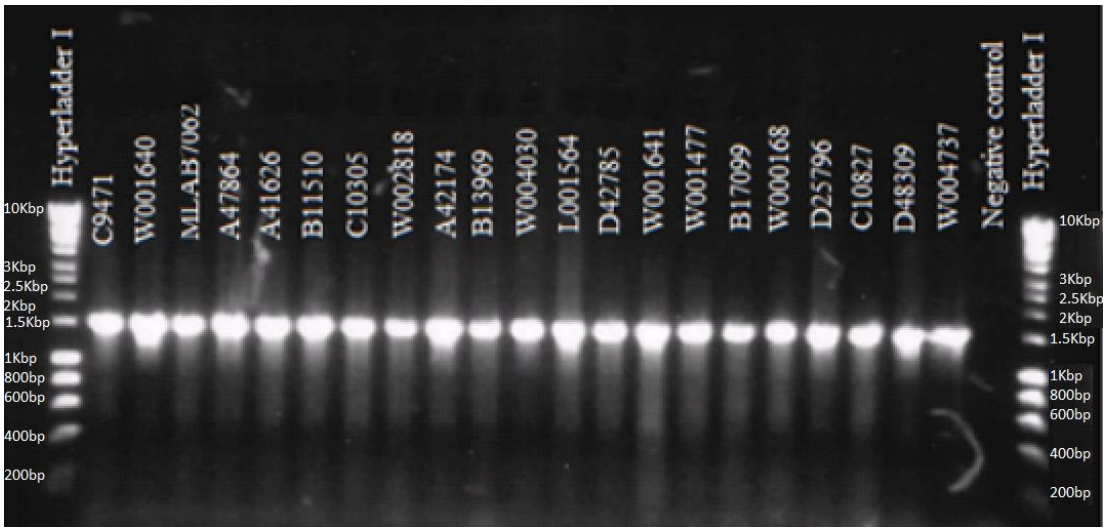

Figure 22. PCR for the pneumolysin gene, shown as the presence or absence of the 1501bp gene product.

3.4. Haemolytic assay

In order to determine the level of production of the active pneumolysin (PLY) protein, the haemolytic activity of pneumolysin produced by ST217 isolates was compared to the haemolytic activity of the pneumolysin produced by the control isolates using a well-established haemolytic assay [73]. The D39 isolate was used as a positive control whilst its isogenic pneumolysin deficient mutant (PLN-A) and the non-haemolytic ST306 strain B915 were used as negative controls.

The haemolytic activity is shown in Figure 23 as the OD_{540} after 30min incubation of different dilutions of the bacterial lysate with a 4% Sheep Red Blood Cell solution. These results clearly demonstrate that the haemolytic activities for all the isolates were broadly similar at the first dilution but differences between isolates began to emerge at 1:2 dilution. A one way ANOVA test was used to analyse the differences between all serotype 1 isolates at 1:2 dilution (Figure 24).

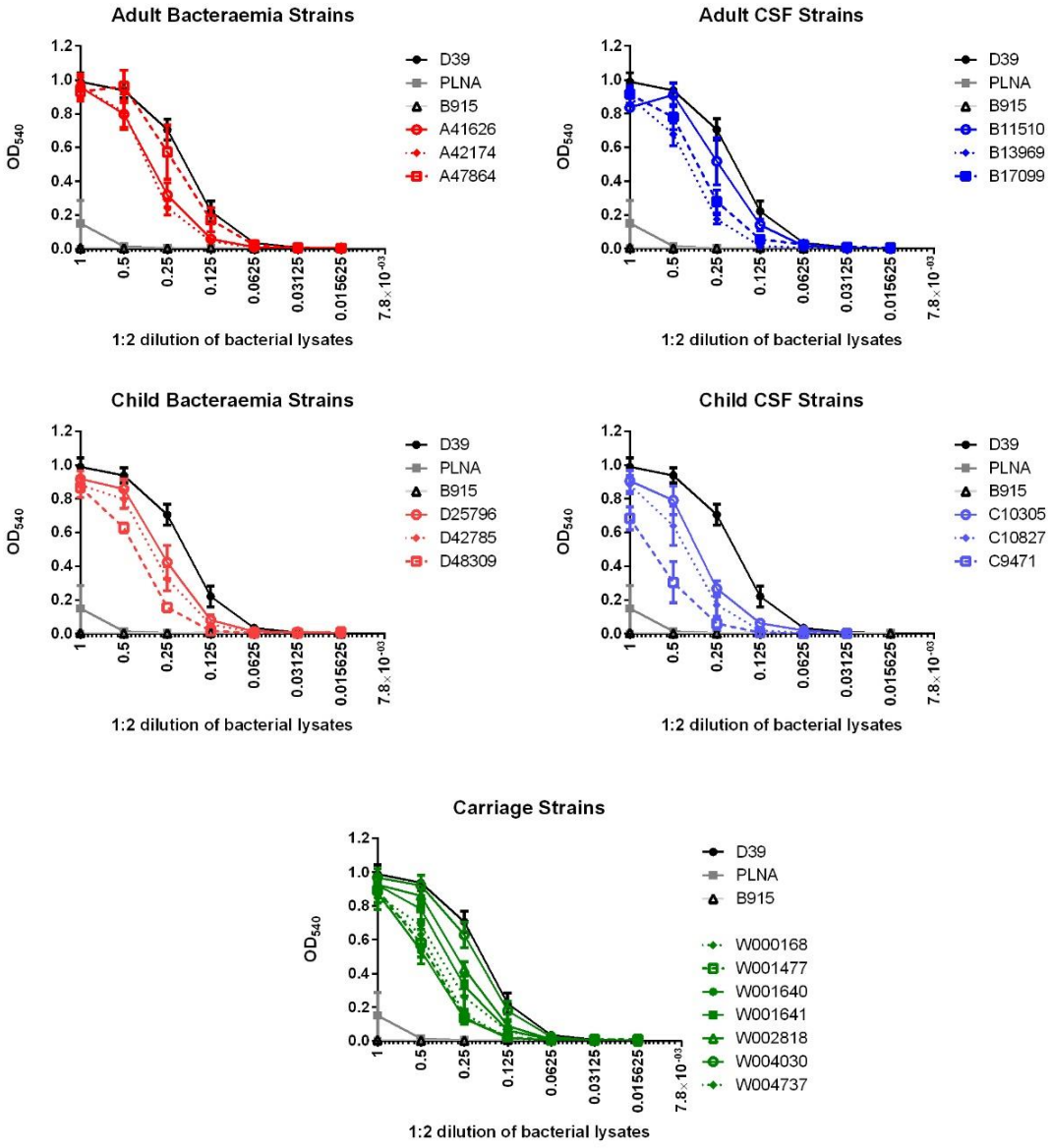

Figure 23. Haemolytic activity of all serotype 1 isolates shown as mean OD_{540} ± SEM of the 1:2 dilutions of bacterial lysates after incubation with a 4% red blood cell solution. The bacteraemia isolates are shown in red, the meningitis isolates are shown in blue and the carriage isolates are shown in green. This experiment was performed in triplicate on three independent days.

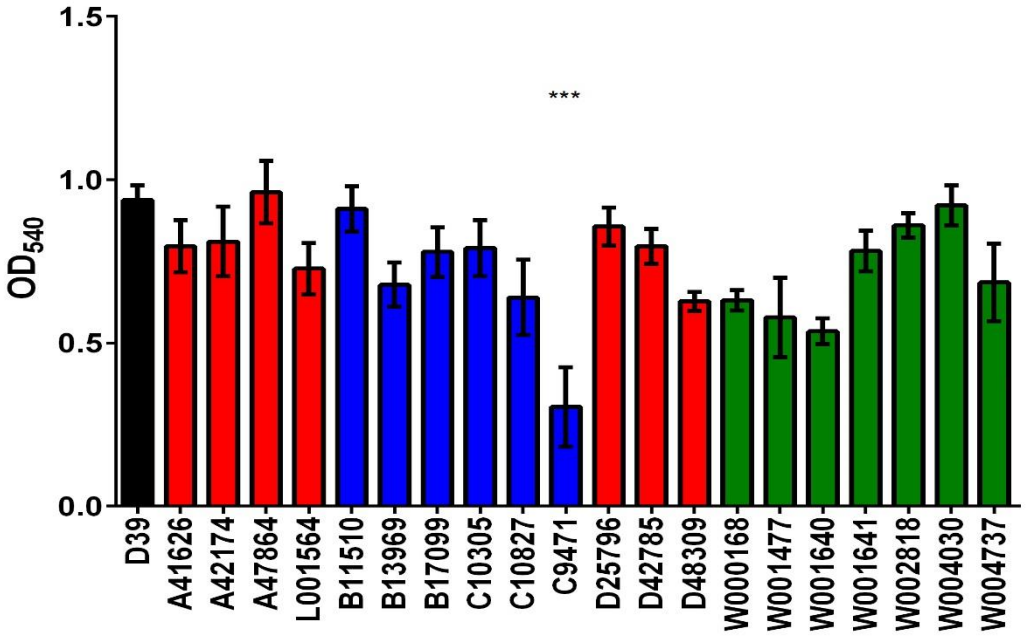

Figure 24. Levels of haemolytic activity of all serotype 1 isolates compared to the haemolytic activity of the serotype 2 D39 at dilution 1:2 shown as mean OD_{540} ± SEM. Black: D39 control, red: bacteraemia isolates, blue: meningitis isolates and green: carriage isolates. ***P-value<0.005 when analysed using a one-way ANOVA test.

Only one isolate had a significantly lower haemolytic activity when compared to the other isolates; the isolate was C9471, which was isolated from the cerebrospinal fluid of a 3 year old child. Since no significant difference in the level of haemolytic activity was observed across the entire set of 20 isolates selected for this study, with the exception of C9471, only three representative isolates were selected for further *in vitro* phenotypic investigations representing adult bacteraemia (A42174), adult CSF (B13969) and child carriage (W004030).

To ensure validity of results, the low haemolytic serotype 1 ST217 isolate (C9471) was selected for direct comparison to the 3 study isolates demonstrating no observable differences in the *in vivo* carriage model of infection. These results can be seen in Section 9.1.

3.5. Growth curves

To determine the fitness of the different bacterial isolates used, a growth rate experiment was undertaken to compare the growth kinetics of the serotype 1 isolates to the well characterised laboratory serotype 2 strain D39 by growing the different bacteria in 20% BHI-serum for 15h and determining the OD_{550} every 30min.

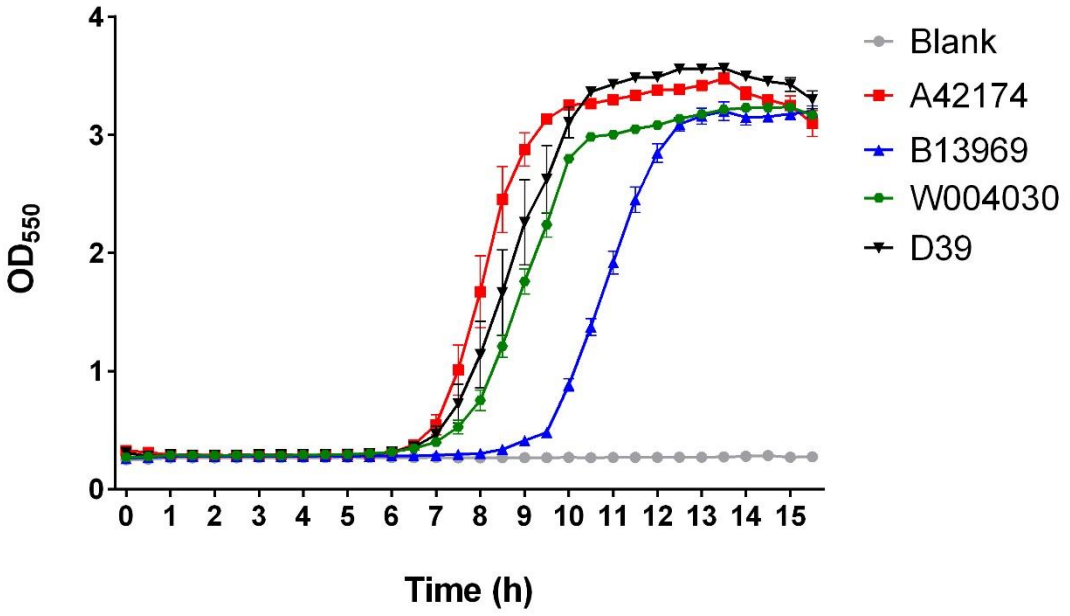

Figure 25. Growth curves of the bacteraemia, meningitis and carriage serotype 1 isolates and the serotype 2 D39 shown as mean OD_{550} ± SEM of bacterial cultures determined every 30 minutes during a 15 hour incubation in 20% BHI-serum. This experiment was done in triplicate on three independent days and no significant differences were observed.

As observed in Figure 25, all the isolates followed the same pattern of growth: there is a static period of time in which the isolates do not grow mainly due to adjustment to the new environment; this phase is also known as the lag phase [328]. Following adaptation to the culture media the isolates begin to replicate in a phase known as the exponential phase [328]. The final bacterial growth phase is known as the stationary phase, where the bacteria stop replication due to the exhaustion of available nutrients, and also due to the increasing accumulation of waste products and toxic metabolites

that are detrimental to bacterial growth. Following the stationary phase the bacteria start to die due to the prolonged lack of nutrients [328].

The doubling times for each bacteria was determined using the OD_{550} values obtained from the growth curve: 50min for the bacteraemia isolate A42174 and 1h for the meningitis isolate B13969, the carriage isolate W004030 and the serotype 2 control strain D39. In summary, no significant differences were observed in the growth patterns of the serotype 1 isolates and the serotype 2 control strain D39.

3.6. Adhesion and invasion assays

To assess the ability of the serotype 1 isolates to adhere to and invade human nasopharyngeal epithelial cells (Detroit-562) and human broncho-alveolar epithelial cells (BEAS-2B), adhesion and invasion assays were performed. Two controls were used in these experiments: the first control was the serotype 2 strain D39 in order to be able to compare the adherence and invasion of the serotype 1 isolates to a well-studied pneumococcal strain. The second control was the non-typeable MLAB7062 pneumococcal strain obtained from Malawi to be able to compare the adhesion and invasion of the serotype 1 isolates to a non-encapsulated wild-type pneumococcal strain.

Figure 26a clearly demonstrates that all the pneumococcal isolates used were able to adhere to nasopharyngeal epithelial cells (Detroits-562), but the percentage of adhesion varied between isolates. No significant differences were observed between the D39 strain and the serotype 1 isolates when analysed by ANOVA, but there was a significant difference in adhesion of D39 and the serotype 1 isolates when compared to the non-typeable strain MLAB7062, for which the adherence levels were much higher than for the rest of the isolates (*P-value<0.05, ***P-value<0.005 ****P-

value<0.0001). Figure 26b shows that some invasion was observed with the non-typeable isolate and the meningitis isolate (B13969), although the level of invasion was not consistently observed throughout independent experimental repetitions, it does suggest that the non-typeable isolate and the meningitis isolate might be better at tissue invasion than the rest of the isolates studied. Furthermore, the levels of invasion were independent of the number of adhered bacteria suggesting that invasion was determined by the bacterial strain and not by the number of adhered bacteria.

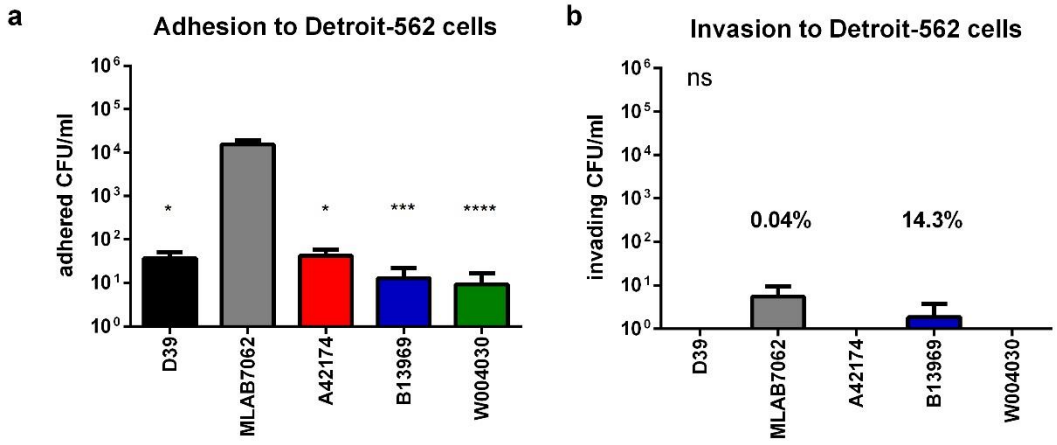

Figure 26. Adhesion and invasion levels of serotype 1 isolates in the Detroit-562 cell line shown as mean CFU ± SEM of adhered bacteria per ml of media with an initial inoculum of 10^6 bacteria/ml. Invasion is also shown as a percentage of adhered bacteria. These experiments were performed in triplicate on three independent days. *P-value<0.05, ***P-value<0.005 and ****P-value<0.001 when compared to the MLAB7062 control using a one-way ANOVA.

When the broncho-alveolar epithelial cells (BEAS-2B) were used no adhesion or invasion was detected for the D39 strain or the serotype 1 isolates. The only strain able to adhere or invade this cell line was the non-typeable strain MLAB7062 (Figure 27a and 27b). The MLAB7062 was significantly more able to adhere to the BEAS-2B cells when compared to the rest of the isolates (*P-value<0.05), but no significant differences were seen for invasion due to the low levels observed.

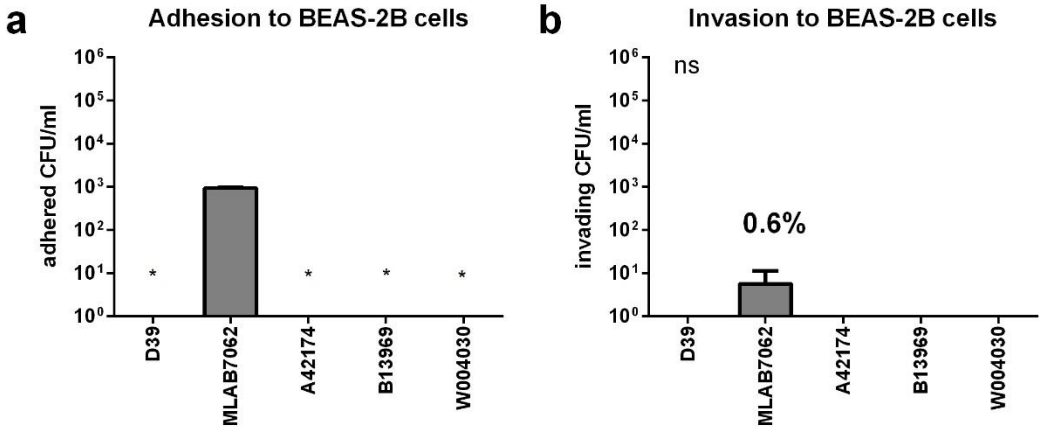

Figure 27. Adhesion and invasion levels of serotype 1 isolates in the BEAS-2B cell line shown as mean CFU ± SEM of adhered bacteria per ml of media with an initial inoculum of 10^6 bacteria/ml. Invasion is also shown as a percentage of adhered bacteria. These experiments were done in triplicate on three independent days. *P-value<0.05 when analysed using a one-way ANOVA.

3.7. Capsule thickness

A major virulence factor of *S. pneumoniae* is the polysaccharide capsule, which provides protection against opsonophagocytosis by the host immune system. The thickness of the capsule can vary between isolates and at different stages of infection. An increase in the capsule thickness can lead to an increased protection against the host immune system, whilst a reduced capsule thickness can lead to an increased ability to adhere to epithelial and endothelial host cells [57, 60, 61]. Therefore by measuring the capsule thickness it is possible to determine the relative ability of the isolate to evade the host immune system and its potential ability to adhere and cause colonisation.

The capsule thickness of the serotype 1 isolates was determined using a FITC-dextran exclusion method and a one-way ANOVA for statistical significance (Figure 28a).

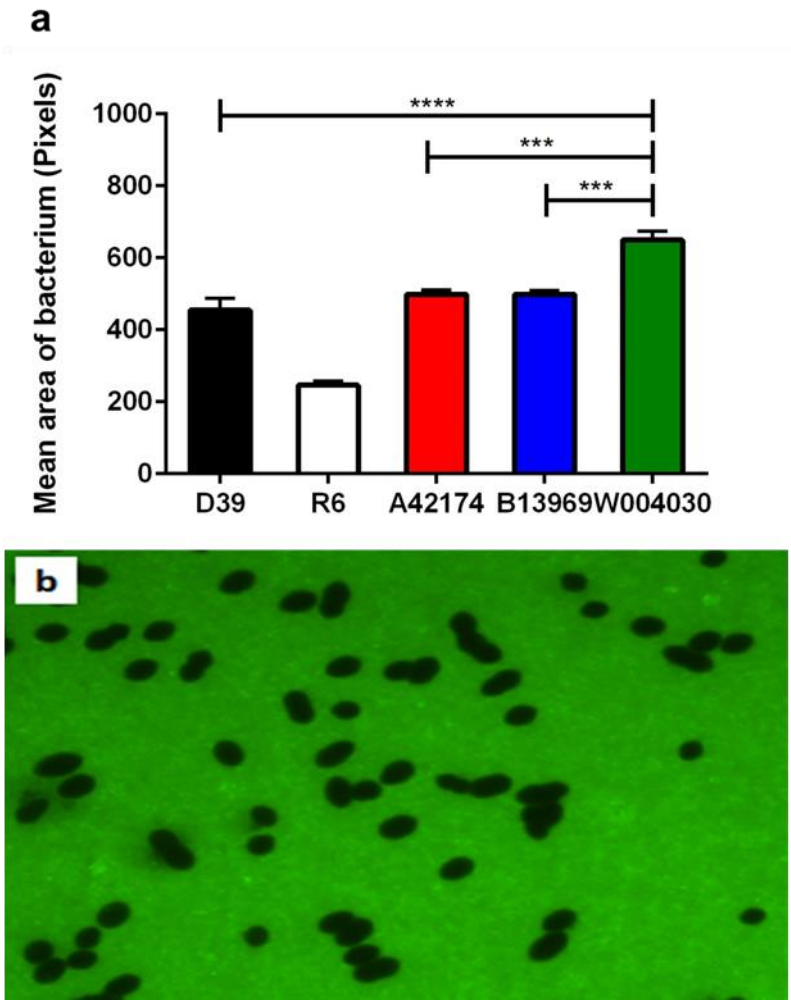

Figure 28. a) Capsule thickness of the serotype 1 isolates directly from frozen stocks shown as the mean area of a minimum of 100 individual bacterium ± SEM. The encapsulated D39 strain and the unencapsulated R6 strain were used as controls. b) *Streptococcus pneumoniae* serotype 1 isolate stained using the FITC-dextran method. ***P-value<0.005 and ***P-value<0.001 when analysed using a one-way ANOVA.

The FITC-dextran exclusion method requires the total surface area of the bacterium to be stained with FITC-dextran and then measured and used to compare the thickness of the capsule of different isolates (Figure 28b). This method is based on the premise that pneumococci are generally uniform in their size, therefore any size differences observed using this method is attributable to differences in capsule thickness. This method is used to measure the size of individual pneumococci not found forming chains. The area, and therefore capsule thickness, measurements showed no differences between the bacteraemia (A42174) and the meningitis (B13969) isolates,

but the carriage isolate (W004030) had a significantly thicker capsule than the other two serotype 1 isolates (***P-value>0.005).

3.8. Opsonophagocytosis killing assay

In order to assess the ability of the serotype 1 isolates to evade phagocytosis an opsonophagocytosis killing assay (OPKA) was performed in which the bacteria were opsonised using intravenous IgG (IVIG) and then incubated with baby rabbit complement and HL-60 derived neutrophils. This experiment was performed using the bacteraemia and the meningitis isolates and the D39 strain as a control. This experiment was not done on the carriage strain due to a sudden reduced availability of HL-60. A high variability in the activity of different batches of differentiated HL-60 cells was the reason why the carriage strain was not further tested during opsonophagocytic killing assays.

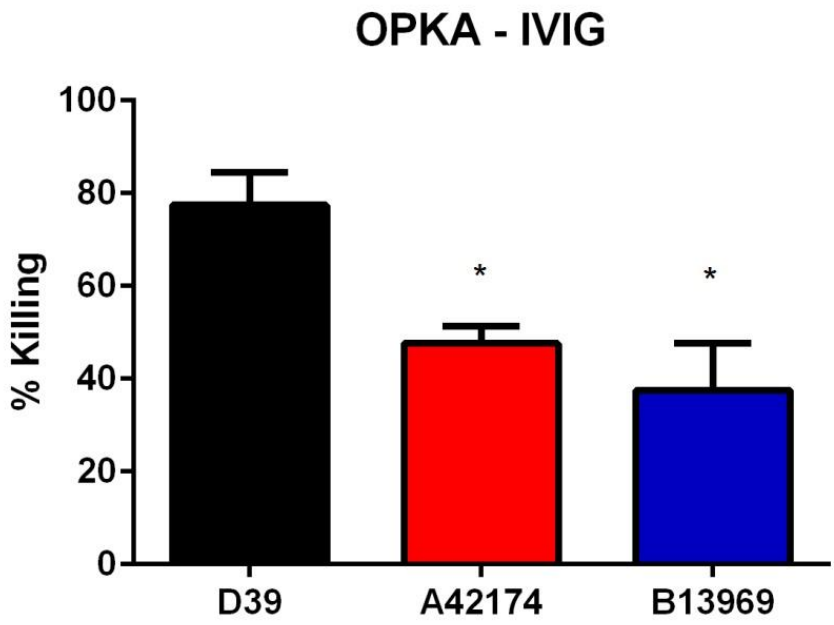

Figure 29. Percentage of IVIG-opsonised bacteria killed by differentiated human neutrophils (HL-60) shown as mean ± SEM. This experiment was done in triplicates on three independent days. *P-value<0.05 when analysed using a one-way ANOVA.

As observed in Figure 29, there were no significant differences in phagocytic killing of the different serotype 1 isolates, but both serotype 1 isolates were significantly less susceptible to phagocytic killing than the serotype 2 D39 strain with approximately 80% killing of D39 and 30-50% killing of the serotype 1 isolates (*P-value<0.05).

3.9. C3 deposition

The activation of the host complement system leads to the deposition of complement protein C3 on the surface of the pneumococcus resulting in recruitment of phagocytes and the phagocytosis of the pathogen [243]. As C3 deposition is closely associated with successful opsonisation and phagocytosis of bacteria, it is important to determine the levels of C3 deposition on different pneumococcal strains to potentially correlate with differences in phagocytosis between isolates. As observed in Figure 30, the three serotype 1 isolates had similar levels of C3 deposition and although all 3 strains had lower C3 deposition levels than the control serotype 2 (D39) strain, this difference was not statistically significant. The reduced levels of C3 deposition on the surface of serotype 1 might explain the reduced killing of the serotype 1 isolates observed in the opsonophagocytic killing assays (Figure 29). The results are shown as fluorescent intensity (FI), and were analysed using a one-way ANOVA.

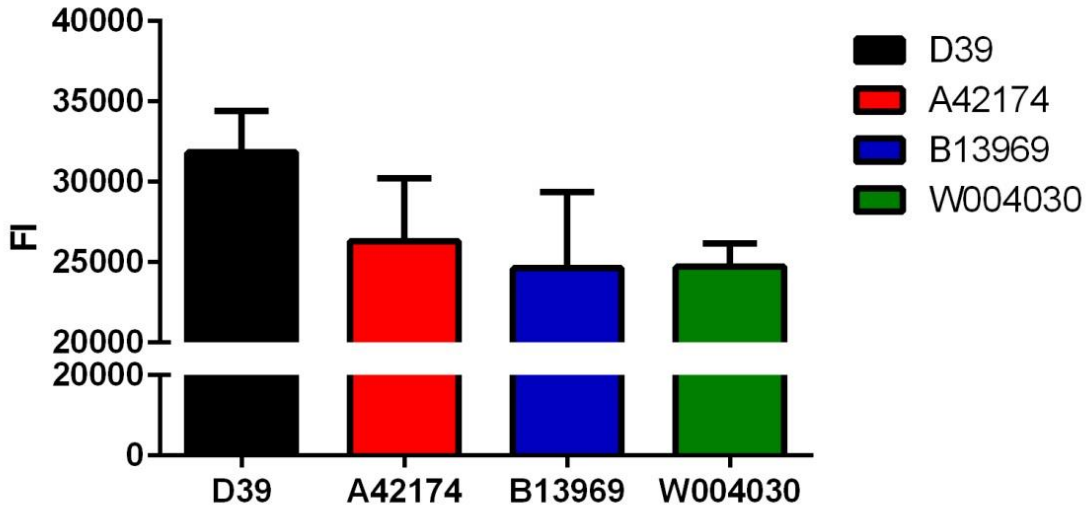

Figure 30. C3 deposition on the surface of serotype 1 isolates and the D39 control strain shown as the mean fluorescent intensity ± SEM. These experiments were done in triplicate on three independent days. No significant differences when analysed using a one-way ANOVA.

3.10. Conclusions

In vitro experiments were performed to characterise the pathogenic phenotype of serotype 1 isolates obtained from cases of bacteraemia (A42174), meningitis (B13969) or carriage (W004030) to assess possible differences in the pathogenesis related to the site of isolation. Experiments that tested bacterial fitness, capsule thickness, adherence and invasion of host respiratory tissue, resistance to phagocytic killing and complement deposition were performed on all 3 serotype isolates and on the serotype 2 strain D39, which was used as a phenotype comparator. The experiments clearly showed that serotype 1 isolates are phenotypically different from the serotype 2 strain D39.

The haemolytic activity of 20 different serotype 1 isolates from bacteraemia, meningitis and carriage was initially tested to try to find isolates with non-haemolytic pneumolysin; however, only one strain was found to produce a significantly less haemolytic pneumolysin. However, the level of haemolysis was not as low as the

ST306 and it had no effect on the pattern of carriage when compared to other serotype 1 isolates with a higher haemolytic activity.

Since no significant differences were found between the haemolytic activity of the different serotype 1 isolates, with the exception of the already mentioned C9471 isolate, one isolate from each infection type (bacteraemia, meningitis and carriage) was selected for further investigations.

Growth curves were performed to assess possible differences in the growth rates of the three serotype 1 isolates. It was observed that all the isolates had similar patterns of growth, although the meningitis isolate was slower during growth when compared to the serotype 2 strain D39 and the other serotype 1 isolates.

Adherence and invasion of the pneumococcus is a key ability needed to cause nasopharyngeal carriage and to progress into invasive disease. For that purpose, adhesion and invasion assays were performed using a human nasopharyngeal epithelial cell line and a human broncho-epithelial cell line to assess the ability of the serotype 1 isolates to adhere and invade those cells. All serotype 1 strains, as well as the control strain D39, were able to adhere to the nasopharyngeal epithelial cells, but not to the broncho-epithelial cells. Adherence of approximately 0.06% of D39 bacteria to broncho-epithelial cells has previously been reported; however, the total concentration of bacteria and the bacteria:cell ratio used in this study were different [313]. Whilst approximately $2x10^7$CFU/ml were used in the published study, only 10^6CFU/ml were used in this study. Moreover, the ratio bacteria:cell was 40:1 in the published study, a ratio of 20:1 was used for this study. These differences in the experimental procedures might explain the low adherence of the pneumococcus to BEAS-2B.

The main finding from the adhesion and invasion assays was that the meningitis isolate was the only isolate, excluding the non-typeable control MLAB7062, able to invade the nasopharyngeal epithelial cells. Moreover, the percentage of invading bacteria relative to the adhered number of bacteria was significantly higher in the meningitis isolate (14.3%) than in the non-typeable isolate (0.04%). These finding suggest that the meningitis isolate might be a better invader and therefore might be more able to translocate from the nasopharynx to the brain by invasion of the nasopharyngeal epithelial tissue.

The analysis of the capsule thickness of the serotype 1 isolates showed no differences in the capsular thickness of the bacteraemia and the meningitis isolates when compared to D39. Nonetheless, the carriage strain showed to have a thicker capsule than the other two serotype 1 isolates and the control strain D39. These observations contradict the hypothesis that a reduction in the thickness of capsule is an adaptation of the pneumococcus during nasopharyngeal carriage to improve adhesion to epithelial cells and to avoid the activation of immune responses. However, no significant differences were observed between the adhesion of the carriage strain to nasopharyngeal epithelial cells (Detroit-562) when compared to the other serotype 1 strains. These observations suggest that although absence of capsule does increase the adhesion ability of the pneumococcus, as observed with the non-typeable MLAB7062 strain, small differences in capsule thickness might not affect adhesion ability providing that a minimum capsule thickness is present.

The main clearance mechanism of the pneumococcus is through phagocytosis, therefore opsonophagocytic killing assays were performed on the serotype 1 isolates and the control strain D39 to assess the ability of serotype 1 to avoid phagocytosis by

host cells. No differences were observed between the percentage of killing of the bacteraemia and the meningitis serotype 1 isolates; however, a higher percentage of killing was observed in the control strain D39. Considering that phagocytosis occurs after opsonisation of the pneumococcus, the levels of C3 deposition of the serotype 1 and the control strain D39 were determined. It was observed that D39 had a higher complement deposition on its surface than the serotype 1 isolates, which could explain the higher killing of this isolate during the opsonophagocytic killing assays when compared to the serotype 1 isolates.

These *in vitro* experiments suggested that serotype 1 might be more adapted to avoid phagocytosis by reducing the C3 deposition on its surface. Moreover, the adhesion and invasion assays suggested that the meningitis isolate may be more invasive than the bacteraemia and the carriage isolates.

The next step was assessing whether the phenotypic differences observed between the serotype 1 isolates *in vitro* contributes to phenotypic differences during nasopharyngeal carriage and invasive disease by assessing their performance *in vivo*. In the next chapter, mouse models of infection will be used to determine the virulence of these isolates and to better understand the mechanisms of infection of African serotype 1 isolates.

In vivo characterisation of the pathogenicity of pneumococcal serotype 1 isolates during invasive pneumococcal disease

4: *In vivo* characterisation of the pathogenicity of pneumococcal serotype 1 isolates during invasive pneumococcal disease

4.1. Introduction

Animal models have been widely used to study infections caused by *Streptococcus pneumoniae*, but whilst certain serotypes have been extensively studied, relatively little is known about the pathogenicity of serotype 1 *in vivo*. The majority of published work is based on infections caused by the serotype 2, strain D39, which in an invasive pneumonia model is able to cause the death by 48h of 80-100% of mice infected intra-nasally, although this effect can be mouse strain dependent [254]. Serotype 1 is known to have a high attack rate, causing the majority of invasive disease outbreaks in Sub-Saharan Africa, while being rarely found in carriage [269, 270]. To understand the mechanisms of serotype 1 infection, its virulence was determined using a well-established *in vivo* pneumonia model [254]. The same model was also used to study the host immune responses to serotype 1 invasive infections.

4.2. Virulence testing

The virulence of each of the serotype 1 isolates was determined by infecting 10 mice with either isolates that caused bacteraemia, meningitis or were recovered from carriage (Figure 31). The mice were culled either when they became lethargic or five days post-infection, which was considered as having survived the infection. Of the mice infected with the 3 serotype 1 isolates, 90% to 100% of them started to show signs of disease 24h post-infection and all became lethargic between 24h and 48h post-infection. Mice infected with the meningitis isolate were all dead by 29h post infection, whereas 10% of the mice infected with the bacteraemia or the carriage isolates were able to survive. Furthermore, by the time the mice infected with the meningitis isolate

had died, only 10% and 30% of mice infected with the carriage and the bacteraemia isolates respectively had succumbed to the infection indicating that the development and progression of disease was significantly faster with the meningitis isolate. Despite this earlier difference though, 90% of mice infected with either the bacteraemia or the carriage isolate succumbed to infection within 48h post-infection. In summary, the meningitis isolate was significantly more virulent than the bacteraemia and the carriage isolates, but no significant differences were observed between the bacteraemia and carriage isolates (*P-value<0.05 and ***P-value<0.005). The data was analysed using the Log-Rank test analysis, which is used for the comparison of survival curves.

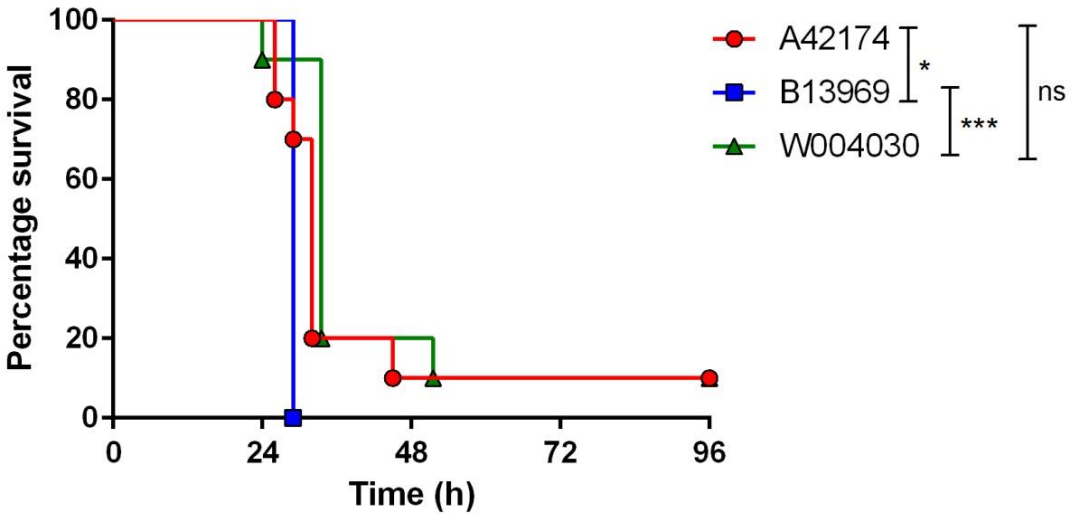

Figure 31. Percentage survival of MF1 mice infected with the bacteraemia (A42174), the meningitis (B13969) and the carriage (W004030) isolates (n=10). *P-value<0.05 and ***P-value<0.005 when analysed using the Log-Rank test.

The blood, lungs, nasopharynx and brain of each mouse were collected to determine the number of bacteria present in each tissue at the time of death.

As observed in Figure 32a, there were no significant differences in the numbers of bacteria found in the nasopharynx and the brain of mice infected with the different

serotype 1 isolates. However, the bacterial load in the lungs of mice infected with the meningitis isolate was significantly higher than in the mice infected with the bacteraemia or carriage isolates (***P-value<0.005 and ****P-value<0.001 respectively) when analysed using a two-way ANOVA. Figure 32b shows the number of bacteria in blood at 24h post-infection, and at the time of death. The values for the mice that survived are highlighted with a circle.

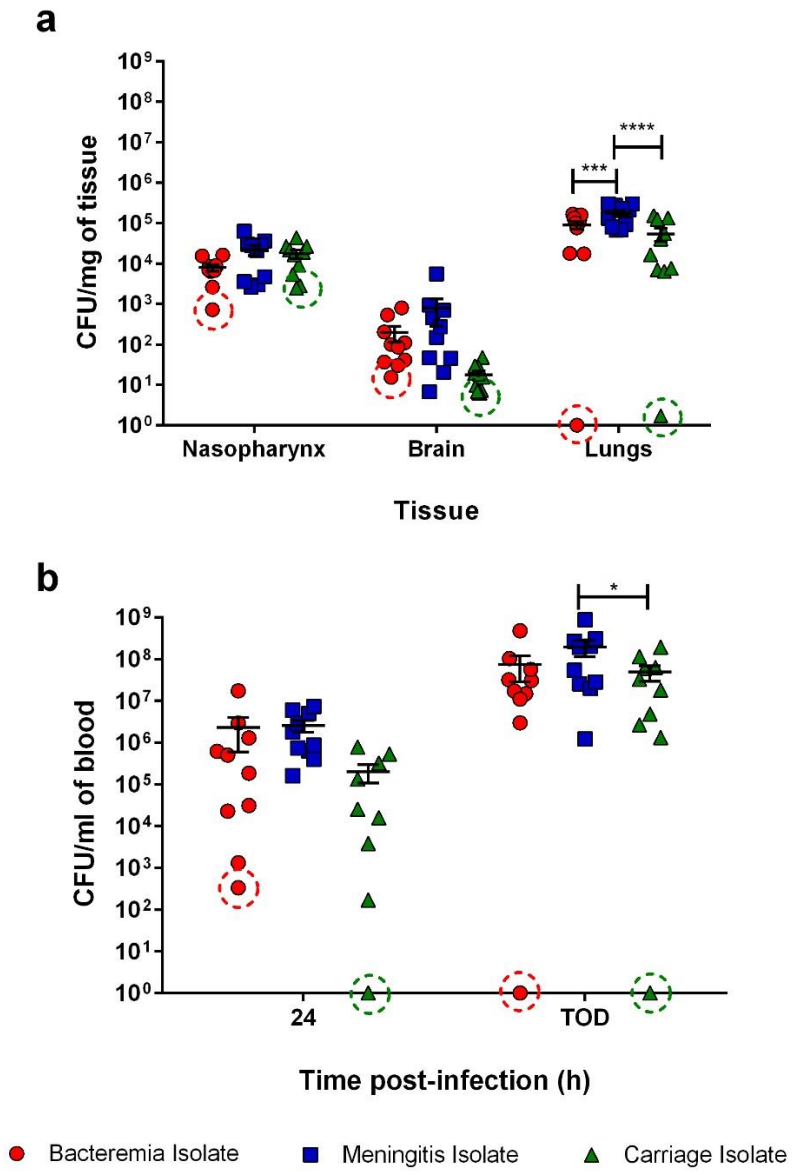

Figure 32. a) Bacterial CFU per mg of nasopharynx, lung and brain tissue of MF1 mice at time of death post pneumococcal infection shown as mean ± SEM (n=10). b) Bacterial CFU per ml of blood in MF1 mice at 24h post-infection and at time of death (TOD) shown as mean ± SEM (n=10). The surviving mice are highlighted with a circle. *P-value<0.05, ***P-value<0.005 and ****P-value<0.001 when analysed using a two-way ANOVA.

These results indicate that although pneumococci were able to translocate into blood in the mice infected with the bacteraemia isolate (A42174), the number of bacteria in blood 24h post-infection was much lower in the mice that survived than in the mice that eventually succumbed to the infection. Furthermore, the mouse was able to clear the infection from the lungs by 96h post-infection, which is when the experiment was terminated. The mouse infected with the carriage isolate (W004030) that survived the infection had no bacteria in the blood 24h post-infection, which suggests that this mouse was able to clear the infection from the lung before the bacteria were able to translocate into the blood.

Although the mice infected with the carriage isolate (W004030) had lower number of bacteria in the blood 24h post-infection this difference was not statistically significant when analysed using a two-way ANOVA. However, the mice infected with the meningitis isolate had significantly higher number of bacteria in blood at time of death than those infected with the carriage isolate (*P-value<0.05). No differences were observed between the bacteraemia and the meningitis isolate or between the bacteraemia and the carriage isolate.

4.3. Virulence comparison between isolates from different geographic locations

As previously mentioned, whole genome sequencing analysis has shown that serotype 1 isolates cluster into four distinct lineages, with each lineage predominantly associated with a single continent [270]. Further phylogeographic analysis determined that African serotype 1 isolates cluster together with the isolates from the same country of origin [270].

In order to determine possible virulence differences between isolates of the same sequence type but from a different geographic location the virulence of an invasive

ST217 isolate isolated from the lung aspirate of a pneumonia patient from The Gambia (PNI830) was determined and compared to the virulence of the ST217 bacteraemia isolate (A42174) from Malawi. No significant differences were observed in the survival of mice infected with either the Malawian or the Gambian ST217 isolates (Figure 33a), or in the bacterial numbers in the nasopharynx, lungs, brain or blood at time of death of mice infected with either the Malawian or the Gambian isolates (Figure 33b). The data was analysed using a Log-Rank test and a two-way ANOVA test respectively.

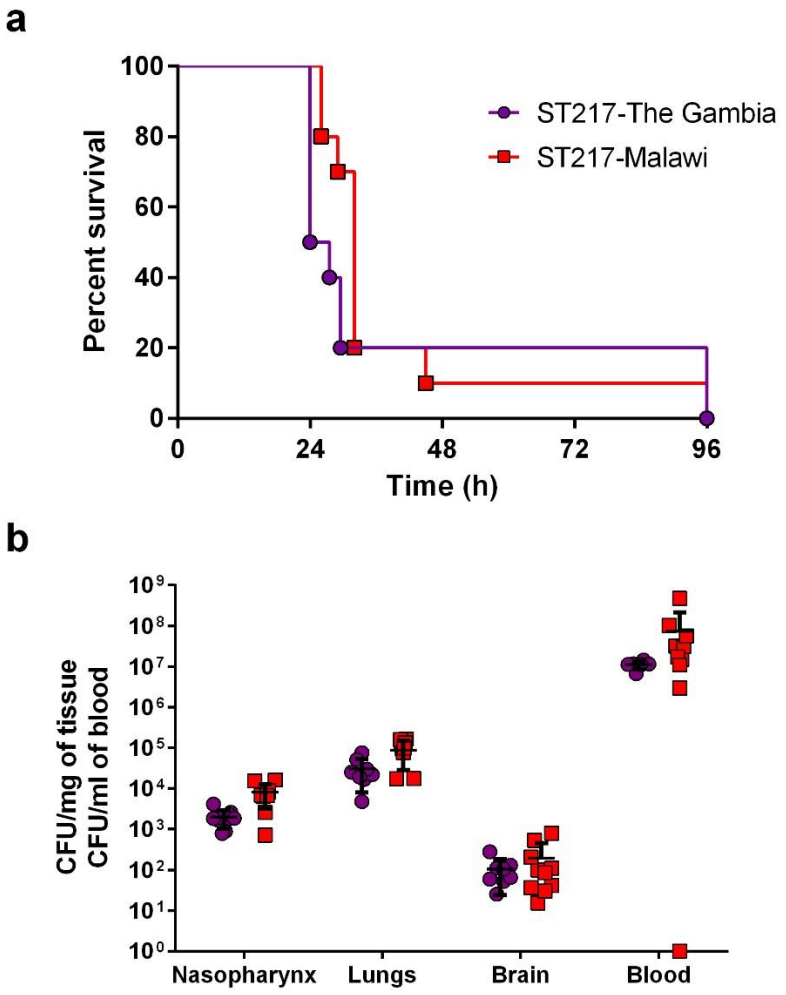

Figure 33. a) Percentage survival of MF1 mice infected with the ST217 bacteraemia strain (A42174) from Malawi (Figures 11 and 12), and the ST217 pneumonia strain (PNI830) from The Gambia (n=10). b) CFU per mg of tissue or ml of blood of the same MF1 mice infected with the ST217 bacteraemia strain (A42174) from Malawi, and the ST217 pneumonia strain (PNI830) from The Gambia (n=10) at time of death shown as mean ± SEM. No significant differences were observed when analysed using the Log-Rank test and a two-way ANOVA, respectively.

4.4. Progression (time-line) of infection during invasive disease by serotype 1

Based on the results from the *in vivo* virulence testing experiments where no major differences between the carriage and the bacteraemia isolates were observed (Section 4.2), only the meningitis and the bacteraemia isolates were selected for further investigation, in order to investigate further any possible differences between these two isolates.

To determine the timeline of infection, 45 mice were intra-nasally infected with 10^6CFU of the bacteraemia isolate and 45 mice were infected with 10^6CFU of the meningitis isolate. Of those, five mice from each group were culled 15 minutes after the infection, and ten mice were culled at each of the following time-points post-infection 3h, 6h, 12h, and 24h to assess the progression of the infection (Figure 34).

As shown in Figure 34, the infection patterns for both isolates were very similar and followed the same patterns as the serotype 2 strain D39 [254]. Moreover, no significant differences were observed between the two isolates at any time-point and in any tissue when analysed using a two-way ANOVA test. For both isolates, there was no major variation of bacterial numbers in the nasopharynx throughout the infection, although a slight increase in number was observed between 12h and 24h post-infection. The number of bacteria in the lungs increased during the first 6h of the infection until they started declining around 12h post-infection, only to go back up by 24h post-infection. The decline in bacterial numbers in the lung correlated with the seeding of bacteria into blood. Although the mean CFU values for the bacterial isolate in blood were higher than for the meningitis isolate at 12h post-infection, this was not statistically significant. Finally, no major differences were observed in the seeding of bacteria into the brain, which did not occur until 24h post-infection. Although not significant, the

number of bacteria in the brain of mice infected with the meningitis isolate was slightly higher than in those infected with the bacteraemia isolate.

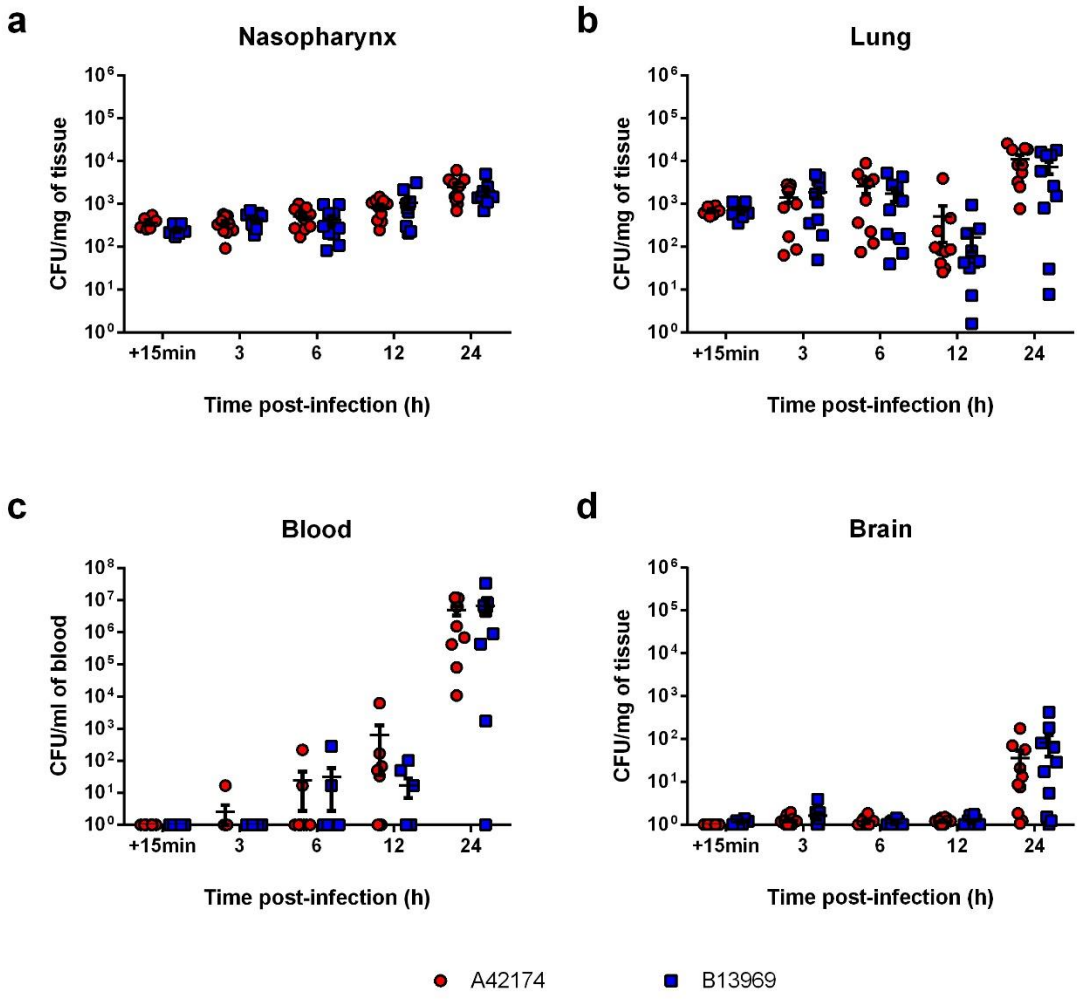

Figure 34. CFU per mg of nasopharynx (a), lung (b), brain (d) and per ml of blood (c) of MF1 mice infected with the bacteraemia isolate (A42174) at times +15min, 3h, 6h, 12h and 24h post-infection shown as mean ± SEM (n=10). Five mice were used for time +15mins and 10 mice were used for the rest of the time-points. No significant differences were observed when analysed using a two-way ANOVA.

In summary, no significant differences were observed in the progression of infection caused by the bacteraemia or the meningitis isolate. These findings suggest that ST217 serotype 1 isolates might follow similar patterns of infection leading to similar pathogenic outcomes over similar time-periods. For that reason, only one of the isolates was selected for further analysis to understand the mechanism of serotype 1 invasive disease. The isolate selected was the bacteraemia isolate (A42174).

4.5. Lung immune cellular profiling during invasive infection with serotype 1

The cellular profiling of the lung of 5 MF1 mice infected with the bacteraemia isolate (A42174) was determined at different stages of infection using flow cytometry. The number of leukocytes (a), B cells (b), T cells (c), T regulatory cells (d), neutrophils (e) and macrophages (f) present in the lung are shown in the following figure:

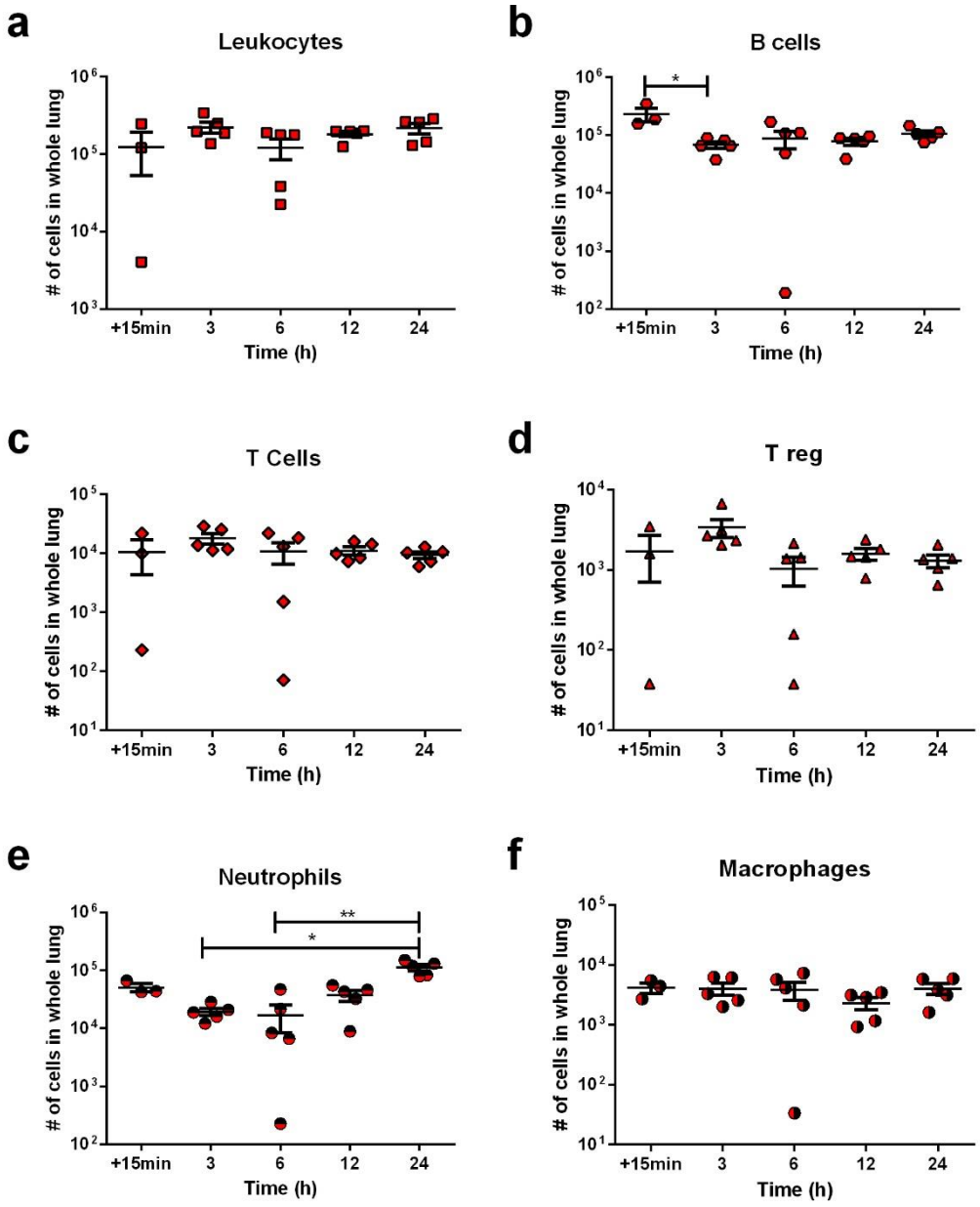

Figure 35. Cellular profiling in the lung of MF1 mice during an invasive infection caused by the bacteraemia isolate (A42174) at times +15min, 3h, 6h, 12h and 24h post-infection shown as mean ± SEM (n=5). *P-value<0.05 and **P-value<0.01 when analysed using a one-way ANOVA.

The CD45 marker was used to gate leukocytes, therefore excluding erythrocytes and platelets, from the rest of the lung cells. Approximately 10^5 cells from the whole lung were detected to be leukocytes and this number did not change significantly throughout the course of the infection (Figure 35a).

There were between 10^5 and 10^6 B cells ($CD19^+$) in the lung at time +15mins post-infection (Figure 35b). Three hours post-infection the number of B cells in the lung were reduced to just below 10^5 and remained at similar levels throughout the course of infection. A one-way ANOVA test showed a significant difference between time +15mins and 3h post-infection, but no other significant differences were observed (*P-value<0.05). The number of T cells ($CD3^+/CD4^+$) and T regulatory cells ($CD4^+/FoxP3^+$) didn't change throughout the duration of the experiment (Figure 35c and 35d). The number of T cells and T regulatory cells in the whole lung was approximately 10^4 and 10^3 cells respectively.

The number of macrophages ($F4/80^+$) were stable at a range of 10^3 to 10^4 during the first 24h of the infection with serotype 1 (Figure 35f). On the other hand, the number of neutrophils in the lung of infected mice varied throughout the duration of infection (Figure 35e). There were below 10^5 neutrophils in the lung 15 minutes post-infection, with numbers decreasing during the first 6 hours of infection. The lowest number of neutrophils was reached 6h post-infection, where the number of neutrophils varied between 10^2 and 10^5 depending on the mouse. In later stages of infection the number of neutrophils significantly increased to approximately 10^5 cells per lung. The number of neutrophils 3h and 6h post-infection were significantly lower when compared to 24h post-infection when compared using a one-way ANOVA test (*P-value<0.05 and **P-value<0.01).

4.6. Cytokine analysis of the lung during invasive infection by serotype 1

Cytokine levels in the lungs of MF1 mice infected with serotype 1 were determined at different stages of infection. This analysis was done on the same lungs used for cellular profiling (Figure 35). A selection of cytokines involved in host immune responses against bacterial infection was analysed: IL-6, TNF-α, MIP-2 (IL-8), IL-17 and INF-γ. The changes in cytokine levels during infection are shown in the following figure:

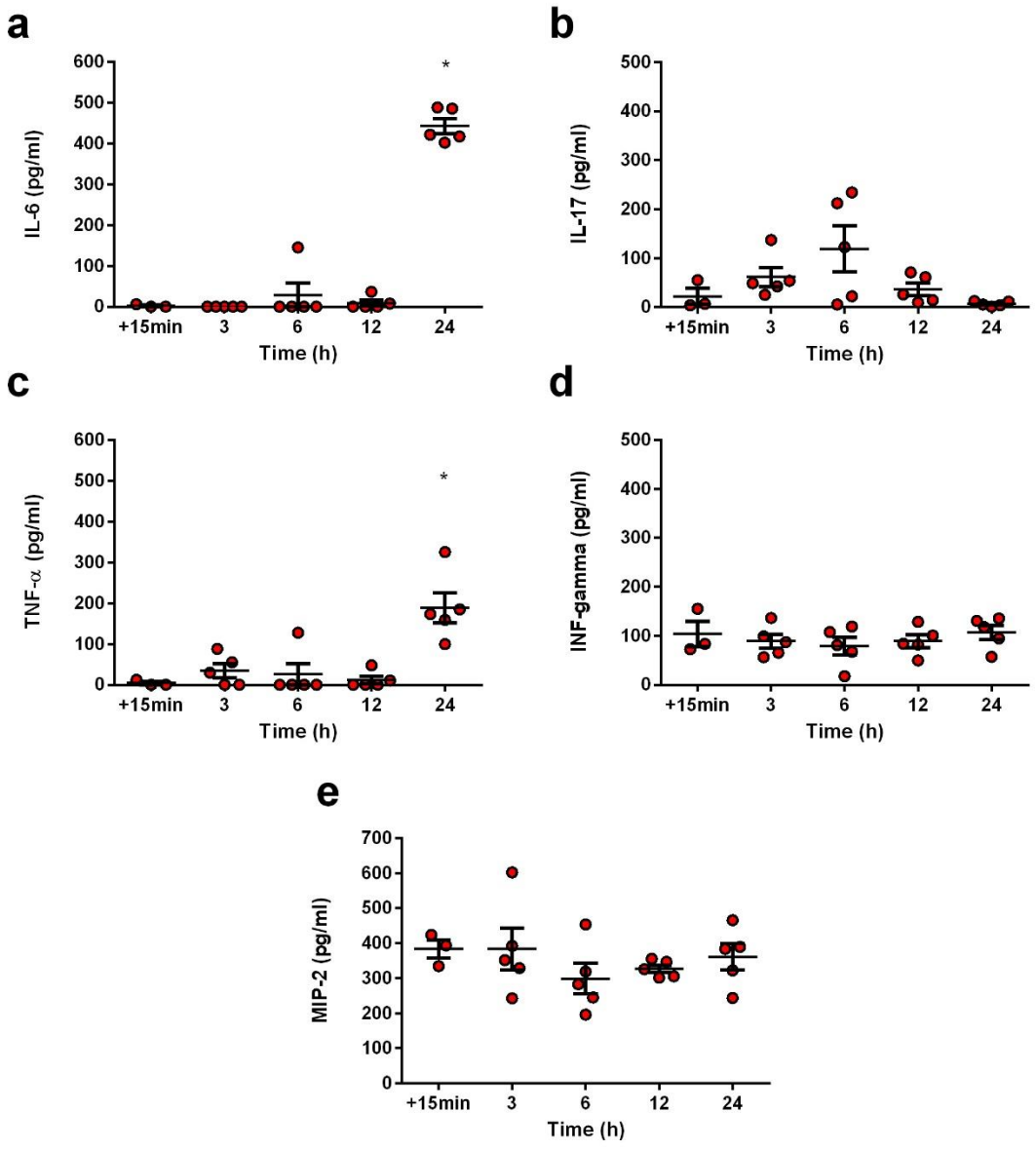

Figure 36. Cytokine analysis of the lung of MF1 mice during an invasive infection caused by the bacteraemia isolate (A42174) at times +15mins, 3h, 6h, 12h and 24h post-infection shown as mean ± SEM (n=5). *P-value<0.05 when analysed using a one-way ANOVA.

As observed in Figure 36a the levels of IL-6 were virtually 0pg/ml throughout most of the infection, except 24h post-infection, when there was a significant increase to 400-500pg/ml coinciding with neutrophil influx (*P-value<0.05). The data was analysed using a one-way ANOVA. IL-6 is secreted by macrophages and T lymphocytes, but the levels of those cell types were not increased, as observed in Figure 35. IL-6 is required for neutrophil influx, therefore the increase in IL-6 levels 24h post-infection relates to the increased number of neutrophils found in the lung 24h post-infection.

The levels of TNF-α followed a similar pattern than IL-6; there were low levels of the cytokine throughout the infection followed by a significant increase in TNF-α 24h post-infection (Figure 36c). TNF-α is a cytokine mainly produced by macrophages and is involved in cellular apoptosis and immune regulation and is an indication of inflammation. Therefore an increase in the levels of TNF-α 24h post-infection is a sign of the inflammation developed in the lung when the bacterial numbers are as high as 10^4cfu/mg of lung (Figure 34).

There were no significant changes in the levels of IL-17, INF-γ and MIP-2 throughout the course of infection (Figures 36b, 36d and 36e). The levels of IL-17 were approximately 0pg/ml at the start of the infection and increased gradually, although not statistically significantly, between 3h and 6h post-infection. IL-17 is responsible of recruiting neutrophils to the site of infection, therefore an increase of this cytokine 6h post-infection could be related to the neutrophil influx observed after 6h. The levels of INF-γ and MIP-2 remained constant at approximately 100pg/ml and 400pg/ml respectively.

4.7. Blood immune cellular profiling during invasive infection with serotype 1

The cellular profiling of the blood during an invasive infection in mice infected with the bacteraemia serotype 1 isolate (A42174) was determined at times +15mins, 3h, 6h, 12h and 24h post-infection. The mice used for the cellular profiling of the blood were the same mice used for the cellular profiling and cytokine analysis of the lung. The percentage of leukocytes (a), B cells (b), T cells (c), T regulatory cells (d), neutrophils (e) and macrophages (f) present in the blood of five mice per time point are shown in Figure 37.

The percentage of leukocytes in blood was, as expected, very high. At time +15mins, approximately 80% of the cells were leukocytes and this percentage increased during the course of infection (Figure 37a). In the last time-points, 12h and 24h, the percentage of leukocytes was between 90% and 100%.

The percentage of B cells was slightly decreased between time +15mins and 3h post-infection, going from 50-60% to 20-40%; however, this difference was not significant (Figure 37b). By 6h post infection the percentage of B cells had returned to the initial levels (50-60%) and remained consistent for the rest of the infection.

Approximately 20% of the leukocytes in the blood were T cells at the beginning of the infection (Figure 37c). An increase in the percentage of T cells was observed at the early stages of infection, which was followed by a steady decline reaching the low percentage of 5-10% by 12h post-infection. The decrease in T cells between 3h and 12h post-infection was statistically significant when analysed using a one-way ANOVA test (*P-value<0.05). The percentage of T cells remained at approximately 10% during the last hours of infection. Less than 10% of the T cells observed at time +15min were T regulatory cells (Figure 37d). The percentage of T cells that were

FoxP3^{+} (T regulatory cells) was significantly increased throughout the course of infection from less than 10% to a range of 10-60% at 12h post-infection (*P-value<0.05).

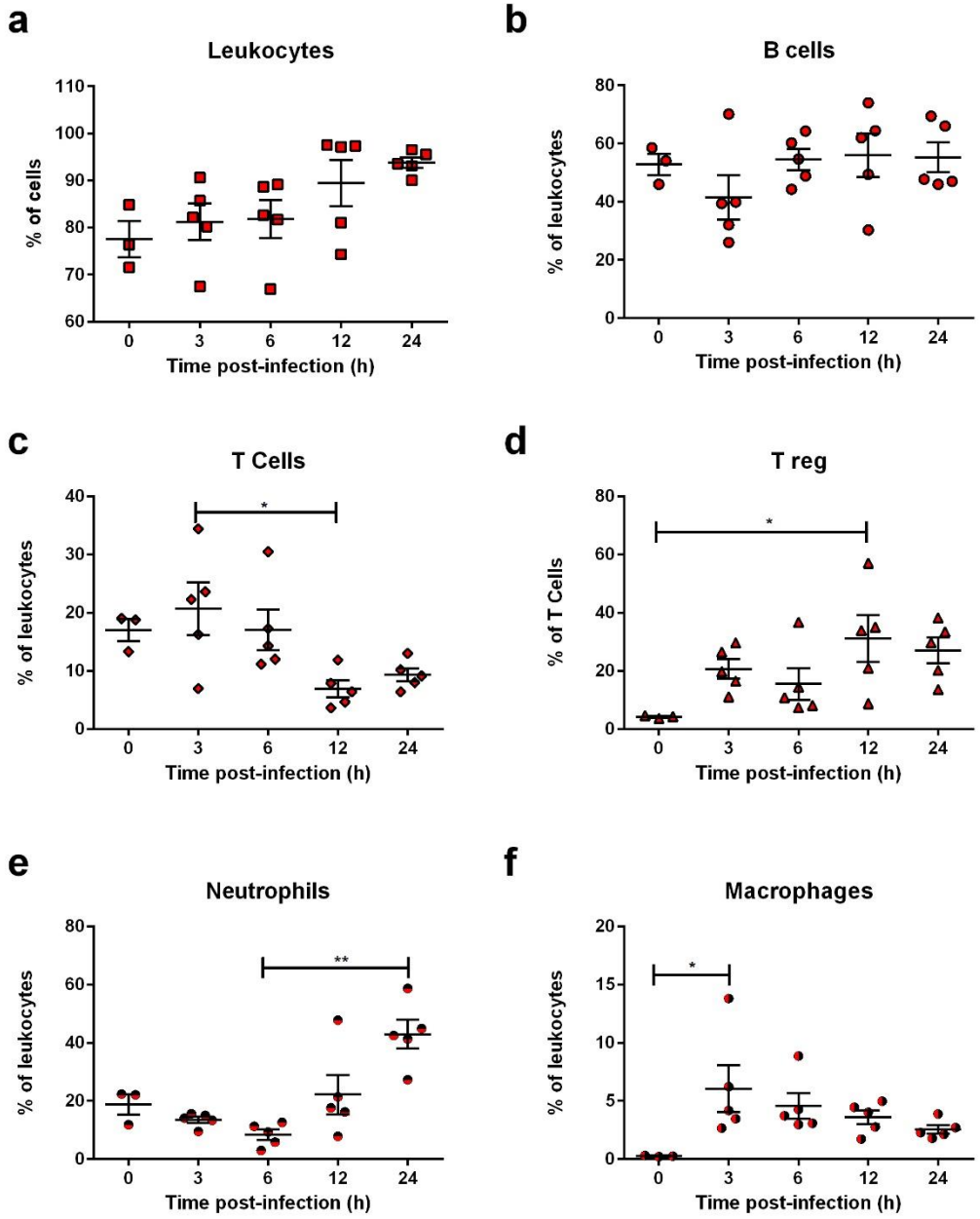

Figure 37. Cellular profiling in blood of MF1 mice during an invasive infection caused by the bacteraemia isolate (A42174) at times +15min, 3h, 6h, 12h and 24h post-infection shown as mean ± SEM (n=5). *P-value<0.05 and **P-value<0.01 when analysed using a one-way ANOVA.

The percentage of neutrophils at time +15min was approximately 20%, which gradually declined to 0-15% by 6h post-infection (Figure 37e). During the last hours of the infection the percentage of neutrophils significantly increased reaching its peak at 24h post-infection at 30-60% (**P-value<0.01).

The percentage of monocytes in blood was consistently low throughout the course of infection (Figure 37f). At time +15mins the percentage of monocytes was approximately 0%. This percentage was significantly increased 3h later to approximately 5%, with the exception of an outlier that reached approximately 15%. The percentage of macrophages remained between 0% and 5% for the rest of the infection.

4.8. Cytokine analysis of serum during invasive infection by serotype 1

The level of selected cytokines in serum of the same MF1 mice used for the cellular profiling of the lung and blood, and the cytokine analysis of the lung was determined during the course of infection at times +15min, 3h, 6h, 12h and 24h post-infection. The cytokines selected were the same set that were studied in the lung: IL-6, IL-17, TNF-α, INF-γ and MIP-2 (Figure 38).

The sera of mice from each time-point were pooled due to the small volume of serum available from each mouse and the large quantity of sample required for each ELISA test. For that reason, a single data set was obtained for each time-point resulting in the inability to perform a statistical test.

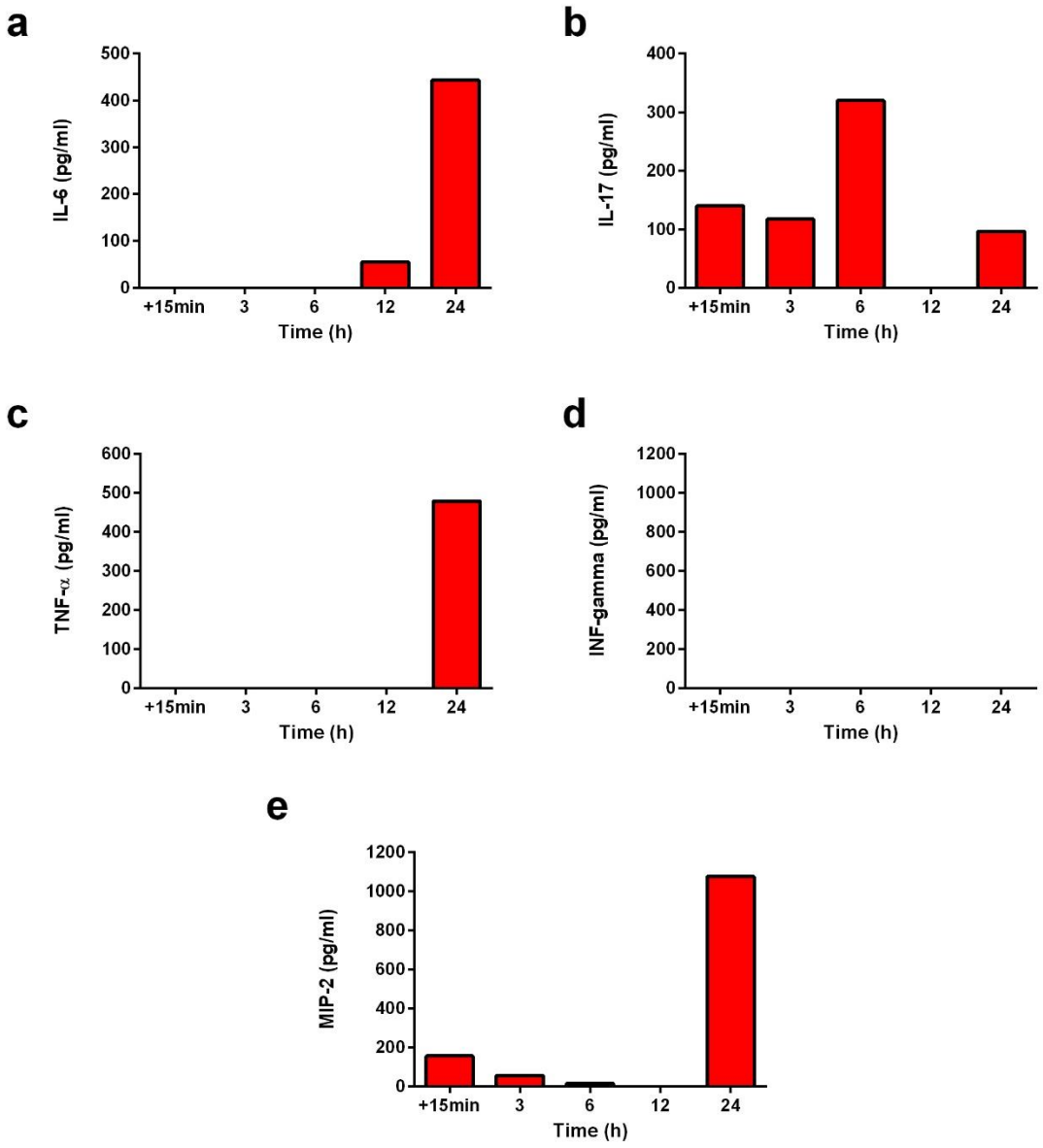

Figure 38. Cytokine analysis of the serum of MF1 mice during an invasive infection caused by the bacteraemia isolate (A42174) at times +15min, 3h, 6h, 12h and 24h shown as mean ± SEM (n=5). No statistical test available for this experiment.

The levels of IL-6 and TNF-α were close to 0pg/ml throughout most of the course of infection, until time-point 24h where the levels of both cytokines increased to approximately 500pg/ml (Figures 38a and 38c), similarly to what occurs in the lung. Contrary to the observation in the lung, the levels of MIP-2 in serum followed the same pattern as IL-6 and TNF-α: the levels of MIP-2 were low throughout most of the course

of infection until 24h post-infection when the levels reach 1000-1200pg/ml indicating high levels of inflammation probably due to the increased number of bacteria in blood which induce a release of MIP-2 from blood endothelial vessels (Figure 38e and Figure 34).

IL-17 followed a similar pattern in serum as it did in the lungs. There were approximately 100-150pg/ml of cytokine in serum at the start of the infection and an increase was observed at 6h post-infection, when there was approximately 300pg/ml. During the last stages of infection, the levels of IL-17 decreased and reached the initial levels of 100-150pg/ml. Therefore, IL-17 probably acted as an early inductor of neutrophils, being itself induced by the presence of bacteria in blood 6h post-infection (Figure 34). The levels of IL-17 12h post-infection were zero, which was probably due to an error in during the detection process, considering that there were a minimum of 100pg/ml at any other given time-point.

No INF-γ was detected in serum throughout the course of infection.

4.9. Brain immune cellular profiling during invasive infection with serotype 1

The same panel of antibodies was used to study the cellular profiling of the brain during an invasive infection in MF1 mice with the bacteraemia serotype 1 isolate (A42174). This analysis was done on the brain of the same mice used for the cellular profiling and cytokine analysis of the lung and the blood.

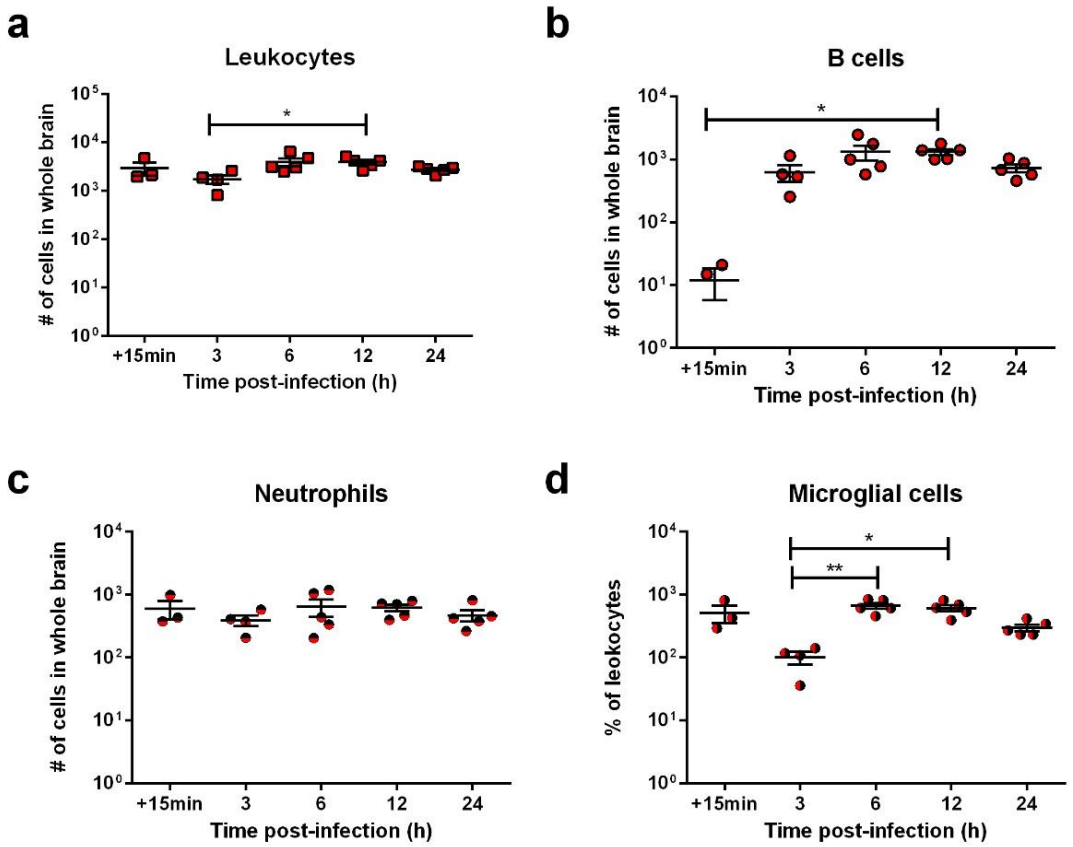

Figure 39. Cellular profiling in the brain of MF1 mice during an invasive infection caused by the bacteraemia isolate (A42174) at times +15mins, 3h, 6h, 12h and 24h post-infection shown as mean ± SEM (n=5). *P-value<0.05 and **P-value<0.01 when analysed using a one-way ANOVA.

As observed in Figure 39, the number of leukocytes in the brain was very low, never exceeding 10^4cells/tissue throughout the duration of the infection; however, there was an increase in leukocytes between time 3h and 12h post-infection (Figure 39a). Due to the low number of leukocytes it was not possible to detect any T cells or T regulatory cells. Approximately 10^1 of the leukocytes were B cells at the beginning of the infection, a number that went up to 10^3cells 3h post-infection and remained at that level for the rest of the infection (Figure 39b). Due to the variability between mice, the increase of B cells when compared to time +15min was only considered significant at 12h post-infection when analysed using a one-way ANOVA test (*P-value<0.05).

Approximately 10^3 of the leukocytes at time +15min were neutrophils (Figure 39c). The number of neutrophils in the brain of infected mice remained constant throughout the course of infection.

However, the number of leukocytes observed to be $F4/80^+$ (microglial cells), was approximately 10^3cells/tissue at the beginning of the infection, a number which decreased between +15min and 3h post-infection only to increase to the original levels by 6h post-infection (Figure 39d). The percentage of microglial cells in the brain of mice infected with the bacteraemia serotype 1 isolate was significantly higher 6h and 12h post-infection than at time 0h when analysed using a one-way ANOVA test (*P-value<0.05 and **P-value<0.01).

4.10. Cytokine analysis of the brain during invasive infection by serotype 1

The levels of IL-6, IL-17, TNF-α, INF-γ and MIP-2 were determined in the brains of MF1 mice infected with the bacteraemia serotype 1 isolate (A42174) (Figure 40). The analysis was done on the same mice used for the cellular profiling of the lung, blood and brain, and cytokine analysis of the lung and serum.

No IL-6 and TNF-α were detected at the start of the infection (Figure 40a and 40b). The levels of these cytokines in the brain did not change throughout the course of the infection, remaining at 0pg/ml.

The levels of MIP-2 at the start of the infection were 200-400pg/ml (Figure 40e). Even though there was a reduction in this cytokine between time 0h and 3h post-infection, this was not significant when analysed using a one-way ANOVA. The levels of MIP-2 remained constant for the rest of the infection at a concentration of 100-300pg/ml.

Similarly to MIP-2, the levels of INF-γ remain constant throughout the course of infection, with the numbers ranging between 0 and 100pg/ml (Figure 40d). On the other hand, the levels of IL-17 were between 0pg/ml and 50pg/ml at the start of the infection, which were increased 3h post-infection to 25-200pg/ml (Figure 40b). After the peak in IL-17 levels at 3h post-infection, there was a gradual reduction of this cytokine in the brain (*P-value<0.05). The data was analysed using a one-way ANOVA test.

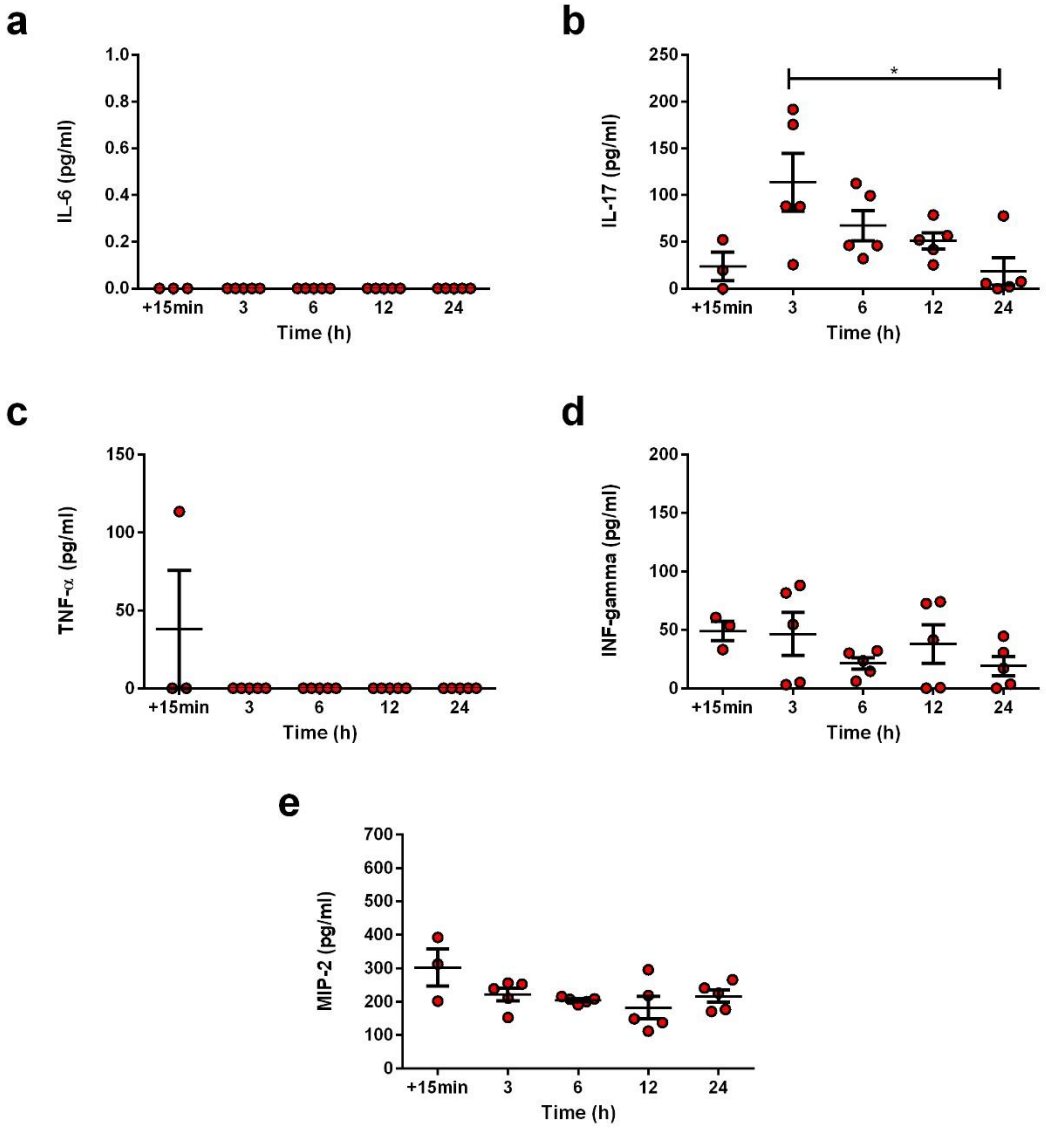

Figure 40. Cytokine analysis of the brains of MF1 mice during an invasive infection caused by the bacteraemia isolate (A42174) at times +15min, 3h, 6h, 12h and 24h shown as mean ± SEM (n=5). *P-value<0.05 when analysed using a one-way ANOVA.

4.11. Conclusions

An *in vivo* model of invasive pneumococcal pneumonia was used to study the pathogenicity and host interactions of serotype 1. Initially, isolates from different sources (carriage, meningitis and bacteraemia) were used to determine potential differences in virulence between isolates. It was observed that 90-100% mice infected with serotype 1, independently of the isolate used, developed septicaemia leading to the death of the mouse. Although most mice succumbed to the infection in the first 48h, the survival time of the mice infected with the meningitis isolate was significantly reduced when compared to mice infected with the bacteraemia or the carriage isolates. Bacterial numbers in different tissues were determined at time of death, showing no significant differences in numbers in the nasopharynx or the brain. However, the number of bacteria in the lungs was significantly higher in mice infected with the meningitis isolate when compared to the other two isolates. No differences were observed in the bacterial load in blood 24h post-infection; on the contrary, more bacteria were detected in blood at time of death of mice infected with the meningitis isolate when compared to the carriage isolate. No differences were observed between the bacteraemia and the meningitis isolate, or between the bacteraemia and the carriage isolate.

No significant differences were observed in the virulence of the bacteraemia and the carriage isolates, and for that reason the bacteraemia and the meningitis isolates were used to further study the progression of serotype 1 infections.

When bacterial numbers were determined in different tissues at specific pre-determined time-points (+15min, 3h, 6h, 12h and 24h) no differences were observed between the bacteraemia and the meningitis isolate suggesting that the progression of infection for the two isolates was similar. Therefore, further experiments were

performed to understand the pathogenicity of serotype 1 isolates focusing on just the bacteraemia isolate. Those experiments included flow cytometry to determine the cellular profiling of different tissues during invasive disease with serotype 1, and cytokine analysis to understand the host immune responses to serotype 1 infections.

The cellular profiling of the lung showed no significant changes during the timecourse of infection with the exception of a small reduction in B cells in the first three hours post infection and a variation in the number of neutrophils. There was a small reduction in the number of neutrophils in the lung during the first hours of infection followed by an increase during the last stages of infection. This suggests that during the initial stages the host is using the neutrophils in the lung to try to control the infection by phagocytosis. In the final stages of infection, the number of bacteria in the lungs increase. An increase in neutrophils is usually required to try to control the infection; however, the increase of neutrophils was not enough to control the infection in this case. The lack of early activation of immune cells suggests that serotype 1 has a mechanism of immune evasion to avoid clearance. Moreover, there was no activation of T regulatory cells, which is observed in other serotypes, which suggests a lack a protective immunity leading to uncontrolled tissue damage [386]. The increase in neutrophils at the final stages of infection was likely to be due to an early increase in IL-17 observed 6h post-infection and an increase in IL-6 observed 24h post-infection. Moreover, there was an increase in TNF-α at 24h post-infection which was probably used as a mechanism to induce neutrophil influx and to immune-modulate to reduce inflammation. Increased IL-6 led to an increase in activated macrophages, but not in the total number of macrophages. The levels of INF-γ and MIP-2 in the lungs remained constant throughout the course of infection.

In blood, there was a small increase in leukocytes during the infection. A reduction of T cells was observed, but the percentage of T cells found to be T regulatory cells increased throughout the infection. An increase was observed in macrophages and neutrophils in the early stages of infection and at the late stages of infection respectively. An increase in IL-6, TNF-α and MIP-2 in blood was probably due to the increased numbers of T cells, macrophages and neutrophils which coincided with the appearance of the first bacteria in blood. There was also an increase in IL-17 by 6h post-infection in blood, which coincided with the translocation of the first bacteria into blood, which is probably responsible for the increase number of neutrophils observed after 6h post-infection. No differences were observed in the levels of INF-γ in blood.

The translocation of bacteria from the blood to the brain occurs between 12h and 24h post-infection; no pneumococci were found in the brain 12h post-infection, but by 24h post-infection there were between 0 to 10^2 bacteria per mg of tissue. As observed in the cellular profiling study and the cytokine analysis of the brain, no immune responses are induced during pneumonia or even during sepsis in the brain. The brain is considered to have limited pathogen-specific immune responses due to the lack of a lymphatic system which would allow the migration of immune cells [314]. However, there was a decrease of microglial cells during the first three hours of infection only to increase to the original levels by 6h post-infection. An increase in the levels of IL-17 observed 3h post-infection might explain the increase of microglial cells observed between 3h and 6h post-infection. No changes were observed in the levels of IL-6, TNF-α, INF-γ and MIP-2. This observation suggests that the immune responses in the brain are limited, with microglial cells being the main innate cell type. The number of bacteria in the brain remained close to zero until 24h post-infection, at which point the

brain is not able to generate a strong immune response due to the large damaged caused in other organs during sepsis.

In summary, the invasive pneumonia model of infection using serotype 1 isolates clearly demonstrated that there were no major differences in virulence between niche specific serotype 1 isolates. The cellular profiling and cytokine analysis during infections with serotype 1 shows that due to the fast progression of infection the adaptive immune system is not involved in controlling the infection; therefore, the innate immune system is the only defence against pneumococcal invasive disease in naive mice. However, the immune responses to serotype 1 infection seem to be delayed when compared to other serotypes, which reflects in the increased virulence of serotype 1 [386].

It is well documented that serotype 1 is rarely detected during carriage, yet it is highly invasive. Therefore the next step was to study the ability of serotype 1 to be carried in the nasopharynx of the mice. The nasopharyngeal carriage model was used to study the patterns of colonisation by serotype 1.

In vivo characterisation of pneumococcal serotype 1 isolates during nasopharyngeal carriage

5. *In vivo* characterisation of pneumococcal serotype 1 isolates during nasopharyngeal carriage

5.1. Introduction

The pneumococcus is a common commensal of the nasopharynx of healthy individuals although some serotypes, like serotype 1, are rarely found during nasopharyngeal carriage. Carriage is considered a pre-requisite for invasive disease, therefore low carriage rates would suggest low invasiveness. Contradictorily, serotype 1 is one of the main causes of pneumococcal invasive disease, accounting for approximately 20% of the cases in sub-Saharan Africa, despite very low carriage rates [269, 270]. These observations have led investigators to question whether serotype 1 is able to establish stable nasopharyngeal colonisation or whether serotype 1 can carry for the same duration and at the same density as other serotypes. The nasopharyngeal carriage mouse model was used in this study to study the carriage pattern of serotype 1 isolates in mice.

5.2. Pneumococcal serotype 1 during nasopharyngeal carriage in mice

The bacteraemia (A42174), the meningitis (B13969) and the carriage (W004030) serotype 1 strains were used in the carriage model of infection in which mice were infected with 10^5 bacteria of either strain. The infection was performed intra-nasally in a total volume of 10µl, allowing the bacteria to stay only in the nasopharynx without seeding into the lungs. Three mice per strain were culled straight after the infection (day 0) and five more were culled at each time-point on days 1, 3, 7 and 14 post-infection. The bacterial numbers per mg of nasopharynx were determined and are shown in Figure 41.

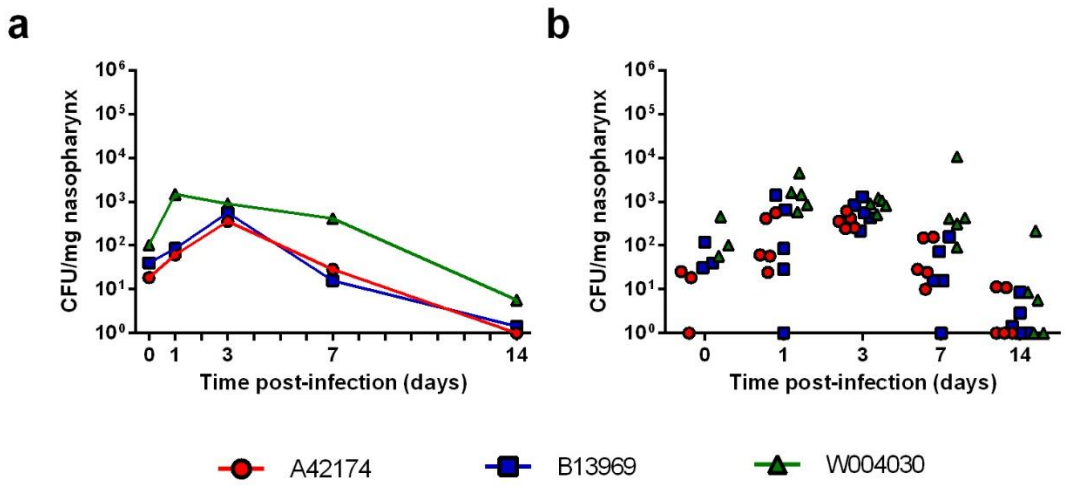

Figure 41. Nasopharyngeal carriage with serotype 1 isolates. Pneumococcal CFU per mg of nasopharyngeal tissue of MF1 mice infected with the bacteraemia (A42174), meningitis (B13969) and carriage (W004030) isolates at days 0, 1, 3, 7 and 14 post-infection shown as median value (a) and CFU of individual mice (b). Three mice were used for the day 0 control, and five mice were used for the rest of the time-points. No significant differences were observed when analysed using a two-way ANOVA.

As observed in Figure 41, the number of bacteria in the nasopharynx of mice on day 0 ranged between 10^1 and 10^2CFU/mg of tissue. One day post-infection the number of bacteria increased approximately half a log for the bacteraemia and the meningitis isolate and approximately one log for the carriage isolate. On day 3 post-infection the nasopharyngeal bacterial numbers reached their peak at approximately 10^3CFU/mg of tissue and started declining after that point to be almost cleared by day 14 post-infection. Although the carriage strain was found at a slightly higher density during days 1, 7 and 14, the differences were not significant when analysed using a two-way ANOVA, probably due to the large variation between mice infected with the same strain. Since there were no significant differences in the patterns of colonisation between the different strains only the bacteraemia and the meningitis isolates were used for further investigation.

Although the number of bacteria in the nasopharynx of mice infected with either the bacteraemia or the meningitis isolates was close to zero at 14 days post-infection, it was necessary to extend the experiment beyond 14 days to see if there would be any recovery of pneumococcal numbers post day 14. For that reason the same experiment was repeated and bacterial numbers were assessed at 0, 1, 3, 7, 14 and 21 days post-infection. The bacterial numbers for each individual mouse and the median for each time point and for each strain is shown in Figure 42. The same pattern of nasopharyngeal colonisation was observed in this experiment. It was found that the bacteraemia isolate was cleared from the nasopharynx of 100% of the mice by day 21, whilst 40% of the mice infected with the meningitis isolate were still carrying the bacteria, although at low density; however, no significant differences were observed between isolates when analysed using a two-way ANOVA test.

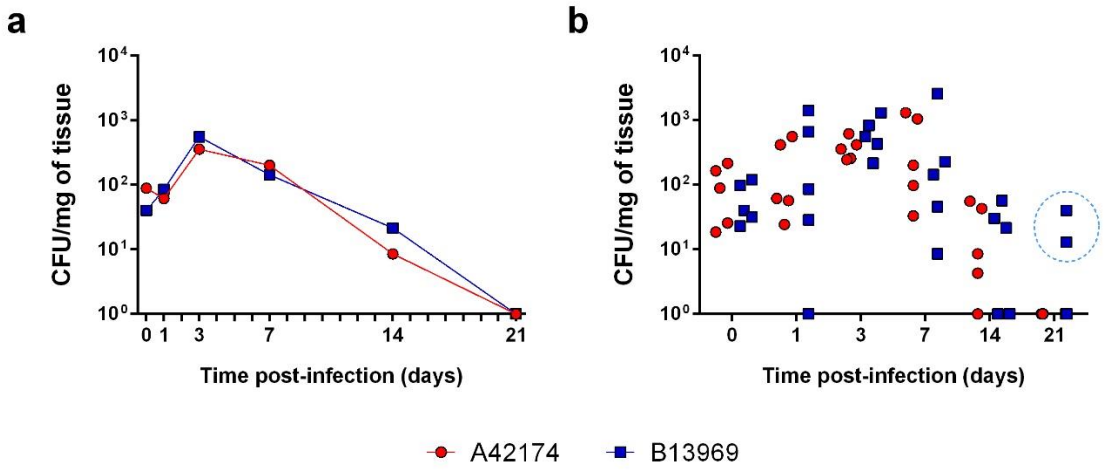

Figure 42. Pneumococcal CFU per mg of nasopharyngeal tissue of MF1 mice infected with the bacteraemia (A42174) and meningitis (B13969) isolates at days 0, 1, 3, 7, 14 and 21 post-infection shown as median (a) and the CFU of individual mice (b). Three mice were used for the day 0 control, and five mice were used for the rest of the time-points. No significant differences were observed when analysed using a two-way ANOVA.

In order to assess whether the presence of bacteria in the nasopharynx of mice infected with the meningitis isolate on day 21 post-infection was due to an improved adapted ability of those bacteria to colonise, the B13969 bacteria recovered after 21 days of carriage (highlighted in Figure 42) were re-tested in the nasopharyngeal carriage model for a further 14 days and compared to the original B13969 strain.

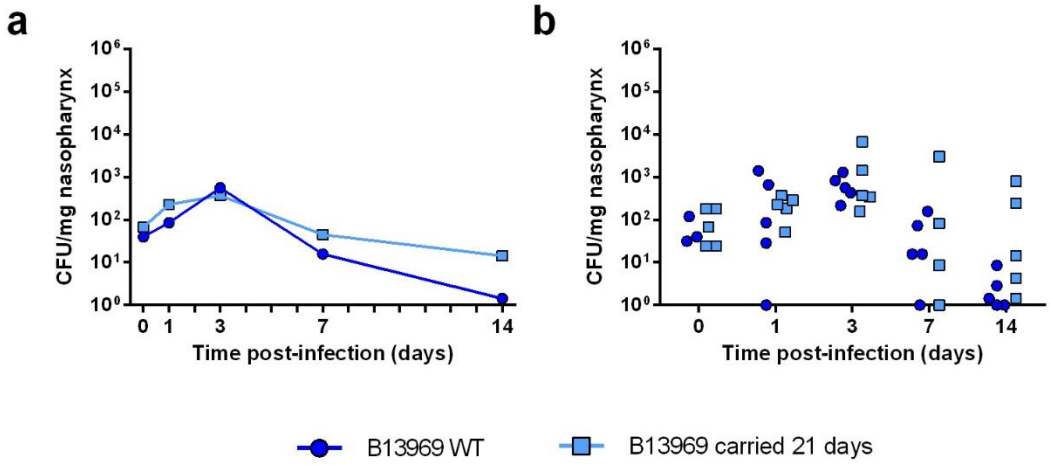

Figure 43. Pneumococcal CFU per mg of nasopharyngeal tissue of MF1 mice infected with the meningitis isolate (B13969) and the same meningitis isolate after carrying for 21 days in the nasopharynx of a mouse (B13969 carried for 21 days) at days 0, 1, 3, 7 and 14 post-infection shown as median (a) and the CFU of individual mice (b). Three to five mice were used for the day 0 control, and five mice were used for the rest of the time-points. No significant differences were observed when analysed using a two-way ANOVA.

As observed in Figure 43, the patterns of infection of both strains were very similar with the exception of day 14 where the bacterial numbers of the B13969 isolates that had been carried for 21 days were almost a log higher than the original B13969 isolate. However, the differences were not significant when analysed using a two-way ANOVA, due to the high variability observed between mice.

5.3. Cellular profiling and cytokine analysis during nasopharyngeal carriage

There are two tissues that have been shown to be important in the regulation of pneumococcal nasopharyngeal carriage in mice: the nasal associated lymphoid tissue (NALT) and the cervical lymph nodes (CLN) [236]. The cellular profiling of these two tissues was studied by flow cytometry in order to study the immune response to pneumococcal nasopharyngeal carriage by serotype 1 in mice. Given that no differences were observed in the patterns of nasopharyngeal carriage of the three serotype 1 isolates studied, only the bacteraemia isolate was used for the study of host immune responses to nasopharyngeal carriage by serotype 1. The cytokine levels in nasopharyngeal homogenates were also studied to better understand the immune response to nasopharyngeal carriage. The bacterial numbers of the carriage experiment used for the cellular profiling and cytokine analysis during nasopharyngeal carriage with the bacteraemia isolate (A42174) are shown in Figure 44.

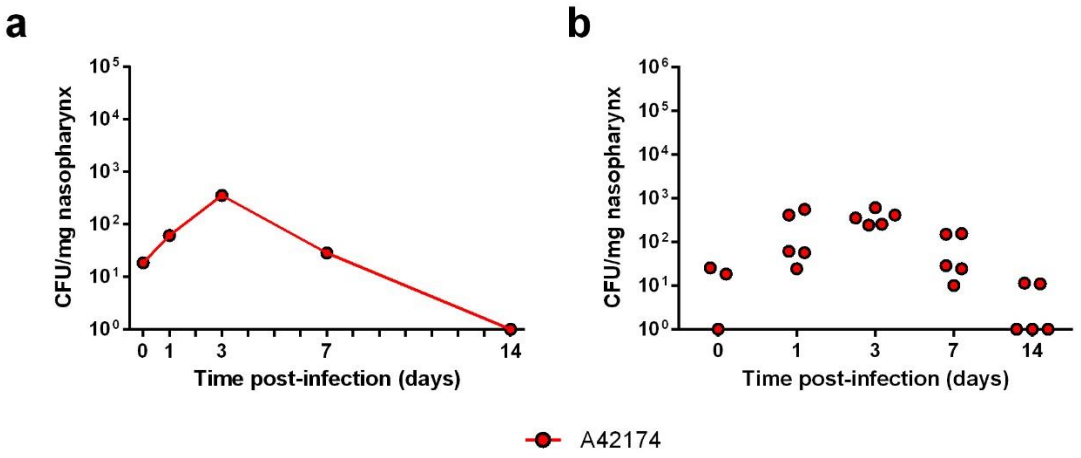

Figure 44. Pneumococcal CFU per mg of nasopharyngeal tissue of MF1 mice infected with the bacteraemia (A42174) isolate at days 0, 1, 3, 7 and 14 post-infection shown as median (a) and the CFU of individual mice (b). Three mice were used for the day 0 control, and five mice were used for the rest of the time-points. No significant differences were observed when analysed using a two-way ANOVA.

5.3.1. Nasal associated lymphoid tissue (NALT)

The nasal associated lymphoid tissue is located in the nasopharynx and has been shown to be important in the regulation of nasopharyngeal carriage in mice [236].

As observed in Figure 45a, the number of leukocytes in the NALT was approximately 10^3 at the time of infection (day 0) and there was 0.5 log increase on days 1 and 3 post-infection, although this increase remained outside statistical difference. The most abundant haematopoietic cell type in this tissue was found to be B cells which remained consistent throughout the duration of nasopharyngeal carriage at a level of approximately 10^3 (Figure 45b).

T cells were found to also remain constant throughout the carriage period at a level of approximately $5x10^2$ per whole tissue, with approximately 50% of those cells belonging to the T regulatory cell subtype (Figures 45c and 45d). No significant differences were observed in the number of T cells and T regulatory cells throughout the duration of carriage.

The numbers of phagocytic cells studied in this experiment (neutrophils and macrophages) were very similar, both of them ranging between 10^2 and 10^3 throughout the carriage period (Figures 45e and 45f). In both cases there was a small (but non-significant) increase in the number of cells during days 1 and 3 post-infection. .

A one-way ANOVA test was used to determine possible significant differences in the number of each cell type throughout the duration of carriage.

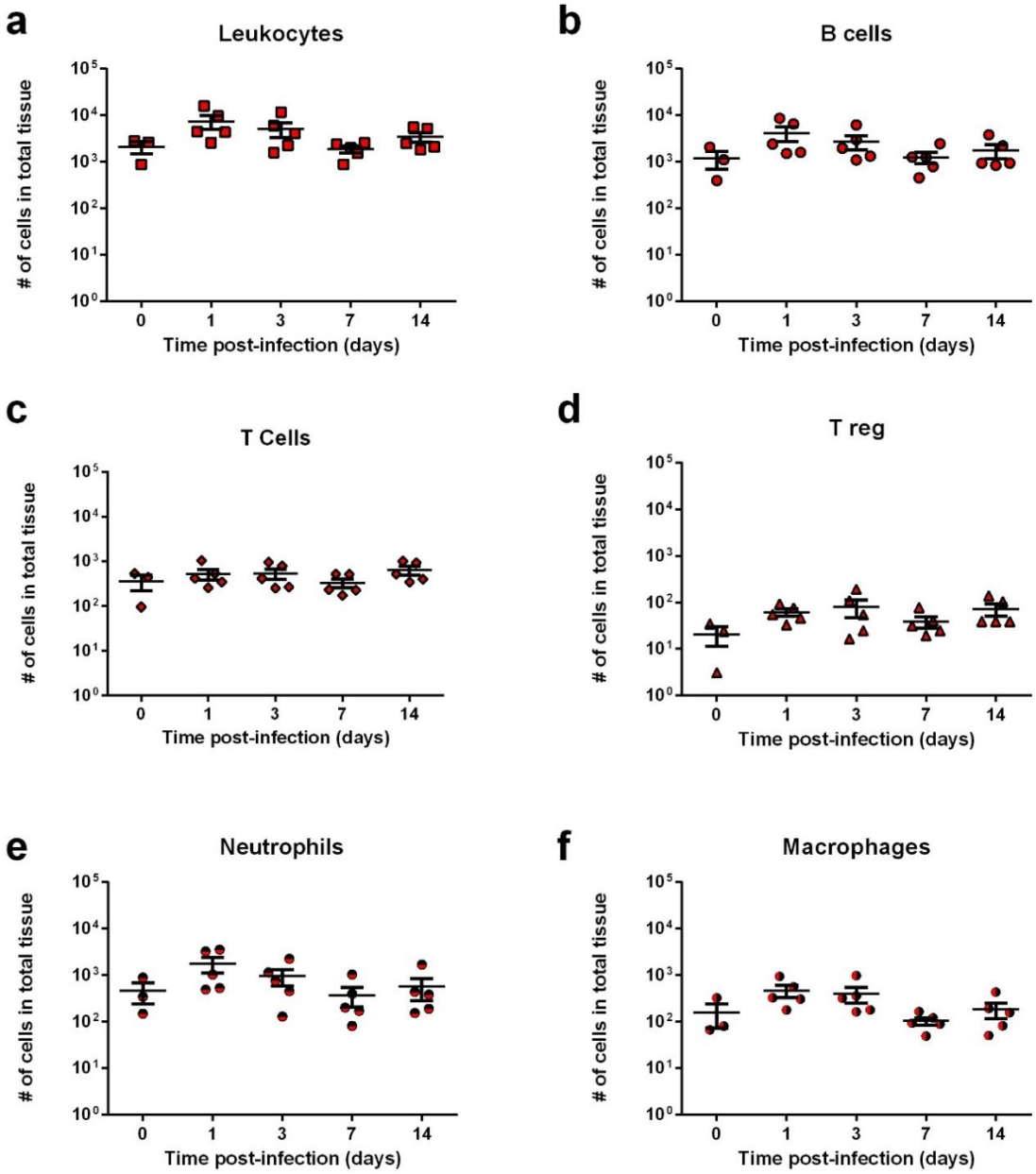

Figure 45. Mean ± SEM of a) leukocytes, b) B cells, c) T cells, d) T regulatory cells, e) neutrophils and f) macrophages in the nasal associated lymphoid tissue during nasopharyngeal carriage with the bacteraemia serotype 1 strain (A42174) in MF1 mice at days 0, 1, 3, 7 and 14 post-infection. Three mice were used for the day 0 control, and five mice were used for the rest of the time-points. No significant differences were observed when analysed using a one-way ANOVA.

5.3.2. Cervical Lymph nodes

The cervical lymph nodes analysed in this study were the mandibular lymph nodes and the superficial parotid lymph nodes, which are situated in the cervical area of mice. As with the nasal associated lymphoid tissue, these lymph nodes have previously been shown to play an important role in the regulation of nasopharyngeal carriage [236, 315].

As observed in Figure 46a, the number of leukocytes in the cervical lymph nodes was higher than in the nasal associated lymphoid tissue ranging between 10^5 and 10^7. Of these leukocytes, the most abundant cell type were the B cells and T cells, both ranging between 10^5 and 10^6 (Figures 46b and 46c). From the T cells, approximately $5x10^4$ were found to be T regulatory cells and no changes were observed in the number of these cells throughout the duration of carriage (Figure 46d).

The number of neutrophils and macrophages was different in this tissue, with the neutrophils ranging between 10^3 and 10^5 and the macrophages ranging between 10^3 and 10^4 (Figures 46e and 46f). Although the number of macrophages in the cervical lymph nodes was very consistent throughout mice and time-points, there was a higher variability in the number of neutrophils in mice on days 3 and 7 post-infection. The high variability in the number of neutrophils in the CLN was probably related to the variability in bacterial numbers found in the nasopharynx during these days. The bacterial number reached a peak three days post-infection, and the number of neutrophils in the CLN could be decreased in those mice that are clearing the infection at day 7 due to the migration of those neutrophils to the site of infection, the nasopharynx.

This data was analysed using a one-way ANOVA test to determine possible significant differences in the number of each cell type between time-points.

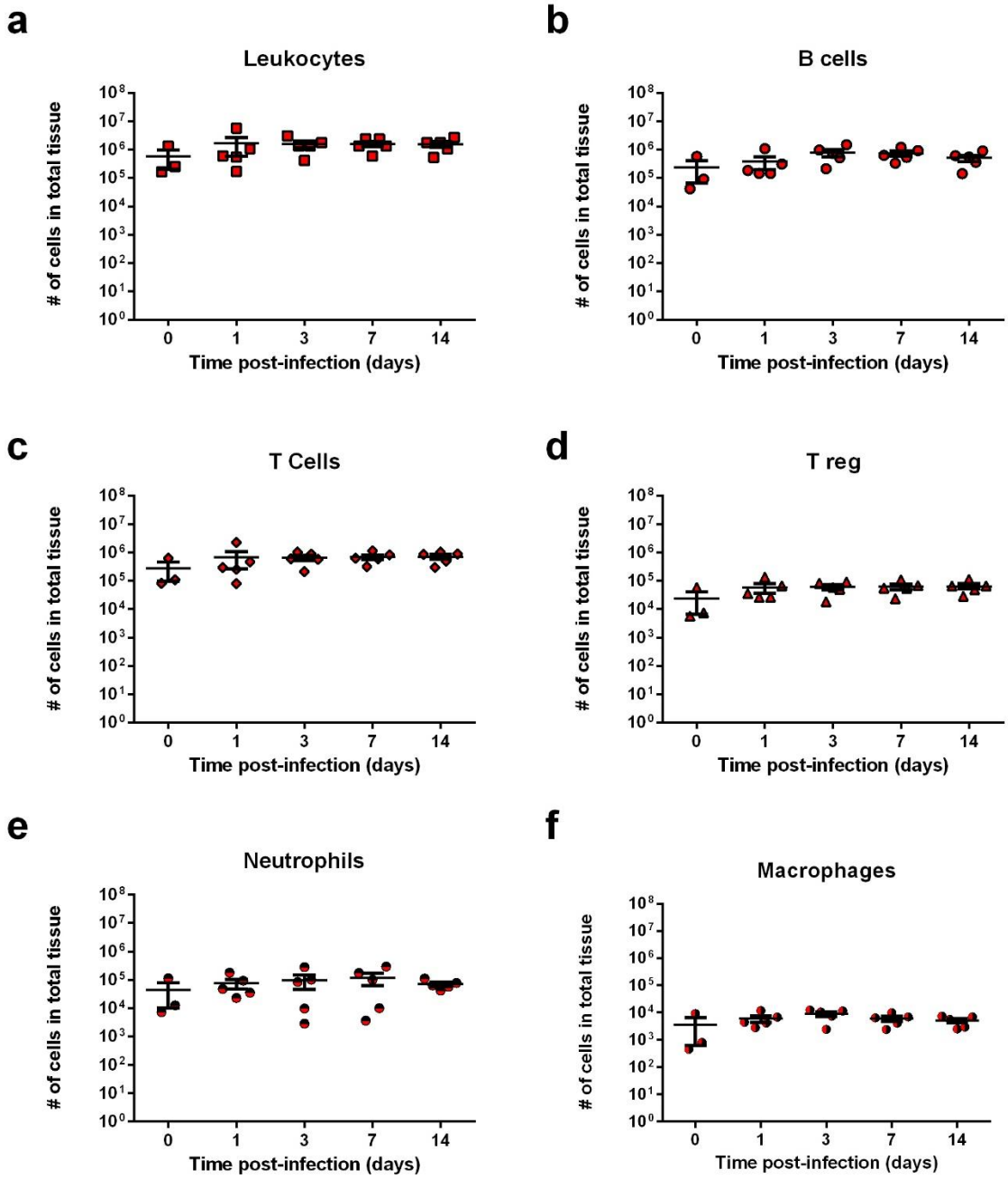

Figure 46. Mean ± SEM of a) leukocytes, b) B cells, c) T cells, d) T regulatory cells, e) neutrophils and f) macrophages in the cervical lymph nodes during nasopharyngeal carriage with the bacteraemia serotype 1 strain (A42174) in MF1 mice at days 0, 1, 3, 7 and 14 post-infection. Three mice were used for the day 0 control, and five mice were used for the rest of the time-points. No significant differences were observed when analysed using a one-way ANOVA.

5.3.3. Cytokine production during nasopharyngeal carriage

Cytokines are small proteins secreted by host cells to communicate between each other allowing the recruitment and activation of other immune cells. There are some cytokines (i.e. IL-10 and TGF-β1) that have previously been shown to be important in maintenance of carriage, and others (i.e. IL-17 and IL-1β) that have been shown to be important for pneumococcal clearance during invasive disease [104, 386].

The concentration of cytokines in the nasopharynx during nasopharyngeal carriage by the bacteraemia serotype 1 isolate (A42174) was studied to determine possible explanations for the early clearance and low duration of carriage by serotype 1. The cytokines studied during nasopharyngeal carriage were IL-6, IL-10, KC, INF-γ, IL-1β, MIP-2, IL-17, TNF-α and active TGF-β1 based on their key roles in inflammation, protective immunity and regulation.

IL-6 was not detected at any stage of serotype 1 strain A42174 carriage in the nasopharynx of mice (Figure 47a). The nasopharyngeal concentration of IL-10 ranged between 250 and 300pg/ml throughout the duration of carriage, with no significant differences observed between time-points (Figure 47b).

As observed in Figures 47c to 47h, the rest of the cytokines analysed (INF-γ, IL-1β, MIP-2, IL-17, TNF-α, active TGF-β and KC) followed similar patterns throughout the nasopharyngeal carriage period starting at low concentrations at time 0 and either gradually increasing throughout the carriage period to reach its peak on day 14 post-infection (INF-γ, MIP-2, IL-17, active TGF-β and KC) or increasing only on day 14 post-infection (IL-1β and TNF-α). Previous studies with the serotype 2 strain D39 have shown that in order for pneumococcal nasopharyngeal carriage to be maintained, the pneumococcus has to induce the production of TGF-β1 in nasopharyngeal cells

resulting in an elevated concentration of TGF-β1 and a high number of T regulatory cells which allow carriage to be maintained [104]. However in these experiments, the induction of active TGF-β1 in mice carrying serotype 1 did not occur until day 14, when the pneumococcus had already been cleared from the nasopharynx suggesting that serotype 1 behaves differently to other pneumococcal serotypes. The lack of TGF-β1 induction together with the lack of a T regulatory cell increase (Figures 45d and 46d) during nasopharyngeal carriage with serotype 1 suggests that this serotype is not able to induce the production of TGF-β1 and T regulatory cells which results in an impaired maintenance and subsequent clearance of nasopharyngeal carriage. In the same study with the serotype 2 strain D39 it was observed that mice carrying the pneumococcus for a prolonged time had increased levels of IL-10 during the early stages of carriage [104], whilst in this study the levels of IL-10 were constant throughout the experimental time. IL-10 is a suppressive cytokine that has anti-inflammatory properties, and like TGF-β1, can be a marker for immunomodulation and tolerance during carriage. Therefore, no variation in the levels of IL-10 in the nasopharynx of mice carrying serotype 1 may explain the early clearance of this serotype from the nasopharynx.

In summary, the lack of induction of modulatory cytokines and T cells that regulate the inflammatory response may lead to the clearance of serotype 1 from the nasopharynx due to an increased number of phagocytes on days 1 and 3 post-infection (Figure 45) induced by tissue damage caused by the high levels of bacteria in the nasopharynx on day 1 and 3 (Figure 44). The lack of induction of modulatory cytokines, such as IL-10 and TGF-β1, lead to an increase of the pro-inflammatory cytokines MIP-2, IL-17, IL-1β, TNF-α and INF-γ which may lead to the clearance of serotype 1 [1, 2, 3, 4].

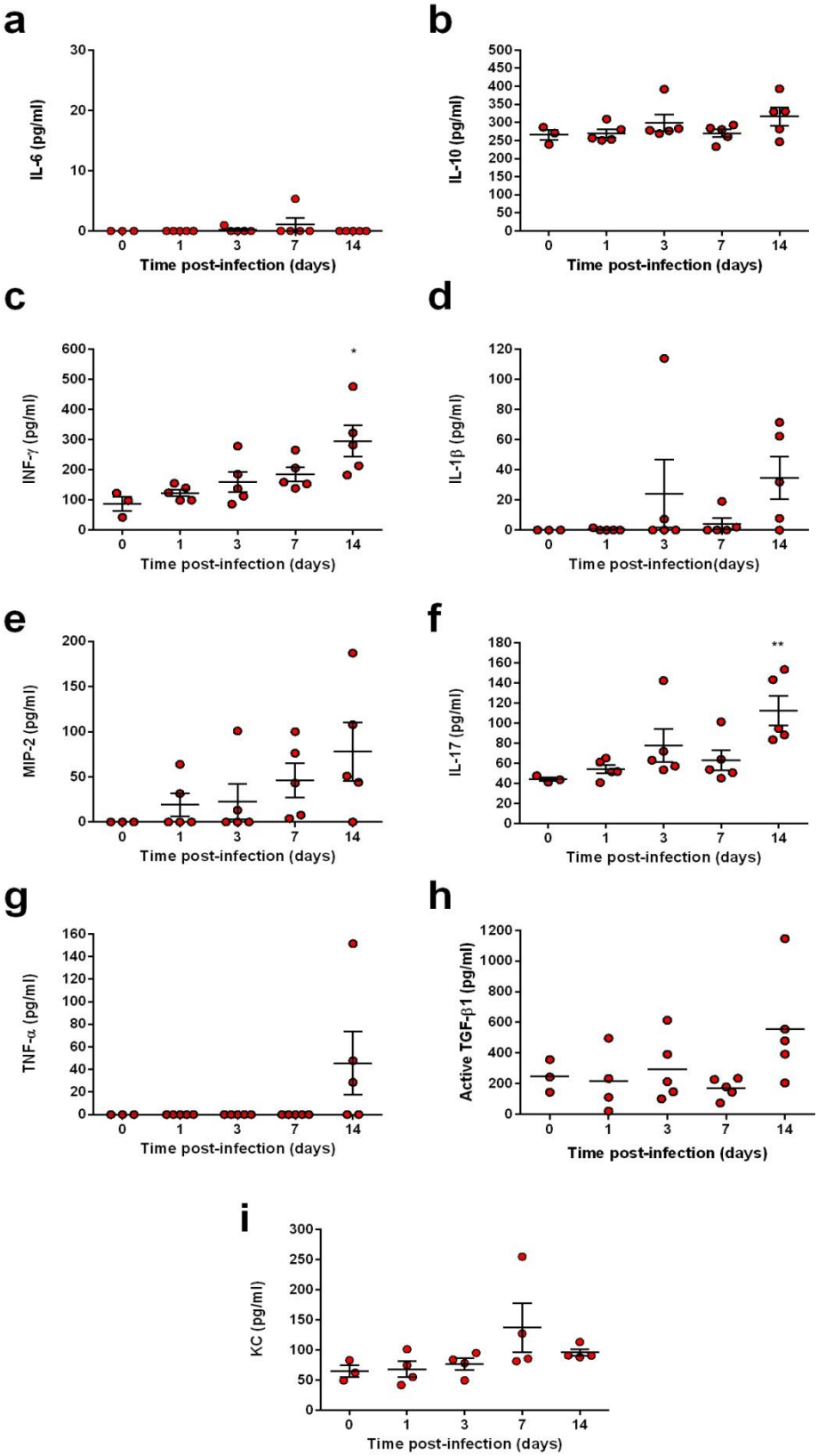

Figure 47. Mean ± SEM of a) IL-6, b) IL-10, c) INF-γ, d) IL-1β, e) MIP-2, f) IL-17, g) TNF-α, h) active TGF-β1 and h) KC in the nasopharynx of mice infected with the bacteraemia strain (A42174) during the nasopharyngeal carriage experiment at days 0, 1, 3, 7 and 14 post-infection. Three mice were used for the day 0 control, and five mice were used for the rest of the time-points. *P-value<0.05 and **P-value<0.01 when analysed using a one-way ANOVA.

5.4. Antibody production during nasopharyngeal carriage

The cellular profiling of the nasal associated lymphoid tissue and the cervical lymph nodes suggested that nasopharyngeal carriage by serotype 1 does not induce a strong host immune response. Considering the short duration of carriage of serotype 1 the concentration of IgA and IgG was determined from the nasopharynx and the serum respectively of mice carrying the serotype 1 strain A42174 to assess the induction of antibody production during carriage up to 28 days.

5.4.1. IgA production

IgA is the main immunoglobulin found in mucosal secretions [153], therefore the concentration of this immunoglobulin in the nasopharynx of mice carrying the bacteraemia isolate (Figure 44) was determined to assess the ability of serotype 1 to induce production of mucosal immunoglobulin.

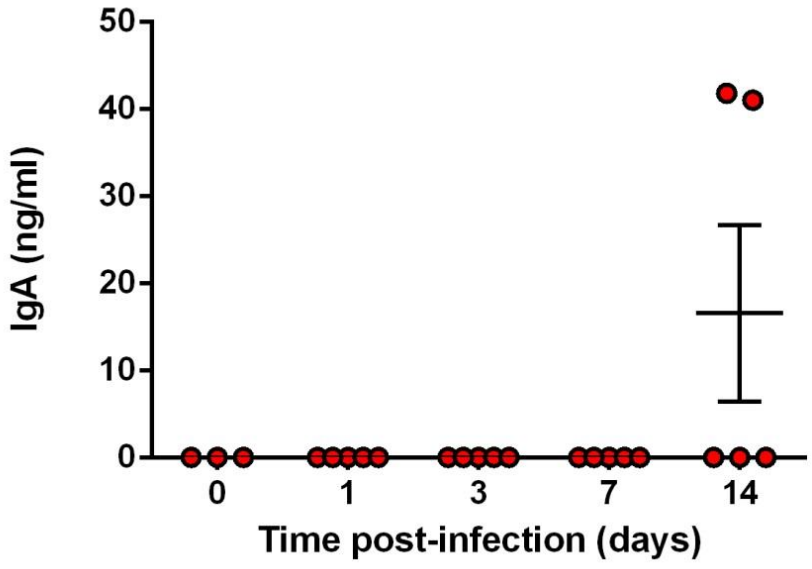

Figure 48. Concentration of IgA in the nasopharynx of MF1 mice carrying the bacteraemia isolate (A42174) at days 0, 1, 3, 7 and 14 post-infection shown as mean ± SEM. Three mice were used for the day 0 control and five mice were used for each of the experimental time-points. No significant differences were observed between time-points when analysed using a one-way ANOVA.

As observed in Figure 48, there were no detectable levels of IgA in the nasopharynx during the first 7 days of carriage. Subsequently, there was an increase from 0 to 40ng/ml, 14 days post-infection in 40% of the mice infected with serotype 1, which were the mice still carrying the pneumococci 14 days post-infection. In previous studies it has been shown that an increase in IgA starts 3 days post-colonisation and peaks approximately 7 days into nasopharyngeal colonisation with the serotype 2 strain D39 [238]. In contrast, induction of IgA production during carriage with serotype 1 is not observed until day 14 post-infection. Furthermore, IgA was only detected in the mice still carrying the pneumococcus by that day indicating that IgA production in serotype 1 is a late response during nasopharyngeal carriage and only occurs if the mouse has been carrying the isolates for approximately 14 days.

5.4.2. Total IgG production

IgG is the most abundant immunoglobulin found in serum and it can neutralise pathogens, activate complement pathways and effector cell functions leading to cytokine secretion and phagocytosis [316].

In this study total IgG levels were determined from the serum of mice carrying the serotype 1 strain A42174 and the Malawian serotype 6B strain D33275, which was used as a control. The 6B isolate was used as a control because it is a serotype commonly associated with carriage [317]. As observed in Figures 49a and 49b, the 6B strain D33275 was able to establish nasopharyngeal colonisation in mice for a longer period of time than the serotype 1 strain A42174, as it did not get completely cleared until 28 days post-infection. Furthermore, the levels of 6B bacteria colonising the nasopharynx was consistent throughout the study period, whilst the number of serotype 1 bacteria increased on day 3 post-infection, being significantly higher than

6B on that time-point when analysed using a two-way ANOVA (****P-value<0.001). When the levels of total IgG in serum were determined it was observed that, although not significant, there was an increase in IgG levels in mice infected with serotype 1 when compared to mice infected with serotype 6B on days 7 and 14 post-infection (Figure 49c).

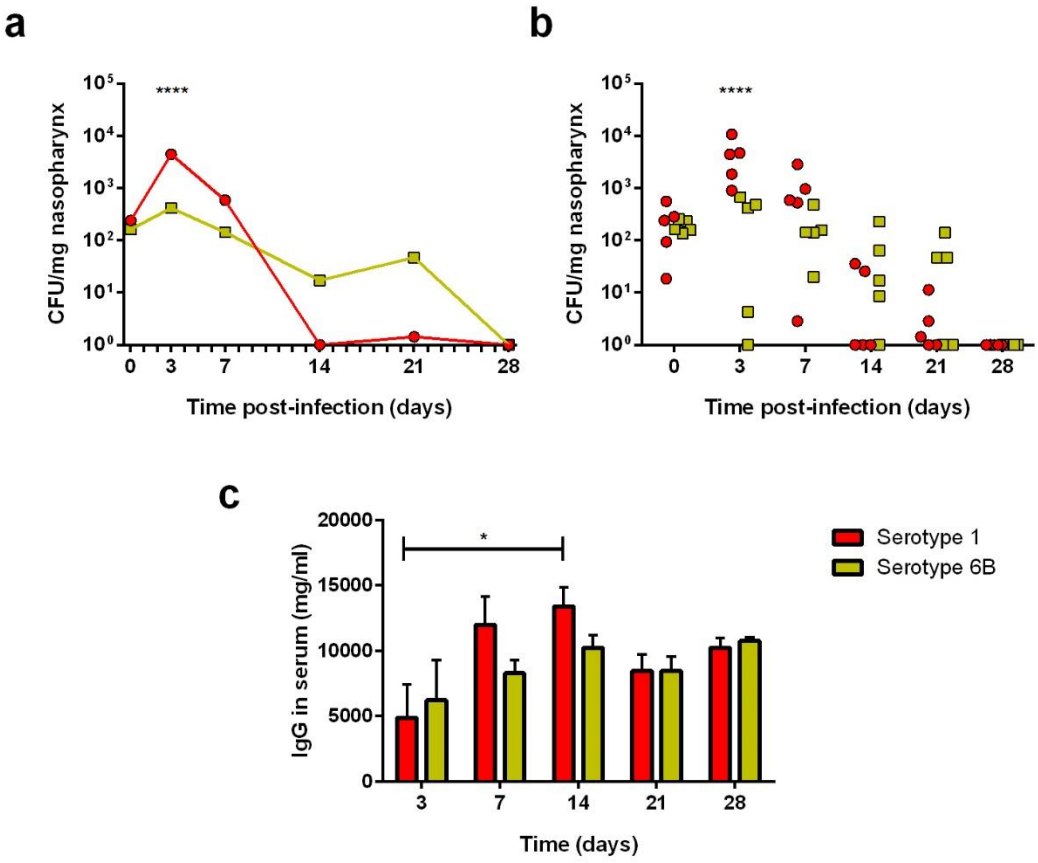

Figure 49. Pneumococcal CFU per mg of nasopharyngeal tissue of MF1 mice infected with the bacteraemia (A42174) serotype 1 isolate and the serotype 6B strain D33275 at days 0, 3, 7, 14, 21 and 28 post-infection shown as median (a),the CFU of individual mice (b) and the levels of total IgG in serum shown as mean mg/ml ± SEM (c). Five mice were used for each time-point. ****P-value<0.001when comparing serotype 1 and serotype 6 in each time point using a two-way ANOVA. *P-value<0.05 when comparing the IgG levels for each serotype during different time points.

In a previous study it was shown that an early increase in the levels of Anti-PspA IgG results in the early clearance of the pneumococcus from the nasopharynx of mice [238]. The increased levels of total IgG in mice infected with serotype 1 coincided

with the time when serotype 1 was getting cleared from the nasopharynx, hence these observations suggest that the increase in the levels of IgG in mice infected with serotype 1 could be associated with the earlier clearance from the nasopharynx of serotype 1 when compared to serotype 6B.

5.4.3. Antibody functionality in vitro

An increase in total IgG from serum of mice carrying the serotype 1 strain A42174 was observed from 7-14 days post-infection. By 21 days post-infection, the concentration of total IgG in serum decreased to return to the same levels observed on day zero. In order to assess the functional activity of increased IgG in serum, an opsonophagocytic killing assay (OPKA) was performed opsonising the bacteria with either the serum from mice carrying the serotype 1 strain B13969 for 10 days or serum from naïve mice. Serum from mice infected with either the bacteraemia or the meningitis strains were going to be used for this experiment but the serum from the bacteraemia infected mice was accidentally destroyed during the heat inactivation process; therefore, only the serum from meningitis infected mice was used. In order to assess whether the IgG was serotype specific or even strain specific, three different strains were used in the OPK assay: the serotype 2 strain D39, the serotype 1 strain A42174 and the serotype 1 strain B13969. The serum used was a pool of 5 mice.

As observed in Figure 50a, mice infected with the meningitis strain (B13969) had approximately double the amount of IgG in serum than naïve mice (*P-value<0.05). When compared to the levels of total IgG in mice infected with the bacteraemia strain (Figure 49b), the mice infected with the meningitis strain seemed to have a lower concentration of immunoglobulin in serum. Considering that the levels of IgG in naïve mice was also lower in this experiment when compared to the bacteraemia experiment

this observation was probably due to a technical problem during the heat inactivation of the serum or during the ELISA process. Nonetheless, an increase was observed in the levels of total IgG in mice carrying either the bacteraemia or the meningitis isolate.

As observed in Figure 50b, when serum from naïve mice was used as opsonin there was no killing of any of the bacterial strains by HL-60-derived neutrophils. When sera from mice carrying the meningitis isolate for 10 days was used as opsonin there was approximately 40-50% killing of the bacteraemia serotype 1 strain (A42174) and approximately 30% killing of the meningitis serotype 1 strain (B13969) (**P-value<0.01, ****P-value<0.001). Interestingly, no killing was observed in the serotype 2 strain D39 when opsonised using the same serum suggesting serotype specific antibody generation. These observations suggest that the increase in total IgG found in serum during nasopharyngeal carriage by serotype 1 is functional and that this functionality is serotype specific.

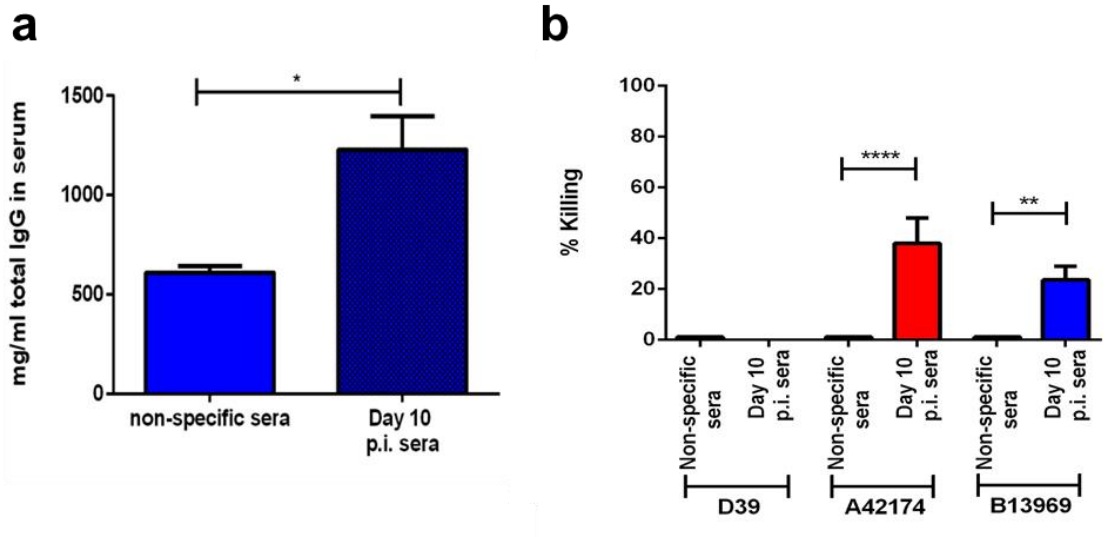

Figure 50. a) Concentration of total IgG found in pooled serum from 5 naïve mice or 5 mice carrying the meningitis serotype 1 strain B13969 for 10 days. b) Percentage of killing by HL-60-derived neutrophils of the serotype 2 strain D39, the bacteraemia serotype 1 strain A42174 and the meningitis serotype 1 strain B13969 after opsonisation with serum from naïve mice or from mice carrying the meningitis strain for 10 days. The OPKA was done in triplicates on three independent days. *P-value< 0.05, **P-value<0.01 and ****P-value<0.001 when analysed using a t-test and a two-way ANOVA respectively.

5.4.4. Assessment of memory responses *in vivo*

The opsonophagocytic killing assay showed that the IgG produced during nasopharyngeal colonisation by serotype 1 is functional, and acts as an opsonin increasing the ability of phagocytes to kill pneumococci in a serotype-specific manner. In this section, the memory immune response in mice was tested *in vivo* by infecting MF1 mice with pneumococci of the bacteraemia serotype 1 strain (A42174) and re-challenging the same mice with the same infective dose 21 days after the first infection. As previously observed (Figure 44), the serotype 1 strain A42174 carries at relatively high concentrations during the first 7 days of carriage but starts getting cleared after day 7 and is almost 100% cleared by day 21 post-infection. For that reason, the second infection was done on day 21 post-infection to ensure that no pneumococci from the first infection were still colonising the nasopharynx of the mice.

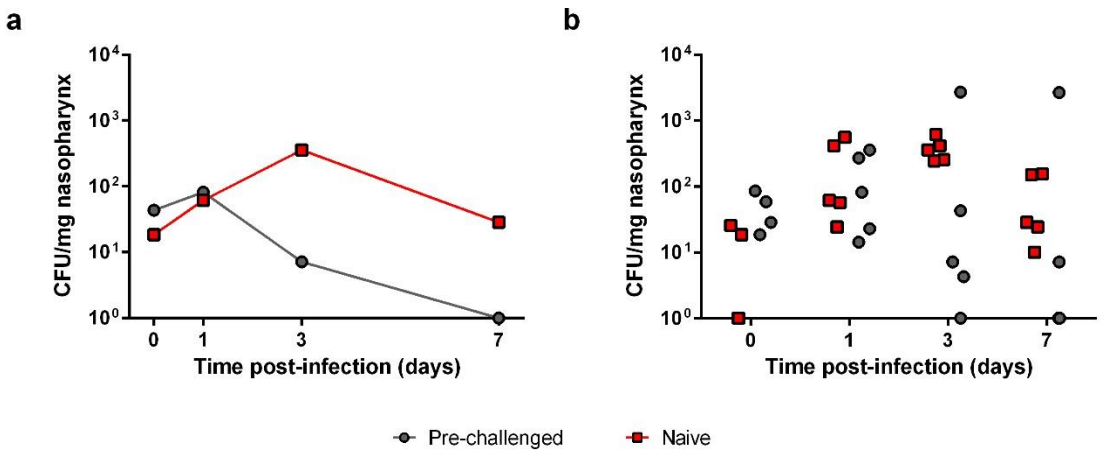

Figure 51. Pneumococcal CFU per mg of nasopharyngeal tissue of MF1 naïve mice or pre-challenged MF1 mice infected with bacteraemia isolate (A42174) at days 0, 1, 3 and 7 post-re-infection shown as median (a) and range of the individual mice (b). Three to four mice were used for the day 0 control, and five mice were used for the rest of the time-points. No significant differences were observed when analysed using a two-way ANOVA.

As observed in Figure 51, naïve mice had a peak in pneumococcal numbers in the nasopharynx on day 3 post-infection. These bacterial numbers started declining after day 3 and by day 7 post-infection were approximately $5x10^1$CFU/mg of tissue, hence equivalent to initial infective inoculum (see day zero). However, mice that had previously carried serotype 1 for 21 days (and had cleared them), started clearing the second infection by day 3 post re-challenge, and had completely cleared all pneumococci by day 7. This clearly shows that nasopharyngeal carriage with serotype 1 induces an adaptive memory immune response upon re-challenge with the same serotype, which prevents future carriage events.

5.5. Capsule switch in serotype 1 during nasopharyngeal carriage

The pneumococcal capsule is an important virulence factor that has been shown to be a key determinant of invasiveness and prevalence; certain serotypes are more commonly associated with carriage and other serotypes are more commonly associated with invasive disease [28, 31, 69]. It has previously been observed that the pneumococcus has the ability to change the serotype of a single clone by altering or exchanging the *cps* locus, which encodes the capsular genes [66, 69]. As shown in this chapter, serotype 1 is able to establish nasopharyngeal colonisation, although for a shorter period of time when compared to other serotypes [238]. Since serotype 1 is rarely found in carriage it was hypothesised that serotype 1 could undergo capsular switching at high rates during nasopharyngeal colonisation therefore being under-detected in the nasopharynx of healthy individuals.

In order to identify possible capsular switching events mice were infected with the serotype 1 strain A42174 and after 3 days co-infected with the Malawian serotype 6B strain D33275, hence both serotypes were co-localised in the nasopharynx. Serotype

6B was chosen for the second infection because it is the fourth most common type found causing nasopharyngeal carriage in Malawian children [317]. The second infection was done on day 3 to ensure that the maximum density of serotype 1 isolates was found in the nasopharynx at the time of co-infection with 6B. A total of 310 single colonies were collected from different time-points: 10 colonies from day 3 before the 6B co-infection which were used as controls, 100 isolates from day 4 (1 day post-co-infection), 100 isolates from day 6 (3 days post-co-infection) and 100 isolates from day 10 (7 days post-co-infection). Phenotypic characterisation of the capsule was done by using a serotype 1 specific latex agglutination test. Genotypic characterisation was done by sequencing one of the seven house-keeping genes used for multi-locus sequencing. The housekeeping gene chosen for the genotypic analysis was the xanthine phosphoribosyltransferase (*xpt*) gene which has 6 allelic differences between serotype 1 and serotype 6B.

146 isolates out of the total 310 isolates were tested using the latex agglutination test to determine whether they expressed a type 1 capsule or not. 47 of the isolates tested from days 1, 3 and 7 post-co-colonisation, as well as day 3 pre-co-colonisation as serotype 1 positive control, were sent to sequencing: 25 serotype 1 positives plus a serotype 1 control and 20 serotype 1 negatives (potentially serotype 6B) and a 6B control. 100% of the serotype 1 positive isolates were found to contain the serotype 1 *xpt* gene and 100% of the serotype 1 negative isolates were found to contain the serotype 6 *xpt* gene. In conclusion, no capsule switch was observed during this experiment. Although no capsule switch was detected in this study, only 15% of the isolates collected were studied. In the future, it would be interesting to increase the number of isolates studied, as capsular switching is a rare event and a very high number of isolates might need to be studied to find a single capsular switching event.

The number of bacteria in the nasopharynx of mice infected with serotype 1 and serotype 6B for the detection of capsular switch can be found in Figure 52. Although the introduction of serotype 6B in the nasopharynx of mice already carrying serotype 1 had no effect on the bacterial numbers of serotype 1, it was observed that the number of serotype 6B bacteria during co-colonisation was lower than in the nasopharynx of naïve mice (Figure 49a and 49b). This observation suggests that co-competition for the nasopharyngeal niche could have important effects on the pattern of carriage of different pneumococcal serotypes. For that reason, co-infection experiments were studied further.

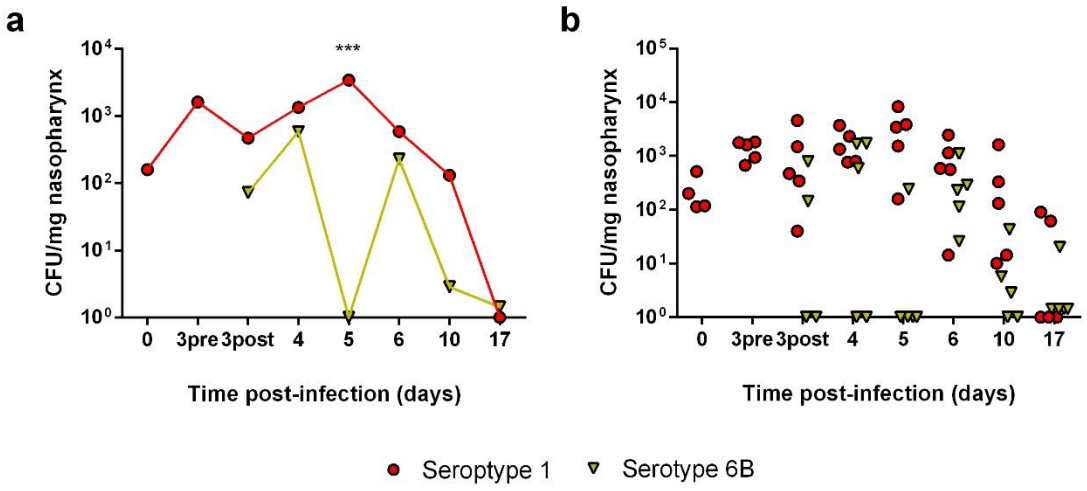

Figure 52. Pneumococcal CFU per mg of nasopharyngeal tissue of MF1 mice infected with the bacteraemia serotype 1 isolate (A42174) on day 0 and re-infected with the serotype 6B isolate D33275 shown as median (a) and range of the individual mice (b) at on days 0, 3 (pre-re-infection), 3 (post-re-infection), 4, 5, 6, 10 and 17 post-infection. Four mice were used for the day 0 control, and five mice were used for the rest of the time-points. ***P-value<0.005 when analysed using a two-way ANOVA.

5.6. *In vivo* competition experiments between serotypes

A recent study has shown that competitive interactions between different serotypes during host colonisation can alter the serotype distribution of nasopharyngeal carriage [318]. In the study by Trzciński et al., multiple serotypes were introduced at the same

time in the nasopharynx of mice and serotype ratios were determined at different stages of colonisation by quantitative PCR [318].

In this study however, the effect of multiple co-colonisation on carriage patterns of serotype 1 was determined by introducing one serotype on day zero and a second serotype 7 days after the first serotype was administered. It is realistic to accept that multiple colonisation events are likely to happen by acquisition of new serotypes through time, rather than by co-colonisation of different serotypes all at the same time; therefore, it was considered that this method was more representative of the clinical situation, than the one used by Trzciński et al.

5.6.1. Co-infections with serotypes 1 and 6B

As observed in Figure 52, when serotype 6B was introduced into the nasopharynx of mice already carrying serotype 1 for 3 days, no significant differences were observed in the nasopharyngeal carriage pattern of serotype 1. However, the carriage pattern of serotype 6B varied when compared to carriage in naïve mice (Figures 49a and 49b) suggesting that secondary colonisation of a niche already colonised by pneumococci significantly affects the newcomer. In this case serotype 6B was almost completely cleared from the nasopharynx by day 7 post-infection when it was introduced into a niche already carrying serotype 1, instead of by day 28 post-infection as it was observed in naïve mice.

In order to determine whether this effect was just a reflection of the order of introduction of the serotypes, another experiment was done where serotype 6B was the one used to initially colonise the nasopharynx, and serotype 1 was the one used as the secondary infection. In this case the second infection was performed on day 7 post-infection to allow the acclimatisation of the firstly introduced serotype.

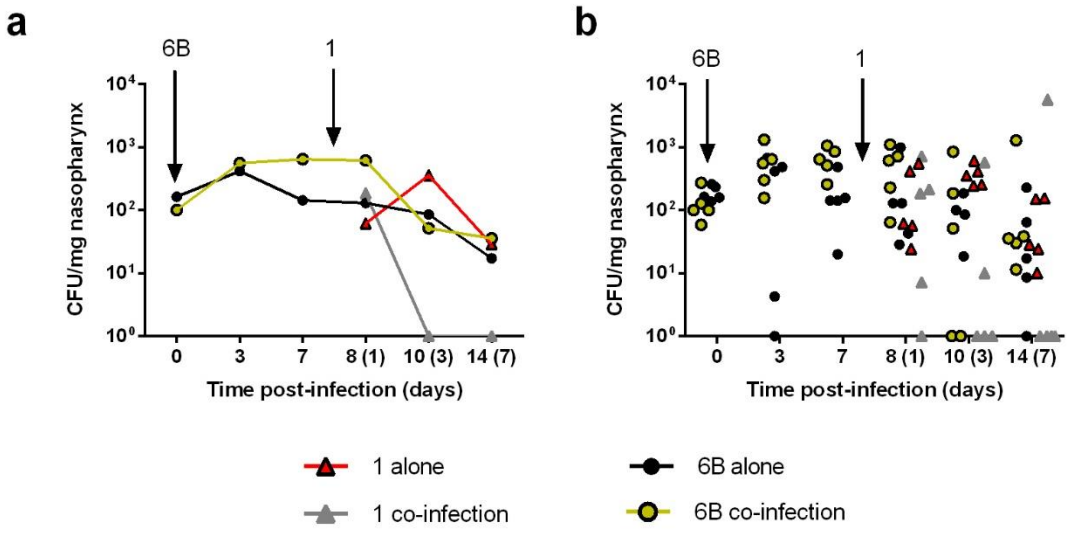

Figure 53. Pneumococcal CFU per mg of nasopharyngeal tissue of MF1 mice infected with the serotype 6B isolate (D33275) on day 0 and re-infected with the bacteraemia serotype 1 isolate (A42174) on day 7 shown as median (a) and range of the individual mice (b) at on days 0, 3, 7 (before re-infection), 8 (1 day post-re-infection), 10 (3 days post-re-infection), and 14 (7 days post-re-infection) post-infection. Five mice were used for each time-point. No significant differences were observed when analysed using a two-way ANOVA.

As observed in Figure 53, no significant differences were observed in the numbers of 6B bacteria in the nasopharynx of mice re-infected with serotype 1 when compared to mice carrying only 6B in the nasopharynx. However, a substantial difference was observed in the number of serotype 1 bacteria when compared to the bacterial numbers in mice only carrying serotype 1. The data was analysed using a two-way ANOVA. Although no significant differences were observed between the number of bacteria in the co-infection and the number of bacteria in naïve mice for each of the serotypes it was clear that when serotype 1 was introduced into the nasopharynx of mice already carrying other pneumococcal serotypes it was not able to establish colonisation at the same density or for the same period of time as it could in naïve mice.

5.6.2. Co-infections with serotypes 1 and 19F

During the competition experiments with serotypes 1 and 6B, it was shown that when one serotype was already colonising the nasopharynx of mice, the introduction of a second serotype did not affect the nasopharyngeal carriage pattern of the original serotype. However, the carriage pattern of the second coloniser was considerably affected with reduced duration and density of colonisation. Hence, acquisition of a co-coloniser would appear to detrimentally affect the ability of the second coloniser to compete in the same niche.

In order to determine whether this observation could be applicable to other serotypes the same experiment was performed using serotype 1 and 19F, which has been shown to be the most common serotype in nasopharyngeal carriage in Malawian children [317]. In this experiment the second serotype was always introduced on day 7 to allow full comparison.

As observed in Figures 54a and 54b, when serotype 19F was introduced 7 days after the introduction of serotype 1 no significant differences were observed in the bacterial numbers of serotype 1 in the nasopharynx of naïve mice or mice carrying both serotypes, although serotype 1 did not seem to clear as quickly during co-infection as it did during single colonisation. When 19F was introduced in the nasopharynx of mice already carrying serotype 1 the number of serotype 19F bacteria during the first 3 days was similar to those in single infected mice. However, 7 days post-re-infection the numbers of 19F bacteria in the nasopharynx were higher in naïve mice than in mice co-infected with serotype 1 and serotype 19F, which had mostly cleared 19F from the nasopharynx.

As observed in Figures 54c and 54d, when serotype 1 was introduced 7 days after the infection with serotype 19F, serotype 1 was not able to establish colonisation at the same density as when infecting naïve mice. Furthermore, the bacterial numbers of serotype 19F were significantly increased after introducing serotype 1 in the nasopharynx of mice already carrying serotype 19F (*P-value<0.05). This increase was only observed one day post-re-infection and the bacterial levels went back to the same levels observed in single infected mice. The data was analysed using a two-way ANOVA.

In summary, these *in vivo* competition experiments clearly show that interactions between different serotypes during nasopharyngeal co-colonisation have clear effects on carriage density and duration. When serotype 1 is carried and a different serotype is subsequently introduced, no significant effect is observed on the carriage pattern of the original coloniser. However, when serotype 1 is acquired in mice already carrying a different serotype, the outcome on the carriage pattern of the original serotype varied amongst serotypes. Whilst there was no significant effect on the carriage pattern of serotype 6B, the introduction of serotype 1 significantly increased the density of serotype 19F. This observation suggests that the additional acquisition of serotype 1 into the nasopharynx affects the pathogenicity of other pre-existing serotypes being carried. Interestingly, independently of the serotype, pneumococci acquired subsequently after the original carrier strain, were always less able to establish successful colonisation when compared to their single infections in naïve mice. Therefore, secondary acquisition is disadvantageous to the new incoming pneumococcal coloniser.

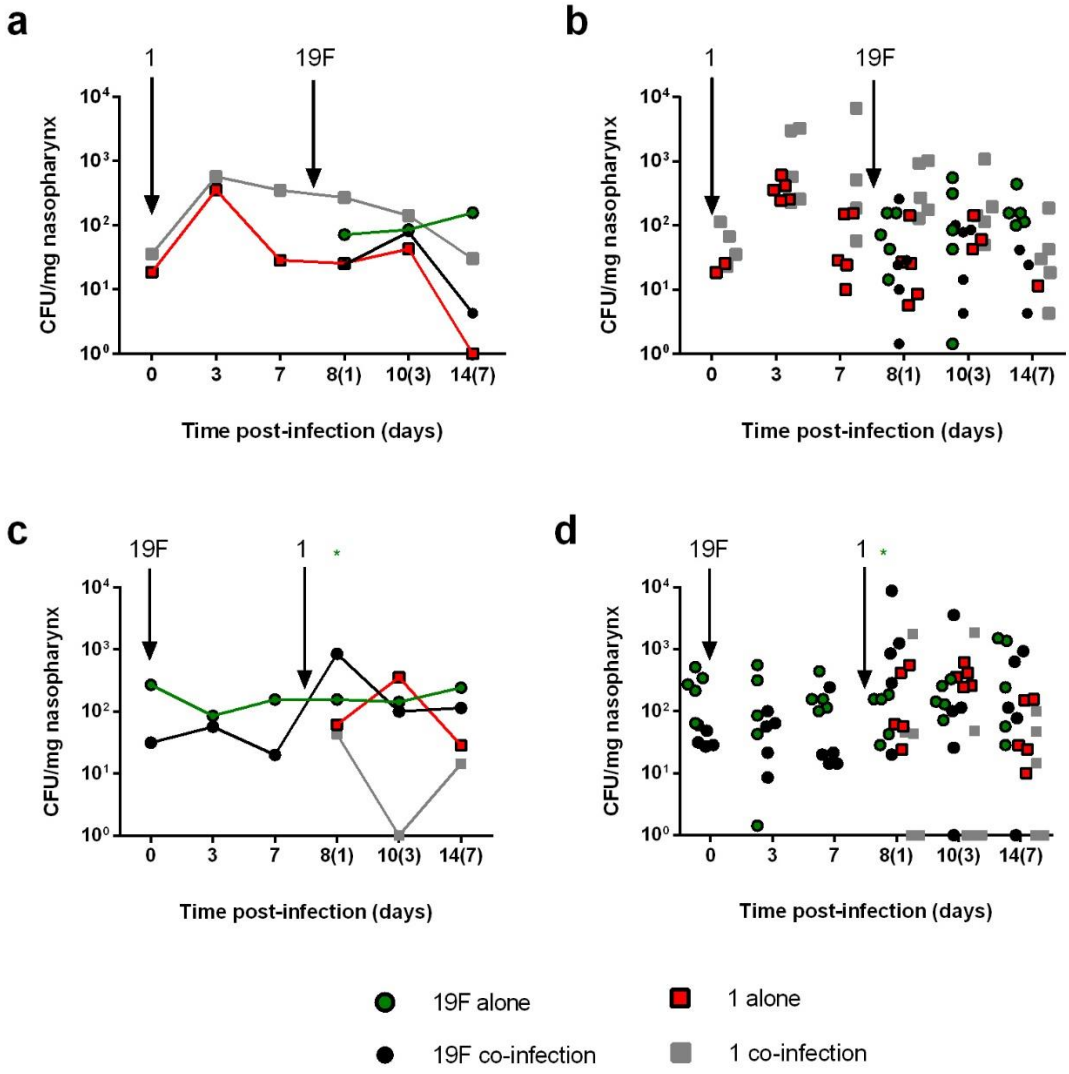

Figure 54. Pneumococcal CFU per mg of nasopharyngeal tissue of MF1 mice infected with either the bacteraemia serotype 1 strain (A42174) or the serotype 19F isolate and re-infected in on day 7 with the serotype 19F isolate or the serotype 1 isolate, respectively. Data shown as median (a) and range of the individual mice (b) at on days 0, 3, 7 (before re-infection), 8 (1 day post-re-infection), 10 (3 days post-re-infection), and 14 (7 days post-re-infection) post-infection. Five mice were used for each time-point. *P-value<0.05 when analysed using a two-way ANOVA.

5.6.3. Host immune responses during co-colonisation

In order to understand the mechanisms behind the different outcomes in the competition experiments the cellular profiling of the nasal associated lymphoid tissue (Figure 55) and the cervical lymph nodes (Figure 57) was analysed from mice that were colonised first with either serotype 6B (6B-1) or 19F (19F-1).

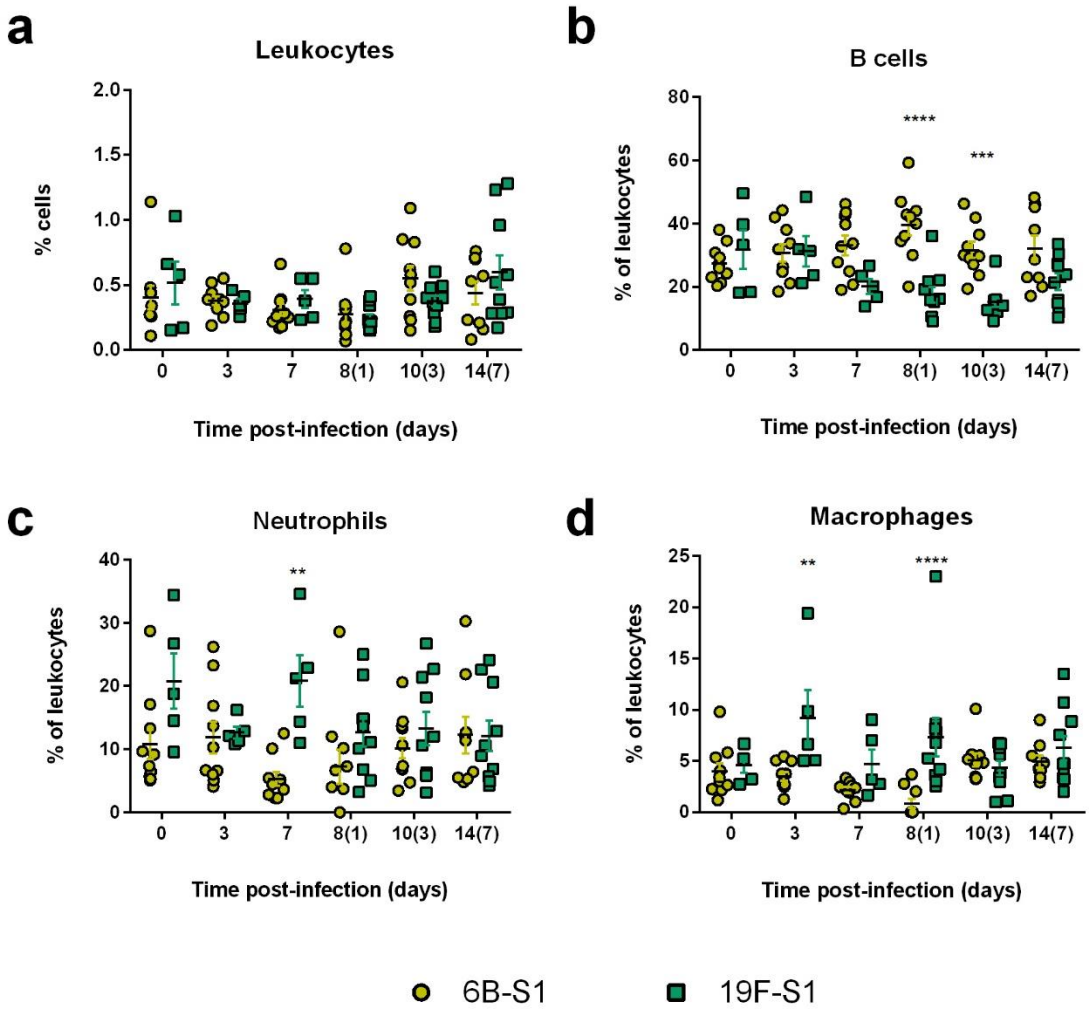

Figure 55. Mean percentage ± SEM of a) leukocytes, b) B cells, c) neutrophils and d) macrophages in the nasal associated lymphoid tissue of mice infected with serotype 1 after carrying serotype 6B (6B-1) or after carrying serotype 19F (19F-1). Five mice were used for each time-point. *P-value<0.05, **P-value<0.01, ***P-value<0.005 and ****P-value<0.001 when analysed using a two-way ANOVA.

There was a significant difference between the two groups studied in the number of macrophages (Figure 55d) and B cells (Figure 55b) in the nasal associated lymphoid. The large variability in the number of macrophages made it difficult to draw any conclusions. However, there was a significant increase in the number of B cells in the 6B-1 group when compared to the 19F-1 group which was already significant before the introduction of serotype 1, suggesting that 6B might induce B cells at a higher level

than 19F and therefore increase the number of antibodies, which might have an effect on the bacterial numbers in the nasopharynx after the introduction of serotype 1. In order to assess this theory the levels of total IgG in serum were assessed.

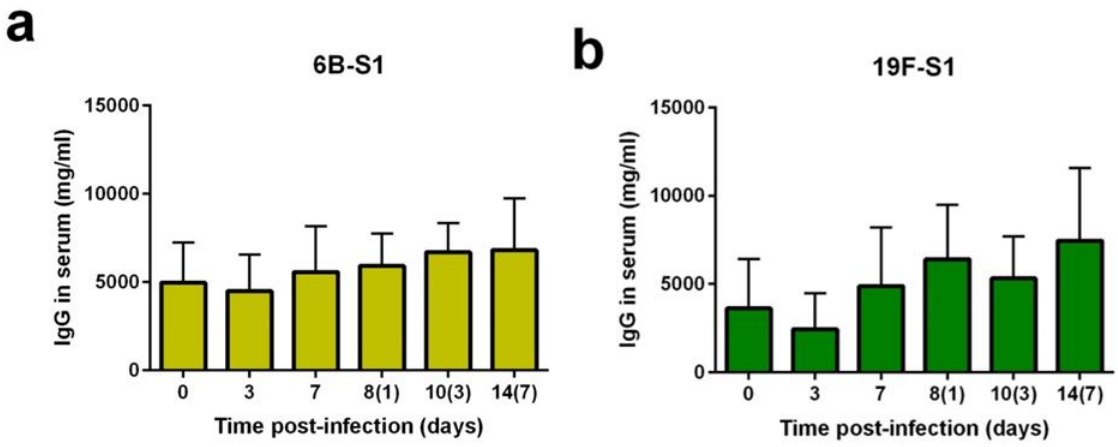

Figure 56. Concentration of total IgG in pooled serum of five mice per time-point form the a) 6B-1 co-infection experiment and the b) 19F-1 co-infection experiment shown as mean ± SEM. No significant differences were observed when analysed using a two-way ANOVA.

As observed in Figure 56, the levels of total IgG in serum were higher in the 6B-1 group than in the 19F-1 group although those differences were only present before the introduction of serotype 1. These observations suggest that the differences observed during competition experiments could be due to the difference in the immune response induced by the serotype that is already colonising the nasopharynx before the acquisition of the second serotype.

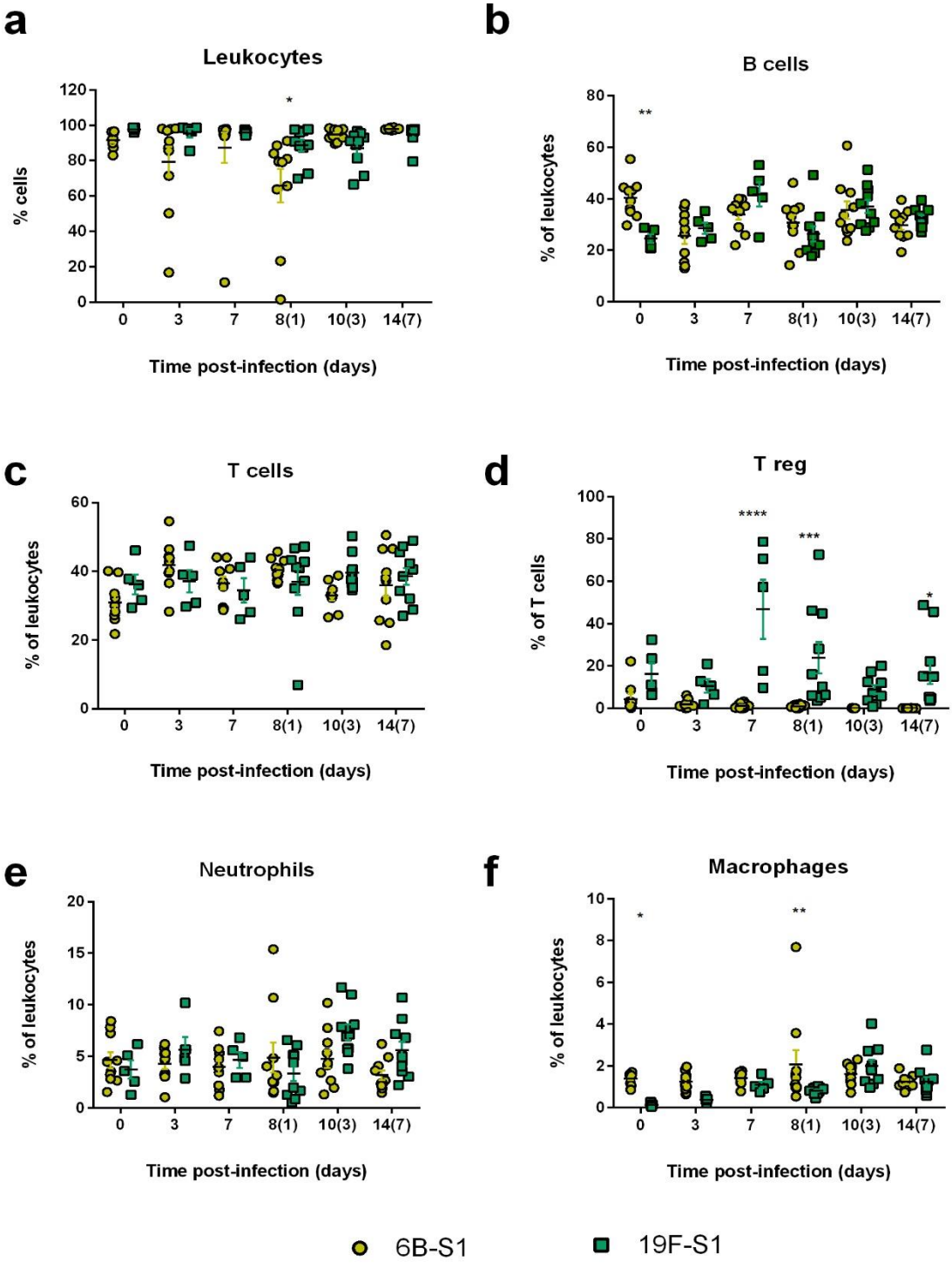

Figure 57. Mean percentage ± SEM of a) leukocytes, b) B cells, c) neutrophils and d) macrophages in the cervical lymph nodes of mice infected with serotype 1 after carrying serotype 6B (6B-1) or after carrying serotype 19F (19F-1). Five mice were used for each time-point. *P-value<0.05, **P-value<0.01, ***P-value<0.005 and ****P-value<0.001 when analysed using a two-way ANOVA.

In the cervical lymph nodes there were significant differences in the numbers of T regulatory cells and macrophages one day after the re-infection. The number of T regulatory cells (Figure 57d) was higher in the 19F-1 infection when compared to the 6B-1 infection, but there were already significant differences between groups before the introduction of serotype 1. This observation suggests that 19F was able to induce the production of T regulatory cells during single carriage which might help the maintenance of carriage. This increased number of T regulatory cells might later on help to control the induction of pro-inflammatory cytokines allowing the bacteria to grow even after the introduction of another serotype. Regarding the increased number of macrophages (Figure 57f) in the 6B-1 group when compared to the 19F-1 group, the high variability observed in the number of macrophages, even at time 0 post-infection, makes it difficult to draw any conclusions.

5.7. Conclusions

There are different hypothesis to explain why serotype 1 is one of the major causes of invasive disease regardless of not being commonly found causing nasopharyngeal carriage:

a) serotype 1 is carried but is undetected by the current methods of detection
b) serotype 1 carries at low density and/or
c) serotype 1 carries for a short period of time.

A mouse model of nasopharyngeal carriage was used in this chapter to understand the patterns of nasopharyngeal carriage by serotype 1.

The bacteraemia, meningitis and carriage strains were used in the *in vivo* nasopharyngeal carriage model to determine the duration and density of carriage of serotype 1, and to assess any possible differences in the carriage patterns of those

isolates. The three strains were able to establish nasopharyngeal colonisation in mice at a density of approximately $5x10^1$ bacteria per mg of tissue. During the first three days of carriage the bacterial numbers increased until reaching their peak on day 3 with a density of $5x10^2$ to 10^3 bacteria per mg of tissue. After this point the bacterial density started declining until being almost cleared by day 14 post-infection. Although the three strains followed the same pattern during carriage, the carriage strain W004030 was carried at a higher density for most of the experiment suggesting that during colonisation serotype 1 is able to acquire certain adaptations allowing it to better colonise at a higher density. Although the density of the carriage strain was slightly higher when compared to the bacteraemia and the meningitis isolates there was no significant difference in the duration of carriage suggesting that the adaptive changes acquired during colonisation are not related to carriage duration. Indeed, when meningitis isolates that had been carrying in the mice for 21 days were used for a subsequent infection, the same results were found: the isolates were able to carry at a higher density than its wildtype but no significant differences were seen in the duration of carriage. These observations suggest that independently of the site of isolation, all serotype 1 ST217 strains are virtually the same isolate which occasionally is able to acquire adaptations to specific niches. These adaptations to specific niches can be permanent changes such as epigenetic changes which are genetic changes that are influenced by the environment, or single point mutations (SNPs) that can be acquired by random mutations. Alternatively, these changes can be transient by up-regulation or down-regulation of the expression of important metabolic pathways or specific virulence factors. Future experiments (i.e. whole genome sequencing) could be done to assess if these adaptations are permanent or transient.

The study of the host immune response to serotype 1 suggests that this pneumococcal serotype induces a late immune response in the nasopharynx which leads to the early clearance from the nasopharynx although it induces a serotype specific IgG production that contributes to protection against future colonisation events by the same serotype. It would be interesting to study the duration of this acquired protection; if this was a long lasting protection it would mean that individuals that had already been carrying serotype 1 would be protected from future carriage events, hence the low detection levels of serotype 1 causing nasopharyngeal carriage.

Finally, it was observed that the bacterial density and the duration of carriage of different serotypes changed during co-infections. The introduction of a serotype in the nasopharynx of mice already carrying a different serotype resulted in a lower bacterial density in the nasopharynx of these mice and a much lower duration of carriage than in naïve mice. In contrast, the density and duration of carriage of the serotype already colonising the nasopharynx varied depending on the serotype. Whilst the carriage pattern of serotypes 1 and 6B was not altered as a result of the introduction of another serotype, the introduction of serotype 1 induced a significant increase in the number of serotype 19F bacteria causing colonisation. These observations suggest that serotype 1 not only is highly able to cause invasive disease but it is also able to alter the pathogenicity of other serotypes during nasopharyngeal co-colonisation.

In summary, it was found that serotype 1 carries for a shorter period of time than other serotypes and although it is not able to induce a strong immune response, it is able to induce the production of serotype specific antibodies that prevent future colonisation events by the same serotype. Therefore, the shorter duration of carriage by serotype 1 and the protective effect of previous colonisation events suggest that serotype 1 is, as observed, likely to be less found causing nasopharyngeal carriage. Furthermore, as a

result of low carriage rates it is likely that if there is a new colonisation event this would happen in a nasopharynx that is already carrying a different serotype. In this case, the carriage density would be even lower and the duration of carriage would be even shorter therefore reducing the chances of further spreading through the community.

In conclusion, although serotype 1 is a highly invasive pneumococcal serotype it is not a good coloniser, especially when other serotypes are already present. Considering that nasopharyngeal carriage is thought to be a pre-requisite for invasiveness, but serotype 1 is not a good coloniser, this serotype might be an opportunistic invader. Unlike other serotypes, serotype 1 is known to cause invasive disease in young adults, which is the age group with lower pneumococcal carriage rates; this is possibly due to the fact that no competition in the nasopharynx with other serotypes leads to a better colonising ability and better chances of spreading and causing disease. Moreover, it was observed that the acquisition of serotype 1 in the nasopharynx of mice already carrying a different serotype was able to induce an increase in density of the original coloniser. Since carriage is a pre-requisite of invasiveness, a higher pneumococcal density may lead to an increased chance of translocation into lungs and therefore an increased chance of causing invasive disease. However, this was only observed in serotype 19F; as there was no effect on density when the original coloniser was serotype 6B. These observations suggest that the interaction of different serotypes in the nasopharynx may lead to different outcomes which might be driven by immune responses to different serotypes during single infection and during co-infection.

Identification of orthologous genes that are differentially expressed between serotype 1 and serotype 2 during *in vitro* growth

6. Identification of orthologous genes that are differentially expressed between serotype 1 and the serotype 2 strain D39 during *in vitro* growth

6.1. Introduction

Streptococcus pneumoniae has historically played an important role in molecular biology, being the bacteria studied in the experiments that led to the discovery of DNA as the genetic material [319, 320]. Since then, many studies have been performed to investigate the genome of the pneumococcus and its influence in virulence.

Although the genomic variability between serotypes has been extensively studied, there is little information on the variability of gene expression between serotypes. The study of bacterial gene expression has been shown to be an important tool for the study of bacterial pathogenicity, by exposing bacterial adaptations to different conditions during infection [321]. At the same time, a comparative transcriptomic approach to compare closely related bacterial pathogens has been shown to be an important tool to understand pathogenic differences between closely related strains [322-324]. Comparative transcriptomics works on the basis that although strains may contain the same virulence and metabolic genes, the levels of expression of certain genes might be significantly different.

Many studies have been done on the gene expression of a small selection of pneumococcal serotypes but little is known about the gene expression of serotype 1, especially of the serotype 1 isolates belonging to lineage B (i.e. ST217) [165, 169, 325]. In this study it was hypothesised that the high virulence of serotype 1, when compared to other serotypes (i.e. serotype 2 strain D39), might be driven by the differential expression of orthologous genes involved in virulence and metabolism.

Orthologous genes are those genes from different strains that evolved from a common ancestor and share the same functions [308].

Most of the pneumococcal gene expression analysis done in the past has been done using either real time PCR or DNA microarrays. Although the microarray technology has greatly evolved since its discovery there are still limitations to the technique: microarrays are only available for the study of known genes since they require the synthesis of specific DNA probes, there is cross-hybridization between similar sequences and there is background noise introduced at different stages which limits the resolution of the technique [326, 327].

In recent years, an alternative to microarrays has been developed that allows the quantification of gene expression by sequencing cDNA fragments which are consequently aligned to the chromosome and quantified, this technique is known as RNA-seq [326]. Although this technology is more expensive than microarrays, it overcomes some of the microarray-associated limitations: RNA-seq allows the quantification of non-annotated genes, and it can detect lower amounts of transcripts due to the absence of background noise [326, 327].

In order to identify differentially expressed genes between the serotype 1 strain A42174 and the serotype 2 strain D39, orthologous genes shared by the two strains were identified and the gene expression of those genes was compared by using the transcriptomic approach.

In this study we compare the gene expression of the ST217 serotype 1 strain A42174 and the serotype 2 strain D39 during five stages of *in vitro* growth using RNA-seq.

6. 2. In vitro growth phases

In this study, the bacterial growth was divided into 5 different stages: early exponential phase (EEP), mid-exponential phase (MEP), late exponential phase (LEP), early stationary phase (ESP) and late stationary phase (LSP). During the three stages of exponential growth the bacteria are rapidly growing until they reach the early stationary phase. At that stage, there is a reduction of the bacterial metabolic rate caused by the decreased nutrient availability in the environment and by the stress caused by the accumulation of toxic metabolites. Finally, during the late stationary phase the metabolic activity stops and there is an activation of the resistance physiology [328]. Virulence genes have been shown to have growth-phase specific expression profiles in other bacterial pathogens such as *Salmonella* [308], for that reason the differential expression of A42174 and D39 was studied at five stages of growth.

The growth curve with the five stages of growth for both A42174 and D39 can be seen in Figure 58. For A42174 the RNA was extracted as described in Section 2.19 when the bacterial cultures reached the following OD_{500} values: 0.130 (EEP), 0.550 (MEP), 1.100 (LEP), 1.250 (ESP) and 5 hours after the early stationary phase (LSP). For D39 the RNA was kindly extracted by Reham Yahya as described in Section 2.19 when the bacterial cultures reached the following OD_{500} values: 0.220 (EEP), 0.650 (MEP), 1.200 (LEP), 1.400 (ESP) and 5 hours after the early stationary phase (LSP).

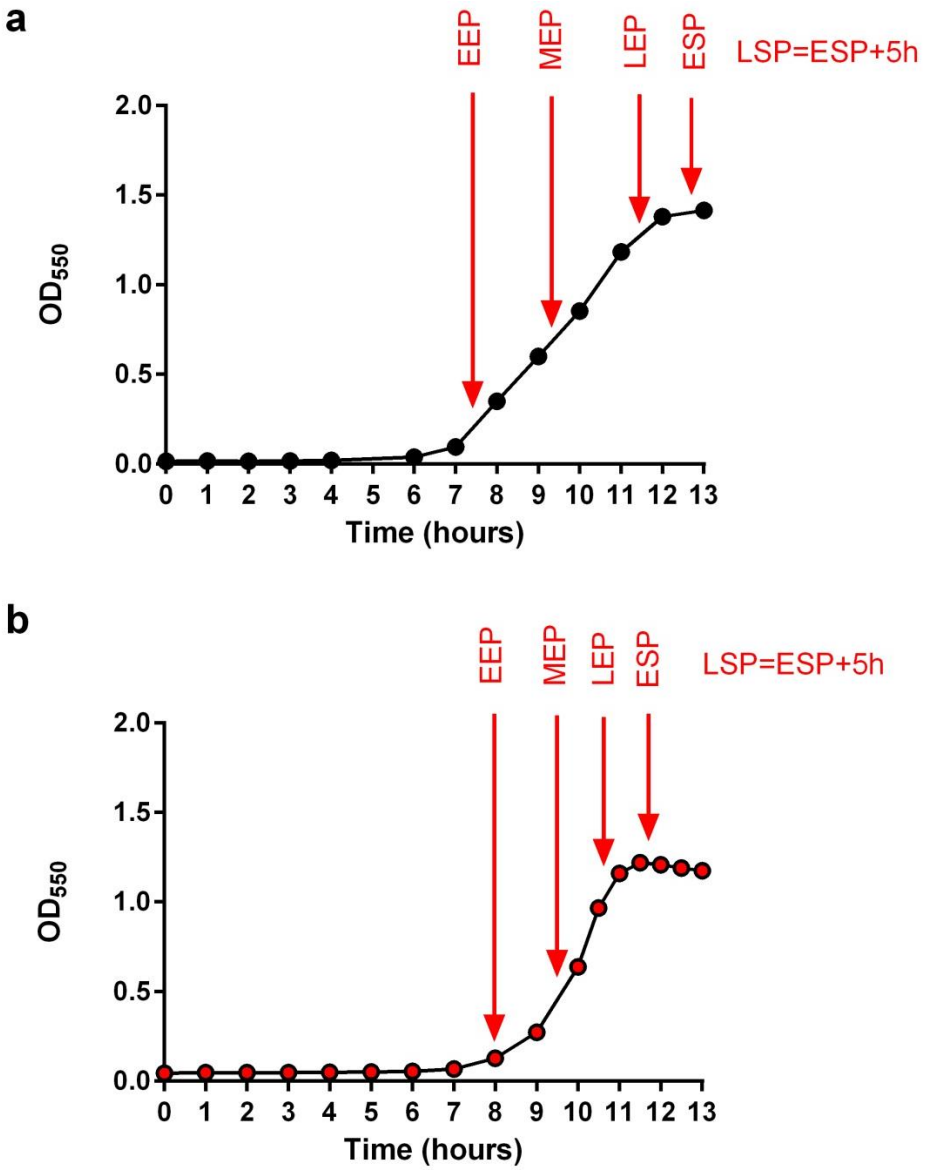

Figure 58. Growth curve of the serotype 1 strain A42174 (a) and the serotype 2 strain D39 (b) in BHI broth (OD_{550}) showing the five distinct growth phases at which bacterial RNA was extracted: early exponential phase (EEP), mid-exponential phase (MEP), late exponential phase (LEP), early stationary phase (ESP) and late exponential phase (LEP).

6. 3. Details of mapping of RNA-seq reads

RNA-seq was performed by Vertis Biotechnologie AG and annotated by Chrispin Chaguza as described in Section 2.20. The mapping of the RNA-Seq reads against the annotated genomes to infer transcripts was done by Dr. Karsten Hokamp at Trinity College, Dublin, as previously described [328]. Finally, the expression analysis of the

transcriptome was done in collaboration with Prof. Jay Hinton (University of Liverpool) using the GeneSpring 7.3 software (Agilent Technologies).

Strain	Growth phase	Total number of reads sequenced	Number of Uniquely Mapped reads (%)	Number of Multi-Mapped reads (%)	Number of Un-Mapped reads (%)
A42174	EEP	13,337,636	12,560,732 (94.2%)	484,212 (3.6%)	292692 (2.2%)
A42174	MEP	13,164,697	12,582,060 (95.6%)	334,810 (2.5%)	247827 (1.9%)
A42174	LEP	12,430,448	11,875,633 (95.5%)	327,528 (2.6%)	227287 (1.8%)
A42174	ESP	9,245,968	8,585,510 (92.9%)	449,433 (4.9%)	211025 (2.3%)
A42174	LSP	7,880,688	6,850,848 (86.9%)	786,438 (10%)	243402 (3.1%)
D39	EEP	29,929,671	6,807,453 (22.7%)	20,924,228 (69.9%)	2,197,990 (7.3%)
D39	MEP	37,829,397	9,045,311 (23.9%)	24,561,956 (64.9%)	4,222,130 (11.2%)
D39	LEP	44,567,392	2,763,509 (6.2%)	37,641,096 (84.5%)	4,162,787 (9.3%)
D39	ESP	38,795,356	2,154,055 (5.6%)	31,435,865 (81%)	5,205,436 (13.4%)
D39	LSP	36,137,954	2,481,893 (6.9%)	30,383,210 (84.1%)	3,272,851 (9.1%)

Table 10. Sequencing depth of the A42174 and D39 samples from different growth phases.

It has been previously shown that a sequencing depth of 5 to 10 million non-rRNA fragments is needed for optimal bacterial transcriptome profiling of *Salmonella*; since the pneumococcal genome is half the size of the *Salmonella* genome, a sequencing depth of 2 to 5 million non rRNA fragments are needed for optimal pneumococcal transcriptome profiling [329]. As observed in Table 10, the sequencing depth of the A42174 and the D39 samples was high enough to provide optimal analysis of the

transcriptomes: the total number of reads sequenced was above 2 million. There was a high percentage of uniquely mapped reads (above 85% in the serotype 1 isolate A42174) which are reads that are mapped to only one location in the genome. The transcriptome analysis in this study was done using only the uniquely mapped reads to assemble a high-quality gene expression dataset.

6. 4. Gene expression of A42174 and D39 at different stages of growth

The study of differential gene expression between different strains is an important tool to understand differences in pathogenicity between different strains, therefore the differential gene expression of A42174 and D39 was done in this study.

A pair-wise comparison of the expression of 2069 orthologous genes was done by normalising the expression of A42174 in each growth phase to the expression of D39 in the same growth stage (Figure 59). The data were filtered by only taking into account TPM (transcript per million) values greater than 10 and fold changes greater than 2. In previous studies it was considered that a gene was expressed when the TPM value was higher than 10 [328]. This cut-off was selected after analysing the expression of a gene essential for the degradation of *myo*-inositol by *Salmonella*. In the absence of the sugar the expression of the gene involved in its degradation was approximately TPM = 4, therefore a conservative cut-off of 10 is used to ensure that the gene is expressed.

As observed in Figure 59, the pair-wise comparison of the expression of orthologous genes between A42174 and D39 showed broadly similar levels of gene expression between the three exponential phases, whilst the two stationary phases followed a different pattern of expression.

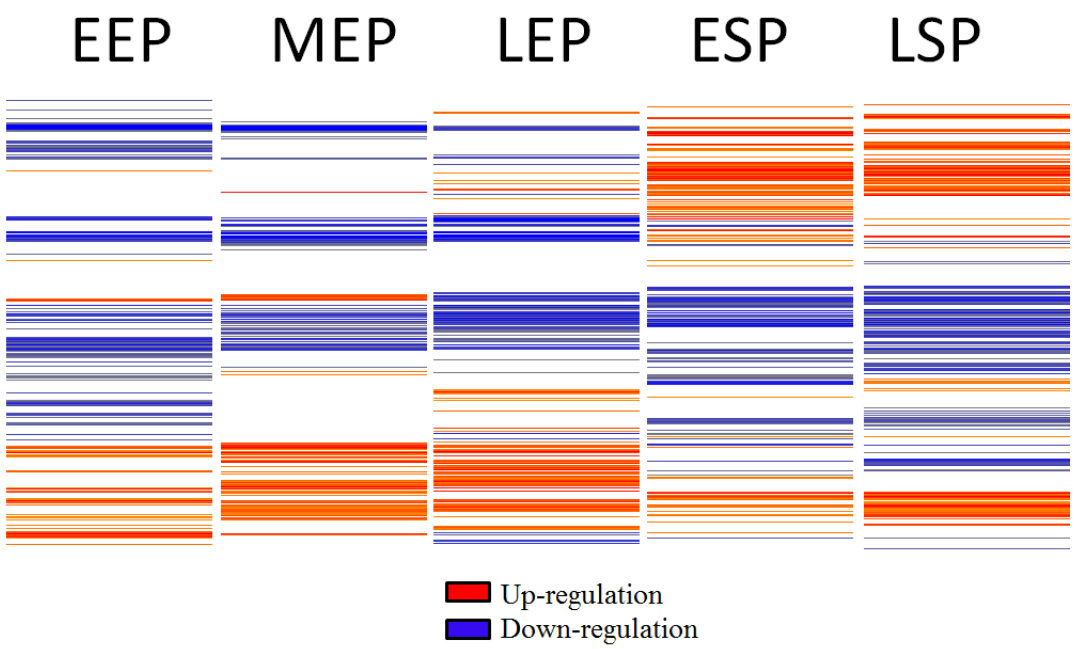

Figure 59. Differential gene expression between strains A42174 and D39 at five stages of growth (EEP: Early Exponential Phase, MEP: Mid Exponential Phase, LEP: Late Exponential Phase, ESP: Early Stationary Phase, LSP: Late Stationary Phase). The 2069 orthologous genes genes were included in this analysis. TPM>10 and >2-fold change. Down-regulated genes in A42174 when compared to D39 are shown in blue, up-regulated genes A42174 when compared to D39 are shown in red, and not differentially expressed genes between the two strains are shown in white.

As observed in Table 11, between 26% and 39% of the orthologous genes were differentially expressed in A4217 compared to D39, with a higher proportion of those being down-regulated than up-regulated.

Growth phase	N° Up-regulated genes (%)	N° Down-regulated genes (%)	Total N° of differentially expressed genes (%)
EEP	168 (8.12%)	375 (18.13%)	543 (26.24%)
MEP	231 (11.16%)	315 (15.22%)	546 (26.39%)
LEP	232 (11.21%)	514 (24.84%)	746 (36.06%)
ESP	329 (15.90%)	390 (18.85%)	719 (34.75%)
LSP	283 (13.68%)	508 (24.55%)	791 (38.23%)

Table 11. Number and percentage of genes differentially expressed in A42174 during the 5 growth phases when compared to D39. 2069 orthologous genes were included in this analysis

6.5. Functional category analysis during the five phases of growth

6.5.1. Selection of functional categories

KEGG (Kyoto Encyclopedia of Genes and Genomes) is a bioinformatic resource that integrates current knowledge on molecular interaction networks such as metabolic pathways [329]. This resource allows the prediction of cellular processes and organism behaviour by classifying genes into different functional categories. The study of functional categories provides the possibility of studying cellular complex processes that are essential for the survival of the pathogen [329].

The functional category analysis of the two pneumococcal strains was done using the information obtained from the KEGG database using DAVID Bioinformatics Resources 6.7 (https://david.ncifcrf.gov/). The hypothetical genes were excluded from the functional category analysis reducing the number of orthologous genes analysed from 2069 to 466. A total of 57 functional categories were categorised by KEGG.

Since the number of genes included in the functional categories obtained from the KEGG database was less than 25% of the whole genome used in the pair-wise comparison of A42174 and D39 (Figure 59), a new pair-wise comparison was done with the reduced set of genes to determine any changes in the proportion of differentially expressed genes for each growth phase. After reducing the number of genes used in the analysis from 2069 to 466 the same patterns of expression were still observed (Figure 60); the gene expression followed broadly similar patterns throughout the three exponential phases, which were distinct from the genes expression patterns seen in the stationary phases.

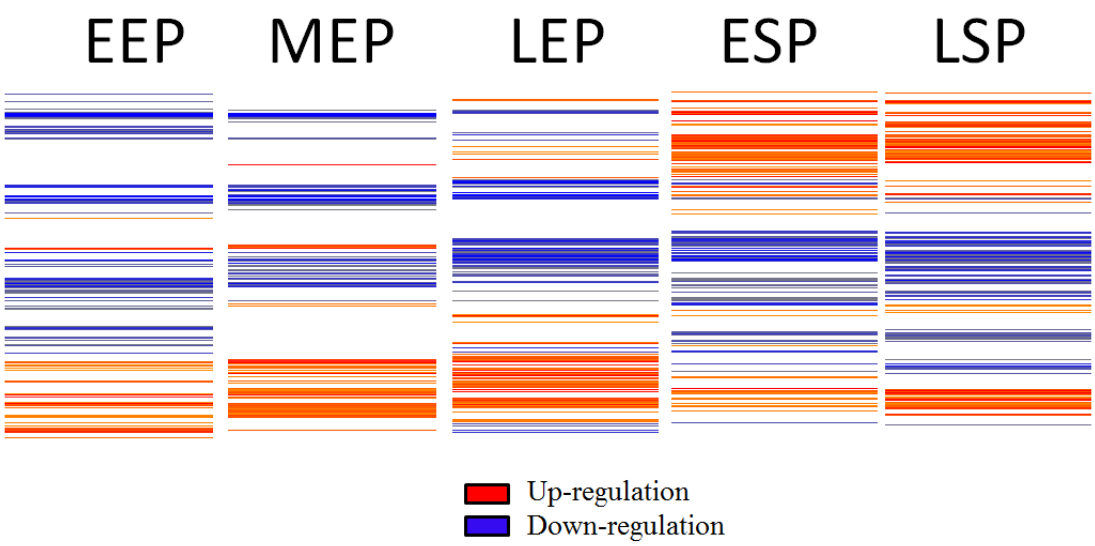

Figure 60. Differential gene expression between strains A42174 and D39 at five stages of growth (EEP: Early Exponential Phase, MEP: Mid Exponential Phase, LEP: Late Exponential Phase, ESP: Early Stationary Phase, LSP: Late Stationary Phase). The 466 genes categorised in the KEGG database were included in this analysis. TPM>10 and >2-fold change. Down-regulated genes in A42174 when compared to D39 are shown in blue, up-regulated genes A42174 when compared to D39 are shown in red, and not differentially expressed genes between the two strains are shown in white.

As observed in Table 12, when the analysis only included the 466 genes detected by the KEGG database there were still more genes that were down-regulated than up-regulated in A42174 when compared to D39 in all phases except early stationary phase. In this analysis the number of differentially-expressed genes varied from 28.11% in the mid exponential phase to 39.06% in the late stationary phase. In summary, although the percentage of genes that were differentially-expressed in A42174 varied when the set of genes used in the analysis is reduced, the percentage still ranged between approximately 25% and 40%.

Growth phase	N° Up-regulated genes (%)	N° Down-regulated genes (%)	Total N° of differentially expressed genes (%)
EEP	42 (9.01%)	96 (20.60%)	138 (29.61%)
MEP	63 (13.52%)	68 (14.59%)	131 (28.11%)
LEP	74 (15.88%)	83 (17.81%)	157 (33.69%)
ESP	91 (19.53%)	76 (16.31%)	167 (35.84%)
LSP	90 (19.53%)	92 (19.74%)	182 (39.06%)

Table 12. Number and percentage of genes differentially expressed in A42174 during the 5 growth phases when compared to D39. The 466 genes detected in the KEGG database were included in this analysis.

For this study, a selection of 11 functional categories was chosen for deep investigation based on their effect on growth and virulence. Nonetheless, the percentage of up-regulated and down-regulated genes in each of the 57 functional categories for each growth phase can be found in Section 9.2.

The categories selected for further investigation were selected based on their importance in virulence and fitness of the pneumococcus (Table 13).

Functional categories	Genes
ABC transporters	*cbiO, cbiO1, adcC, adcB, adcA, malD, malC, malX, pstB, pstA, pstC, pstS, rafE, rafF, rafG, amiA, amiC, amiD, amiE, amiF, proWX, psaC, psaB, aliB, potA, potC, potD, lmb, ftsX, ftsE, livF, livG, livM, livH, livJ, aliA*
Bacterial secretion system	*yajC-2, secAm ffh, ftsY, secG, yajC-1, secY*
Fatty acid biosynthesis	*accA, accD, accC, fabZ, accB, fabF, fabD, fabK, fabH*
Fructose and mannose metabolism	*fucl, scrK, tpiA, pgm, rpml, pfkA, fba, manL, manM*
Galactose metabolism	*galU, nagA, aga, galK, galT-2, scrB, galE-1, lacA, lacB, lacC, lacD, lacF-2, lacE-2, lacG-2, pfkA, bgaA, lacE-1, lacG-1, lacF-1, agaS, bgaC*
Glucolysis / gluconeogenesis	*pgi, gap, tpiA, ldg, acoA, lpdA, eno, gapN, celA, pyk, pfkA, exp5, gki, fba, pgk, galM*
Peptidoglycan biosynthesis	*pbp1B, pbp2A, penA, ddlA, murF, murE, murC, murB, dacC, murG, murD, fibB, bacA, pbp1A, mraY, pbpX*
Phosphotransferase system (PTS)	*lacF-2, lacE-2, ptsl, celB, lacE-1, lacF-1, manL, manM*
Pyruvate metabolism	*ackA, ldh, acoA, lpdA, ppc, gloA, nspC, pyk, spxB, pflB, accA, accD, accC, accB*
Starch and sucrose metabolism	*malP, galU, pgi, gtfA, scrB, scrK, amy, glgA, glgD, glgC, glgB, pulA, bglA-2, bglA-1*
Two-component system	*pgi, tkt, trpE, zwf, pfkA, deoC, deoB, rpiA, fba, grpE, gnd, eda, talC, prsA*

Table 13. Functional categories selected for the study and genes included in each category.

Given the limitations of the KEGG databases the genes in Table 13 are not the only genes involved in these pathways, but are the only ones recognised by the KEGG database and therefore the only ones used for this analysis.

Figure 61 shows the variation during the five stages of growth of the proportion of differentially expressed genes for each functional category. Each functional category was analysed in detail (Figures 62-74).

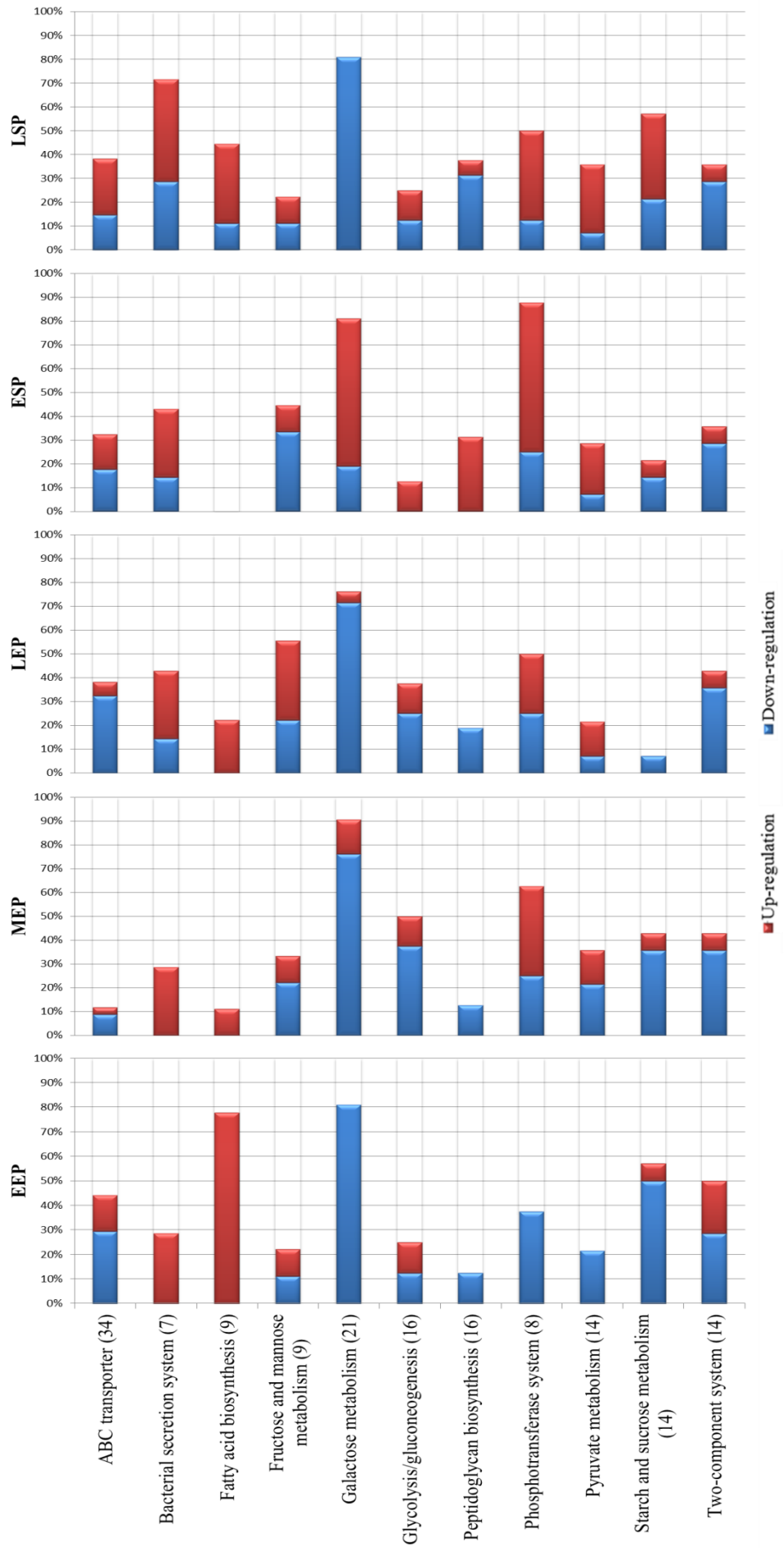

Figure 61. Pair-wise comparison of the functional category analysis of the different growth phases (EEP: Early Exponential Phase, MEP: Mid Exponential Phase, LEP: Late Exponential Phase, ESP: Early Stationary Phase, LSP: Late Stationary Phase) shown as percentage of genes from the functional category that are up-regulated (red) or down-regulated (blue) in A42174 compared to D39. TPM>10 and >2-fold change.

6.5.2. Transport mechanisms

6.5.2.1. Bacterial secretion system

In bacteria, proteins need to be transported, secreted and/or inserted into membranes and this process occurs via the secretion system (Sec), formed by Sec protein conducting channels. The two main subunits forming the Sec machinery in *S. pneumoniae* are *secA* and *secY* [330, 331].

In the pneumococcus, most virulence factors and some proteins involved in cell division are transported to the cell surface by the Sec system, and it has previously been observed that mutations in cardiolipin synthase (responsible to promote the associations of the different components of the Sec pathway) lead to growth impairment and fragility of pneumococcal chains [331].

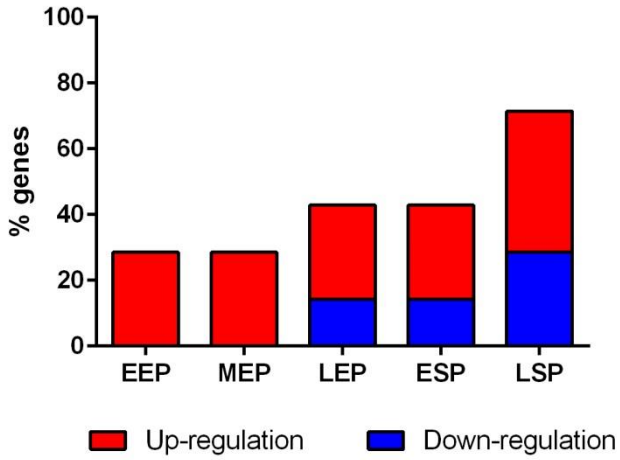

Figure 62. Differential expression of the genes involved in bacterial secretion shown as percentage of up-regulated (red) and down-regulated (blue) genes in A42174 compared to D39.

During all growth phases it was observed that the genes involved in this pathway were mainly up-regulated, although some genes were down-regulated at the later stages of growth (Figure 62). These results may suggest that serotype 1 requires up-regulation

of protein transport genes during growth to compensate the down-regulation of other transport pathways.

6.5.2.2. ABC transporter

The ATP-binding cassette (ABC) transporters are transmembrane proteins that use adenosine triphosphate (ATP) for the translocation of substances across the cytoplasmatic membrane. A critical requirement for the pneumococcus to be able to colonise the nasopharynx and cause invasive disease is to have an efficient nutrient acquisition system; the ABC transporters have been shown to be involved in the transport of a wide range of substances such as cations, sugars, aminoacids, oligopeptides, polyamines, and minerals [332-334]. In previous studies an increase in the expression of genes involved in the ABC transporter system was associated with antibiotic resistant pneumococcal strains [335, 336].

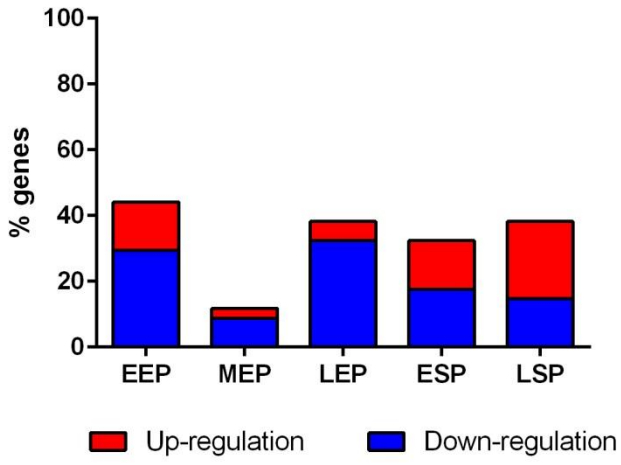

Figure 63. Differential expression of the genes involved in the ABC transporter pathway shown as percentage of up-regulated (red) and down-regulated (blue) genes in A42174 compared to D39.

More ABC transporters genes were down-regulated in A42174 throughout the different growth phases than up-regulated (Figure 63). During exponential growth the

percentage of genes down-regulated was higher than the up-regulated ones, but this observation changed in the last two phases of growth. The growth phases in which the pneumococcus is growing and dividing are the three exponential phases. Down-regulation of the ABC transporters in serotype 1 during these phases may lead to reduced acquisition of nutrients and therefore reduced growth.

6.5.2.3. Phosphotransferase system

There are two main transport systems in the pneumococcus used for the transport of carbohydrates: the phosphoenolpyruvate phosphotransferase system (PTS) and the ATP-binding cassette (ABC) transporters [332]. The PTS transports sugars and can be classified into 6 different families: glucose- and sucrose-PTS, mannitol- and fructose-PTS, lactose- and cellobiose-PTS, mannose-PTS, glucitol-PTS, and galactitol-PTS [337, 338]. Although the PTS is mainly known for its role in sugar phosphorylation, it also regulates a wide variety of metabolic processes and controls the expression of numerous genes [339].

Approximately 38% of the genes involved in the phosphotransferase system were down-regulated in serotype 1 during the early exponential phase (Figure 64). For other growth phases there was a high percentage of differentially expressed genes: 20-60% of the genes were up-regulated and 12-25% of the genes were down-regulated genes in this pathway. As previously mentioned, different types of PTS families are involved in the transport of different types of sugars. The high variability in the gene expression of the PTS genes by serotype 1 may suggest that this serotype might be more successful at using only certain types of sugars, which could be a strategy by serotype 1 to compensate for the down-regulation of other genes involved in the pathway.

Although the main known function of the PTS pathway is sugar transport, it is also involved in other processes in the bacterium. Given the diverse functionality of the pathway it is difficult to know the exact repercussions of the up-regulation or down-regulation of the genes from this pathway without looking into the differential expression of individual genes.

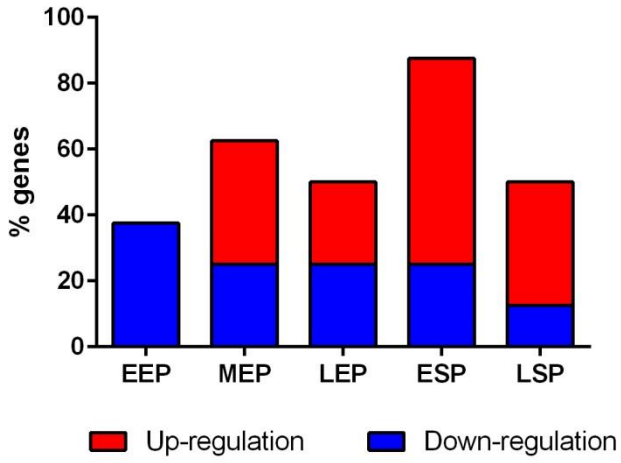

Figure 64. Differential expression of the genes involved in the phosphotransferase system pathway shown as percentage of up-regulated (red) and down-regulated (blue) genes in A42174 compared to D39.

6.5.3. Metabolism of carbohydrates

S. pneumoniae is a strictly fermentative bacterium that relies on glycolytic metabolism to obtain energy; this makes the ability to uptake and metabolise sugars a crucial factor for the fitness of the pneumococcus [340]. Glucose is the preferred source of carbon of the pneumococcus, but the concentration of this sugar in the nasopharynx is low, which makes glycoproteins, such as mucin, good candidates as alternative carbon sources [340, 341]. The pneumococcal surface contains a number of glycosidases, such as NanA, which break down the glycosidic bonds releasing free sugars into the environment [342]. The attenuation of these glycosidases in the pneumococcus leads to a reduced colonisation ability [127, 343].The following figure shows the pathways

proposed by Paixão et.al. for the decomposition of monosaccharides originated from the deglycosilation of host glycans by the *Streptococcus pneumoniae* strain D39 [340].

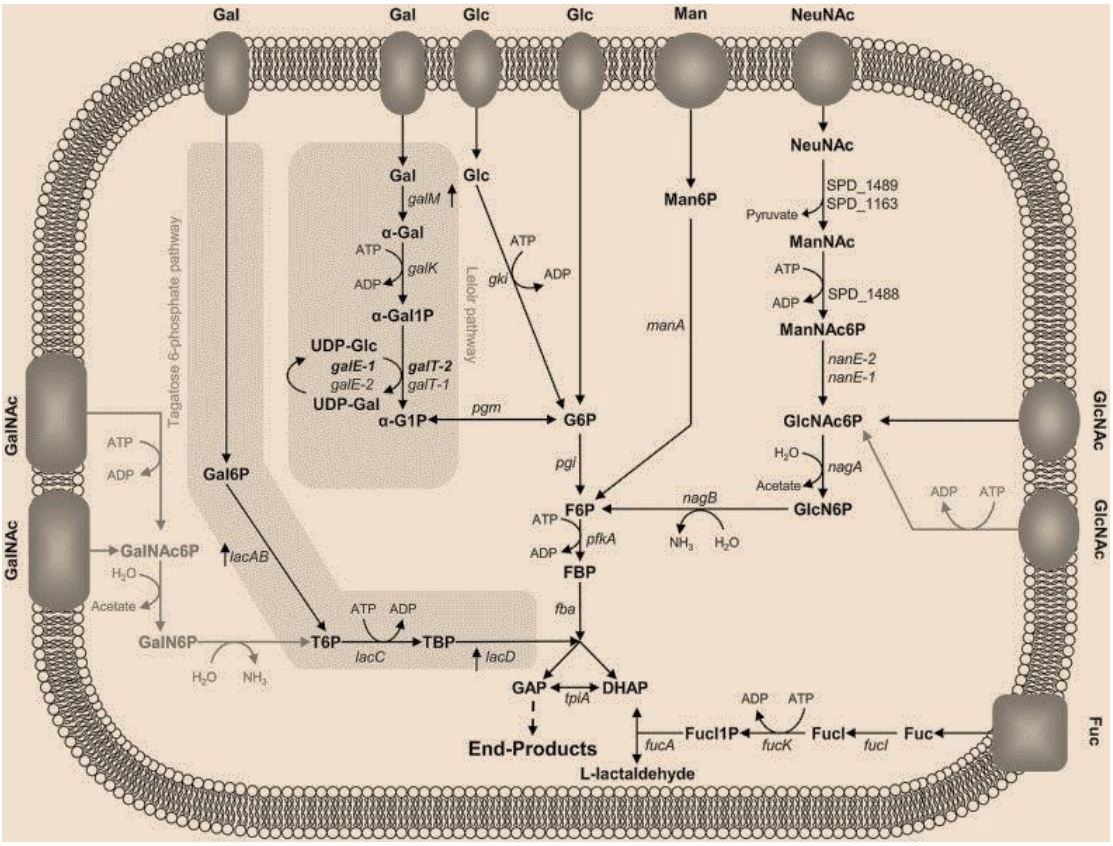

Figure 65. Proposed pathways for the decomposition of monosaccharides originated from the deglycolysation of host glycans by the *Streptococcus pneumoniae* strain D39 [340].

6.5.3.1. Fructose and mannose metabolism

Between 10% and 30% of the genes involved in the metabolism of fructose and mannose were down-regulated in A42174 when compared to D39, with only 10% of the genes being up-regulated in all growth phases with the exception of the late stationary phase where the percentage of up-regulated genes increased to approximately 30% (Figure 66). These observations may suggest that serotype 1 is less able to use fructose and mannose as a source of carbon throughout all the growth phases and therefore less efficient in the uptaking of sugar which would be detrimental in an environment with sugar limitations.

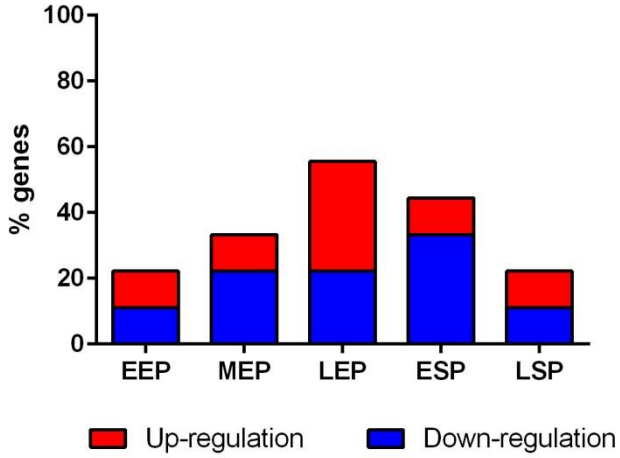

Figure 26. Differential expression of the genes involved in the metabolism of fructose and mannose shown as percentage of up-regulated (red) and down-regulated (blue) genes in A42174 compared to D39.

6.5.3.2. Galactose metabolism

Galactose is the main sugar involved in pneumococcal metabolism [340]. A high proportion (20-80%) of genes involved in metabolism were found to be significantly down-regulated in serotype 1 when compared to D39 in all growth phases except the early stationary phase (Figure 67). These observations may suggest that, as well as mannose and fructose, galactose is not easily metabolised by serotype 1.

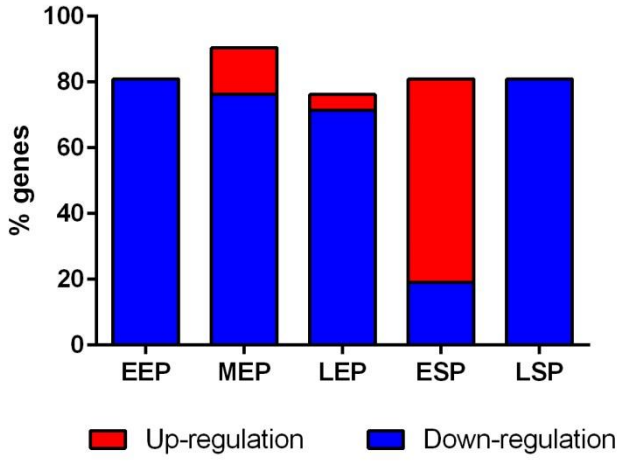

Figure 67. Differential expression of the genes involved in the metabolism of galactose shown as percentage of up-regulated (red) and down-regulated (blue) genes in A42174 compared to D39.

6.5.3.3. Starch and sucrose metabolism

Similar patterns of differential expression were observed in the starch and sucrose metabolism pathway, where a big proportion of genes (10-50%) were down-regulated throughout most of the stages of growth (Figure 68).

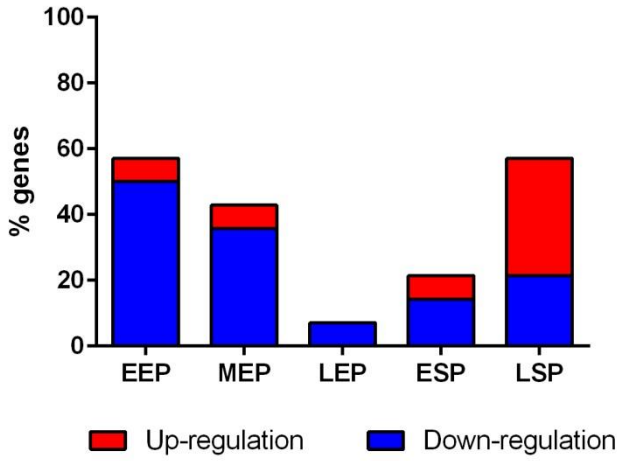

Figure 68. Differential expression of the genes involved in the metabolism of starch and sucrose shown as percentage of up-regulated (red) and down-regulated (blue) genes in A42174 compared to D39.

The late exponential phase showed the lowest percentage of down-regulated genes involved in the starch and sucrose metabolism which may suggest that serotype 1 is more capable of metabolising starch and sucrose during the late exponential phase. A high percentage of genes involved in the metabolism of starch and sucrose (approximately 40%) were up-regulated in the late stationary phase probably induced by an increase in sugar release during the death of bacteria in this phase.

6.5.3.4. Glycolysis and gluconeogenesis

Finally, the gene expression of genes involved in the glycolysis and gluconeogenesis pathways was studied. As observed in Figure 69, the gene expression pattern of this pathway showed an important down-regulation of genes throughout all stages of

growth (10-35%) and a consistent up-regulation of approximately 12% of the genes throughout all the stages of growth.

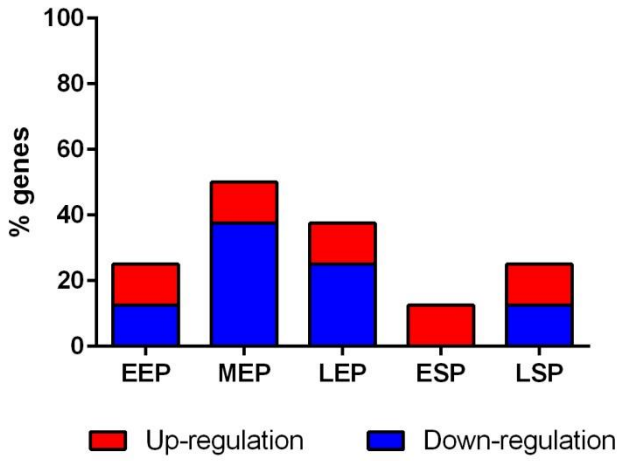

Figure 69. Differential expression of the genes involved in glycolysis and gluconeogenesis shown as percentage of up-regulated (red) and down-regulated (blue) genes in A42174 compared to D39.

In summary, the analysis of the expression of genes involved in the metabolism of sugars shows a high percentage of down-regulated genes during exponential growth. These observations may suggest that serotype 1 could be less capable of causing nasopharyngeal colonisation due to the reduced ability of the bacterium to metabolise the sugars present in the human nasopharynx [342]. Since the concentration of sugars in blood has been reported to be up to 6 times higher and 12 times higher than in nasal secretions and in lower airway secretions respectively, this reduced expression of genes involved in the metabolism of sugar might not have an effect on the growth of serotype 1 in blood [341].

6.5.3.5. Pyruvate metabolism

The absorption of sugars is an essential process for the growth and survival of the pneumococcus. Sugars are uptaken through different transport mechanisms (i.e.

phosphotransferase system) and metabolised by the classical Embden-Meyerhof-Parnas pathway which produces pyruvate and NADH and a net gain of 2 ATP molecules per mole of glucose [344]. Lactic acid is the main metabolic end product of this pathway (Figure 70) [344].

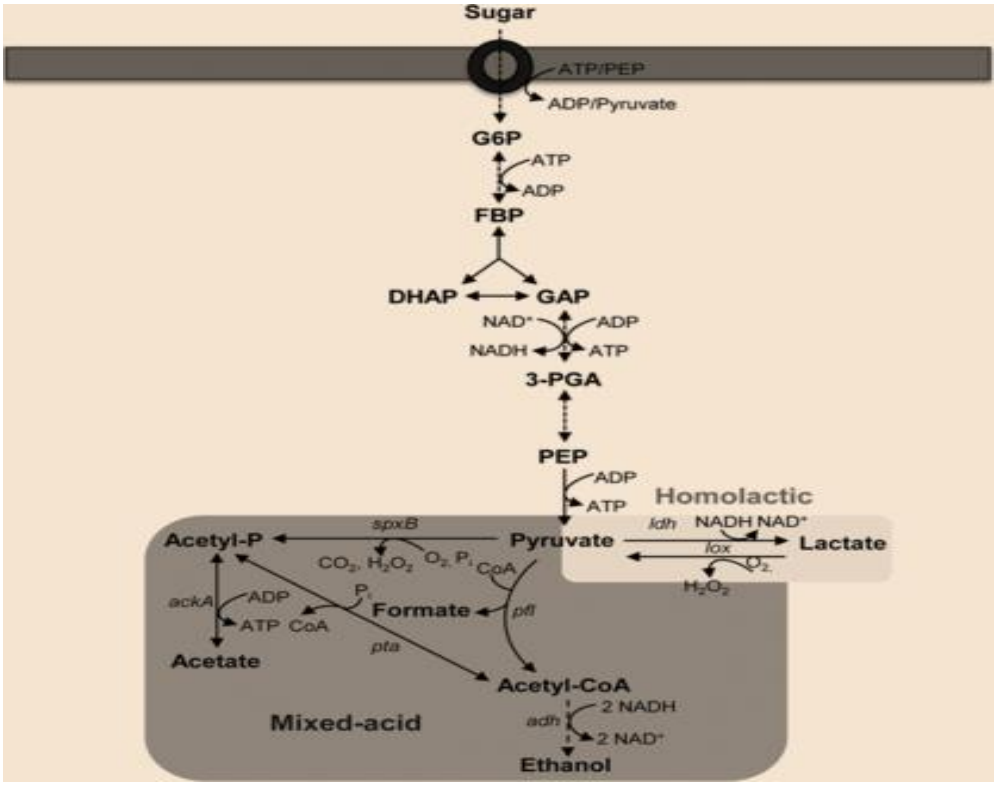

Figure 70. Schematic representation of the metabolism of sugar by *S. pneumoniae* [344].

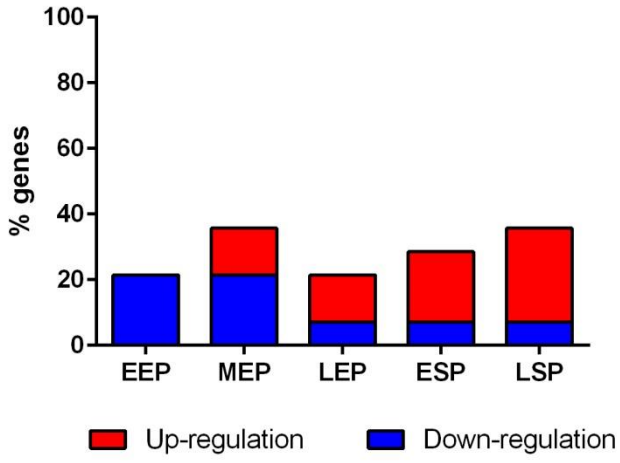

Figure 71. Differential expression of the genes involved in the metabolism of pyruvate shown as percentage of up-regulated (red) and down-regulated (blue) genes in A42174 compared to D39.

As observed in Figure 71, approximately 20% of the genes from this pathway were down-regulated in the first two growth phases. On the contrary, there was a higher proportion of up-regulated genes in serotype 1 during the last three phases of growth. As previously observed, the transport of sugars by serotype 1 is less efficient than in D39 which is detrimental for the growth of the bacterium. In serotype 1, the up-regulated genes in the pyruvate metabolism pathway might be an effort from the bacterium to overcome the reduced income of sugars by metabolising those sugars more efficiently.

6.5.4. Biosynthesis

6.5.4.1. Fatty acid biosynthesis

There are different genes involved in the biosynthesis of fatty acids, and the fatty acid composition of the pneumococcal membrane is closely regulated by the *fab* gene cluster [345]. It has been observed in previous studies that up-regulation of the *fab* gene cluster causes changes in the membranes which makes the bacteria more sensitive to low pH [345].

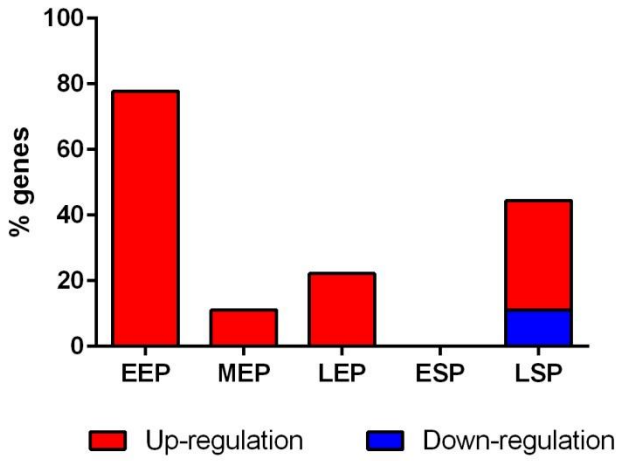

Figure 72. Differential expression of the genes involved in the biosynthesis of fatty acids shown as percentage of up-regulated (red) and down-regulated (blue) genes in A42174 compared to D39.

As observed in Figure 72, the genes in the fatty acid pathway were mostly up-regulated in the serotype 1 strain A42174 throughout all the growth phases, compared to D39. As previously mentioned, up-regulation of the *fab* gene cluster leads to higher sensitivity to low pH which means that serotype 1 is likely to be more sensitive in low pH environments. Previous publications report that the pH of the human nasopharynx is approximately 6.2, whilst the pH of blood is approximately 7.4 [346, 347]. Therefore, up-regulation of the *fab* gene cluster in serotype 1 may explain why serotype 1 is not very efficient during nasopharyngeal colonisation but commonly causes invasive disease.

6.5.4.2. Peptidoglycan biosynthesis

Peptidoglycan, also known as murein, is an essential and specific component of the bacterial cell wall that provides protection to the bacterium; degradation or inhibition of its biosynthesis results in the lysis of the bacterium [348]. In the pneumococcus there are mainly two groups of genes that are responsible for the cell wall synthesis: the *mur* genes which encode enzymes that are involved in the synthesis of peptidoglycan precursors, and the *pbp* genes which encode peptidoglycan synthases [349-351].

As observed in Figure 73, approximately 10-30% of the genes involved in this pathway were down-regulated in serotype 1, when compared to D39, except in the early stationary phase where 30-40% of the genes were up-regulated. Since peptidoglycan is an essential component of the pneumococcal cell wall these observations may suggest that the growth rates of serotype 1 could be reduced when compared to D39 in all phases except in the early stationary phase.

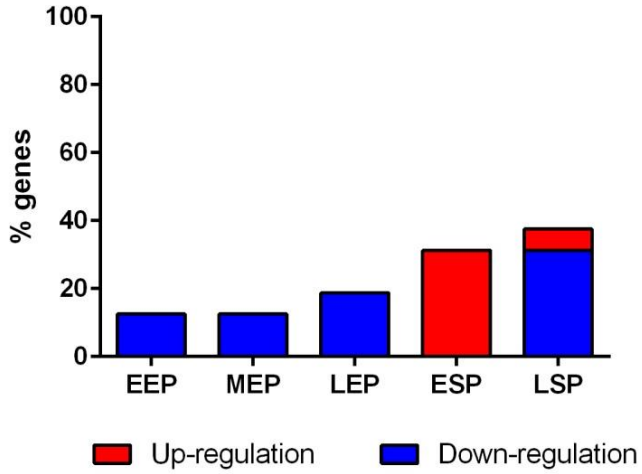

Figure 73. Differential expression of the genes involved in the biosynthesis of peptidoglycan shown as percentage of up-regulated (red) and down-regulated (blue) genes in A42174 compared to D39.

6.5.5. Two-component system

The two-component systems, also known as two-component signal transduction systems, are an important mechanism through which bacteria are able to perceive and respond to environmental stimuli [352]. The two-component systems are composed of two proteins: a membrane associated sensor which receives the stimuli from the outside and a cytoplasmatic cognate response regulator which initiates the cell response to the stimuli [353]. There are 13 known two-component systems in the pneumococcus, and previous studies have shown that some of them have a key role in virulence [353-355]. It is believed that two-component systems may regulate the expression of pneumococcal virulence factors depending on the external stimuli [356, 357].

The genes involved in the two-component system pathways were significantly down-regulated in serotype 1 when compared to D39 and this observation was consistent throughout all the stages of growth (Figure 74). These observations may suggest that serotype 1 might be less effective at responding to external stimuli than D39 which could explain its lower ability to cause nasopharyngeal colonisation. The nasopharynx

is a complex system where different species co-exist and compete for the niche, therefore the reduced ability of serotype 1 to quickly respond to environmental stimuli might result in out-competition by other serotypes or species.

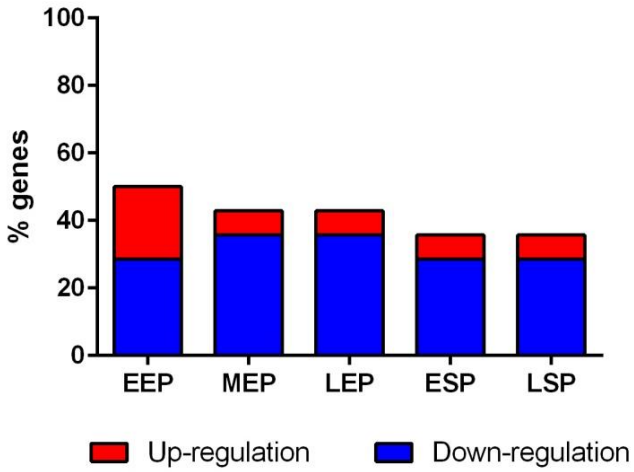

Figure 74. Differential expression of the genes involved in the two-component systems pathways shown as percentage of up-regulated (red) and down-regulated (blue) genes in A42174 compared to D39.

6.6. Analysis of gene expression of virulence factors in A42174 compared to D39

There are a great number of pneumococcal genes that are required for the virulence of the pneumococcus; the products of these genes are known as virulence factors [12]. Absence of certain virulence factors in the pneumococcus has been associated with reduced virulence [12]. Nonetheless, although the presence or absence of these genes might determine the level of virulence, it has previously been reported that differential expression of virulence factors is an important tool to understand differences in virulence between different strains or serotypes [322]. A list of well-characterised virulence factors was selected for this analysis based on their reported role in nasopharyngeal carriage and/or invasive disease (Table 14) [12, 36].

Gene name	D39 identifier	A42174 identifier	Gene Product
bgaA	SPD_0562	SPQ_00804	Beta-galactosidase
cbpA	SPD_2017	SPQ_00113	Choline binding protein A
cbpC	SPD_0345	SPQ_00583	Choline binding protein C
cbpD	SPD_2028	SPQ_00123	Choline binding protein D
cbpE	SPD_0821	SPQ_00865	Choline binding protein E
cbpF	SPD_0357		Choline binding protein F
cbpG	SPD_0356	SPQ_00593	Choline binding protein G
cps2A	SPD_0315	SPQ_00548	Integral membrane regulatory protein
cps2B	SPD_0316	SPH_0456	Tyrosine-protein phosphatase
cps2C	SPD_0317		Chain length determinant protein/polysaccharide export protein, MPA1 family protein
cps2D	SPD_0318		Tyrosine-protein kinase Cps2D cytosolic ATPase
cps2E	SPD_0319		Undecaprenylphosphate glucosephosphotransferase Cps2E
cps2F	SPD_0321		Glycosyl transferase family protein
cps2G	SPD_0322		Glycoside hydrolase family protein
cps2I	SPD_0324		Glycoside hydrolase family protein
cps2K	SPD_0326	SPQ_00559	UDP-glucose 6-dehydrogenase
cps2L	SPD_0328		Glucose-1-phosphate thymidylyltransferase
cps2P	SPD_0327		UDP-galactopyranose mutase
cps2T	SPD_0320		Glycoside hydrolase family protein
cps2H	SPD_0323		Polysaccharide polymerase
eno	SPD_1012	SPQ_01975	Binds to plasminogen/phosphopyruvate hydratase
hyl	SPD_0287	SPQ_00521	Breaks down hyaluronan-containing extracellular matrix components
igA	SPD_1018	SPQ_01969	Cleaves human IgA1
lytA	SPD_1737	SPQ_01440	Autolysin - digests the cell wall
nanA	SPD_1504	SPD_1504	Sialidase A
nanB	SPD_1499	SPQ_01320	Sialidase B
pavA	SPD_0854	SPQ_00899	Binds to fibronectin /adherence and virulence protein A
pcpA	SPD_1965		Choline binding protein PcpA
ply	SPD_1726	SPQ_01427	Pneumolysin
psaA		SPQ_01284	Component of the ABC transport system
psaB	SPD_1461	SPQ_01282	Manganese ABC transporter ATP-binding protein
psaC	SPD_1462	SPQ_01283	Manganese ABC transporter permease
pspA	SPD_0126	SPQ_00349	Surface protein - prevents binding of C3 onto pneumococcal surface
spxA	SPD_0178	SPQ_00409	Transcriptional regulator Spx
spxB	SPD_0636	SPQ_01798	Pyruvate oxidase
srtA	SPD_1076	SPQ_01907	Sortase
strH	SPD_0063	SPQ_00287	Beta-N-acetylhexosaminidase - adherence

Table 14. Virulence factors selected for gene expression analysis. Genes in red are genes that were not detected during the annotation process in either A42174 or D39.

6.6.1. Differential expression of virulence factors between A42174 and D39

The level of expression of each of the virulence factors at different growth phases is shown in Figure 75, and the absolute levels of expression for both D39 and A42174 can be found in Section 9.3. The data were normalised to the levels of expression of D39 for each time point.

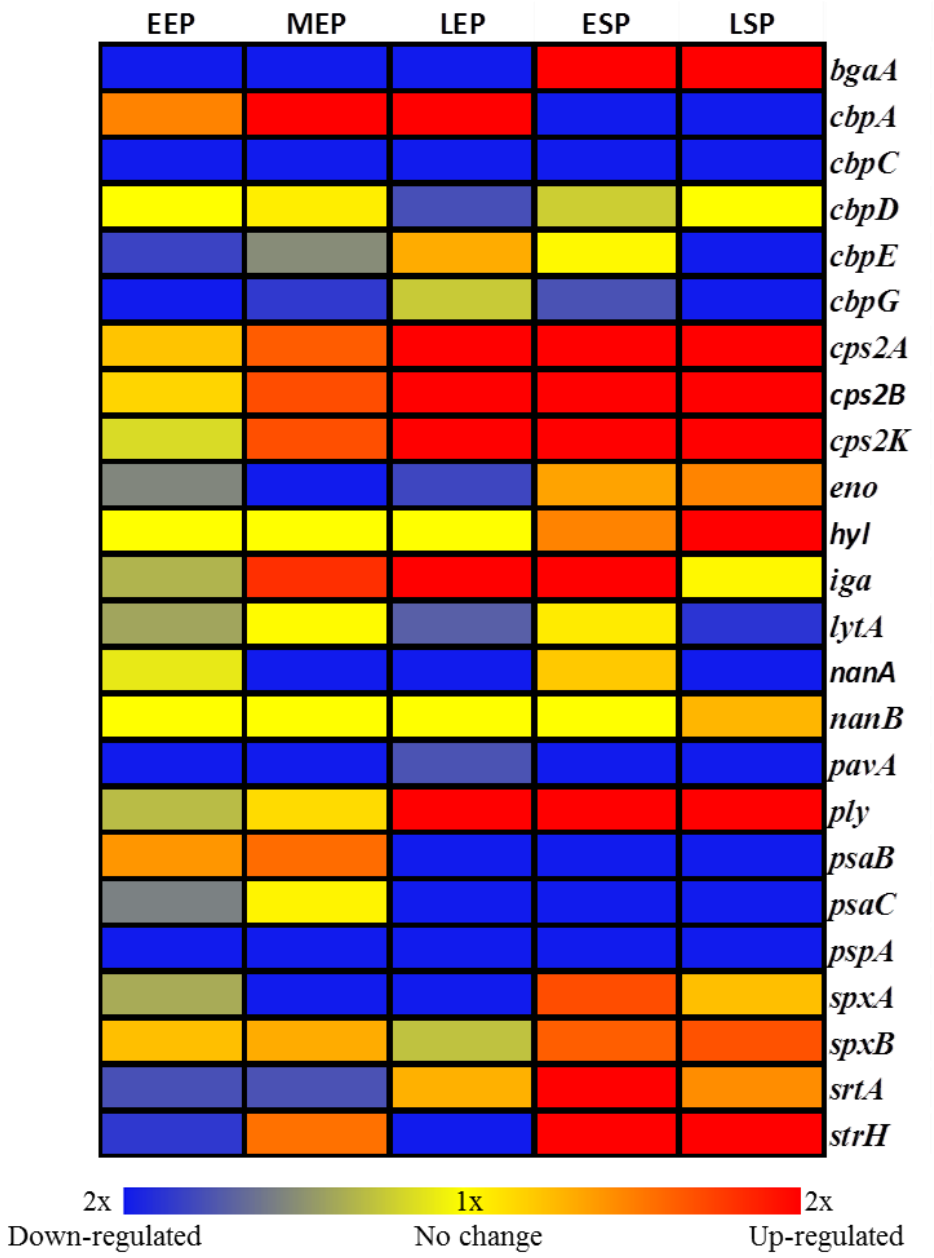

Figure 75. Virulence factors that are differentially expressed in A42174 compared to D39. TPM>10 and >2-fold change. Red is >2-fold up-regulation, yellow is no differential expression and blue is >2-fold down-regulation.

As shown in Table 15, more genes were down-regulated than up-regulated in A42174 compared to D39. In summary, the growth phases with a higher number of differentially-expressed genes were the late exponential phase, the early stationary phase and the late stationary phase. The gene expression of each virulence factor was analysed in detail (Figures 76-86).

	EEP	MEP	LEP	ESP	LSP
Number of down-regulated genes	5	7	8	6	9
Number of up-regulated genes	0	1	6	8	7
Number of differentially expressed genes	5	8	14	14	16

Table 15. Number of virulence genes that are down-regulated and up-regulated in A42174 when compared to D39 in the different stages of growth.

6.6.2. Capsule genes: *cps2A, cps2B* and *cps2K*.

The polysaccharide capsule is known to be the major pathogenicity determinant in the pneumococcus. All virulent strains express capsule and there are over 90 different capsular types. In serotype 2 there is a series of capsule genes which are called the *cps2* genes [358]. Genes *cps2A* to *cps2J* share a high similarity with those of other serotypes, as well as cps2K which shares 89% similarity with serotype 1 [358]. Due to the variation between the capsule genes of serotype 2 and serotype 1, only the ones with high similarity were found in the annotation process and therefore analysed. Those genes were *cps2A*, *cps2B* and *cps2K*.

As observed in Figure 76, the three capsule genes analysed in this study were up-regulated throughout the whole growth of A42174 when compared to D39, although only significantly in the last three stages of growth. Previous studies have shown that

up-regulation of capsule genes *in vitro* is linked to invasiveness and is inversely proportional to the percentage of carriage prevalence [359]. This finding is consistent with the fact that serotype 1 is, compared to D39, more capable of causing disease but less able to cause nasopharyngeal carriage.

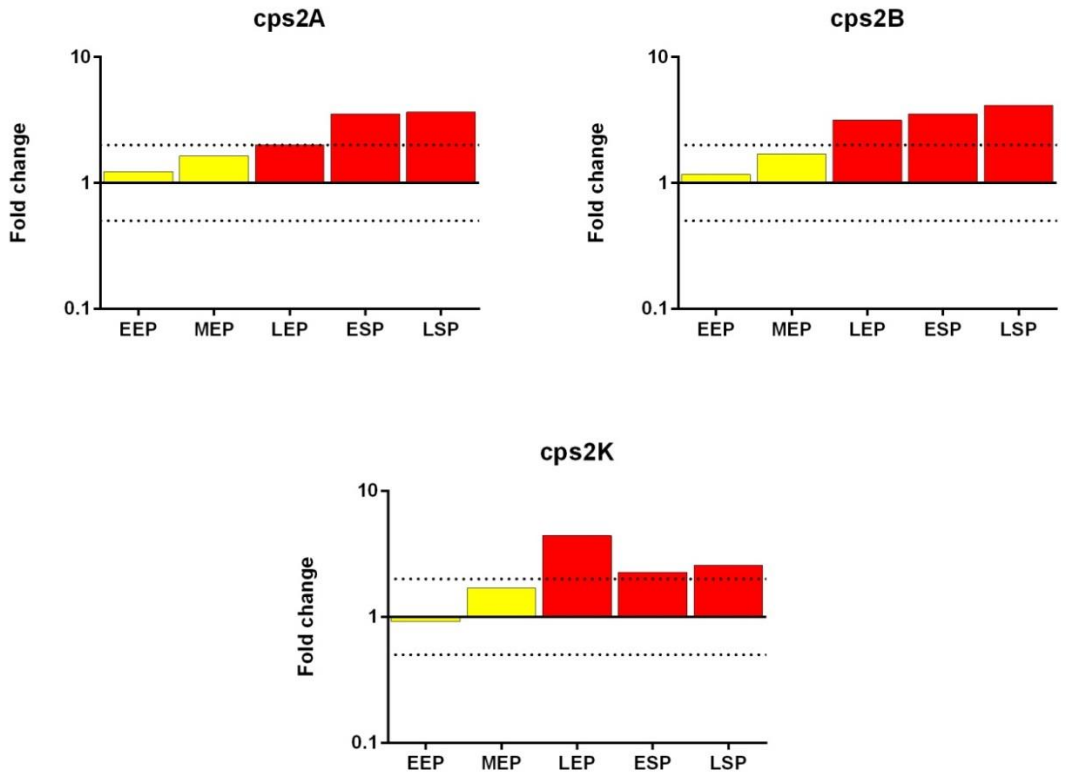

Figure 76. Differential expression of the capsule genes *cps2A*, *cps2B* and *cps2K* in A42174 compared to D39. Data shown as fold change after filtering for TPM values greater than 10. Red = up-regulation, blue = down-regulation and yellow = not significant differential expression.

6.6.3. Pneumolysin

Together with the capsule, pneumolysin is the most studied virulence factor of the pneumococcus [360]. This CDC is not actively secreted by the bacterium, but is released after the lysis of the bacterium by either autolysis or lysis induced by external factors such as antibiotics causing the lysis of eukaryotic cells by forming pores on their membrane [360]. Pneumolysin is able to induce inflammation which aids

bacterial survival and dissemination [360]. Previous studies have shown that increased levels of pneumolysin are linked to increased virulence [361].

As observed in Figure 77, the expression of pneumolysin was significantly up-regulated in serotype 1 during the late exponential phase, the early stationary phase and the late stationary phase. These observations may suggest that up-regulation of pneumolysin might induce inflammation in the nasopharynx leading to its early clearance. On the contrary, during invasive disease the up-regulation of pneumolysin might cause major damage in the tissues leading to increased virulence and spreading of the bacteria.

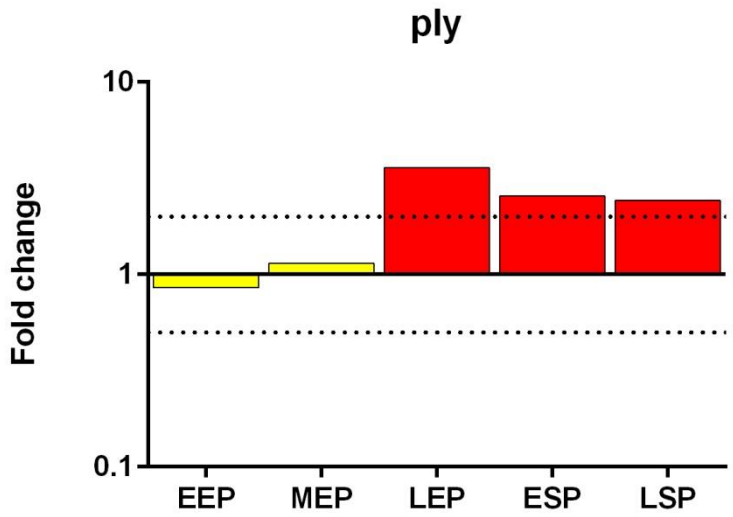

Figure 77. Differential expression of the pneumolysin gene in A42174 compared to D39. Data shown as fold change after filtering for TPM values greater than 10. Red = up-regulation, blue = down-regulation and yellow = not significant differential expression.

6.6.4. Surface proteins

6.6.4.1. Choline binding proteins: CbpA, CbpC, CbpD, CbpE, CbpG, PspA and LytA.

The choline binding proteins are a family of approximately 15 surface proteins that bind to choline residues of lipoteichoic acid and teichoic acid present in the cell wall making the pneumococcus able to interact with host cellular receptors [362, 363]. As

observed in Figure 78, the expression of the relevant protein coding genes was quite variable.

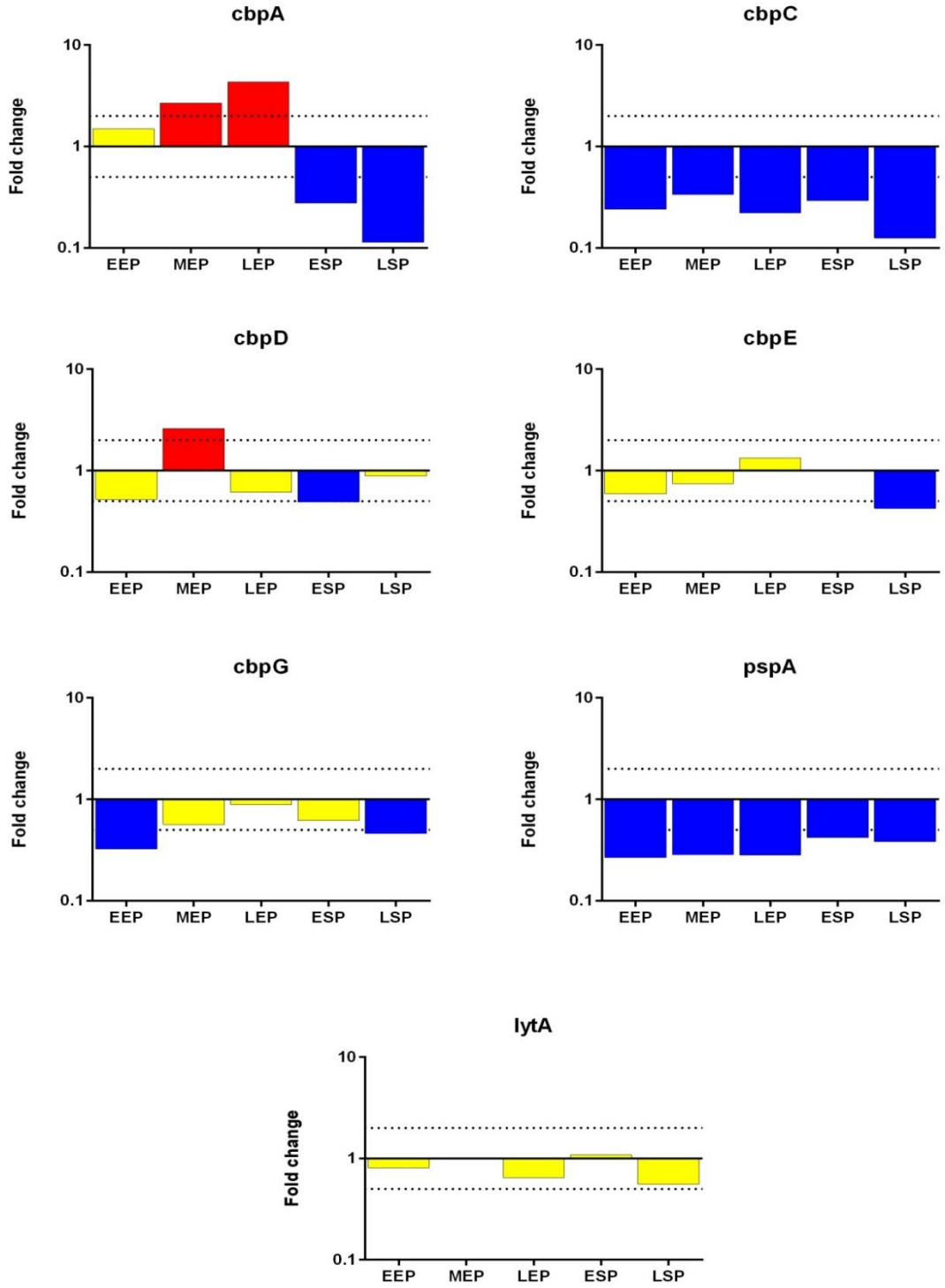

Figure 78. Differential expression of the choline binding proteins genes *cbpA*, *cbpC*, *cbpD*, *cbpE*, *cbpG* and *pspA* in A42174 compared to D39. Data shown as fold change after filtering for TPM values greater than 10. Red = up-regulation, blue = down-regulation and yellow = not significant differential expression.

CbpA has been reported to bind to factor H preventing C3b deposition and therefore preventing opsonisation of the bacterium [363, 12]. CbpA has also been shown to bind to the polymeric immunoglobulin receptor, usually responsible for the secretion of IgA, suggesting that CbpA might be a key protein in the translocation of the pneumococcus through tissues. [12]. The expression of CbpA was up-regulated in A42174 during the mid-exponential phase and the late exponential phase, but down-regulated during the stationary phases of growth (Figure 78). The variability of expression of CbpA during the different growth phases suggests that *in vivo*, the expression of this gene might be determined by environmental conditions given that the nutrient availability during the stationary phases is considerably lower than during the exponential phases.

Similarly to CbpA there was a high variability in the expression of CbpD throughout the different growth phases, making it hard to draw any conclusions from these data (Figure 78).

The *cbpC*, *cbpE*, *cbpG* and *pspA* genes were seen to be mainly down-regulated in serotype 1 (Figure 78). These proteins have been shown to be important in adherence having a direct effect on nasopharyngeal carriage and virulence. These observations suggest that the low ability of serotype 1 to carry could be due to the down-regulation of these adherence proteins [108].

Finally, LytA is an amidase responsible for the cleavage of the N-acetylmuramoyl-L-alanine bond of the pneumococcal peptidoglycan, consequently LytA causes the autolysis of the pneumococcus [12]. As observed in Figure 78, no significant differences were observed in the expression of this protein between the serotype 1

strain A42174 and the serotype 2 strain D39. These observations may suggest that LytA might not be involved in the increased virulence of serotype 1.

6.6.4.2. Pneumococcal surface adhesins: PsaB and PsaC.

The pneumococcal surface adhesins PsaB and PsaC are members of the ABC transporter family and have been shown to be involved in adhesion, which is probably linked to the acquisition of nutrients through the ABC transporter system [12, 152, 222, 364]. The study of the gene expression of *psaB* and *psaC* showed a significant down-regulation of the expression of these proteins in serotype 1 during the late exponential phase, the early stationary phase and the late stationary phase (Figure 79). These observations raise the possibility that serotype 1 might be less capable of adhering and acquiring nutrients than serotype 2 which could have a negative effect on its growth and therefore virulence.

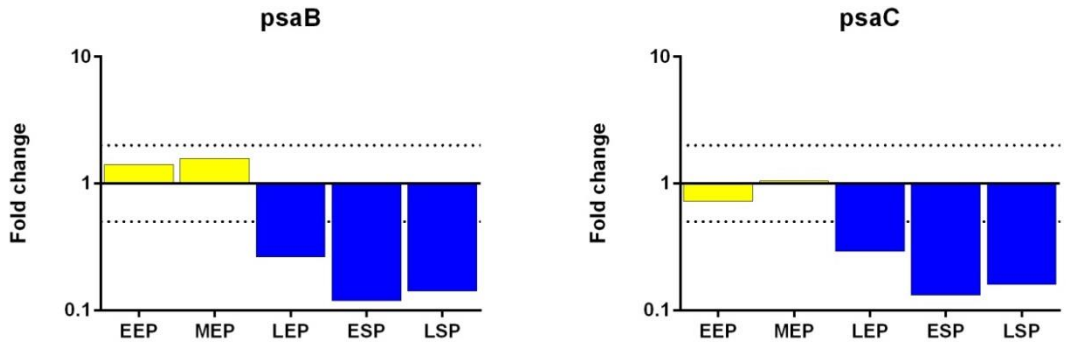

Figure 79. Differential expression of the pneumococcal surface proteins genes *psaB* and *psaC* in A42174 compared to D39. Data shown as fold change after filtering for TPM values greater than 10. Red = up-regulation, blue = down-regulation and yellow = not significant differential expression.

6.6.4.3. Surface exoglycosidases: NanA, NanB, BgaA and StrH.

The nasopharynx is usually the first host niche that the pneumococcus encounters during infection. The concentration of sugars, the main nutrient for the pneumococcus, is low in the nasopharynx hence the pneumococcus needs a mechanism to efficiently

use the sugars available in the environment [340, 341]. The pneumococcus expresses proteins responsible for cleaving extracellular sugars and making the sugars more available for the bacterium; these proteins are the exoglycosidases [342, 365, 366].

The gene expression of the exoglycosidases NanA, NanB, BgaA and StrH is shown in Figure 80.

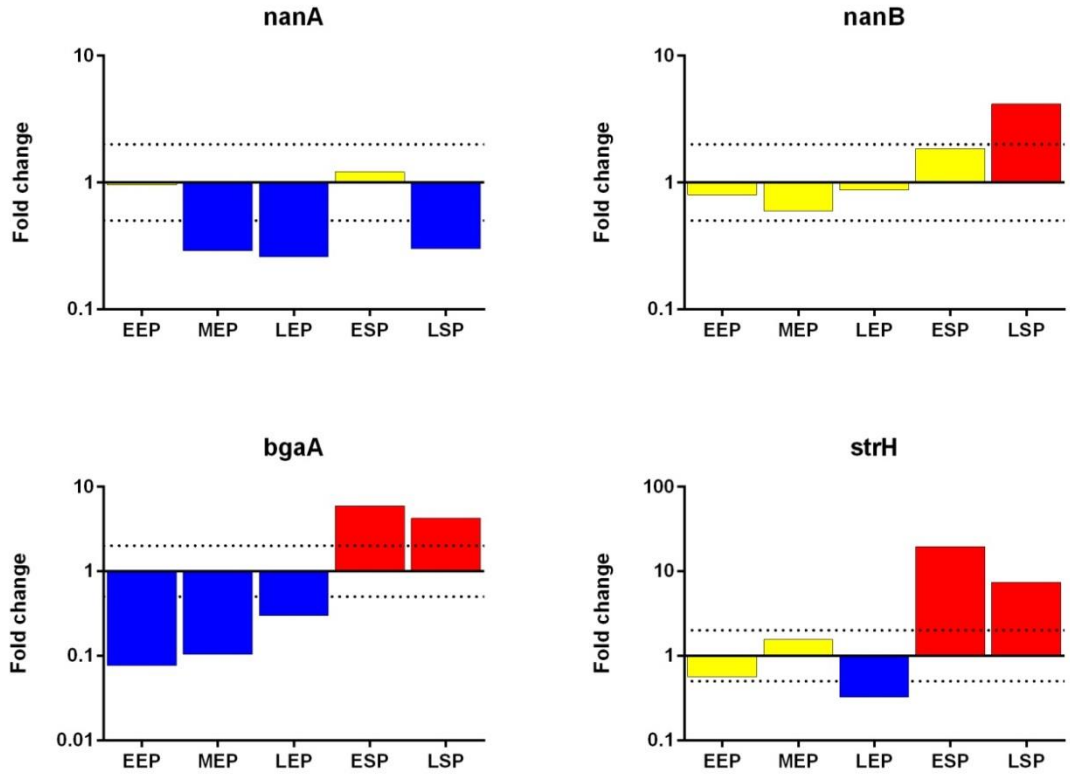

Figure 80. Differential expression of the surface exoglycosidases genes *nanA*, *nanB*, *bgaA* and *strH* in A42174 compared to D39. Data shown as fold change after filtering for TPM values greater than 10. Red = up-regulation, blue = down-regulation and yellow = not significant differential expression.

The expression of the exoglycosidase genes tended to be down-regulated in the first three stages of growth and up-regulated in the stationary phases, with the exception of NanA which was mainly down-regulated throughout all the growth phases. These observations may suggest a lower ability of serotype 1 to break down the sugars of the

environment and therefore a reduced ability to survive in nutritionally poor environments such as the nasopharynx.

6.6.4.4. Enolase

Enolase is a pneumococcal protein that allows the pneumococcus to bind to human plasminogen [136]. It is believed that enolases bind to plasminogen leading to an increased production of plasmin, which is responsible for the degradation of the extracellular matrix [367]. For this reason, enolases promote the adhesion of the pneumococcus to the extracellular matrix in the host inducing its degradation [367, 368]. As shown in Figure 81, *eno* was not differentially-expressed in serotype 1, except for the mid-exponential phase. Down-regulation of enolases in the pneumococcus may suggest a reduction in adherence and therefore virulence, although the expression of enolases could be induced by host factors during colonisation which might help the bacterium become invasive.

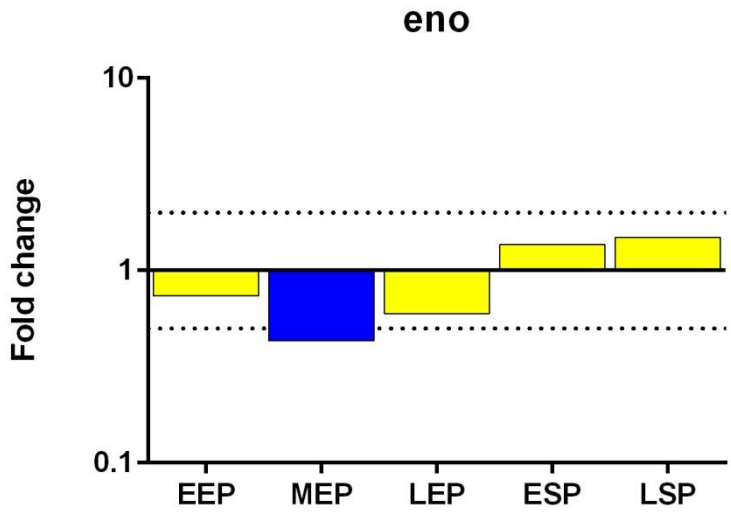

Figure 81. Differential expression of the enolase gene in A42174 compared to D39. Data shown as fold change after filtering for TPM values greater than 10. Red = up-regulation, blue = down-regulation and yellow = not significant differential expression.

6.6.4.5. Hyaluronidase lyase

Hyaluronidase lyase is a surface protein that degrades hyaluronic acid, which is found in mammalian connective tissue and in the extracellular matrix [85]. The degradation of hyaluronic acid by hyaluronidase lyase increases the permeability of mammalian tissues, hence playing an important role in the translocation of the pneumococcus between tissues [36, 85, 152, 369]. As observed in Figure 82, no differential expression was observed during the exponential phases of growth. In contrast, the expression of *hyl* was increased during the stationary phases, especially in the late stationary phase. An increase in the expression of hyaluronidase lyase might play an important role in the increased virulence of serotype 1.

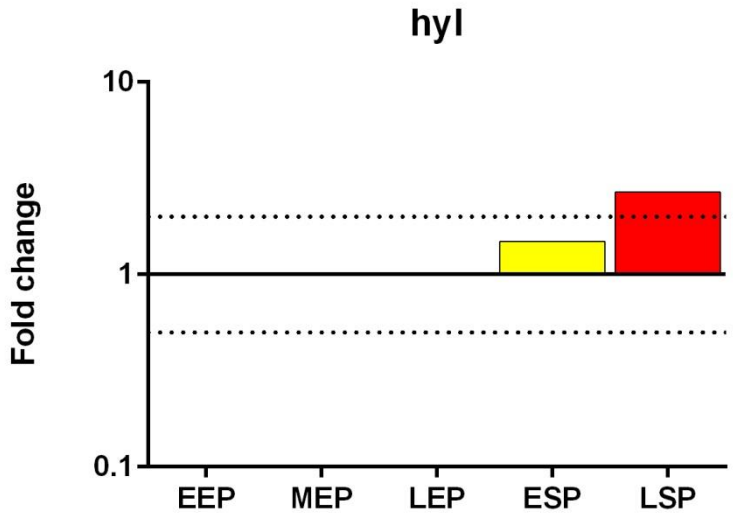

Figure 82. Differential expression of the hyaluronidase lyase gene in A42174 compared to D39. Data shown as fold change after filtering for TPM values greater than 10. Red = up-regulation, blue = down-regulation and yellow = not significant differential expression.

6.6.4.6. Pneumococcal adherence and virulence factor A: PavA

PavA is a pneumococcal protein known to bind to fibronectin and is associated with adherence and virulence in the pneumococcus [132, 370]. Despite binding to fibronectin, it is believed that this protein is not the only pneumococcal protein able to bind to fibronectin since PavA-deficient pneumococci were able to retain 50% of their

fibronectin binding ability [132, 133, 370]. The pathogen-fibronectin interaction mediates the attachment of the pneumococcus to host cells and it has been shown that PavA mutants have a decreased ability to adhere and cause invasive disease [133, 136, 370]. Furthermore, PavA has been shown to protect against recognition and phagocytosis by dendritic cells [133, 370].

As observed in Figure 83, the expression of *pavA* in serotype 1 is down-regulated throughout all the stages of growth. This observation may suggest that down-regulation of *pavA* may reduce the ability of serotype 1 to adhere to epithelial cells during nasopharyngeal colonisation. Nonetheless, PavA-deficiencies have been shown to be important during invasiveness therefore *pavA* might be down-regulated in serotype 1 during nasopharyngeal carriage and an external stimuli could induce the expression of the gene encoding for PavA allowing the bacteria to become invasive. In other words, the expression of pavA could be dependent on external host factors.

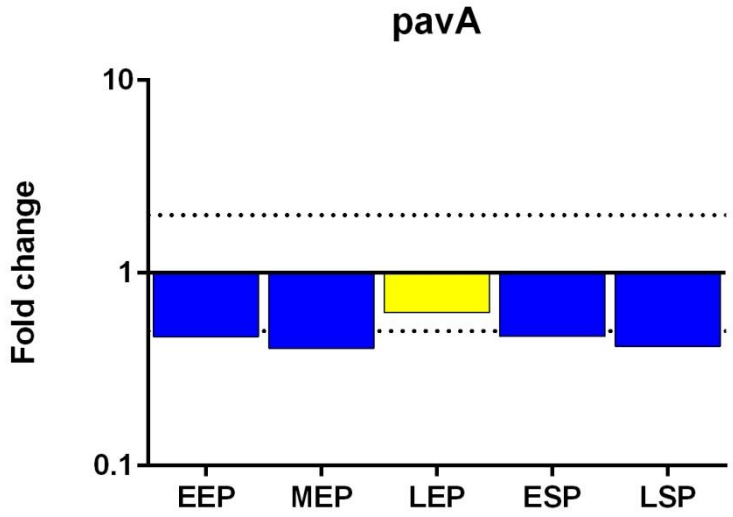

Figure 83. Differential expression of the *pavA* gene in A42174 compared to D39. Data shown as fold change after filtering for TPM values greater than 10. Red = up-regulation, blue = down-regulation and yellow = not significant differential expression.

6.6.5. Sortase A

SrtA is a highly conserved pneumococcal transpeptidase enzyme, also known as sortase A, responsible for the connection of the pneumococcal pili and other specific proteins to the cell wall [132, 371]. The effect of SrtA in colonisation and pathogenesis of the pneumococcus has been previously studied, showing that SrtA contributes to adherence of the pneumococcus to human cells but has no important effect on pathogenesis [372-375]. As observed in Figure 84, sortase A was only up-regulated in early stationary phase of the serotype 1 growth. The up-regulation of this protein in only one growth phase makes it difficult to draw any conclusions on the possible effect of this protein in the virulence of serotype 1.

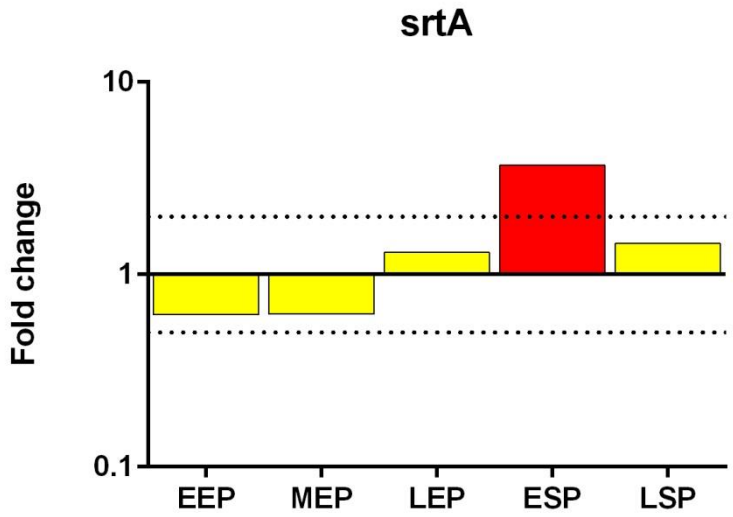

Figure 84. Differential expression of the sortase A gene in A42174 compared to D39. Data shown as fold change after filtering for TPM values greater than 10. Red = up-regulation, blue = down-regulation and yellow = not significant differential expression.

6.6.6. IgA protease

IgA is a pneumococcal protease that specifically cleaves human IgA1 antibodies [136, 377]. Human IgA1 is present in blood at low concentrations, but it is the main antibody present in human mucosal surfaces such as the nasopharynx [376]. The activity of IgA1 proteases lead to the inactivation of the antibodies in the nasopharynx therefore

aiding colonisation of the nasopharynx [377]. As observed in Figure 85, the expression of *igA* in serotype 1 is up-regulated when compared to D39 during the late exponential phase and the early stationary phase. This observation may suggest that serotype 1 clearance might be strongly mediated by the innate immune system and not the acquired immune system.

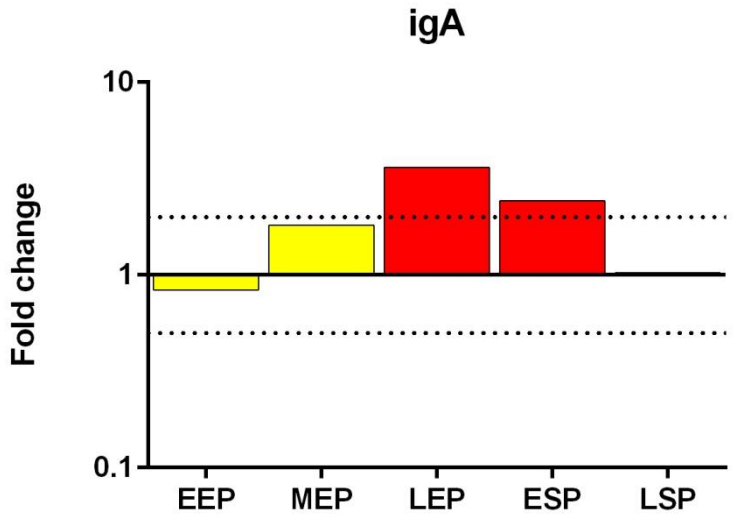

Figure 85. Differential expression of the IgA protease gene in A42174 compared to D39. Data shown as fold change after filtering for TPM values greater than 10. Red = up-regulation, blue = down-regulation and yellow = not significant differential expression.

6.6.7. Pyruvate oxidases

SpxB is a pyruvate oxidase involved in the metabolism of sugar by decarboxylating pyruvate and producing acetyl-phosphate [378]. This process produces oxygen peroxide (H_2O_2) in quantities high enough to kill other members of the nasopharyngeal flora [378].

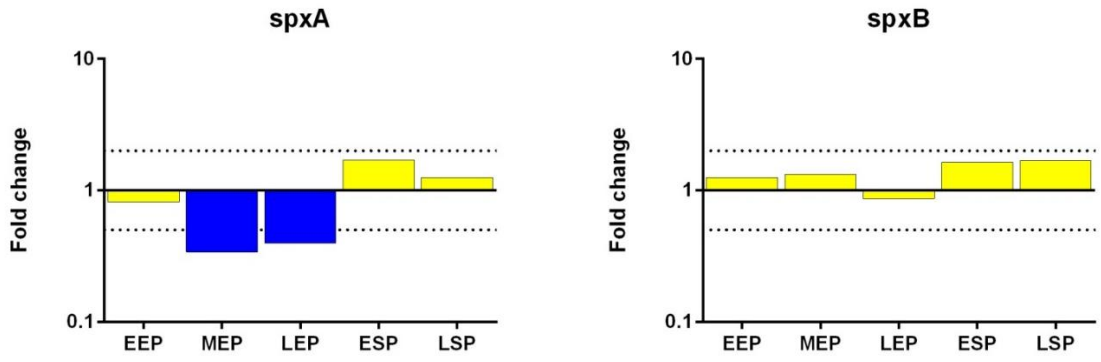

Figure 86. . Differential expression of the pyruvate oxidase spxB and its regulator *spxA* in A42174 compared to D39. Data shown as fold change after filtering for TPM values greater than 10. Red = up-regulation, blue = down-regulation and yellow = not significant differential expression.

As observed in Figure 86 the levels of expression of *spxB* were not significantly different from those in D39. On the contrary, *spxA*, the transcription regulator of *spxB*, was significantly differentially-expressed and was down-regulated in the mid and late-exponential phases in serotype 1 when compared to serotype 2. The down-regulation of the transcription regulator of *spxB* did not translate into a down-regulator of the pyruvate oxidase SpxB.

6.7. Conclusions

The *in vitro* analysis of the gene expression of the serotype 1 strain A42174 in comparison to the serotype 2 strain D39 showed that 25-40% of the genes were differentially expressed in the two strains. The patterns of expression were different between growth phases but there were two clear sub-groups: the three exponential phases shared similar patterns of expression, as well as the two stationary phases, which also shared similar patterns of expression.

The differential expression of different functional categories was studied to understand possible differences in the important cellular functions leading to differences in virulence and survival of the two strains studied.

A high percentage of genes involved in the ABC transporter were down-regulated in A42174 when compared to D39, as well as the genes involved in the metabolism of carbohydrates such as starch, sucrose and galactose. These observations suggest that serotype 1 might be less capable of metabolising sugars than serotype 2. On the contrary, there was a high percentage of up-regulated genes involved in the phosphotransferase system in the A42174 strain, which would be consistent with a mechanism of the bacteria to compensate the down-regulation of the ABC transporter. Moreover, the genes involved in the metabolism of pyruvate were considerably up-regulated in A42174 when compared to D39. Pyruvate is one of the metabolites from the acid lactic biosynthesis pathway, therefore, an increase in the pyruvate pathway could be a mechanism of serotype 1 to compensate for the low sugar intake due to the down-regulation of the ABC transporter genes and the sugar metabolism pathway genes.

The biosynthesis of peptidoglycan was also down-regulated in serotype 1, supporting the idea of a lower growth ability by serotype 1 when compared to serotype 2.

Additionally, there was an up-regulation of the genes involved in the biosynthesis of fatty acids in A42174, which may increase the sensitivity of the pneumococcus to environments with low pH. The pH in the nasopharynx is known to be lower than in the blood, hence serotype 1 is probably more capable of growing in blood than in the nasopharynx, which could explain why it is rarely found in carriage but commonly found causing invasive disease. Furthermore, there was a down-regulation in the genes

involved in the two-component systems which helps the pneumococcus to respond to external stimuli. The nasopharynx is a complex system with a high diversity of species found causing asymptomatic colonisation [13]. As observed in Chapter 5, the interaction between different species or strains in the nasopharynx can have a high impact in the nasopharyngeal carriage patterns of the pneumococcus. Therefore, the down-regulation of genes involved in the ability to respond to external stimuli could be detrimental for serotype 1 reducing the ability of this serotype to cause successful nasopharyngeal carriage.

The differential expression of 24 important pneumococcal virulence factors was analysed in the A42174 and the D39 strains. The number of differentially-expressed genes varied between growth phases, ranging between 5 and 16 differentially expressed genes. The majority of differentially expressed genes were down-regulated in A42174 when compared to D39, although the number of up-regulated genes increased in the last stages of growth.

The analysis of differentially expressed genes in the A42174 and D39 strains showed that the expression of genes involved in invasive disease (i.e. capsule genes, pneumolysin and hyaluronidase lyase) were up-regulated in A42174 when compared to D39. On the other hand, the genes involved in adherence and colonisation (i.e. choline binding proteins, pneumococcal surface adhesins, pyruvate oxidase, pneumococcal adherence and virulence factor A and enolases) were found to be down-regulated in A42174 when compared to D39. These observations suggest that serotype 1 might be more adapted to cause invasive disease than nasopharyngeal carriage. Since nasopharyngeal carriage is a pre-requisite for invasive disease, the reduced ability of serotype 1 to adhere and cause asymptomatic colonisation suggests that serotype 1

might be an opportunistic pathogen that is not good at colonisation but has evolved to be successful during invasive disease. Therefore to compensate the reduced expression of adherence and colonisation related genes, serotype 1 may increase the expression of genes involved in increased invasiveness in the pneumococcus to ensure successful invasiveness.

Although there is a pattern of down-regulation of genes involved in nasopharyngeal colonisation and up-regulation in genes involved in invasiveness it is important to consider that some of the proteins previously mentioned are often involved in both carriage and invasive disease.

In the future, the findings from this study should be validated by analysing the proteomic profile of serotype 1 compared to D39. Furthermore, the *in vivo* analysis of the differential gene expression of A42174 and D39 should be done from bacteria obtained during nasopharyngeal colonisation or form invasive disease to understand the progression of infection and the pathogenic mechanisms of serotype 1.

Discussion

7. Discussion

7.1. Introduction

Streptococcus pneumonaie is a human pathogen that causes severe invasive diseases such as pneumonia, septicaemia and meningitis, as well as less serious non-invasive diseases such as otitis media, bronchitis and sinusitis [379]. The World Health Organisation (WHO) estimates that the pneumococcus accounts for 15% of all deaths of children under 5 years old, killing an estimated 922,000 children in 2015 [2]. Nonetheless, the cases of pneumococcal disease are believed to be underestimated because the incidence statistics are largely based on bacteraemic infections, as it has been estimated that for every bacteraemic infection caused by *S. pneumoniae* there are three non-bacteraemic infections caused by the same pathogen [379]. The high incidence of pneumococcal disease and the high mortality and morbidity associated with this pathogen results in a significant burden for the healthcare systems of both developed and developing countries [5].

Serotype 1 is a highly invasive serotype that is rarely found during nasopharyngeal carriage [269-272]. This serotype is however responsible for approximately 10% of invasive pneumococcal disease cases globally, increasing to up to 30% in some developing countries [200]. Despite the addition of this capsular type to the pneumococcal conjugate vaccine formulation, it has been observed that vaccine efficacy against serotype 1 is limited [192, 294]. For that reason, studying the mechanisms involved in the pathogenicity of pneumococcal serotype 1 is of key importance, to understand how, despite the low carriage rates observed for this serotype, it is still one of the leading causes of invasive pneumococcal disease. Understanding the mechanisms of pathogenesis of serotype 1 will help elucidate how

serotype 1 transmission occurs and may lead to the development of more efficient vaccines against this highly invasive serotype.

In this study, the pathogenesis of African serotype 1 isolates belonging to sequence type 217 (the most common sequence type in Africa), was investigated due to the high incidence of invasive disease in Sub-Saharan Africa caused by serotype 1.

7.2. *In vitro* characterisation of serotype 1

The first approach to understanding the pathogenicity of serotype 1 was to study different aspects of the pneumococcus *in vitro* that have been shown to have an impact on pathogenesis, such as: haemolytic activity, ability to adhere to and invade epithelial cells, capsule thickness, ability to evade phagocytosis and complement deposition.

7.2.1. Haemolytic activity

Pneumolysin is a cholesterol-binding cytolysin that is produced by the pneumococcus causing, at high concentrations, the disruption of mammalian cell membranes leading to the lysis of the cell [74, 75]. Pneumolysin is produced by all clinical isolates, and its haemolytic activity has been shown to be a key factor in immune activation, pro-inflammation and disease progression. Hence, the haemolytic activity of the African ST217 serotype 1 isolates was determined to assess the levels of haemolytic activity in this sequence type as compared to other serotypes. Out of the 20 isolates tested, only the C9741 strain (which was isolated from the cerebro-spinal fluid of a child), was found to have a significantly lower haemolytic activity than the control serotype 2, D39 strain, used as the comparator strain. Considering that the haemolytic activity of the majority of isolates was similar to that of D39, it was considered that ST217 serotype 1 isolates have a fully-functioning pneumolysin, and any variation in the

haemolytic activity between strains is due to inter-strain variation. Moreover, the similar levels of haemolysis observed in the pneumolysin produced by the D39 strain and the serotype 1 strains used in this study suggest that pneumolysin by itself may have little influence on the increased virulence of the ST217 serotype 1 isolates.

7.2.2. Adhesion and invasion

The pneumococcus is able to adhere to and invade epithelial and endothelial cells [133]. Many pneumococcal virulence factors have been shown to be involved in adherence and invasion: CbpA (binds to glycoconjugates from human cells and is involved in translocation between tissues by binding to the IgA receptor), hyaluronate lyase (breaks down the extracellular matrix increasing tissue permeabilisation), neuraminidases (cleaves terminal sugars from host surfaces revealing receptors for adherence), PavA (binds to fibronectin from the extracellular matrix), enolase (binds to human plasminogen), PsaA (acts as an adhesion) and pili (adheres to human cells) amongst others. The absence of any of these virulence factors have been shown to reduce the ability of the pneumococcus to adhere and therefore to become invasive [380, 381]. Since the ability to adhere is a determinant of the success of the pneumococcus to cause colonisation and to eventually cause invasive disease, the adhesion of three serotype 1 ST217 isolates (an isolate from a bacteraemia patient, an isolate from the cerebro-spinal fluid of a meningitis patient, and an isolate form the nasopharynx of a healthy individual) was compared to adhesion and invasion by the well-characterised serotype 2, D39 strain, and a non-typeable clinical isolate. The non-typeable isolate used in this study was unencapsulated, and lack of capsule has previously been associated with increased adherence to epithelial surfaces given the increased exposure of adhesins [60, 61]. Human nasopharyngeal epithelial cells and

human broncho-alveolar epithelial cells were used in this study to assess the ability of the serotype 1 isolates to adhere to tissue in comparison to other serotypes and to assess possible differences between the different serotype 1 isolates. No differences were observed in the ability of the different serotype 1 isolates to adhere to nasopharyngeal epithelial cells. Furthermore, the ability to adhere to these cells was similar in serotype 1 isolates and the D39 strain, although significantly lower than the non-typeable strain. However, there was a significant difference in their ability to invade nasopharyngeal epithelial cells, with the meningitis isolate being the only one able to cause invasion. Although invasion was only seen in one of the three repeats done for the experiment, these observations suggested that the meningitis isolate may be better at invading host cells and therefore could be better adapted to translocate between tissues. Although invasion was also observed for the non-typeable strain, the numbers of invading bacteria for this strain were very low when compared to the number of adhered bacteria. Whilst 14% of the adhered meningitis bacteria were able to invade the nasopharyngeal epithelial cells, only 0.04% of the adhered non-typeable bacteria were able to cause invasion. Considering that the levels of adhesion were similar in all of the encapsulated strains, the increased ability of the meningitis isolate to invade is most likely to be an adapted characteristic particular to this strain and clearly associated with enhanced invasiveness, a potential key factor in its ability to translocate through the brain-blood barrier. The genetic changes leading to this acquired characteristic, remain to be determined.

In broncho-alveolar epithelial cells no adhesion or invasion was observed for any of the encapsulated isolates, the exception being the non-typeable isolate, which was the only strain able to adhere and invade in those cells. Previous studies have shown that the pneumococcus is able to adhere to broncho-epithelial cells; however, the

bacteria:cell ratio in the published experiments was different from the ratio used in this study, a variable that has previously been show to influence the adhesion and invasion rates *in vitro* [313, 382].

7.2.3. Capsule thickness

An important virulence factor that has been shown to have an impact in the adherence of the pneumococcus is the capsule. The exposure of adhesins and other surface molecules is increased in the absence of capsule, therefore, unencapsulated isolates are more able to adhere to epithelial cells. Furthermore, the ability to modify capsule thickness in encapsulated isolates has been shown to be an adaptation of the pneumococcus to increase adherence to host surfaces (by reducing capsule) or to evade the host immune system during invasiveness (by increasing capsule) [56, 58, 60, 61]. Nonetheless, the thickness of the capsule is not the only variable having an effect on adherence and invasiveness since it has been shown that capsular type also determines the interaction of pneumococcal surface proteins and adhesins with the host surfaces [70, 71]. Furthermore, capsular type influences the levels of C3 deposition on the bacterial surface and determines the vulnerability of the pneumococcus to phagocytosis [71]. It has been proposed that the biochemical structure of the different capsular types influence the accessibility and functionality of surface proteins; therefore, differential expression of surface proteins might be altered to overcome these differences [70-72].

The capsule thickness, the vulnerability to phagocytosis and the levels of complement deposition were determined in the three serotype 1 isolates and compared to the serotype 2 strain D39. It was observed that the capsule thickness of the bacteraemia and the meningitis isolates was similar to D39, nonetheless, the capsule of the carriage

serotype 1 isolate was significantly thicker than the bacteraemia and the meningitis serotype 1 isolates as well as the serotype 2 strain D39. These observations contradict the current dogma that suggests that carriage isolates reduce the expression of their capsule to increase their ability to adhere to host surfaces. Since the variation of capsule thickness is a transient adaptation to a specific niche, it is likely that the differences in capsule thickness are only temporary and not reproducible *in vitro*. In order to compare the capsule thickness of the different isolates it would be interesting to determine and compare the thickness of the capsule in the different niches *in vivo* and at different stages of infection, which might also have an effect on polysaccharide production.

7.2.4. Opsonophagocytic killing assay

During an opsonophagocytic killing assay where the bacteria were opsonised using commercial intra-venous IgG, it was observed that the serotype 1 strains were more resistant to phagocytosis by HL-60 neutrophils than the serotype 2 strain. However, no differences were observed between the two serotype 1 isolates used in the OPKA experiments; the bacteraemia and the meningitis isolates. Since no differences were observed between the capsule thickness of the meningitis and bacteraemia serotype 1 isolates and the serotype 2 strain, the levels of C3 deposition were determined to elucidate the mechanisms through which the serotype 1 isolates were more resistant to phagocytosis by HL-60-derived neutrophils than the serotype 2 strain.

7.2.5. Complement deposition

The C3 deposition on the surface of the different isolates showed that the three serotype 1 isolates (meningitis, bacteraemia and carriage) had a lower deposition of

the complement protein C3 on their surface when compared to the serotype 2 strain. No differences were observed between the serotype 1 isolates suggesting that the overall biochemical structure of the type 1 capsule might have an effect on the binding of complement proteins to the bacterial surface. Whilst most pneumococcal serotypes have a neutral or a negatively charged capsule, the serotype 1 capsule contains both negative and positive charges [383]. The positive charges found in the serotype 1 capsule is due to the presence of amino groups (NH_3^+) which have previously been linked to reduced complement deposition [384,385].

In summary, *in vitro* characterisation of serotype 1 isolates showed that the capsule of serotype 1 may have a role in reducing the levels of surface binding by complement proteins resulting in reduced phagocytosis by the host, therefore making this serotype more resistant to host killing and hence more virulent. Moreover, it was observed that the meningitis isolate was the only encapsulated isolate able to invade nasopharyngeal epithelial cells, although only at very low rates. This observation suggests that this isolate might have developed mechanisms to be more invasive by increasing its ability to translocate between tissues. For that reason, *in vivo* models of invasive disease and nasopharyngeal carriage were used to determine possible differences in the virulence of the different serotype 1 isolates.

7.3. Serotype 1 during invasive disease

7.3.1. Progression of infection

The virulence of the different serotype 1 isolates was determined using a well-established *in vivo* model of invasive pneumonia in mice [254]. When serotype 2 strain D39 is used in this model, mice start showing signs of disease approximately 24h post-

infection and become moribund by 48h post-infection with only up to 10-20% surviving the infection. In this study, when the serotype 1 isolates were used in this same infection model, there was also a final survival of approximately 10-20%. However, the progression of disease in serotype 1 infected mice was substantially faster than in mice infected with the serotype 2 strain; approximately 80-100% of the mice succumbed to the infection between 24h and 48h post-infection [254].

When the different serotype 1 isolates were compared, it was observed that the meningitis isolate was significantly more virulent than the bacteraemia and the carriage isolates. However, when the progression of infection was analysed using a timecourse experiment, no significant differences were observed in the bacterial load in the tissues analysed in mice infected with the bacteraemia and the meningitis serotype 1 isolates.

Pneumococcal meningitis is believed to occur by either translocation of bacteria from blood to the meninges through the blood-brain barrier, or through direct translocation from the nasopharynx and the middle ear to the meninges during asymptomatic carriage or during otitis media [13]. The mechanisms that determine whether the pneumococcus is able to translocate from the blood or the nasopharynx to the meninges are, however, unclear [380]. A recent genome comparison of pneumococcal isolates collected from the blood of bacteraemia patients or from the CSF of meningitis patients has concluded that presence or absence of disease associated genes alone does not explain why some isolates are able to reach the meninges and others are not [380]. Nonetheless, in this study it was observed that the bacteraemia serotype 1 isolate tended to be able to translocate into blood quicker than the meningitis isolate, and that the meningitis isolate tended to be present in the brain at higher rates than the

bacteraemia isolate 24h post-infection, even though the bacterial numbers in blood were lower on that specific time-point for the meningitis isolate than for the bacteraemia isolate. Although these observations were not significant, they correlate with the invasion patterns observed in nasopharyngeal epithelial cells, where the meningitis isolate was the only encapsulated isolate able to cause invasion. For that reason, it would be interesting to study possible single nucleotide polymorphisms (SNPs) differences between the bacteraemia and the meningitis isolate, as well as possible differences in the gene expression of key virulence factors *in vivo* which may explain the increased invasiveness of the meningitis isolate when compared to other serotype 1 isolates.

7.3.2. Host immune responses against invasive pneumococcal disease

The host immune response against serotype 1 invasive disease in mice was studied and it was observed that mice succumbed to infection before the adaptive immunity could be effective. Therefore, the mechanisms activated in mice to fight against infection were those associated with innate immunity. However, although innate, it was a delayed response which failed to clear the infection.

In the lung, there was an increase in the levels of IL-17 6h post-infection, which started the recruitment of neutrophils to the site of infection. This was followed by an increase of TNF-α and IL-6 24h post-infection, which are key markers of inflammation and the recruitment of neutrophils, respectively. These observations suggest that by 24h post-infection it was highly likely that there was tissue damage, which would correlate with an increase of bacteria in the lung. In other serotypes such as serotype 2, D39 strain, there is an increase in T regulatory cells during invasive disease, but this study shows

that serotype 1 is not able to induce an increase of T regulatory cells, which may explain increased tissue damage caused by uncontrolled inflammation [386].

In blood, there was also an increase in the number of neutrophils in the last stages of infection which correlates with the increase observed in levels of IL-6, TNF-α and MIP-2, 24h post-infection. Similarly to lungs, there was an increase in the levels of IL-17, 6h post-infection, after which the number of neutrophils started increasing. The percentage of T regulatory cells in blood significantly increased throughout the course of infection; however, the percentage of T cells was reduced.

In the brain, a low level of inflammation was observed with the levels of most cytokines remaining constant throughout the course of infection. Although, pneumococci started seeding into blood as soon as 3h post-infection, there was no translocation into the brain until 12h to 24h post-infection. These observations suggest that the brain is kept clear of pneumococcal infection (at least until the infection becomes systemic by 24hrs) in order to avoid inflammation, which clearly can be significantly detrimental for the survival of the individual [314].

In summary, serotype 1 is a highly invasive serotype that has been shown to be more virulent than the serotype 2 strain used in this study. Intra-serotype virulence differences were observed, with the meningitis isolate being significantly more invasive than the bacteraemia and the carriage isolates, which correlates with the increased levels of invasion previously observed with the meningitis isolate. Furthermore, unlike other serotypes, serotype 1 induces a delayed innate immune response in mice during invasive pneumococcal disease, which also fails to clear the infection and eventually leads to their death through lack of control of infection in

lungs and seeding of pneumococci into blood, causing uncontrollable systemic disease. Moreover, unlike other serotypes, serotype 1 fails to induce T regulatory cells which is likely to lead to uncontrolled inflammation resulting in increased host tissue barrier damage and therefore increased spread and invasiveness of the pneumococcus.

7.4. Serotype 1 during nasopharyngeal carriage

7.4.1. Bacterial progression

Serotype 1 is rarely found during nasopharyngeal carriage [269, 270]. Since nasopharyngeal carriage is a pre-requisite for invasive disease, it is key to understand the patterns of infection by serotype 1 to understand how it is transmitted and therefore, possibly reduce the incidence of disease caused by this serotype.

The three serotype 1 isolates were tested in a well-characterised *in vivo* model of long term nasopharyngeal carriage in mice to determine the pattern of colonisation of serotype 1 and to determine possible differences between the isolates from different host niches. The serotype 2 strain D39 is able to colonise the nasopharynx of mice for a minimum of 28 days at a relatively stable density [238]; however, the serotype 1 isolates were only able to establish colonisation for an average of 14 days. Other serotypes were tested in this study such as 6B and 19F which are serotypes highly associated with nasopharyngeal carriage [29]. Similarly to serotype 2, 6B and 19F isolates were able to establish stable colonisation at a constant density of 10^2 to 10^3 bacteria/mg of nasopharynx throughout the duration of carriage which varies for each serotype (28 days for serotype 2, 21 days for serotype 6B and a minimum of 14 days for serotype 19F): however, the density of serotype 1 was highly variable throughout the same colonisation period. Initially, serotype 1 colonised at a density of 10^1 to 10^2

bacteria per mg of nasopharyngeal tissue, which increased up to 10^3 during the first three days post-infection, only to start declining until eventually cleared, by 14 - 21 days post-infection. No significant differences were observed in the patterns of nasopharyngeal carriage of the three serotype 1 isolates suggesting that, independently of the site of isolation, ST217 serotype 1 isolates might behave similarly during nasopharyngeal colonisation *in vivo*. In summary, serotype 1 is able to establish nasopharyngeal colonisation, but for a shorter period of time and at lower bacterial density than other serotypes (2, 6B and 19F).

7.4.2. Host immune responses to pneumococcal nasopharyngeal colonisation

Detailed host immune responses during nasopharyngeal carriage have been previously studied, leading to the conclusion that the induction of immuno-modulatory cytokines such as IL-10 and TGF-β1 by the pneumococcus, as well as an increased numbers of T regulatory cells, leads to the longer term maintenance of pneumococcal carriage [104]. However, if immune-regulatory responses are not instigated the pneumococcus can be cleared from the nasopharynx by pro-inflammatory responses instead. In this study it was observed that serotype 1 failed to induce immune-regulatory responses, and increases in cytokines associated with pneumococcal clearance was observed which correlated with a decline in bacterial numbers in the nasopharynx. Hence, these observations suggest that serotype 1 is not able to induce the production of immune-modulatory cytokines or T regulatory cells and instead drives an increase of pro-inflammatory cytokines involved in pneumococcal clearance. However, previous studies have shown that purified serotype 1 capsule can activate $CD4^+$ T cells after intra-peritoneal injection inducing IL-10 and TGF-β, which provide immune tolerance [296, 297]. This contradictory result can be explained by the different experimental

protocols used. The studies that suggest induction of T regulatory cells and IL-10 and TGF-β1 cytokines were done using injected purified capsule, and it has been shown that although capsular type has an important effect on the host immune response against the pneumococcus, the interaction of the capsule with bacterial surface proteins anchored to the cell wall can alter the host immune response [70, 71]. The pneumococcus produces many virulence factors that have been shown to have an effect on immune activation, pro-inflammation and disease progression; moreover, there are differences in presence and absence of certain virulence factors and in the expression of those. Therefore, studying the effect of purified capsule *in vivo* might not be reflective of the host immune responses to intact bacteria.

As previously mentioned, serotype 1 is able to establish nasopharyngeal carriage but for a shorter period of time and at a lower density than other serotypes. However, it was still able to induce serotype-specific IgG in mice between days 7 and 14 post-infection. During carriage with the serotype 6B there was also an increase in IgG between days 7 and 14 post-infection, but the levels of IgG were not as high as in mice carrying serotype 1. The levels of IgG in serum returned to base-line levels by day 21 post-infection suggesting that independently of serotype, the production of IgG occurs between days 7 and 14 post-infection. The increased IgG production in serotype 1-carrying mice was probably due to the increased nasopharyngeal pneumococcal density when compared to serotype 6B. Therefore, IgG is always produced during the first 2 weeks of carriage, independently of serotype, and is likely to be proportional to the density of carriage. Nonetheless, the levels of IgG are in every case reduced back to initial levels. Although the levels of IgG in serotype 1-carrying mice was reduced in the last stages of carriage, re-infected mice were able to clear a newly-acquired serotype 1 infection much earlier than naïve mice, indicating that the IgG produced

during serotype 1 carriage is protective against future colonisation events. IgG generated during pneumococcal nasopharyngeal carriage has been proven to also be protective against invasive disease; however, this needs to be proven for serotype 1 [238].

An increase in nasopharyngeal IgA by day 14 was observed only in the mice still carrying the bacteria at that stage, suggesting that production of IgA in serotype 1 is a later immune response. Hence, it was observed than nasopharyngeal carriage with serotype 1 induces an adaptive memory response which prevents from further colonisation events. However, the long-term duration of this protection was not assessed in this study.

7.4.3. Serotype 1 during nasopharyngeal co-colonisation with other serotypes

Many studies have investigated the effect of inter-species nasopharyngeal co-colonisation on the density, duration and serotype distribution of pneumococcal carriage [214, 381, 387, 388]. In addition, co-colonisation with multiple pneumococcal serotypes is also considered an important factor which affects progression into invasive disease, horizontal gene transfer and host-to-host transmission [389-391]. However, the incidence of multiple pneumococcal carriage is considered to be underestimated, with a study suggesting that the incidence of multiple carriage in Bangladesh can range from 3% when using the WHO recommended detection method, to 22% when using a novel molecular technique [392]. In sub-Saharan Africa, up to 40% of the infants studied carried two or more serotypes at the sampling time [317, 277].

Co-colonisation is believed to have an effect on acquisition of new serotypes, rather than in clearance of carriage [393]. However, in this study it was observed that although acquisition of a new serotype during co-colonisation is reduced, the acquisition of a new serotype can also change the carriage patterns of serotypes already colonising the nasopharynx. These observations were serotype-specific, given that only the density of serotype 19F increased with the acquisition of serotype 1 whilst this acquisition had no effect on the density of serotype 6B when this was the already pre-colonising serotype.

In this study, it was observed that serotype 1 is able to colonise the nasopharynx of mice for a shorter period of time and at a lower density than other serotypes; it is likely therefore, that the transmission of serotype 1 occurs during the early stages of colonisation and is also likely to be acquired when a different serotype is already present in the nasopharynx. As the co-infection experiments suggest, if serotype 1 is acquired when another serotype is already colonising the nasopharynx, the duration and density of carriage by serotype 1 is even lower than during single-serotype colonisation suggesting that serotype 1 might be more successful at colonisation when no other serotypes are present in the nasopharynx. Considering that there is a high incidence of pneumococcal carriage in infants and the elderly, serotype 1 should be able to be more efficient during nasopharyngeal carriage in individuals that are not carrying the pneumococcus, i.e. young adults [8]. These observations may explain why the incidence of invasive pneumococcal disease caused by serotype 1 is higher in this age group, as opposed to other serotypes that affect mainly infants and the elderly [270, 271, 272].

The limited carriage ability of serotype 1 is likely to reduce transmission; however, its high virulence results in high incidence of invasive disease by serotype 1 despite the low carriage rates. If the carriage rates of serotype 1 increased, this would lead to an even higher incidence of invasive disease caused by serotype 1. Since PCV13 has been shown to have limited efficacy against serotype 1 it is important to consider how the effect of the vaccine in the carriage incidence of other serotypes might lead to an increase of serotype 1 carriage and therefore, an overall increase in invasive disease cause by this serotype. These observations highlight the importance to develop a more efficient vaccine against serotype 1.

7.5. Gene expression of serotype 1

Differential gene expression is a relatively new technique that allows the comparison of levels of expression of genes that are key for virulence or for the metabolism of bacteria [164]. Some studies have been performed on the differential gene expression of a limited number of virulence factors in serotype 1 compared to other serotypes; however, a more extensive analysis was done in this study. The *in vitro* gene expression in serotype 1 of 24 key pneumococcal virulence factors and 11 pathways involved in metabolism was studied and compared to the *in vitro* gene expression in the serotype 2 strain D39. In the mouse models used in this study it was observed that serotype 1 is more virulent than serotype 2, but is less efficient at nasopharyngeal carriage; however, differential gene expression between these two serotypes was studied *in vitro* due to difficulties in extracting intact RNA from mouse.

Between 25% and 40% of the orthologue genes were differentially expressed between the two serotypes, with a general down-regulation of pathways related to bacterial growth such as the metabolisms of sucrose, starch and galactose. It has previously been

shown that increased growth is linked to increased prevalence of carriage; therefore, the down-regulation of mechanisms involved in growth might explain the reduced ability of serotype 1 to carry [305].

It was also observed that the biosynthesis of fatty acids is up-regulated in serotype 1 when compared to serotype 2. Previous studies show that up-regulation of this metabolic pathway can increase the sensitivity of the pneumococcus to external low pH. The pH varies in different parts of the body; whilst the nasopharynx has a pH of approximately 6.2, the pH in blood is closer to 7.4. Therefore, the up-regulation observed in the biosynthesis of fatty acids in serotype 1 can increase the sensitivity of the pneumococcus in the nasopharynx when compared to blood, suggesting that for this serotype, the nasopharynx is a more hostile environment than blood. Moreover, a down-regulation was observed in genes involved in two-component systems, which are an important mechanism through which the pneumococcus is able to perceive and respond to environmental stimuli. Considering that the nasopharynx is a host niche where a variety of species are present, it is important to have an efficient mechanism to be able to respond to the constant external stimuli produced by other commensals of the nasopharynx. Therefore, the down-regulation of two-component systems in serotype 1 may reduce the ability of this serotype to change and adapt to the constant external stimuli present in the nasopharynx. These observations suggest that serotype 1 is likely to survive more comfortably in a stable niche such as blood, instead of the nasopharynx where there is constant competition with other pneumococcal serotypes as well as other species.

The analysis of the differential expression of the 24 virulence factors showed that virulence factors that are associated with carriage (i.e. choline binding proteins,

pneumococcal surface adhesins, pyruvate oxidase, pneumococcal adherence and virulence factor A and enolases) are down-regulated in serotype 1 when compared to serotype 2. In contrast, virulence factors associated with invasiveness, such as pneumolysin, capsule and hyaluronidase lyase, were found to be up-regulated in serotype 1 when compared to serotype 2.

These observations suggest that serotype 1 is less adapted to colonisation and adherence to nasopharyngeal cells and is more likely to be successful at surviving in blood than in the nasopharynx, which is a more dynamic environment. Therefore, not only does serotype 1 increase the expression of genes encoding key virulence factors that are involved in invasiveness and protective against host immunity (such as pneumolysin and capsule) but it also decreases the expression of those genes related to adherence and nasopharyngeal carriage, adapting itself to conditions more favourable to survival in blood for example than in the nasopharynx.

7.6. Summary

This study shows that serotype 1 is a highly invasive serotype that is able to cause nasopharyngeal colonisation but for a shorter period of time and at a lower density than other serotypes. The zwiterionic capsule it has, exhibits both negative and positive charges and these positive charges might prevent the deposition of the complement protein C3b on the surface of the bacteria therefore reducing its susceptibility to phagocytosis and therefore increasing its virulence.

Moreover, differential gene expression of serotype 1 and serotype 2 showed that those virulence factors involved in nasopharyngeal carriage were down-regulated in serotype 1, whilst virulence factors involved in invasiveness were up-regulated when

compared to serotype 2. These observations suggest that serotype 1 is not adapted to be a successful coloniser, for which it has had to develop mechanisms to overcome the reduced spreading opportunities. Nasopharyngeal carriage is the main reservoir for the pneumococcus and therefore essential for transmission; however, the pneumococcus is also able to transmit during pneumonia. Given the reduced ability to establish nasopharyngeal carriage, serotype 1 may have developed mechanisms to transmit during pneumonia episodes instead of during asymptomatic colonisation. Pneumonic spread of bacteria has been shown to be more virulent than carriage spread, probably due to the up-regulation during invasiveness of key virulence factors [394]. Although the mechanisms leading to this increased virulence remains unclear, it is likely to be a consequence of increased expression of major virulence factors during pneumonia; when the pneumococcus is then transmitted during coughing or sputum it is already highly virulent and is therefore ready to invade. This theory may explain why serotype 1 is a major cause of disease outbreaks and is commonly isolated from communities where a recent outbreak occurred [269].

Additionally, it was observed that the three ST217 serotype 1 isolates used in this study showed no differences during adherence *in vitro* and during nasopharyngeal carriage *in vivo*; however, significant differences were observed in invasiveness, both *in vitro* and *in vivo*. These observations suggest that the ability to cause nasopharyngeal carriage in serotype 1 might be determined by capsule whilst the ability to cause invasive disease might be determined by other non-capsular determinants such as pneumolysin, IgA protease, neuraminidases, hyaluronidase lyase or the pneumococcal adherence and virulence factor PavA.

7.7. Research limitations

Although the aims of this study were accomplished, there were some limitations that need to be taken into account. First, only one isolate form each collection site (i.e. blood, CSF, nasopharynx) was used for investigation; a bigger sample size would have been more representative. There was also a limitation in the number of mice that could be used for each *in vivo* experiment. According to the principle of the 3Rs, animal work should be replaced when possible, the number of animals should be reduced when possible, and the methods used during animal work should be refined to minimise animal suffering. For that reason, a small number of animals were used for these experiments which compromised, in some cases, the statistical analysis of the results. Finally, financial limitations lead to reduced in-depth studies of the gene expression of serotype 1 by limiting the transcriptomic study to only the bacteraemia isolate.

7.8. Future work

In this study it was found that the host immune responses to ST217 serotype 1 isolates are different from those to other serotypes, although, the mechanisms leading to these different responses are still unknown. For that reason, further study is needed to understand the mechanisms that lead to the differential induction of host immunity in this serotype compared to other clinical serotypes.

It was observed that nasopharyngeal co-colonisation had an effect on the carriage patterns of the pre-colonising serotype and also on the carriage patterns of the newly-acquired serotype; however, this effect was different depending on the serotype combinations used during co-colonisation. A deeper investigation is needed on the interaction of serotypes and their effect on carriage and invasiveness, for that reason a

wider range of serotypes should be studied in co-colonisation studies to further understand the mechanisms that lead one serotype to enhance the density of one serotype but not others.

The *in vitro* study of the differential gene expression of the bacteraemia serotype 1 isolate compared to the serotype 2 reference strain D39 showed important differences in the metabolism and the expression of virulence factors of those isolates.

It would be interesting to study the differential genes expression of serotype 1 and serotype 2 *in vivo*; however, there are currently limitations in the process of extraction of good quality and importantly quantity of RNA from tissue to be able to undertake RNAseq analysis. When the extraction of RNA from tissue is optimised it would be interesting to do the analysis of gene expression of serotype 1 *in vivo* at different stages of infection and using bacteria collected from different sites of infection (nasopharynx, lung, blood and brain). Moreover, considering the findings of this study it would be interesting to study the gene expression of the meningitis isolate to understand the mechanisms that make this isolate more invasive than the bacteraemia and the carriage isolates.

In summary, the unique nature of the capsule of serotype 1 is likely to influence its inability to maintain longer periods of nasopharyngeal colonisation; however, this inability may also have allowed serotype 1 to develop other mechanisms of survival, such as causation of invasive disease, with the progression of pneumonia acting as an alternative method of transmission for this serotype.

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8. References

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Appendix

9. Appendix

9.1. Appendix 1: Nasopharyngeal carriage by the serotype 1 strain C9174

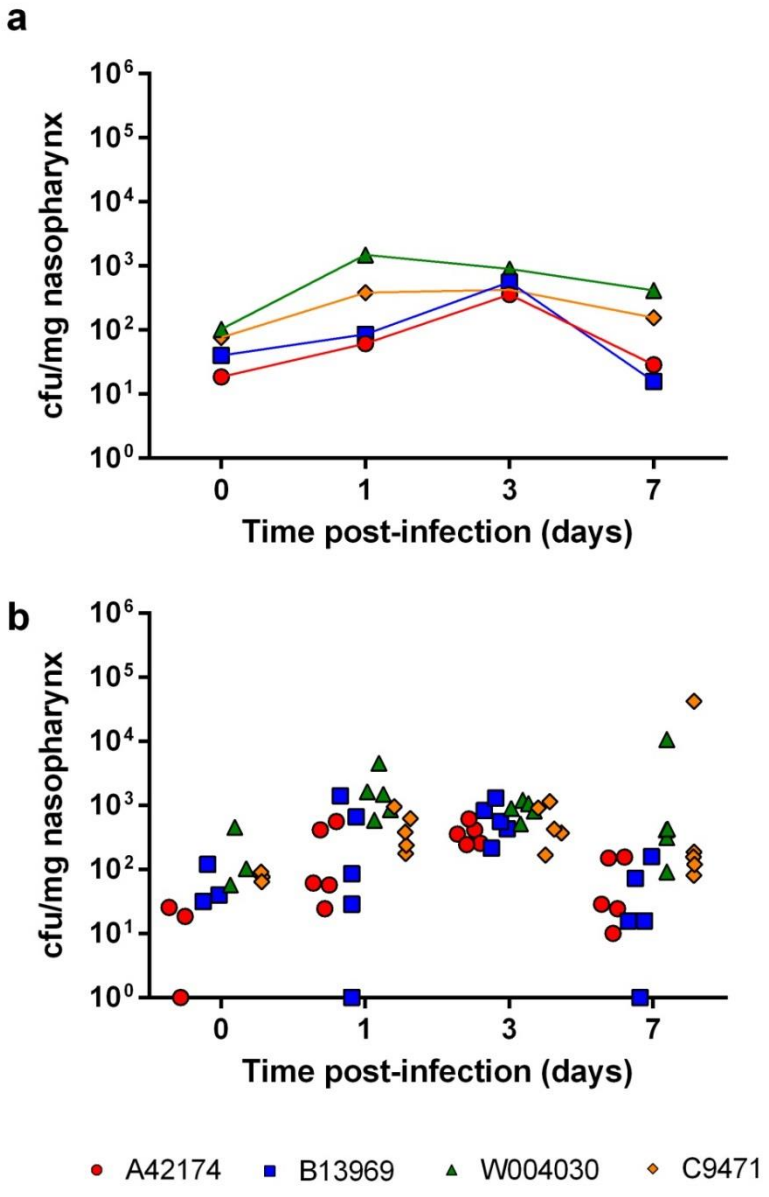

Figure 87. Pneumococcal CFU per mg of nasopharyngeal tissue of MF1 mice infected with the low haemolytic serotype 1 isolate (C9471), the bacteraemia (A42174), the meningitis (B13969) and the carriage (W004030) isolates at days 0, 1, 3 and 7 post-infection shown as median value (a) and CFU of individual mice (b). Three mice were used for the day 0 control, and five mice were used for the rest of the time-points. No significant differences were observed when analysed using a two-way ANOVA.

9.2. Appendix 2: Functional category analysis of the different growth phases

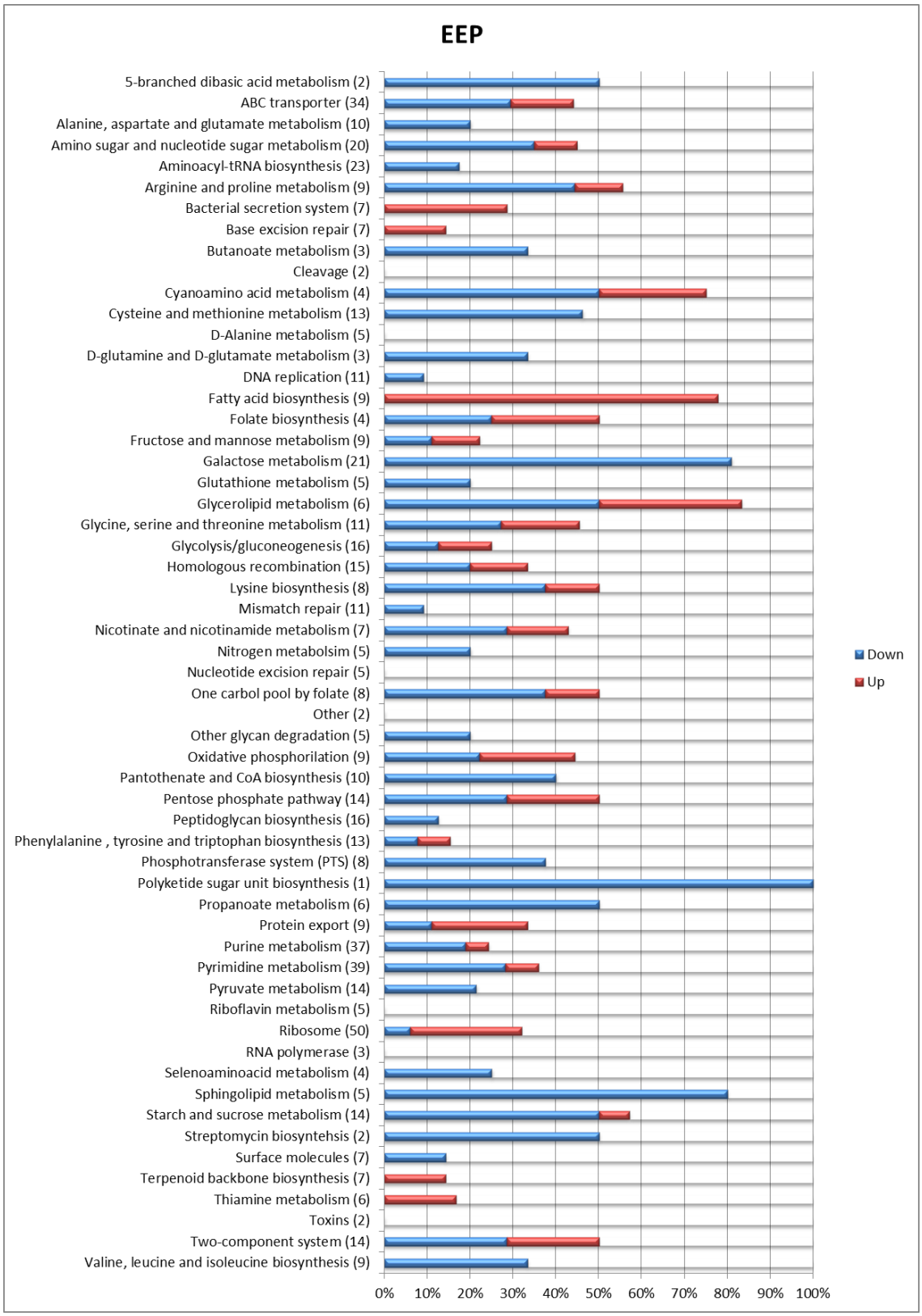

Figure 88. Pairwise comparison showing the percentage of genes in each pathway that are up or down-regulated in A42174 when compared to D39 during the Early Exponential Phase

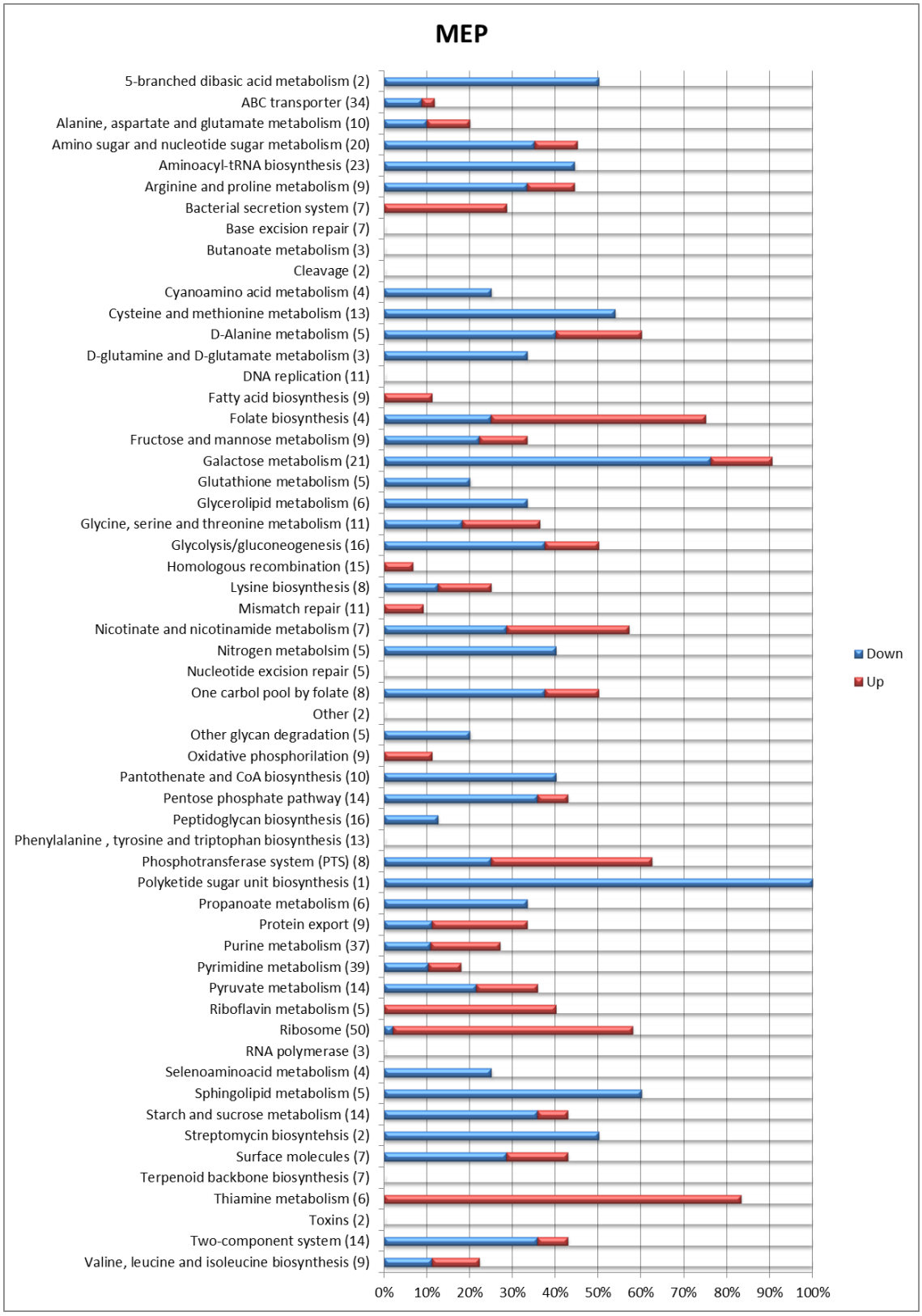

Figure 8927. Pairwise comparison showing the percentage of genes in each pathway that are up or down-regulated in A42174 when compared to D39 during the Mid Exponential Phase

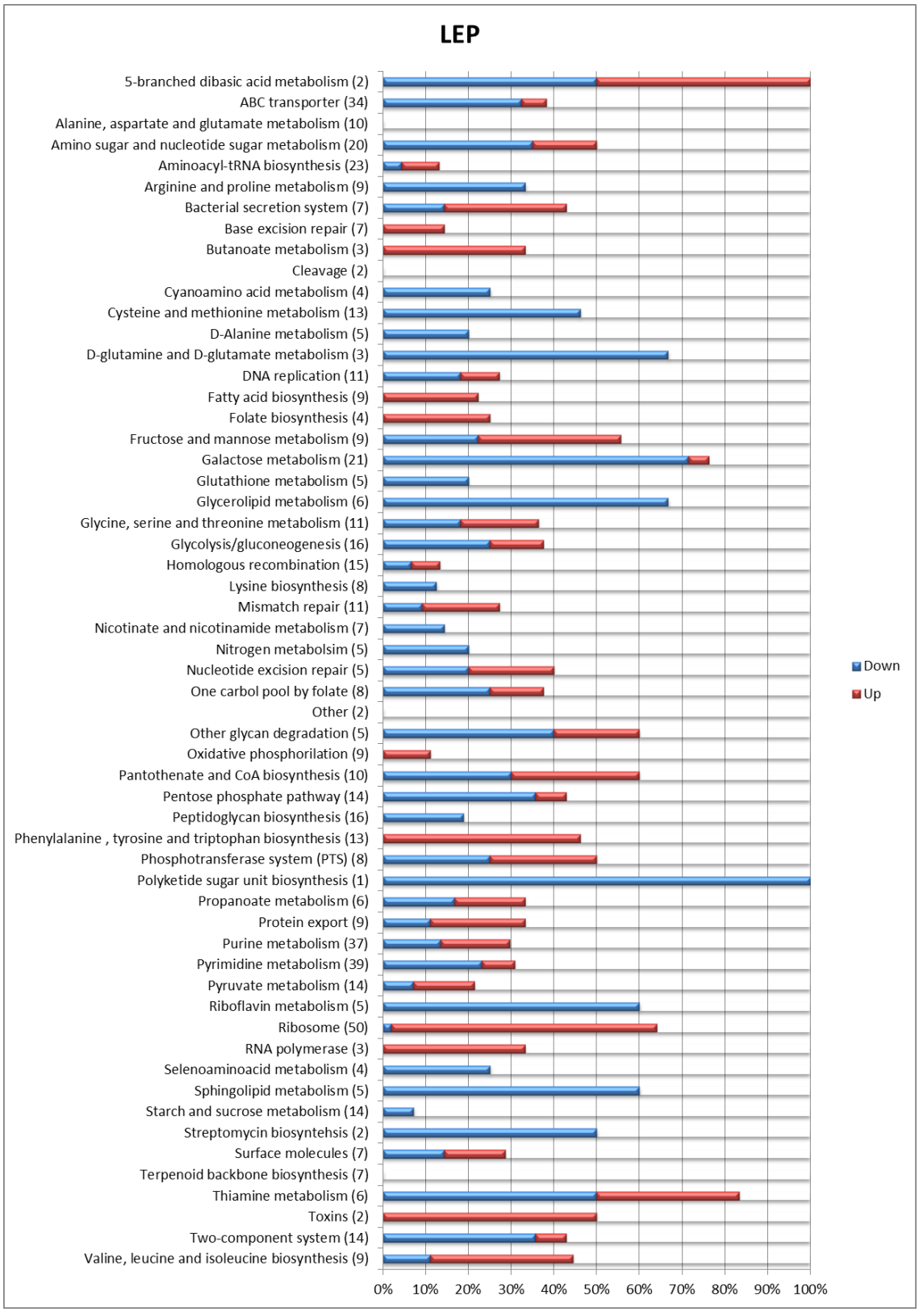

Figure 9028. Pairwise comparison showing the percentage of genes in each pathway that are up or down-regulated in A42174 when compared to D39 during the Late Exponential Phase

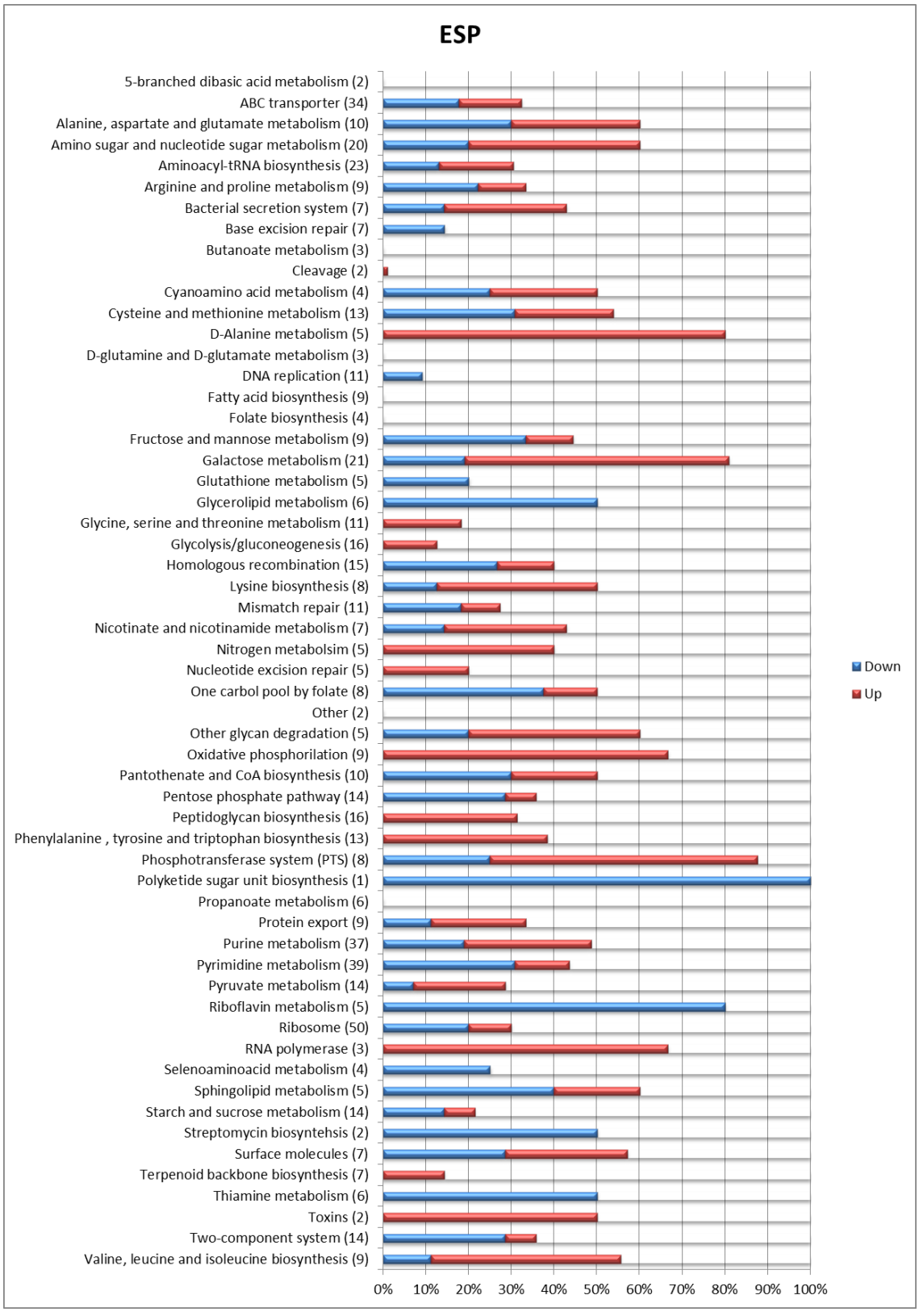

Figure 91. Pairwise comparison showing the percentage of genes in each pathway that are up or down-regulated in A42174 when compared to D39 during the Early Stationary Phase

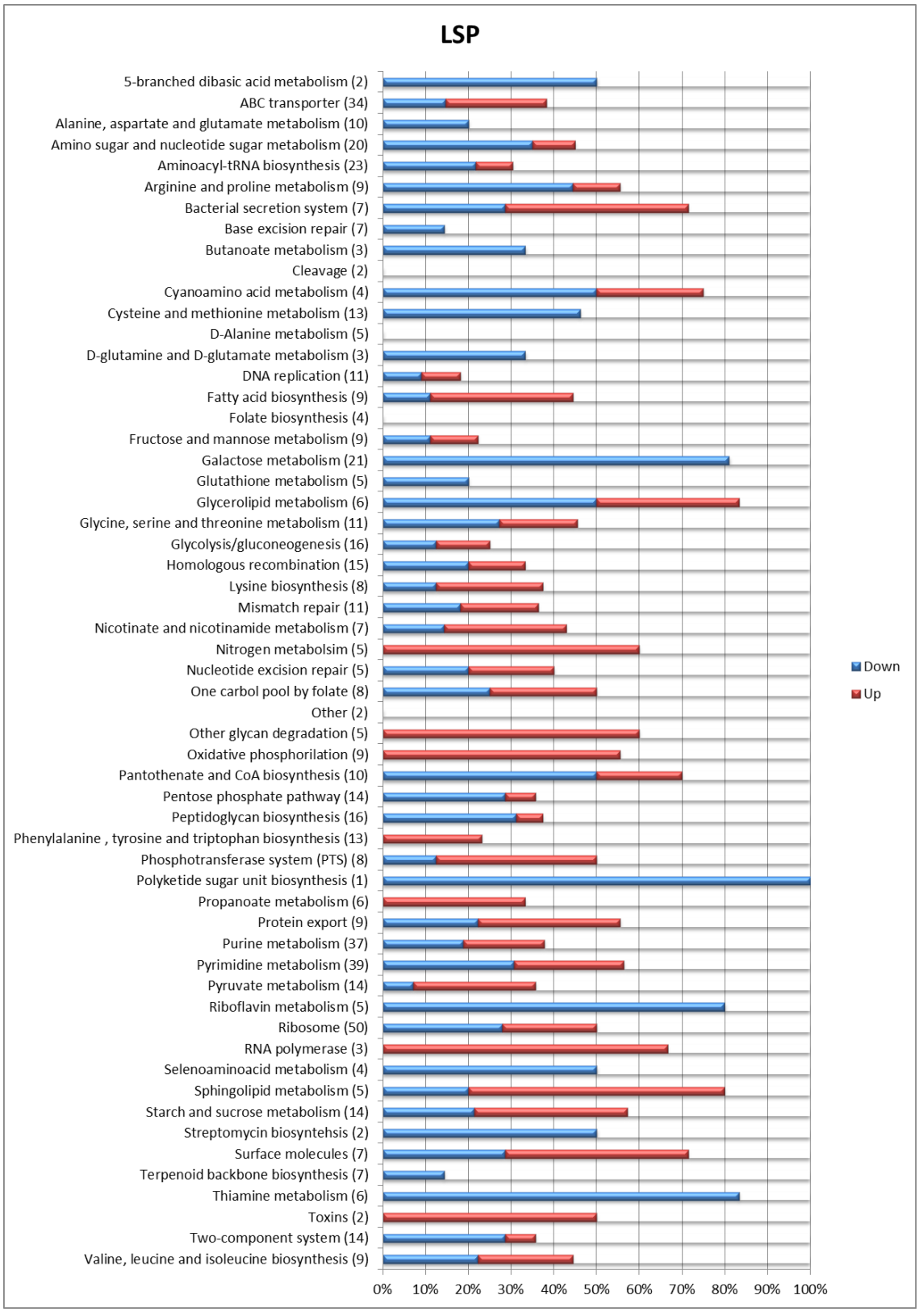

Figure 92. Pairwise comparison showing the percentage of genes in each pathway that are up or down-regulated in A42174 when compared to D39 during the Late Stationary Phase

9.3. Appendix 3: Absolute expression of virulence factors in D39 and A42174.

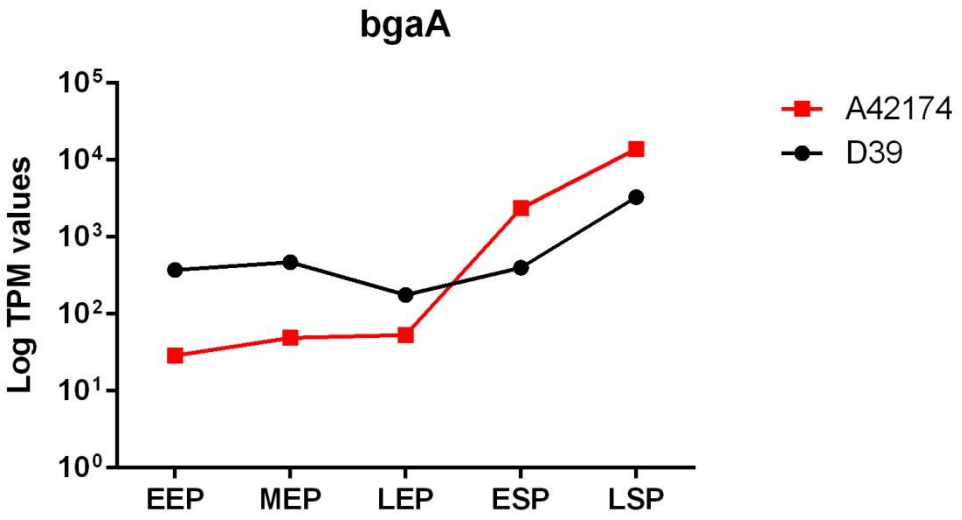

Figure 9329. Absolute expression values of *bgaA* in D39 and A42174.

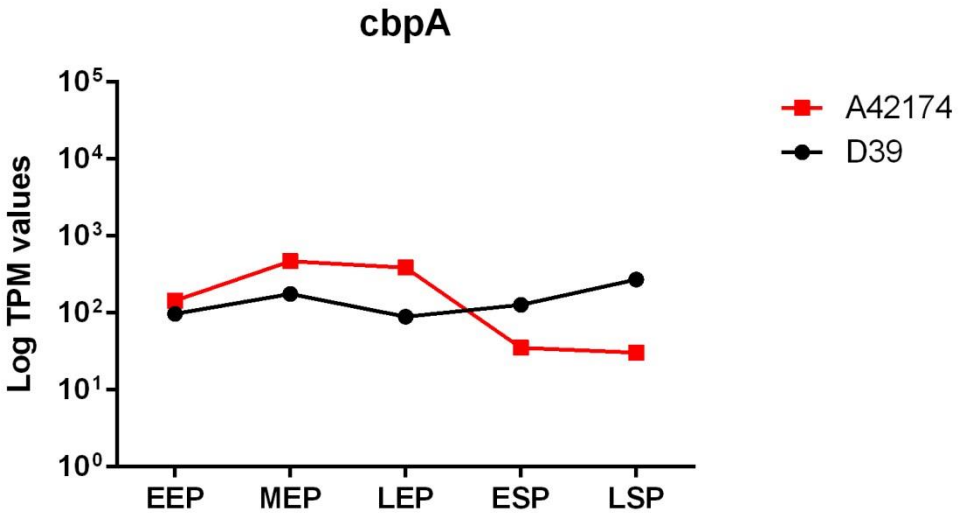

Figure 94. Absolute expression values of *cbpA* in D39 and A42174.

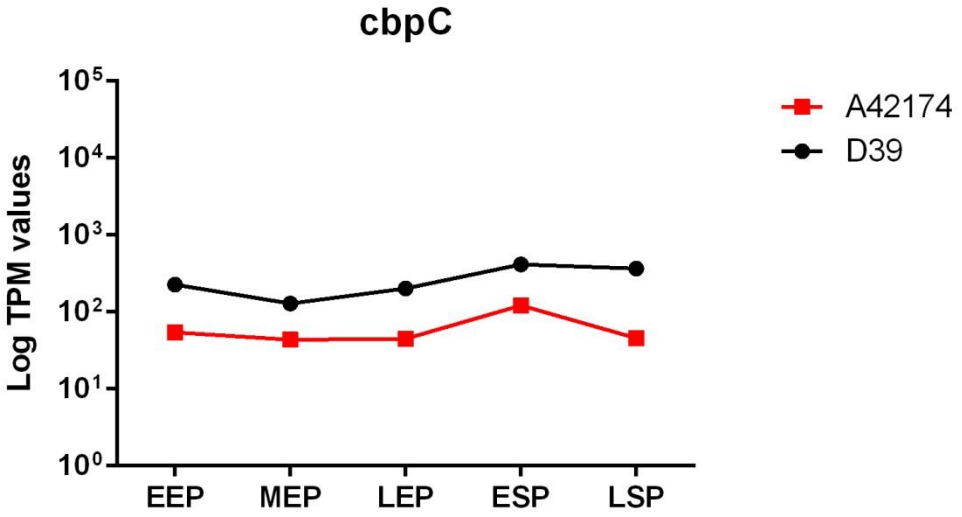

Figure 95. Absolute expression values of *cbpC* in D39 and A42174.

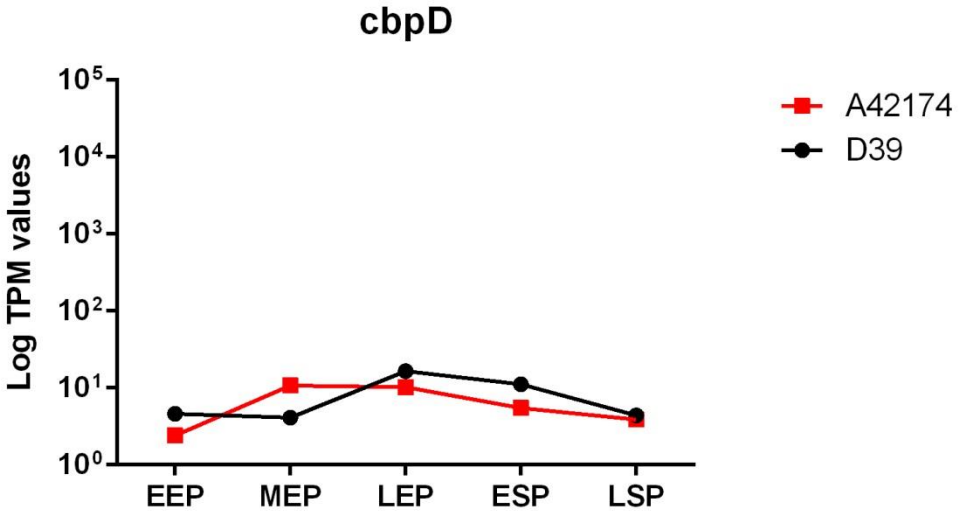

Figure 96. Absolute expression values of *cbpD* in D39 and A42174.

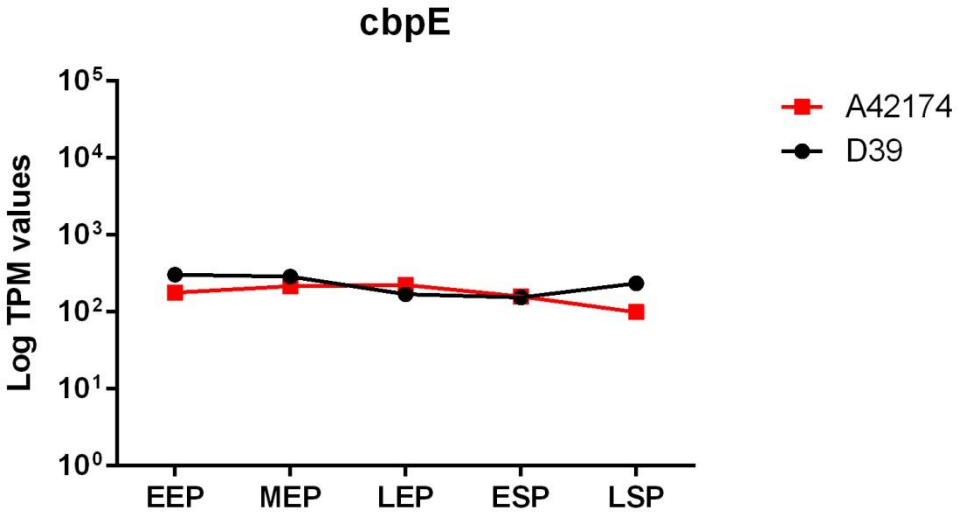

Figure 97. Absolute expression values of *cbpE* in D39 and A42174.

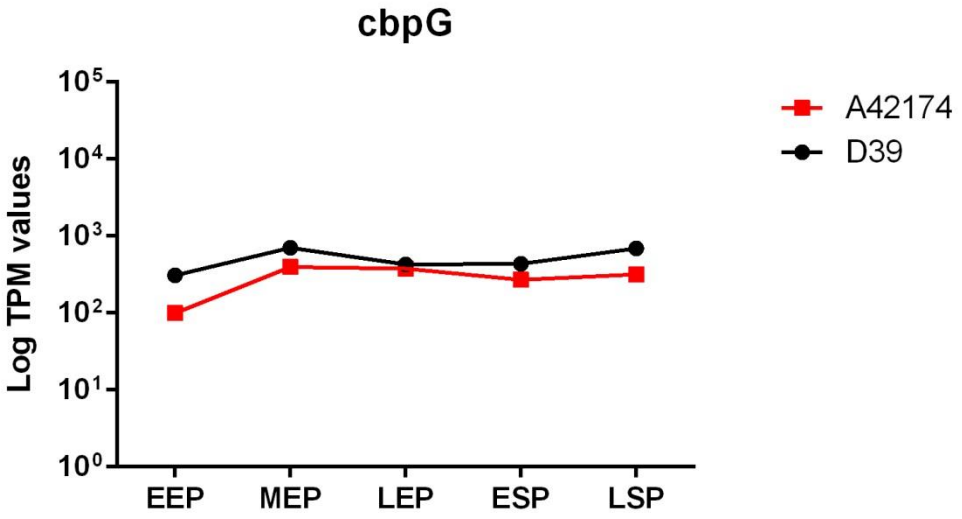

Figure 98. Absolute expression values of *cbpG* in D39 and A42174.

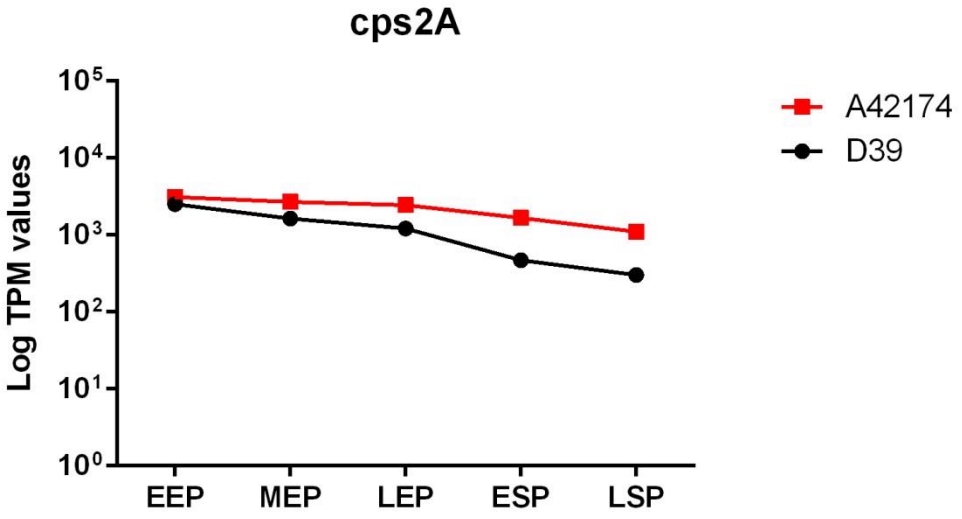

Figure 9930. Absolute expression values of *cps2A* in D39 and A42174.

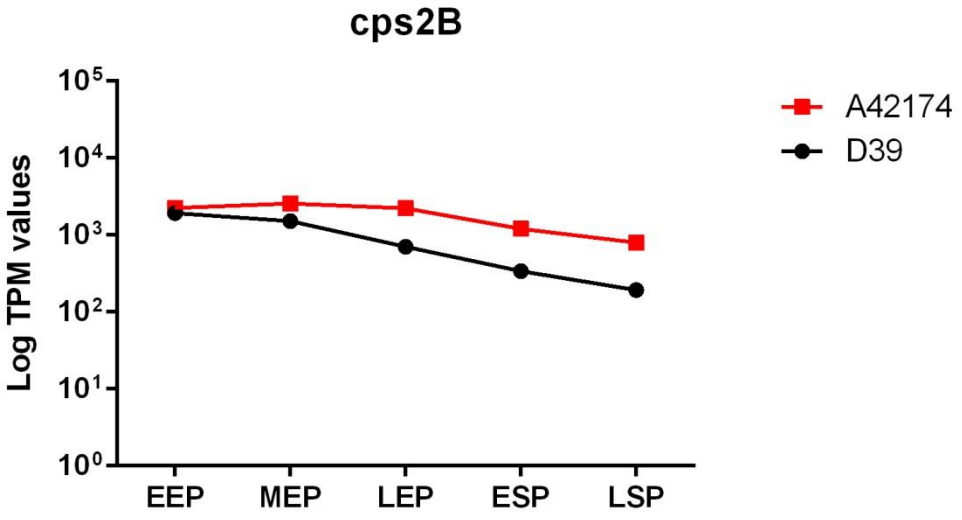

Figure 100. Absolute expression values of *cps2B* in D39 and A42174.

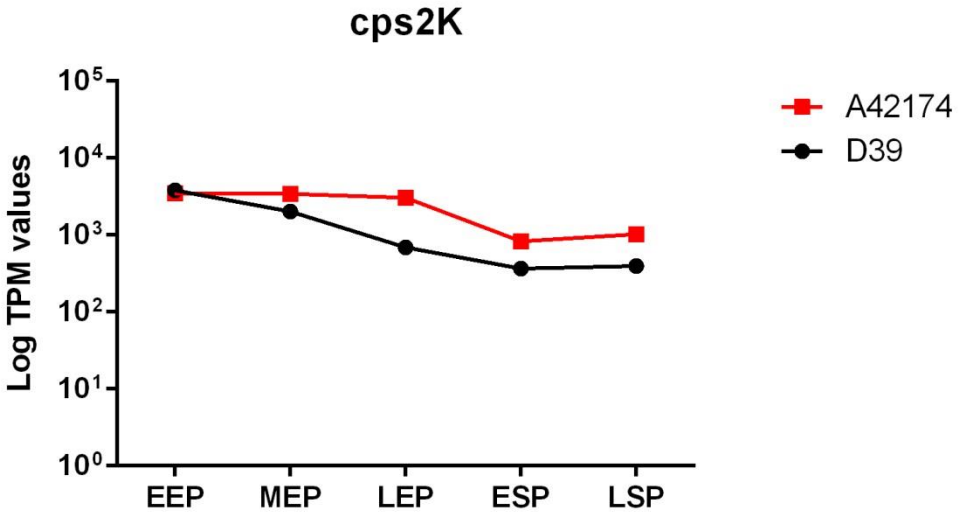

Figure 10131. Absolute expression values of *cps2K* in D39 and A42174.

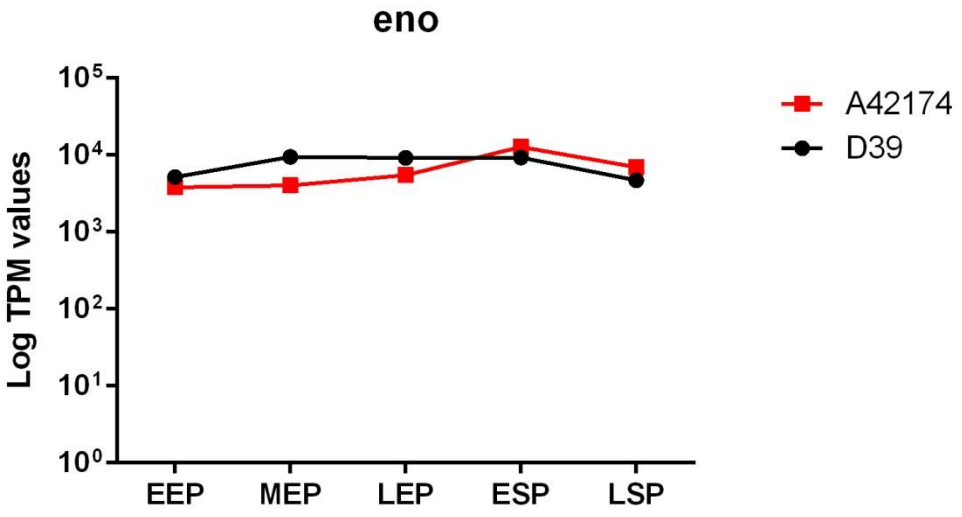

Figure 10232. Absolute expression values of *eno* in D39 and A42174.

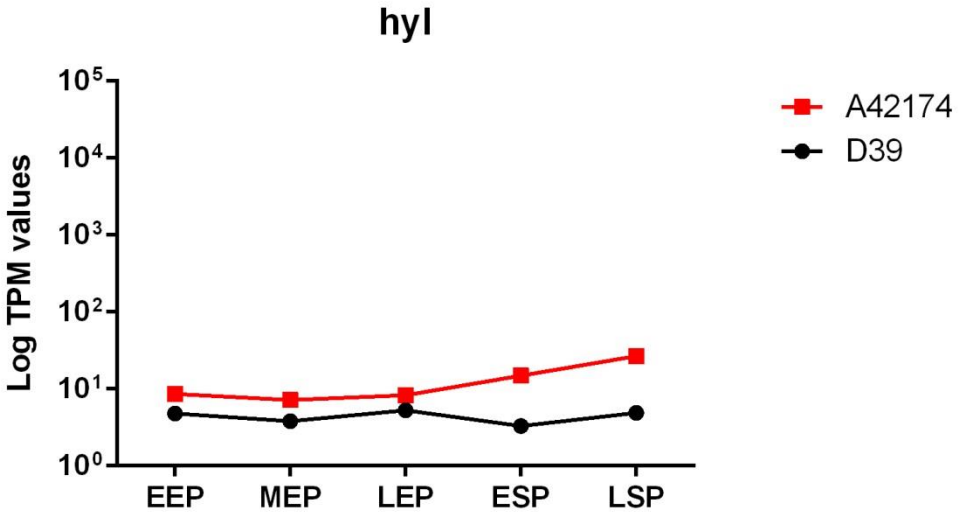

Figure 103. Absolute expression values of *hyl* in D39 and A42174.

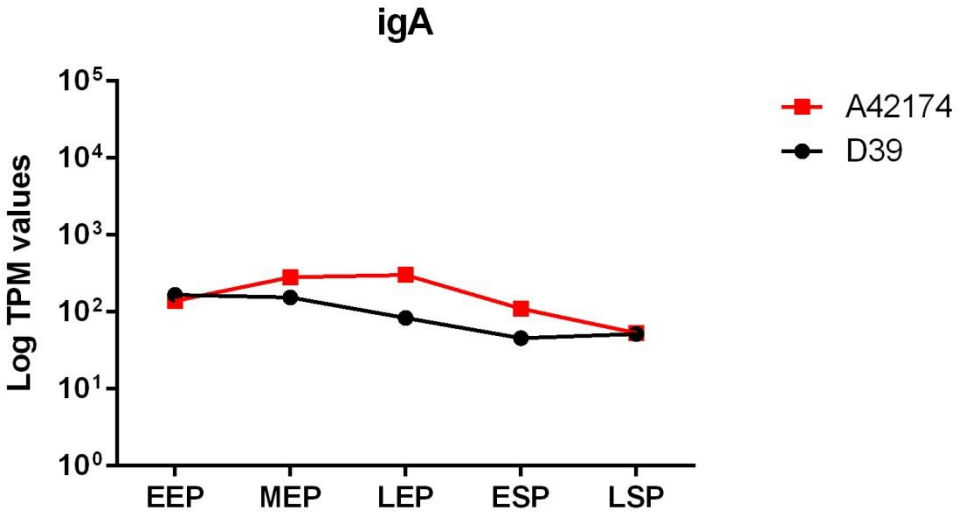

Figure 10433. Absolute expression values of *igA* in D39 and A42174.

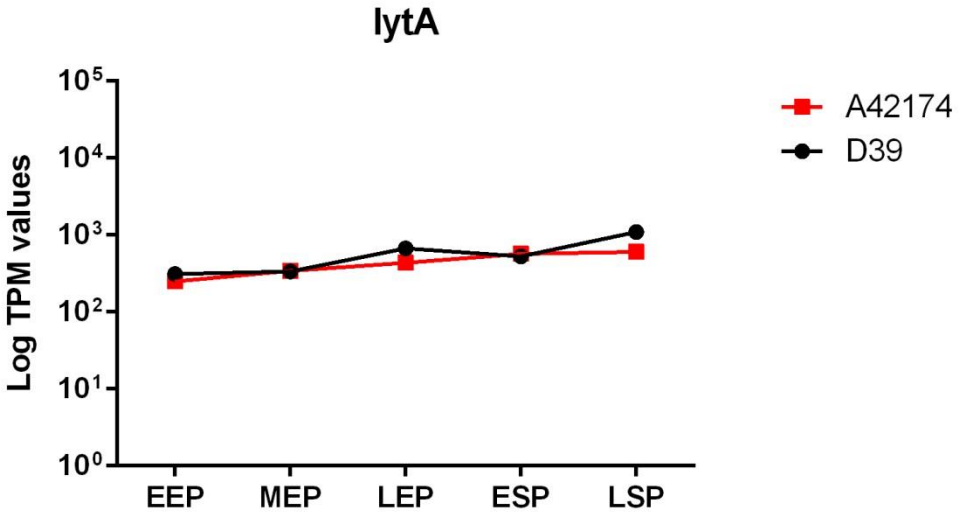

Figure 105. Absolute expression values of *lytA* in D39 and A42174.

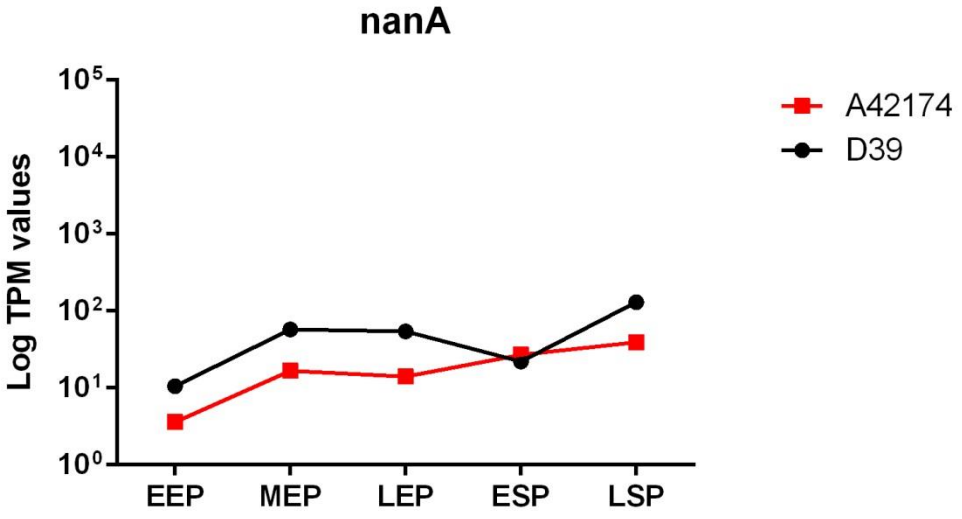

Figure 106. Absolute expression values of *nanA* in D39 and A42174.

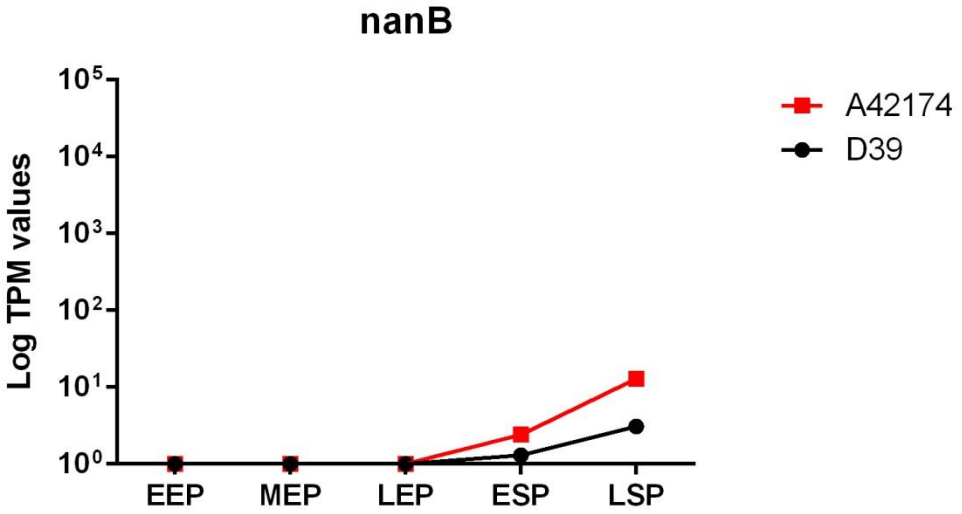

Figure 10734. Absolute expression values of *nanB* in D39 and A42174.

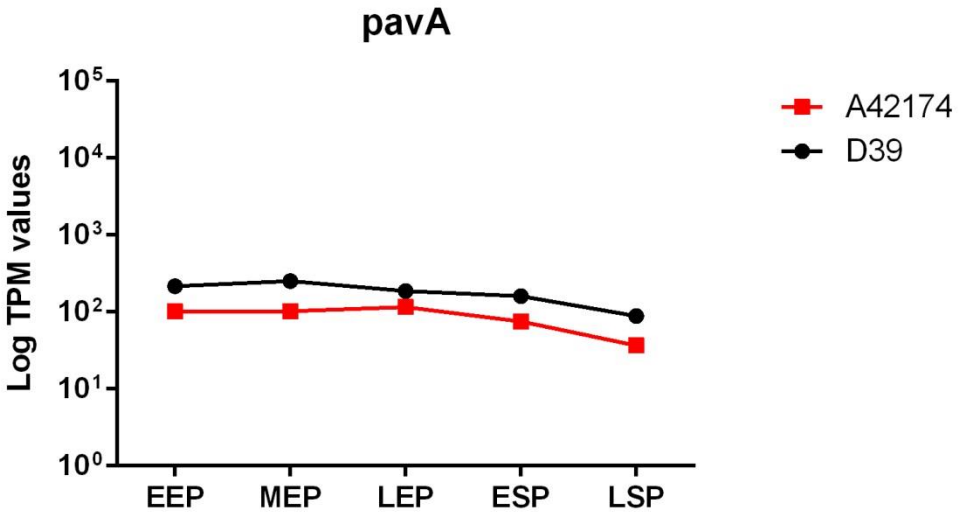

Figure 10835. Absolute expression values of *pavA* in D39 and A42174.

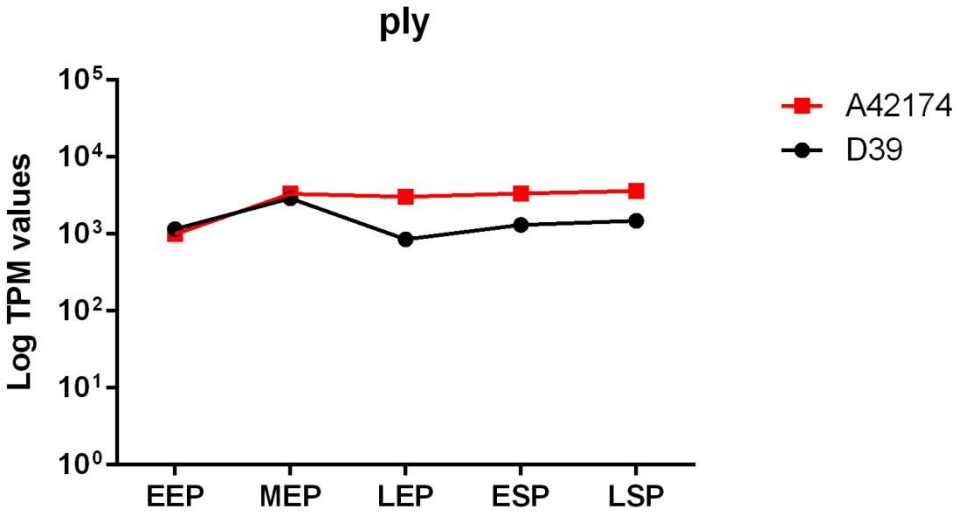

Figure 109. Absolute expression values of *ply* in D39 and A42174.

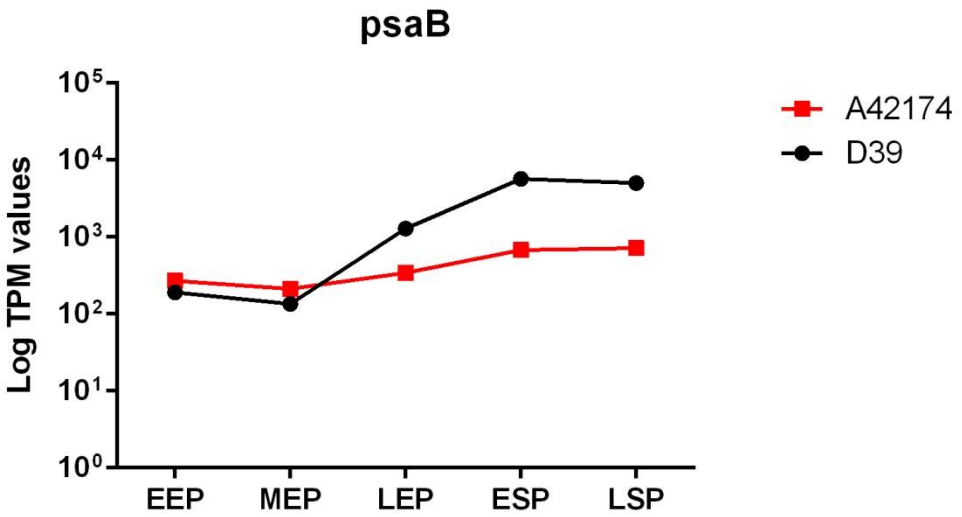

Figure 110. Absolute expression values of *psaB* in D39 and A42174.

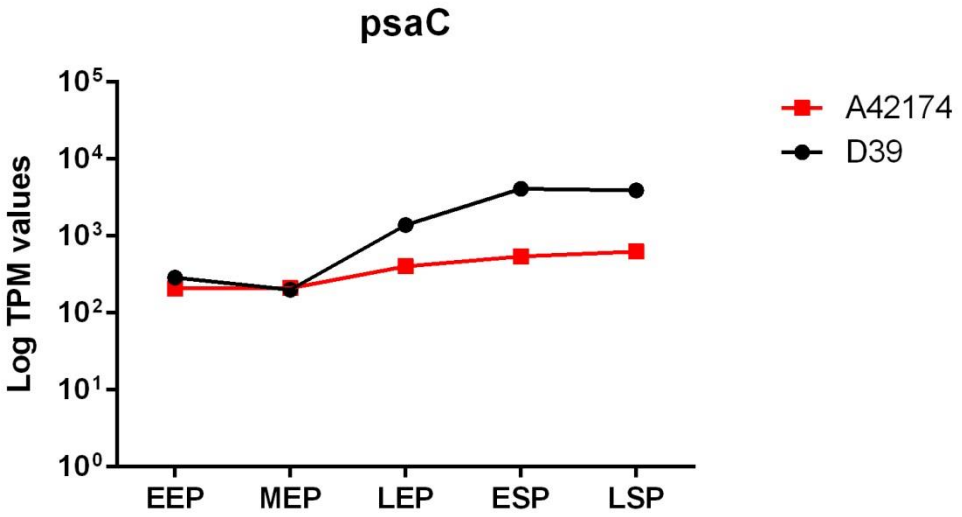

Figure 11136. Absolute expression values of *psaC* in D39 and A42174.

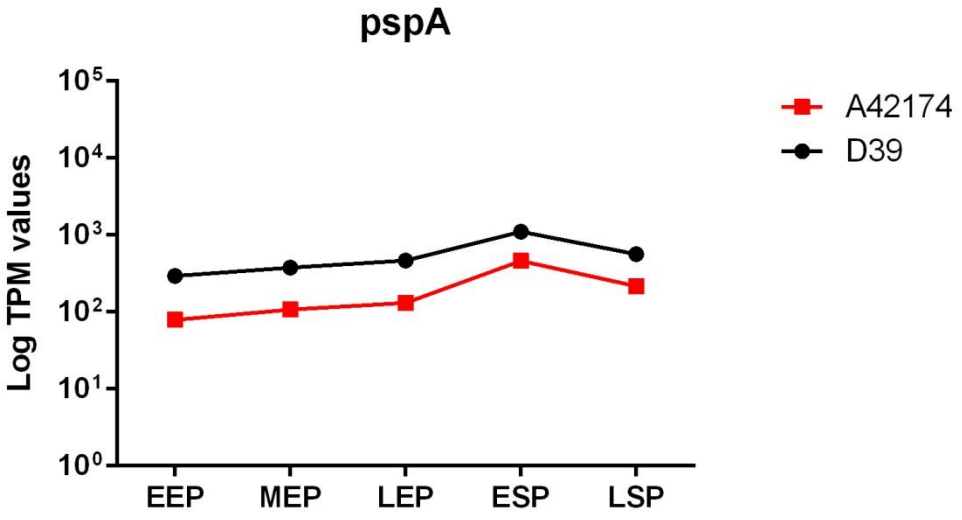

Figure 112. Absolute expression values of *pspA* in D39 and A42174.

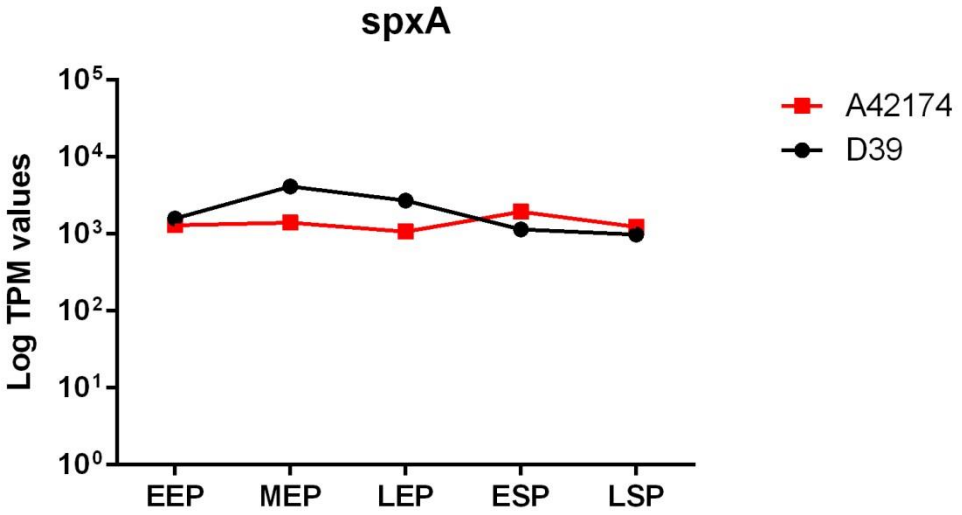

Figure 113. Absolute expression values of *spxA* in D39 and A42174.

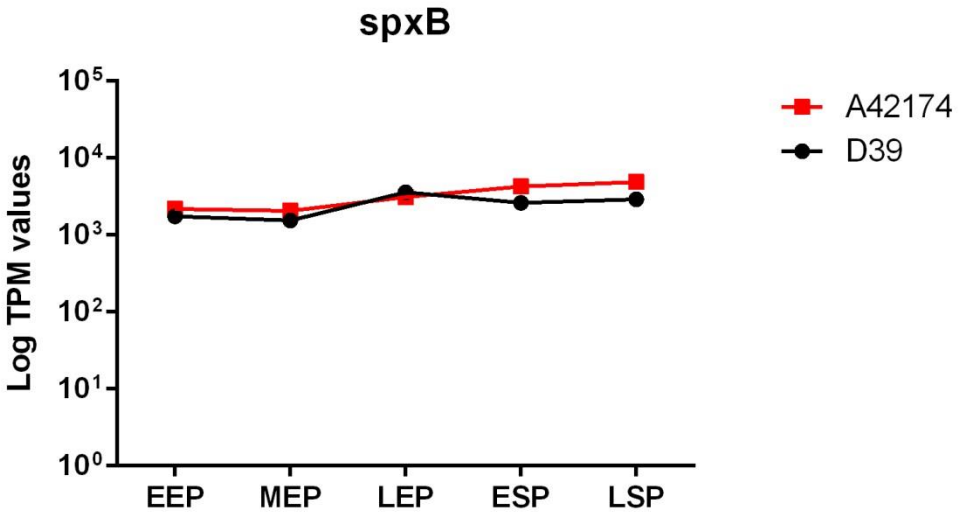

Figure 11437. Absolute expression values of *spxB* in D39 and A42174.

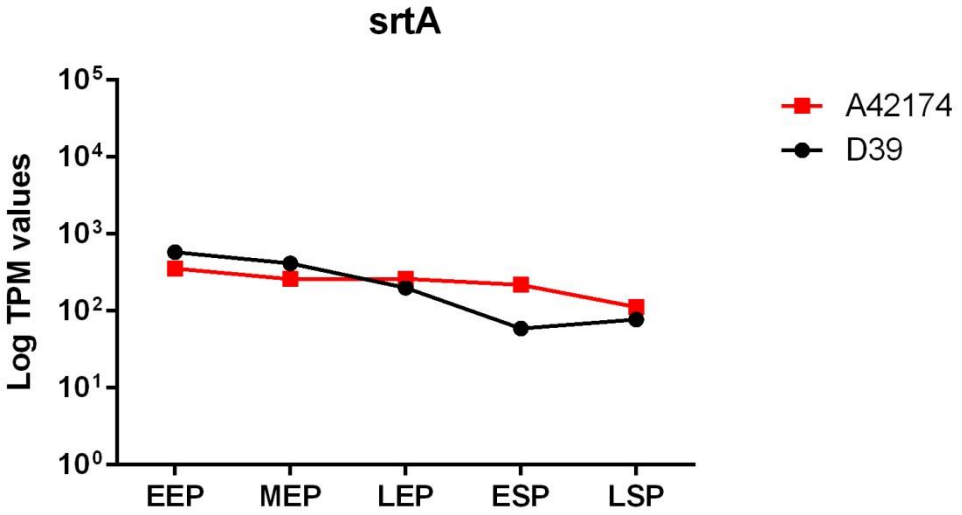

Figure 115. Absolute expression values of *srtA* in D39 and A42174.

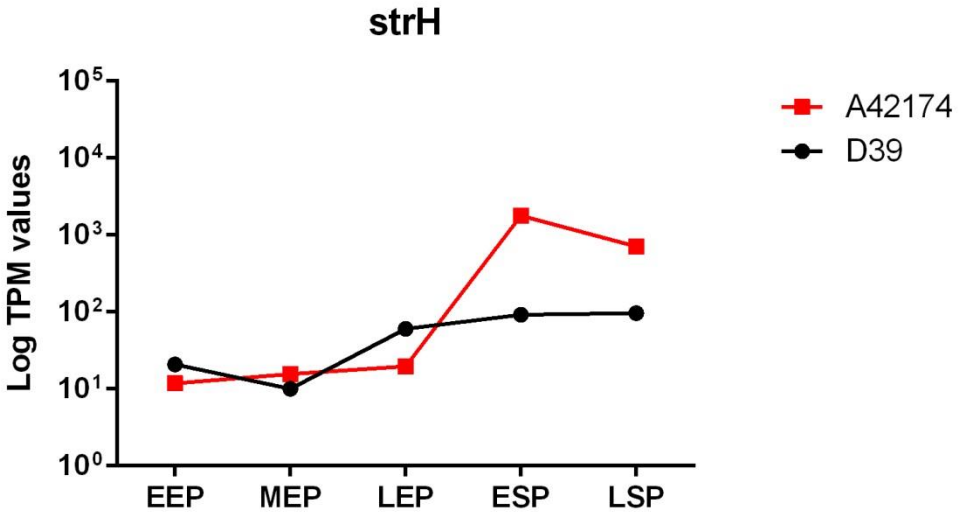

Figure 116. Absolute expression values of *strH* in D39 and A42174.