Analysis of the Bactericidal Activity of Naive Rabbit Serum against *Staphylococcus aureus*

by

Beatriz González Muñoz
Molecular Biotechnology Engineer, MSci
(Universidad de Chile)

A thesis submitted for the degree of Doctor of Philosophy
November 2015

Department of Molecular Biology and Biotechnology, University of Sheffield,
Firth Court, Western Bank, Sheffield, S10 2TN

Summary

Staphylococcus aureus is a commensal bacterium that can also act as an opportunistic pathogen, causing a wide range of diseases in humans and in economically important livestock such as cows and rabbits. *S. aureus* quickly develops resistance against antibiotics and also evades the immune system of the host. As current treatments are difficult and expensive, new antibiotics, vaccines and the use of elements of the immune system are currently being studied as novel control approaches.

A novel bactericidal mechanism able to kill *S. aureus* present in naive rabbit serum (NRS) was previously discovered in our laboratory. The bactericidal activity of the NRS had been characterized and the importance of the wall teichoic acids (WTA) on the bacteria for susceptibility was identified previously, but the mechanism involved in the bacterial killing remained unknown.

In this study the role of the WTA as the bacterial receptor that interacts with the 'killing factor' present in the NRS was established. The importance of WTA modifications were also determined in pathogen resistance to the NRS killing mechanism. Use of bacterial cell wall material as an affinity matrix led to the identification of secreted phospholipase A2 (sPLA2) as a potential part of the bactericidal mechanism. The use of a range of inhibitors and specific antibodies confirmed sPLA2 as part of the killing mechanism.

Purified sPLA2 from a number of species was tested and cobra sPLA2 (cvPLA2) was able to kill *S. aureus* in buffer but led to bacterial growth when added to NRS. This suggests that cvPLA2 interacts with rabbit serum components and this results in inactivation of both cvPLA2 and the bactericidal activity of the NRS. Annexin A1, a known regulator of PLA2, was identified through mass spectrometry as a serum protein also bound to the cell wall affinity matrix. This protein enhanced the bactericidal activity of cvPLA2 against *S. aureus* by direct protein-protein interaction. A model of NRS staphylococcal activity is presented.

Acknowledgements

First, I would like to thank Professor Simon Foster for all the support and guidance throughout this project, for allowing me be part of his research group and for all his help and understanding about everything during these four years.

I would also like to thank Dr. Jorge García-Lara and Dr. Stephane Mesnage for their support and technical assistance, and for all the times I just needed a 'science question' answered; Dr. Fiona Shackley from the Royal Hallamshire Children's Hospital for the samples of infant's blood; Dr. Suzan Roiijakkers from University Medical Centre Utrecht (The Netherlands) for sharing her results confirming my hypothesis; Dr. Richard Beniston from the biOMICS Facility from the University of Sheffield for the LC-MS analysis; Dr. Cristina Teles and John Connolly for the gifts of different types of mammalian serum; and everybody in F18 who donated their blood for my studies.

I need to thank all of the friends I made in the F18 and F25 labs, who made these years awesome (and not just for the science), especially Kasia Wacnik and Bartek Salamaga, Victoria Lund, Hannah Kelly, Felix Weihs, Bryony Cotterell, Kalpita Padwal and Joe Kirk. I need to also thank my friends from Chile (Astrid, Cristina, Daniela, Alejandra, Ximena, Inés y Maca), who where just a text away no matter the time difference.

Finally and most importantly, I need to thank my family. They have supported me my whole life, and these four years in the UK have not been different. Thank you for all the pacience, the support, the love, and all the things that they did for me, from Skype calls to plane tickets so I could spent every Christmas with them. This is for my parents, Mónica and Alejandro, and my siblings, Javier and Lorena.

Publications from this study

Viana, D., Comos, M., McAdam, P.R., Ward, M.J., Selva, L., Guinane, C.M., González-Muñoz B.M., Tristan, A., Foster, S.J., Fitzgerald, J.R., Penadés, J.R. (2015) A single nucleotide mutation alters bacterial pathogen host tropism. *Nature Genetics*, 47, p.361-366.

Abbreviations

ºC	Degree Celsius
A_X	Absorbance at indicated wavelength x (nm)
$AACOCF_3$	Arachinodyl trifluoromethyl ketone
ADP	Adenosine diphosphate
AGC	Automatic gain control
AMP	Adenosine monophosphate
Anx-A1	Annexin A1
APS	Ammnoium persulphate
ATP	Adenosine triphosphate
BCR	B cells receptor
BHI	Brain heart infusion
BLAST	Basic local alignment search tool
BODIPY	Boron-dipyrromethene
BPB	4-bromophenacyl bromide
BPI	Bactericidal/permeability increasing protein
BSA	Bovine serum albumin
C5aR	Complement component C5a receptor
C55-P	Undecaprenol phosphate
CC	Clonal complex
CID	Collision induced dissociation
CFU	Colony forming units
CHIPS	Chemotaxis inhibitory protein of *S. aure*us
ClfA	Clumping factor A
Cm	Chloramphenicol
cPLA2	Cytosolic phospholipase A2
CRIT	C2 receptor inhibitor trispanning
cvPLA2	Cobra venom phospholipase A2
D-ala	D-alanine
DGlcDAG	Diglucosyl-diacylglycerol
dH_2O	Distilled water
DNA	Desoxyribonucleic acid
DOPC	1,2-Dioleoyl-sn-glycero-3-phosphocholine
DOPG	1,2-Dioleoyl-sn-glycero-3-phospho-rac-(1-glycerol)
DTT	Dithiothreitol

Eap	Extracellular adherence protein
Ecb	Extracellular complement binding protein
ECL	Enhanced chemiluminescent
Efb	Extracellular fibrinogen binding protein
EGTA	Ethileneglycol tretaacetic acid
Ery	Erytromycin
ETV	Electron transfer dissociation
FPR	Formyl peptide receptor
FRET	Fluorescence resonance energy transfer
g	Grams
Gal	Galactose
Glc_2DAG	Diglycosyl-diacylglycerol
GlcNac	N-acetylglucosamine
Gro-P	Glycerophosphate
HF	Hydrofluoric acid
hr	Hour
HRP	Horseradish peroxidase
HS	Human serum
ICAM-1	Intracellular adhesion molecule-1
Ig	Immunoglobulin
IL	Interleukin
iPLA2	Calcium-independent phospholipase A2
Kb	Kilobase pairs
kDa	Kilodaltons
kV	Kilovolts
L	Litre
LB	Luria-Bertani
LC-MS	Liquid Chromatography Mass Spectrometry
LPS	Lipopolysaccharide
LTA	Lipoteichoic acid
µg	Microgram
µl	Microlitre
µm	Micrometre
µM	Micromolar
M	Molar
MAC	Membrane attack complex
ManNac	N-acetylmannoseamine

Map19	MBL-associated plasma protein of 19 kDa
MASPs	MBL-associated serine proteases
MBL	Mannose-binding lectin
Mbp	Megabase pairs
MES	2-(N-morpholino) ethanesulphonic acid
mg	Miligram
MGE	Mobile genetic element
MHC	Major histocompatibility complex
MIC	Minimum inhibitory concentration
min	Minute
ml	Mililitre
MLST	Multilocus sequence typing
mM	Milimolar
MRSA	Methicillin-resistant *S. aureus*
MS	Mass spectrometry
MSCRAMM	Microbial surface components recognizing adhesive matrix molecules
MurNac	N-acetylmuramic acid
MWCO	Molecular weight cut-off
m/z	mass to charge ratio
nm	Nanometres
NRS	Naive rabbit serum
OD_X	Optic density at indicated wavelength x (nm)
PAF	Platelet activating factor
PAF-AH	Platelet activating factor acetylhydrolase
PAMPs	Pathogen-associated molecular pattern
PBPs	Penicillin binding proteins
PBS	Phosphate buffered saline
PG	Peptidoglycan
PGRP	Peptidoglycan recognition protein
PLA2	Phospholipase A2
ppm	Parts per million
PRRs	Pathogen- or pattern-recognition receptor
rhPLA2	Recombinant human phospholipase A2
RNA	Ribonucleic acid
rpm	Revolutions per minute
RT	Room temperature

SB	Slotblot
Sbi	Staphylococcal immunoglobulin binding protein
SCIN	Staphylococcal complement inhibitor
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
sMAP	Small MBL-associated protein
SNP	Single nucleotide polymorphism
Spec	Spectinomycin
sPLA2	Secreted phospholipase A2
SSL	Staphylococcal superantigen-like protein
STs	Sequence types
TBS	Tris buffered saline
TCR	T cell receptor
TEMED	Tetramethylethylenediamine
Tet	Tetracyclin
TNF	Tumour necrosis factor
Tris	Tris (hydroxymethyl) aminomethane
TSA	Tryptic soy agar
TSB	Tryptic soy broth
V	Volts
v/v	Volume for volume
VISA	Vancomycin-intermediate *S. aureus*
VRSA	Vancomycin-resistant *S. aureus*
w/v	Weight for volume
WB	Western blot
WTA	Wall teichoic acid

Table of Contents

List of Figures

List of Tables

CHAPTER 1:

Introduction

1.1 Staphylococci

Staphylococci were first identified and named by Sir Alexander Ogston in 1881 (Archer, 1998). They are Gram-positive, spherical, non-motile and facultative anaerobic bacteria. They are non-spore forming and have a diameter of approximately 0.5-1.5 µm (Harris et al, 2002; Hennekinne at al, 2012). The cells can divide in three perpendicular planes, forming grape-like clusters (Tzagoloff and Novick, 1977).

Staphylococci have relative complex nutritional requirements that vary from strain to strain, but in general they require 5-12 essential amino acids, an organic source of nitrogen and B vitamins, including thiamine and nicotinamide (Kloos and Schleifer, 1986; Wilkinson, 1997; Harris et al, 2002). Staphylococci can resist desiccation and heat (Kloos and Lambe, 1991), and are tolerant to high concentrations of salt (Wilkinson, 1997). They are catalase-positive and oxidase-negative, which can distinguish staphylococci from other Gram-positive cocci like streptococci (Wilkinson, 1997; Madigan et al, 2006).

Staphylococci are important pathogens with more than 50 species having been described. They are differentiated by their ability to produce coagulase, a protein that can react with prothrombin and clots the blood (Hennekinne et al, 2012). *Staphylococcus aureus*, a coagulase positive strain (Bergdoll, 1990), is significantly important due to its interaction with humans.

1.2 Staphylococcus aureus

Staphylococcus aureus is the most frequent human pathogen of the *Staphylococcus* genus and is important due to the rise of antibiotic resistant

strains (Lowy, 1998; Harris et al, 2002). This pathogen has also been associated with infections in economically important livestock such as cows, sheep and rabbits (Viana et al, 2015).

This bacterium can be distinguished from other staphylococcal species by the golden yellow pigmentation of its colonies on agar, coagulase activity, mannitol-fermentation and deoxyribonuclease activity (Wilkinson, 1997; Lowy, 1998).

The genome of several strains has been sequenced. The average size of the *S. aureus* chromosome is 2.8 Mbp (Kuroda et al, 2001) and there is a high degree of sequence similarity between strains (Lowy, 2010). Comparison of genomic sequences shows that large fragments of DNA have been acquired by horizontal transfer from other species. These mobile elements contain many antimicrobial resistance and virulence factor encoding genes (Lowy, 2010; Stefani et al, 2012).

1.2.1 Antibiotic resistance in *S. aureus*

Cells resistant to antibiotics and other drugs have emerged due to the selective pressure that their widespread use has caused (McKeegan et al, 2002). *S. aureus* is naturally susceptible to every antibiotic that has been developed (Chambers and DeLeo, 2009) but it has the ability to quickly acquire resistance (Demerec, 1945; Lowy, 2003; Gordon and Lowy, 2008; Chambers and DeLeo, 2009) (Figure 1.1). This ability to gain resistance to antibiotics makes *S. aureus* an important issue for worldwide health not only because the treatment of the bacteria has become increasingly difficult and expensive, but because the mortality rate due to *S. aureus* infection has increased to about 30% (Dancer, 2008). Resistance to antibiotics is often acquired by horizontal transfer of genes from outside sources, but chromosomal mutations and antibiotic selection are also important (Demerec, 1945; Livermore, 2000; McKeegan et al, 2002).

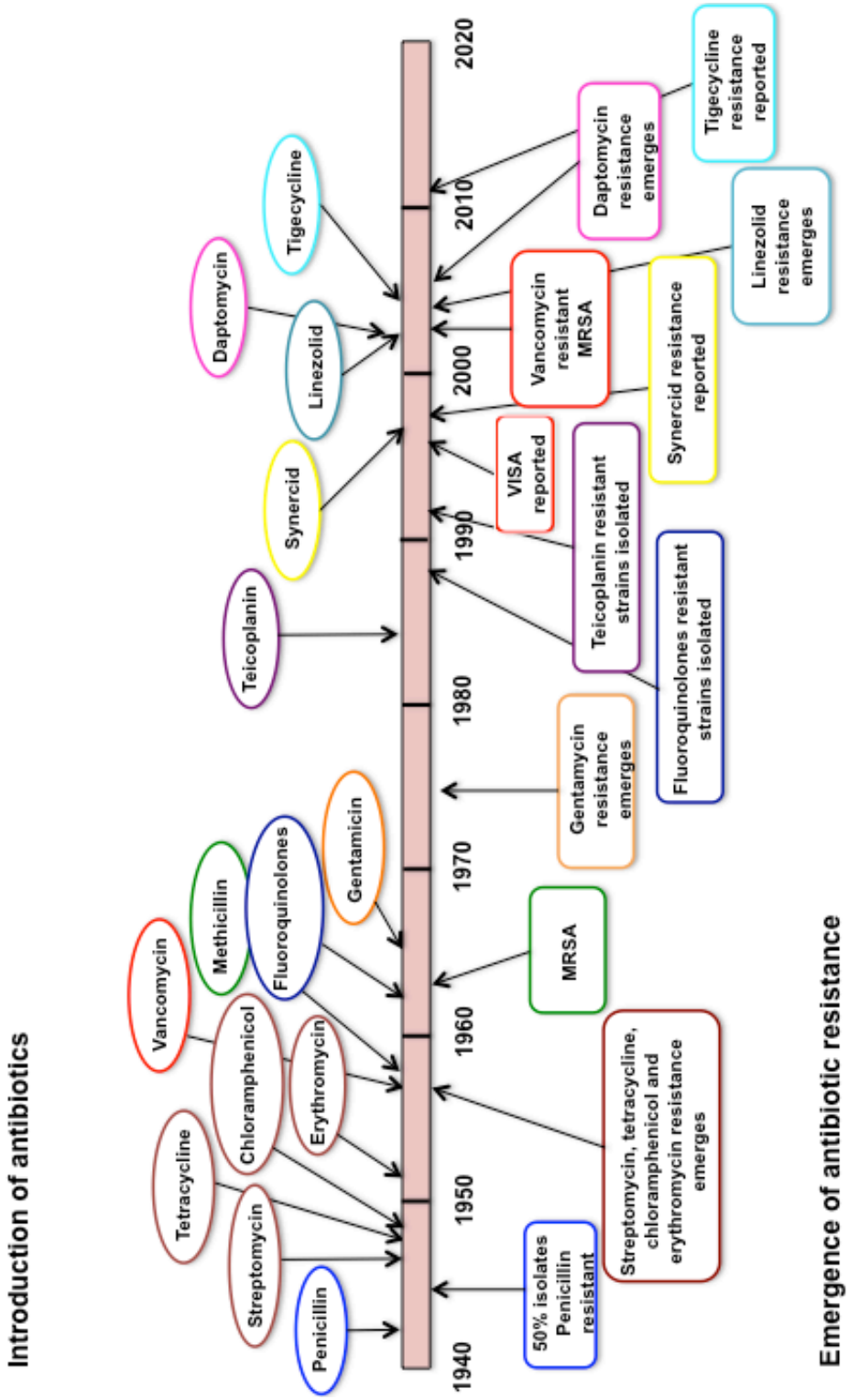


Figure 1.1 Chronology of the introduction of antibiotics and subsequent emergence of resistance in *S. aureus*

Figure adapted from (Kabli, 2013)

Resistance to penicillin and other β-lactam antibiotics is caused by the inactivation of the antibiotic by β-lactamase, which hydrolyses the β-lactam ring (Lowy, 1998). This resistance was identified soon after the introduction of penicillin and by 1948 approximately 50% of isolates were resistant (Barber and Rozwadokawa-Dowzenko, 1948; Lowy, 1998). Approximately 5% of *S. aureus* isolates now show sensitivity to penicillin (Lowy, 1998).

New antibiotics such as streptomycin, erythromycin, chloramphenicol and tetracycline were developed during the 1950s and staphylococcal isolates resistant to these antibiotics were discovered shortly after their introduction into clinical practice (Demerec, 1948; Lacey, 1975; Livermore 2000). The mode of action of these antibiotics is to block protein synthesis and resistance mechanisms are diverse: ribosome modification to alter antibiotic binding affinity, including acetylation of the antibiotics to reduce its toxicity and action of efflux pumps (Lacey and Chopra, 1972; Courvalin and Fiandt, 1980; Burdett, 1991; Guay et al, 1993; Murray and Shaw, 1997; Roberts et al, 1999, Witte et al, 2008).

Methicillin, cloxacillin and oxacillin are β-lactamase-resistant semi-synthetic penicillins that were developed to treat penicillin-resistant *S. aureus* (Rice, 2006). However, methicillin-resistant *S. aureus* (MRSA) strains were isolated just two years after the introduction of methicillin into the United Kingdom (1961) and a few years later in the United States (1969) (Jevons, 1961; Rice, 2006). The resistance to methicillin derives from the acquisition of the *mecA* gene, not native in this species (Kono and Arakawa, 1995; Stefani et al, 2012). This gene encodes for modified penicillin-binding protein (PBP2a) that is able to replace the function of the essential PBPs in the presence of the antibiotics and has low affinity for them (Lim and Strynadka, 2002; Stefani et al, 2012). MRSA infections were first detected in hospitals, but in recent years infections have emerged in the community and livestock (Stefani, 2012).

Gentamicin was introduced to treat MRSA in the United Kingdom in 1964, but by the mid-1970s reports of gentamicin-resistant *S. aureus* were published (Naidoo and Noble, 1978). Fluoroquinolone antibiotics were introduced in the

late 1980s, but *S. aureus* and MRSA easily developed resistance against them (Schaefler, 1989; Ng et al, 1994; Ferrero et al, 1995; Muñoz-Bellido et al, 1999). Vancomycin is a glycopeptide that has an important role in treating MRSA cases. Glycopeptides bind with high affinity to the D-ala-D-ala terminus of peptidoglycan precursors, blocking their incorporation into peptidoglycan (Loll and Axelsen, 2000; Courvalin, 2006). *S. aureus* with intermediate vancomycin resistance (VISA, MIC 8mg/L) were first reported in Japan in 1996 (Hiramatsu et al, 1997) and in the United States in 1999 (Hageman et al, 2001). This resistance is due to a thickening in the cell wall with reduced cross-linking, allowing the bacteria to sequester the vancomycin and reduce its diffusion across the cell wall (Cui et al, 2000; Cui et al 2006). In 2002, vancomycin-resistant *S. aureus* (VRSA, with MIC > 32 mg/L) was first isolated (Avison et al, 2002; Cui et al, 2006), but the incidence of VRSA is still very rare.

Antibiotics like linezolid, daptomycin, synercid and tigecycline have been introduced recently for the treatment of MRSA and VRSA, but resistant strains have already been identified for all these antibiotics (Dowzicky et al, 2000; Tsiodras et al, 2001; Werner et al, 2001; Hayden et al, 2005; Hope et al, 2010; Kreis et al, 2013).

1.2.2 *S. aureus* infections

S. aureus is considered an opportunistic pathogen that can be found in the nasal cavities, skin, vagina, axilla, perineum and oropharynx (Lowy, 1998; Peacock et al, 2001; Lowy, 2010). Several biotypes have been isolated from different hosts, demonstrating the close adaptation of the bacterium to its host (Hennekinne et al, 2012; Viana et al, 2015.)

This bacterium is part of the normal human flora as a commensal with approximately 20% of the healthy population are permanent carriers and 30-50% transient (Lowy, 1998). People who are colonized by *S. aureus* have an increased risk of subsequent infection than those who are not carriers, and the host is usually infected with their own carriage isolate (Lowy, 1998; Peacock et al, 2001). *S. aureus* can also be acquired from other people or from

environmental exposures (Lowy, 2010). The risk of *S. aureus* infection is higher for intravenous drug users, diabetic and immunocompromised patients (Archer, 1998; Lowy, 1998).

This pathogen commonly enters the body through a wound and an abscess is formed at the initial site of infection to allow the bacteria to avoid the host defences (Clements and Foster, 1999). After colonization the bacteria replicates at the initial site of infection and produces enzymes that facilitate bacterial survival and local spread across tissues (Lowy, 2010). *S. aureus* is responsible for a wide range of diseases such as local skin infections including acne, mastitis, folliculitis, impetigo and cellulitis, but it can also cause systemic illnesses such as wound infections and metastatic complications, endocarditis, musculoskeletal and respiratory infections, and toxin-mediated diseases like toxic shock syndrome and food poisoning. Once toxin has been incorporated into food, food poisoning can develop without any viable bacteria (Archer, 1998; Lowy, 1998; Foster, 2005; Lowy 2010; Hennekinne et al 2012).

1.2.3 *S. aureus* virulence factors

It has been shown that the wide range of diseases that are caused by *S. aureus* are the result of several secreted and cell-surface associated virulence factors expressed by the bacteria. Expression of these virulence determinants is growth-phase dependent *in vitro* (Projan and Novick, 1997). In the exponential phase the bacteria synthetizes surface-associated virulence factors, switching to extracellular virulence factors such as toxins in the post-exponential phase. This corresponds with the formation of the abscess *in vivo*. Once high cellular density is reached within the abscess, the cells produce enzymes and toxins that allow their dispersal to other sites (Elek, 1956; Projan and Novick, 1997; Foster, 2005; Lowy, 2010; Johannessen at al, 2012).

S. aureus expresses these determinants in order to help spread and perpetuate the infection by avoiding the immune system. They facilitate colonization and bacterial migration to other tissues, tissue destruction, cytolytic effects on host cells and protect the bacteria from the host immune system (Tompkins et al,

1990; Bhakdi and Tranum-Jensen, 1991; Thakker et al, 1998; Navarre and Schneewind, 1999; Foster, 2005).

Virulence factors include surface proteins like protein A, iron-regulated surface determinant A (IsdA) and clumping factors A and B that are covalently bound to the bacterial peptidoglycan (Foster, 2005; Brown et al, 2014). These proteins promote the adhesion to damaged tissue and to the surface of host cells, and can also act as immune evasion molecules (Harris et al, 2002; Foster, 2005; Lowy, 2010). *S. aureus* also produces several toxins that can lyse eukaryotic cells and induce pro-inflammatory responses in the host, such as cytotoxins, leukotoxins and haemolysins (Lowy, 1998; Foster 2005). α-toxin is the best characterized and potent of the cytotoxins and is capable of pore formation in different types of host cells at low concentrations (Bhakdi and Tranum-Jensen, 1991).

Extracellular enzymes including proteases, catalase, lipases and coagulase are also produced and released by *S. aureus* and are involved in the spread of the infection and damage to host tissue (Foster, 2005).

Superantigens bind to major histocompatibility class II proteins. They are recognized by the β-chain variable regions of the T cell receptors, stimulating up to 20% of all T-cells (McCormick et al, 2001; Llewelyn and Cohen, 2002; Schlievert et al, 2008; Lowy, 2010; Kim et al, 2012). This large T-cell proliferation results in a massive release of cytokines, producing host-mediated tissue damage that can lead to hypotension, shock, multi-organ failure and even death (Kotb, 1995). Superantigens have also been implicated in autoimmune disorders including multiple sclerosis and rheumatoid arthritis (McCormick et al, 2001).

Finally, *S. aureus* produces capsular polysaccharides that protect the bacteria from phagocytosis (Foster, 2005). There are several capsular polysaccharides described, but most human infections are caused by serotypes 5 and 8 (Dassy et al, 1993; Foster, 2005; Lowy, 2010). The *S. aureus* capsule also plays a role in the induction of abscess formation (Lowy, 2010).

1.3 The bacterial cell wall

The bacterial cell wall serves as the interface between a bacterium and its environment, and performs several functions that are essential for its growth, division and survival (Scheffers and Pinho, 2005; Hayhurst et al, 2008). The main functions are the preservation of cell integrity and cell shape (Errington et al, 2001; Pichoff and Lutkenhaus, 2007), maintenance of the internal turgor pressure (Osborn and Rothfield, 2007), biofilm formation (Lévesque et al, 2005) and providing a physical barrier for protection from external influences (Silhavy et al, 2010). The cell wall also plays an important role in sensing external signals, acquiring nutrients and in the resistance to the host defences through the proteins that can be found embedded in it (Schneewind and Missiakas, 2012).

Bacteria can be separated into two groups based on differences in the cell envelope structure, Gram-positive bacteria and Gram-negative bacteria (Figure 1.2) (Madigan et al, 2006). Bacteria from the *Thermoplasma* genus are able to live without a cell wall as protoplasts in osmotically protected environments, such as animals (Madigan et al, 2006; Vinogradon et al, 2012), and only recently it has been demonstrated that the *Chlamydiales* possess functional peptidoglycan (Liechti et al, 2014; Jacquier et al, 2015).

1.3.1 Gram-negative bacteria

The cell envelope of Gram-negative bacteria is comprised of the cytoplasmic or inner membrane, the outer membrane and the peptidoglycan cell wall (Figure 1.2A) (Silhavy et al, 2010). The inner membrane works as a barrier between the cytoplasm and the external environment. In addition to a thin layer of peptidoglycan, these bacteria contain an outer membrane comprised of proteins, lipoproteins and lipopolysaccharide (Madigan et al, 2006; Silhavy et al, 2010). The inner and outer membranes delimit a protein-rich compartment called the periplasm, which contains the peptidoglycan and also serves as a scaffold for proteins and polysaccharides that mediate the interactions between

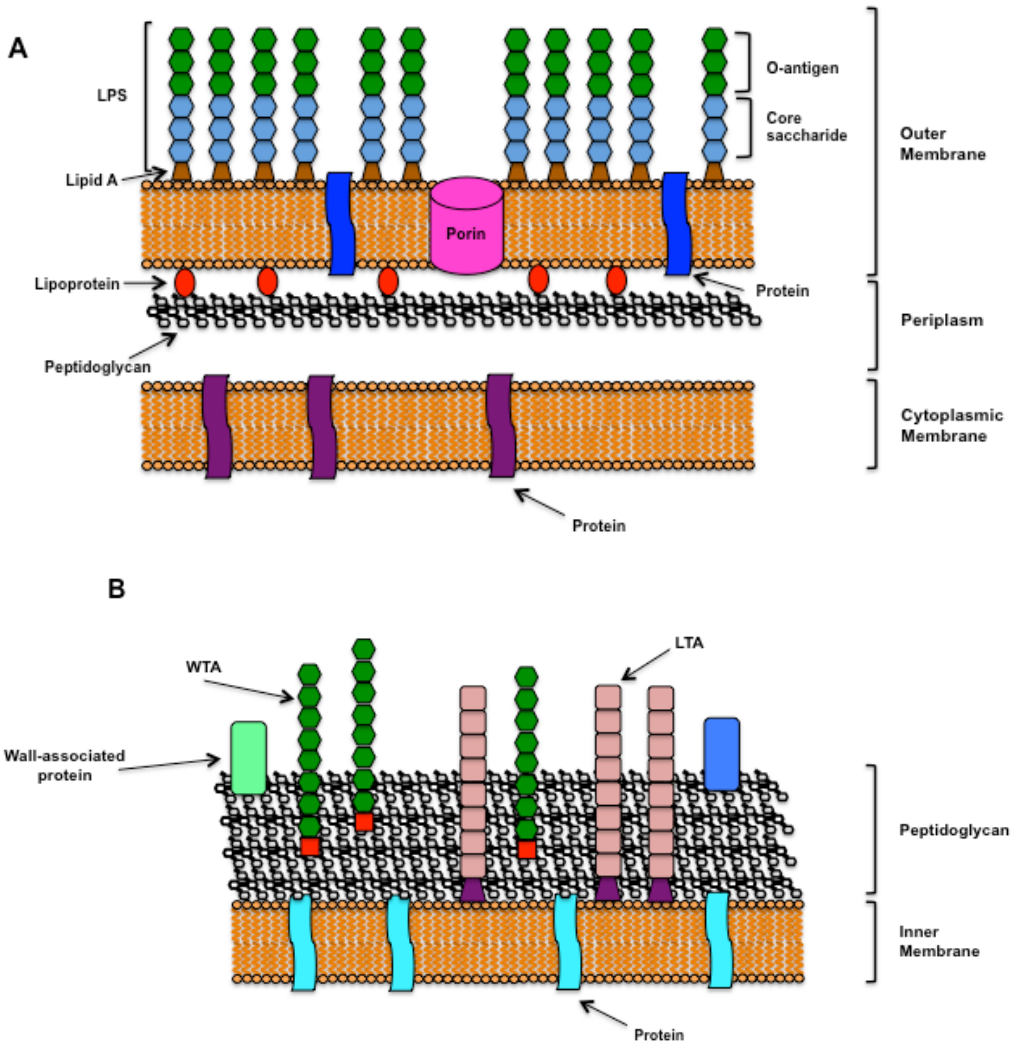


Figure 1.2 General structure of Gram-negative and Gram-positive cell envelope

The major structural components of the **A)** Gram-negative and **B)** Gram-positive cell envelope are shown. LPS: lipopolysaccharide; LTA: Lipoteichoic acid; WTA: wall teichoic acid. Adapted from (Silhavy et al, 2010).

the organism and its environment (Mullineax et al, 2006; Silhavy et al, 2010; Swoboda et al, 2010).

1.3.2 Gram-positive bacteria

S. aureus has a characteristic Gram-positive cell wall structure, which lacks an outer membrane and a distinct periplasm (Swoboda et al, 2010). Gram-positive bacteria have very thick peptidoglycan layers (20-35 nm) (Vollmer and Seligman, 2010) which can account for up to 60-90% of the cell wall mass (Madigan et al, 2006; Silhavy et al, 2010). Peptidoglycan stabilizes the cell membrane and also works as a scaffold for other molecules like bacterial surface proteins (Silhavy et al, 2010; Schneewind and Missiakas, 2012). The peptidoglycan is modified with carbohydrate-based anionic polymers called wall teichoic and lipoteichoic acids (Figure 1.2B) (Neuhaus and Baddiley, 2003) that appear to perform some of the functions of the outer membrane, influencing membrane permeability, extracelullar interactions and also act as a scaffold for extracytoplasmic proteins (Swoboda et al, 2010).

1.3.3 Peptidoglycan

Peptidoglycan (or murein) is an essential component of the cell wall that can be found on the outside of the cytoplasmic membrane of almost every bacterium (Mengin-Lecreulx and Lemaitre, 2005; Vollmer et al, 2008). Its main function is the preservation of cell integrity and any inhibition of its biosynthesis or degradation will result in cell lysis (Mengin-Lecreulx and Lemaitre, 2005). Peptidoglycan is also involved in maintaining cell shape (Vollmer et al, 2008), interaction with the host (Mengin-Lecreulx and Lemaitre, 2005; Turner at al, 2014) and in the anchoring of proteins (Dramsi et al, 2008) and teichoic acids (Neuhaus and Baddiley, 2003).

Peptidoglycan is a polymer made of glycan strands that are cross-linked via short peptide stems (Vollmer et al, 2008). The glycan strands are made up of repeating units of β- (1,4) linked N-acetylglucosamine (GlcNac) and N-

acetylmuramic acid (MurNac) (Vollmer et al, 2008) with different degrees of polymerization and terminating with MurNac residues at the reducing ends, forming relatively short glycan strands for *S. aureus* (Boneca et al, 2000).

The composition of the glycan strands is highly conserved between species but its length varies considerably between organisms, from short glycan strands (e.g. *S. aureus* mean length is 6 disaccharides) to very long (e.g. *B. subtilis* mean length between 50-250 disaccharides, with 25% of strands with a mass of 500 disaccharides) (Hayhurst et al, 2008; Vollmer et al, 2008; Vollmer and Seligman, 2010).

The MurNac or GlcNac can be modified by *O*-acetylation, *N*-deacetylation and *N*-glycolylation (Vollmer, 2008). While the presence of *N*-deacetylation reduces the activity of autolysins and the susceptibility to lysozyme (Vollmer and Tomasz, 2002; Vollmer, 2008), the *O*-acetylation is not only associated with resistance to lysozyme and other hydrolysins, but also pathogenesis and modulation of the host immune response (Boneca et al, 2007; Vollmer, 2008). *N*-glycolylation is mainly found in bacteria with mycolic acids in their envelope (Sutcliffe, 1998; Vollmer, 2008) and while its role is not known it appears to be involved in lysozyme resistance and stability of the cell envelope (Brennan and Nikaido, 1995; Vollmer, 2008).

During synthesis covalently attached to the MurNac of *S. aureus* there is a pentapeptide chain consisting of L-alanine, D-glutamine, L-lysine and D-alanyl-D-alanine moiety, called the stem peptide (Vollmer et al, 2008). In *S. aureus* the stem peptide chain is connected to other side chain via a pentaglycine cross-bridge, forming a mesh-like layer (Figure 1.3) (Madigan et al, 2006; Silhavy et al, 2010). The stem peptides are involved in the association of surface proteins and resistance to β-lactam antibiotics (Chambers, 2003; Silhavy, 2010).

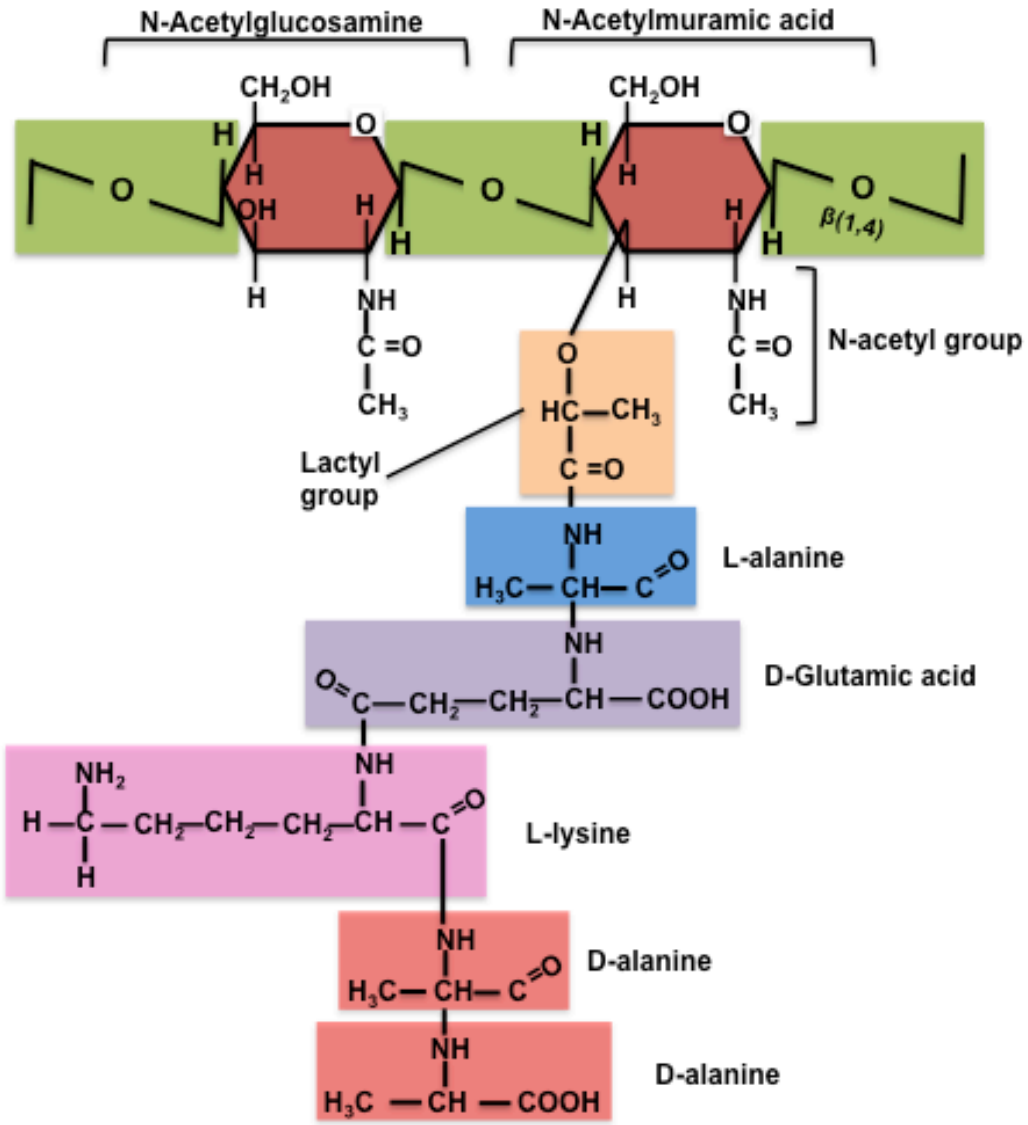


Figure 1.3 Structure of peptidoglycan

The figure shows the structure of one peptidoglycan disaccharide pentapeptide with a typical pentapeptide, even though its composition may vary. Adapted from (Fournier and Philpott, 2005; Madigan et al, 2006).

1.3.4 Surface proteins

S. aureus expresses several virulence factors to aid the colonization of the host, including surface proteins that interact with the environment and facilitate adherence to tissue (Tompkins et al, 1990; van Belkum et al, 2009). The adherence is mediated by the microbial surface components recognizing adhesive matrix molecules (MSCRAMM) family, which can be covalently attached to the peptidoglycan (Foster and Höök, 1998). They can interact with host proteins such as fibronectin, fibrinogen and elastin (Clarke and Foster, 2006; Silhavy et al, 2010). Adhesins are also surface proteins but they are attached to the peptidoglycan or teichoic acids via non-covalent ionic interactions (Dramsi et al, 2008;Silhavy et al, 2010).

1.3.5 Teichoic acids

Teichoic acids are anionic polymers that can be found on the cell surface of a wide range of Gram-positive organisms, including *S. aureus*. Teichoic acids are capable of performing some of the functions of the outer membrane, such as scavenging divalent cations, influencing membrane permeability, providing additional stability to the plasma membrane, mediating extracellular interactions and invasion of the host, and, along with peptidoglycan, act as a frame for extra-cytoplasmic enzymes (Silhavy et al, 2010; Hanson and Neely, 2011). There are two major types: wall teichoic acids (WTA) and lipoteichoic acids (LTA) (Swoboda et al, 2010; Hanson and Neely, 2011).

Even Gram-positive bacteria that lack WTA and LTA have functionally similar anionic polymers on their cell wall, illustrating the importance of these polymers (Neuhaus and Baddiley, 2003). For example, *Micrococcus luteus* has lipomannan instead of LTA (Powell et al, 1975; Neuhaus and Baddiley, 2003), and *B. subtilis* grown in phosphate-limited medium has teichuronic acid instead of WTA (Ellwood and Tempest, 1972; Neuhaus and Baddiley, 2003).

Whilst mutants without lipoteichoic or wall teichoic acids exist, they have morphological or growth defects (Weidenmaier et al, 2005b; Fedtke et al, 2007; Koprivnjak et al, 2008). Mutants lacking both LTA and WTA are non-viable, suggesting that one type of teichoic acid can compensate the other to some extent (Schirner et al, 2009; Xia et al, 2010a).

1.3.5.1 Lipoteichoic acids

Lipoteichoic acids (LTA) are anionic cell surface polymers that are anchored to the cell membrane through their glycolipid fraction and the hydrophilic chain extends into the cell wall (Figure 1.4) (Neuhaus and Baddiley, 2003: Silhavy et al, 2010). In most of the Gram-positives, including *Staphylococcus aureus*, this chain is made of poly-[glycerophosphate] (Gro-P), but in others like *Lactococcus garvieae* and *Clostridium innocuum* the repeating units are (GalGal-Gro-P) or (Gal-Gro-P), respectively (Fisher, 1994; Neuhaus and Baddiley, 2003). The length of the hydrophilic chain also varies, as well as the degree of D-alanylation and the kind and extent of glycosyl substitution (Iwasaki et al, 1986; Fischer, 1993; Roethlisberger et al, 2000; Neuhaus and Baddiley, 2003).

During biosynthesis LTA is assembled at the outer surface of the cytoplasmic membrane by the LTA polymerase (LtaS) directly on the glycolipids that will serve as anchor structures using phosphoglyceryl units from the phosphatidylglycerol present in the bacterial membrane as building blocks to elongate the LTA. Once the biosynthesis of the polymer is completed the LTA can be modified by D-alanylation or glycosylation (Figure 1.5) (Glaser and Lindsay, 1974; Neuhaus and Baddiley, 2003; Grundling and Schneewind, 2007a; Weidenmaier and Peschel, 2008; Xia et al, 2010a).

LTA has a crucial role in cell division, which might explain why it is indispensable for the viability of the cell (Weidenmaier and Peschel, 2008). Whilst mutants for some of the genes involved in the synthesis of LTA exist, they display aberrant cell shape and increased size (Kiriukin et al, 2001;

Grundling and Schneewind, 2007b; Richter et al, 2013). Loss of LTA has been associated with decrease in autolysin activity in *S. aureus* (Hanson and Neely, 2011). LTA is also involved in the interaction of *S. aureus* with the host (Weidenmaier and Peschel, 2008).

1.3.5.2 Wall teichoic acids

Wall teichoic acids (WTA) are anionic glycopolymers that are covalently attached to the peptidoglycan through a link to the 6-hydroxyl of the N-acetylmuramic acid residues via a disaccharide composed of N-acetylglucosamine-1-P and N-acetylmannosamine, followed by two units of glycerol phosphate (Neuhaus and Baddiley, 2003; Swoboda et al, 2010; Sewell and Brown, 2014).

The composition of the repetitive subunits varies between species and even between clonal groups, and typically comprises glycerol or ribitol phosphate (Qian et al, 2006; Xia et al, 2010a). *S. aureus* WTA are composed of D-ribitol connected by a 1,5 phosphodiester bond (Baddiley et al, 1962; Swoboda et al 2010). The subunits are modified by cationic D-alanine esters or monosaccharides (Figure 1.4) (Neuhaus and Baddiley, 2003; Swoboda et al, 2010). The length of the main chain of the wall teichoic acid polymers can vary, with 10-40 residues long for *S. aureus*, 40 residues for *B. subtilis* W23 and 45-60 residues for *B. subtilis* 168 (Foster and Popham, 2002).

The biosynthesis of WTA uses undecaprenol phosphate (C55-P) as lipid carrier during the assembly process and it starts with C55-P at the inner leaflet of the cytoplasmic membrane (Neuhaus and Baddiley, 2003; Xia et al, 2010a). The process has several stages that can be summarized as follows: i) linking of the disaccharide linkage unit $(Gro\text{-}P)_3$-ManNac-GlcNac to the C55-P, ii) polymerization of the subunits on the lipid intermediate, iii) glycosylation of the moiety, and iv) attachment of the WTA linkage unit to the peptidoglycan (Figure 1.6) (Neuhaus and Baddiley, 2003; Brown et al, 2008; Xia et al, 2010a; Brown et al, 2013).

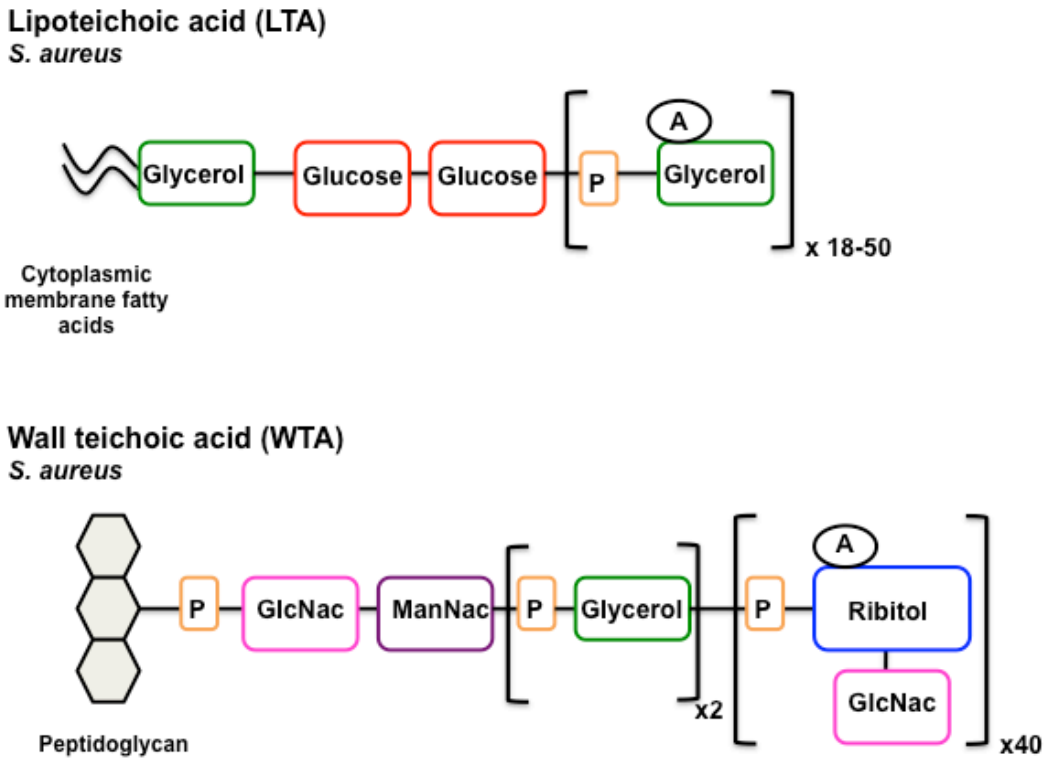


Figure 1.4 Schematic structure of lipoteichoic and wall teichoic acid of *S. aureus*
Lipoteichoic acids are anchored to the cytoplasmic membrane of the bacteria through their glycolipid fraction (fatty acids). The length of the poly-(glycerolphosphate) chain varies in different species. Wall teichoic acids are covalently attached to the peptidoglycan, with repeating units of poly-(ribitol) connected through phosphodiester bonds. The teichoic acids can be modified by D-alanine esters or monosaccharides. P: phosphate; A: D-alanine; GlcNac: N-acetylglucosamine; ManNac: N-acetylmannoseamine. Adapted from (Xia et al,2010b).

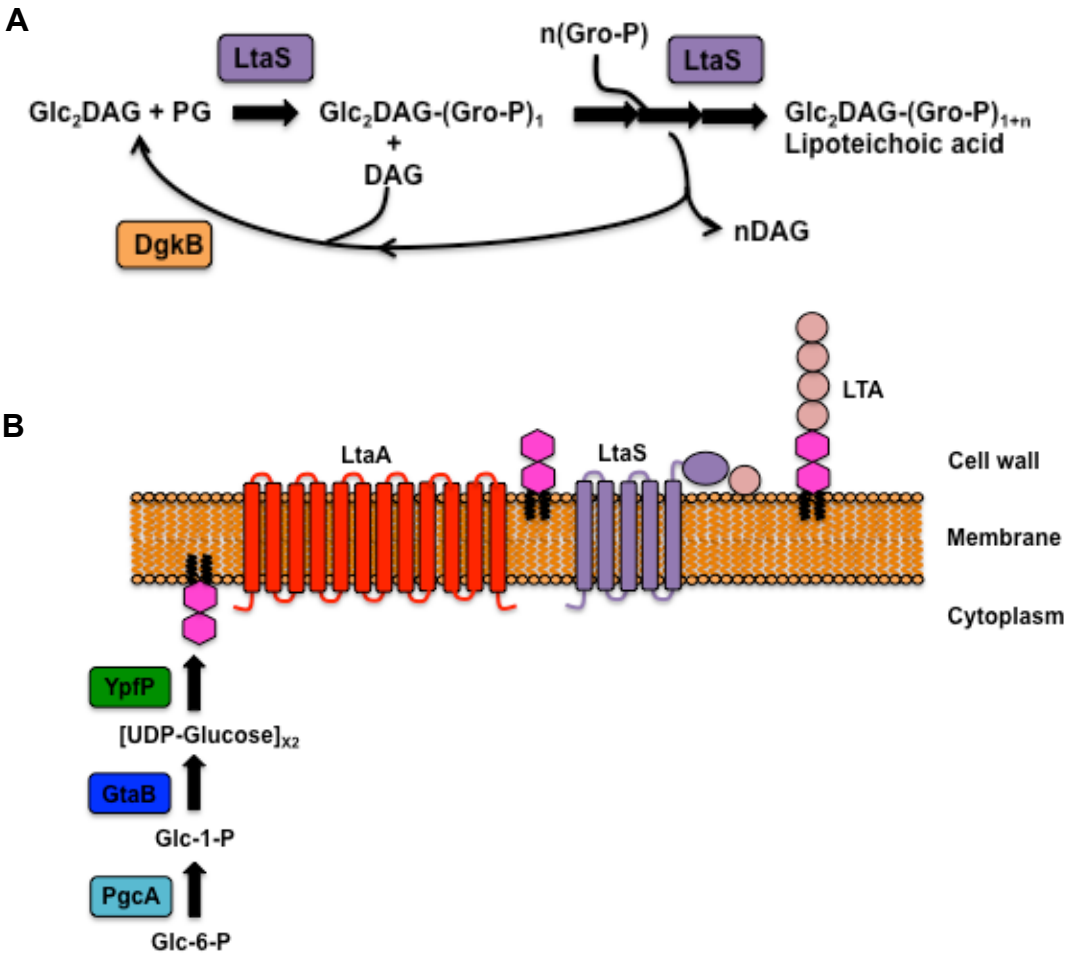


Figure 1.5 Synthesis of lipoteichoic acid in *S. aureus*

A. LtaS assembles the lipoteichoic acid at the outer surface of the cytoplasmic membrane. The first glycerolphosphate subunit is cleaved from phosphatidyl glycerol (PG) and is attached to diglycosyl-diacylglycerol (Glc_2DAG), the glycolipid anchor. The reaction leads to the release of DAG.

B. PgcA, GtaB and YpfP synthesize the Glc_2DAG glycolipid anchor and the transmembrane protein LtaA moves it to the outside of the cytoplasmic membrane. LtaS adds glycerolphosphate residues to the distal end of the growing glycerolphosphate chain.

Adapted from (Percy and Gründling, 2014; Schneewind and Missiakas, 2014).

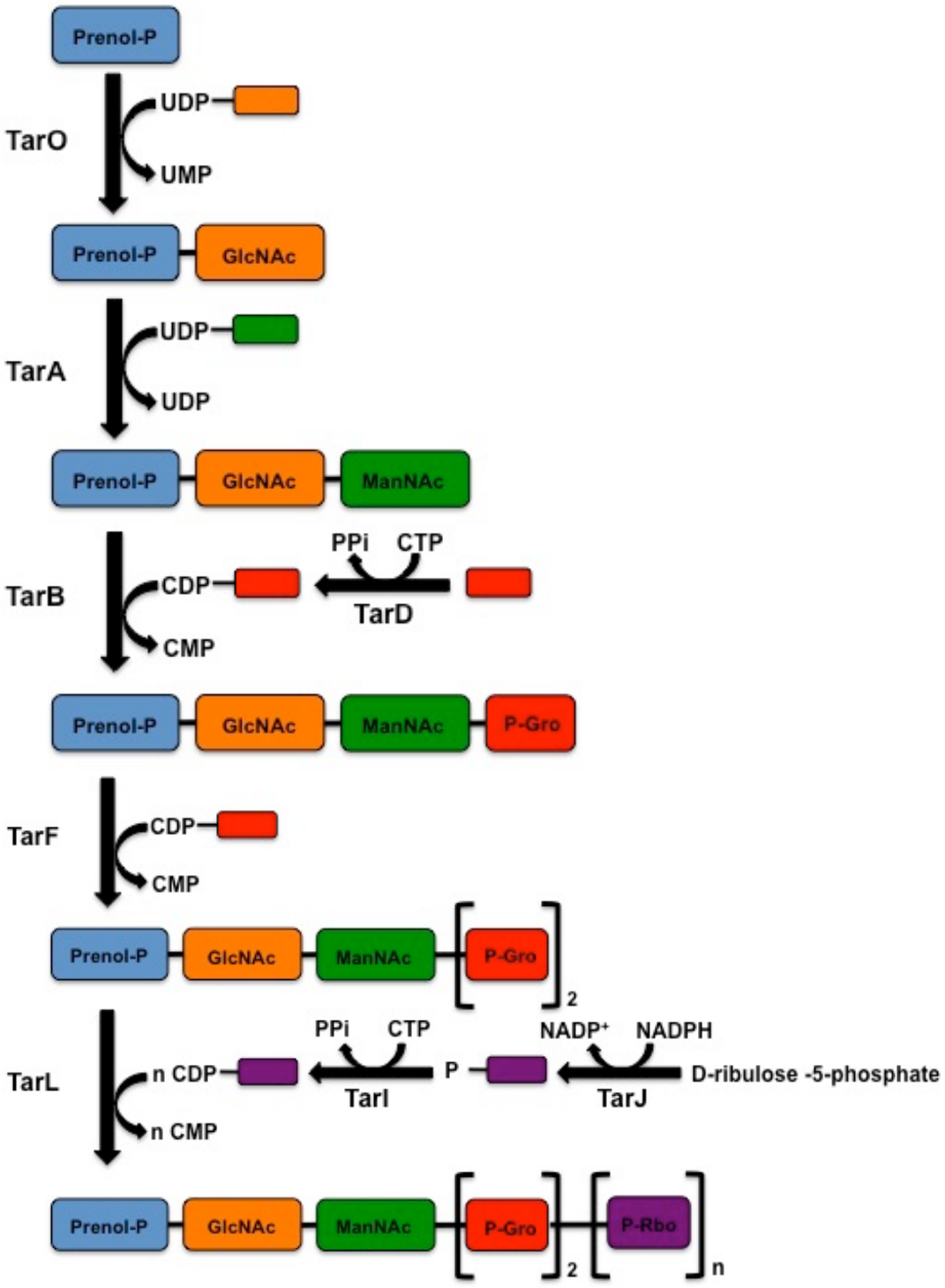

Prenol-P
TarO
UDP
UMP
Prenol-P
GlcNAc
TarA
UDP
UDP
Prenol-P
GlcNAc
ManNAc
TarB
PPi
CTP
CDP
TarD
CMP
Prenol-P
GlcNAc
ManNAc
P-Gro
TarF
CDP
CMP
Prenol-P
GlcNAc
ManNAc
P-Gro
2
TarL
PPi
CTP
NADP+
NADPH
n CDP
P
D-ribulose -5-phosphate
TarI
TarJ
n CMP
Prenol-P
GlcNAc
ManNAc
P-Gro
2
P-Rbo
n

Figure 1.6 Proposed polyribitol-phosphate WTA biosynthetic pathway for *S. aureus*.

The genes encoding the enzymes for the assembly of WTA have been isolated and characterized in *B. subtilis* 168 (*tag* genes, for teichoic acid glycerol). Functions were assigned to the *tar* genes (for teichoic acid ribitol) of *S. aureus* based on sequence homology to the *tag* genes. This wall teichoic acid is linked to a nascent peptidoglycan chain.

Adapted from (Neuhaus and Baddiley, 2003; Brown et al, 2008; Pereira et al, 2008; Brown et al, 2013)

S. aureus is capable of modulating the structure of WTA according to bacterial cell density and environmental changes through the repression of some of the WTA biosynthesis genes by the *agr* quorum sensing system (Meredith et al, 2008). The genes encoding enzymes for the assembly of WTA have been isolated and characterized in *B. subtilis* and *S. aureus*, and it was shown that they are organized in several operons. The regulatory elements of the operons are modulated by signals coupled to cell division and growth phase (Neuhaus and Baddiley, 2003; Brown et al, 2008).

The WTA can be modified via D-alanylation or glycosylation. The D-alanylation occurs after the completion of the biosynthesis of the glycopolymer via the activity of the *dltABCD* operon (Perego et al, 1995; Clemans et al, 1999; May et al, 2005). Glycosylation is modulated by TarM and TarS with α-GlcNac or β-GlcNac transferase activity, respectively (Xia et al, 2010a; Sobhanifar et al, 2015). The modifications affect cation homeostasis, antibiotic susceptibility and survival in the host, among other properties (May et al, 2005; Swoboda et al, 2010).

The presence of WTA in most of the Gram-positive bacteria suggests it has an important role (Weidenmaier and Peschel, 2008). Mutants lacking WTA have altered localization, assembly and activation of cell the wall elongation and division machinery (Bhavsar et al, 2001; D'Elia et al, 2006; Campbell et al, 2011; Brown et al, 2013). Wall teichoic acids are also important in cation homeostasis, provide a reservoir for ions that might be required for enzyme activity, create localized changes in the pH and modulate the function of enzymes, and help in the prevention of fluctuations in osmotic pressure between the inside and outside of the cell (Swoboda et al, 2010; Biswas et al, 2012; Brown et al, 2013).

WTA protects the cell from damage caused by cationic antibiotics (Peschel and Sahl, 2006), lysozymes, surfactants and antimicrobial peptides (Weidenmaier and Peschel, 2008; Silhavy et al, 2010; Swoboda et al, 2010). They have a role in the adherence to biomaterials and biofilm formation in *in vitro* and *in vivo* models (Gross et al, 2001; Fabretti et al, 2006; Xia et al, 2010b), and are

required for adhesion to the host tissue (Weidenmaier et al, 2004; Weidenmaier et al, 2005b; Swoboda et al, 2010). WTA are a potent immunogenic agent, and are recognized by the adaptive immune system and can be targeted effectively by antibodies (Neuhaus and Baddiley, 2003). WTA can also be a target for bacteriophages to adhere to the bacterial cell surface (Chatterjee, 1969; Weidenmaier and Peschel, 2008).

1.3.5.3 D-alanylation of the teichoic acids

The teichoic acids can be modified by D-alanylation once their synthesis is complete (Perego et al, 1995; Clemans et al, 1999; May et al, 2005) by the proteins encoded by the *dltABCD* operon (Figure 1.7) (Perego et al, 1995; Peschel et al, 1999).

In a two-step reaction that occurs in the cytoplasm of *S. aureus*, the product of the *dltA* gene, a D-alanyl carrier protein ligase (Dcl), activates the D-alanine by hydrolysis of ATP and transfers this high energy D-alanyl AMP intermediate to the phosphopantetheine cofactor of the D-alanine carrier protein (Dcp), which is encoded by *dltC* (Perego et al, 1995; Peschel et al, 1999; Koprivnjak et al, 1996). The roles of DltB and DltD are not completely clear, and several models exist (Percy and Gündling, 2014). The Fischer model suggests that DltB helps with the movement of D-alanine across the membrane while DltD is involved in the final transfer of the D-alanine to the LTA on the outside of the cell (Figure 1.8A) (Perego et al, 1995; Reichman et al, 2013). On the other hand, the Neuhaus and Baddiley model suggests that the DltD protein helps in the delivery of the activated Dcl-D-alanyl AMP intermediate complex to the Dcp protein (Debabov et al, 2000; Koprivnjak et al, 2006), while DltB is a transmembrane protein that is involved in the passage of the D-alanyl-Dcp complex through the cytoplasmic membrane and the incorporation of the D-alanine into the teichoic acids (Figure 1.8B) (Koprivnjak et al, 2006; Reichman et al, 2013).

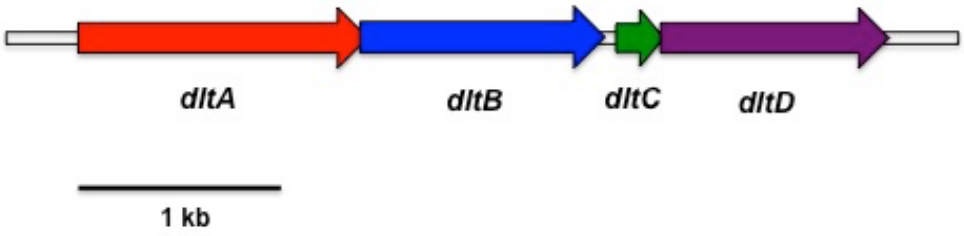


Figure 1.7 Organization of the *dlt* operon in *S. aureus*

This operon consist of *dltA*, *dltB*, *dltC* and *dltD*, which encodes for proteins of 485, 404, 78 and 391 amino acids, respectively (Peschel et al, 1999). The structure of the *dlt* operon is conserved in other organisms such as *E.faecalis*, *L. rhamnosus*, *B. subtilis* and *S. agalactiae* (Neuhaus and Baddiley, 2003; Fabretti et al, 2006). Adapted from (Peschel et al, 1999).

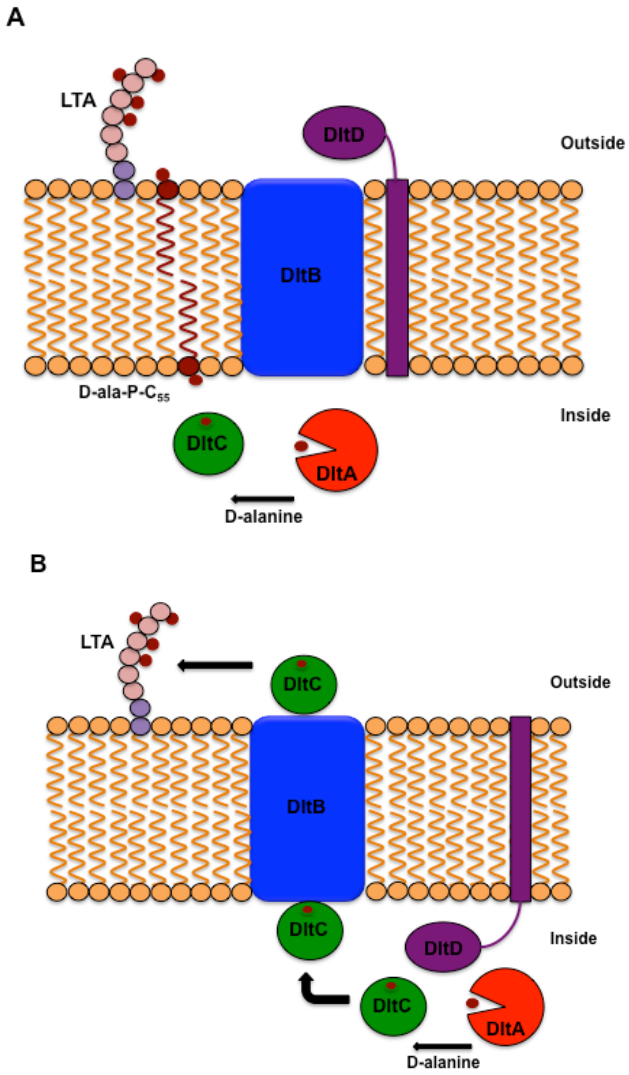


Figure 1.8 Models for the D-alanylation of teichoic acids in *S. aureus*

A. Fischer model: DltA activates D-alanine and transfers it to the carrier protein DltC. The DltB protein helps with the transfer of the D-alanine from DltC to undecaprenyl phosphate, while DltD is involved in the transfer of the residue to the LTA on the outside of the cell (Perego et al, 1995; Reichman et al, 2013).

B. Neuhaus and Baddiley model: the DltA and DltC proteins maintain the same function, but DltD increases the efficiency of the delivery of the activated D-alanine from DltA to DltC. The DltB protein helps the charged DltC to cross the membrane and in the incorporation of the D-alanine into the teichoic acids (Debabov et al, 2000; Neuhaus and Baddiley, 2003; Koprivnjak et al, 2006; Reichman et al, 2013).

Models adapted from (Reichmann et al, 2013).

It has been reported that D-alanine esterified to lipoteichoic acids is the precursor for the D-alanylation of the WTA (Haas et al, 1984; Koprivnjak et al, 2006), and that they are transferred to the wall teichoic acids by transacylation (Perego et al, 1995; Fabretti et al, 2006).

Changes in the degree of D-alanylation can affect the conformation and charge of the surface of *S. aureus*. The D-alanyl ester content of WTA is variable and can be modulated by environmental influences such as pH, temperature and salt concentrations (Neuhaus and Baddiley, 2003; Koprivnjak et al, 2006).

Considering that the amino group of the D-alanine is protonated at neutral pH (predicted pKa of 8.42), the D-alanine moiety has a positive charge (Bernal et al, 2009). Due to the positive charge of the amino acid, the esterification of the cell wall leads to a decrease in the negative charge of the cell surface, conferring resistance to cationic antimicrobial peptides (Peschel et al, 1999; Fedtke et al, 2004) and other components of the immune system of the host such as defensins and phospholipase A2 (Peschel et al, 1999; Koprivnjak et al, 2002).

The inactivation of the *dlt* operon affects the susceptibility of *S. aureus* to glycopeptide antibiotics such as vancomycin (Peschel et al, 2000). Mutant strains for these genes have altered the activity of autolysins (Peschel et al, 2000), biofilm formation (Gross et al, 2001; Fabretti et al, 2006) and virulence in the mouse model (Weidenmaier et al, 2004). The lack of D-alanylation also leads to increased susceptibility to phagocytic cells (Poyart et al, 2003) and to neutrophil killing (Collins et al, 2002), suggesting a role for the D-alanylation of teichoic acids in the modulation of the immune response (Fournier and Philpott, 2005; Chan et al, 2007).

1.4 Host response and defence mechanisms

Evolutionary evidence suggests that mechanisms of host defence existed by the time the ancestors of plants and animals diverged. This system is the Toll pathway of NFκB activation of gene function, and can be found in fruit flies such as *Drosophila* and in vertebrates, and evidence exists that occurs in plants (Wilson et al, 1997; O'Neil and Greene, 1998; Valanne et al, 2011).

Recent evidence suggests that the emergence of vertebrates with jaws (gnathostomes) was accompanied by a change in the structure and function of the immune system that led to the appearance of the adaptive immune system (Flajnik and Du Pasquier, 2004; Cooper and Alder, 2006; Boehm et al, 2012). The vertebrate immune system can be classified into two sub-systems: the innate and adaptive immune systems. Innate immunity is evolutionary ancient and immediate, and is considered a non- specific response, while the adaptive system mediates highly specific but delayed response (Vivier and Malissen, 2005).

1.4.1 Innate immune system

The innate immune system is capable of recognizing molecules that are evolutionary conserved and shared by many infectious agents, such as lipopolysaccharides, peptidoglycan, non-methylated CpG, mannan in yeast and double-stranded RNA (Beutler, 2004; Vivier and Malissen, 2005). These molecules are called "pathogen-associated molecular patterns" (PAMPs) and can be recognized due to a set of defined receptors called pathogen- or pattern-recognition receptors (PRRs) (Ausubel, 2005; Akira et al, 2006).

This first line of defence against infections is composed of the skin, the secreted mucus layer that overlays the epithelium in the respiratory, gastrointestinal and genitourinary tracts, soluble proteins such as antimicrobial peptides, bacterial permeability increasing proteins and complement system components, pattern-recognition receptors that recognize and bind to molecular patterns expressed

on the surface of pathogens, and by cells like NK cells, macrophages and neutrophils (Figure 1.9) (Parkin and Cohen, 2001; Beutler, 2004; Carroll, 2004; Chaplin, 2010; Kumar and Sharma, 2010).

1.4.1.1 Cellular components

Neutrophils, mast cells, natural killer cells, macrophages and dendritic cells comprise the cellular components of the innate immune system (Kumar and Sharma, 2010). Vertebrates are largely dependent upon these cells for the engulfment and destruction of pathogens (Beutler, 2004). These cells can defend the organism on their own but they have evolved to work better with the elements of the adaptive immune system.

Mast cells function as immune 'sentinel' cells that can respond directly to pathogens through phagocytosis and the production of reactive oxygen species and antimicrobial peptides (Dawicki and Marshall, 2007; Abraham and St John, 2010). Mast cells also send signals that can modulate the innate and adaptive immune responses (Parkin and Cohen, 2001; Urb and Sheppard, 2012).

Dendritic cells also work as 'sentinels' and are known as the link between innate and adaptive immunity. They can capture and process antigens, which cause their differentiation, which is called 'maturation'. This maturation influences the type of the following immune response (Th1 vs Th2) (Clark et al, 2000; Steinman and Hemmi, 2006).

Natural killer cells eliminate virally infected cells using cytotoxic proteins (Topham and Hewitt, 2009). Macrophages eliminate pathogens via phagocytosis, and they can also regulate lymphocyte activation and proliferation (Elhelu, 1983; Parkin and Cohen, 2001).

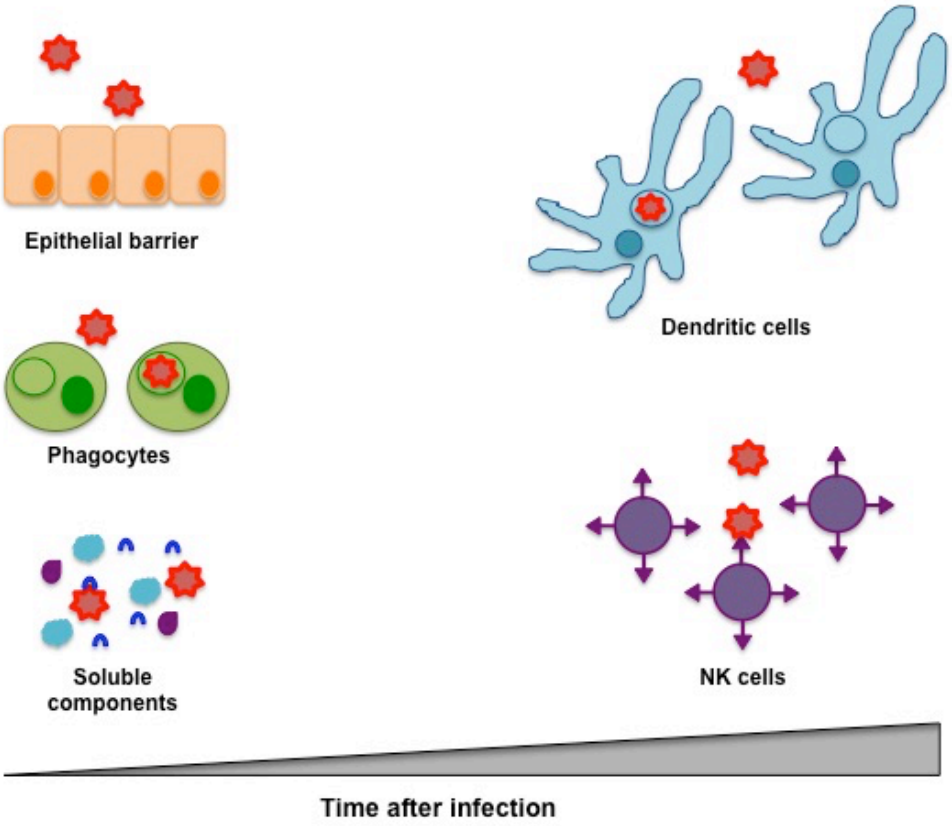


Figure 1.9 Components of the innate immune response in humans

When a pathogen gets in contact with the host several elements work together in order to stop the infection. Some of these elements are shown here. Adapted from (Beutler, 2004) and University of California San Francisco, Immunology Module (http://missinglink.ucsf.edu/lm/immunology_module/prologue/objectives/ obj02.html).

Neutrophils are dynamic cells that survey connective tissue, mucosal membranes, skeletal muscle and lymphatic organs for any sign of tissue damage, inflammation or invading microorganisms (Rigby and De Leo, 2012). Neutrophils can migrate to the site of infection for containment and clearance of the pathogens through the engulfment of the infectious particles, followed by destruction using large quantities of reactive oxygen species that are cytotoxic to pathogens (Beutler, 2004; Chaplin, 2010; Kumar and Sharma, 2010). These cells also produce enzymes that are involved in tissue repair after injury (Kumar and Sharma, 2010), as well as cytokines and chemokines, expanding the role of neutrophils to regulators of the immune response (Chaplin, 2010; Kumar and Sharma, 2010; Mantovani et al, 2011; Rigby and De Leo, 2012).

1.4.1.2 Soluble components

Cells are not the only components of the innate immune system. The soluble components of the innate immunity can be separated into two groups: sensors and effectors. Different molecules are responsible for sensing pathogens. For example, mannose-binding protein can recognize the terminal mannosyl residues on the surface of pathogens and activates the complement system via the lectin pathway (Matsushita and Fujita, 1992; Matsushita and Fujita, 1995; Thiel et al, 1997). C-reactive protein and other pentraxins can also bind directly to pathogens via lectin-like interactions (Beutler, 2004; Shishito et al, 2012).

Collectins are calcium-dependent lectins that can be found in mammals and target and bind to carbohydrates in the surface of bacteria, fungi, viruses and even potential allergens. This binding leads to the agglutination of the target particle, mediating phagocytosis through specific receptors on the phagocytes (Hakansson and Reid, 2000; Beutler, 2004). Lipopolysaccharide-binding protein and CD14, in its soluble form, recognize lipopolysaccharide from several species of Gram-negative bacteria. This interaction leads to the activation of monocytes and macrophages with the subsequent secretion of inflammatory cytokines and other mediators (Schumann et al, 1994; Gutsmann et al, 2001; Beutler, 2004).

The previously discussed proteins are able to sense the pathogens in the host, but they are not able to eliminate them. Other extracellular molecules take care of that. Human skin is able to secrete fatty acids into the sebum and these molecules are extremely effective killing agents against *S. aureus*. They can prevent colonization by the bacteria, reduce the severity of the infection and kill *S. aureus* within abscesses (Miller et al, 1988; Wille and Kydonieus, 2003; Georgel et al, 2005; Cartron et al, 2014; Neumann et al, 2015).

Human cells can produce proteins that are able to destroy the bacterial envelope like lysozyme and phospholipase (Femling et al, 2005), and iron-binding proteins like transferrin and lactoferrin that are able to restrict the iron available for bacterial growth (Bullen, 1981; Bullen et al, 2005; Ling and Schryvers, 2006). Lactoferrin can also affect the motility and biofilm formation of certain bacteria (Singh et al, 2002). The bactericidal/permeability increasing protein (BPI) is produced by polymorphonuclear leukocytes and acts against Gram-negative bacteria, binding to the cells and causing growth arrest and outer membrane alterations, increasing its permeability (Mannion et al, 1990; Holweg et al, 2011). This protein can work together with phospholipase and kill Gram-negative bacteria such as *E. coli* and *S. typhimurium* (Elsbach et al, 1979; Nevalanien et al, 2008).

Mammals are able to synthesize two kinds of antimicrobial peptides: defensins and cathelicidins, which are expressed in neutrophils and in epithelia, respectively (Beutler, 2004). These antimicrobial peptides are normally short with an overall positive charge and a substantial proportion of hydrophobic residues (≥ 30%) (Zasloff, 2002; Hancock and Sahl, 2006). Because they are cationic peptides they can electrostatically interact with the bacterial membrane, which usually comprises negative polymers such as lipopolysaccharides and wall teichoic acids (Brodgen, 2006; Hancock and sahl, 2006; Thomas et al, 2010). These peptides cause cell death through the disruption of the integrity of bacterial cell membrane, or the inhibition of extracellular polymer synthesis (like peptidoglycan synthesis) or intracellular functions (Ong et al, 2002; Yeaman and Yount, 2003; Hancock and Sahl, 2006).

1.4.1.3 Complement system

The complement system is a family of about 30 serum and cell membrane proteins that can recognize and kill pathogens such as bacteria, virus infected cells and parasites (Gasque, 2004), and also takes part in the enhancement of B and T cells responses (Barrington et al, 2001; Carroll et al, 2004; Gál et al, 2007; Ansari and Sayegh, 2008) and in tissue regeneration (Kimura et al, 2003; Shishido et al, 2012).

Phylogenetically, complement is an ancient arm of the innate immunity (Gasque, 2004). While the adaptive immune system is present only in jawed vertebrates, complement components can also be found in jawless fish and non-vertebrate deuterostomes. The liver is the major source of most of the complement components in mammals, with the exception of C1q, Factor D and C7 (Morgan and Gasque, 1997; Gasque, 2004): C1q can be synthesized by epithelial cells, monocytes and fibroblasts (Tenner and Volkin, 1986), Factor D is mainly produced by adipocytes (White et al, 1992) and the major sources of C7 are monocytes and macrophages (Würzner et al, 1994).

The components circulate in the serum as pro-proteins and can be activated through cleavage, which removes the inhibitory fragment of the molecule and exposes the active site. This activation occurs in a cascade-like manner, with components activating each other (Gál et al, 2007). Once activated, these components have a short half- life before being inactivated again.

When activated, the complement system generates a potent response and causes intense local inflammation that can severely damage host tissues. To control the correct activation of the complement system the host cells are protected by regulatory molecules that inhibit the assembly of the central C3-cleaving enzymes or the formation of the membrane attack complex (Morgan, 1995; Miwa and Song, 2001; Gasque, 2004; Kim and Song, 2006). These regulatory molecules can be soluble plasma proteins or can be expressed on the cell membrane (Chaplin, 2010).

Three separate pathways can activate the complement system: classical, alternative (or properdin) and lectin pathways (Figure 1.10). The classical pathway is activated by antibodies; the lectin pathway is activated by the recognition of the mannose binding protein target in the bacteria; and the alternative pathway is spontaneously activated by C3 and the interaction between Factor B and the microbial surface (Beutler, 2004; Carroll, 2004; Gasque, 2004). The three activation pathways include the activation of C3b, which is able to bind to pathogens and opsonize them for phagocytosis, and the generation of C5a (inflammatory and chemotactic molecule) and C5b (Beutler, 2004). The activation and sequential deposition of C5b through C9 is essential for the formation of the membrane attack complex (MAC), a ring shaped assembly of protein subunits that can kill Gram-negative bacteria through osmotic lysis but is ineffective against Gram-positive bacteria due to their thick peptidoglycan layer (Muller-Eberhard, 1986; Bestebroer et al, 2010; Yongqing et al, 2012).

1.4.1.3.1 Classical Pathway

The classical pathway can be activated by the formation of a soluble antigen-antibody complex or by the binding of an antibody to an antigen on a suitable target, like a bacterial cell (Figure 1.10) (Gasque, 2004).

The first component of this pathway is C1q, a very versatile pattern recognition molecule (Ricklin et al, 2010). The C1q component can interact with these immune complexes and cause the sequential activation of the C1r and C1s proteases, forming the C1 complex ($C1qC1r_2C1s_2$) (Ricklin et al, 2010). C1q can also interact and be activated by bacterial, fungal and virus membrane components (Brown et al, 2002; Gasque, 2004). Activated C1s can cleave C2 and C4, activating them and forming a C3 convertase, C4bC2a. This C3 convertase can cleave C3 into C3a and C3b.

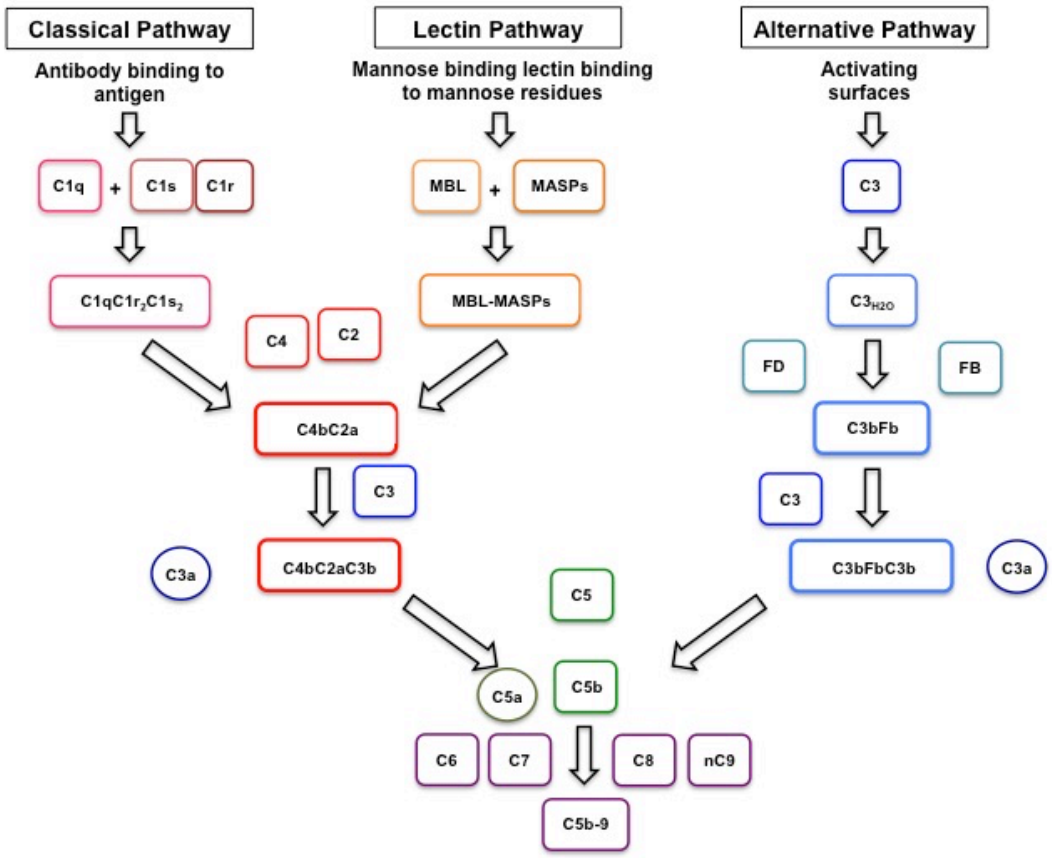


Figure 1.10 Activation pathways of the complement system

The complement system can be activated by three pathways: classical (CP), lectin (LP) or alternative (AP). The CP is activated by immune complexes, LP by the recognition of carbohydrates present on the cell surface and AP is spontaneously activated by activating surfaces, water and factor B. These three pathways include the activation of C3b through the C3 convertases, the generation of C5b by the C5 convertases and the liberation of C3a and C5a, inflammatory and chemotactic molecules. The three pathways lead to the formation of the membrane attack complex (MAC). Adapted from (Gasque, 2004; Ricklin et al, 2010).

The C3a fragment is a small cationic pro-inflammatory peptide (Klos et al, 2009) that also has potent antimicrobial activity by itself (Nordahl et al, 2004). Along with C5a, anaphylatoxin produced by the cleavage of the component C5 by the C5 convertases, the C3a fragment is involved in the regulation of vasodilation and permeability of small blood vessels (Ember et al, 1998), tissue regeneration (Mastellos et al, 2001; Klos et al, 2009), tissue fibrosis (Hillebrandt et al, 2005) and brain development (Klos et al, 2009). As an anaphylatoxin C3a acts as a chemoattractant for neutrophils, eosinophils, monocytes, mast cells and macrophages towards the sites where the complement system has been activated (Hartmann et al, 1997; Haas and van Strijp, 2007; Klos et al, 2009; Ricklin et al, 2010). This molecule is also able to modulate the synthesis of IL-6 and TNF-α from B cells and monocytes (Fischer and Hugli, 1997; Fischer et al, 1999).

The cleavage of the complement component C3 also produces the C3b molecule. This cleavage exposes an intramolecular thioester bond present in C3b that allows it to bind covalently to amines and carbohydrates found in the bacterial surface (Law and Dodds, 1997; Rooijakkers and van Strijp, 2007; Ricklin et al, 2010; Berends et al, 2013).

This deposition of the C3b molecule on the bacterial surfaces acts as a signal for phagocytic uptake by neutrophils and macrophages (Rooijakkers and van Strijp, 2007; Shishido et al, 2012). The opsonisation of the bacteria also leads to the amplification of cleavage of C3 via the activation of the alternative pathway (Rooijakkers et al, 2005c; Laarman et al, 2011) and the formation of its C5 convertase (Petersen et al, 2000; Ricklin et al, 2010). The binding of C3b to C4bC2a shifts the substrate specificity from C3 to C5 and leads to the formation of the lectin and classical pathway C5 convertase (C4bC2aC3b) (Petersen et al, 2000; Ricklin et al, 2010).

The C5 convertase cleaves C5 producing C5a, another protent anaphylatoxin (Haas and van Strijp, 2007; Klos et al, 2009), and C5b. The C5b molecule leads to the generation of the C5b-C9 complex (also known as membrane attack complex or MAC), which is capable of inserting itself into the bacterial

membrane and generate pores, leading to lysis (Müller-Eberhard, 1986; Laarman et al, 2011; Berends et al, 2013).

This convertase initiates the late stages of complement activation, cleaving C5 to form C5a and C5b. The C5-cleavage product C5a is a proinflammatory molecule that attracts phagocytes to the site of infection and primes them for bacterial uptake (Bestebroer et al, 2010), while C5b is necessary for formation of the membrane attack complex in the cell membrane (Rawal and Pangburn, 2001). C5b interacts with C6 and then with C7, which induces a hydrophilic-amphiphilic transition that allows this trimolecular complex to bind to the cell membrane (Müller-Eberhard, 1986). C8 binds next and this C5b-C8 complex is able to lyse erythrocytes but not nucleated cells. When C9 binds to the C5b-C8 complex this induces the binding of more units of C9, forming a poly-C9 that can form disrupt and form a large transmembrane pore that leads to the lysis of the target cell (Müller-Eberhard, 1986; Ricklin et al, 2010).

Due to the strong response and inflammation that this pathway can generate, a tight regulation system is in place. The activation of the classical pathway can be stopped from the start by the C1 inhibitor (C1-inh), a secreted glycoprotein that is able to break the C1qC1rC1s complex and turn it into C1rC1s$(C1\text{-inh})_2$ complexes, leaving the C1q protein bound to the activator (Ziccardi and Cooper, 1979; Sim et al, 1979). The C2 receptor inhibitor trispanning (CRIT) can bind to C2 and inhibit its activation by the C1s protease (Inal et al, 2005).

1.4.1.3.2 Lectin pathway

Mannose-binding lectins (MBL) and ficolins (H-ficolin and L-ficolin) act as the pattern recognition molecules that activate the lectin pathway (Schwaeble et al, 2002; Gasque, 2004; Ma et al, 2004; Seelen et al, 2005; Ricklin et al, 2010). They are able to recognize and bind to carbohydrates present in the bacterial cell wall, such as mannose and N-acetylglucosamine (Figure 1.10) (Matsushita et al, 2000b).

This binding induces the activation of the MBL-associated serine proteases or MASPs. These proteases share structural similarity with the C1s and C1r proteases from the classical pathway (Ricklin et al, 2010) and the MBL-MASPs complex has a strong structural and functional similarity with C1qC1rC1s, the recognition complex of the classical pathway (Roos et al, 2003). In human blood three types of MASPs have been identified, MASP-1, MASP-2, and MASP-3, and a truncated form of MASP-2 called sMAP (small MBL-associated protein) or Map19 (MBL-associated plasma protein of 19 kDa) (Takahashi et al, 1999; Matsushita et al, 2000a; Ma et al, 2004; Takahashi et al, 2006).

The binding of MBL to the ligands present in the target cells induces the activation of the MASPs pro-enzymes, and this activated complex is capable of cleaving the complement components C2 and C4, leading to the formation of the C3 convertase C4bC2a (Matsushita et al, 2000a; Petersen et al, 2000; Schwaeble et al, 2002; Ricklin et al, 2010). As in the classical pathway, this C3 convertase is able to cleave C3, which leads to the formation of the C5 convertase C4bC2aC3b. This leads to the late stages of complement activation, ending with the formation of the membrane attack complex (Matsushita et al, 2000a; Petersen et al, 2000; Roos et al, 2003; Seelen et al, 2005).

C1-inh can associate with the MBL complex and inhibit its activity (Matsushita et al, 2000a). The complement Factor I is one of the regulatory proteins of the complement system, a serine protease that can inactivate C3b and C4b through the cleavage of alpha chains in the components (Schlaf et al, 2001; Gasque, 2004; Ricklin et al 2010).

1.4.1.3.3 Alternative pathway

This pathway of complement activation is also known as the properdin parthway, due to the important role that this plasma component has. The complement component C3 is relatively inert in its native form and has few ligands. In plasma C3 can turn into $C3_{H2O}$ via spontaneous hydrolysis, exposing new binding sites (Figure 1.10) (Harboe and Mollnes, 2008; Ricklin et al, 2010).

This form of C3 is unstable, and is either inactivated by hydrolysis or can form covalent bonds with the cell surface of a nearby target, allowing the complement activation to continue when deposited on a non-self surface or on immune complexes (Harboe and Mollnes, 2008). This binding to a cell surface favours the binding of the Factor B protease to $C3_{H2O}$, and Factor D cleaves Factor B into Ba and Bb. The C3bBb complex is formed on the cell surface. The half-life of this complex is short under physiological conditions but it can be stabilized 5- to 10-fold by association with properdin (Fearon and Austen, 1975; Kemper et al, 2010). This spontaneous activation of C3 is controlled and inhibited by Factor H, a glycoprotein that prevents the binding of Factor B and promotes the dissociation of the C3Bb complex (Schlaf et al, 2010).

This C3bBb complex is the C3 convertase of the alternative pathway, capable of cleaving C3 into C3a and C3b (Beutler, 2004; Gasque, 2004). This creates an efficient cycle of C3 cleavage and convertase assembly that markedly amplifies the immune response of the complement system. This amplification leads to increased density of C3b, helping the formation of the C4bC2aC3b and C3bBbC3b complexes, the C5 convertases. As stated previously, the formation of these convertases shifts the substrate specificity from C3 to C5, initiating the late stages of complement activation (Petersen, 2000; Seelen et al, 2005; Ricklin et al, 2010). The formation of C5b initiates the assembly of the terminal complement complex, leading to cell lysis.

1.4.1.4 Phospholipase A2

The phospholipase A2 (PLA2) superfamily consist in a varied group of enzymes that are capable of hydrolysing phospholipids in the sn-2 position of the ester bond. This reaction generates a free fatty acid and a lysophospholipid (Figure 1.11) (Six and Dennis, 2000; Balsinde et al, 2002; Wu et al, 2010). The fatty acids, such as arachidonic acid and oleic acid, play a role in the storage of energy (Six and Dennis, 2000). Arachidonic acid can also be converted into eicosanoids, signalling molecules that play a role in immune responses, inflammation and pain perception (Balsinde et al, 1999; Balsinde et al, 2002; Schaloske and Dennis, 2006; Dennis et al, 2011). Lysophospholipids, the other

molecule generated by PLA2, can serve as a precursor for lipid mediators such as lysophosphatidic acid and platelet activating factor (PAF), and is involved in phospholipid remodeling, cell signalling and membrane perturbation (Six and Dennis, 2000; Schaloske and Dennis, 2006; Dennis et al, 2011).

This superfamily can be divided into five kinds of enzymes based on their catalytic mechanism as well as functional and structural features: secreted PLA2 (sPLA2); cytosolic PLA2 (cPLA2); calcium-independent PLA2 (iPLA2); platelet activating factor acetylhydrolase (PAF-AH) and lysosomal PLA2 (Schaloske and Dennis, 2006; Dennis et al, 2011). The cytosolic PLA2 (cPLA2) are large cytosolic proteins with a molecular weight of 85 kDa that have a serine residue in the catalytic site (Marshall et al, 2000, Chakraborti, 2003). These enzymes utilize calcium not for catalysis but for translocation to the intracellular membrane. These enzymes have a preference for phospholipids containing arachidonic acid and it has been suggested that they might be associated with signalling cascades associated with receptors (Six and Dennis, 2000; Chakraborti, 2003; Dennis et al, 2011).

iPLA2 are enzymes with a molecular weight of 85 kDa and a serine residue in the catalytic site as for cPLA2 (Burke and Dennis, 2009). They are intracellular enzymes with no substrate specificity for phospholipids containing arachidonic acid, have no need for calcium and contain eight ankyrin repeats in the N-terminal half of the molecule (Balsinde et al, 1999; Schaloske and Dennis, 2006; Burke and Dennis, 2009). It has been suggested these enzymes are involved in bone formation, sperm development, insulin secretion, and monocyte recruitment and directionality (Mishra et al, 2008; Burke and Dennis, 2009).

Unlike the other PLA2s with catalytic dyads in their active sites, the PAF-AH has a catalytic triad formed by a serine, histidine and aspartic acid (Tjoelker et al, 1995; Burke and Dennis, 2006). This enzyme can act on oxidized lipids and platelet activating factor present in the sn-2 position, and it has been shown that

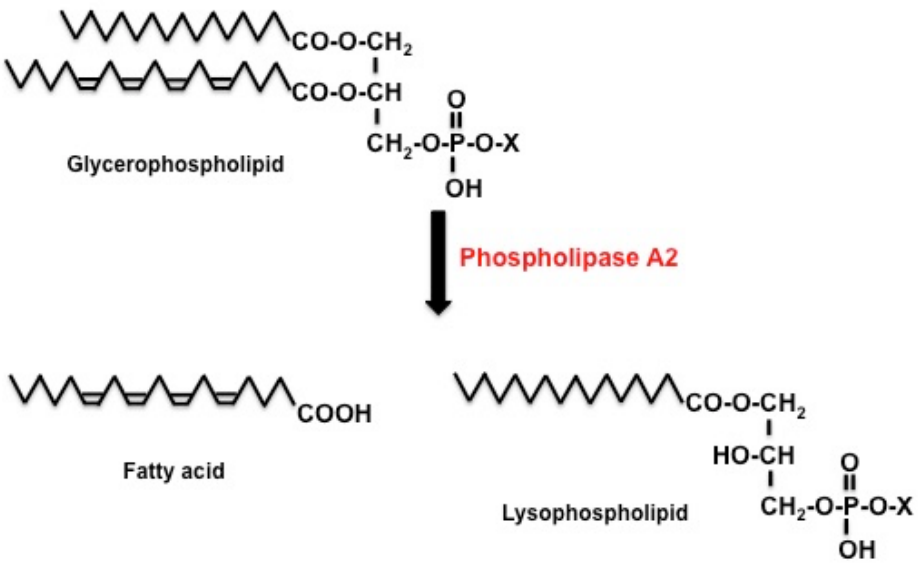


Figure 1.11 Hydrolysis of phospholipid by phospholipase A2

PLA2 enzymes are able to hydrolyse phospholipids in the *sn*-2 position generating fatty acids and lysophospholipids. X: head group. Adapted from (Murakami et al, 2011).

this enzyme has pro-inflammatory activity (Chakraborti, 2003; Burke and Dennis, 2006). The lysosomal PLA2 has calcium-independent PLA2 and transacylase activities, the same catalytic triad as PAF-AH, it localizes in lysosomes and shows preference for phosphatidylcholine and phosphatidylethanolamine (Schaloske and Dennis, 2006).

The secreted PLA2 (sPLA2) is a group of small secreted proteins (14-16 kDa) that usually contains 6 to 8 sulphide bonds (Schaloske and Dennis, 2006; Dennis et al, 2011). Calcium is required for their activity due to the histidine/aspartic acid catalytic dyad (Dennis et al, 2011; Murakami et al, 2011; Murakami and Lambeau, 2013). There are other conserved residues that participate in the active site hydrogen bonding networks, including tyrosines and glycines in the calcium binding loop and a second aspartate residue that can activate and orient the catalytic histidine (Six and Dennis, 2000; Murakami et al, 2011).

The sPLA2 enzymes can be found in mammals and snake venoms, and have also been isolated from plants, invertebrates and fungi (Schaloske and Dennis, 2006; Nevalainen et al, 2008). In mammals these enzymes can be found in several fluids, including tears, blood, seminal plasma, inflammatory exudates and the intestinal lumen (Kunze and Bohn, 1978; Tayakama et al, 1991; Weinrauch et al, 1996; Qu and Lehrer, 1998; Mennschikowski et al, 2006). They are capable of acting on cellular membranes, microbial membranes, lipoproteins, pulmonary surfactant and microvesicles (Murakami et al, 2011), but show differential selectivity towards different cell types: Gram-positive bacteria > Gram-negative bacteria >> eukaryotic cells (Nevalainen et al, 2008). sPLA2 do not show specificity for particular sn-2 position fatty acids, but instead for certain head groups of the phospholipids (Schaloske and Dennis, 2006; Dennis et al, 2011).

These enzymes play a role in inflammatory diseases such as rheumatoid arthritis (Schaloske and Dennis, 2006), in the development of atherosclerosis (Webb, 2005), exocytosis (Lambeau and Gelb, 2008; Harris and Scott-Davey, 2013) and are involved in coagulation (Schaloske and Dennis, 2006; Lambeau

and Gelb, 2008). They also play a role in the immune system of the host due to their antifungal (Moerman et al, 2002; Samy et al, 2007) and antiviral activities (Dennis et al, 2011), but mostly through their well known antibacterial activity against Gram-positive and Gram-negative bacteria (Weiss et al, 1994; Weinrauch et al, 1996; Qu and Lehrer, 1998; Laine et al, 1999; Buckland and Wilton, 2000).

1.4.2 Adaptive immune system

As stated above (Chapter 1.4.1), the innate immune system can fight infection very efficiently, but is also capable of causing important collateral damage. The emergence of vertebrates with jaws (gnathostomes) was accompanied by the appearance of the adaptive immune system (Cooper and Alder, 2006; Boehm et al, 2012). The adaptive immune system increases the efficacy of the immune response by focusing the response in an antigen-specific manner using highly specific receptors, minimizing collateral damage (Palm and Medzhitov, 2009). This system can recognize bacteria, viruses, fungi and parasites (Cooper and Alder, 2006).

An important difference with the innate immunity is that adaptive immunity is able to generate long lasting immunological memory. This provides protection from future infections with the same pathogen via an accelerated response upon a new encounter (Usharauli, 2010). Another difference is that the adaptive immune response is relatively slow in comparison with the innate immunity (Hedrick, 2004). One problem with the adaptive system is that because the receptors are randomly generated they can potentially lead to autoimmunity or to allergies (Palm and Medzhitov, 2009).

The adaptive immune system uses randomly generated, clonally expressed and highly specific receptors of unlimited specificity to recognize microbial invaders (Palm and Medzhitov, 2009), allowing this system to focus the response and minimize the collateral damage. The lymphocytes are the specialized cell type

of the adaptive immune system, they generate from progenitor cells within the bone marrow with receptors in their cell surface that are able to recognize different antigen configurations of specific pathogens (Parking and Cohen, 2001). Once this recognition takes place a clonal amplification is triggered, leading to cell differentiation and antibody production that has the same antigen binding specificity of the receptor in the lymphocyte that interacted with the pathogen in the first place (Figure 1.12) (Parkin and Cohen, 2001; Cooper and Alder, 2006).

The response of the adaptive immune system is a multistep process with several checkpoints that can control different aspects of the response and integrate information from different signals. These checkpoints help the response to determine the origin of the infection and its type, the extent and duration of the infection and the requirement for immediate or future defence (Iwasaki and Medzhitov, 2004; Pasare and Medzhitov, 2004; Palm and Medzhitov, 2009).

Adaptive immunity is mediated by a large number of immunoglobulins and B and T cell receptors (BCRs and TCRs, respectively), which are generated through recombinaton of variable (V), diversity (D) and joining (J) gene segments (Litman et al, 2010). Molecules encoded by the major histocompatibility complex (MHC) alleles control the TCR-mediated response through the presentation of the antigenic peptides (Vivier and Malissen, 2005). Each TCR has an *a priori* ligand (Vivier and Malissen, 2005). The immunoglobulins function as membrane-bound receptors on the B cells and their precursor cells, and they are selected by their antigen-binding specificity and affinity during their development (Vivier and Malissen, 2005; Palm and Medzhitov, 2009).

It is known that the complement system is able to modulate adaptive immunity (Ricklin et al, 2010). The activation and deposition of complement components within immune complexes helps to target them to cells that have receptors against these antigens, such as B lymphocytes (Parkin and Cohen, 2001). The

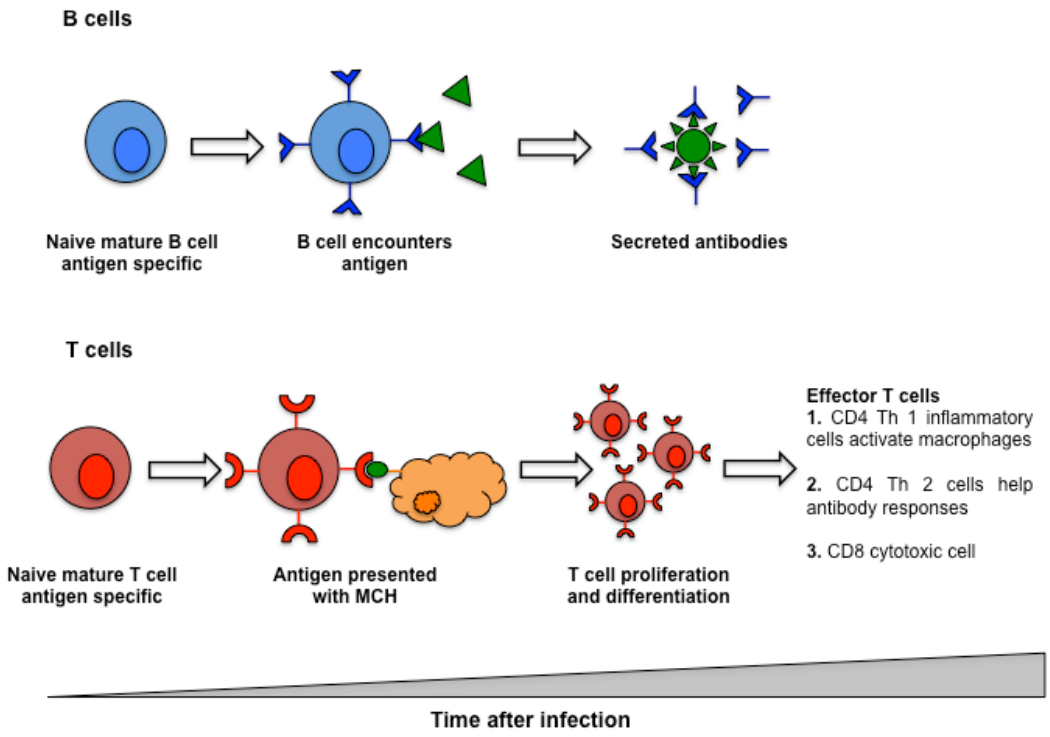


Figure 1.12 Components of the adaptive immune system

When antigens are recognized by specific receptors present in B and T cells, these cells are activated and differenciated within lymphoid tissue. This leads to the effector response through antibodies released into the blood by activated B cells or through effector T cells travelling to the infection site.

Adapted from (Parkin and Cohen, 2001).

antibody response is also controlled by complement-derived fragments, as shown in experiments where animals without C3 had a reduced formation of antibodies against T cell-dependent antigens (Pepys, 1974; Gasque, 2004). It has also been reported that the alpha chain of the C4b protein can activate B-lymphocytes through the CD40 receptor (Brodeur et al, 2003). The C1q component of the classical pathway of complement activation also acts in adaptive immunity after binding to natural IgM complexes to antigens (Heyman, 2000).

1.4.3 Rabbit immune system

The European laboratory rabbit (*Oryctolagus cuniculus*) belongs to the *Leropidae* family of the *Lagomorpha* order (Okerman, 1989). Originally they could be found in the Iberian Peninsula, western France and northern Africa (Gibb, 1990), but currently their presence is widespread throughout the world (Thompson and King, 1994).

The rabbit was an important model species during the early times of immunology and the first studies about allelic exclusion and inheritance of idiotypes were carried out in rabbits (Davie et al, 1971; Hague and Kindt, 1998). They were also used in early studies of transplantation and histocompatibility (Ehlers and Ahrons, 1971; Goldstein et al, 1975; Hague and Kindt, 1998). This species has also been used as a model for the study of rheumatoid arthritis (Beischer et al, 2002), atherosclerosis (Yanni, 2004; Phinikaridou et al, 2009), syphilis (Froberg et al, 1993), herpes virus (Schlitt et al, 1986), adult T cell leukemia-lymphoma (Simpson et al, 1996) and human immunodeficiency virus (Kulaga et al, 1989), among others. Currently rabbit is the preferred animal for antibody production due to their convenient size, life span, ease of handling and bleeding, and because the rabbit can produce sufficient high-titer, high-affinity, precipitating antiserum (Hanly et al, 1995; Leenaars and Hendriksen, 2005).

The immune system of the rabbits is similar to the human, with small differences. It has been shown that mammals with gestation periods longer than 60 days have mature immune systems before birth (Solomon, 1970). Rabbits

have relatively short gestation periods of 31-32 days (Patton, 1994) and at the time of birth they are physiologically immature with not fully developed lymphoid organs (Jeklova et al, 2007).

The role of the appendix is another difference between humans and rabbits. The appendix is an appendage located from the postero-medial wall of caecum without any known function in humans and is regarded as a vestigial remnant of our herbivorous ancestors (Malla, 2003; Ansaloni et al, 2009). On the other hand, in rabbits the appendix has a role as primary lymphoid organ involved in the maturation and production of B cells (Hague and Kindt, 1998; Drouet-Viard and Fortun-Lamothe, 2002). These newly generated B cells undergo a selection process in the appendix, involving self and foreign antigens (Pospisil and Mage, 1998). Studies of rabbit blood lymphocytes showed that they have a very heterogenous population spite their morphologically homogenous appearance (Sell, 1979).

Differences also can be found between human and rabbit immunoglobulins. In human serum five immunoglobulin classes can be found: IgG, IgM, IgA, IgE and IgD (Janeway et al, 2001). Rabbit immunoglobulins include IgE and IgM, with only one gene for IgE and 13 IgA genes with no IgD gene reported (Hague and Kindt, 1998). An important feature of these immunoglobulins are the differences in structure between allelically determined immunoglobulins. Somatic gene conversion is responsible for antibody diversity in rabbits (Hague and Kindt, 1998; Drouet-Viard and Fortun-Lamothe, 2002).

1.5 *Staphylococcus aureus* immune evasion strategies

In order to successfully colonize the host, *S. aureus* expresses many virulence factors that interact with the environment and facilitate the adherence to host tissue (Tompkins et al, 1990; van Belkum et al, 2009). *S. aureus* also produces several wall associated and excreted proteins that contribute to its escape from the immune system of the host (Foster, 2004). The bacterium is capable of defensin evasion, neutrophil migration evasion, complement evasion and

reactive oxygen species attack evasion (Rooijakkers et al, 2005a). The majority of the described evasion proteins appear to be involved in avoiding complement attack (Rooijakkers et al, 2005a).

Antimicrobial peptides are an essential part of the host innate immune system and they are responsible for the first line of defence against pathogens (Jin et al, 2004; Ryu et al, 2014). Most of these peptides are cationic and are able to interact with the anionic microbial cell wall, leading to the disruption of the integrity of bacterial cell membrane (Rooijakkers et al, 2005a; Ryu et al, 2014). *S. aureus* can change the composition and the net charge of its cell wall allowing the repulsion of the cationic peptides through the incorporation of D-alanine and L-lysine into its teichoic acids or phospholipids using the *dltABCD* operon and the *mprF* gene product, respectively (Peschel et al, 1999; Collins et al, 2002; Yeaman and Yount, 2003; Bera et al, 2005; Rooijakkers et al, 2005a).

Staphylokinase is a protein secreted by *S. aureus* that has fibrinolytic activity and can bind to defensins from human neutrophils, inhibiting its bactericidal effect (Jin et al, 2004; Peschel and Sahl, 2006; Ryu et al, 2014). This protein also has anti-opsonic properties, preventing Fc receptor-mediated phagocytosis (Rooijakkers et al, 2005b). *S. aureus* produces peptidases and proteases, such as metalloproteinase and aureolysin, that have the ability to cleave cationic antibacterial peptides (Shaw et al, 2004; Sieprawska-Lupa et al, 2004; Ryu et al, 2014).

S. aureus is also resistant to lysozyme via the *O*-acetylation of the *N*-acetylmuramic acid on its peptidoglycan (Bera et al, 2005; Bera et al, 2007). Resistance to lysozyme allows the bacteria not only to avoid direct peptidoglycan degradation, but also the production of IL-1β. Peptidoglycan degradation leads to the secretion of IL-1β by macrophages and the primary role of this interleukin in host defence is the regulation of the recruitment of neutrophils to the sites of infection (Shimada et al, 2010).

As one of the major components of the innate immune system, neutrophils are the primary defence against invading organisms and provide a fast, non-specific

and potent response (Rigby and De Leo, 2012). *S. aureus* produces surface components and secreted molecules that can act as neutrophil chemotactic molecules directly or via complement cascade components (Standiford et al, 1994; Yao et al, 1996; McLoughlin et al, 2006). Simultaneously, *S. aureus* can evade host defence used by neutrophils, including the ability to modulate normal neutrophil turnover, causing a delay in neutrophil apoptosis or accelerated neutrophil lysis (De Leo, 2004; Rigby and De Leo, 2004). This alteration facilitates the survival of the pathogen and promotes infection (Rigby and De Leo, 2012).

Inside the neutrophils, pathogens are killed by reactive oxygen species contained within granules (Beutler, 2004; Chaplin, 2010; Kumar and Sharma, 2010). *S. aureus* produces several molecules that help with the evasion of reactive oxygen species attack, such as catalase. This protein is able to inactivate toxic hydrogen peroxide and free radicals that are produced within phagocytic cells (Rooijakkers et al, 2005a; Kim et al, 2012). *S. aureus* produces a yellow carotenoid pigment that can scavenge oxygen free radicals (Foster, 2005, Liu et al, 2005), and also expresses two superoxide dismutase enzymes that remove the superoxide radicals (O_2^-) (Karavolos et al, 2003). *S. aureus* also secretes cytolytic toxins that can damage the neutrophils that attempt to engulf and kill this pathogen (Foster, 2005).

S. aureus can produce proteins that are capable of inhibiting neutrophil recruitment to the site of infection (Foster, 2005). One of these proteins is the extracellular adherence protein (Eap), capable of blocking recruitment by interfering with several interactions of adhesion receptors and ligands, such as intracellular adhesion molecule-1 (ICAM-1) present on the surface of endothelial cells (Chavakis et al, 2002). This binding prevents leukocyte adhesion, diapadesis and extravasation (Foster, 2005; Hammel et al, 2007a). This suggests that this protein has anti-inflammatory activity (Chavakis et al, 2002; Chavakis et al, 2005).

Another protein that can affect the recruitment of neutrophils is chemotaxis inhibitory protein of *S. aureus* (CHIPS). CHIPS can bind the formyl peptide

receptor (FPR) and the C5a receptor (C5aR) present in neutrophils, inhibiting the binding of their normal agonist (de Haas et al, 2004; Postma et al, 2004; Foster, 2005). It has been suggested that CHIPS plays a role in the early stages of bacterial infection (de Haas et al, 2004).

Another *S. aureus* immune evasion strategy is its ability to minimize or inhibit host recognition by modifying its surface (Rigby and De Leo, 2012). The expression of capsule has been associated with an increase in the virulence in animal models. The capsular polysaccharides are able to modify the negative charge of the bacterial surface (O'Riordan and Lee, 2004; Vuong et al, 2004) and *in vitro* assays have revealed that the presence of capsule reduces the phagocytosis of the pathogens by neutrophils in the presence of normal serum opsonins, indicating that the capsule has anti-opsonic properties (Thakker et al, 1998; Foster, 2005).

S. aureus produces Protein A, which has the ability to bind to the Fc region of immunoglobulins, coating the bacteria with antibodies in a nonopsonic orientation. This provides a disguise that disrupts the opsonization and phagocytic uptake (Nizet, 2007; Rigby and De Leo, 2012). Clumping factor A (ClfA), a fibrinogen-binding protein that is present in the surface during stationary phase, is produced by *S. aureus* (McDevitt et al, 1997) and this helps the bacteria to become coated with fibrinogen inhibiting the access and deposition of opsonins (Foster, 2005). This protects *S. aureus* from phagocytosis by macrophages and neutrophils (Palmqvist et al, 2004; Higgins et al, 2006). *S. aureus* produces serine proteases (SspA), cysteine proteases (SspB) and metalloproteases that are also capable of cleaving the immunoglobulins in solution and bound to the cell surface (Shaw et al, 2004; Nizet et al, 2007; Fernández Falcon et al, 2011).

S. aureus makes cytolytic toxins that target the leukocytes and form pores that lead to cell lysis (Foster, 2005; Nizet, 2007). Whilst the Panton-Valentine leukocidin (PVL) is toxic just for leukocytes, the γ-hemolysyn or γ-toxin also affects erythrocytes (Foster, 2005). It has been shown that *S. aureus* can also avoid the action of leukocytes via the production of adenosine from AMP, ADP

or ATP, as adenosine receptor-mediated signalling on phagocytes interferes with phagocytosis by polymorphonuclear leukocytes (Kim et al, 2012).

S. aureus has a large number of complement system evading strategies. Protein A and its ability to bind antibodies at the bacterial surface can also block the classical pathway of complement activation by interrupting pathogen recognition. Protein A can bind to the Fc region of immunoglobulin G, blocking the Fc-receptor-mediated complement activation via C1q (Rooijakkers et al, 2005a; Rooijakkers and van Strijp, 2007).

The bacteria excrete extracellular fibrinogen binding protein (Efb). This molecule is able to bind to the C3d region of complement component C3, blocking its deposition on the bacterial cell surface and subsequent phagocytosis of the pathogen (Lee et al 2004a; Jongerius et al, 2007), or further activation beyond C3b (Lee et al, 2004b). This allows Efb to block the alternative pathway convertase (C3bBb) and the C5 convertases of the lectin/classical pathway (C4b2a3b) and of the alternative pathway (C3bBb3b) (Rooijakkers et al, 2005a; Jongerius et al, 2007). Another excreted protein involved in immune evasion is the extracellular complement-binding protein (Ecb), also known as Ehp (Hammel et al, 2007b; Jongerius et al, 2012). This protein is a homologue of Efb that can also bind to C3b and inactivate the C3b containing convertases, blocking all the activation pathways of complement activation (Jongerius et al, 2007; Jongerius et al, 2012).

The surface protein SdrE of *S. aureus* has been implicated in human platelet aggregation (O'Brien et al, 2002) and is capable of recruiting the complement regulatory protein Factor H and inhibiting the alternative pathway of complement (Sharp et al, 2012). Factor H is able to accelerate the dissociation of the alternative pathway C3 convertase (C3bBb) and acts as a cofactor for the Factor I mediated cleavage of C3b into C3bi, the inactive form of this complement component (Foster 2005; Jozsi y Zipfel, 2008). The bacteria use the surface protein ClfA to recruit Factor I (Hair et al, 2010) and it has been shown that the Ecb protein enhances the acquisition of Factor H from the serum (Amdahl et al, 2013).

Another immune evasion protein produced by *S. aureus* is the staphylococcal Ig-binding protein (Sbi) (Upadhyay et al, 2008). This protein has four domains and is able to affect the adaptive and innate immune systems of the host. The two N-terminal domains (Sbi-I and Sbi-II) are IgG-binding-domains that allow the protein to interfere directly with adaptive immunity (Zhang et al, 1998; Atkins et al, 2008), while the Sbi-III and Sbi-IV domains can modulate the complement system through binding with C3 (Burman et al, 2008; Upadhyay et al, 2008).

SCIN, or staphylococcal complement inhibitor, is a protein secreted by *S. aureus* and is considered the most efficient complement inhibitor because is able to block all the complement pathways, efficiently preventing the phagocytosis of *S. aureus* and the production of C3a (Rooijakkers et al, 2005c; Rooijakkers et al, 2006; Rooijakkers and van Strijp, 2007). SCIN is produced during the early stages of growth, making the protein an effective immune modulator (Rooijakkers et al, 2006). SCIN can block complement through the binding and stabilization of the membrane-bound C3 convertases (C4bC2a and C3bBb), which affects the complement cascade in different ways (Foster, 2005; Rooijakkers et al, 2005c).

The dissociation of the C3 convertases leaves the membrane-bound components C4b and C3b available to act as cofactors for the cleavage of C2 and Factor B, respectively. The stabilization of C3bBb and C4b2a on the surface of *S. aureus* prevents the generation of additional convertases (Foster, 2005; Rooijakkers et al, 2005c; Ricklin et al, 2009). The binding of SCIN also affects the enzymatic activity of these complexes, making the convertases no longer able to cleave C3 (Rooijakkers and van Strijp, 2007). Finally, SCIN is able to prevent further C3b deposition in the bacterial surface, interfering with the amplification loop of the alternative pathway of complement activation (Foster, 2005; Rooijakkers et al, 2005c). As the C5 convertases are no longer being formed, SCIN attenuates the neutrophil response induced by the release of C5a and also the formation of the membrane attack complex on the bacteria (Rooijakkers et al, 2006; Rooijakkers et al, 2007).

The staphylococcal superantigen-like proteins (SSLs) have structural similarity to superantigens (Rooijakkers et al, 2005a; Hermans et al, 2012). While superantigens bind to T-cell receptors and major histocompatibility complex class II molecules, SSLs bind to components of the innate immunity such as complement, Fc receptors and myeloid cells (Rooijakkers et al, 2005a; Bestebroer et al, 2010; Hermans et al, 2012). For example, SSL-10 is able to bind to the Fc region of human IgG1 blocking phagocytosis mediated by the FcγR (Patel et al, 2010), while SSL5 and SSL11 are able to bind to neutrophils and inhibit neutrophil rolling (Bestebroer et al, 2007).

The staphylococcal superantigen-like 7 (SSL-7) can bind to IgA, blocking its binding to FcαR, inhibiting the IgA-mediated anti-staphylococcal mechanism at the mucosal surface of epithelial cells (Langley et al, 2005; Langley et al, 2010). SSL-7 is also able to bind to C5, inhibiting the formation of the membrane attack complex of the complement and the production of C5a (Langley et al, 2005; Bestebroer et al, 2010; Langley et al, 2010).

1.6 Mechanisms of host adaptation by *S. aureus*

The ability of a microbial pathogen to colonize and infect a host organism is known as host specificity or tropism (Pan et al, 2014). Pathogenic bacteria exhibit different levels of host specificity, from extreme diversity such as *Yersinia pestis* (Perry and Fetherston, 1997; Anisimov et al, 2004), intermediate levels of host tropism like *Listeria monocytogenes* (Lecuit, 2005; Hamon et al, 2006) or strict host selectivity, like *Streptococcus pneumoniae* and *Neisseria meningitidis* have for humans (Lu et al, 2005; Hill et al, 2010; Achila et al, 2015).

Along with being a major human pathogen, *S. aureus* is able to infect domestic animals such as cows, sheep, poultry and rabbits (Vancraeynest et al, 2006; Fitzgerald, 2012; Viana et al, 2015). It has been reported that host-specialized clones of *S. aureus* resulted from human to animal host jumps, but the

molecular basis for this adaptation is not completely understood (Herron-Olson et al, 2007; Sung et al, 2008; Viana et al, 2015). However, it has been reported that most of the host tropisms are associated with genes involved in interactions with the immune system such as virulence factors and cell adhesion proteins (Smyth et al, 2009; Guinane et al, 2010; Shepheard et al, 2013).

As stated above, most of the mutations that are involved in host tropisms can be found in genes involved in interactions with the immune system, like toxins, complement inhibitors and proteins involved in biofilm formation. It has been found that mobile genetic elements (MGE), such as bacteriophages and pathogenicity islands play a role in this process (Herron-Olson et al, 2007; Viana et al, 2010; McCarthy et al, 2011; Resch et al, 2013).

The use of different sequencing techniques, in particular Multilocus Sequence Typing (MLST), revealed strains mostly associated with a single host species (Enright and Spratt, 1999; Fitzgerald, 2012; Shepheard et al, 2013). Mutations found in the sequenced genes allow identification of sequence types (STs), which represent different *S. aureus* genotypes. However, some STs have the ability to infect more than one host organism, such as the ST121 strain capable of infecting both humans and rabbits (Viana et al, 2007; Viana et al, 2015) and the ST398 MRSA strain, generally pig-associated but also found in cows, poultry and humans (van Loo et al, 2007; Fitzgerald, 2012). The STs can be grouped into clonal complexes (CC), which are comprised by STs that differ in no more than two of the seven MLST alleles (McGavin et al, 2012).

1.7 Aims of this project

This project aimed to identify the bacterial component(s) that act as a receptor for the bactericidal activity of the naive rabbit serum and the mechanism that is responsible for the antibacterial properties of the serum.

2 CHAPTER 2:

Materials and Methods

2.1 Growth Media

All media were prepared using distilled water and sterilized by autoclaving at 121ºC, 15 pounds per square inch for 20 minutes.

2.1.1 Blood Agar

Blood agar base #2 (Oxoid)	40 g/L

The agar was resuspended in dH_2O, boiled to ensure dissolving and autoclaved. The agar was cooled to 50ºC before adding 7% (v/v) Oxoid defibrinated sheep blood.

2.1.2 Brain Heart Infusion (BHI)

Brain heart infusion (Oxoid)	37 g/L

1.5% (w/v) Oxoid Agar was added to make BHI agar.

2.1.3 Luria-Bertani (LB)

Tryptone (Oxoid)	10 g/L
Yeast extract (Oxoid)	5 g/L
NaCl	5 g/L

The pH was adjusted to 7.2 using 5M NaOH. 1.5% (w/v) Oxoid Agar was added to make LB agar.

2.1.4 Tryptic Soy Broth (TSB)

Tryptic soy broth (Oxoid)	30 g/L

1.5% (w/v) Oxoid Agar was added to make Tryptic soy agar (TSA).

2.2 Antibiotics

The antibiotics that were used in this study are listed in Table 2.1. They were prepared from stock solutions, which were prepared in different solvents, filter sterilised (0.2µm pore size) and stored in aliquots at -20ºC. For liquid media, antibiotic stock solution was added just before use. For agar plates, the media were cooled to approximately 50ºC before adding the antibiotic stock.

2.3 Bacterial strains

2.3.1 *S. aureus* strains

The bacteria used in this study are listed in Table 2.2. For growth in liquid media, a single colony was used to inoculate 5 ml of media and the culture was grown overnight aerobically at 37ºC shaking at 250 rpm. This culture was used to inoculate fresh media until it reached a known OD_{600}. The strains were also cultured on BHI agar plates with the appropriate antibiotics for an overnight at 37ºC and stored at 4ºC for up to three weeks. For long-term storage, a single colony was used to inoculate 5 ml of media and grown up to an OD_{600} of 0.5. Two ml of the culture were then centrifuged and the pellet was used to inoculate Microbank beads that were stored at -80ºC.

2.3.2 *E. coli* strains

The *E. coli* strain used in this study is shown in Table 2.3. The growth and storage conditions were the same as for the *S. aureus* strains (Chapter 2.3.1), except that LB broth or agar was used as growth media.

2.4 Buffers and solutions

All buffer and solutions were prepared using distilled water unless otherwise stated. They were sterilized if necessary and stored at room temperature or 4ºC.

Phosphate buffered saline (PBS)

NaCl	8 g/L
Na_2HPO_4	1.4 g/L
KCl	0.2 g/L
KH_2PO_4	0.2 g/L

The pH was adjusted to 7.4 using NaOH

Table 2.1 Antibiotic stock solutions and concentrations

Antibiotic	Solvent	Stock concentration (mg/ml)	Working concentration (µg/ml)
Chloramphenicol (Cm)	100% (v/v) ethanol	5	10
Erythromycin (Ery)	100% (v/v) ethanol	5	2.5
Spectinomycin (Spec)	dH_2O	50	100
Tetracycline (Tet)	dH_2O	5	5

Table 2.2 *S. aureus* strains used in this study

Strain	Relevant genotype/Marker	Source
SH1000	Functional *rsbU*$^+$ derivative of 8325-4	Horsburgh *et al*, 2002
CH 3934	J strain, ST121 clone rabbit origin	Viana *et al*, 2015
CH 9982	J strain, *dlt*B deleted	Viana *et al*, 2015
CH 8348	J strain, *rot* from human isolates	Viana *et al*, 2015
CH 9548	J strain, *dltB* from human isolates	Viana *et al*, 2015
CH 7705	F strain, ST121 clone human origin	Viana *et al*, 2015
CH 8569	F strain, *rot* mutation present in rabbit isolates (K103*)	Viana *et al*, 2015
CH 9692	F strain, *dltB* 1SNP (*405Y)	Viana *et al*, 2015
CH 9700	F strain, *rot* 1SNP (K103*) and *dltB* 1SNP (*405Y)	Viana *et al*, 2015
CH 9897	F strain, *rot* 1SNP (K103*) and *dltB* 1SNP (*405Y), cured from the prophage inserted in the β-toxin gene	Viana *et al*, 2015
CH 10310	F strain, *dltB* 3SNPs (T113K, Y250H, *405Y)	Viana *et al*, 2015
CH 10311	F strain, *dltB* 3SNPs (T113K, Y250H, *405Y) and *rot* 1SNP (K103*)	Viana *et al*, 2015
CH 10320	F strain, *dltB* 2SNPs (T113K, Y250H)	Viana *et al*, 2015
DL 9	ST96 clone, rabbit origin.	Viana *et al*, 2015
BK 4337	ST96 clone, human origin	Viana *et al*, 2015

RN4220	Restriction deficient derivative of 8325-4	Kreiswirth *et al*, 1983
RN4220 Δ*tarM*	RN4220 with deletion of *tarM*, unmarked	Brown *et al*, 2012
RN4220 Δ*tarS*	RN4220 with deletion of *tarS*, unmarked	Brown *et al*, 2012
RN4220 Δ*tarM* Δ*tarS*	RN4220 with deletion of *tarM* and *tarS*, unmarked	Brown *et al*, 2012
RN4220 Δ*tarM* Δ*tarS* pRB-tarS	RN4220 with deletion of *tarM* and *tarS*, with complementation of tarS, Cm^R	Brown *et al*, 2012
Sa113	Restriction deficient mutant of NCTC 8325.	Iordanescu and Surdeanu, 1976
Sa113 Δ*dltA*	Sa113 with *dltA* gene replaced by spectinomycin resistance gene, $Spec^R$	Peschel *et al*, 1999
Sa113 Δ*mprF:ermB*	Sa113 with *mprF* gene replaced by erythromycin resistance cassette (*ermB*), Ery^R	Weidenmaier *et al*, 2005a
Sa113 Δ*mprF:ermB* pRB474-*mprF*	Sa113 with *mprF* replaced by *ermB*, complemented. Ery^R Cm^R	Weidenmaier *et al*, 2005a
Sa113 Δ*ypfP:ermB*	Sa113 with *ypfP* gene replaced by erythromycin resistance cassette (*ermB*), Ery^R	Fedtke *et al*, 2007
Sa113 Δ*ypfP:ermB* pRB473-*ypfP*	Sa113 with *ypfP* replaced by *ermB*, complemented. Ery^R Cm^R	Fedtke *et al*, 2007
15981	Clinical strain, biofilm positive	Lab collection, gift from I. Lasa
15981 Δ*tagO*	15981 with deletion of *tagO*, unmarked	Vergara-Irigaray *et al,* 2008.

Cm^R, chloramphenicol resistant; Ery^R, erythromycin resistant; $Spec^R$, spectinomycin resistant

Table 2.3 *E. coli* strains used in this study

Strain	Relevant genotype/Marker	Source
MC1061	F^- Δ(*ara-leu*)7697 [*araD*139]$_{B/r}$ Δ(*codB-lacI*)3 *galK*16 *galE*15 λ^- $e14^-$ *mcrA0 relA1 rpsL*150(*strR*) *spoT1 mcrB1 hsdR2*(r^-m^+)	Lab collection

2.4.1 Peptidoglycan purification

2.4.1.1 5% (w/v) SDS solution

Sodium dodecyl sulphate	5% (w/v)

Sterile MilliQ water was used to reach the desired volume.

2.4.1.2 4% (w/v) SDS solution

Sodium dodecyl sulphate	4% (w/v)

Sterile MilliQ water was used to reach the desired volume.

2.4.1.3 Washing solution

Tris-HCl	50 mM

The pH was adjusted to 7.0 using NaOH and sterile MilliQ water was used to reach the desired volume.

2.4.2 Wall teichoic acid purification

2.4.2.1 MES Buffer 1

2-(N-morpholino) ethanesulfonic acid (MES)	50 mM

The pH was adjusted to 6.5 using NaOH

2.4.2.2 MES Buffer 2

2-(N-morpholino) ethanesulfonic acid (MES)	50 mM
SDS	4% (w/v)

The pH was adjusted to 6.5 using NaOH

2.4.2.3 MES Buffer 3

2-(N-morpholino) ethanesulfonic acid (MES)	50 mM
NaCl	2% (v/v)

The pH was adjusted to 6.5 using NaOH

2.4.3 Lipoteichoic acid purification

2.4.3.1 Sodium citrate washing solution

Sodium citrate	0.1M

The pH was adjusted to 4.7 with HCl.

2.4.3.2 Dialysis buffer

Sodium citrate	20mM

The pH was adjusted to 4.7 with HCl.

2.4.3.3 Hydrophobic Interaction Chromatography

The buffers were filtered and degassed before use.

Buffer A (0.1M sodium citrate (pH 4.7), 15% (v/v) 1-propanol)

0.5 M sodium citrate (pH 4.7)	200 ml
1-propanol	150 ml
dH_2O	650 ml

Buffer B (50mM sodium citrate (pH 4.7), 65% (v/v) 1-propanol)

0.5 M sodium citrate (pH 4.7)	100 ml
1-propanol	650 ml
dH_2O	250 ml

2.4.4 SDS-PAGE solutions

2.4.4.1 SDS-PAGE Reservoir buffer (10x)

Glycine	144 g/L
Tris Base	30.3 g/L
SDS	10 g/L

The buffer was diluted with dH_2O to 1:10 before use.

2.4.4.2 Loading buffer (5x)

Tris-HCl pH 6.8	250 mM

SDS	10% (w/v)
Bromophenol blue	0.5 % (w/v)
Glycerol	50% (v/v)
2-mercaptoethanol	10% (v/v)

2.4.4.3 Coomassie Blue stain

Coomassie Blue	0.1% (w/v)
Methanol	5% (v/v)
Glacial acetic acid	10% (v/v)

2.4.4.4 Coomassie Blue destain

Methanol	5% (v/v)
Glacial acetic acid	10% (v/v)

2.4.4.5 Silver Staining

The Pierce Silver Staining Kit from Life Technologies was used and the Sensitizer Solution, Stain Solution and Developer Solution were prepared using the reagents and protocol provided. The rest of the solutions were prepared as follows:

Fixing Solution

Acetic acid	10% (v/v)
Ethanol	30% (v/v)
MilliQ water	to 50 ml

Ethanol Solution

Ethanol	10% (v/v)
MilliQ water	to 50 ml

Stop Solution

Acetic acid	5% (v/v)
MilliQ water	to 50 mL

2.4.5 Native polyacrylamide gel electrophoresis solutions

2.4.5.1 Running buffer

Tris Base	3.02 g/L
Glycine	14.41 g/L
Distilled water	to 1L

2.4.5.2 Loading buffer (2x)

Tris-HCl pH 6.8	62.5 mM
Glycerol	25% (v/v)
Bromophenol blue	1% (w/v)

2.4.6 Western Blotting buffers

2.4.6.1 Blotting buffer

Tris Base	2.4 g/L
Glycine	11.26 g/L
Ethanol	20% (v/v)

The buffer was chilled at 4ºC for 60 minutes before use.

2.4.6.2 TBST buffer (20x)

Tris-HCl pH 7.4	48.4 g/L
NaCl	20 g/L
Tween-20	2% (v/v)

The Tris-HCl solution was autoclaved before adding the rest of the components. The pH of the solution was adjusted to 7.6 and the buffer was diluted 1:20 with dH_2O before use.

2.4.6.3 Blocking Solution

Skim milk powder (Oxoid)	5% (w/v)

The powder was dissolved in 1xTBST Buffer.

2.4.7 Purified Phospholipase A2 Buffer

Tris Base	12.11 g/L
$CaCl_2$	0.14 g/L
Fatty acid free BSA	1.0 g/L

The solution was prepared with sterile MilliQ water and its pH was adjusted to 8.0 with HCl. The solution was filtered (0.2µm pore size) before use and stored at room temperature.

2.4.8 Fluorescence Microplate Assay for Phospholipase A2

2.4.8.1 Assay Buffer

Tris Base	6.06 g/L
NaCl	5.80 g/L
$CaCl_2$-$2H_2O$	0.15 g/L

The solution was prepared using sterile MilliQ water and the pH was adjusted with HCl to 8.9. The solution was filtered (0.2µm pore size) before use and stored at 4ºC.

2.5 Stock solutions

All the chemicals used in this study were of analytical grade quality and were purchased from Sigma, Fisher Scientific, MP Biomedicals or Roche unless otherwise stated. Concentrations and storage conditions of the stock solutions are shown in Table 2.4.

The origins of the different types of mammalian sera tested in this study are shown in Table 2.5. Protein ladders for SDS-PAGE were purchased from New England Biolabs. The antibodies used in this study were purchased from Sigma, Abcam and Genscript and are shown in Table 2.6 and Table 2.7.

Table 2.4 Chemical stock solutions used in this study

Stock Solution	Concentration	Dissolved in	Storage conditions
2,4-dinitrophenylhydrazine	0.1% (w/v)	2M HCl	RT, aluminium-covered
Ammonium acetate	1 M	MilliQ water	RT
Ammonium carbonate	1 M	MilliQ water	RT
Ammonium chloride	1 M	MilliQ water	RT
Ammonium hydroxide	1 M	MilliQ water	RT
Ammonium persulfate (APS)	10%(w/v)	MilliQ water	-20ºC
Calcium chloride ($CaCl_2$)	0.1 M	MilliQ water	RT
D-amino acid oxidase	2.5 mg/ml	0.2M Tris-HCl pH 8.4	RT, aluminium-covered
Dithiothreitol (DTT)	100 mM	MilliQ water	4ºC
1,2- Dioleoyl-sn- glycero-3-phosphocholine (DOPC)	10 mM	Ethanol	-20ºC
1,2-Dioleoyl-sn-glycero-3-phospho-rac-(1-glycerol) (DOPG)	10 mM	Ethanol	-20ºC
Ethylene glycol tetraacetic (EGTA)	0.1 M	MilliQ water	RT
Honey Bee (*Apis mellifera*) PLA2	5 mg/ml	Sterile PBS	-20ºC
Magnesium chloride ($MgCl_2$)	0.1 M	MilliQ water	RT
Pronase	2 mg/ml	MilliQ water	-20ºC
Proteinase K	20 µg/ml	20mM TrisHCl (pH 8.0), 0.5% (w/v) SDS	-20ºC
Red/Green BODIPY PC-A2	1 mM	DMSO	-20ºC, aluminium covered
Spitting Cobra (*Naja mossambica mossambica*) PLA2	1 mg/ml	Sterile PBS	-20ºC
Trypsin	1 mg/ml	1mM HCl	4ºC
Zymosan	10 mg/ml	Sterile PBS	-20ºC

RT: Room temperature.

Table 2.5 Mammalian sera used in this study

Animal source	Source
Naive rabbit blood	New England White female rabbits as explained in Chapter 2.8
Adult human blood	Healthy adult volunteers as explained in Chapter 2.13
Infant human blood	Healthy infant volunteers under 12 months of age as explained in Chapter 2.14
Mouse blood	Balb/c strain, gift from Dr Cristina Teles
Defibrinated Horse blood	E&O Laboratories
Defibrinated Sheep blood	E&O Laboratories
Bovine calf blood	Fisher Scientfics
Porcine serum	Sigma-Aldrich
Bovine adult serum	MP Biomedicals
Rat blood	Sigma-Aldrich

Table 2.6 Primary antibodies used in this study

Antibody	Dilution used
Anti- MBL rabbit complement component, raised in chicken (Genscript)	WB: 1:1000 SB: 1: 5000
Anti-C4b rabbit complement component, raised in chicken (Genscript)	WB: 1:1000 SB: 1: 5000
Anti-C5b rabbit complement component, raised in chicken (Genscript)	WB: 1:1000 SB: 1: 5000
Anti-C9 rabbit complement component, raised in chicken (Genscript)	WB: 1:1000 SB: 1: 5000
Chicken IgY control, whole molecule (Genscript)	SB: 1:10,000
Chicken IgY control immunoglobulin (Promega)	Inactivation assays only
Anti-MBL human complement component, raised in mouse (Abcam)	WB: 1:5000 SB: 1:10,000
Anti-C4b human complement component, raised in mouse (Abcam)	WB: 1:5000 SB: 1:10,000
Anti-C5b human complement component, raised in mouse (Abcam)	WB 1:5000 SB: 1:10,000

Anti-C9 human complement component, raised in mouse (Abcam)	WB: 1:5000 SB: 1:10,000
Anti-low molecular weight phospholipase A2 antibody (Merk Millipore)	WB: 1:5000 SB: 1:10,000
Anti-low molecular weight phospholipase A2 antibody (Abcam)	WB: 1:5000 SB: 1:10,000
Rat control antibody, whole molecule (Sigma-Aldrich)	Inactivation assays only
Anti-annexin A1 antibody, mouse polyclonal (Sigma-Aldrich)	WB: 1:5000 SB: 1:10,000
Anti-lipoteichoic acid monoclonal antibody, raised in mouse (Hycult Biotechnology)	SB: 1:1,000
Anti-cobra venom polyclonal antibody, raised in duck (Abcam)	WB: 1:5,000

WB: Western blot; SB: Slot blot

Table 2.7 Secondary antibodies used in this study

Antibody	Dilution used
Anti-chicken IgG (H+L), F(ab) fragment, HRP antibody produced in goat (Sigma-Aldrich)	1:10,000
Anti-mouse antibody, HRP antibody produced in goat (Sigma-Aldrich)	1:10,000
Anti-rabbit IgG1 (H) HRP antibody produced in goat (Sigma-Aldrich)	1:10,000
Anti-bird IgY H&L HRP antibody produced in goat (Abcam)	1:10,000

2.6 Centrifugation

The following centrifuges were used to harvest cells and precipitated material:

I. Eppendorf centrifuge 5418, rotor capacity 18 x 1.5/2 ml, max speed 16,873g (14,000 rpm).
II. Eppendorf centrifuge 5424, rotor capacity 24 x 2ml, max speed 20,238g (14,680 rpm).
III. Sigma 4K15C centrifuge, max capacity 4x500 ml, max speed 15,000 rpm.
IV. Sigma 3K15 centrifuge, max capacity 4x200 ml, max speed 5410g.
V. Avanti High Speed J25 centrifuge, Beckman.
    a. Rotor JA-20: max capacity 8x50ml, max speed 48,400g (20,000 rpm).
    b. Rotor JLA-10.500: max capacity 3L, max speed 18.500g (10,000 rpm)

The temperature at which the centrifugation was carried out is noted in each protocol.

2.7 Determination of bacterial density

To quantify the bacterial yield spectrophotometric measurements were carried out at 600 nm (OD_{600}) using WPA Biowave DNA Spectrophotometer and Semi-micro PS cuvettes (Fisherbrand). Culture samples were diluted 1:10 and 1:20 in sterile culture medium when necessary.

2.8 Preparation of Naive Rabbit Serum (NRS)

Blood was collected directly in 50 ml Falcon tubes from New Zealand White female rabbits immediately post mortem and left at room temperature for one hour before being placed at 4ºC overnight on ice. The tubes containing the blood were then centrifuged twice (5000 rpm, 10 minutes, 4°C) and the supernatant was collected, filtered (0.2µm pore size), aliquoted (1 ml) and stored at -80ºC.

2.9 Effect of Naive Rabbit Serum (NRS) on *S. aureus*

One colony of the bacterial strain to test was used to inoculate 5 ml of media in a 25 ml sterile universal and grown overnight at 37ºC on a rotary shaker at 250 rpm. This culture was used to inoculate 5 ml of fresh media and grown at 37ºC and 250 rpm until it reached exponential phase. The cells were washed twice with sterile PBS by centrifugation (14,000 rpm, 3 minutes, room temperature) and then resuspended in PBS.

An aliquot of NRS was inoculated with 10^5-10^6 cells/ml of this culture and incubated at 37ºC with rotation. Samples were taken from this culture at appropriate times (0, 0.5, 1, 2, 3 and 4 hr), 10-fold serial dilutions were made in sterile PBS and two 10 µl aliquots of each dilution were directly spotted on BHI plates. The 'zero' time point represents to a time point between 5 or 10 minutes after the inoculation of the bacteria into the serum, considering the time that took to set each experiment. After overnight incubation at 37ºC the number of colony forming units was determined (CFU/ml). Unless otherwise stated, all experiments were carrieds out in biological triplicates.

2.9.1 Statistical Analysis

Survival curves from bactericidal killing experiments were compared using Student t-test and the analysis were carried out using GraphPad Prism (version 6). Statistical significance was determined using the Holm-Sidak method as recommended by GraphPad, and results were considered significant at a p-value below 0.05. One asterisk (*) denotes p-value below 0.05; ** a p-value below 0.01; and *** a p-value below 0.001. Experiments were carried out on triplicate and error bars correspond to the standard deviation of the means.

2.10 Inactivation of NRS

2.10.1 Heat treatment

The rabbit serum was heated at 56ºC for 30 minutes in a water bath before inoculating with 10^5 cells/ml of an exponential phase bacterial culture. Killing assays as described in Chapter 2.9 were carried out.

2.10.2 Ethylene glycol tetraacetic acid (EGTA) treatment

The stock solution of EGTA was prepared using dH_2O and it was autoclaved before being mixed with the serum. An aliquot 150 µl of rabbit serum was gently mixed with 15 µl of 0.1M EGTA to a final concentration of 10 mM, and incubated for 60 minutes at 37ºC before the addition of 10^5 cells/ml of an exponential phase culture of *S. aureus* SH1000 to the mix. Killing assays were carried out as explained in Chapter 2.9.

For the experiments with the *E. coli* MC1061 strain, 50µM EGTA was used instead and the samples were spotted on LB agar plates.

2.10.3 Pre-incubation with zymosan A from *Saccharomyces cerevisiae*

Zymosan A was activated before the pre-incubation with NRS. Zymosan was resuspended to a concentration of 0.1% (w/v) in 0.15 M sodium chloride and boiled for 60 minutes in a water bath. This solution was centrifuged for 30 minutes at 4000 rpm and the supernatant was discarded. The insoluble material was resuspended in sterile PBS to the desired stock concentration of 10 mg/ml. This material was stored at 4ºC until further use.

An aliquot of 100 µl of rabbit serum was mixed with 10 µl of the activated zymosan A to a final concentration of 1 mg/ml, and incubated for 60 minutes at 37ºC with rotation. This mixture was then centrifuged for 5 minutes at 4000 rpm at room temperature and the supernatant was recovered for killing assays using 10^5 cells/ml of an exponential phase culture of *S. aureus* SH1000 for the inoculation and following the protocol explained in Chapter 2.9.

2.10.4 Ammonium salts and ammonium hydroxide pre-incubation

The ammonium solutions were prepared using sterile MilliQ water and sterilised using a 0.2 µm pore size filter. Aliquots of 150 µl of rabbit serum was treated with 25 µl of ammonium acetate, ammonium carbonate, ammonium chloride and ammonium hydroxide (final concentration 150 mM) for 45 minutes at 37ºC, and afterwards these solutions had their pH adjusted to 7.4 using HCl. The

mixtures where then inoculated with 10^5 cells/ml of an exponential phase culture of *S. aureus* SH1000 and killing assays were carried out (Chapter 2.9)

2.10.5 Dithiothretiol pre-incubation

A 100 mM stock solution of dithiothretiol (DTT) was prepared with sterile MilliQ water and it was filtered using a 0.2 µm pore size filter. Aliquots of 100 µl of rabbit serum were treated with 10 µl of the stock solution of DTT (10 mM final concentration) and preincubated at 37ºC for 30 minutes. This mixture was inoculated with 10^5 cells/ml of an exponential phase culture of *S. aureus* SH1000. Killing assays were carried out (Chapter 2.9)

2.11 Reactivation of NRS with magnesium chloride and calcium chloride

The different stock solutions used in the reactivation of the bactericidal activity of rabbit serum experiments were prepared using dH_2O and were autoclaved before used.

For the reactivation of rabbit serum with magnesium chloride, a liquid culture of *S. aureus* SH1000 or *E. coli* MC1061 was grown at 37ºC and 250 rpm until it reached exponential phase (OD_{600}= 0.5). An aliquot of NRS was mixed with equimolar concentrations of EGTA and magnesium chloride (final concentration 10 mM for *S. aureus* and 50 µM for *E. coli*) and was incubated for 60 minutes at 37ºC before the addition of 10^5 cells/ml, after which killing assays were carried out (Chapter 2.9).

The same protocol was used for the reactivation of NRS with magnesium chloride and calcium chloride. The only difference was that the rabbit serum was mixed with EGTA, $MgCl_2$ and $CaCl_2$ (final concentration 50 µM or 10 mM) before incubation of this mixture for one hour at 37ºC, after which the solution was inoculated with *E.coli* MC1061 or *S. aureus* SH1000, respectively.

2.12 Inactivation of the bactericidal activity of NRS using pre-incubation with antibodies

2.12.1 Antibodies against complement components

Stock solutions at 2 mg/ml of antibodies against complement components (Table 2.6) were used for inactivation of rabbit serum experiments. Aliquots 100 µl of NRS were mixed with 5, 10, 15 and 20 µl of the stock solution of each antibody (final concentration 100, 200, 300 and 400 µg/ml, respectively) and incubated for 1 hour at 37ºC with rotation. After this incubation the solution was inoculated with 10^5 cells/ml of an exponential phase culture of *S. aureus* and killing assays were carried out (Chapter 2.9).

The same protocol was followed for the inactivation with the control whole chicken molecule antibodies (Table 2.6).

2.12.2 Antibodies against phospholipase A2

Stock solutions at 1 mg/ml of antibodies against phospholipase A2 (Table 2.6) were used to inactivate rabbit serum. Aliquots of 100 µl of rabbit serum were inoculated with different volumes of the stock solution of anti-phospholipase A2 antibodies (final concentrations 10-100 µg/ml) and incubated for 60 minutes at 37ºC with rotation. The solution was then inoculated with 10^5 cells/ml of an exponential phase culture of *S aureus* and killing assays were carried out (Chapter 2.9).

The same protocol was followed for the inactivation with the whole rabbit control antibody (Table 2.6).

2.13 Preparation of Adult Human Serum and effect on *S. aureus*

2.13.1 Preparation of adult human serum

The South Sheffield Ethics Committee approved the protocol for phlebotomy (Reference Number STH13927). 100 ml of blood was collected directly from four healthy volunteers in 50 ml Falcon tubes and the samples were left at room temperature for 60 minutes before transferring the tubes to 4ºC on ice for an

overnight. The supernatant was taken from the tubes and centrifuged twice at 4ºC for 10 minutes at 5000 rpm. The supernatant was then collected, filtered (0.2 µm pore size), aliquoted (1 ml) and stored at -80ºC.

2.13.2 Effect of adult human serum on *S. aureus*

Aliquots of 100 µl of adult human serum were inoculated with 10^5 cells/ml of an exponential phase culture of different strains of *S. aureus* and killing assays were carried out (Chapter 2.9)

2.14 Preparation of Infant Human Serum and effect on *S. aureus*

2.14.1 Preparation of infant human serum

Blood was collected from healthy infants under 12 months of age with their parents' authorization from the Royal Hallamshire Children's Hospital with the help of Dr Fiona Shackley under Case Number CA11011. The samples were left at room temperature for 60 minutes before transferring the tubes to 4ºC on ice for 60 minutes. The supernatant was taken and centrifuged at 4ºC for five minutes at 4000 rpm and then for one minute at 5000 rpm. The serum was then removed from the tubes, filtered (0.2 µm pore size), aliquoted (50 µl) and stored at -80ºC.

2.14.2 Effect of infant human serum on *S. aureus*

Aliquots of 50 µl of infant human serum were incubated with 10^5 cells/ml of an exponential phase culture of *S. aureus* SH1000 and killing assays were carried out (Chapter 2.9).

2.15 Purification of human and rabbit serum components by centrifugal filtration

2.15.1 Fractionation of human and rabbit serum

2 ml of NRS were used to obtain different fractions using Amicon Ultra 0.5ml centrifugal filters, Ultracel (Millipore). The filters used had a molecular weight cut-off of 10, 30 and 50 kDa. For this purification the protocol from Millipore was

followed with a few modifications. The sample reservoir with the filter was placed into the vial. 0.5 ml of serum was placed into the sample reservoir without touching the membrane and the cap was sealed. The sample was centrifuged for 10 minutes at 14000 rpm at room temperature, and the fraction collected in the vial (components smaller than the cut-off) was taken. The sample reservoir with the components bigger than the cut-off molecular weight was carefully placed upside down in a new vial and centrifuged for 3 minutes at 3000 rpm and room temperature. The vials were carefully labelled and they were stored at -80ºC until further use in NRS killing assays and protein analysis. The same protocol was used for the fractionation of human serum.

2.15.2 Bactericidal activity of purified fractions of human and rabbit serum against *S. aureus*

The Amicon Ultra 0.5ml centrifugal filters, Ultracel (Millipore) also concentrated the fraction containing the bigger component for each filter. This concentration of components was taken into consideration before inoculating the fractions with the bacteria: the fraction containing the components smaller than the molecular weight cut-off was used directly but the fractions containing the components bigger than the molecular weight cut-off were diluted accordingly using sterile PBS.

Aliquots of 100 µl of the different fractions were inoculated with 10^5 cells/ml of an exponential phase culture of *S. aureus* SH1000 and killing assays were carried out as explained in Chapter 2.9.

2.15.3 Combination of different purified fractions from rabbit and human serum

Appropriate volumes of the different fractions were mixed and rabbit serum killing assays were carried out as explained in Chapter 2.9. Aliquots of 100 µl of total volume of combined fractions were inoculated with 10^5 cells/ml of an exponential phase culture of *S. aureus* SH1000 and killing assays were carried out (Chapter 2.9).

2.16 Purification of peptidoglycan from *S. aureus* strains

2.16.1 Purification of peptidoglycan

A single colony of *S. aureus* from a BHI agar plate was used to inoculate 10 ml of BHI broth that was incubated at 37ºC with 250 rpm for an overnight. The OD_{600} of this overnight culture was determined and an appropriate volume was used to inoculate 500 ml of fresh BHI broth with an initial OD_{600}=0.01. This culture was grown until it reached exponential phase (OD_{600}=0.5) at 37ºC and 200 rpm shaking. The culture was then chilled to 4ºC on ice for 10 minutes to stop growth. The cells were then harvested by centrifugation (15000g, 10 minutes, 4ºC) and the pellet was resuspended in 30 ml of ice cold sterile PBS. This mixture was boiled in a water bath for 7 minutes and then chilled to 4ºC on ice. The cells were centrifuged (13000g, 10 minutes, 4ºC) and the pellet was resuspended in 3 ml of PBS sterile.

Next, the cells were broken by homogenization using lysing matrix B, RNAse/DNAse free tubes with silica beads (MP Biomedicals) in a Fast Prep-24 machine (MP Biomedicals): 24x at speed 6 for 30 seconds with 60 seconds pause on ice between each run. To remove the silica beads the tubes were centrifuged (2000 rpm, 30 seconds, room temperature) and the broken cell suspension was carefully transferred to a 15 ml Falcon tube. The suspension was centrifuged (5000 rpm, 8 minutes, 4ºC), the pellet was fully resuspended into 7 ml of pre-warmed (55ºC) 5%(w/v) SDS and then boiled for 30 minutes in a water bath, after which the solution was chilled on ice. The insoluble material was collected by centrifugation (5000 rpm, 8 minutes, 4ºC), resuspended into 7 ml of pre-warmed (55ºC) 4% (w/v) SDS and then boiled for 15 minutes in a water bath.

The solution was chilled on ice and the insoluble material was collected by centrifugation (5000 rpm, 8 minutes, 4ºC). The pellet was washed five times with 7 ml of sterile MilliQ water (60ºC) until free of SDS (500 rpm, 8 minutes, 4ºC). Covalently attached proteins were removed by treatment with 4 ml of 50 mM Tris-HCl (pH 7.0) containing 2mg/ml pronase for 90 minutes at 60ºC. The sacculi were washed repeatedly, once with 50 mM Tris-HCl (pH 7.0) and five

times with cold sterile MilliQ water. The peptidoglycan was then lyophilized using a Freeze Dryer Scanvac Cool Safe.

The stripped peptidoglycan (peptidoglycan without wall teichoic acid) used during this project came from laboratory stocks. To obtain it, the wall teichoic acids were removed using 48% (v/v) hydrofluoric acid (HF) treatment. This acid was added to the peptidoglycan and incubated for 24 hours at 4ºC, after which the mixture was repeatedly washed by centrifugation (9000g, 10 minutes at room temperature) using 50 mM Tris-HCl (pH 7.0) once and cold sterile MilliQ water five times, until the pH of the solution was neutral. This pure peptidoglycan was lyophilized using a Freeze Dryer Scanvac Cool Safe, resuspended in sterile PBS, aliquoted and stored at -20ºC until used.

2.16.2 Treatment of NRS with purified peptidoglycan

The lyophilized peptidoglycan samples were resuspended in PBS in order to obtain stock solutions with a final concentration of 10 mg/ml. An aliquot of NRS was mixed with this purified cell wall material (final concentration 1 mg/ml) and incubated for 30 minutes at 4ºC with rotation. The tubes were then centrifuged (10000 rpm, 3 minutes, room temperature) and the supernatant was collected and used for rabbit serum killing assays as described in the Chapter 2.9. The insoluble material obtained was washed three times with sterile MilliQ water and after a final centrifugation (14000 rpm, 3 minutes, room temperature) was resuspended in 30 µl of MilliQ water. These samples were stored at -20ºC until further use.

2.17 Purification of wall teichoic acid (WTA) from *S. aureus* strains

2.17.1 Purification of wall techoic acid

Following an overnight culture of *S. aureus* in BHI broth, the OD_{600} was determined and an appropriate volume was used to inoculate 500 ml of fresh BHI broth with an initial OD_{600}=0.01. This culture was grown until exponential phase (OD_{600}=0.5) at 37ºC with 200 rpm shaking. The culture was chilled on ice for at least 10 minutes to stop growth. The cells were harvested (15000g, 10 minutes, 4ºC), the pellet was washed with 60 ml of MES Buffer 1 and the cells

were centrifuged at 13000g for 10 minutes at room temperature. The cells were resuspended in 60 ml of MES Buffer 2 and placed in a boiling water bath for 60 minutes. The cells were chilled on ice before centrifugation (13000g, 10 minutes, 4ºC) and the pellet was resuspended in 10 ml of MES Buffer 2, transferred to Eppendorf tubes and centrifuged for 10 minutes at room temperature.

Next, the pellet was washed once with 20 ml of MES Buffer 2, once with MES Buffer 3 and once with MES Buffer 1 (4600g, 10 minutes, 4ºC), and then the sample was treated with 20 µg/ml Proteinase K at 50ºC for 4 hours. The sample was then washed with 20 ml of MES Buffer 3 and three times with sterile MilliQ water.

To release the wall teichoic acid, the sample was thoroughly resuspended in 10 ml 0.1 M NaOH and mixed by inversion at room temperature for 16 hours. The insoluble cell wall fragments were removed by centrifugation (13000g, 10 minutes, 4ºC) and the supernatant was recovered. Neutralization was carried out with 0.1 M acetic acid and then extensively dialysed against MilliQ water using Spectra/Por CE Dialysis Tubing, 500-1000 MWCO from Spectrum (VWR Jencons) and then lyophilized to obtain a white powder using a Freeze Dryer Scanvac Cool Safe.

2.17.2 Treatment of NRS with purified wall teichoic acid

The lyophilized wall teichoic acid samples were resuspended in PBS in order to obtain stock solutions with a final concentration of 10 mg/ml. Aliquots of rabbit serum were mixed with 1 mg/ml (final concentration) of purified WTA and incubated for 30 minutes at 4ºC with rotation. The tubes were then centrifuged (10000 rpm, 3 minutes, room temperature). Due to the soluble nature of WTA there was no visible pellet after the centrifugation step. The supernatant was carefully collected and used for rabbit serum killing assays as described in the Chapter 2.9. Any insoluble material was washed three times with sterile MilliQ water and after a final centrifugation (14000 rpm, 3 minutes, room temperature)

was resuspended in 30 µl of MilliQ water. These samples were stored at -20ºC until further use.

2.18 Purification of lipoteichoic acids (LTA) from *S. aureus* strains

2.18.1 Purification of lipoteichoic acids

This protocol was modified from Grundling and Schneewind, 2007a. A single colony was used to inoculate 10 ml of BHI broth that was incubated overnight at 37ºC. The OD_{600} of this culture was determined and an appropriate volume was used to inoculate 500 ml of fresh BHI broth with an initial OD_{600}= 0.01. The culture was incubated at 37ºC with 200 rpm shaking until it reached exponential phase ($0D_{600}$=0.5). The cells were placed on ice for 10 minutes to stop bacterial growth and then they were centrifuged (6000g, 10 minutes, 4ºC). The pellet was resuspended in 90 ml of 0.1 M sodium citrate (pH 4.7), centrifuged (6000g, 10 minutes, 4ºC) and the pellet was stored at -80ºC until used.

To extract the LTA, the pellet was thawed on ice and resuspended in 4 ml of 0.1 M sodium citrate (pH 4.7). The cells were broken using a Fast Prep-24 (MP Biomedicals) and lysing matrix B, RNAse/DNAse free tubes with silica beads (MP Biomedicals): 24 times at speed 6 for 30 seconds with 60 seconds pause on ice between each run. The silica beads were removed by centrifugation (2000 rpm, 30 seconds, room temperature) and the broken cells were carefully recovered into a new centrifugation tube. The silica beads were washed once with 1ml of 0.1 M sodium citrate (pH 4.7) and the supernatant was mixed with the broken cells. This suspension was centrifuged at 13000g and 4 ºC for 60 minutes.

The pellet was washed once with 0.1 M sodium citrate (pH 4.7) and centrifuged again at 13000g and 4 ºC for 60 minutes. The pellet was washed with 10 ml of 0.1 M sodium citrate (pH 4.7) and 10 ml of 1-butanol was added. This solution was mixed by inversion at room temperature for 30 minutes. The insoluble material was separated by centrifugation (13000g, 20 minutes, 4ºC), the extract was transferred into a new tube and the phases were separated by centrifugation (13000g, 20 minutes, 4ºC).

The aqueous phase contains the LTA and it was recovered and dialysed thoroughly using 20 mM sodium citrate (pH 4.7) and Spectra/Por CE Dialysis Tubing, 500-1000 MWCO from Spectrum (VWR Jencons). The protein content of the dialysed samples was determined and the samples were adjusted to contain 0.088 mg/ml of protein in a 0.1 M sodium citrate (pH 4.7) and 15% (v/v) 1-propanol buffer. The samples were then filtered using Acrodisc 13 mm Syringe Filter with 0.45µm pore size Nylon Membrane, HPLC certified (Life Sciences). The samples were loaded in a HiTrap Octyl Sepharose Fast Flow Column (GE Healthcare) for the hydrophobic interaction chromatography step. The LTA was eluted using a linear 15-65% (v/v) 1-propanol gradient in 50 mM sodium citrate (pH 4.7) and 0.5 ml/min flow rate. 750 µl fractions were collected. The fractions were then lyophilized to obtain a white powder using a Freeze Dryer Scanvac Cool Safe. To verify that LTA was present in the fractions, they were analysed through immunoblot using a specific anti-LTA antibody.

2.18.2 Treatment of NRS with purified lipoteichoic acids

The lyophilized lipoteichoic acid samples were resuspended in sterile PBS to obtain 10mg/ml stock solutions. Aliquots of rabbit serum were mixed with 1mg/ml of this purified material and incubated at 4ºC for 30 minutes with rotation. The samples were centrifuged (10000 rpm, 3 minutes, room temperature), the supernatant was recovered and used for NRS killing assays as described in Chapter 2.9. The insoluble material was washed three times with sterile MilliQ water and after a final centrifugation (14000 rpm, 3 minutes, room temperature) was resuspended in 30 µl of MilliQ water. These samples were stored at -20ºC until further use.

2.18.3 Measurement of D-alanylation of WTA and LTA

In order to release teichoic acids from peptidoglycan to measure the D-alanine content of the SH1000 strain, the peptidoglycan was incubated in trichloroacetic acid 10% (final concentration) for 18 hrs at 4ºC, after which it was removed by centrifugation (Bernal et al, 2009; Bertsche et al, 2013; Reichmann et al 2013). The D-alanine content of the supernatant was measure as follows.

This protocol was modified from Peschel *et al*, 1999. Wall teichoic acid and lipoteichoic acid samples were adjusted to pH 9-10 with sodium hydroxide and these 10 µl were incubated for 60 minutes at 37ºC. 20 µl of 0.2 M Tris-HCl (pH 8.4) containing 2.5 mg of D-amino acid oxidase/ml was added to the solution and the samples were incubated for one hour at 37ºC. This reaction was stopped with 10 µl of 30% (v/v) trichloroacetic acid and the precipitated material was removed by centrifugation (10000 rpm, 5 minutes, room temperature). The supernatant was placed in a new tube, 10 µl of a 0.1% (w/v) 2,4-dinitrophenylhydrazine prepared in 2 M HCl was added and incubated for 5 minutes at room temperature. After the addition of 20 µl of 2.5 M NaOH to the solution, the absorbance at 525 nm (A_{525}) was determined using WPA Biowave DNA Spectrophotometer and a Ultra-micro cell 105.202-QS LP 10mm, vol.50 µl, CH 15mm quartz cuvette. For the calibration curve, a stock solution of 100mg/ml D-alanine (98% w/v) was prepared using sterile MilliQ water (Figure 2.1).

2.19 Use of purified cell wall material as affinity matrices

The lyophilized purified cell wall material was resuspended in sterile PBS to obtain 10 mg/ml stock solutions. 1 ml of rabbit serum was mixed with 1 mg/ml (final concentration) of the different purified material and incubated for 30 minutes at 4ºC with rotation. After incubation the samples were centrifuged (10000 rpm, 3 minutes, room temperature) and the insoluble material was washed three times with sterile MilliQ water. After a final centrifugation (14000 rpm, 3 minutes, room temperature) the insoluble material was resuspended in 30 µl of MilliQ water. The samples were stored at -20ºC until further use in protein analysis via mass spectrometry, SDS-PAGE and 24-well slot blotting.

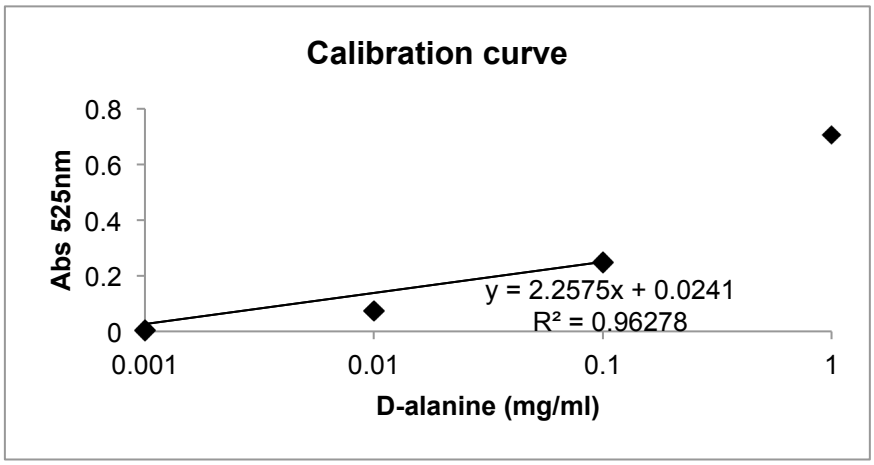


Figure 2.1 Calibration curve used for the determination of D-alanine content

The calibration curve was prepared using a stock solution of 100 mg/ml D-alanine (98% [w/v], Sigma-Aldrich) and 10-fold serial dilutions between 10 mg/ml to 1µg/ml in sterile MilliQ water. The different solutions were made in duplicate and the absorbance was determined at 525 nm using the WPA Biowave DNA Spectrophotometer and Ultra-micro cell 105.202-QS LP 10mm, vol.50 µl, CH 15mm quartz cuvette.

2.20 Mass Spectrometry

2.20.1 Preparation of samples for MS

Aliquots of 50 µl of the samples were boiled in a water bath for 5 minutes. They were cooled to room temperature before adding 150 µl of 50 mM ammonium bicarbonate. 1mg/ml of trypsin from porcine pancreas resuspended in 1mM HCl was added to the mixture and the tubes were incubated for 16 hours at 37ºC. The samples were then centrifuged (10000 rpm, 5 minutes, room temperature), the supernatant was transferred to a new tube and kept at -20ºC until they were used.

2.20.2 Liquid Chromatography-Mass Spectrometry Analysis (LC-MS)

The LC-MS analysis was carried out by Dr. Richard Beniston at the biOMICS Facility from the University of Sheffield. After the tryptic digestion of the samples, the peptides were vacuum dried and subsequently solubilized in Switchoss Solution (0.1% [v/v] formic acid, 3% [v/v] acetonitrile).

10% of the material was injected, using a Dionex Ultimate 3000 uHPLC, onto a PepMap100 C18 2cm x75µm I.D. trap column (ThermoFisher Scientific) at 5µL/min in 0.1% (v/v) formic acid, 2% (v/v) acetonitrile and 35°C in the column oven and 6°C in the autosampler. The sample was separated, over a 64 minute uHPLC run of increasing acetonitrile from 2.4% (v/v) up to 72% (v/v), in 0.1% (v/v) formic acid, using a EasySpray 15cm PepMap100 C18 analytical column (2µm particle size, 100Å pore size 75µm I.D) (ThermoFisher Scientific) at 250nL/min and 35°C.

Table 2.8 Sample separation parameters

Data collection occurred between retention time 8.1 to 63.1 minutes, and the main sample separation took place for 28 minutes, between RT 13 to 41 minutes. Buffer A: Ultrapure water with 0.1% (v/v) formic acid; Buffer B: 20% (v/v) ultrapure water, 80% (v/v) acetonitrile with 0.1% formic acid (v/v).

Retention Time (RT) (min)	Flow rate (µL/min)	% Buffer B	Column Valve

0	0.250	3.0	1-2
8.0	0.250	3.0	10-1
8.1	0.250	3.0	10-1
13	0.250	10.0	10-1
41	0.250	60.0	10-1
42	0.250	90.0	10-1
47	0.250	90.0	10-1
48	0.250	3.0	10-1
61	0.250	3.0	1-2
63.1	0.250	3.0	1-2
64	0.250	3.0	1-2

The mass spectrometer analyser used was the electron transfer dissociation (ETD) enabled Thermo Fisher-Scientific Orbitrap Elite, equipped with an Easyspray ESI source (ThermoFisher Scientific). Nanospray ionization was carried out at 1.8 kV, with the ion transfer capillary at 250°C, and S-lens setting of 60%. MS^1 spectra were acquired at a resolving power of 60,000 with an automatic gain control (AGC) target value of $1x10^6$ ions by the Orbitrap detector, with a range of 350-2000m/z. Following MS^1 analysis the top 20 most abundant precursors were selected for data dependant activation (MS^2 analysis) using collision induced dissociation (CID), with a 10ms activation time, and an AGC setting of 10,000 ions in the dual cell linear ion trap on normal scan rate resolution, with wideband isolation active and an ion intensity threshold of 5000. Ion injection time prediction and FTMS preview mode were enabled. Charge state screening and monoisotopic precursor selection were enabled with precursor ions of single charge rejected, and a 30 second dynamic exclusion window setting was used after a single occurrence of an ion with a +/- 10ppm tolerance.

The resulting spectra were searched with Mascot (Matrix Science) against the Swissprot database (Rodentia taxonomic filter) and a decoy database, within the Proteome Discoverer 1.3 software package (ThermoFisher Scientific). Full trypsin enzymatic specificity was required with up to 2 missed cleavages permitted. Instrument was set to ESI-TRAP. A mass tolerance of 5ppm was

used for precursors and 0.2Da for fragment ions. Carbamidomethylation of cysteine (+57.021Da) was specified as a fixed modification and oxidation of methionine (+15.995Da) was specified as variable modification. False discovery rates (FDRs) were set at 1% (strict) and 5% (relaxed) by Target Decoy PSM Validator (workflow node within Proteome Discoverer) and were used to distribute the confidence indicators for the peptide spectral matches. Proteins required a minimum of two peptides with a 95% confidence interval or above in order to be reported.

2.21 Protein Analysis

2.21.1 24-well slot blot system

The Amersham^{TM} Hybond-ECL nitrocellulose blotting membrane (GE Healthcare) was cut to the same size as the 24-well slot blotting apparatus (CORE Life Sciences) and equilibrated in blotting buffer for 15 minutes before use. The wet membrane was placed between the two parts of the apparatus, and the screws of blotting apparatus were secured before turning on the vacuum. 200 µl of PBS were passed through the slots as preparation and then 200 µl of the samples were loaded into the slots, ensuring that the sample really goes through the membrane.

After loading the membrane, it was removed from the 24-well slot blotting apparatus and incubated in blocking buffer for 1 hour at room temperature with gentle shaking. The membrane was washed three times with TBST before incubating it for 1 hour at room temperature with blocking buffer containing the primary antibody at appropriate dilution (Table 2.6). After the incubation the membrane was washed 3 times with TBST before incubating it for 1 hour at room temperature and shaking with blocking buffer containing the suitable horseradish peroxidase conjugated secondary antibody (Table 2.7) at 1:10,000 dilution. The membrane was washed three times with TBST before its development.

For the development of the membrane equal volumes of SuperSignal® West Pico (Thermo Scientific) Enhanced Chemiluminescent (ECL) substrate reagent

1 and ECL reagent 2 were mixed and poured over the entire surface of the air-dried membrane and incubated for 5 minutes at room temperature in a dark room. The membrane was then dried and placed between two sheets of plastic that were inside of a film cassette. Amersham™ Hyperfilm-ECL (GE Healthcare) was placed on top of the nitrocellulose membrane and exposed for different times, depending on optimal detection of the proteins. After the exposure the film was washed in developer solution until the bands appeared, then the film was submerged immediately in fixer solution and rinsed in distilled water twice. The developed films were then air-dried.

2.21.2 SDS-PAGE

The samples were prepared as follows. The protein concentration was measured using Bradford protocol and adjusted using sterile PBS. These samples were mixed with Loading Buffer and boiled for five minutes in a heating block, after which they were cooled down to room temperature before loading them in the gels. The samples were stored at -20ºC between uses.

The resolving gels were prepared as follows. The ammonium persulfate (APS) and tetramethylethylenediamine (TEMED) were added immediately before pouring the gels.

10% (w/v) resolving gel	
dH_2O	3.2 ml
50 mM Tris-HCl (pH 8.4)	2.0 ml
SDS 10% (w/v)	80 µl
30% (w/v) acrylamide/2.6% (w/v)bis (BioRad)	2.67 ml
10% (w/v) APS	80 µl
TEMED	10 µl

12% (w/v) resolving gel	
dH_2O	1.75 ml
50 mM Tris-HCl (pH 8.4)	1.25 ml
SDS 10% (w/v)	50 µl
30% (w/v) acrylamide/2.6% (w/v) bis (BioRad)	2.0 ml

10% APS	25 µl
TEMED	10 µl

The components were gently mixed in a Falcon tube and loaded into the BioRad Mini-Protean II gel casting apparatus, selecting a spacer plate of the desired gel thickness. A layer of isopropanol was pipetted on the top of the gel to isolate it from the air and to make sure it was even. Once the gel had solidified, the isopropanol was drained using filter paper. The stacking gel was made up as follows and applied on top of the resolving gel.

4% (w/v) stacking gel

dH_2O	3.05 ml
50 mM Tris-HCl (pH 8.4)	1.25 ml
SDS 10% (w/v)	50 µl
30% (w/v) acrylamide/2.6% (w/v) bis (Biorad)	0.65 ml
10% (w/v) APS	25 µl
TEMED	10 µl

A BioRad plastic comb was placed immediately into the gel to create the wells and to isolate the gel from the air. After the stacking gel solidified, it was transferred to a Protean II (BioRad) protein gel-running tank and submerged in 1x SDS PAGE reservoir buffer. The comb was removed and 5-20 µl of samples were loaded. 5 µl of 0.2 mg/ml ColorPlus Prestained Protein Ladder, Broad Range (New England Biolabs), Color Prestained Protein Standard, Broad Range (New England Biolabs) or Unstained Protein Ladder, Broad Range (New England Biolabs) protein size markers were also loaded. The proteins were separated by electrophoresis at 100V until the blue dye front of the sample buffer was at the base of the gel plate.

2.21.3 Native polyacrylamide gel electrophoresis

The samples were prepared by mixing them with Native Loading Buffer. The samples were stored at -20ºC between uses.

The resolving gels were prepared as follows. Ammonium persulfate (APS) and tetramethylethilenediamine (TEMED) were added immediately before pouring the gels.

8% (w/v) resolving gel	
30% (w/v) acrylamide/2.6% (w/v) bis (BioRad)	2.6 ml
0.375 M Tris-HCl pH 8.8	7.29 ml
10% (w/v) APS	100 µl
TEMED	10 µl

10% (w/v) resolving gel	
30% (w/v) acrylamide/2.6% (w/v) bis (BioRad)	3.4 ml
0.375 M Tris-HCl pH 8.8	6.49 ml
10% (w/v) APS	100 µl
TEMED	10 µl

The components were gently mixed in a Falcon tube and loaded into the BioRad Mini-Protean II gel casting apparatus, selecting a 1mm spacer plate. No stacking gel was used in these gels, so the BioRad plastic comb was placed immediately into the resolving gel to create the wells and to isolate the gel from the air. After the gel solidified, it was transferred to a Protean II (BioRad) protein gel-running tank and submerged in 1x Native PAGE Running buffer. The comb was removed and 10 µl of samples were loaded. The proteins were separated by electrophoresis at 100V for 5 hours and the Protean II (BioRad) protein gel-running tank was placed on ice for the duration of the electrophoresis.

2.21.4 Coomassie Blue staining

After the electrophoresis the SDS gels were placed in Coomassie Blue stain for 60 minutes with light shaking in order to visualise the proteins. The gels were then destained using 25 ml of destain solution overnight and at least three washes with 25 ml of destain solution after that until the background was clear. The gels were then stored in distilled water before being scanned and dried.

The molecular masses of the proteins present were estimated by comparison to protein standards of known molecular mass.

2.21.5 Silver Staining

The procedure from Thermo Scientific Pierce Silver Staining Kit (Life Technologies) for silver staining of proteins in polyacrylamide gels was followed. After the electrophoresis, the gels were washed twice with MilliQ water before starting the staining process. The gels were washed twice for 15 minutes with Fixing Solution, twice with Ethanol Solution for 5 minutes and then with MilliQ water twice for 5 minutes. The gels were then incubated for exactly one minute with Sensitizer Working Solution and then washed twice with MilliQ water for 1 minute. The gels were incubated for 30 minutes at room temperature and light shaking with Stain Working Solution. After this incubation the gels were washed twice with MilliQ water for 30 seconds, and immediately after Developer Working Solution was added. The gels were incubated between 30 seconds and 3 minutes in this solution, until the protein bands appeared. Once the desired band intensity was reached, the Developer Working Solution was replaced with Stop Solution. After a 2 minutes incubation, the gels were incubated in fresh Stop Solution until they were dried.

2.21.6 Gel Drying

The SDS gels stained with Coomassie Blue or Silver were dried between two sheets of DryEase® Mini Cellophane (Novex by Life Technologies) that had been pre-soaked in Gel-Dry™ Drying Solution (Invitrogen). A gel-drying frame was used to hold the gel and the drying sheets at room temperature for 24 hours or until completely dry, after which the gels were scanned.

2.21.7 Western Blot

The protein samples were separated by SDS-PAGE or Native PAGE as described in Chapter 2.21.2 and 2.21.3, respectively. The gel was equilibrated in blotting buffer for 15 minutes before the transfer began. Amersham™ Hybond-ECL nitrocellulose blotting membrane (GE Healthcare) was cut to the same size as the gel and equilibrated in blotting buffer for 15 minutes before

use. The proteins were transferred from the gel to the membrane by wet transfer using the BioRad Mini-PROTEAN® Tetra System for 90 minutes at 100V with ice-cold ethanol blotting buffer.

After the transfer the membrane was washed twice with TBST for 5 minutes and once for 15 minutes. The membrane was then incubated with blocking buffer for 1 hour at room temperature with shaking, after which it was washed with TBST twice for 5 minutes and once for 15 minutes. The membrane was incubated overnight at 4ºC with blocking buffer containing the primary antibody at an appropriate dilution (Table 2.6). After the incubation the membrane was thoroughly rinsed at least 3 times with TBST, and then incubated for 60 minutes at room temperature and shaking with blocking buffer containing horseradish peroxidase conjugated secondary antibody (Table 2.7) at 1:10,000 dilution. The unbound secondary antibody was then removed by washing the membrane with TBST once for 15 minutes and twice for 5 minutes.

For the development of the membrane, the following steps were carried out in a dark room. Equal volumes of SuperSignal® West Pico (Thermo Scientific) Enhanced Chemiluminescent (ECL) substrate reagent 1 and ECL reagent 2 were mixed and poured over the entire surface of the air-dried membrane and incubated for 5 minutes at room temperature. The membrane was dried and placed between two sheets of plastic that were inside of a film cassette. Amersham™ Hyperfilm-ECL (GE Healthcare) was placed on top of the nitrocellulose membrane and exposed for different times, depending on optimal detection of the proteins. After exposure, the film was washed in developer solution until the bands appeared, then the film was submerged immediately in fixer solution and rinsed in distilled water twice. The developed films were then air-dried before scanning.

2.21.8 Bradford estimation of protein concentration

Different concentrations of bovine serum albumin (BSA) were used to prepare the standard curve. 0, 2, 5, 10 and 20 µg of BSA were added to distilled water to a final volume of 800 µl in a Semi-micro PS spectrophotometer cuvette

(Fisherbrand). 200 µl of BioRad Protean Assay Dye was added and mixed. The absorbance at 595 nm (A_{595}) was immediately measured using a WPA Biowave DNA Spectrophotometer. The spectrophotometer was calibrated to zero using the sample containing 0 µg of BSA.

To determine the concentration of the protein samples, the samples were diluted in distilled water to a final volume of 800 µl in a spectrophotometer cuvette. Then 200 µl of BioRad Protean Assay Dye were added to the cuvette and mixed by inversion. The A_{595} was measured and the protein concentration was calculated from the standard curve equation.

2.22 Effect of purified Phospholipase A2 (PLA2) on *S. aureus*

Aliquots of 100 µl of PLA2 Buffer (Chapter 2.4.7) were inoculated with different concentrations of recombinant human PLA2 (Sigma-Aldrich), spitting cobra (*Naja mossambica mossambica*) PLA2 (Sigma Aldrich) and honey bee (*Apis mellifera*) PLA2 (Sigma-Aldrich) before the addition of 10^5 cells/ml of an exponential phase culture of *S. aureus.* Killing assays were carried out as explained in Chapter 2.9. Aliquots of rabbit serum, heated rabbit serum and adult human serum were also used following the protocol just described.

2.23 Fluorescence microplate assay for PLA2 activity

Stock solutions of assay buffer (50mM Tris-HCl, 100mM NaCl, 1mM $CaCl_2$, pH8.9), DOPC, DOPG and Red/Green BODIPY PC-A2 were prepared as explained in Chapter 2.4.8 and Table 2.4, respectively. 30 µl of 10 mM DOPC, 30 µl of 10 mM DOPG and 30 µl of Red/Green BODIPY PC-A2 were mixed in a conical microcentrifuge tube, and 77 µl of this ethanolic lipic mix was slowly and steadly injected over a minute into 5 ml of assay buffer that was being stirred. This results in 5 ml of liposomally-incorporated substrate. This mixture was aliquoted in amber Eppendorf tubes and kept at -20ºC until used.

The Victor^{TM} X3 2030 Multilabel Reader (Perkin Elmer) fluorescence reader was used and set up according to the specifications of the BODIPY PC-A2 substrate (excitation: 485nm; emission: 535 nm, instructions from manufacturer)

before starting the reaction. 50 µl of the PLA2-containing samples to test were pipetted into a well from a Nunc® 96-well Microwell Plate, flat (Thermo Scientific), as well as the appropriate controls. This was mixed with 50 µl of the liposomally-incorporated substrate, which marked the time point zero for the reaction. The plate was incubated at 37ºC and measurements of the fluorescence were taken at appropriate times. Each sample was prepared in triplicate.

For the standard curve samples containing known enzyme concentrations in assay buffer were prepared, and 50 µl of these samples were mixed with 50 µl of the BODIPY/DOPC/DOPG mixture per well. The time points recorded were the same as with the phospholipase A2 containing samples.

2.24 Inactivation of purified PLA2 by purified cell wall material

10mg/ml stocks of the different purified wall material (Chapter 2.16 and 2.17) were used for the inactivation of purified cobra (*Naja mossambica mossambica*) and purified honey bee (*Apis mellifera*) phospholipase A2. Aliquots of the purified phospholipase A2 were mixed with purified cell wall material (1 mg/ml final concentration) and incubated for 30 minutes at 4ºC with rotation. The tubes were then centrifuged (10000 rpm, 3 minutes, room temperature) and the supernatant was used to inoculate NRS at the same concentration as untreated phospholipase.

This rabbit serum was then used in killing assays as described in the Chapter 2.9, inoculating with 10^5 cell/ml of an exponential phase culture of *S. aureus* SH1000.

2.25 Effect of annexin A1 on the activity of PLA2

To test the bactericidal activity of annexin A1 on its own, aliquots of 40 µl of PLA2 Buffer (Chapter 2.4.7) were inoculated with 1.5 µl of human annexin A1 at a final concentration 50 µg/ml (Abcam) before the addition of 10^5 cells/ml of an exponential phase culture of *S. aureus* (Chapter 2.9)

To test the effect of the annexin A1 on the activity of purified cobra phospholipase A2, aliquots of 40 µl of PLA2 buffer were inoculated with 1.5 µl of human annexin A1 (final concentration 50 µg/ml) and then different concentrations of purified cobra phospholipase A2 were added to the solution. Killing assays were carried out as explained in Chapter 2.9.

3 CHAPTER 3:

Identification of the bacterial elements involved in the killing mechanism of naive rabbit serum

3.1 Introduction

Evolutionary evidence suggests that mechanisms of immune defence existed by the time the ancestors of plants and animals diverged (Wilson et al, 1997; O'Neil and Greene, 1998). Whilst invertebrates and plants survive with innate immunity mechanisms only (O'Neil and Greene, 1998; Leulier et al, 2003; Beutler, 2004), in vertebrates the innate immunity acts as the first line of defence and evolution led to the development of the highly specific adaptive immunity (Beutler, 2004; Guan and Mariuzza, 2007; Chaplin, 2010).

The innate immune system is able to recognize self-cells from microbial pathogens through the recognition of evolutionary conserved molecules that are specific to microorganisms and are not produced by the host (Medzhitov and Janeway, 2002; Vivier and Malissen, 2005). These conserved molecules are called 'pathogen-associated molecular patterns' (PAMPs) and are perceived as molecular markers of infection by the immune system (Medzhitov and Janeway, 2002). These PAMPs are 'molecular signatures' of different types of pathogen, allowing the immune system to choose the most efficient mechanism against this particular class (Medzhitov and Janeway, 2000). Examples of these microbial components that are recognized include lipopolysaccharides of Gram-negative bacteria and lipoteichoic acid of Gram-positive bacteria, along with peptidoglycan, mannan, flagellin and non-methylated CpG sequences present in both types of bacteria, as well as double-stranded RNA for viruses (Medzhitov and Janeway, 2002; Beutler, 2004; Guan and Mariuzza, 2007)

These molecules can be recognized by a set of defined receptors called pathogen- or pattern-recognition receptors (PRRs) (Ausubel, 2005; Akira et al, 2006). PRRs are germ-line encoded receptors and soluble molecules, and even

though their expression may be restricted to some cell types, they are not clonally distributed (Vivier and Malissen, 2005; Dziarski and Gupta, 2006). On Chapter 1.4.1.2 several molecules responsible for sensing the pathogens such as mannose-binding protein and collectins were discussed, but they are not the only ones.

Peptidoglycan is an essential component of the bacterial cell wall responsible for the maintenance of cell integrity (Mengin-Lecreulx and Lemaitre, 2005; Vollmer et al, 2008) and interactions with the host (Mengin-Lecreulx and Lemaitre, 2005). This polymer that can be found on the outside of the cytoplasmic membrane (Mengin-Lecreulx and Lemaitre, 2005; Vollmer et al, 2008), and whilst the length of the glycan strands can vary considerably its composition is highly conserved (Hayhurst et al, 2008; Vollmer et al, 2008; Vollmer and Seligman, 2010). Due to the conservation of its composition and its essential role in bacteria, peptidoglycan is a good target for recognition by the innate immune system (Dziarski and Gupta, 2005).

Several pathogen-recognition molecules can detect peptidoglycan. It has been shown that peptidoglycan can activate macrophages in a CD14-dependent manner (Gupta et al, 1996; Dziarski et al, 1998). Toll-like receptor 2 (Yoshimura et al, 1999) and nucleotide-binding-oligomerization domain-containing proteins (NODs) (Franchi et al, 2002; Fournier and Philpott, 2005) also detect peptidoglycan, along with peptidoglycan recognition proteins (PGRPs) (Michel et al, 2001; Guan and Mariuzza, 2007).

Teichoic acids are anionic polymers that are associated with the cell wall of Gram-positive organisms (Silhavy et al, 2010; Hanson and Neely, 2011). Wall teichoic and lipoteichoic acids are the two major types (Swoboda et al, 2010; Hanson and Neely, 2011). Whilst lipoteichoic acids can be recognized by the CD14 receptor, leading to the activation of macrophages (Cleveland et al, 1996), wall teichoic acids limit the access of the pathogen-recognition molecules to peptidoglycan (Atilano et al, 2011).

Protein A is one of *S. aureus* surface proteins that can interact with pathogen-recognition molecules. Protein A can interact directly with tumour necrosis factor-receptor 1 (TNFR1) in airway epithelium, reproducing the effects of TNF-α, the ligand of TNFR1 (Gomez et al, 2004; Fournier and Philpott, 2005).

These recognition molecules are able to sense the pathogen, but they are not able to eliminate them from the host. Other extracellular molecules produced by the immune system of the host take care of that, such as cationic antimicrobial peptides and skin fatty acids, complex protein systems like the complement cascade and enzymes as lysozyme and phospholipase A2. These molecules can be found through out the host, specially in the skin (Miller et al, 1988; Wille and Kydonieus, 2003; Georgel et al, 2005), tears (Qu and Lehrer, 1998; Girgis et al, 2003; Fluckinger et al, 2004; McDermott, 2013), serum (Taylor, 1983; Crokaert et al, 1988; Grönroos et al, 2002; Gasque, 2004; Menschikowski et al, 2006; Ricklin et al, 2010) and seminal plasma (Kunze and Bohn, 1978; Nevalainen et al, 1993).

The interaction between the bacterial cell wall components and the elements of the immune system is necessary for the recognition and elimination of the pathogens. This led us to suggest that the bacterial receptor for the antibacterial activity of naive rabbit serum (NRS) against *Staphylococcus aureus* had to be a surface component.

3.1.1 Aims of this chapter

- Study the role of wall teichoic acids in the bactericidal mechanism of NRS.
- Study the role of other cell wall components through the use of mutant strains and purified cell wall material.
- Analysis of the role of modifications of the cell wall, such as D-alanylation and glycosylation, on the interaction of *S. aureus* with NRS.

3.2 Results

3.2.1 Effect of mammalian serum on *S. aureus* SH1000

Previous studies carried out in our laboratory showed that naive rabbit serum (NRS) has the ability to kill *S. aureus* SH1000 (Bozakouk, 2011). To verify this, the bactericidal properties of rabbit and six other types of mammalian sera (mouse, calf, cow, pig, sheep, horse and rat, Table 2.5) against *S. aureus* were tested.

To test their bactericidal activity, killing assays using the mammalian sera were carried out as explained in Chapter 2.9. An aliquot of the mammalian serum was inoculated with 10^5-10^6 cells/ml of an exponential phase culture of *S. aureus* SH1000 and incubated at 37ºC. Samples were taken at specific times, 10-fold serial dilutions were made and aliquots of each dilution were spotted on BHI plates. Naive rabbit serum was the only serum that showed bactericidal activity against the bacteria (Figure 3.1). A 10-fold decrease of the bacterial numbers was observed after just 30 minutes of incubation in NRS, with bacterial count lower than the detection limit (10^3 cfu/ml) after 2 hours of incubation.

3.2.2 Effect of adult human serum on *S. aureus* SH1000

It has been well documented that human serum has bactericidal and bacteriolytic activity against Gram-negative (Waisbren and Brown, 1961; Oling, 1977; Taylor, 1983) and Gram-positive (Lehrer and Ganz, 1996; Steinberg et al, 1997; Noore et al, 2013) bacteria due to the action of several soluble components, such as complement, phospholipase, lysozyme and cationic antimicrobial peptides.

The blood of four healthy adult volunteers was collected and prepared as explained in Chapter 2.13.1. To test the bactericidal activity of each serum against *S. aureus*, killing assays using 10^5 cell/ml of an exponential phase growth culture of *S. aureus* SH1000 were carried out as described (Chapter 2.13.2).

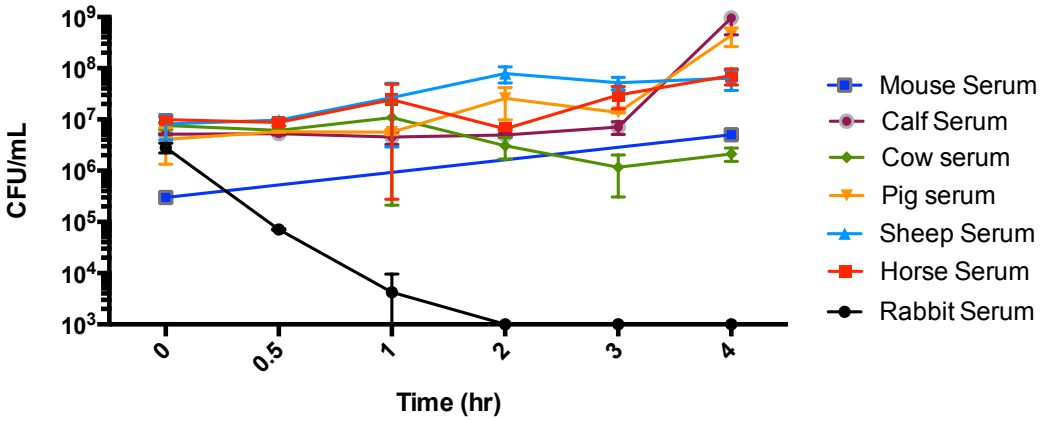


Figure 3.1 Effect of mammalian serum on *S. aureus* SH1000

The bactericidal activity of serum from different domestic mammals (Table 2.5) was tested against *S. aureus* SH1000. The colours represent different samples and these experiments were carried out in triplicate (biological triplicates), apart from the mouse serum where only 0 and 4 hr were analysed in duplicate due to the availability of the sample. Error bars represent the standard deviation of the means.

No bactericidal activity against the *S. aureus* was observed and a significant difference can be seen when compared to the effect of NRS on the bacteria (Figure 3.2, $p<0.001$).

3.2.3 Effect of infant human serum on *S. aureus* SH1000

The use of the *S. aureus* Δ*tagO* mutant showed that wall teichoic acid (WTA) is involved in the activity of NRS on the bacteria, and complement component C4b and mannose-binding lectin (MBL) were able to bind to purified WTA (Bozakouk, 2011).

It has been reported that the *S. aureus* cell wall components are able to activate complement pathways (Neth et al, 2000; Lynch et al, 2004; Shi et al, 2004). Also that whilst serum mannose-binding lectin from adults is not able to recognize WTA on the cell surface due to the presence of anti-WTA antibodies, serum MBL from infants is able to do so (Park et al, 2010). This binding also serves as an antigen in the development of anti-WTA IgGs (Park et al, 2010). As a result it was hypothesized that infant serum might be able to carry out complement-mediated lysis of *S. aureus* due to the low levels of antibodies present in infants during the first 18 months of life.

To confirm this hypothesis the bactericidal activity of baby serum was studied. Blood was obtained from four healthy infants at the Royal Hallamshire Hospital and the serum was purified according to the protocol in Chapter 2.14.1. Aliquots of the serum were incubated with an exponential phase culture of *S. aureus* SH1000, samples were taken at certain times, diluted on sterile PBS and spotted on BHI plates.

As shown in Figure 3.3, while naive rabbit serum was able to reduce the bacterial count lower than the detection limit, the incubation of *S. aureus* in infant human serum led to bacterial growth. Therefore, just as seen with adult

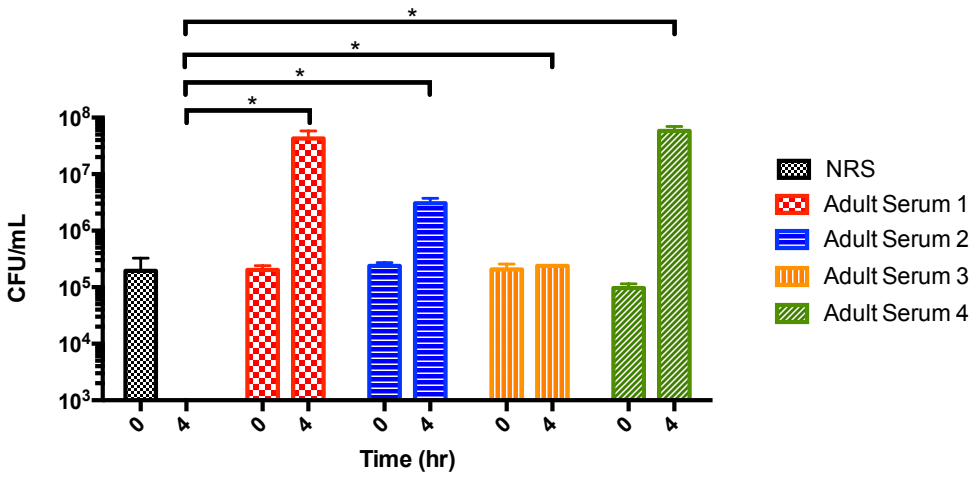


Figure 3.2 Effect of adult human serum on *S. aureus* SH1000

The bactericidal activity of serum from four different adult healthy volunteers was tested against *S. aureus* SH1000 and compared to naive rabbit serum (black bars). Samples were taken at appropriate times, 10-fold dilutions were made using PBS and spotted on BHI plates. After an overnight incubation at 37ºC the colony forming units/ml for each serum was determined. Only the results obtained after the inoculation with *S. aureus* (T= 0 hr) and at the end of the experiments (T= 4 hr) are shown to facilitate the comparison. The colours represent different samples and these experiments were carried out in triplicate (i.e. biological triplicates), error bars represent the standard deviation of the means. Student's t-tests were used and significant differences are marked with an asterisk.

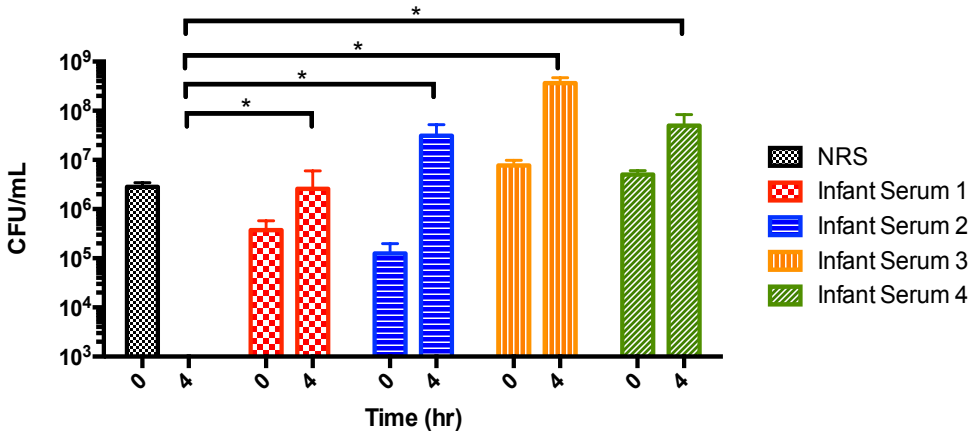


Figure 3.3 Effect of infant human serum on *S. aureus* SH1000

The bactericidal activity against *S. aureus* SH1000 of serum from four infant (younger than 18 months old) human healthy volunteers was tested and compared to the activity of rabbit serum. Each sample was inoculated with between 10^5 and 10^7 cell/ml of the bacteria and aliquots were taken at the moment of the inoculation (T= 0 hr) and after 4 hours of incubation at 37ºC (t= 4 hr). The colours represent the different samples and these experiments were carried out in triplicate (i.e. biological triplicates), with error bars as the standard deviation of the means. Student's t-tests were used and significant differences are marked with an asterisk.

human serum, infant human serum has no bactericidal activity against *S. aureus*.

3.2.4 Effect of NRS on different laboratory strains of *S. aureus*

Previous experiments carried out in the laboratory showed differences in the effect of NRS on several clinical isolates of *S. aureus* (Bozakouk, 2011). To test the possible strain specificity of the effect of NRS several laboratory strains were used. Killing assays were carried out as described before using serum from the same rabbit in all cases. The rabbit serum had the ability to kill all the tested strains in a similar manner, with fewer colonies than the detection limit after 2 hours of incubation (Figure 3.4), suggesting that the receptor for the bactericidal activity of NRS is present in all of these strains.

Even though the rabbit aliquots were inoculated with the same initial concentration of cells, a 10-fold difference was observed between the RN4220 and the rest of the strains suggesting that that an enhanced bactericidal activity of NRS for this strain.

3.2.5 Effect of heat treatment in bactericidal activity of NRS

It has been reported that heating the serum for 30 minutes at 56ºC can inactivate the bactericidal activity of mammalian serum (Beckerdite-Quagliata et al, 1975; Redelman and Russell, 1980; Taylor, 1983). This led us to test if this treatment was able to inactivate the bactericidal activity of NRS against *S. aureus* SH1000.

An aliquot of rabbit serum was incubated in a water bath at 56ºC for 30 minutes before inoculating this heated serum with 1 an exponential growth phase culture of *S. aureus* SH1000, just as described in Chapter 2.10.1. This treatment was repeated every time fresh rabbit serum was prepared. Just as expected

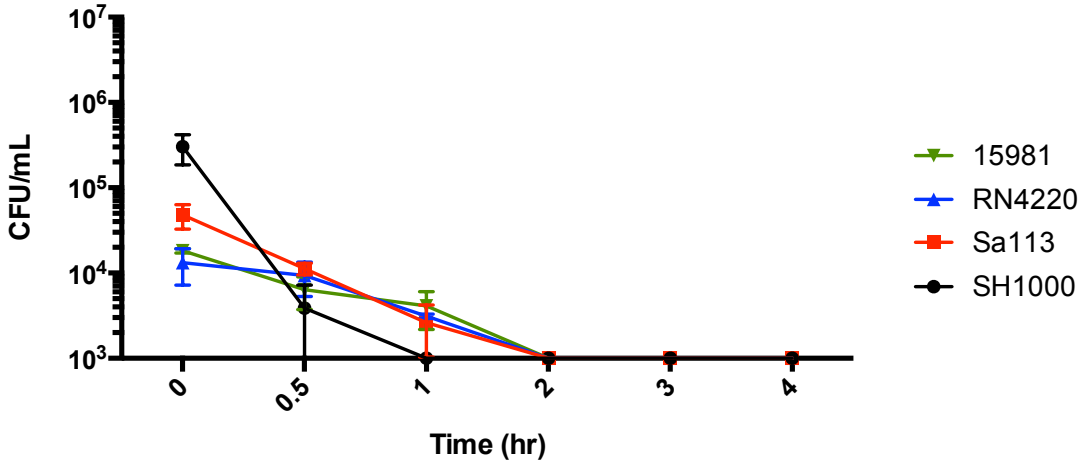


Figure 3.4 Comparison of the effect of NRS on *S. aureus* laboratory strains

The bactericidal activity of NRS against several strains of *S. aureus* (Table 2.2) was tested, inoculating an aliquot of rabbit serum with 10^5-10^6 cells/ml of each strain. Cultures were incubated at 37ºC and aliquots were taken at determined times, diluted in PBS and spotted onto BHI plates. After an overnight incubation at 37ºC the colony forming units/ml on each case was determined. The colours represent the different strains. These experiments were carried out on triplicate (i.e. biological triplicates) and the error bands represent the standard deviation of the means.

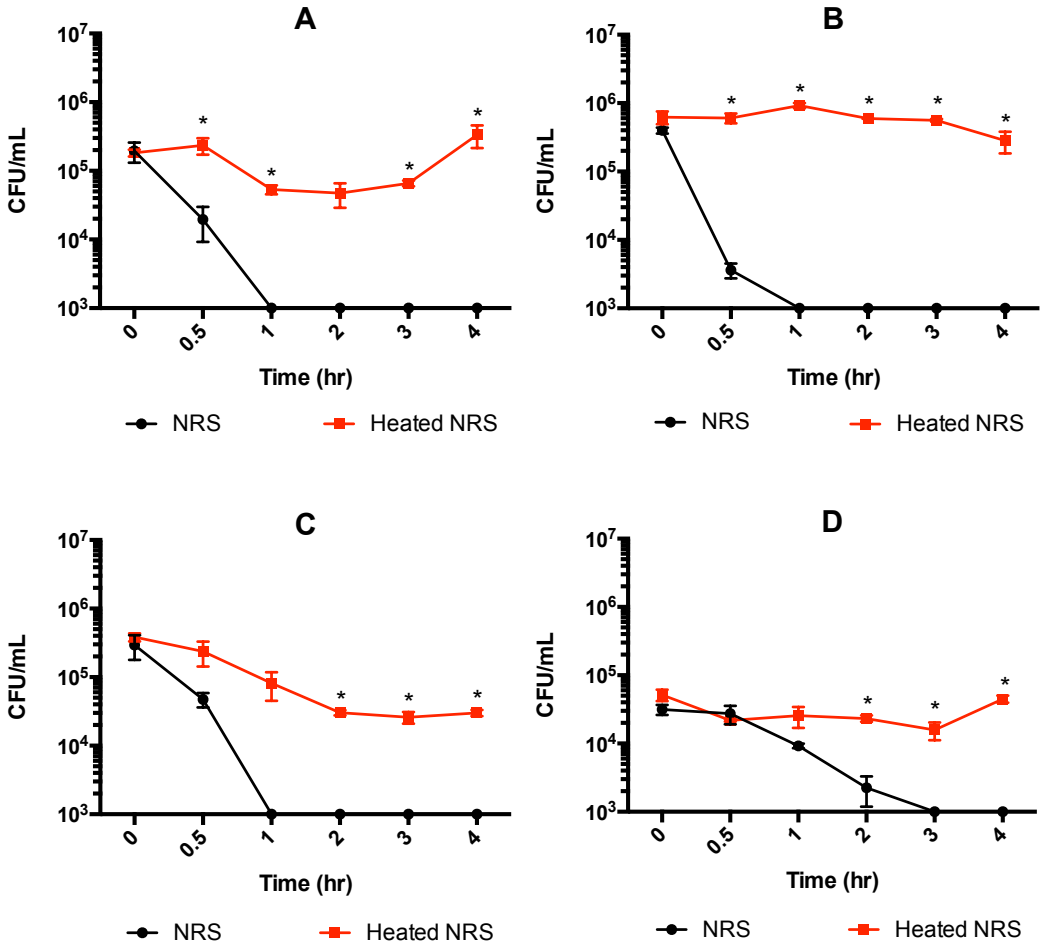


Figure 3.5 Inactivation of the bactericidal activity of NRS using heat treatment

Serum from four different rabbits (panel A, B, C and D) was prepared as described in Chapter 2.8 and aliquots were heated in a water bath for 30 minutes at 56ºC before the inoculation with 10^5-10^6 cells/ml of the bacteria for killing assays as described before. All the experiments were carried out using exponential growth phase cultures of *S. aureus* SH1000. The black line corresponds to untreated NRS, while the red line shows the heat-treated serum. These experiments were done in triplicate (i.e. biological triplicates), Student's t-tests were used and significant differences are marked with an asterisk ($p<0.05$). The error bars represent the standard deviation of the means.

from the literature, the heat treatment of the serum before the addition of the bacteria inactivates its bactericidal activity (Figure 3.5, all panels). This was observed with all the rabbit sera that have been used during this project, suggesting that the bactericidal activity of NRS is temperature sensitive.

3.2.6 Role of wall teichoic acid (WTA) in the bactericidal activity of NRS

Wall teichoic acids play an important role on the colonization of the host by *S. aureus* (Weidenmaier et al, 2004; Weidenmaier et al, 2005b; Bera et al, 2007; Weidenmaier et al, 2008), and they are involved in the resistance of the bacteria to the host immune system (Kohler et al, 2009; Carvalho et al, 2015). Previous results obtained in the laboratory showed that wall teichoic acids were involved on the bactericidal activity of NRS (Bozakouk, 2011), suggesting that this component may have a role as the receptor for this activity.

In order to verify the role of the wall teichoic acids, killing assays were carried out using the Δ*tagO* strain, mutant with a deletion of the first gene in the biosynthesis pathway of wall teichoic acids (Figure 1.6; Vergara-Irigaray et al, 2008; Swoboda et al, 2010). Killing assays using NRS were carried out using this mutant and the parent as a control, following the protocol described previously (Chapter 2.9). Whilst the parental strain was killed during the course of the experiment, the mutant was resistant to the bactericidal activity of the serum (Figure 3.6). These results suggest that due to the lack of wall teichoic acids in the cell wall of the mutant strain the 'killing factor' present in rabbit serum was not able to interact with the bacteria, confirming the role of WTA as a receptor for the killing activity of the rabbit serum.

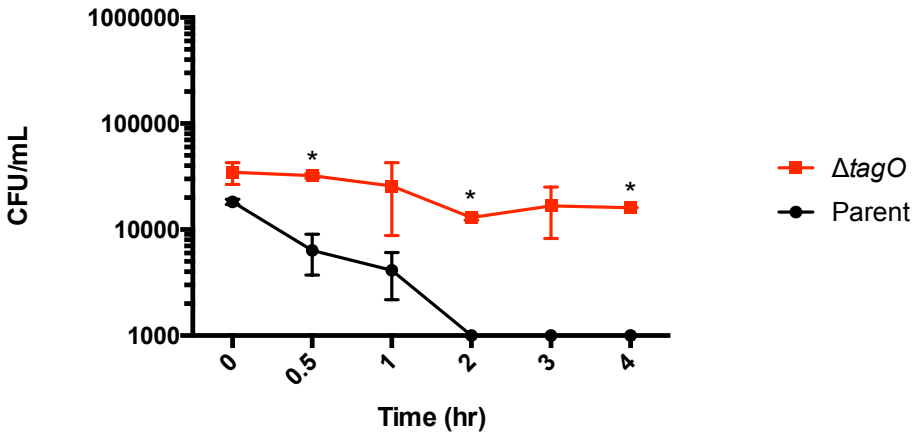


Figure 3.6 Role of wall teichoic acids in the bactericidal activity of NRS

The 15981 parental (black line) and Δ*tagO* (red line) strains were grown to exponential phase before inoculating an aliquot of NRS with 10^5 cells/ml. These experiments were carried out in triplicate (i.e. biological triplicates). Student's t-tests were used and significant differences are marked with an asterisk ($p<0.05$). Error bars correspond to the standard deviation of the means.

3.2.7 Role of lipoteichoic acid (LTA) in the bactericidal activity of NRS

After confirming the role of wall teichoic acids as a potential receptor for the bactericidal activity of NRS, lipoteichoic acid appeared as another possible candidate. This molecule is the other class of teichoic acid that can be found in *S. aureus* (Neuhaus and Baddiley, 2003; Hanson and Neely, 2011) and is involved in virulence (Weidenmaier et al, 2004), amongst other functions.

This molecule is essential for cell viability (Weidenmaier and Peschel, 2008), making it impossible to generate an LTA-deficient mutant in an unsupressed background. A mutant strain that has only 13% of LTA content when compared to the parental strain has been created by deletion of the *ypfP* gene (Fedtke et al, 2007). The YpfP protein mediates the synthesis of diglucosyl-diacylglycerol (DGlcDAG), the anchor to which the poly- [glycerophosphate] repeating unit is attached in *B.subtilis* and *S. aureus* (Figure 1.5; Jorasch et al, 2000).

The Δ*ypfP* mutant showed a different phenotype than the parental strain, with slower growth in liquid medium and smaller colonies on BHI plates (data not shown). When killing assays were carried out using this mutant strain no colonies were obtained in any of the time points, suggesting that the bactericidal elements of the serum kill the bacteria faster than the parental strain (Figure 3.7A). To test if this death was caused by the element studied in this project and not by another problem in the cell wall of this mutant killing assays were carried out using heat-treated NRS. The Δ*ypfP* mutant behaved in the inactivated serum in the same way as in the active NRS while the parental strain was able to grow in it (Figure 3.7B), suggesting that the extremely fast death of this mutant strain on rabbit serum was caused by other elements of the serum that are not affected by the heat treatment. Therefore, it was not possible to grant LTA any function in this killing mechanism of the serum using this partial mutant strain.

To test that the bacterial death observed for this mutant was not caused by other properties of the mammalian serum that could affect this LTA-deficient mutant, such as the osmolarity of the serum (Percy and Gründling, 2014), the

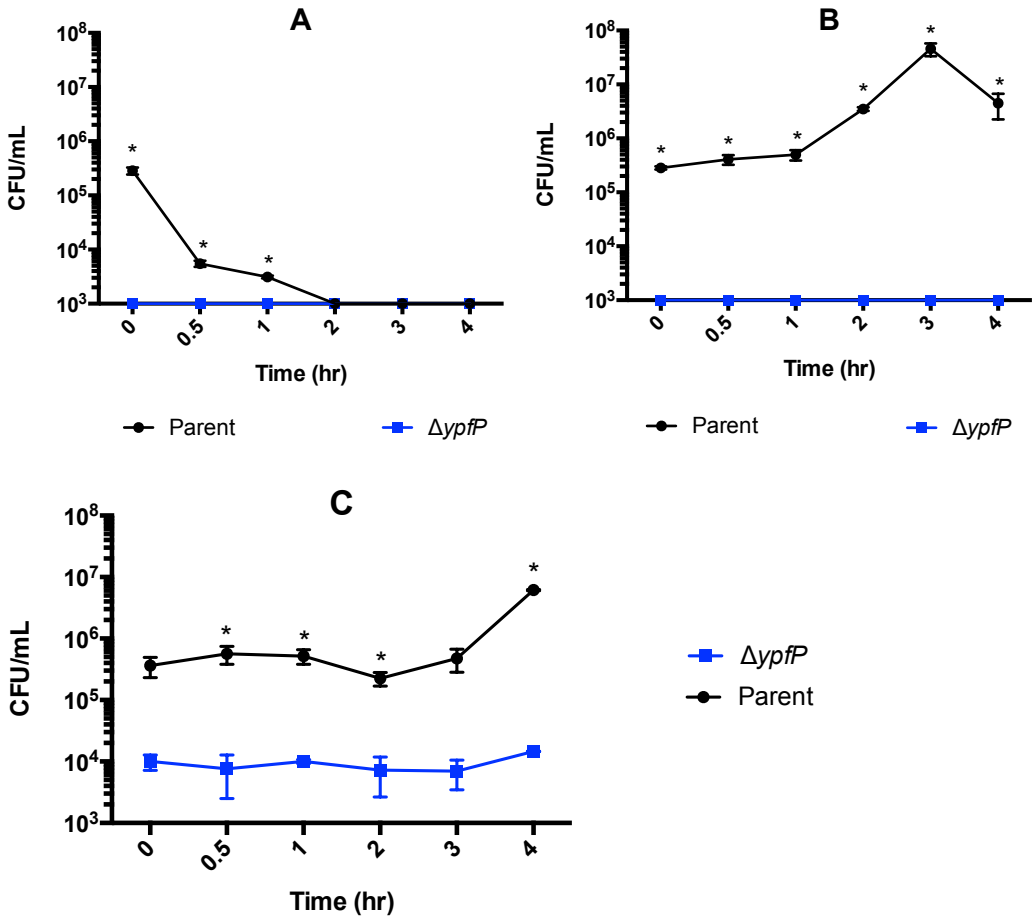


Figure 3.7 Role of lipoteichoic acids in the bactericidal activity of NRS

The Sa113 parent (black line) and the Sa113 Δ*ypfP* mutant (blue line) strains were used in killing assays using **A)** rabbit serum, **B)** heat inactivated rabbit serum, and **C)** human serum as previously described in Chapter 2.9. The same initial inoculum of both strains (10^6 cells/ml) was used in each experiment. These experiments were carried out in triplicate (i.e. biological triplicates). Student's t-tests were used and significant differences are marked with an asterisk ($p<0.05$). Error bars represent the standard deviation of the means.

parent and the Δ*ypfP* mutant strain were incubated in human serum. Both strains were able to survive in the human serum (Figure 3.7C), suggesting that the human not only differs from the rabbit serum in the mechanisms that is being studied in this project, but also in other elements that may change its properties (Waymouth, 1970). However, even though the initial inoculum was the same for both strains the LTA-deficient strain showed a 10-fold difference in the colony forming units when compared to the parent, suggesting that the mutant is still sensitive to some of the elements of this otherwise non-bactericidal human serum.

3.2.8 Role of D-alanylation of teichoic acids on the bactericidal activity of rabbit serum

Both lipoteichoic and wall teichoic acids from *S. aureus* can be modified by D-alanylation once their synthesis is complete (Perego et al, 1995; Clemans et al, 1999; May et al, 2005). The proteins encoded by the *dltABCD* operon are responsible for the translocation and incorporation of the D-alanine into the teichoic acids as it was explained in Chapter 1 (Chapter 1.3.5.3) (Perego et al, 1995; Peschel et al, 1999).

After obtaining evidence showing the role of wall teichoic acid as the receptor of the bactericidal activity of NRS, the function of the modification of the teichoic acids was studied. A *dltA* mutant where the spectinomycin resistance gene replaces the *dltA* gene was used in these experiments (Peschel et al, 1999; Table 2.2).

The *dltA* strain exhibited similar growth in liquid culture when compared to its parent strain, as well as when plated on BHI agar plates. The *dltA* strain was also used in killing assays as previously described (Chapter 2.9). An enhanced bactericidal rate of the rabbit serum on the mutant was observed when compared to the parental strain (Figure 3.8). While the reduction of colony forming units under the detection limit was observed after two hours in the

parental strain, this only took 30 minutes for the *dltA* strain. This suggests that the absence of the D-alanylation in the teichoic acids of *S. aureus* makes the bacteria more susceptible to the bactericidal elements of the rabbit serum.

Even though the aliquots of rabbit serum were inoculated with the same amount of cells initially, a 10-fold difference in the colony forming units (CFU) count was seen between the parental and the mutant strain at the beginning of the experiment. This suggested that the bactericidal elements of the rabbit serum had higher affinity for the cell wall of the mutant strain, allowing the rabbit serum to start killing the bacteria immediately.

To be able examine this difference between the mutant and the parental strain, killing assays were carried out using diluted rabbit serum. It has been shown in this laboratory that NRS can be diluted up to four times without losing its bactericidal activity (Bozakouk, 2011). Different dilutions of the rabbit serum were made using sterile PBS and the protocol to test the bactericidal activity of this solution was carried out as explained in Chapter 2.9. No survival was observed for the Δ*dltA* mutant in any of the dilutions used, whilst the rabbit serum diluted 2 times led to a 100-fold decrease in the parental strain (Figure 3.9). This supports the suggestion that the elements of the serum that are responsible for the bacterial killing have a higher affinity for the cell wall of the D-alanylation mutant than the parental strain.

3.2.9 Role of glycosylation of teichoic acids in the bactericidal activity of NRS

After obtaining results suggesting that the D-alanylation of the teichoic acids plays a role in the survival of *S. aureus* in NRS, other modifications of the bacterial cell wall were studied.

Once the biosynthesis of wall teichoic acids is completed, they can be modified by D-alanylation or glycosylation (Neuhaus and Baddiley, 2003; Weidenmaier

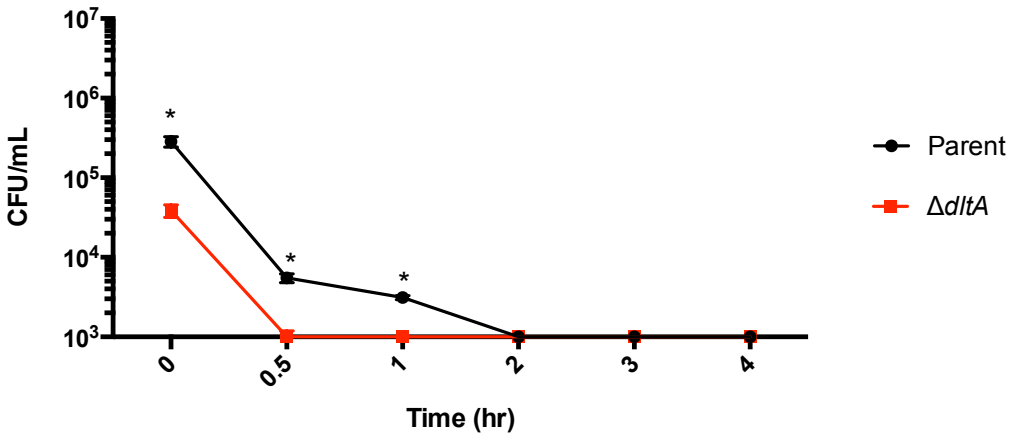


Figure 3.8 Role of the D-alanylation of teichoic acids in the bactericidal activity of NRS against *S. aureus*

The Sa113 Δ*dltA* mutant was used for killing assays using NRS as described in Chapter 2.9. The parent (black line) and the Δ*dltA* (red line) were grown until exponential phase before inoculation into rabbit serum by 10^5 cells/ml. These experiments were carried out in triplicate (i.e. biological triplicates) and Student's t-tests were used. The significant differences are marked with an asterisk ($p<0.05$) and error bars represent standard deviations of the means.

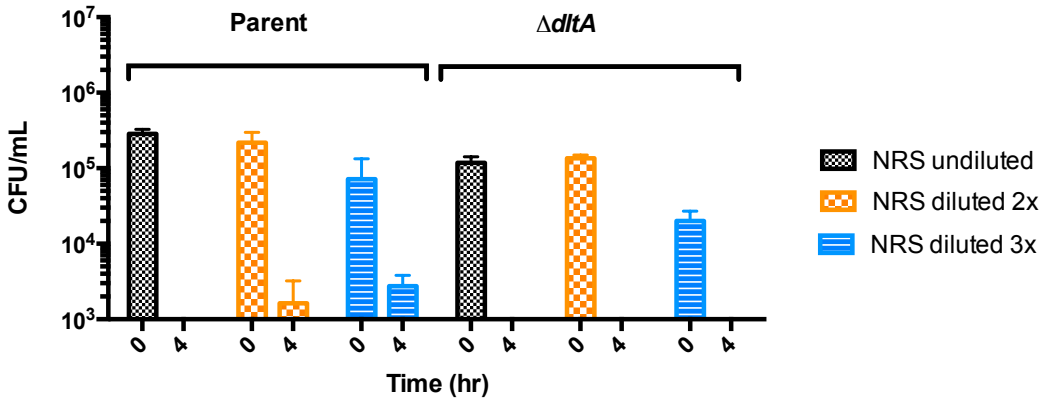


Figure 3.9 Effect of the dilution of rabbit serum in the bactericidal activity against Δ*dltA* strain

NRS was diluted 2- and 3-fold using sterile PBS before inoculation with 10^5 cells/ml of the Sa113 parent or Δ*dltA* mutant strain. Killing assays were carried out as described (Chapter 2.9). For an easier comparison of the dilutions for different dilutions and strains, only the starting and ending time points are shown. These experiments were carried out in triplicate (i.e. biological triplicates). Significant differences are shown with asterisks ($p<0.05$, Student's t-test). Error bars correspond to standard deviation of the means.

and Peschel, 2008; Xia et al, 2010b). As explained previously, the *dltABCD* operon is responsible for the modification of the wall teichoic acids via the addition of D-alanine esters into the C2 hydroxyl position (Neuhaus and Baddiley, 2003). On the other hand, the glycosylation adds an N-acetylglucosamine residue into the C4 hydroxyl of the poly-ribitolphosphate unit (Sobhanifar et al, 2015). This monosaccharide can be added through a α- or a β- linkage via the TarM or TarS proteins, respectively (Xia et al, 2010b; Brown et al, 2012). Whilst the *tarS* gene co-localize with wall teichoic acid biosynthesis gene clusters (Brown et al, 2012), *tarM* does not co-localizes with wall teichoic acid gene clusters and is not present in every *S. aureus* genome (Winstel et al, 2014).

The configuration of this glycosidic linkage varies and it has been possible to identify strains of *S. aureus* solely with α - or β-O linked monosaccharide, whilst others display a mixture of linkages with variable abundance between individual strains (Torii et al, 1964; Xia et al, 2010b; Sobhanifar et al, 2015).

It has been suggested that these sugar modifications are involved in the host antibody response to wall teichoic acids (Juergens et al, 1963; Xia et al, 2010b) and in the binding of bacteriophages to *S. aureus* (Chaterjee, 1969; Winstel et al, 2014). It has been shown that the lack of β-O-GlcNac renders MRSA strains susceptible to β-lactam antibiotics, suggesting that this sugar might work as a scaffold for proteins that are involved in PBP2a activity (Brown et al, 2012).

Mutant strains with deletions for the α-O-glycosyltransferase (Δ*tarM*) and β –O-glycosyltransferase (Δ*tarS*) genes, along with Δ*tarM*Δ*tarS* and Δ*tarM*Δ*tarS* complemented with *tarS* (Δ*tarM*Δ*tarS* pRB-*tarS*) were used to test the role of the glycosylation of the wall teichoic acids on the survival of *S. aureus* against rabbit serum. No significant differences between the mutant strains, or between the mutants and the parental strain, were found when growth curves of all the strains were made in BHI. When the killing assays were carried out it was found that all the mutant strains were susceptible to the bactericidal elements present in the serum, without significant differences compared to the parental strain (Figure 3.10, panels A, B, D). However, while all the strains reached bacterial

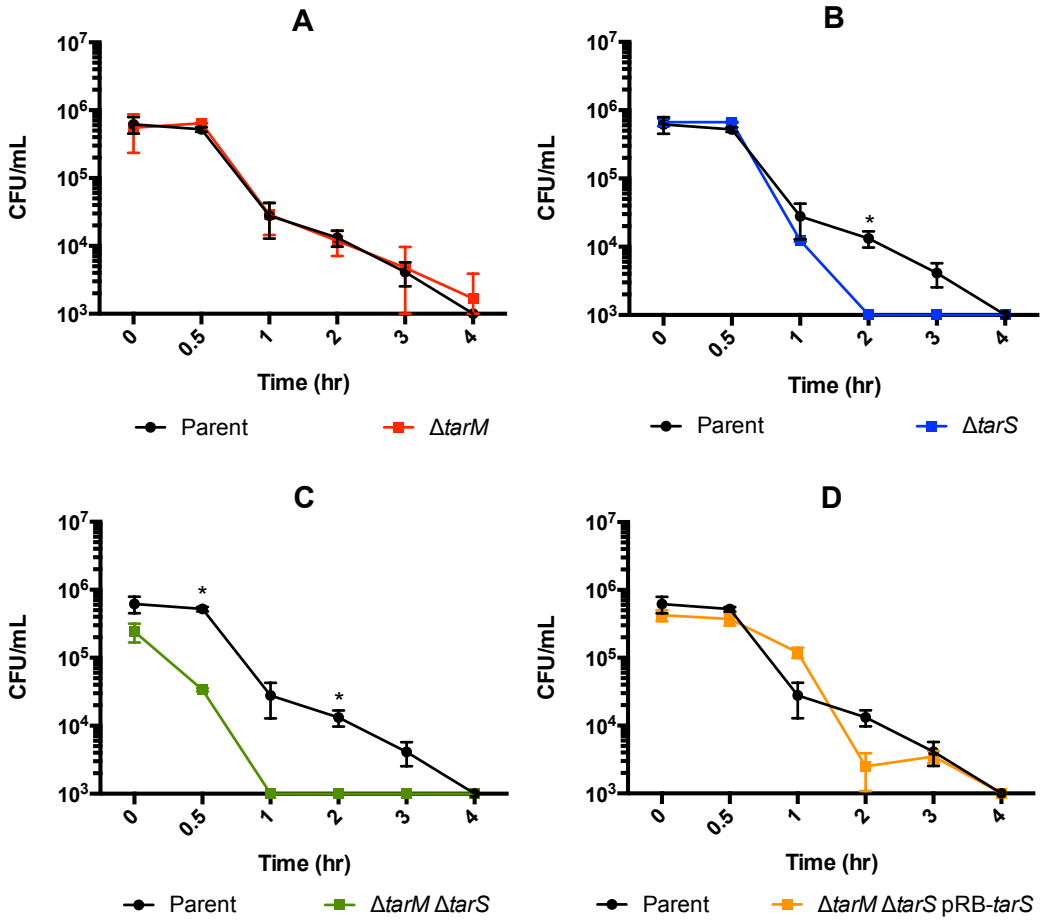


Figure 3.10 Role of the glycosylation of teichoic acids in the bactericidal activity of NRS

An initial inoculum of 10^6 cells/ml of the *S. aureus* RN4220 mutant strains **A)** Δ*tarM*, **B)** Δ*tarS*, **C)** Δ*tarM*Δ*tarS* and **D)** Δ*tarM*Δ*tarS* pRB-*tarS*, as well as the parental strain (black line) were used for killing assays to test the bactericidal activity of NRS. These assays were carried out as described in Chapter 2.9. These experiments were carried out in triplicate (i.e. biological triplicates) and Student's t-tests were carried out. Significant differences are shown with asterisks ($p<0.05$). Error bars correspond to the standard deviation of the means.

numbers lower than the detection limit after 4 hours of incubation, significant differences were found for the double mutant only after 30 and 120 minutes of incubation, suggesting that this strain is slightly less sensitive than the other strains to the NRS killing (Figure 3.10C).

In order to examine more closely the effect of the serum on the different mutant strains, diluted serum was used to carry out killing assays. As in Chapter 3.2.8, 2- and 3-fold dilutions were made using sterile PBS and the strains were grown until they reached exponential growth phase before inoculating the serum. The undiluted and the 2-fold diluted serum was able reduce the bacterial count under the detection limit (10^3 cfu/ml) in all the strains used, confirming that diluting the serum 2 times does not affect its bactericidal activity (Figure 3.11). The 3-fold diluted serum was not able to kill all the Δ*tarM* or the Δ*tarS* mutants, but a significant reduction of 1000-fold in the bacterial count was found for the parent, the double mutant and the double mutant complemented with the β–O-glycosyltransferase (Figure 3.11, $p<0.05$). Even though the differences between the single mutants and the double mutant strains were not significant ($p<0.05$), they indicate that the role of this modification of the wall teichoic acids in the activity of the bactericidal elements of the rabbit serum against S. aureus remains unclear.

3.2.10 Role of L-lysine modification of phospholipids in the bactericidal activity of NRS

The D-alanylation modification of the bacterial cell wall protects *S. aureus* from cationic antimicrobial peptides and other soluble components of the host immune system because the incorporation of the positively charged residues are capable of neutralizing the negative charge of the cell wall. Other modifications of the cell surface help in this protection by the introduction of positive charges (Kovacs et al, 2006), such as the deacetylation of N-acetylglucosamine in peptidoglycan by the PgdA protein in *S. pneumoniae*

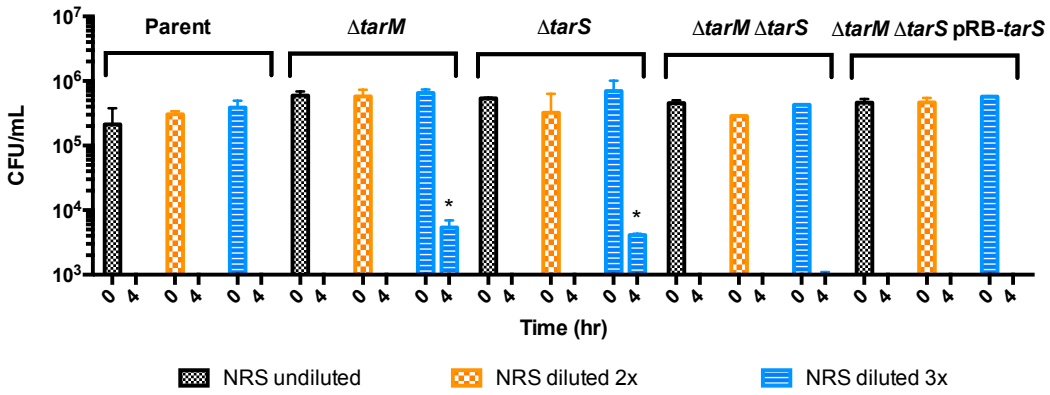


Figure 3.11 Effect of diluted NRS on its bactericidal activity against *S. aureus* glycosylation mutants

The *S. aureus* RN4220 mutant strains for the genes responsible for the glycosylation, Δ*tarM*, Δ*tarS*, Δ*tarM*Δ*tarS* and Δ*tarM*Δ*tarS* pRB-*tarS*, as well as the parental strain, were used for killing assays to test their survival in naive rabbit serum diluted 2- and 3-fold on sterile PBS. The undiluted and diluted serum were inoculated with 10^5-10^6 cells/ml of the different strains and the cultures were incubated at 37ºC. Only the results for the beginning and end of the experiment are shown in order to make the comparison easier. The experiments were carried out in triplicate (i.e. biological triplicates). Student's t-tests were carried out and the asterisk show significant differences to the parental strain ($p<0.05$). Error bars represent the standard deviation of the means.

(Vollmer and Tomasz, 2000; Kovacs et al, 2006) and the lysinylation of membrane lipids by the MrpF protein in *S. aureus* (Peschel et al, 2001).

MprF (multiple peptide resistance factor) is a *S. aureus* virulence factor that is involved in the escape from the innate host defences (Peschel et al, 2001). This protein is able to add a L-lysine residue to the anionic phosphatidylglycerol, synthesizing lysyl-phosphatidylglycerol (Weidenmaier et al, 2005a). This modification neutralizes the negative charge and enhances the positive charge of the bacterial surface, facilitating the repulsion of the cationic antimicrobial peptides (Weidenmaier et al, 2005a), defensins (Peschel et al, 2001) and other molecules such as Group IIA phospholipase A2 (Koprivnjak et al, 2002).

In order to test if this modification was involved in the bactericidal mechanism of the NRS, a Δ*mprF* mutant and the complemented strain were tested. The *mprF* gene was replaced by the erythromycin resistance gene (*ermB*) via homologous recombination in this mutant strain (Weidenmaier et al, 2005a). The parent and mutant strains showed similar growth curves in liquid culture and colonies when plated in BHI agar plates. When compared to the parental strain, the rabbit serum has similar bactericidal activity against the deletion mutant and the complemented strain (Figure 3.12A and B).

When the killing assays were carried out using 2- and 3- fold diluted rabbit serum (as explained in Chapter 3.2.9), differences were observed between the strains when the rabbit serum was diluted 3-fold (Figure 3.13). Whilst a reduction in the bacterial count of the mutant strain lower than the detection limit (10^3 cfu/ml) was observed in all the assays, the parent showed some survival in the diluted serum and the complemented strain was completely resistant to the bactericidal activity of the serum when it was diluted 3-fold. The differences between the parent and the complemented strain might be caused by the multiple copies of the *mprF* gene present in the plasmid-complemented strain (Yang et al, 2013). However, while the differences between strains with and without this modification in 3-fold diluted NRS showed a trend they were not significant ($p<0.05$), suggesting that the role of L-lysine in the resistance of *S. aureus* to NRS was not as important as the D-alanylation.

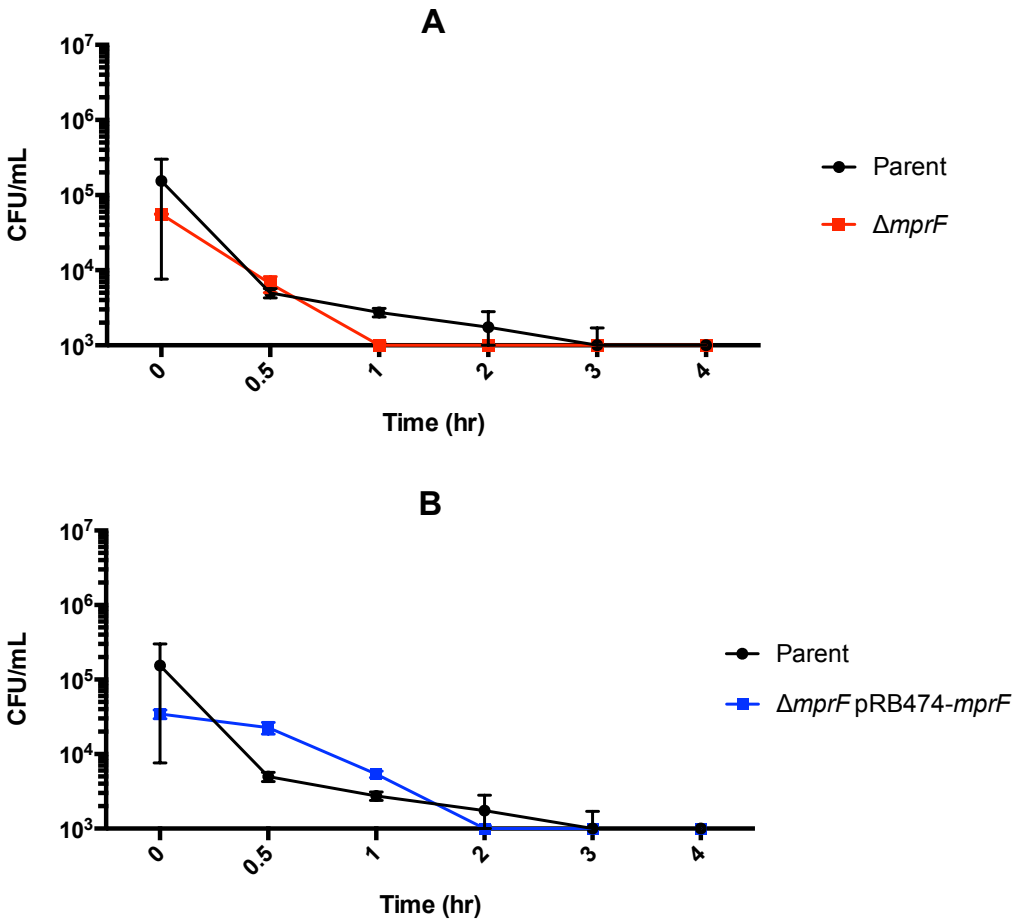


Figure 3.12 Role of the L-lysine modification of phospholipids in the bactericidal activity of NRS against *S. aureus*

An initial inoculum of 10^5 cells.ml of the *S. aureus* Sa113 mutant strains **A)** Δ*mprF* and **B)** Δ*mprF pRB474-mprF* were used for killing assays to test the bactericidal activity of rabbit serum. The black line represents the parent strain in both graphs. These experiments were carried out in triplicate (i.e. biological triplicates). Student's t-tests were carried out and no significant differences were found ($p>0.05$). Error bars correspond to the standard deviation of the means.

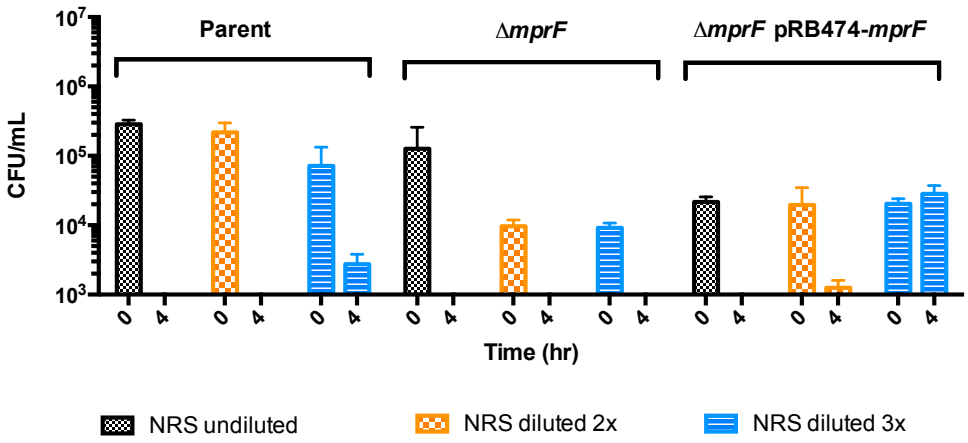


Figure 3.13 Effect of dilution of rabbit serum in its bactericidal activity against *S. aureus* Δ*mprF* mutant strain

An initial inoculum of 10^4-10^5 cells/ml of the Sa113 Δ*mprF* and Δ*mprF* pRB474-*mprF* strains, as well as the parent, were used for killing assays to test their survival in NRS diluted 2- and 3-fold in sterile PBS. These assays were carried out as described in Chapter 2.9 and the experiments were carried out in triplicate (i.e. biological triplicates). Student's t-tests were carried out and no significant differences were found ($p>0.05$). Error bars correspond to the standard deviation of the means.

3.2.11 Effect of rabbit serum on rabbit and human evolved strains of *S. aureus*

The ability of a microbial pathogen to colonize a host is defined as host specificity (Pan et al, 2014). This host specificity is determined by interactions between the pathogen and the host (Pan et al, 2014). *S. aureus* is a major resident and transient colonizer of the skin and mucosa of humans and primates and can occasionally live on domestic animals (McCarthy et al, 2012; Pantosti, 2012). It has been shown that *S. aureus* uses the haemoglobin-binding protein IsdB to capture haemoglobin for heme-iron acquisition, and that the bacteria has a preferential binding to human haemoglobin when compared to murine haemoglobin (Torres et al, 2006; Pishchany et al, 2010; Pan et al, 2012). This suggests that the iron availability in human contributes to the host specificity of *S. aureus* (Pishchany et al, 2010). However, this pathogen has also been associated with infections in economically important livestock such as cows, sheep and rabbits (Viana et al, 2015)

Bacterial pathogens can switch and adapt to different host species, but the molecular mechanisms involved are not well understood (Herron-Olson et al, 2007; Guinane et al, 2010). It has been shown that while some specific genetic backgrounds, known as lineages, are associated with specific mammalian hosts (Sung et al, 2008), others can cause infections in a broad number of animal species (Pantosti, 2012).

S. aureus clonal complex CC121, also known as clone ST121, can cause skin infections and necrotizing pneumonia in humans (Viana et al, 2015). A highly virulent epidemic ST121 clone associated with skin abscesses and mastitis in rabbits has recently emerged (Vancraeynest et al, 2006). This colonization of humans and rabbits can be explained by the existence of host-specific subtypes of the ST121 clone (Viana et al, 2015). This host adaptation was not caused by the acquisition of mobile genetic elements, but by a single mutation in the *dltB* gene. This mutation was sufficient and required to turn the human strain, which is incapable of infecting rabbits, into a strain that can cause epidemics in rabbit farms (Viana et al, 2015). In addition to this mutation, the rabbit isolates require

a mutation in *rot* (repressor of toxins) gene regulator to infect the skin. Two additional mutations in the *dltB* gene enhanced the infectivity and bacterial proliferation within rabbit skin lesions (Viana et al, 2015).

As stated above, the rabbit *dltB* gene shows three single nucleotide polymorphisms (SNPs) when compared to the human evolved strain. Two of these SNPs change amino acids (T113K and Y250H), while the third one changes the stop codon of the protein to a tyrosine, after which a new stop codon appears (*405Y). In other words, the rabbit DltB protein has two substitutions and is one residue longer that the human protein. The rabbit isolates also present a nonsense mutation in the global virulence regulator *rot* (K103*). The role of these mutations in the rabbit infectivity was tested by their introduction into the human ST121 strain F (Viana et al, 2015).

Rabbit infectivity was not the only parameter tested using these rabbit and human ST121 strains. Their cell wall composition was studied and the peptidoglycan structure was not affected, and neither was the protein profile of the proteins bound to the bacterial cell wall (Viana et al, 2015). The mutations present in the DltB might have an effect on the interaction of the bacteria with the host immune system, and this was tested by killing assays using naive rabbit serum.

These experiments were also carried out to confirm the importance of the D-alanine esters present in the bacterial cell wall on the survival of the bacteria against rabbit serum. This role was confirmed in Chapter 3.2.8 using a Δ*dltA* mutant strain. Those results suggested that the absence of these residues make the bacteria more susceptible to the bactericidal elements of the rabbit serum because they have a higher affinity for this bacterial cell wall when compared to the parental strain.

As previously stated, the function of the DltB protein in the D-alanylation pathway is not completely clear. It has been suggested that this protein is involved in the movement of either the D-alanine residue across the cytoplasmic membrane (Perego et al, 1995; Reichman et al, 2013), or the Dcp

Table 3.1 Rabbit and human evolved *S. aureus* strains

All of the strains were constructed and their sequence was analysed in Viana *et al* (2015). Data on infectivity were taken from Viana *et al* (2015): I: highly pathogenic; NI: not highly pathogenic; NA: no data available.

Strain	Genotype	Rabbit infectivity	Survival in rabbit serum
CH 3934	Parental J strain, rabbit origin	I	Yes
CH9982	J strain, Δ*dltB*	NA	No
CH 8348	J strain with human version of DltB protein due to reversion of the 3SNPs present in the rabbit protein ($DltB^H$)	NI	Yes
CH 9548	J strain with human version of Rot regulator due to reversion of the SNP present in the rabbit protein (Rot^H)	NI	Yes
CH 7705	Parental F strain, human origin	NI	No
CH 8569	F strain with mutation in *rot* present in the rabbit isolates (*rot* K103*)	NI	No
CH 9602	F strain with mutation in *dltB* present in the rabbit isolates (*dltB* *405Y)	I	No
CH 9700	F strain with mutations in rot (*rot* K103*) and dltB (*dltB* *405Y) present in rabbit isolates	I	Yes
CH 9897	F strain with mutations in rot (*rot* K103*) and dltB (*dltB* *405Y) present in rabbit isolates, also cured from the prophage inserted in the β-toxin gene	I	Yes
CH 10310	F strain with mutations in *dltB* present in the rabbit isolates (*dltB* T113K, Y250H, *405Y)	I	No
CH 10311	F strain with mutations in *dltB* (*dltB* T113K, Y250H, *405Y) and *rot* (*rot* K103*) present in the rabbit isolates	I	No
CH 10320	F strain with mutations in *dltB* present in the rabbit isolates (*dltB* T113K, Y250H)	NA	No
DL 9	Parental ST96 clone, rabbit origin with 2 SNPs in *dltB* when compared to the human clone (*dltB* K402R, *405Y)	I	Yes
BK 4337	Parental ST96 clone, human origin	NI	No

complex through the cytoplasmic membrane and in the incorporation of the D-alanine into the teichoic acids (Koprivnjak et al, 2006; Reichman et al, 2013).

To study the effect of the different mutations on the survival of *S. aureus* against rabbit serum, killing assays were carried out using these strains (Table 3.1). The bactericidal effect of rabbit serum was tested first on the ST121 clone parent or J strain (CH 3934), and compared to the Δ*dltB* mutant (CH 9982), the strain with the Rot regulator from human isolates (Rot^H; CH 8348) and the strain with DltB protein from human isolates ($DltB^H$; CH 9548). Killing assays were carried out as explained in Chapter 2.9, inoculating aliquots of rabbit serum with 10^6 cell/ml of cultures of each strain in exponential growth phase.

Even though the rabbit serum had the ability to kill the J parental strain, this bactericidal effect was not as strong as with the other laboratory strains tested in this project (as shown in Chapter 3.2.4). After four hours of incubation in rabbit serum only a 100-fold decrease in the bacterial numbers was observed. When compared to the J mutant strains, no significant difference was found for the strain with the Rot from human isolates (Figure 3.14B) or with the DltB protein from human isolates (Figure 3.14C). The Δ*dltB* mutant, on the contrary, was strongly affected by the bactericidal elements of the rabbit serum, reaching numbers lower than the detection limit by 30 minutes of incubation in the NRS (Figure 3.14A). This behaviour was similar to that of the Δ*dltA* mutant, confirming the important role of this modification of the teichoic acids in the survival of the bacteria in rabbit serum.

For the ST121 human clone or F strains, the bacterial count of the parental strain was lower than the detection limit after 2 hours of incubation. When the effect of NRS on the parent was compared to the effect on the different human mutant strains significant differences were found for two strains: CH 9700 and CH 9897 (Figure 3.15C and D, respectively). Both of these strains have a mutation in *rot* (resulting in K103*) and in *dltB* (resulting in *405Y), and the CH 9897 strain also was cured from the prophage inserted in the β-toxin gene. As explained in table 3.1, these mutations in the *dltB* and *rot* human genes allowed

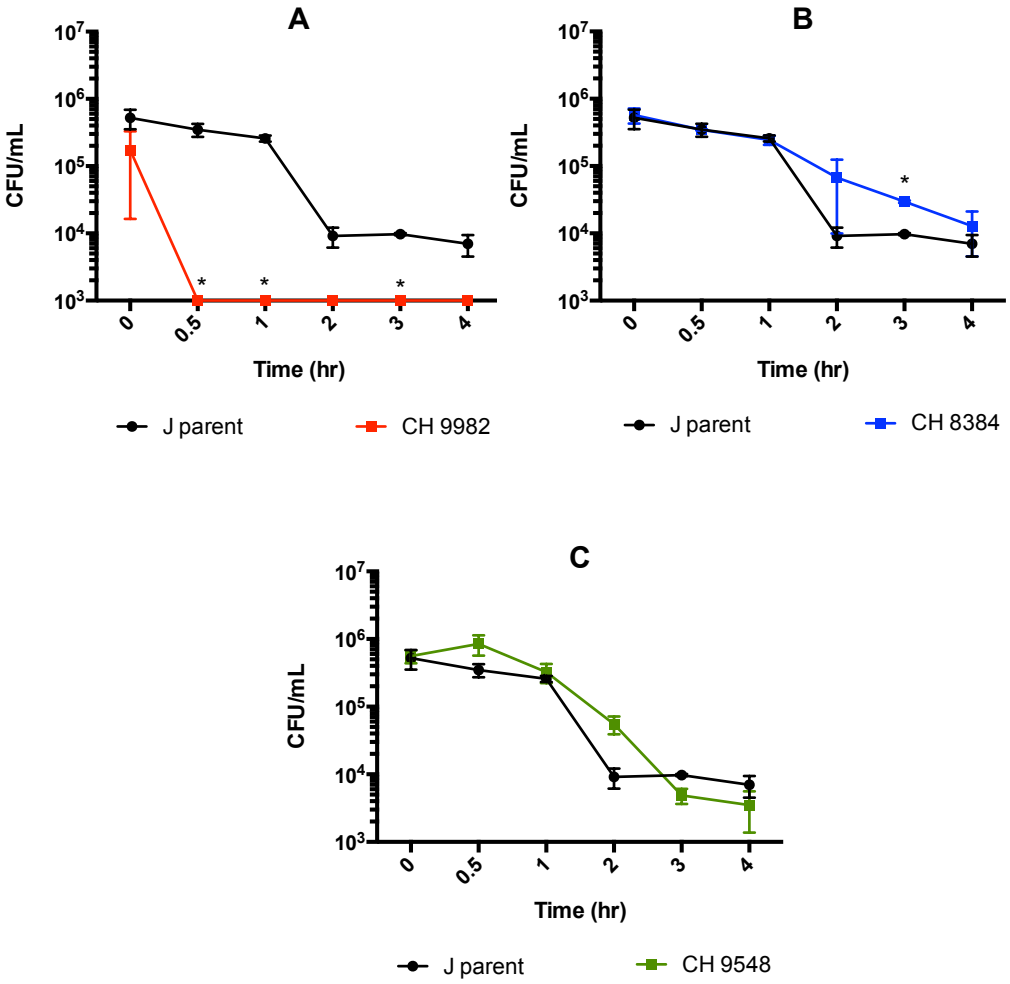


Figure 3.14 Effect of NRS on the survival of ST121 clone rabbit strains

The bactericidal effect of NRS was tested on the J strain, the ST121 clone rabbit origin strain (CH 3934, black line) and compared with **A)** J Δ*dltB* (CH 9982), **B)** J Rot^{H} (CH 8384), and **C)** J DltB^{H} (CH 9548). All the strains were grown until they reached exponential growth phase before inoculating a rabbit serum aliquot with 10^{6} cell/ml. Killing assays were carried out as explained in Chapter 2.9. These experiments were carried out in triplicate (i.e. biological triplicates). Student's t-tests were carried out and asterisks show significant differences ($p<0.05$). Error bars represent the standard deviation of the means.

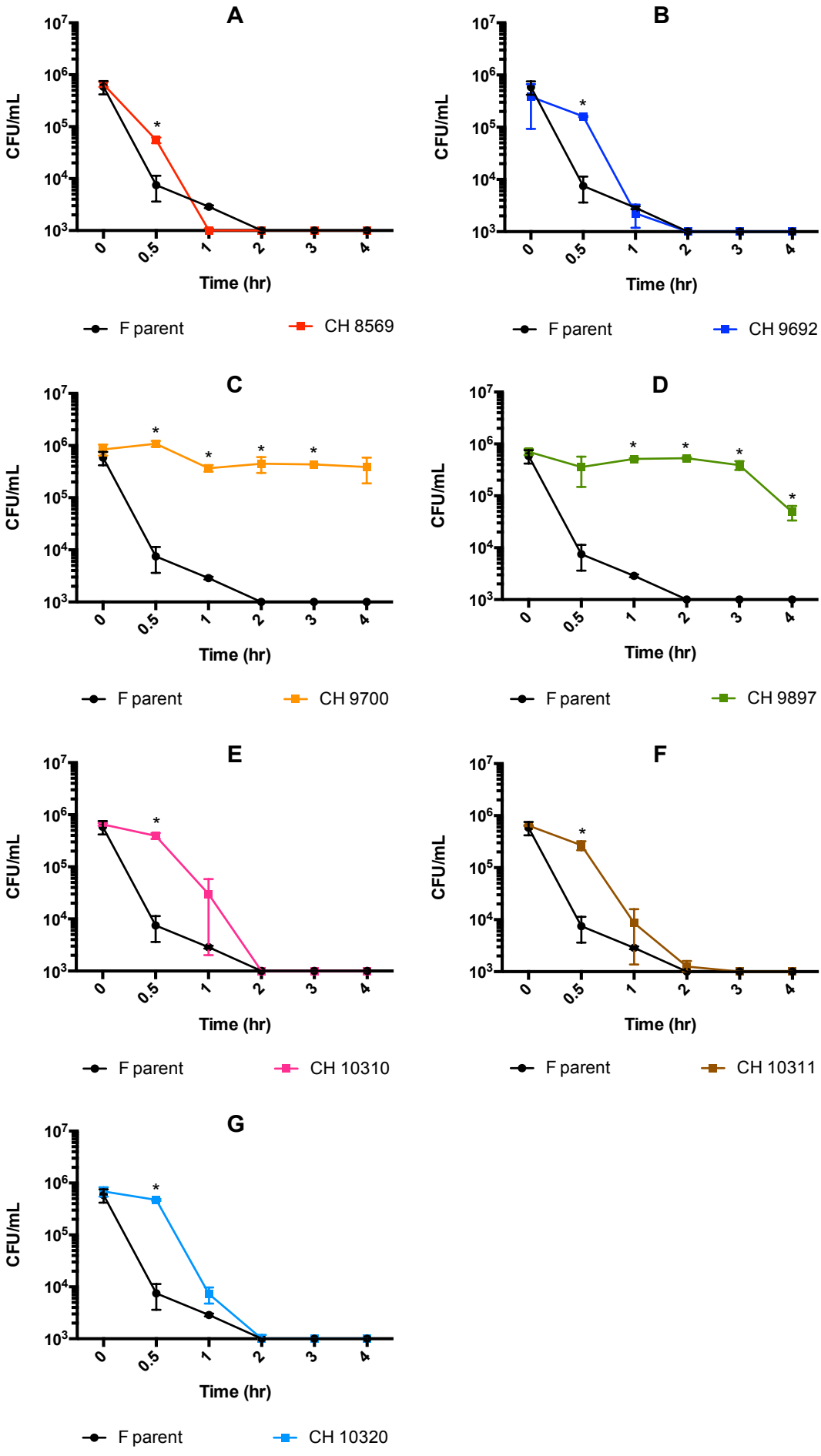

A
B
C
D
E
F
G
CFU/mL
Time (hr)
F parent
CH 8569
CH 9692
CH 9700
CH 9897
CH 10310
CH 10311
CH 10320

Figure 3.15 Bactericidal effect of NRS on ST121 clone human strains

The bactericidal activity of rabbit serum was tested against the F strains, ST121 human clone. The F parental strain (CH 7705, black line in all panels) was compared to **A)** CH 8569 (*rot* K103*), **B)** CH 9692 (*dltB* *405Y), **C)** CH 9700 (*rot* K103*; *dltB* *405Y), **D)** CH 9897 (*rot* K103*; *dltB* *405Y; cured from the prophage inserted in the β-toxin gene), **E)** CH 10310 (*dltB* T113K, Y250H, *405Y), **F)** CH 10311 (*rot* K103*; *dltB* T113K, Y250H, *405Y), **G)** CH 10320 (*dltB* T113K, Y250H).

10^6 cells/ml of each strain were inoculated into aliquots of rabbit serum and killing assays were carried out in triplicate (i.e. biological triplicates, Chapter 2.9). Student's t-tests were carried out and significant differences are marked with asterisks ($p<0.05$). Error bars represent the standard deviation of the means.

the bacteria to infect rabbits (Viana et al, 2015). This suggests that the mutations not only have an effect in the infectivity of the strains but also in the interactions with the host immune system.

Another *S. aureus* rabbit isolate, ST96, was also tested against rabbit serum. This clone has two SNPs when compared to the human clone (Table 3.1), and *dltB* one of them is different than the SNPs present in the ST121 rabbit clone. Just as with the ST121 isolates, the ST96 human strain is killed faster by the NRS when compared to the rabbit strain (Figure 3.16). This supports the idea of host adaptation by the rabbit isolates.

3.2.12 Pre-incubation of NRS with different types of purified cell wall material and its effect on the killing activity

In order to further analyze the role of the WTA as receptor for NRS and to test the importance of the interaction between WTA and peptidoglycan for the bactericidal activity, different types of fragmented cell wall material were purified from *S. aureus* SH1000 as explained in Chapter 2.16. Aliquots of rabbit serum were mixed with 1 mg/ml of unstripped (PG+WTA) or hydrofluoric acid stripped (just PG) peptidoglycan. This mixture was incubated for 30 minutes at 4ºC with rotation and then centrifuged. The supernatant was collected and placed in a new tube to be used for killing assays with *S. aureus* SH1000, while the insoluble material was washed with sterile MilliQ water at least three times, after which was resuspended in 30 µl of MilliQ and stored at -20ºC for further use.

As expected, whilst the pre-incubation with pure peptidoglycan (stripped PG) was able to partially inactivate the killing activity (Figure 3.17A), the cell wall sample that contained wall teichoic acid (unstripped) was able to completely inactivate the bactericidal activity of the rabbit serum (Figure 3.17B).

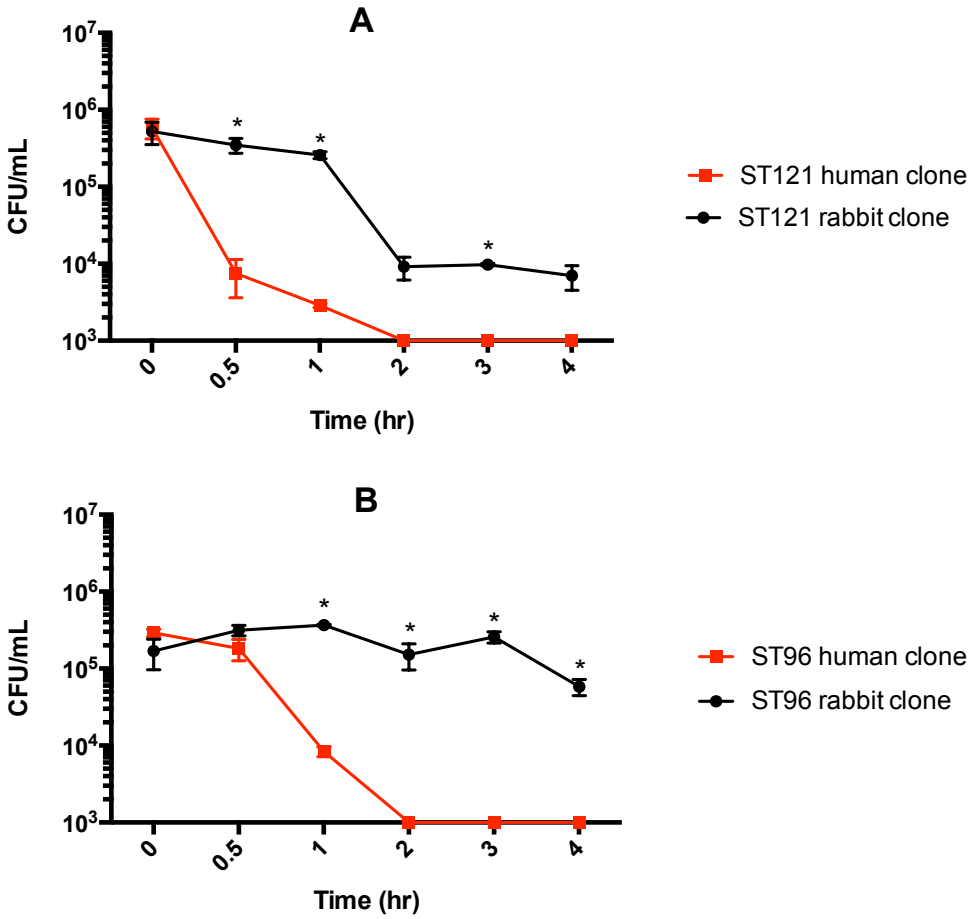


Figure 3.16 Effect of NRS on rabbit and human *S. aureus* isolates

Aliquots of rabbit serum were inoculated with **A)** 10^6 cell/ml of ST121 clones and **B)** 10^5 cells/ml of ST96 clones and killing assays were carried out. Rabbit isolates are shown as black lines and human isolates red lines. These experiments were carried out in triplicate (i.e. biological triplicates) and significant differences are shown with asterisks ($p<0.05$). Error bars represent the standard deviation of the means.

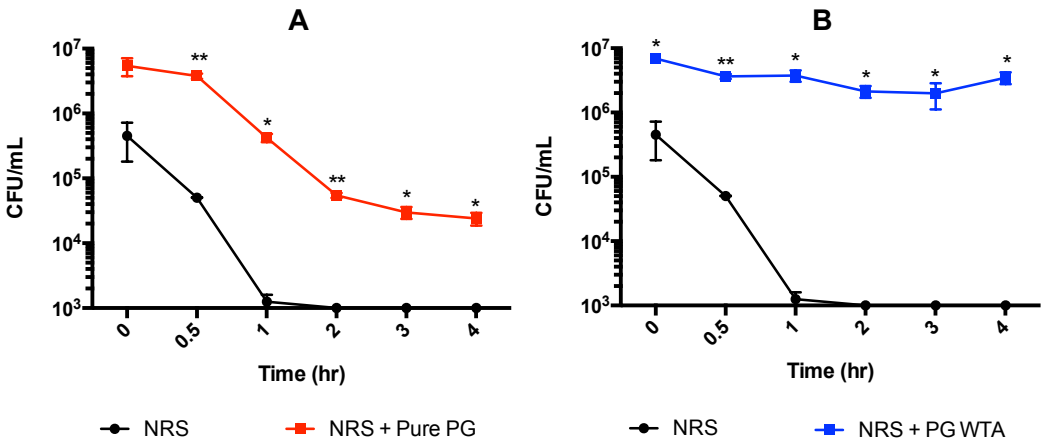


Figure 3.17 Effect of pre-incubation of NRS with cell wall material on its bactericidal activity

Aliquots of NRS were incubated for 30 minutes at 4ºC with 1mg/ml of stripped peptidoglycan [Pure PG] and unstripped peptidoglycan [PG+WTA] after which they were centrifuged. The insoluble material was washed and stored at -20ºC, whilst the supernatant was used for killing assays as described before (Chapter 2.9). **A)** Pure PG and **B)** PG+WTA pre-treated serum was inoculated with 10^6 cells/ml of an exponential growth phase culture of SH1000 and killing assays were carried out as explained previously. The experiments were carried out in triplicate (i.e. biological triplicates) and the error bars correspond to the standard deviation of the means. Significant differences are shown with asterisks ($p<0.05$).

3.2.13 Effect of pre-incubation of rabbit serum with purified wall teichoic acid in its bactericidal activity against *S. aureus*

The previous results gave more evidence in the role of wall teichoic acids as the receptor for the killing activity of the rabbit serum. To confirm this role, the ability of pure wall teichoic acids to deplete the antibacterial activity of the rabbit serum was tested. In order to do this wall teichoic acid from the SH1000 wild type strain of *S. aureus* was purified as described in Chapter 2.17. The purity of the WTA was tested for lack of proteins and exopolysaccaride by protein gel stained by silver stain and Alcian blue, respectively.

To test the effect of this purified cell wall material, aliquots of rabbit serum were mixed with 1 mg/ml of purified wall teichoic acid. The samples were incubated for 30 minutes at 4ºC and the tubes were then centrifuged. The purified wall teichoic acid is soluble so there was no 'pellet' after the centrifugation step. The supernatant was collected very carefully and used for killing assays as described before (Chapter 2.9).

When the bactericidal activity against *S. aureus* of this pre-treated serum was compared to the activity of untreated naive rabbit serum, a partial inactivation of this activity could be observed (Figure 3.18). This partial inactivation can be compared to the inactivation obtained by using pure peptidoglycan on the rabbit serum (Figure 3.17A), while the total inactivation of the antibacterial activity of the serum obtained after the treatment of peptidoglycan with wall teichoic acid (PG+WTA) suggests that the WTA needs to be bound to peptidoglycan in order to work as the receptor for the killing elements present in the rabbit serum.

3.2.14 Purification of cell wall material from different *S. aureus* mutant strains and its effect on the antibacterial activity of NRS

After establishing the role of the wall teichoic acids as the receptor for the killing elements present in the rabbit serum, the effect of other types of purified cell

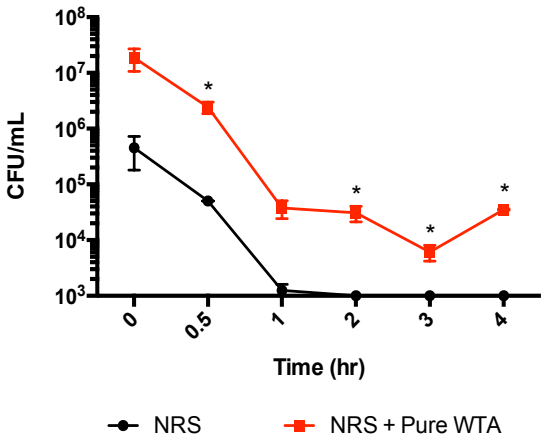


Figure 3.18 Effect of purified WTA in rabbit serum bactericidal activity

An aliquot of rabbit serum was pre-incubated with 1 mg/ml of purified wall teichoic acid for 30 minutes at 4ºC, after which the mixture was centrifuged and the supernatant was used for killing assays as previously described using an initial inoculum of 10^6 cells/ml of *S. aureus* SH1000. The antibacterial activity of untreated rabbit serum (black line) and treated rabbit serum (red line) were compared. These experiments were carried out in triplicate (i.e. biological triplicates). Student's t-tests were carried out and no significant differences were found ($p>0.05$). Error bars correspond to the standard deviation of the means.

wall material was tested. By purifying peptidoglycan from the different mutant strains the role of cell wall components could be studied.

As shown in Figure 3.6 of this chapter the Δ*tagO* strain, which lacks wall teichoic acids, is resistant to the killing activity of the rabbit serum. This suggests that the killing element present in the serum cannot bind to these bacteria without wall teichoic acid in their cell wall. The peptidoglycan from the Δ*tagO* strain was purified following the protocol explained in Chapter 2.16.

As shown in Figure 3.8, the Δ*dltA* strain, which lacks the D-alanylation modification in the teichoic acids, was highly sensitive to the bactericidal activity of the rabbit serum. This suggests that the lack of the D- alanylation modification in the teichoic acid increases the affinity of the 'killing factor' from the rabbit serum for the bacteria, allowing the serum to kill the bacterial cells faster. Hence, a complete inactivation of the bactericidal activity of the rabbit serum would expected after the pre-incubation of the serum with this type of purified cell wall material.

Cell wall material from the Δ*tagO* and Δ*dltA* mutant strains was purified and it was used for the inactivation of the rabbit serum killing activity (Chapter 2.16.2). Rabbit serum was incubated for 30 minutes with 1 mg/ml of the cell wall material, after which the mixture was centrifuged. The insoluble material was washed with sterile MilliQ water and stored at -20ºC until used, while the supernatant was used for killing assays.

The pre-incubation of rabbit serum with cell wall from the Δ*tagO* mutant had no effect on the bactericidal activity of the serum when compared to untreated rabbit serum (Figure 3.19A), showing that the killing factor present in the rabbit serum is not able to effectively bind to this peptidoglycan. Pre-incubation with cell wall material from the Δ*dltA* mutant leads to the complete loss of the killing activity of the rabbit serum (Figure 3.19B). The level of inactivation of the antibacterial activity is higher in this case than when using cell wall with wall teichoic acid from the parental strain (Figure 3.17B), confirming that the lack of

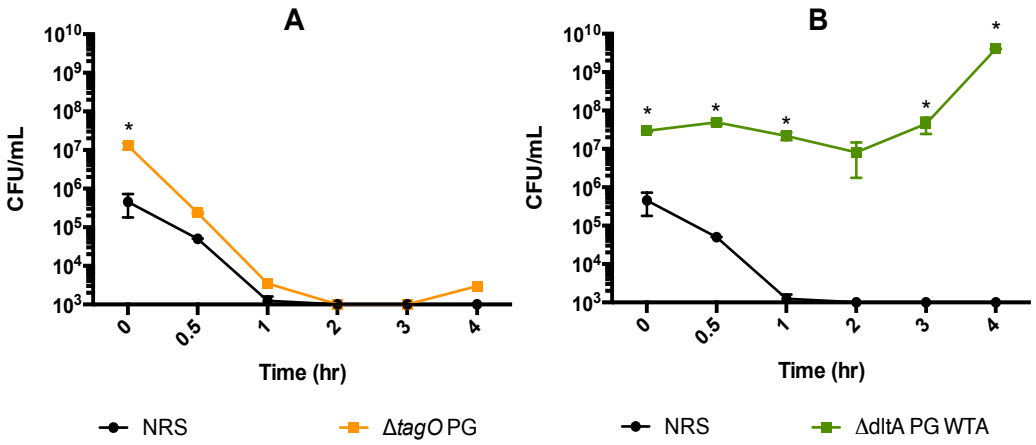


Figure 3.19 Pairwise comparison of the effect of pre-incubation of NRS with purified cell wall fragments from different *S. aureus* strains

NRS was incubated with purified peptidoglycan from the Δ*tagO* and Δ*dltA* mutants for 30 minutes at 4ºC before being centrifuged. The insoluble material was washed and stored at -20ºC for further use, while the supernatant was used for killing assays with an initial inoculum 10^6 cells/ml of an exponential growth phase culture of SH1000. The experiments with **A)** Δ*tagO* and **B)** Δ*dltA* purified cell wall material were carried out in triplicate (i.e. biological triplicates), showing significant differences with asterisks ($p<0.05$). The error bars correspond to the standard deviation of the means.

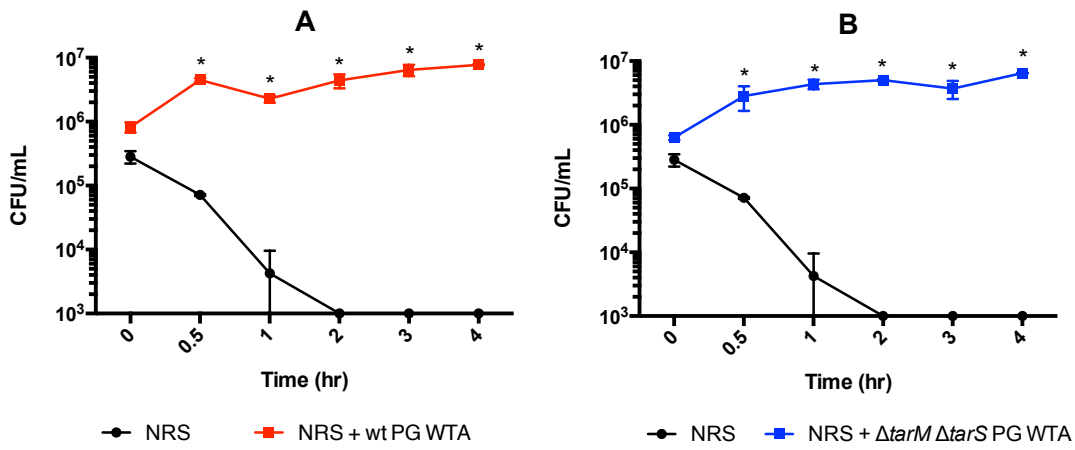


Figure 3.20 Role of WTA glycosylation on the effect of pre-incubation of NRS with purified cell wall material

Killing assays were carried out using the supernatant of rabbit serum incubated with purified **A)** peptidoglycan with wall teichoic acid from the parental strain and **B)** peptidoglycan with wall teichoic acid from the Δ*tarM* Δ*tarS* strain as explained previously. The insoluble material was washed and stored until further use at -20ºC. These experiments were carried out in triplicate (i.e. biological triplicates) and error bars represent the standard deviation of the means. Significant differences are shown with asterisks (Student's t-test, $p<0.05$).

D-alanylation leads to an increased affinity for the killing factor(s) present in the rabbit serum. A 100-fold increase in one hour in the bacterial numbers was observed for the bacteria incubated in NRS treated with this purified cell wall material. This could be due to a mistake during the dilution process, as the cell doubling time cannot explain this.

Cell wall from RN4220 Δ*tarM* Δ*tarS* was also purified following this protocol, as well as cell wall material from RN4220. Purified cell wall material from both strains was able to completely inactivate the serum when compared to untreated rabbit serum (Figure 3.20A and B, respectively). This suggests that glycosylation of the teichoic acids has no effect on the bactericidal activity of the rabbit serum (Figure 3.10).

3.2.15 Effect of pre-incubation of naive rabbit serum with purified lipoteichoic acid on its antibacterial activity against *S. aureus*

To confirm that lipoteichoic acids had no effect over the bactericidal activity of the rabbit serum, LTA was purified from *S. aureus* using 1-butanol extraction followed by hydrophobic interaction chromatography as explained in Chapter 2.18. The presence of lipoteichoic acids after the purification was verified using specific monoclonal antibodies against LTA (Figure 3.21).

Just as expected from the results obtained using the LTA- deficient mutant strain (Figure 3.7A), the pre-incubation of rabbit serum with purified lipoteichoic acids had no significant effect on the bactericidal activity of rabbit serum against *S. aureus* when compared to untreated rabbit serum (Figure 3.22).

3.2.16 Comparison of the wall teichoic and lipoteichoic acid D-alanine content

The D-alanine content of different types of purified cell wall was measured in order to verify the importance of this modification in the bactericidal activity of

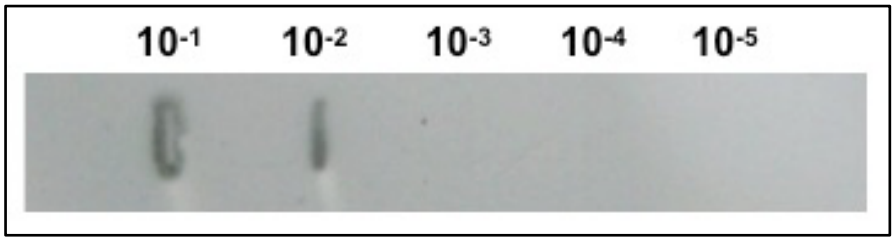


Figure 3.21 Reactivity of purified LTA with specific antibodies

Lipoteichoic acid was purified as explained previously (Chapter 2.18), diluted 10-fold using sterile PBS and 200 µl of each sample was loaded into each well. The number on top of the figure shows the dilution loaded in each well. The membrane was incubated with blocking buffer for 60 minutes, washed and then incubated for 60 minutes with blocking buffer containing the primary antibody at 1:1,000 dilution (mouse monoclonal anti-LTA antibody). The membrane was then incubated with the secondary antibody (1:10,000 dilution) for 60 minutes, washed and developed using the SuperSignal® West Pico Enhanced Chemilunminescent Kit. The membrane was developed for 30 minutes.

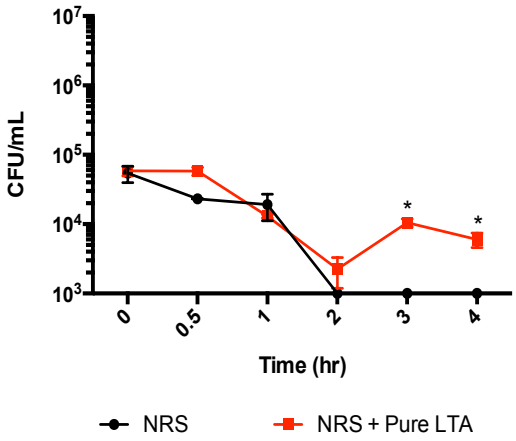


Figure 3.22 Effect of purified LTA on rabbit serum bactericidal activity

Lipoteichoic acids were purified from *S. aureus* SH1000 using 1-butanol extraction followed by hydrophobic interaction chromatography (Grundling and Schneewind, 2007a). Aliquots of rabbit serum were incubated with 1mg/ml of this purified material and killing assays were carried out using the supernatant after centrifugation and 10^5 cells/ml of an exponential growth phase culture of SH1000. These experiments were carried out in triplicate (i.e. biological triplicates). The error bars represent the standard deviation of the means. No significant differences were found ($p>0.05$).

the rabbit serum. These experiments were carried out using purified wall teichoic and lipoteichoic acid from different *S. aureus* strains, which were purified as explained in Chapter 2.17 and 2.18, respectively.

First, the D-alanine content of different components of the cell wall of SH1000 was determined. As expected, the D-alanine content detected for 1 mg/ml of peptidoglycan with wall teichoic acids and for 1 mg/ml of pure wall teichoic acid was similar, while no D-alanine esters were detected in the sample where WTA has been removed. No significant difference was found between the D-alanine content of the purified lipoteichoic acid when compared to the content of purified wall teichoic acid (Table 3.2). These values match previously reported data (Jenni and Berger-Bächi, 1998; Giaouris et al, 2008). D-alanine in peptidoglycan did not contribute to this assay as it was removed by centrifugation after trichloroacetic acid extraction.

Next, the D-alanine content was measured for different strains of the rabbit and human isolates studied in Chapter 3.2.11. Mutant strains for the *dltB* gene were chosen for both isolates. For the rabbit isolates, CH 3934 (parent strain), CH 9982 (Δ*dltB* strain) and CH 9548 (Δ*dltB* strain with human DltB protein) were studied, while CH 7705 (parent strain), CH 9692 (*dltB* *405Y) and CH 10310 (*dltB* T113K, Y250H, *405Y) were studied for the human ST121 isolate. Wall teichoic and lipoteichoic acids were purified as explained previously (Chapter 2.17.1 and 2.18.1, respectively) for each of these six strains and the D-alanine content was determined using the same protocol as with the SH1000 strain.

Significant differences ($p<0.05$) were found in the D-alanine content of the purified wall teichoic acid from the wild type and mutant strains for both the rabbit and human isolates (Table 3.3A), with 88% decrease in the D-alanine content between CH 3934 and CH 9982 (34±9.4 µg/ml and 4.1±0.2 µg/ml, respectively) and 78% decrease between CH 7705 and CH 10310 (24±0.1 µg/ml and 7.6±1.0 µg/ml, respectively). However, no significant differences were found in the D-alanine levels of the lipoteichoic acid between the parental and mutant strains (Table 3.3B).

Table 3.2 D-alanine content of cell wall material from *S. aureus* SH1000

The D-alanine content of different purified cell wall fractions from SH1000 was measured using a modified protocol from Peschel *et al*, 1999. These experiments were carried out in duplicate and the values shown here were obtained using the equation from the calibration curve (Figure 2.1). (-): No significant values.

Cell wall purification	D-alanine content (µg/ml)
PG+WTA	34
Pure PG	0.0
Pure WTA	50
Pure LTA	47

Table 3.3 D-alanine content of teichoic acids from rabbit and human strains of *S. aureus*.

D-alanine content of **A)** purified wall teichoic acid and **B)** purified lipoteichoic acid. The experiments were carried out on triplicate and these values were obtained using the equation from the calibration curve (Figure 2.1). ANOVA tests were carried out using Bonferroni adjustment as shown in (Viana et al, 2015)

A

Strain	Genotype	D-alanine (µg/ml)	% compared to parent
CH 3934	ST121 rabbit, parent strain	34 ± 9.4	-
CH 9982	ST121 rabbit, Δ*dltB*	4.1 ± 0.02	12.05
CH 9548	ST121 rabbit, human DltB	21 ± 0.8	61.70
CH 7705	ST121 human, parent strain	24 ± 0.1	-
CH 9692	ST121 human, DltB *405Y	9.0 ± 0.6	42.80
CH 10310	ST121 human, DltB 3 SNPs	7.6 ± 1.0	22.35

B

Strain	Genotype	D-alanine (µg/ml)	% compared to parent
CH 3934	ST121 rabbit, parent strain	52 ± 1.3	-
CH 9982	ST121 rabbit, Δ*dltB*	42 ± 2.6	80.82
CH 9548	ST121 rabbit, human DltB	55 ± 5.4	105.31
CH 7705	ST121 human, parent strain	44 ± 3.2	-
CH 9692	ST121 human, DltB *405Y	29 ± 8.3	66.01
CH 10310	ST121 human, DltB 3 SNPs	52 ± 2.3	118.91

3.3 Discussion

Previous work carried out in the laboratory showed that naive rabbit serum (NRS) has antibacterial activity against *S. aureus* (Bozakouk, 2011), however the mechanism involved in this bacterial killing remained unknown. The aim of this project is to identify not only the element(s) of the rabbit serum that are responsible for this bactericidal activity against *S. aureus*, but also the bacterial components that play a role as receptors for this killing mechanism.

The innate immune system of vertebrates is able to identify pathogens from self-cells through the recognition of evolutionary conserved molecules produced only by pathogens, called 'pathogen-associated molecular patterns' (PAMPs) (Medzhitov and Janeway, 2002; Vivier and Malissen, 2005). The host immune system is able to identify these PAMPs not only as signs of infection but also as 'molecular markers' specific for different types of pathogens, allowing the immune system to choose the most efficient defence mechanism (Medzhitov and Janeway, 2000).

Due to the conservation of its composition and the essential role that plays in the bacteria (Dziarski and Gupta, 2005), several pathogen-recognition molecules can detect peptidoglycan, including a specific group of proteins called peptidoglycan-recognition proteins (PGRPs) (Michel et al, 2001; Guan and Mariuzza, 2007). The teichoic acids found in the cell wall of Gram-positive bacteria can also be recognized by the immune system of the host (Cleveland et al, 1996; Atilano et al, 2011).

It is known that the interaction between elements of the bacterial cell wall and the immune system of the host is essential for the recognition and clearance of pathogens (Cleveland et al, 1996; Janeway and Medzhitov, 2002). Therefore, the bacterial component that acts as a receptor for the antibacterial activity of the naive rabbit serum is likely to be a surface component present in the *S. aureus* cell wall.

Previous work carried out in the laboratory identified wall teichoic acid (WTA) as a candidate for this role as the bactericidal activity receptor (Bozakouk, 2011). Wall teichoic acids play a role in the resistance of the bacteria to the host immune system (Kohler et al, 2009; Carvalho et al, 2015) and also in colonization (Weidenmaier et al, 2004; Weidenmaier et al, 2005b; Bera et al, 2007; Weidenmaier et al, 2008). TagO is the first protein involved in the biosynthesis of wall teichoic acids in *S. aureus* (Figure 1.6) (Neuhaus and Baddiley, 2003; Brown et al, 2008; Pereira et al, 2008; Xia et al, 2010a) and a deletion in the corresponding gene results in the complete absence of wall teichoic acids from the bacterial cell wall (Weidenmaier et al, 2005b; Vergara-Irigaray et al, 2008). A Δ*tagO* strain was resistant to the bactericidal activity of the NRS, whilst a decrese in the bacterial count under the detection limit was found for the parent (Figure 3.6). This suggested that the lack of wall teichoic acids in the bacterial cell wall allowed the bacteria to survive in the serum because the 'killing factor' present in the rabbit serum was not able to interact with the bacteria or it was not able to reach its final target. Cell wall material purified from Δ*tagO* had no effect on the bactericidal activity of the NRS (Figure 3.19A). As no WTA is present in this material the killing component(s) present in the rabbit serum was not able to bind to this affinity matrix. This confirmed the role of WTA as receptor for the killing activity of the rabbit serum.

This essential role of WTA was verified by the complete inactivation of the bactericidal activity caused by peptidoglycan with wall teichoic acids, whilst peptidoglycan stripped of WTA was able to only partially inactivate NRS (Figure 3.17). Overall this strogly suggested that WTA is the receptor for the 'killing factor' in the NRS.

The role of lipoteichoic acid (LTA), the other teichoic acid present in *S. aureus*, as a potential receptor for the NRS bactericidal activity was studied. As LTA is essential for the bacteria it was impossible to create an LTA-deficient mutant in an unsuppressed background. However, the deletion of the *ypfP* gene allowed the creation of a mutant strain with only 13% of LTA content when compared to the parental strain (Fedtke et al, 2007). The YpfP protein mediates the

synthesis the anchor to which the poly-[glycerophosphate] repeating unit is attached during the synthesis of LTA in *S. aureus* (Jorasch et al, 2000).

The Δ*ypfP* strain was affected by the NRS immediately after inoculation as no colonies were obtained in any of the time points studied, while decrease of colony forming units under the detection limit for the parent strain was observed only after two hours of incubation (Figure 3.7A). This suggests that the mutant was strongly affected by the bactericidal elements of the rabbit serum from the beginning of the experiment. Immediate bacterial death after inoculation was also observed when the mutant was incubated with heat-inactivated rabbit serum (Figure 3.7B), suggesting that the LTA-deficient mutant is sensitive to other elements of the rabbit serum that are not affected by the heat-inactivation. Consequently, it was not possible to determine if the LTA had a function in this heat sensitive NRS. The Δ*ypfP* mutant was also partially susceptible to human serum compared to the parent (Figure 3.7C), demonstrating its overall sensitivity. Also pre-incubation of purified LTA with NRS had no effect on the antibacterial activity (Figure 3.22). Thus it is unlikely that LTA is a major requirement for the bactericidal mechanism of NRS.

After determination of the role of wall teichoic acid as the component of the bacterial cell wall that interacts with the 'killing factor' present in the rabbit serum, the different modifications of this molecule were studied. Once the biosynthesis of this polymer is complete, wall teichoic acids can be modified by D-alanylation or glycosylation (Perego et al, 1995; Clemans et al, 1999; May et al, 2005; Xia et al, 2010a; Sobhanifar et al, 2015). Killing assays to test the bactericidal effect of the rabbit serum on different mutant strains for the D-alanylation (Δ*dltA*) and glycosylation (Δ*tarM*, Δ*tarS* and Δ*tarM* Δ*tarS*) were carried out, and peptidoglycan from the Δ*dltA* strain and the Δ*tarM* Δ*tarS* double mutant was purified to test its effect on the bactericidal activity of the NRS.

The Δ*dltA* strain was more sensitive to the bactericidal effect of the rabbit serum than the parent strain from the beginning of the experiment and its bacterial counts was lower than de detection limit after 30 minutes of incubation (Figure 3.8). As the addition of the D-alanine esters to the teichoic acids changes the

net charge of the cell surface, the stronger effect of the bactericidal activity of the rabbit serum on the Δ*dltA* strain could be explained as a higher affinity of the 'killing factor' for the cell wall of the mutant. This higher affinity of the 'killing factor' was confirmed by the complete inactivation of all the bactericidal activity of the rabbit serum after 30 minutes of pre-incubation with purified cell wall from the Δ*dltA* strain (Figure 3.19B). This suggested that the interaction between the 'killing factor' and *S. aureus* might be electrostatic and that the D-alanylation of the teichoic acids is important for the survival of the bacteria in NRS.

Conversely, deletion of the α- and β-glycosylation had no effect on the bactericidal activity of NRS (Figure 3.11). However, some differences were observed between the single and the double mutant strain in diluted serum (Figure 3.11), suggesting that its role in the bactericidal activity of NRS is not completely understood. Also pre-incubation of NRS with cell wall material from the Δ*tarM* Δ*tarS* strain had the same effect as that from the parent (Figure 3.20). All of this suggests that glycosylation of WTA has no impact on the NRS killing mechanism.

The MprF protein is a virulence factor of *S. aureus* that is able to add a L-lysine residue to membrane lipids (Peschel et al, 2001; Weidenmaier et al, 2005a), introducing positive charges. Whilst no differences in the effect of undiluted NRS on a Δ*mprF* mutant, its complemented strain and the parental strain were found (Figure 3.12), the differences observed in the effect of 3-fold diluted serum between the strains with and without the modification showed a trend (Figure 3.13). This might indicate that whilst it has been reported that this modification affects the response of the bacteria to some elements of the immune system of the host, the addition of this amino acid is not as important as the addition of D-alanines to the teichoic acids in this killing mechanism of the NRS.

The adaptation of *S. aureus* to humans as hosts is demonstrated by many the different strategies including facilitation of the adherence to host tissue (Tompkins et al, 1990; van Belkum et al, 2009), evasion of the host immune system and nutrient acquisition (Torres et al, 2006; Pishchany et al, 2010; Pan

et al, 2012). However, *S. aureus* is also associated with infections in economically important livestock such as cows, sheep and rabbits (Viana et al, 2015).

S. aureus clonal complex CC121 (clone ST121) can cause skin infections and necrotizing pneumonia in humans and recently a highly virulent ST121 capable to infect rabbits has emerged (Vancraeynest et al, 2006; Viana et al, 2015). This host adaptation is caused by a single mutation in the *dltB* gene that is sufficient and required to turn this human strain (which is unable to infect rabbits) into a strain that causes epidemics in rabbit farms (Viana et al, 2015). Three other single mutations are necessary to infect the skin and to enhance the infectivity, two of which are in the *dltB* gene. It was hypothesized that the mutations present in the *dltB* gene might have an effect on the interaction of the bacteria with the host immune system. This hypothesis led us to study these rabbit and human strains by *in vitro* killing assays using rabbit serum.

Whilst the human isolates were susceptible to the bactericidal activity of the NRS, the rabbit isolates were able to survive in the rabbit serum (Figure 3.16), confirming that the adaptation of the rabbit strains to the host involves resistance to the killing mechanism that is being studied in this project.

Although the rabbit serum had bactericidal activity against the different mutant rabbit strains (Figure 3.14), this effect was observed as a 100-fold decrease in the bacterial numbers instead of complete bacterial death. The Δ*dltB* rabbit strain, however, was extremely sensitive to the rabbit serum and was completely killed after just 30 minutes of incubation (Figure 3.14A), just as the Δ*dltA* strain, confirming the importance of this modification in the survival of the bacteria.

On the other hand, most of the human isolates were completely killed by the rabbit serum (Figure 3.15). Only two human strains, both with mutations in the *rot* (K103*) and *dltB* (*405Y), were able to survive in the rabbit serum (Figure 3.15C [CH 9700] and D [CH 9897]). These mutations allow human isolates to infect as the rabbit isolates do (Viana et al, 2015), suggesting that the mutations

are not only involved in the infectivity of the bacteria but in survival in the rabbit serum, where they are in contact with all the components of the immune system the host.

The D-alanine content was measured for WTA and LTA purified from rabbit and human isolates with different mutations in the *dltB* gene. No significant difference was found for the D-alanine content in LTA (Table 3.3B), while significant differences between the mutant and parental strains were found for D-alanine content of WTA (Table 3.3A). From these results we can propose that whilst the lipoteichoic acids are not involved in the bactericidal mechanism of the rabbit serum, the wall teichoic acids seem to be involved in the bactericidal mechanism as both the receptor that interacts with the 'killing factor' and through the presence of the D-alanine esters.

4 CHAPTER 4:

Identification of naive rabbit serum components responsible for antibacterial activity

4.1 Introduction

In Chapter 3 wall teichoic acid was identified as the likely bacterial component that acts as the receptor for the bactericidal activity of the rabbit serum. The importance of the D-alanylation of teichoic acids was also shown, suggesting that the first interaction between the bacteria and the elements of the serum responsible for this killing might be of an electrostatic nature.

In order to understand the bactericidal mechanism, the serum elements responsible for it must to be identified. This mechanism is independent of the cellular components of the immune system due to the use of 0.2μm pore size filters during the purification process of the rabbit serum. All of the cells present in the mammalian blood are bigger than the pore size of the filters used, resulting in sterilized serum without cellular components. This suggests that killing mechanism of the NRS is not cell mediated.

Immunoglobulins are proteins produced and secreted by differentiated B cells (Parkin and Cohen, 2001). Immunoglobulins are able to recognize specific parts of an antigen, allowing binding. This antigen-antibody binding is known as opsonisation and targets the antigen containing structure for phagocytosis (Chaplin, 2010; Kumar and Sharma, 2010). The immunoglobulins are also involved in the activation of the complement system through the classical pathway. This pathway is activated by soluble antigen-antibody complexes or by antibodies bound to bacterial cells (Gasque, 2004). The complement component C1q recognizes these antigen-antibody complexes, which leads to the formation of the C1 complex and further activation of the classical pathway (Ricklin et al, 2010). It has been reported that immunoglobulins also participate in activation of the lectin pathway (Roos et al, 2010). However, previous results

obtained in our laboratory established that the NRS bactericidal mechanism is immunoglobulin-independent (Bozakouk, 2011).

'Pathogen-associated molecular pattern' (PAMPs) molecules, such as lipopolysaccharide and peptidoglycan, are molecules conserved and shared by many infectious agents that the host is able to recognize by using specific pattern recognition receptors (Beutler, 2004; Ausubel, 2005; Akira et al, 2006). Some of these pattern recognition receptors are soluble proteins, such as C-reactive protein, collectins and mannose-binding lectin (Matsushita and Fujita, 1992; Beutler, 2004).

Mammalian cells also produce soluble components that are able to kill pathogens. For example, antimicrobial peptides, cationic peptides that can interact with the bacterial membrane (Zasloff, 2002; Beutler, 2004; Hancock and Sahl, 2006), cause bacterial death by inhibition of extracellular polymer synthesis or intracellular functions, or by disruption of the integrity of the cell membrane (Ong et al, 2002; Yeaman and Yount, 2003; Hancock and Sahl, 2006).

The complement system, formed by serum and cell membrane proteins, is essential for the innate immune response against bacterial infecions. It is able to recognize and kill pathogens (Gasque, 2004) and enhances the B and T responses (Barrington et al, 2001; Carroll et al, 2004; Gál et al, 2007; Ansari and Sayegh, 2008). While it is known that the membrane attack complex is able to kill Gram-negative bacteria through the formation of pores on the bacterial membrane, which leads to lysis (Taylor, 1983; Frank et al, 1987), Gram-positive bacteria is not affected by the membrane attack complex due to the presence of the thick layer of peptidoglycan of the cell wall (Joiner et al, 1984).

However, recent papers show that several components of the complement system can interact with Gram-positive bacteria. It has been reported that mannose-binding lectin can bind to the surface of *S. aureus* and lead to the activation of the lectin pathway (Ma et al, 2004; Park et al, 2010). Lipoteichoic acids are able to activate the lectin pathway through binding with L-ficolin

(Lynch et al, 2004), and the C5b-9 complex can be found on the surface of several Gram-positive bacteria including *S. aureus* (Berends et al, 2013).

Up to this point, results have shown that the bactericidal activity of the NRS against the bacteria must be caused by a soluble component of the immune system that is able to interact with the wall teichoic acids present in the cell wall. Taking into consideration the results obtained previously in the laboratory, which showed that complement components, mannose-binding lectin and C4b, bound to purified cell wall from *S. aureus*, it was decided to start the identification of the rabbit serum elements by determining the role of the complement system in the bactericidal activity against *S. aureus*.

4.1.1 Aims of this chapter

- Analysis of the role of the complement system, especially of the lectin pathway, in the bactericidal mechanism of the rabbit serum.
- Identification of elements of the NRS that are able to interact with *S. aureus* purified cell wall material by mass spectrometry.
- Study of the role of the identified components in the antibacterial mechanism of the NRS against *S. aureus*.

4.2 Results

4.2.1 Inactivation of the bactericidal activity of NRS by ethyleneglycoltetraacetic acid (EGTA) treatment

Previous experiments carried out in the laboratory suggested that mannose-binding lectin and the complement component C4b might be involved in the killing mechanism of the rabbit serum against *S. aureus* (Bozakouk, 2011). As demonstrated in Chapter 3, heating the serum at 56ºC for 30 minutes inactivates the antibacterial activity. It is known that the complement components C2 and Factor B are affected by heat (Ecker et al, 1943a; Kerr and Porter, 1978; Servais et al, 1991; Morgan, 2000). These results suggested that the lectin pathway of complement activation might be involved in the bactericidal mechanism.

To confirm this involvement of the lectin pathway, several inactivation protocols were used. Ethyleneglycoltetraacetic acid (EGTA) is a chelating agent that has a 10^5-times higher affinity for calcium than for magnesium (Bryan, 1974), and it has been reported that pre-incubation of serum with this chelating agent inactivates its bactericidal activity by blocking the classical (Des Prez et al, 1975; Fine, 1978; Morgan, 2000) and the lectin (Schwaeble et al, 2002; Roos et al, 2003; Takahashi et al, 2006) pathways of complement activation.

To test if this chelating agent was able to inactivate the rabbit serum, aliquots of NRS were incubated with 10 mM of sterile EGTA for 60 minutes at 37ºC before the inoculation of 10^5 cell/ml of an exponential phase culture of *S. aureus* SH1000. After the inoculation of the bacteria into the serum killing assays were carried out (Chapter 2.10.2). *E. coli* MC1061 was used as a control for the inactivation of the complement system in the serum. Only 50μM EGTA was used instead as a higher concentration was found to be intrinsically inhibitory for *E. coli* (data not shown).

Even though the addition of EGTA reduced the bactericidal activity of the rabbit serum against both *S. aureus* and *E. coli* (Figures 4.1A and B), the inactivation

was not total when tested against the *E. coli* strain. This suggests that calcium is necessary for the activity of the rabbit serum components that are capable of killing *S. aureus*, whilst *E. coli* is affected by other calcium-independent elements also present in the rabbit serum.

4.2.2 Effect of EGTA-magnesium chloride and EGTA-magnesium chloride/calcium chloride on NRS antibacterial activity

After confirming that the components of NRS responsible for the bactericidal activity against *S. aureus* need calcium in order to be active, the reactivation of the killing activity was tested by adding magnesium and calcium ions into the solutions.

As explained previously, whilst the alternative pathway only needs magnesium to be active (for the assembly of the C3bFb complex) (Des Pres et al, 1975; Morgan, 2000), the classical and lectin pathways need magnesium (for the assembly of C4b2a complex) (Des Pres et al, 1975; Morgan, 2000) and calcium (for the C1 complex assembly and for MBL activity, respectively) (Platts-Mills and Ishizaka, 1974; Morgan, 2000; Schwaeble et al, 2002; Takahashi et al, 2006; Gál et al, 2009). Therefore, only the alternative pathway of complement activation is active when the rabbit serum is pre-incubated with equimolar concentrations of EGTA and magnesium chloride. The addition of calcium chloride to this mixture reactivates the classical and the lectin pathways.

In order to identify the complement pathway involved in the bactericidal activity of NRS, magnesium and magnesium with calcium were added to aliquots of EGTA-inactivated NRS as explained previously (Chapter 2.11). Magnesium chloride was added to test the effect of the alternative pathway, while magnesium chloride and calcium chloride was added to recover the activity of the classical and the lectin pathways of complement activation.

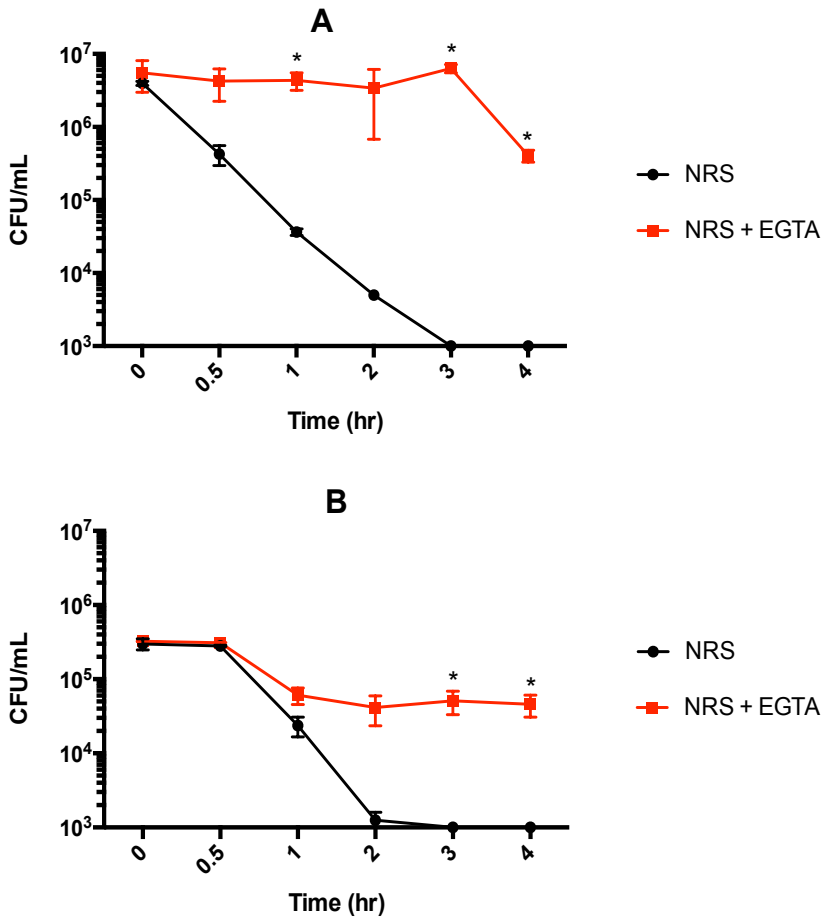


Figure 4.1 Effect of EGTA pre-incubation in the bactericidal activity of NRS

Aliquots of NRS were preincubated with EGTA for 60 minutes at 37ºC, and then inoculated with **A)** 10^7 cells/ml of *S. aureus* SH1000, or **B)** 10^5 cells/ml of *E. coli* MC1061. Samples were taken at appropriate time points, diluted on sterile PBS and spotted onto agar plates. The bactericidal activity of rabbit serum (black line) was compared with the activity of the serum treated with the chelating agent (red line). These experiments were carried out in triplicate (i.e. biological triplicates) and significant differences are shown with asterisks (Students t test, $p<0.05$). The error bars represent the standard deviation of the means.

As the three complement pathways can kill *E. coli*, this bacterium was used as a positive control to test if the addition of the divalent cations to the EGTA-inactivated NRS reactivated the killing activity. Just as expected, the addition of magnesium chloride is enough to reactivate the rabbit serum (Figure 4.2A) and similar results were obtained when magnesium and calcium ions were added into the serum in presence of EGTA (Figure 4.2B). The bactericidal activity of the serum reactivated by the addition of magnesium and of magnesium along with calcium has no significant difference compared to the bactericidal activity of the untreated serum (Figure 4.2C), confirming than the addition of these divalent cations counteract the chelating action of the EGTA.

After confirming the reactivation of bactericidal activity of the serum by the addition of the divalent cations by the use of *E. coli*, this killing activity was tested for *S. aureus*. Neither the addition of magnesium chloride or magnesium chloride along with calcium chloride resulted in a reactivation of the bactericidal activity of the NRS (Figure 4.3A and B, respectively). The addition of magnesium and calcium even caused bacterial growth. A significant difference in the bactericidal activity was observed between untreated and treated serum (Figure 4.3C). No reactivation of the bactericidal activity was observed when the concentration of divalent cations was doubled (data not shown), suggesting that the effect of EGTA on the mechanism involved in the killing of *S. aureus* is different than for *E. coli*.

4.2.3 Effect of zymosan A on the bactericidal activity of naive rabbit serum

The complement component C3 plays an important role in the complement system. The three complement pathways are activated by different signals (Beutler, 2004) but come together through the generation of the C3 convertases (C4b2a for the classical and lectin pathways, and C3bFb for the alternative pathway), which are able to cleave C3, generating C3a and C3b (Rooijakkers

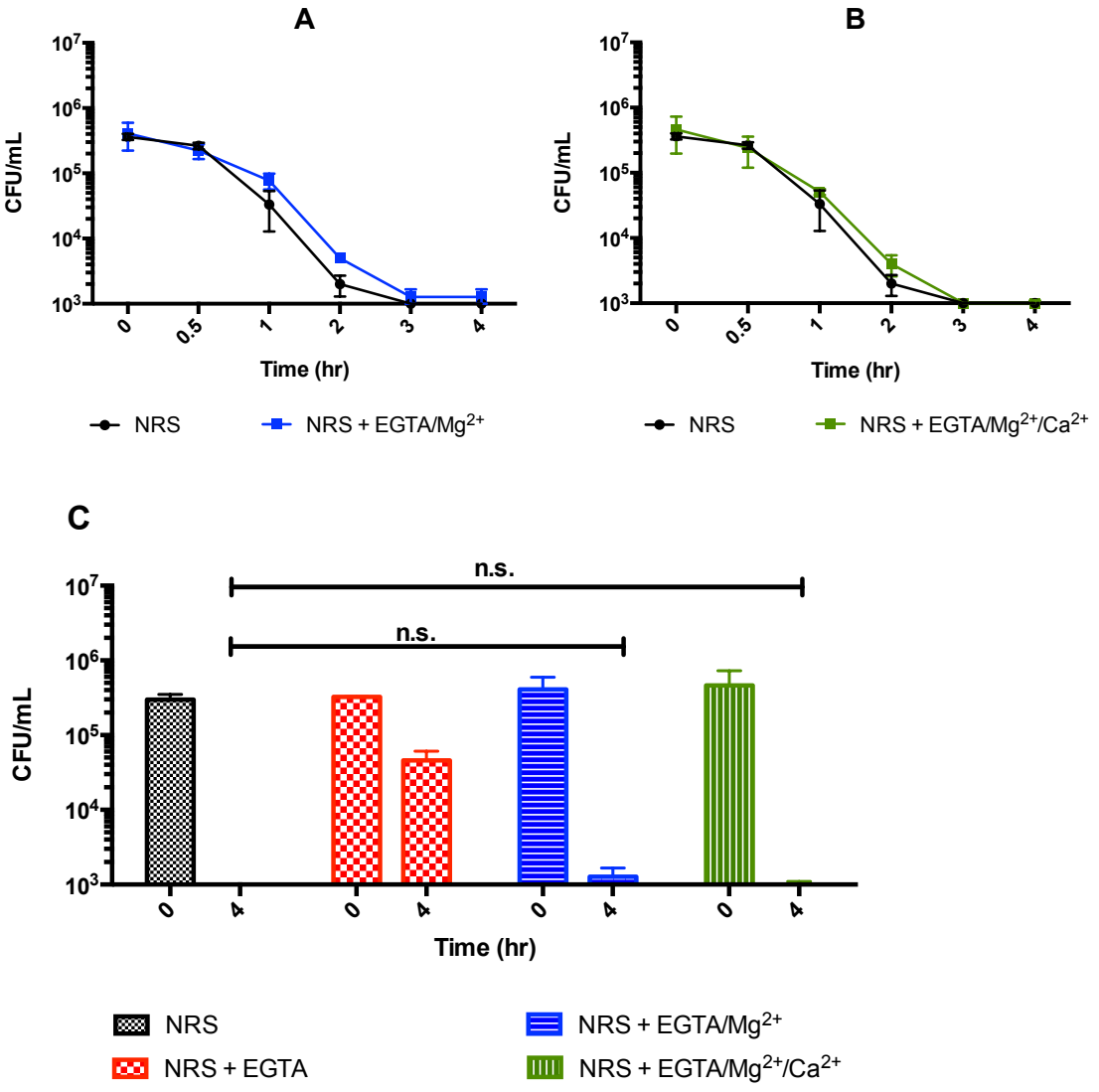


Figure 4.2 Effect of pre-incubation of NRS with EGTA-magnesium chloride and EGTA-magnesium chloride/calcium chloride on its bactericidal activity against *E. coli*

Aliquots of rabbit serum were pre-incubated with equimolar concentrations of **A)** EGTA and magnesium chloride or **B)** EGTA, magnesium chloride and calcium chloride for 60 minutes before inoculating this solution with an exponential growth phase culture of *E. coli* MC1061. **C)** Comparison of the bactericidal activities of untreated and treated rabbit serum against the bacteria. These experiments were carried out in triplicate (i.e. biological triplicates) and significant results are shown with asterisks (Students t test, $p<0.05$). Error bars correspond to the standard deviation of the means.

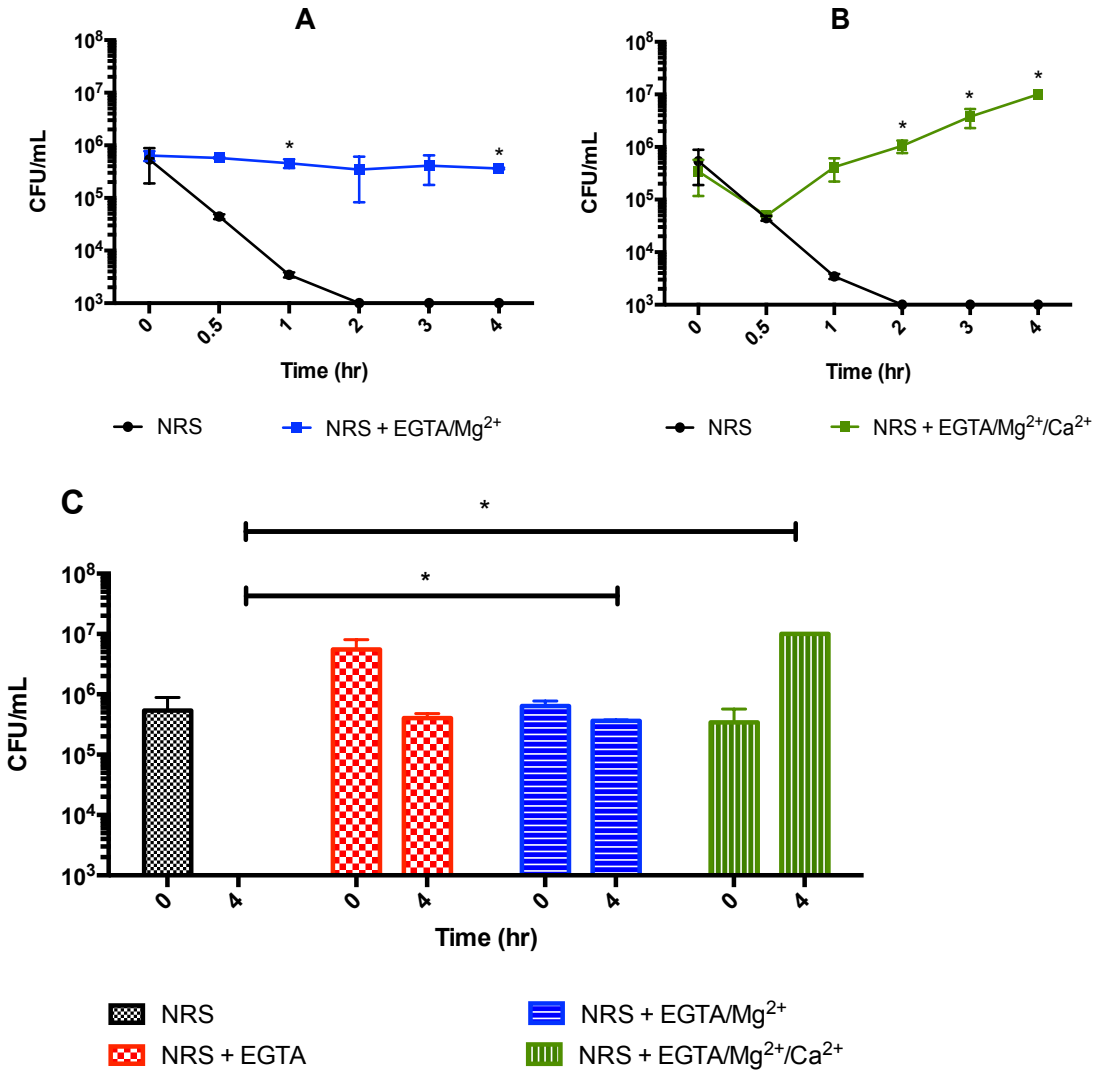


Figure 4.3 Effect of pre-incubation of NRS with EGTA-magnesium chloride and EGTA-magnesium chloride/calcium chloride on its bactericidal activity against *S. aureus*

Aliquots of rabbit serum were pre-incubated with 10 mM of **A)** EGTA and magnesium chloride or **B)** EGTA, magnesium chloride and calcium chloride for 60 minutes at 37ºC before inoculating this solution with 10^6 cell/ml of an exponential growth phase culture of *S. aureus* SH1000. **C)** Comparison of the bactericidal activities of untreated and treated rabbit serum against the bacteria. These experiments were carried out in triplicate (i.e. biological triplicates) and the error bars correspond to the standard deviation of the means. Significant results are shown with asterisks (Students t test, $p<0.05$).

and van Strijp, 2007; Ricklin et al, 2010; Laarman et al, 2011; Shishido et al, 2012).

The C3a fragment is a small cationic pro-inflammatory peptide (Klos et al, 2009) that also has potent antimicrobial activity by itself (Nordahl et al, 2004). The C3b fragment deposits on the bacterial surface and acts as a signal for phagocyte uptake by neutrophils and macrophages (Rooijakkers and van Strijp, 2007; Shishido et al, 2012). The opsonisation of the bacteria also leads to the amplification of cleavage of C3 via the activation of the alternative pathway (Rooijakkers et al, 2005c; Laarman et al, 2011) and the formation of the C5 convertase (Petersen et al, 2000; Ricklin et al, 2010).

Due to the important role of the complement component C3, it has been shown that its depletion leads to the complete inactivation of the alternative pathway and also affects the rest of the complement system (Des Prez et al, 1975; Smith et al, 1982; Rooijakkers et al, 2005c; Fritzinger et al 2009). It has been reported that the use of zymosan A, a polysaccharide from the cell wall of *S. cerevisiae* composed by glucan and mannan residues, can be used to efficiently deplete C3, partially C5 and other terminal complement components from the serum (Smith et al, 1982; Morgan, 2000; Zhang et al, 2003).

Thus, the effect of zymosan A on the bactericidal activity of rabbit serum against *S. aureus* was tested. As explained in Chapter 2.10.3, the zymosan A was activated before being used in the rabbit serum following instructions from the manufacturer. Once it was activated, an aliquot of rabbit serum was incubated with 1 mg/ml of zymosan A and incubated for 60 minutes at 37ºC. After the incubation this mixture was centrifuged and the supernatant was used for killing assays as explained previously (Chapter 2.9). As expected, the serum treated with zymosan A had no bactericidal activity against *S. aureus* SH1000 when compared to untreated serum (Figure 4.4).

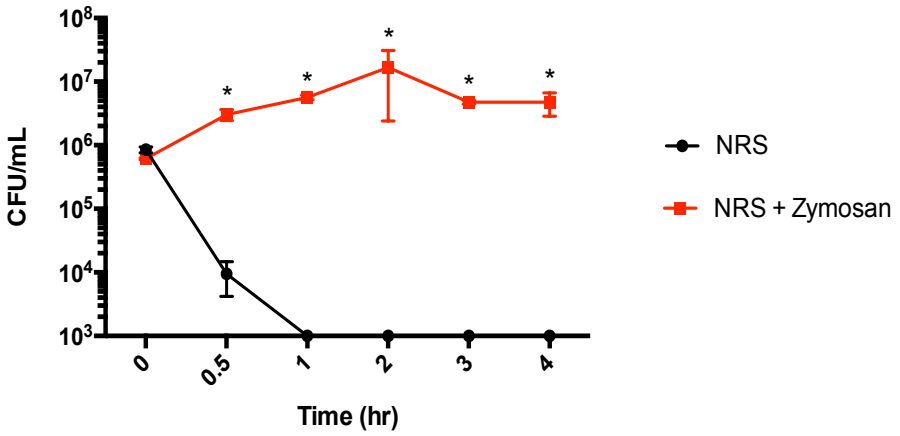


Figure 4.4 Effect of Zymosan A from *S. cerevisiae* pre-incubation on the antibacterial activity of rabbit serum

Activated zymosan A from *S. cerevisiae* was used to inactivate the killing activity of rabbit serum. Aliquots of NRS were treated with 1 mg/ml activated zymosan A and preincubated for 60 minutes, after which the mixture was centrifuged and the supernatant was used for killing assays. The bactericidal activity was tested on 10^6 cells/ml of *S. aureus* SH1000 and compared against the activity of untreated rabbit serum. The experiments were carried out in triplicate (i.e. biological triplicates). The error bars represent the standard deviation of the means and significant differences are shown with asterisks (Students T-test, $p<0.05$).

4.2.4 Effect of ammonium salts and ammonium hydroxide on the bactericidal activity of rabbit serum

The complement components C3 and C4 posses an intramolecular thioster group that is essential for their activity (Law et al, 1980; Law and Dodds, 1997; Wouter et al, 2005). When these components are cleaved they become active, leaving the reactive thioster group available to bind covalently to elements found on bacterial surfaces, such as carbohydrates (Law and Dodds, 1997; Wouters et al, 2005; Rooijakkers and van Strijp, 2007).

It has been known for many years that the addition of ammonia and other strong nucleophiles are able to inactivate the activity of these components (Gordon et al, 1926; Ecker et al, 1943a; Ecker et al, 1943b; Law et al, 1980; von Zabern et al, 1981; Morgan, 2000). The inactivation occurs because the ammonia is able to interact with the proteins, breaking the thioester group and causing a conformational change (Isenman et al, 1981; Pangburn, 1992; Law and Dodds, 1997). Even though this change can be reversed and the thioester reformed, the inactive form of C3 (C3b-like) can be cleaved by the complement components factor H and factor I (Pangburn, 1992).

Due to the important role of C3 and C4 in the formation of the C3 convertases (Beutler, 2004; Ricklin et al, 2010), the effect of incubation with ammonium solutions on the bactericidal activity of the NRS was tested. Aliquots of rabbit serum were mixed with 150 mM of the different solutions used and incubated at 37ºC, before adjusting the pH back to 7.4. The mixtures were then inoculated with *S. aureus* and killing assays were carried out as explained previously (Chapter 2.9). The effect of the ammonium solutions on bacterial growth was also tested and no significant effect was observed (data not shown).

As expected from literature, the preincubation of the rabbit serum with ammonia led to an inactivation of the bactericidal activity against *S. aureus* (Figure 4.5, all the panels). These results, along with the results from the experiments using heat, EGTA and zymosan A, suggested that the complement system might be

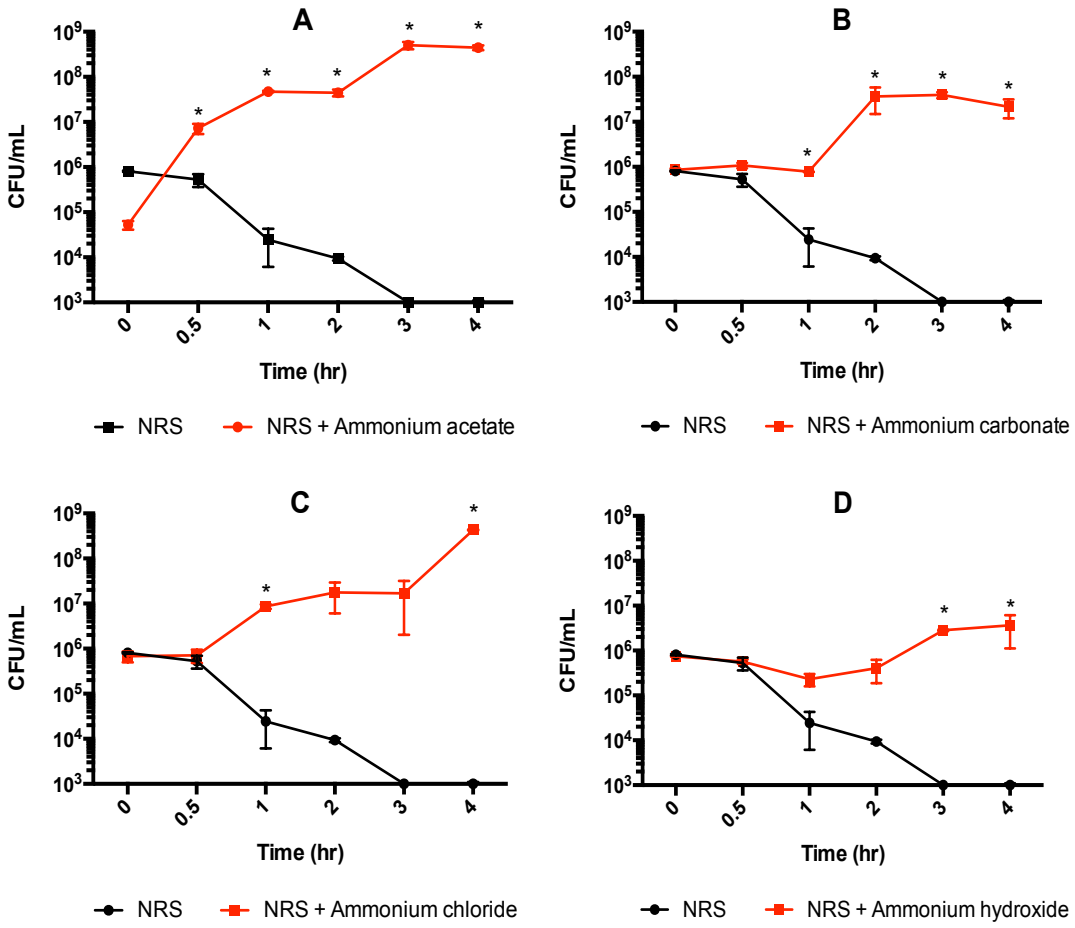


Figure 4.5 Effect of pre-incubation with ammonium compounds in the bactericidal activity of rabbit serum

The solutions used were prepared as explained in Chapter 2.10.4 of Materials and Methods. Aliquots of NRS were preincubated for 45 minutes at 37ºC with 150 mM of **A)** Ammonium acetate, **B)** Ammonium carbonate, **C)** Ammonium chloride, and **D)** Ammonium hydroxide, after which the pH was adjusted at 7.4. 10^5-10^6 cells/ml of *S. aureus* were then added. The experiments were carried out in triplicate (i.e. biological triplicates) and significant differences between the untreated (black line in all panels) and treated (red line) serum are shown with asterisks (Student's t-test, $p<0.05$). The error bars represent the standard deviation of the means.

involved in the killing mechanism of naive rabbit serum against *S. aureus* and that further experiments were needed in order to confirm this hypothesis. The 100-fold increase in the bacterial numbers observed between 3 and 4 hours in figure 4.5C could be due to an error in the dilution process. This explanation could be used for other experiments that showed this increased growth.

4.2.5 Inactivation of the naive rabbit serum antibacterial activity using antibodies against complement components

The results obtained from the complement inactivation protocols and evidence from work previously carried out in the laboratory (Bozakouk, 2011) led to the hypothesis that the complement system was involved in the bactericidal activity of the NRS against *S. aureus*. However these inactivation protocols were not specific against the complement and they could be affecting another serum component that was involved in the antibacterial activity of the rabbit serum.

To gather more evidence to confirm the role of the complement in the bacterial death caused by the rabbit serum, more specific protocols were needed. It has been reported that the use of antibodies against complement components leads to the inactivation of the complement system (Whitehead et al, 1981; Gupta-Bansal et al, 2000; Mastellos et al, 2004; DiLillo et al, 2006). Thus, antibodies against specific components of the rabbit complement system were used to inactivate the killing activity of the naive rabbit serum.

Four complement components were chosen for this inactivation: mannose-binding lectin, component C4b, C5b and C9. The first two components were identified by mass spectrometry as components of the rabbit serum bound to purified cell wall (Bozakouk, 2011). All the activation pathways lead to the formation of the membrane attack complex and components C5b and C9 are essential for its deposition and formation, which led us to also pick them for the inactivation.

As explained previously, the mannose-binding lectin (MBL) is a recognition molecule that binds to carbohydrates present in the bacterial cell wall and leads to the activation of the lectin pathway through the activation of the MBL-associated serine proteases (MASPs) (Matsushita et al, 2000b; Gasque, 2004; Ricklin et al, 2010). This MBL-MASPs complex cleaves the complement components C2 and C4, leading to the formation of the C3 convertase C4bC2a (Matsushita et al, 2000a; Petersen et al, 2000; Schwaeble et al, 2002; Ricklin et al, 2010). The C4bC2a complex is also the C3 convertase of the classical pathway of complement (Petersen et al, 2001; Ricklin et al, 2010). This complex not only cleaves C3 but is also used for the formation of C4bC2aC3b, the C5 convertase of the classical and lectin pathways (Petersen et al, 2001; Seelen et al, 2005; Ricklin et al, 2010)

4.2.5.1 Design and synthesis of antibodies against rabbit complement components

Whilst antibodies against all the human complement components have been developed and are currently available via many companies, the antibodies, however, show no cross-reactivity with rabbit proteins. Another problem was that the anti-human protein antibodies were developed using rabbit as a host, which could lead to serious cross-reactivity between the secondary antibody in the Western blots used to confirm the identity of the complement components and the endogenous immunoglobulins present in the rabbit serum. This led to the design of our own anti-rabbit complement antibodies and synthesize them using chicken as the host organism.

The first step was to identify the sequences of the proteins chosen for the antibody production. The predicted sequences were obtained from the protein database from the National Center for Biotechnology Information (NCBI) and their identity was confirmed using the Basic Local Alignment Search Tool (BLAST) from Uniprot.org (http://www.uniprot.org/blast/). Conserved domains within the proteins were identified using the Conserved Domains Database from NCBI, further confirming both their identity and function.

Once the proteins and sequences were chosen, companies are able to produce the antibodies were identified. Most companies produce antibodies using purified proteins. While most human complement components can be purchased purified from several companies, the rabbit versions are not available. The purification protocols that can be found in the literature used large volumes of human blood to obtain these components. When compared to the amount of blood obtained from one rabbit (70 ml of blood maximum), the number of rabbits needed to purify each component was too large and very expensive. This meant that only the sequences of the proteins were available.

GenScript was the only company that offered custom polyclonal antibody production from protein sequences using a variety of animal hosts. Due to the interest in avoiding any cross-reaction between the antibodies and the immunoglobulins present in the rabbit serum in the inactivation experiments and in the western blots, chicken was chosen as the animal host for the antibody production due to its distant phylogenetic relationship with rabbit. Chicken was also chosen as the host because high concentration of immunoglobulins can be found in the egg yolk. As they lay multiple eggs a high yield of purified polyclonal antibody by the end of the production was obtained.

From the protein sequences, GenScript was able to design several peptides for each protein using their GenScript OptimumAntigen^{TM} design tool. All of the peptides were considered easy to synthesize and purify, and had an extra cysteine added to the C- or N- terminus to facilitate conjugation to carrier proteins. Several characteristics of the peptides were analysed and reported, including hydrophobicity, flexible regions, signal peptides, homology, helix, sheet and transmembrane regions. If more than one peptide met the criteria, the company also reported about the expected antigenicity, hydrophilicity and surface probability. These parameters were taken into consideration at the moment of choosing the peptides that were synthesized.

From the list of peptides provided by Genscript, one peptide was chosen for each complement component. The sequences of the peptides are shown in Table 4.1. Alignment of the peptides and the rabbit (*Oryctolagus cuniculus*)

genome showed not only 100% of identity between these sequences and the predicted complement components, but also their location within the protein (Figure 4.6).

Once GenScript synthesized the desired peptides, they were used to produce the antibodies using chickens as host organisms. The whole process took four months, from preparation of the antigen to the purification and quality control of the antibody. The chickens were immunized four times with the antigen, with two weeks between the fist and second immunization and three weeks for the third and fourth. By the end of the process GenScript sent not only the affinity-purified antibodies, but also the synthesized antigen, serum from pre-immmunized chickens and antiserum obtained after the fourth immunization. As two chickens were used for each protein, GenScript sent two vials of affinity-purified antibodies.

4.2.5.2 Effect of anti-rabbit complement components antibodies in the bactericidal activity of NRS

One of the many immune evasion strategies of *S. aureus* involves the secretion of molecules that are able to inactivate different components of the complement cascade. These include the staphylococcal complement inhibitor (SCIN), which binds to the membrane-bound C3 convertases blocking all the complement pathways (Foster, 2005; Rooijakkers et al, 2005c; Rooijakkers et al, 2006; Rooijakkers and van Strijp, 2007); the extracellular fibrinogen binding protein (Efb), able to bind to C3, blocking its deposition on the bacterial surface and its activation beyond C3b (Lee et al 2004a; Lee et al, 2004b; Jongerius et al, 2007); and staphylococcal syperantigen-like protein 7 (SSL-7), molecule that is able to bind to C5, inhibiting the formation of the membrane attack complex and the production of C5a (Langley et al, 2005; Bestebroer et al, 2010; Langley et al, 2010).

Table 4.1 Sequence of peptides used for antibody production

GenScript designed different peptides for each of the rabbit proteins. The peptides used for antibody production are shown here. The extra cysteine residue added for conjugation to carrier proteins is bolded.

Protein	Peptide sequence
MBL	**C**TSVADSERGALRAE
C4	IQEKGVEWSDPLPK**C**
C5	**C**SETLTREAREERLL
C9	**C**KEKLLTKAKMVDVT

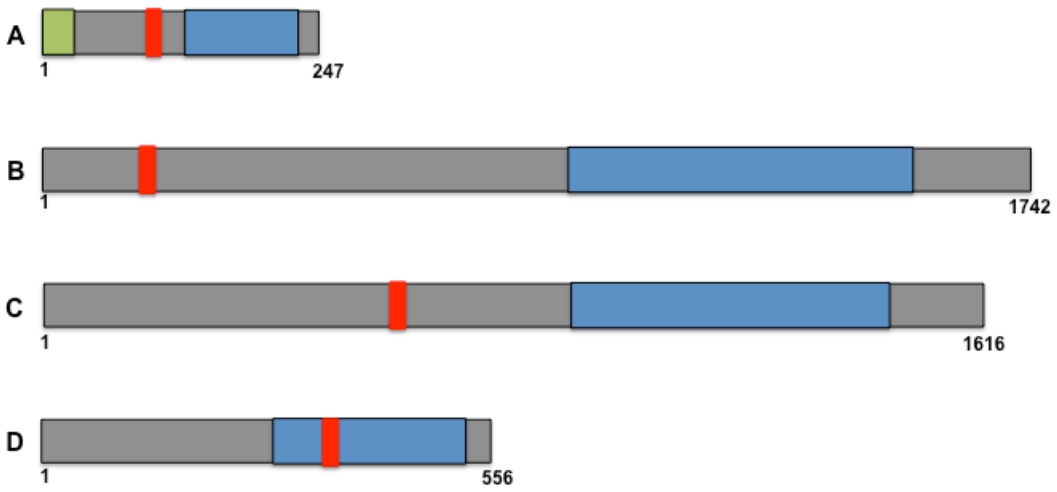


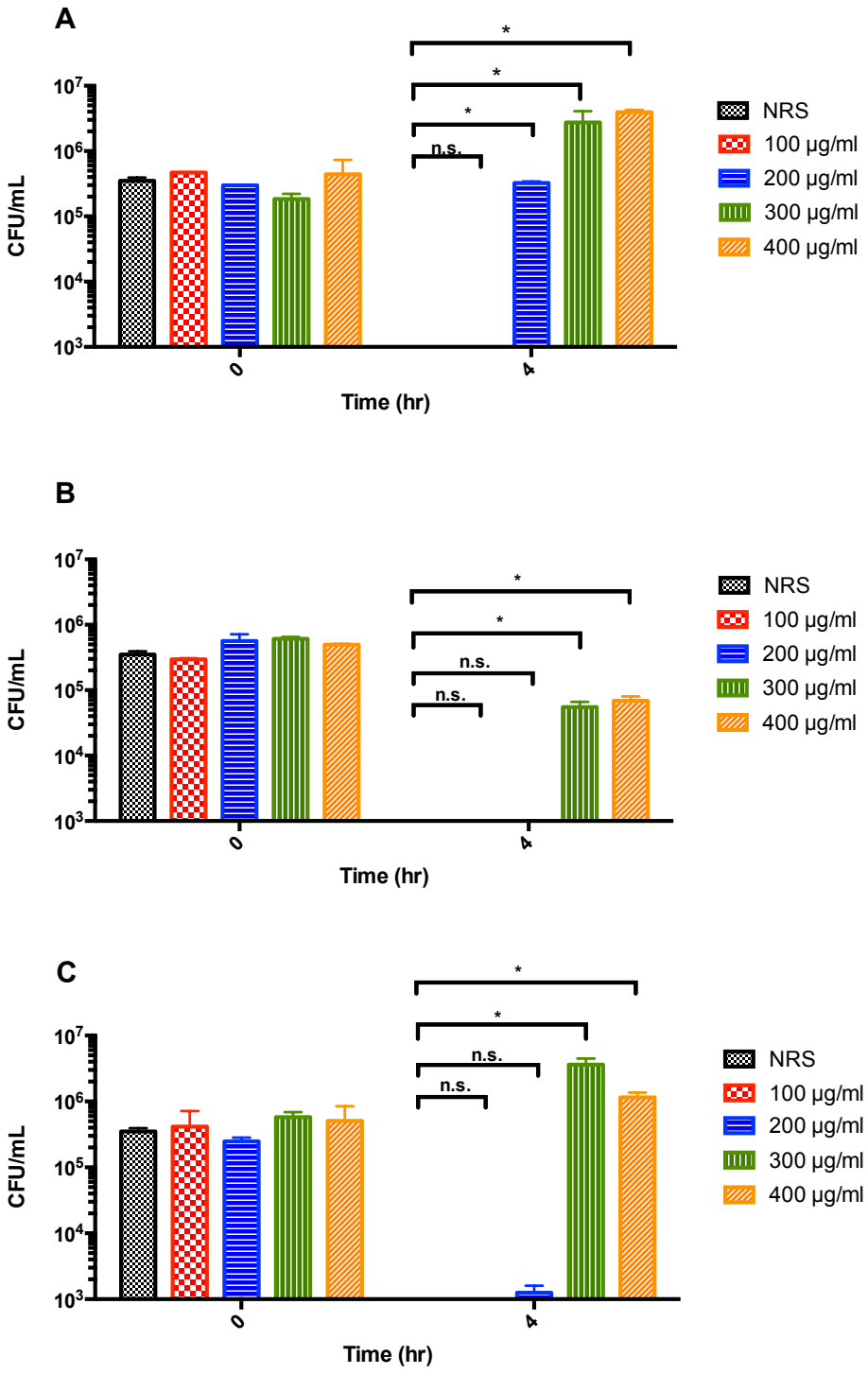
Figure 4.6 Location of synthesized peptides within rabbit complement components sequences

The location of the peptides used for the production of anti-complement components antibodies is shown in red, whilst blue shows the location of the active domain of each protein. Green shows the transmembrane region. **A.** Mannose-binding lectin (Accession number XP_002718411.1), **B.** complement component C4 (XP_002714341.1), **C.** complement component C5 (XP_002722895.1), **D.** complement component C9 (NP_001075815.1).

These molecules synthesized by bacterias are not the only way to block the complement system. It has been reported that the use of monoclonal antibodies blocks the complement system. As the component C5 is common for all the complement pathways and is essential for the formation of the membrane attack complex, several antibodies against this component have been developed and showed results that suggest they may be used to prevent collagen-induced arthritis (Wang et al, 1995) and complement-mediated damage of xenogeneic organs (Rollins et al, 1995). Eculizumab, a humanized monoclonal antibody against C5 developed by Solaris, blocks both the pro-inflammatory and the cytolytic effects of the C5 activation and is used to treat paroxysmal nocturnal hemoglobinuria disease (Rother et al, 2007).

For the inactivation experiments using the anti-rabbit complement components antibodies, stock solutions of the antibodies developed by GenScript were prepared and aliquots of rabbit serum were mixed with different concentrations of the antibodies. These mixtures were incubated for 60 minutes and then inoculated with 10^5-10^6 cells/ml of an *S. aureus* culture in exponential phase, after which killing assays were carried out (Chapter 2.9).

No inactivation was observed when the control antibody was added to the rabbit serum (Figure 4.7E). The pre-incubation of rabbit serum with 300 µg/ml and 400 µg/ml of antibodies against mannose-binding lectin, complement components C4, C5 and C9 led to a significant inactivation of the antibacterial activity when compared to the activity of untreated serum (Figure 4.7A, B, C and D, green and orange bars respectively). These results suggested the role of the lectin pathway of complement in the bactericidal activity of the serum and the importance of the membrane attack complex in bacterial death.


A
B
C
CFU/mL
Time (hr)
0
4
n.s.
*
NRS
100 µg/ml
200 µg/ml
300 µg/ml
400 µg/ml

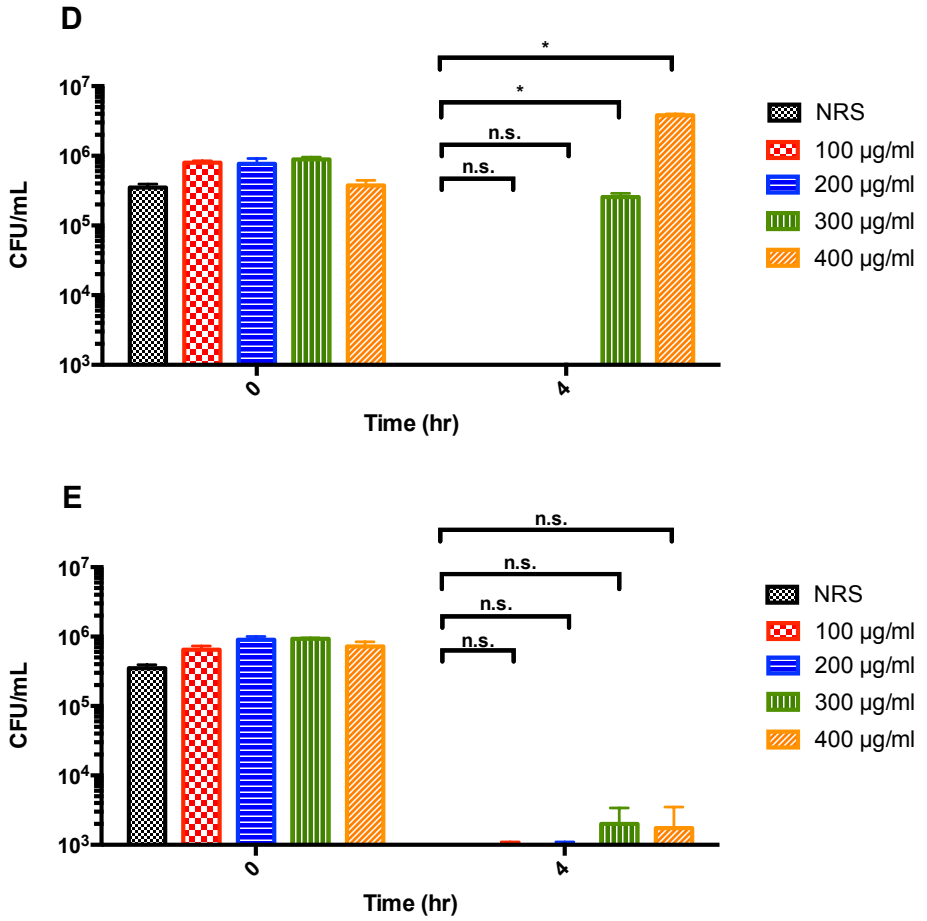


Figure 4.7 Effect of anti-complement antibodies on the bactericidal activity of rabbit serum

Aliquots of rabbit serum were incubated with different concentrations (100-400µg/ml) of antibodies against **A.** MBL, **B.** C4, **C.** C5, **D.** C9, **E.** control chicken antibody. The bactericidal activity after 60 minutes pre-incubation was tested against an exponential phase culture of *S. aureus* SH1000. Results for the start and final time point (0 and 4 hr, respectively) are shown in order to make comparisons easier. These experiments were carried out in triplicate (i.e. biological triplicates) and the error bars correspond to standard deviation of the mean. Significant differences between the untreated serum (black bars in all panels) and the treated serum are shown with asterisks (Students t-test, $p<0.05$). n.s. : non significant.

4.2.5.3 Study of the specificity of the anti-rabbit complement components antibodies

Not only the ability of these antibodies to inactivate the bactericidal activity of the rabbit serum was tested, but also their capacity to recognize the proteins they were raised against through Western blot. As explained before, these antibodies were raised using peptides from the rabbit proteins and they are not expected to crossreact with the human proteins. Comparison of the protein sequences shows that while some residues are fully conserved, most of the peptides only have a weak conservation with the human protein sequences (Figure 4.8).

The affinity of the antibodies against the peptides they were raised against was tested through immunoblots using nitrocellulose membranes and chemiluminescence kits for visualization of the signal. While a strong signal was obtained in each case, this signal was weaker when using the C4 peptide/antibody pair (Figure 4.9). This weaker interaction with the antibody could explain the level of inactivation of the bactericidal activity of the serum when it was pre-incubated with this antibody in comparison to the other antibodies.

To verify the presence of the complement proteins in the rabbit serum and their interaction with the antibodies Western blots were carried out. Each of the antibodies was tested against samples prepared using serum from four different rabbits and from four different human volunteers. The synthesized peptides were used as positive controls for the antibodies. The samples were prepared and resolved as explained in Chapter 2.21.6, and the membranes were developed using a chemiluminescence kit.

No reactive proteins against any of the antibodies were observed in rabbit or human serum (Figure 4.10, all panels). The antibodies against the complement components MBL, C5 and C9 were only able to interact with the sample containing the synthesized peptide (Lane 2 in Fig. 4.10 A, C and D,

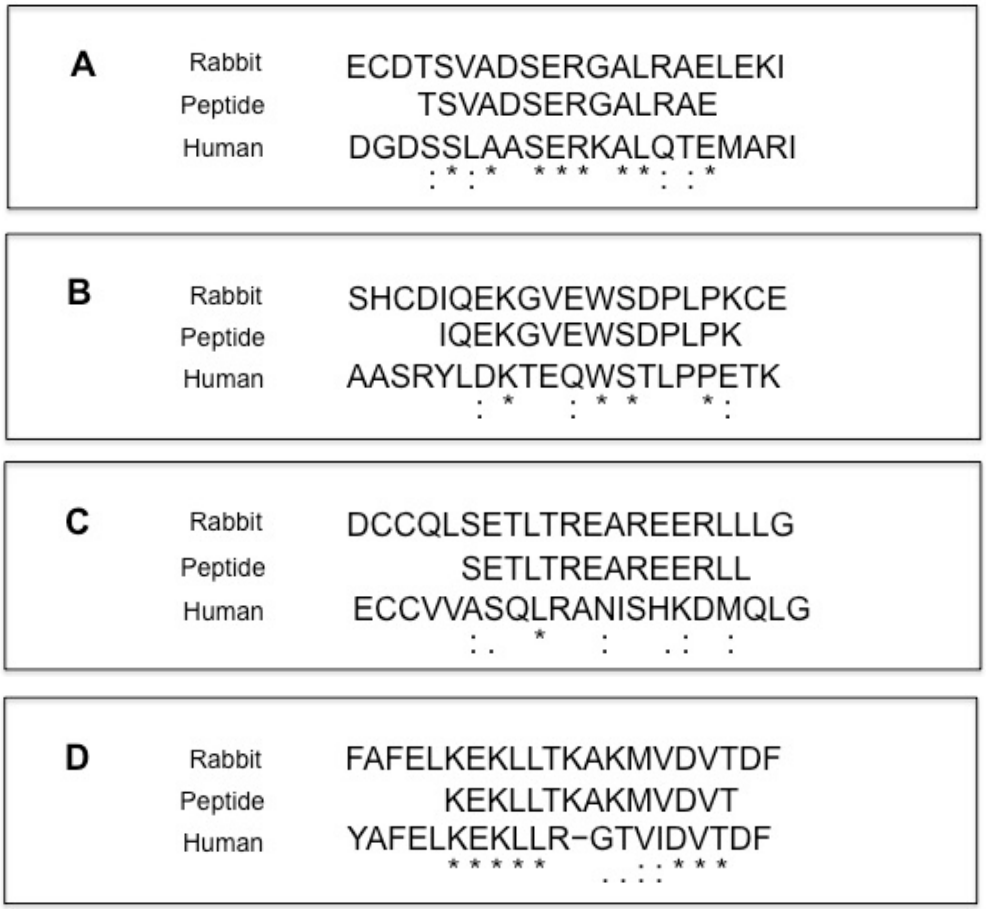


Figure 4.8 Sequence alignments between complement components from human (*Homo sapiens*) and rabbit (*Oryctolagus cuniculus*)

Alignment of the sequences of rabbit, human and peptide sequences of **A.**MBL, **B.** C4, **C.** C5 and **D.** C9. The alignments were made using ClustalW, with the EBLOSUM62 matrix. The residues that are marked with an asterisk (*) are fully conserved; colon (:) indicates strongly conserved substitutions and full stop (.) indicates weakly conserved residue substitutions. The extra cysteine added to the peptide sequence is not shown.

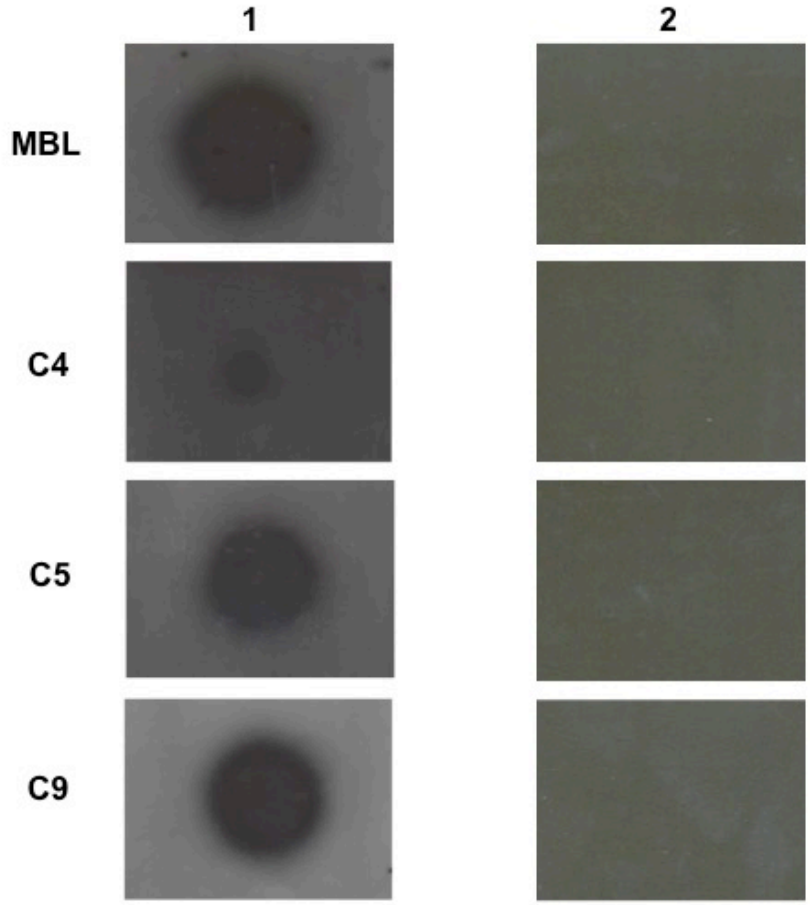


Figure 4.9 Enhanced chemiluminescence signal from immunoblots using synthesized peptides and chicken antibodies

A 10 µl aliquot (5µg) of each synthesized peptide MBL was placed directly into a nitrocellulose membrane, then air dried before following the slot blot system protocol from Chapter 2.21.1. The membranes were incubated with **1.** Specific antibody and **2.** Chicken control antibody. The signal was obtained after 5 minutes of incubation for development.

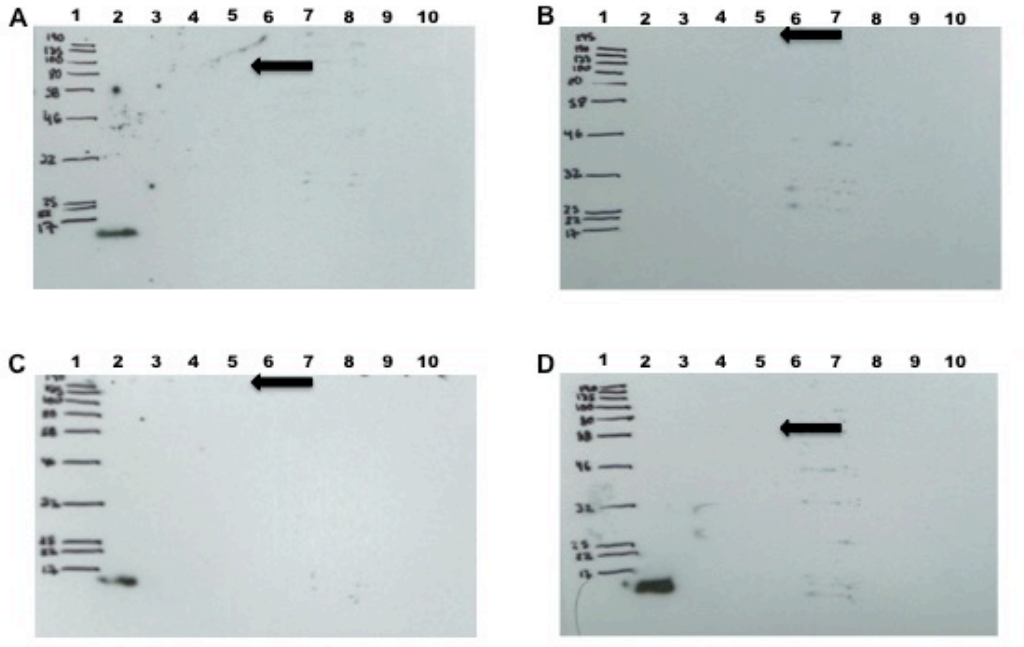


Figure 4.10 Reactivity of the anti-rabbit complement component antibodies

Aliquots of 10 µg of each synthesized peptide (lane 2), serum from four different rabbits (lanes 3, 4, 5 and 6) and serum from four different human volunteers (lanes 7, 8, 9 and 10), along with 5 µl of Color Prestained Protein Standard, Broad Range from New England Biolabs (lane 1), were analysed in 10% (w/v) SDS-PAGE and Western blot. The blots were incubated with the chicken produced antibodies against rabbit **A.** MBL, **B.** C4, **C.** C5 and **D.** C9 at a 1: 5,000 dilution. The arrows show the expected size for each protein: 96kDa for MBL, 210kDa for C4, 180kDa for C5 and 69kDa for C9. The signal was obtained after 20 minutes of incubation for development.

respectively). No signal was obtained using the antibody against C4 (Figure 4.10B).

As three of the chicken antibodies against the rabbit complement components (MBL, C5 and C9) were only able to react with the synthesized peptide they were raised against but not with any protein present in the samples prepared with sera from different rabbits, a problem could be the concentration of the proteins in the samples loaded into the gel and thus a 24-wells slot blot system (CORE Life Sciences) was used.

To test the reactivity of the antibodies for proteins in the serum, a 10^{-1}- to 10^{-4}-fold dilution of rabbit and human serum were used. 50 µl of each sample was loaded into the wells, the membranes were incubated with each of the antibodies according to the protocol explained in Chapter 2.21.1 and signals were obtained only after 30 minutes of development using chemiluminescence.
No signal was obtained for rabbit serum, confirming that these antibodies are not able to react with proteins in this serum. On the contrary, all of the antibodies were even able to interact with human proteins present in the sera diluted 10^{-3}-fold (Figure 4.11, all panels). Similar results were obtained when anti-human complement antibodies were used (data not shown).

These experiments indicated that the antibodies produced in chicken by GenScript were not able to interact with the complement proteins present in the rabbit serum. They only showed reactivity with the peptides they were raised against. This suggests that the linear peptides do not contain epitopes held by the full length protein. This could be due to modifications or other structural features. Without a clear signal identifying the rabbit complement proteins it cannot be said that the inactivation that was obtained is due to a specific interaction between that antibody and its target protein.

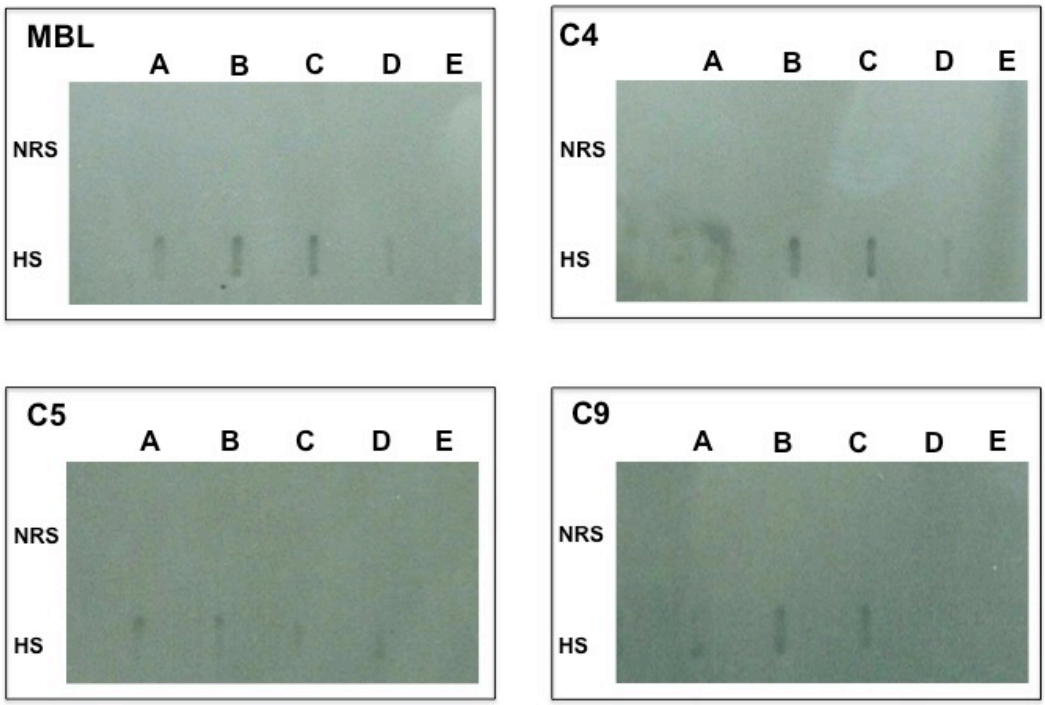


Figure 4.11 Study of reactivity of anti-rabbit complement components antibodies with rabbit and human sera using a 24-well slot blot system

Rabbit (NRS) and human (HS) serum was diluted 10-fold using sterile PBS and 50 µl of each sample was loaded into each well: **A.** undiluted sera, **B.** 10^{-1} dilution, **C.** 10^{-2} dilution, **D.** 10^{-3} dilution, **E.** 10^{-4} dilution. The membrane was incubated with blocking buffer for 60 minutes, after which was washed and then incubated for 60 minutes with blocking buffer containing the primary antibody at 1:10,000 dilution (MBL, C4, C5 and C9, as indicated in the figure). The membranes were then incubated with the secondary antibody (1:10,000 dilution) for 60 minutes, washed and developed using the SuperSignal® West Pico Enhanced Chemilunminescent kit. The membranes were developed for 30 minutes.

4.2.6 Purification of human and rabbit serum components by centrifugal filters and study of their bactericidal activity

To characterize the elements of the NRS that were responsible for its bactericidal activity against *S. aureus*, the rabbit serum was separated into different fractions according to molecular mass and the fractions were tested on their ability to affect the growth of the bacteria.

In order to obtain the different fractions, the sera were separated using Amicon Ultra 0.5ml centrifugal filters, Ultracel (Millipore) with different molecular mass cut-off: 10, 30 and 50 kDa. Rabbit serum was placed into the sample reservoir and centrifuged following the instructions of the manufacturer (Chapter 2.15.1) By the end of this separation, eight different fractions of rabbit serum were collected: smaller and larger than 10 kDa, smaller and larger than 30 kDa, smaller and larger than 50 kDa, between 10 and 30 kDa, and between 30 and 50 kDa. All the fractions were stored at -80ºC until further use. The same protocol was used with human serum.

To confirm that the filtration process itself had no negative effect on the overall bactericidal activity of the fractions, all the fractions obtained using each of the filters were mixed together. Before mixing the fractions it was taken into consideration that this separation process also concentrated the fraction containing the component with larger molecular mass than the cut-off of each filter. No significant difference between the killing activity of untreated rabbit serum and the reconstituted serum was observed (Figure 4.12), suggesting that this separation process did not have an effect on the bactericidal activity of the serum components. The same experiments were carried out with the human serum fractions and no difference between the activity of the untreated and reconstituted serum was found (data not shown).

After confirming that filtration had no effect on the bactericidal activity of the rabbit serum, the bactericidal activity of the different fractions was tested. As explained previously, it was necessary to take into consideration the concentration of the fraction containing components with larger molecular mass

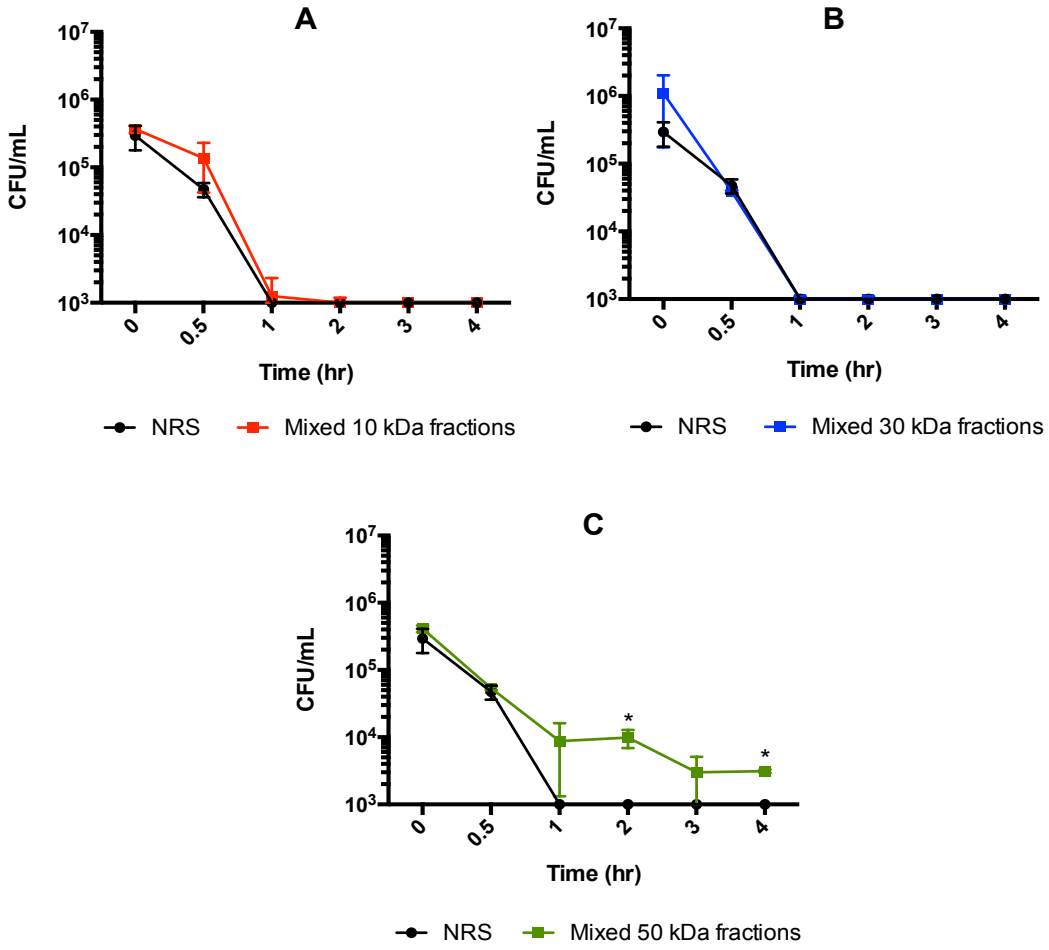


Figure 4.12 Pairwise comparison of the reconstitution of the bactericidal activity of the fractioned NRS against *S. aureus*

Aliquots of the rabbit serum fractions were mixed to reconstitute the serum before being inoculated with $5x10^5$ to 10^6 cells/ml of an exponential phase culture of *S. aureus* SH1000. Killing assays were carried out as explained previously (Chapter 2.9). Experiments were carried out in triplicate (i.e. biological triplicates). Student's t-tests were carried out and no significant differences were found ($p>0.05$). Error bars correspond to the standard deviation of the means.

than the cut-off of each filter. Therefore, the fraction containing the components smaller than the cut-off of each filter was used directly in the experiments, while the fraction containing the components bigger than the cut-off were diluted accordingly using sterile PBS.

The experiments showed that none of the serum fractions had a bactericidal activity comparable to the antibacterial activity of the untreated serum (Figure 4.13). Two of the rabbit serum fractions, containing components smaller than 30 kDa and components smaller than 50 kDa (Figure 4.13C and E, respectively), showed an effect on the bacteria similar to bacteriostatic effect, while all the other fractions allowed bacterial growth. The fractions containing components between 10-30 kDa and between 30-50 kDa also allowed bacterial growth (Figure 4.14). These results suggested that the elements of the rabbit serum responsible for the killing activity might be found in the fractions containing components smaller than 30 kDa and smaller than 50 kDa in a concentration that affects the bacteria but not high enough to kill them completely. These antibacterial elements might be also present in other fractions but they are not able to kill the bacteria due to a concentration issue, or because they need another element in order to be able to kill the bacteria and this element was not present in the fractions.

No bactericidal activity against *S. aureus* was observed when killing assays were carried out using the fractions obtained from the human serum. Previous work carried out in the laboratory showed that the combination of fractions from human and rabbit serum resulted in a 'reconstituted serum' capable of killing the bacteria (Bozakouk, unpublished). Here a finer separation took place and the human serum used was obtained directly from healthy human volunteers. When these experiments were repeated no bactericidal activity was observed for any of the combinations studied (Figure 4.15), suggesting that the rabbit serum fractions cannot augment the human serum.

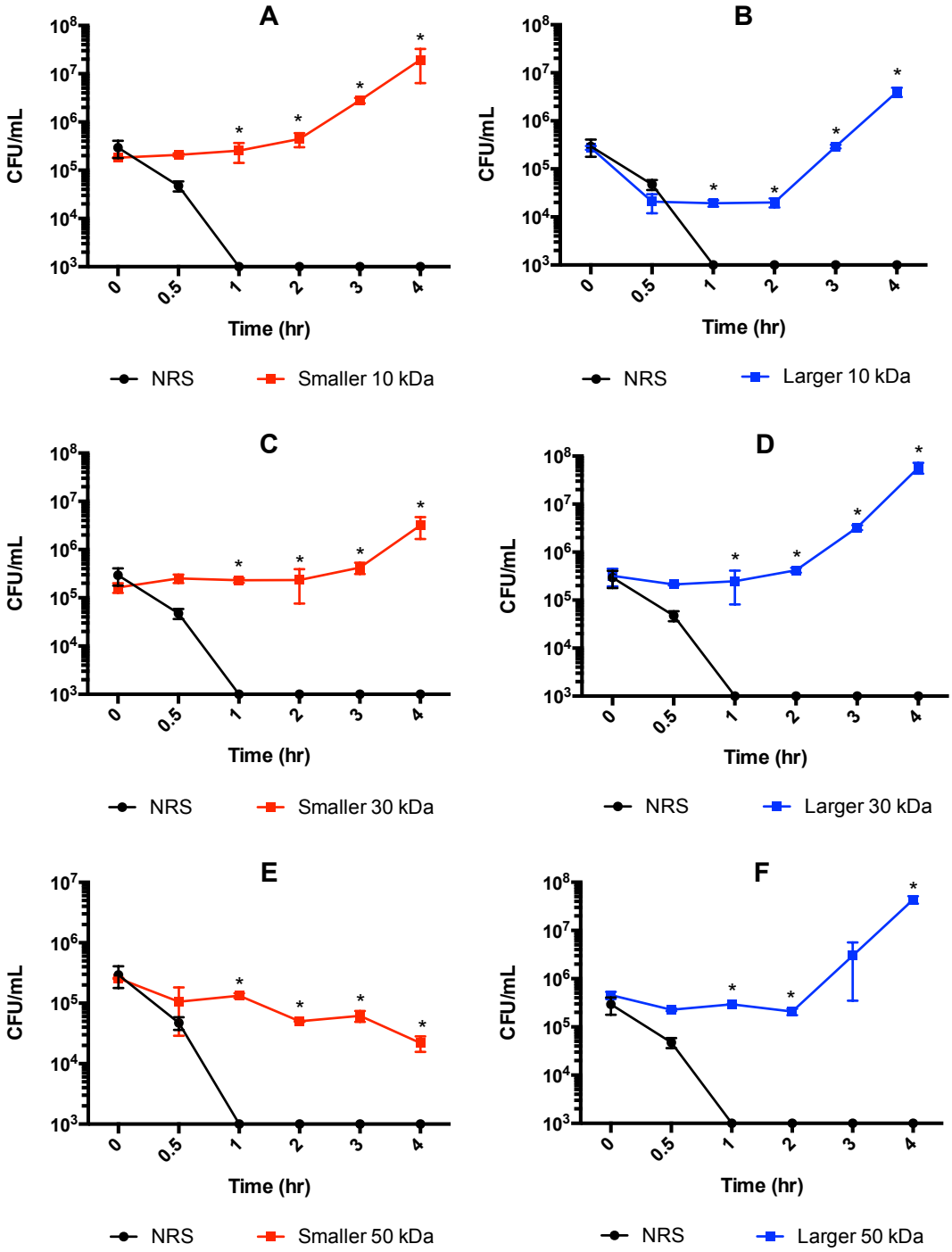

A
B
C
D
E
F
CFU/mL
Time (hr)
NRS
Smaller 10 kDa
Larger 10 kDa
Smaller 30 kDa
Larger 30 kDa
Smaller 50 kDa
Larger 50 kDa

Figure 4.13 Pairwise comparison of the effect of fractionated rabbit serum on *S. aureus*

Aliquots of the fractionated rabbit serum were inoculated with 10^5 cells/ml of *S. aureus* SH1000 and killing assays were carried out to test the bactericidal activity of each fraction. **A.** Smaller than 10 kDa, **B.** Larger than 10 kDa, **C.** Smaller than 30 kDa, **D.** Larger than 30 kDa, **E.** Smaller than 50 kDa, **F.** Larger than 50 kDa. These experiments were carried out in triplicate (i.e. biological triplicates) and significant differences are shown with asterisks (Student's t-test, $p<0.05$). The error bars represent the standard deviation of the means.

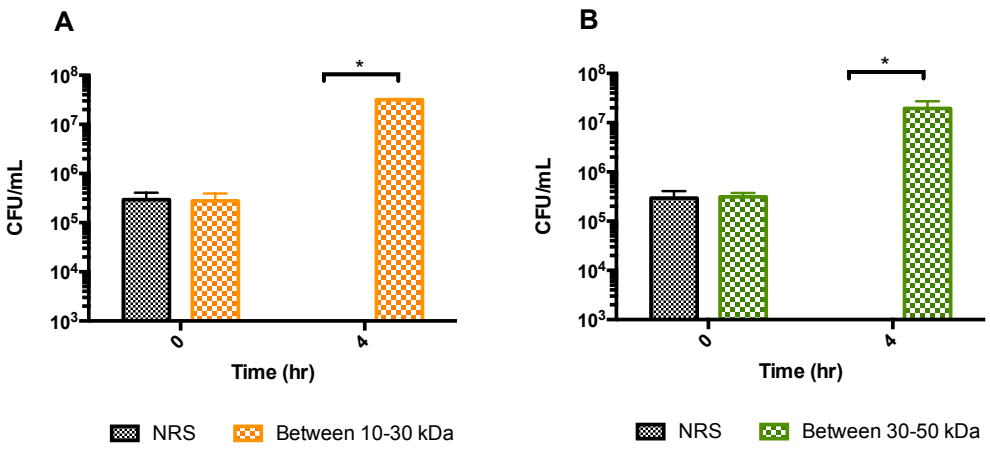


Figure 4.14 Comparison of the bactericidal activity of fractionated and untreated rabbit serum

Aliquots of the fractionated and untreated rabbit serum were inoculated with 10^5 cells/ml of an exponential phase culture of *S. aureus* SH1000 and killing assays were carried out to test the bactericidal activity of each fraction. **A.** Components between 10-30 kDa, **B.** Components between 30-50 kDa. These experiments were carried out in triplicate (i.e. biological triplicates), the error bars correspond to the standard deviation of the means and the asterisks show significant differences (Student's t-test, $p<0.05$).

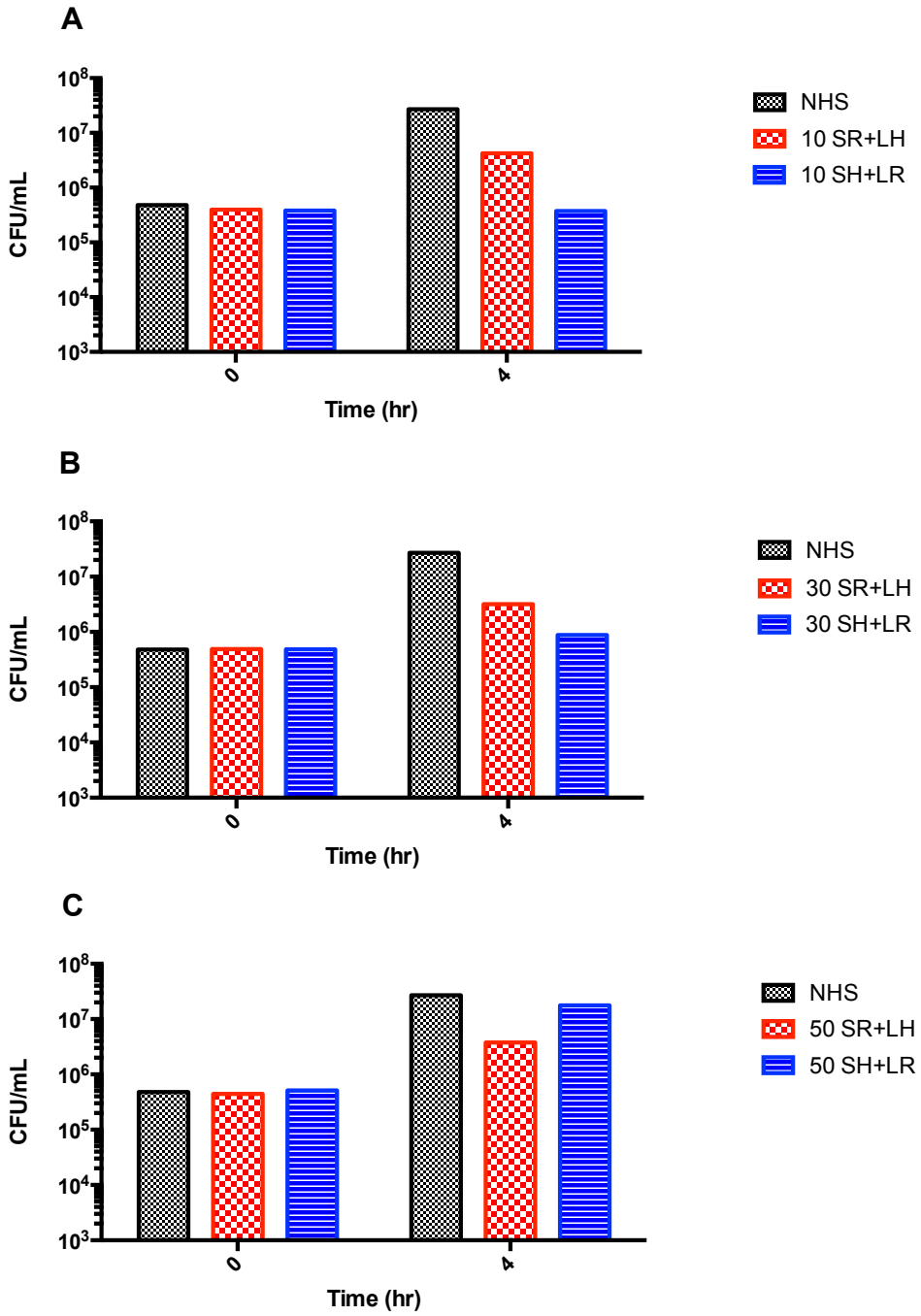


Figure 4.15 Bactericidal activity of the mixed human and rabbit serum fractions

Aliquots of smaller (SR) and larger (LR) fractions of rabbit serum were mixed with smaller (SH) and larger (LH) fractions of human serum according to the filters used, obtaining 'reconstituted' serum. The bactericidal activity of this 'reconstituted' serum was tested through killing assays using $5x10^5$ cells/ml of *S. aureus* SH1000. Fractions were obtained using **A.** 10 kDa, **B.** 30 kDa, or **C.** 50 kDa filters as explained previously. The activity of untreated human (black bars) serum is present in every graph for comparison. These experiments were carried out in duplicate.

4.2.7 Identification of rabbit serum components that bind to *S. aureus* purified cell wall

To continue with the identification of the rabbit serum components responsible for its bactericidal activity against *S. aureus*, purified cell wall material was used. In the previous Chapter it was shown that the NRS lost its antibacterial activity when it was pre-incubated with different types of purified cell wall material. These results suggested that the binding of the serum elements responsible for the killing to the purified cell wall caused the inactivation of the bactericidal activity of the serum.

4.2.7.1 Visualization of protein profiles of affinity matrices pre-incubated with NRS

In Chapter 3 the NRS pre-incubated with cell wall material was used for killing assays and the experiments highlighted the role of the wall teichoic acids in this process, along with the importance of D-alanylation. The insoluble material obtained after the incubations was washed with sterile MilliQ water and stored at -20ºC.

Due to the ability of the purified material to inactivate the NRS, this material was used as an affinity matrix for the serum components that are able to kill *S. aureus*. To check if there was a difference between the bound elements, the cell wall bound material was studied and protein patterns were compared through SDS-PAGE and silver staining of the resulting gel.

As shown in Figure 4.16, the protein patterns of the individual samples are different from each other. The cell wall material from the incubations that were able to completely inactivate the bactericidal activity of NRS showed more protein bands (lanes 3 and 6) than the material from serum that was only partially inactivated (lanes 2 and 4). These results supported the hypothesis

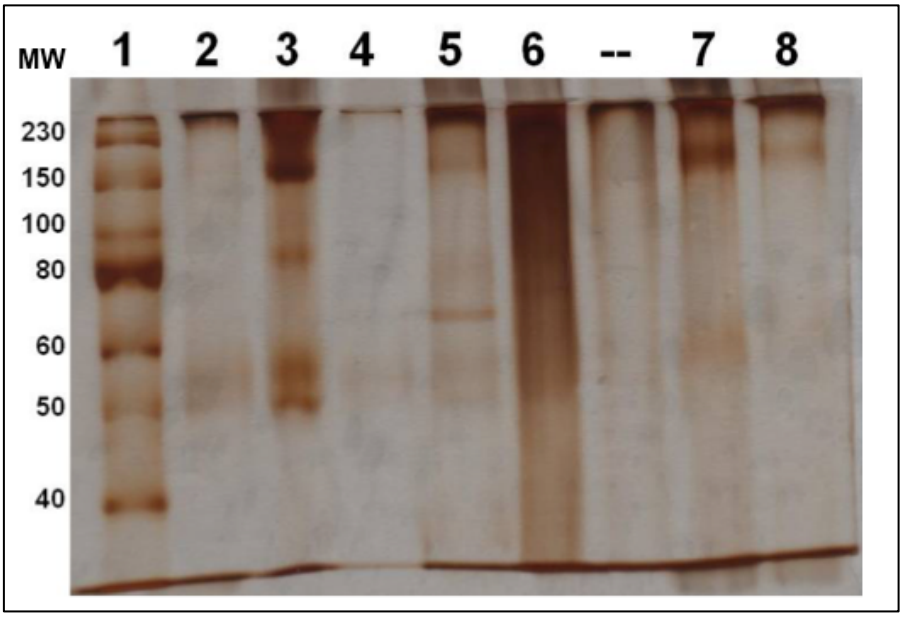


Figure 4.16 Silver staining of 10% (w/v) SDS-PAGE of pre-incubated cell wall material samples

1mg/ml of each cell wall purification was pre-incubated with 1ml of NRS for 30 minutes at 4ºC before being centrifuged. The insoluble material was washed three times with sterile MilliQ. Samples were prepared as explained previously (Chapter 2.21.2), diluting the samples 10-fold beforehand in order to facilitate the loading of the sample into the gel. Lane **1**: Protein ladder (molecular weight (MW): 10-230 kDa range); **2**: stripped PG [hydrofluoric acid treated PG, no WTA]; **3**: unstripped PG [PG+WTA]; **4**: purified WTA; **5**: cell wall material from Δ*tagO*; **6**: cell wall material from Δ*dltA*; **7**: cell wall material from RN4220; **8**: cell wall material from RN4220 Δ*tarM* Δ*tarS*.

that the elements of the serum that are responsible fot the killing of the bacteria were absorbed out of the rabbit serum by the purified cell wall material.

4.2.7.2 Mass spectrometry analysis of rabbit serum proteins bound to purified cell wall material

The different protein profiles observed by silver staining of the gels led to the next step: the identification of the different proteins. This identification was carried out by mass spectrometry analysis of the proteins bound to the cell wall material obtained after the pre-incubation with NRS. For this analysis three types of affinity matrices were used: stripped (peptidoglycan without WTA) and unstripped (peptidoglycan with WTA) peptidoglycan from *S. aureus* SH1000 cell wall material from the Δ*dltA* strain. These samples were chosen as they showed differential absorptive capacity for the NRS killing activity (Figures 3.17 and 3.19).

Mass spectrometry (MS) is a technique used for qualitative and quatitative analysis of proteins. This method provides information about the identity, structure, molecular mass, amount and purity of a sample (Ardrey, 2003; Becker and Bern, 2011; Cappadona et al, 2012). It has been widely reported that different types of mass spectrometry can be used to analyse proteins present in blood. This technique has been used to characterize the full proteome of human blood (Adkins et al, 2002; Shen et al, 2005), but also to identify low-molecular mass and low-abundance proteins that would go unnoticed using other methods (Merrel et al, 2002; Jaros et al, 2013). For example, MS analysis is currently used to identify biomarkers for different types of cancer (Zhang et al, 2004; De Bock et al, 2010; Mook et al, 2010; Timms et al, 2011; Tessitore et al, 2013).

A mass spectrometer has three main components: the ionization source, the mass analyser and the ion detector. The sample to be analysed is ionized and broken into charged fragments, which are separated in a mass analyser

according to their mass to charge ratio (m/z). This separation typically occurs by accelerating the charged fragments by application of a magnetic or an electric field. Different types of mass analysers can be used, each with different resolutions and useful for the analysis of different types of sample. The ion detector recognizes the charged fragments and the results are shown as a spectrum of the abundance of these ions as a function of the mass-to-charge ratio. It is possible then to identify the molecules by comparing the results obtained to known masses and characteristic or theoretical fragmentation patterns (Trauger et al, 2002; Glish and Vachet, 2003; Wysocki et al, 2005).

In order to obtain the samples that were analysed, aliquots of 1 ml of rabbit serum were incubated with 1 mg/ml of each of the cell wall purifications that were used as affinity matrices. After the incubation, the samples were centrifuged and the supernatant was used in killing assays to test that the bactericidal activity of these samples was affected by the treatment. The recovered insoluble material was washed several times with MilliQ, resolved by 10% (w/v) SDS-PAGE and stained using silver to demonstrate the different profile of proteins bound to each matrix. The insoluble material was then prepared for MS as explained in Chapter 2.20.1 using 16 hours of digestion with 1mg/ml of trypsin to completely digest the proteins.

Liquid Chromatography Mass Spectrometry (LC-MS) analysis of the three samples was carried out (Chapter 2.20.2) and the resulting spectra was searched with Mascot against the Swissprot Database using ‘Rodentia’ as taxonomic filter. Proteins with at least two peptides identified and with a confidence interval of 95% or higher were reported.

Between 245 and 317 proteins were identified through LC-MS in each preparation. Eleven proteins showed differential presence in the samples and only three proteins were present in the samples capable of completely inactivating the serum (unstripped peptidoglycan from SH1000 and unstripped peptidoglycan from Δ*dltA*) and not in the sample that only caused partial inactivation (stripped peptidoglycan) (Table 4.2). These proteins were immunoglobulin kappa chain V- region AH80, immunoglobulin lambda chain C

Table 4.2 Comparison of protein profiles from LC-MS analysis

The eleven proteins that were not present in all of the samples are shown here.

(**+**): present; (**-**): not present

Protein	Stripped cell wall material	Unstripped cell wall material	Unstripped *ΔdltA* cell wall material
Alpha-1-antiproteinase F	+	-	-
Hemoglobin subunit beta-1/2	-	+	-
Cholisteryl ester transfer protein (fragment)	-	+	-
Complement component C9	-	+	-
Ig kappa chain V region AH80-5	-	+	+
Ig lambda chain C region	-	+	+
Cystatin-C	-	+	-
Lysozyme C	-	+	-
Phospholipase A2 (fragment)	-	+	+
Ig kappa chain V region BS-1	-	-	+
Annexin A1	-	-	+
Metalloproteinase inhibitor 3 (fragments)	-	-	+

region, and phospholipase A2. These immunoglobulin fragments belong to the light chains of mammalian antibodies and they were considered unlikely to be part of the bactericidal mechanism.

Phospholipase A2 (PLA2) is part of a superfamily of enzymes capable of hydrolysing phospholipids in the sn-2 position, leading to the generation of arachidonic acid and lysophospholipid (Balsinde et al, 2002; Wu et al, 2010). Arachidonic acid can be converted into eicosanoid, a signalling molecule that plays a role in immune responses, inflammation and pain perception. The lysophospholipids can serve as a precursor for lipid mediators such as lysophosphatidic acid and platelet activating factor (Schaloske and Dennis, 2006).

These proteins can be divided into five kinds of enzymes based on their catalytic mechanism as well as functional and structural features (Schaloske and Dennis, 2006; Dennis et al, 2011). One of these groups is the secreted PLA2 (sPLA2), small secreted proteins (14-16 kDa) that usually contains 6 to 8 sulphide bonds (Schaloske and Dennis, 2006) and require of μM levels of calcium for catalysis due to their His/Asp catalytic dyad (Dennis et al, 2011; Murakami and Lambeau, 2013). It is known that the secreted PLA2 (sPLA2) has antibacterial activity against Gram-positives, including *S. aureus*. Its activity is affected by heat and zymosan, and the initial binding of PLA2 to *S. aureus* is based upon electrostatic interactions between the protein and the cell surface (Weinrauch et al, 1996; Qu and Lehrer, 1998; Santagelo et al, 1999; Buckland and Wilton, 2000; Koduri et al, 2002; Dennis et al, 2011). This led to the new hypothesis that sPLA2 might be the element responsible for the bactericidal activity of the NRS against *S. aureus*.

4.2.8 Effect of the inactivation of phospholipase A2 (PLA2) on the bactericidal activity of NRS against *S. aureus*

Thus far it has been shown that the antibacterial activity of the rabbit serum is affected by temperature, calcium concentration and by pre-incubation with zymosan A, ammonium salts and ammonium hydroxide. All of these treatments resulted in the complete inactivation of the bactericidal activity of the serum against *S. aureus* but they are non-specific and can affect several elements of the serum. Some of the elements that could be affected by these protocols were part of the complement system, which led us to study the role of this system in the bactericidal activity of the rabbit serum. However, in Chapter 4.3.5 it was confirmed that the complement system was likely not involved.

After identifying PLA2 as a candidate for the rabbit serum component responsible for the killing activity of the NRS, the effect of its inactivation on the antibacterial activity was studied. It has been reported that the activity of PLA2 is affected by heat treatment (Paliyath et al, 1999; Santangelo et al, 1999; Merchant et al, 2011), zymosan A from *S. cerevisiae* (Daum et al, 1998; Harboe et al, 2012) and by pre-incubation with a chelating agent (Harwig et al, 1995; Qu and Lehrer, 1998; Dennis et al, 2011), concurring with the NRS results obtained previously.

It has been reported that the activity of the PLA2 can be affected by the use of arachinodyl trifluoromethyl ketone ($AACOCF_3$) and 4-bromophenacyl bromide (BPB) (Balsinde et al, 1999; Ouyang and Kamisnki, 1999; Dennis et al, 2011). These compounds interact with specific types of phospholipases, which could help elucidate if the secreted phospholipase A2 (sPLA2) is really the bactericidal element of the rabbit serum.

The arachinodyl trifluoromethyl ketone is an analogue of arachidonic acid that interacts directly with the phospholipase and acts as a tight-binding inhibitor of the cytoplasmic (cPLA2) and the calcium-independent phospholipase (iPLA2) (Street et al, 1993; Ackerman et al, 1995; Loweth et al, 1996, Dennis et al, 2011). 4-bromophenacyl bromide is able to covalently bind to an exposed histidine residue present on the sPLA2, inhibiting the catalytic activity of the protein (Roberst et al, 1977; Mayer et al, 1993, Kim and Bonventre, 1993). This compound does not affect cPLA2 or iPLA2 (Balsinde et al, 1999).

In order to test the effect of $AACOCF_3$ and BPB on the bactericidal activity of the rabbit serum, these compounds were added to aliquots of NRS mixed with *S. aureus*. To determine possible effects of the inhibitors on *S. aureus* directly growth curves were carried out. The inhibitors had a significant negative effect on bacterial growth in each case (data not shown). This negative effect on the survival of *S. aureus* SH1000 was also observed when these inhibitors were added to heat inactivated rabbit serum (data not shown). This precluded the use of $AACOCF_3$ and BPB to confirm the role of PLA2 in the bactericidal activity of NRS.

Another molecule that is able to inhibit the activity of PLA2 is dithiothreitol (DTT). DTT is a strong reducing agent used to differentiate sPLA2 from the other types of phospholipase (Lambeau and Gelb, 2008). sPLA2 has a high number of disulphide linkages (between 6 and 8) that DTT is able to reduce, destabilizing the protein structure and affecting its activity (Castle and Castle, 1981; Mizushima et al, 1989; Singer et al, 2002; Lambeau and Gelb, 2008).

After confirming that DTT had no negative effect on *S. aureus* growth (data not shown), phospholipase A2 inactivation experiments were carried out. Aliquots of rabbit serum were mixed with DTT and pre-incubated for 30 minutes at 37ºC before the addition of the bacteria. This treatment was capable to inactivate the bactericidal activity of NRS against *S. aureus*, suggesting that sPLA2 was involved in this killing mechanism (Figure 4.17). A 100-fold increase in the bacterial numbers was observed between 3 and 4 hours of incubation in the NRS treated with this reducing agent. This increase could be due to a mistake in the dilution process prior to the plating, as the cell doubling time cannot explain this.

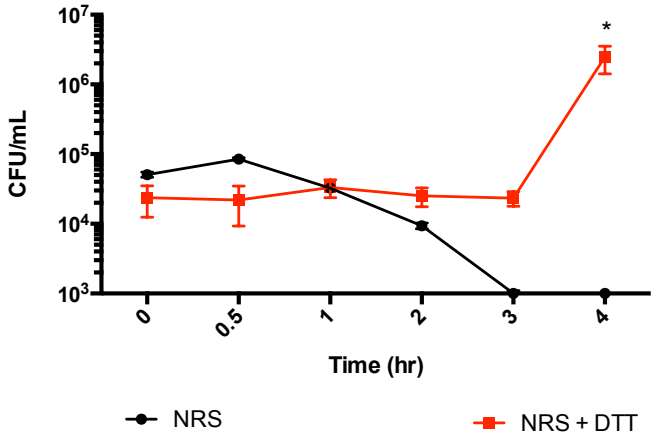


Figure 4.17 Effect of dithiothreitol pre-incubation on the bactericidal activity of NRS

Aliquots of rabbit serum were treated with 10 mM DTT and pre-incubated for 30 minutes at 30ºC, and then inoculated with $5x10^4$ cell/ml of an exponential phase culture of *S. aureus* SH1000. The activity of the treated serum (red line) was compared with the activity of untreated rabbit serum (black line). This experiment was carried out in triplicate (i.e. biological triplicates) and significant differences are shown with asterisks (Students t-test, $p<0.05$). The error bands correspond to the standard deviation of the means.

4.2.9 Effect of anti-phospholipase A2 antibodies in the bactericidal activity of rabbit serum

All the serum inactivation protocols used so far were non-specific and could affect other components of the serum as well as sPLA2. It has been reported that antibodies can be used not only to identify sPLA2, but also to inactivate its phospholipase activity (Murakami et al, 1988; Mizushima et al, 1989; Koduri et al, 1998). This approach was used to analyse the role of sPLA2 in the bacterial killing caused by the NRS. Rat monoclonal antibodies able to recognize the low molecular PLA2 were selected and purchased, as this low molecular protein corresponds to the secreted PLA2.

Rabbit serum was pre-incubated with different concentrations of monoclonal antibodies (10-100 µg/ml) before inoculating the mixtures with *S. aureus* SH1000 to test if there was any effect on the bactericidal activity. No significant difference between the bactericidal activity of untreated and treated serum was observed using the rat control antibody for any of the concentrations studied (Figure 4.18A). Whilst some effect on the antibacterial activity was observed when the serum was pre-incubated with 50 µg/ml of anti-PLA2 antibody, significant inactivation of the rabbit serum was observed when the rabbit serum was pre-incubated with 75 µg/ml or higher concentrations of the anti-PLA2 antibodies ($p < 0.05$; Figure 4.18B).

As a significant inactivation of the bactericidal activity was observed when the serum was pre-incubated with 75 µg/ml of the anti-PLA2 antibodies, complete killing assays were carried out using this concentration. Just as expected, the bactericidal activity was inactivated when the NRS was treated with the monoclonal anti-PLA2 antibodies, while treatment with the control antibody had no effect (Figure 4.19). This confirms the role of the secreted phospholipase A2 in the bactericidal activity of naive rabbit serum against *S. aureus*.

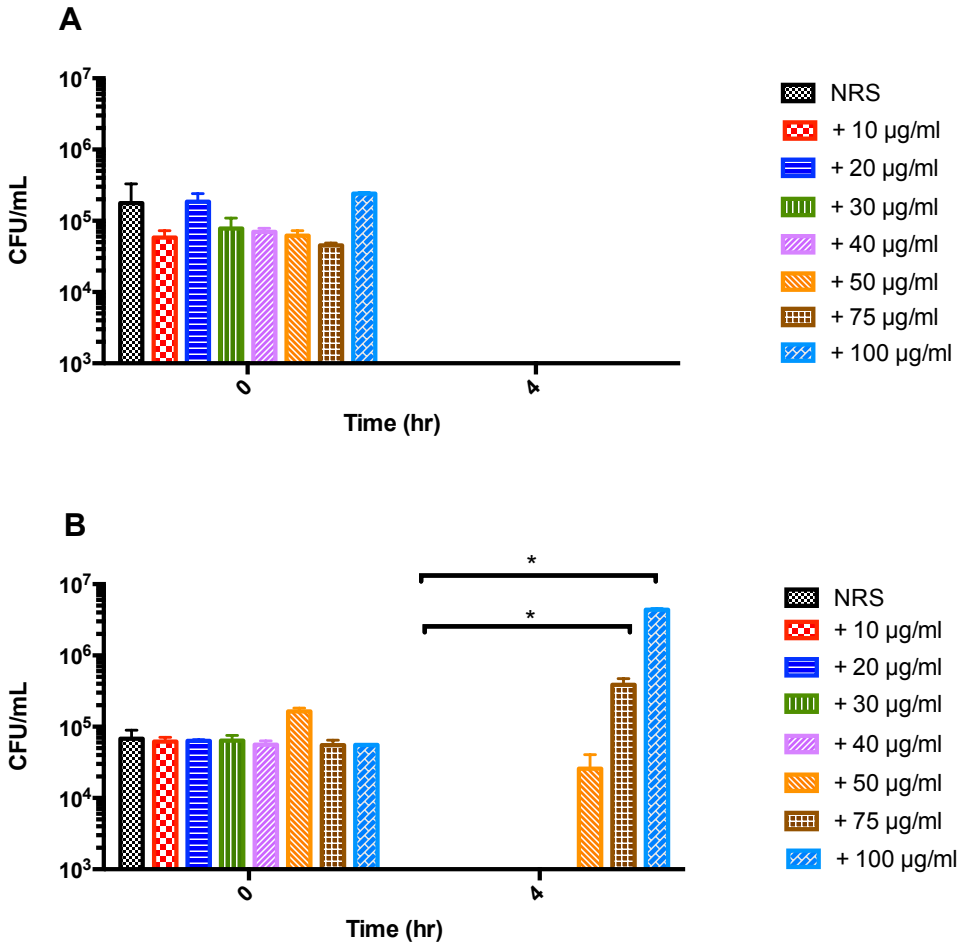


Figure 4.18 Effect of anti-PLA2 antibodies on the bactericidal activity of NRS

Aliquots of rabbit serum were pre-incubated with 10-100 µg/ml of **A.** rat control antibody, **B.** rat monoclonal anti-PLA2 antibodies before inoculating with 10^5 cells/ml of an exponential phase culture of *S. aureus* SH1000 to test their effect on the bactericidal activity of NRS. Treated serum was compared with untreated rabbit serum (black bars) and significant differences are marked with asterisks (Student t-test, $p<0.05$). These experiments were carried out in triplicate (i.e. biological triplicates). Error bars represent the standard deviation of the means.

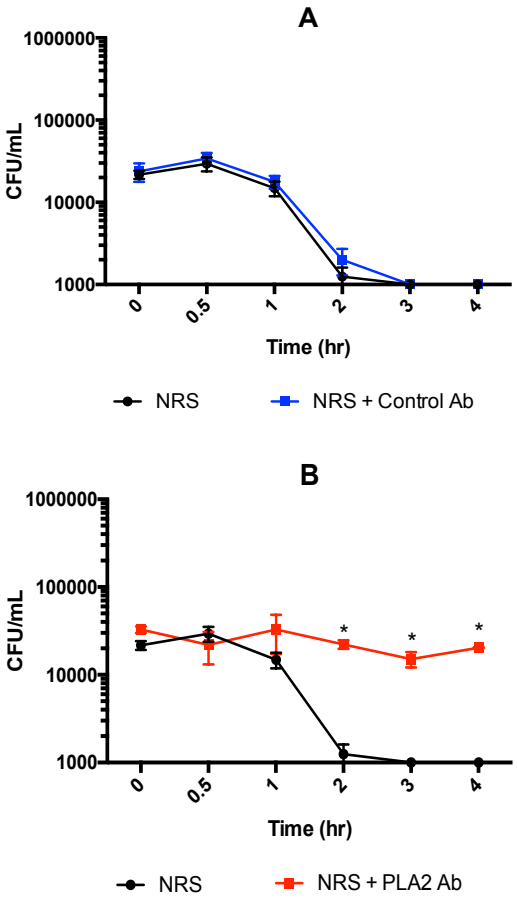


Figure 4.19 Pairwise comparison of the effect of anti-PLA2 antibodies on the antibacterial activity of rabbit serum

Aliquots of rabbit serum were incubated for 30 minutes with 75µg/ml of **A.** rat control antibody, **B**. rat monoclonal anti-phospholipase A2 antibody, before inoculating with 10^4-10^5 cells/ml of *S. aureus* SH1000. These experiments were carried out on triplicate (i.e. biological triplicates) and significant differences between the bactericidal activity of untreated serum (black line) and the treated serum are shown with asterisks (Students t-test, $p<0.05$). Error bars correspond to the standard deviation of the means.

4.2.10 Study of the specificity of anti-phospholipase A2 antibodies

To verify the reactivity of the antibodies against the secreted phospholipase A2 present in the rabbit serum, 10-fold dilutions up to 10^{-10} of rabbit and human serum were prepared using sterile PBS. 200 µl of each sample was loaded into the wells of the 24-wells slot blot system. The membrane was incubated with the monoclonal anti-PLA2 antibodies and signals were obtained after 10 seconds of development using the chemiluminescence kit. As expected, the antibodies were able to interact with proteins present in the rabbit serum but not in the human serum confirming the specificity for the rabbit PLA2 (Figure 4.20).

To verify that the anti-PLA2 antibodies were interacting with the desired protein, samples using human and rabbit and human serum were prepared and Western blots were carried out as explained in Chapter 2.21.6, developing the membrane using a chemiluminescence kit.

None of the proteins present in the human serum were able to interact with the anti-PLA2 antibody (Figure 4.21, lanes 2-5), while a 16kDa band was observed in all the samples prepared with serum from different rabbits (Figure 4.21, lanes 7-10). The band has the expected molecular weight of the sPLA2, demonstrating that the antibody interacts in a specific manner. The other bands that are present in this Western blot might correspond to dimers and other polymeric forms of the protein, which can also be exist in the serum in the presence of calcium (Castle and Castle, 1981).

4.2.11 Reactivity of anti-PLA2 antibodies to proteins bound to purified cell wall material after incubation with NRS

Previously (Chapter 4.2.7), differential binding of the NRS killing factor to purified cell wall fractions revealed sPLA2 as the potential active component. To determine if this correlates with specific sPLA2 levels the monoclonal antibody was used. The 24-well slot blot system was used and serial dilutions of stripped peptidoglycan (PG without WTA), unstripped cell wall material (PG+WTA) from

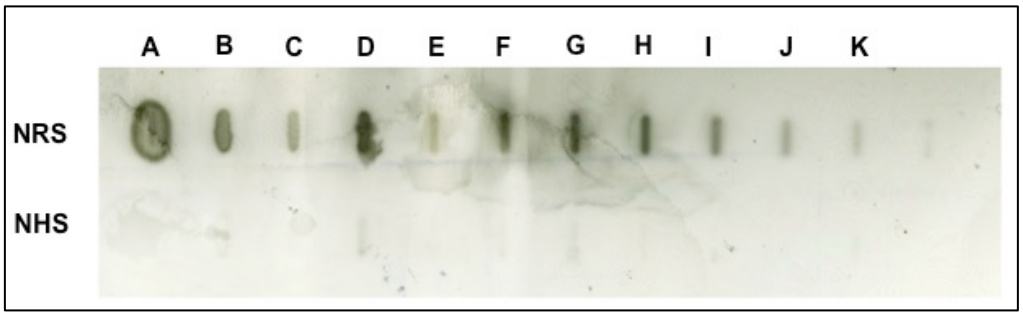


Figure 4.20 Reactivity of anti-phospholipase A2 antibodies with rabbit and human sera

Rabbit (NRS) and human (HS) serum was diluted 10-fold using sterile PBS and 200 µl of each sample was loaded into each well: **A.** undiluted sera, **B.** 10^{-1} dilution, **C.** 10^{-2} dilution, **D.** 10^{-3} dilution, **E.** 10^{-4} dilution, **F.** 10^{-5} dilution, **G.** 10^{-6} dilution, **H.** 10^{-7} dilution, **I.** 10^{-8} dilution, **J.** 10^{-9} dilution, **K.** 10^{-10} dilution. The membrane was incubated with blocking buffer for 60 minutes, washed and incubated for 60 minutes with blocking buffer containing the primary antibody at 1:10,000 dilution. The membrane was then incubated with the secondary antibody (1:10,000 dilution) for 60 minutes, washed and developed using the SuperSignal® West Pico Enhanced Chemilunminescent kit. The membranes were developed for 10 seconds.

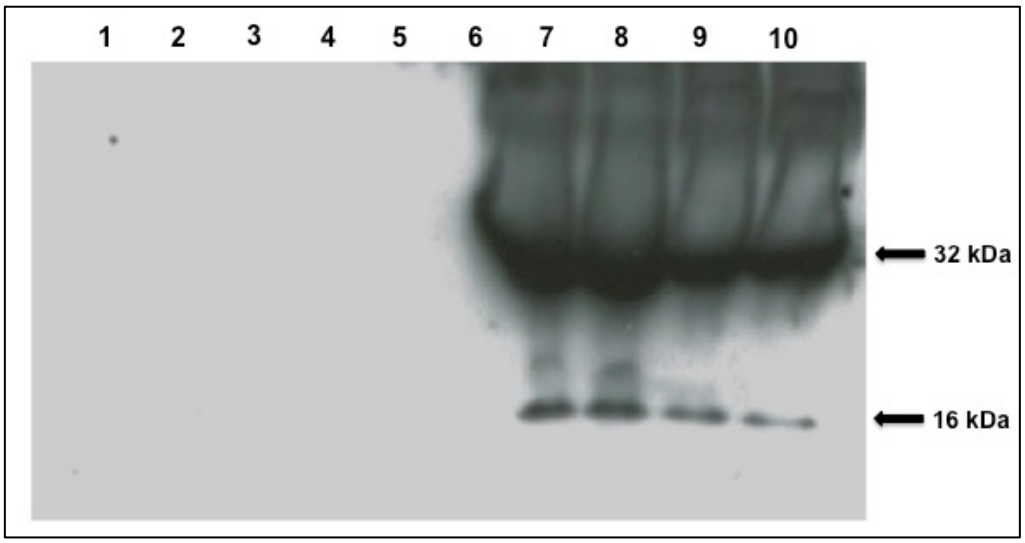


Figure 4.21 Specificity of the anti-phospholipase A2 antibody for rabbit serum proteins

Serum from four healthy human volunteers (lanes 2, 3, 4 and 5) and from four different rabbits (lanes 7, 8, 9 and 10), along with the Color Prestained Protein Standard, Broad Range from New England Biolabs (lane 1) were analysed by 10% (w/v) SDS-PAGE and Western blot. The membrane was incubated with the anti-PLA2 antibodies at a 1:5,000 dilution. The signal was obtained after 30 seconds of incubation for development.

SH1000 and unstripped material purified from Δ*dltA* pre-incubated with NRS were loaded into each well.

PLA2 was present in all the samples but at different concentrations (Figure 4.22). The signal obtained using stripped peptidoglycan was weaker than the signal for the unstripped cell wall material from SH1000 and for material purified from Δ*dltA*. This qualitatively supports the hypothesis that the differences in the degree of inactivation of the rabbit serum are caused by the variation in the affinity of the bactericidal component for different types of cell wall components.

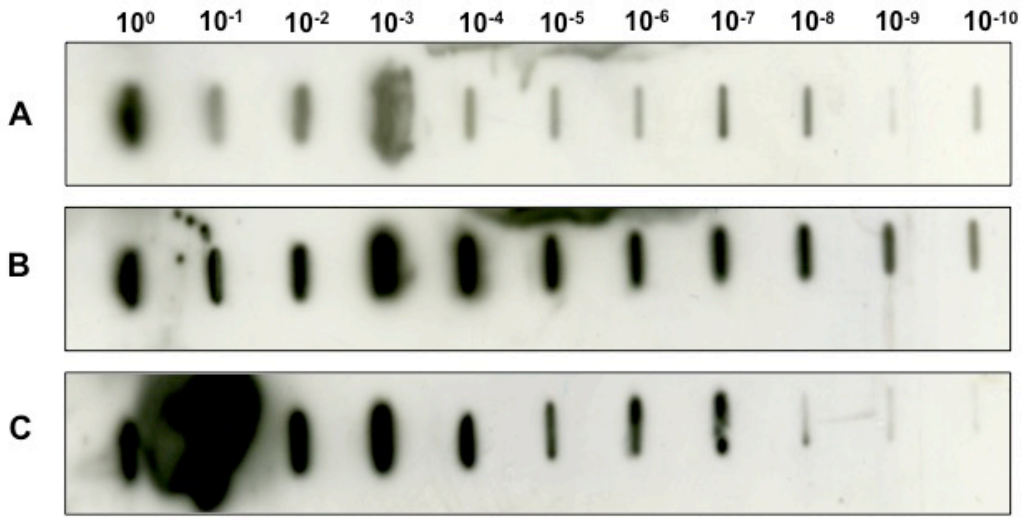


Figure 4.22 Identification of phospholipase A2 bound to affinity matrices after pre-incubation with rabbit serum

Bound material obtained from the incubation of rabbit serum with 1 mg/ml of **A.** Pure peptidoglycan, **B.** Unstripped cell wall material from parental strain, **C.** Cell wall material from the Δ*dltA*, was diluted 10-fold using sterile PBS and 200 µl of each sample was loaded into each well. The number on top of the figure shows the dilution present in each well, from undiluted (10^{-0}) to 10^{-10}-fold diluted (10^{-10}).

The membranes were incubated with blocking buffer, then washed and incubated for 60 minutes with blocking buffer containing the monoclonal anti-phospholipase A2 antibodies at 1:10,000 dilution. The membrane was then incubated with the secondary antibody (1:10,000 dilution) for 60 minutes, washed and developed using the SuperSignal® West Pico Enhanced Chemilunminescent kit. All the membranes were developed for 10 seconds, except the membrane with lipoteichoic acid (C) that was developed for 5 minutes.

4.3 Discussion

Components of the complement system were initially identified as possible candidates for the bactericidal element of the rabbit serum as previous work carried out in the laboratory identified mannose-binding lectin (MBL) and complement component C4b as proteins bound to purified cell wall material (Bozakouk, 2011). This hypothesis was supported by the inactivation of the antibacterial activity of the rabbit serum by several complement inactivation protocols such as the use of calcium chelating agents (Des Prez et al, 1975; Morgan, 2000; Schwaeble et al, 2002; Roos et al, 2003; Takahashi et al, 2006), heat treatment (Ecker et al, 1943a; Kerr and Porter, 1978; Servais et al, 1991; Morgan, 2000) and pre-incubation with zymosan A from *S. cerevisiae* (Smith et al, 1982; Morgan, 2000; Zhang et al, 2003).

However, these protocols were non-specific for the complement system and can also affect other elements present in the rabbit serum. Dr. Suzan Roiijakkers from University Medical Centre Utrecht (The Netherlands) performed experiments using specific inhibitors against the central complement components C3 and C5, and found that the bactericidal activity of the serum against *S. aureus* was not affected, suggesting that the killing activity was not mediated by complement (Personal Communication).

To clarify the role of complement, antibodies against the rabbit proteins were synthesized and used for inactivation experiments and to identify the components in the NRS. MBL and C4 were chosen due to the results observed previously in the laboratory (Bozakouk, 2011), and C5 and C9 were chosen due to their role in the formation of the membrane attack complex.

During the project the annotation of the predicted C5 sequence was altered to C3 (NCBI accession number XP_002722895.1). As explained previously the complement component C3 is as essential as C5, so this change in the protein that the antibody targets should not affect the experiments that were carried out to analyse the role of the complement system.

Anti-MBL, C4, C5 and C9 antibodies affected the bactericidal activity of the NRS. These results suggested that the lectin pathway of the complement was involved in the bacterial killing observed in rabbit serum. However, no interaction was found between the antibodies and the proteins present in the NRS as shown by the lack of signal when the rabbit samples were resolved by Western blot or when the 24-well slot blot system was used.

This lack of reactivity with rabbit proteins suggested that the peptides used to produce these antibodies were not the ones that were chosen. This means that the inactivation of the bactericidal activity observed in killing assays could be caused by interaction of the antibodies with proteins or other elements of the rabbit serum that were not identified because the real target for these antibodies is unknown.

The mass spectrometry analysis of the proteins bound to the different types of purified cell wall showed the absence of complement components bound to the affinity matrices capable of inactivating the NRS, proving that the complement system is likely not involved in the antibacterial activity of the NRS.

Once the complement system was discarded as responsible for the bactericidal effect of NRS, other methods such as fractionation by centrifugal filters were used to try to identify the ‘killing factor’. The antibacterial activity of each fraction of NRS was studied in order to distinguish which of them contained the bactericidal element, and also to clarify if the bactericidal activity was caused by one or more elements of the serum.

None of the fractions studied had bactericidal activity against the bacteria and only the fractions containing components ‘smaller than 30 kDa’ and ‘smaller than 50 kDa’ showed a bacteriostatic effect, suggesting that these fractions contain elements of the rabbit serum capable of affecting the bacterial growth but not concentrated enough to kill the bacteria as the whole serum does. This ‘killing element’ might be also present in other fractions but at a low concentration. The concentration of other elements necessary for the

bactericidal activity, such as small molecules like calcium or other proteins that may form a complex with the killing element, might also be affected.

To confirm the species specificity of the killing mechanism, human serum was also separated using these centrifugal filters. Bacterial growth was observed in all the fractions, and when rabbit and human fractions were mixed to reconstitute a 'chimeric' serum no bactericidal effect against *S. aureus* was observed in any case. This suggests that the phenomenon is NRS specific.

In the previous chapter it was shown that purified cell wall material from *S. aureus* acts as an affinity matrix that binds the component(s) present in NRS responsible for its bactericidal activity. When the protein profiles of NRS elements bound to the affinity matrices were studied, more proteins were found bound to the matrices capable of completely inactivating the serum than to the ones that caused only partial inactivation.

The mass spectrometry analysis of the proteins bound to stripped peptidoglycan, to peptidoglycan with WTA and to peptidoglycan from the mutant with WTA but lacking D-alanylation (Δ*dltA* strain) identified different proteins attached to each affinity matrix. Due to the ability of the unstripped peptidoglycan and the unstripped peptidoglycan from Δ*dltA* to completely inactivate the NRS, proteins present in both of these samples were examined in order identify the 'killing factor'.

One of the identified proteins was phospholipase A2 (PLA2), part of a superfamily of enzymes that can bind to membrane surfaces and hydrolyze membrane phospholipids at the sn-2 position (Berg et al, 2001; Koduri et al, 2002). They are present in several bodily fluids including tears and serum (Weinrauch et al, 1996; Qu and Lehrer, 1998). These enzymes play a role in inflammation and immune response (Nevalainen et al, 2008). It has been reported that these proteins have a bactericidal effect against Gram-negative (Harwig et al, 1995) and Gram-positive (Qu and Lehrer, 1998) bacteria, and that secreted phospholipase A2 (sPLA2) has a potent antibacterial activity against *S. aureus* (Weinrauch et al, 1996; Qu and Lehrer, 1998; Dominiecki and Weiss,

1999; Laine et al, 1999; Koprivnjak et al, 2002). It has also been reported that the activity of sPLA2 is affected by calcium concentration, heat and zymosan A (Daum et al, 1998; Paliyath et al, 1999; Santangelo et al, 1999; Merchant et al, 2011; Harboe et al, 2012), making sPLA2 a good candidate for the 'killing factor' present in NRS.

In order to have an effect on the phospholipids present in the cell membrane of Gram-positive bacteria, PLA2 needs to be able to recognize and bind to the bacterial cell wall and move through it in order to gain access to these phospholipids (Foreman-Wykert et al, 1999; Koprivnjak et al, 2008). This process can be separated into four steps: PLA2 binds to the bacterial surface, penetrates through the peptidoglycan, degrades the phospholipids present in the membrane and activates bacterial autolysins (Foreman-Wykert et al, 1999).

It has been reported that the initial binding of the phospholipase A2 to the cell surface of *S. aureus* is based upon electrostatic interactions between the protein and the bacterial cell surface (Foreman-Wykert et al, 1999; Koprivnjak et al, 2002). This protein is highly cationic, which allows it to interact and penetrate the anionic bacterial cell wall (Buckland et al, 2000; Beers et al, 2002; Koprivnjak et al, 2002). Lipoteichoic and wall teichoic acids are anionic molecules present in the bacterial cell wall that are capable of interact with this enzyme, helping PLA2 to reach the phospholipids in the membrane (Koprivnjak et al, 2002). This interaction between the teichoic acids and PLA2 might cause the inactivation of the bactericidal activity of the rabbit serum when pre-incubated with this purified components.

As the D-alanylation modification of the teichoic acids results in the addition of positive charges into the cell wall (Peschel et al, 1999; Koprivnjak et al, 2002; Fedtke et al, 2004), this modification also may affect the interaction of PLA2 with the cell surface of *S. aureus*. This could explain the important role of this modification in the survival of the bacteria in rabbit serum shown in the previous chapter (Chapter 3.2.8). It has been reported that the presence of D-alanylation increases 100-fold the bacterial resistance to sPLA2 (Koprivnjak et al, 2002; Hunt et al, 2006). The lack of this modification in the Δ*dltA* mutant might also

explain the total inactivation of the bactericidal activity observed when the rabbit serum was pre-incubated with purified cell wall from this strain: the positive PLA2 was able to interact more with this bacterial cell wall with negative net charge.

Cations such as magnesium and calcium are able to bind to the bacterial cell wall, with half of the binding sites in the polyphosphate groups of the teichoic acids and the other half in the carboxyl units of the peptidoglycan (Thomas and Rice, 2014). It seems plausible that the strain without WTA (Δ*tagO* mutant) binds less calcium ions on the bacterial surface than the parental strain, affecting the availability of this cation for the calcium-dependent secreted phospholipase A2 (Qu and Lehrer, 1998; Balsinde et al, 1999). The level of calcium available can also be affected by the D-alanylation modification of the teichoic acids (Koprivnjak et al, 2002). The absence of D-alanylation in the Δ*dltA* strain might increase the binding of calcium to the cell surface, increasing the availability for PLA2.

Once the PLA2 is bound to the bacterial surface, it needs to penetrate the peptidoglycan in order to reach the phospholipids present in the bacterial membrane. It has been previously shown that mutant strains of *S. aureus* lacking wall teichoic acids show resistance against the action of PLA2 due to the inability of the bound PLA2 to access and act on the phospholipids present in the bacteria (Koprivnjak et al, 2008). These results could explain why wall teichoic acid deficient mutants (Δ*tagO* strain) are resistant to the bactericidal activity of the NRS.

It has also been reported that the D-alanylation might have a role in the ability of PLA2 to reach the bacterial membrane (Koprivnjak et al, 2002; Hunt et al, 2006). The teichoic acids inserted into the peptidoglycan form a negatively charged network that connects the bacterial cell wall and the membrane (Neuhaus and Baddiley, 2003). In the Δ*dltA* mutant strain both WTA and LTA lack the D-alanine modification, turning this network more negative. This change in the charge of the ‘ladder’ used by PLA2 to reach the phospholipids might facilitate the penetration of the enzyme.

The role of PLA2 was supported by the use of a specific monoclonal antibody, which caused a complete inactivation of the bactericidal activity of NRS. The antibody was also able to specifically identify the protein in the serum obtained from different rabbits. Additionally, it was possible to relate the differential level of protein binding with the degree of inactivation caused by treatment with an specific affinity matrix, detecting a higher amount of PLA2 bound to the cell wall material that was able to completely inactivate the NRS than to the purifications that only caused a partial inactivation of the killing activity.

Together the evidence is compelling that secreted phospholipase A2 is the component of the rabbit serum responsible for its antibacterial activity against *S. aureus*. However, the results obtained from the fractionated serum experiments suggest that PLA2 is not the only element involved in this bactericidal activity. It is necessary to identify other possible elements in order to be able to explain the killing mechanism. Experiments using purified phospholipase A2 were carried out to confirm that this enzyme was able to kill *S. aureus* and to find the minimal system necessary for the bacterial killing. All of this will be explained in the next chapter.

5 Chapter 5:

Bactericidal mechanism of NRS against *S. aureus*

5.1 Introduction

In the previous chapters wall teichoic acid was identified as the bacterial component that acts as a likely receptor for the bactericidal activity of the rabbit serum, and secreted phospholipase A2 (sPLA2) as the element of the NRS responsible for the bacterial killing. However, experiments carried out using fractionated rabbit serum suggested that sPLA2 was not the only component involved in this bactericidal activity.

In order to explain the antibacterial mechanism that allows rabbit serum to kill *S. aureus*, it is necessary to identify any other components involved. These could affect activity of phospholipase directly or assist in interactions at the bacterial surface. The presence of these additional components could explain why the bactericidal activity against *S. aureus* is only observed with rabbit serum and not with other mammals.

Due to the involvement of PLA2 in many biological processes such as inflammation, immune response, reproduction and skin homeostasis (Balsinde et al, 1999; Lambeau and Gelb, 2008; Murakami et al, 2010; Murakami et al, 2013), and its association with several diseases including septic and allergic shock, rheumatoid arthritis, adult respiratory distress syndrome and pancreatitis (Scheuer, 1998; Murakami et al, 2010), PLA2 needs to be regulated. There have been reports showing that phosphorylation cascades (Lin et al, 1993; Qiu et al, 1993; Qiu et al, 1998), phosphatidylinositol 4,5-biphosphate levels (Monsior et al, 1998) and intracellular calcium elevations (Glover et al, 1995; Qiu et al, 1998) are able to regulate the activation of the cytosolic PLA2. It has also been reported that the calcium-independent PLA2 can be regulated by

different mechanisms, including ATP binding, caspase cleavage and possible ankyrin repeat mediated protein aggregation (Burke and Dennis, 2009; Dennis et al, 2011)

Calcium can affect the activity of the secreted phospholipase A2 (sPLA2) as its catalytic activity is dependent on this cation (Qu and Lehrer, 1998; Balsinde et al, 1999; Koprivnjak et al, 2002) and it has been reported that the levels of calcium in rabbit blood are higher than in other mammals, including humans (Brown, 1928; Redrobe, 2002; Rosenthal, 2006). However, as none of the NRS fractions studied in Chapter 4 had bactericidal activity on their own, but the reconstituted serum was able to kill *S. aureus*, there is another factor in the rabbit serum apart from calcium that is essential for the activity of the enzyme and the antibacterial activity of the NRS.

Pancreatic PLA2 is also regulated by proteolysis as it is synthesized in the acinar cells and secreted as a zymogen, only becoming active after trypsin cleavage of an N-terminal heptapeptide (Volwerk et al, 1984; Lenting et al, 1988; Scheuer, 1989; Murakami et al, 2010). However, this kind of irreversible activation has only been observed for pancreatic PLA2.

PLA2 is not only present in mammals, but also in the venom of bees, scorpions and snakes (Moreira et al, 2002; Lizano et al, 2003; Lambeau and Gelb, 2008; Petras et al, 2010; Stábeli et al, 2012). These venomous animals have to regulate not only the enzymatic activity of PLA2, but also its myotoxic, cytotoxic, cardiotoxic, edematogenic and anticoagulating activities (Faure, 2000; Machiah and Gowda, 2006; Stábeli et al, 2012; Harris and Scott-Davey, 2013). It has also been reported that animals resistant to snake venom such as the mongoose (*Herpestes edwardsii*) and the opossum (*Dedelphis marsupialis*) showed the presence of protein factors in their blood which inhibit the activity of venom components (Qi et al, 1994; Neves-Ferreira et al, 2000; Lizano et al, 2003), including metalloproteinase and PLA2 inhibitors (Perales et al, 1992; Fox and Bjarnason, 1998; Pérez and Sánchez, 1999; Lizano et al, 2003).

5.1.1 Aims of this chapter

- Analysis of the bactericidal activity of purified phospholipase A2 against *S. aureus*.
- Identification of the other component(s) of the rabbit serum involved in the bactericidal activity against *S. aureus*.
- Study of the interaction between phospholipase A2 and other NRS components.

5.2 Results

5.2.1 Effect of purified PLA2 on *S. aureus*

In Chapter 4 sPLA2 was identified as the component of the rabbit serum responsible for its bactericidal activity against *S. aureus*. These results were obtained using rabbit sPLA2 in the context of the rest of the rabbit serum. In order to further analyse the role of PLA2 purified material was used.

Purified sPLA2 from rabbit serum is unavailable and it was not practical to purify it due to the lack of availability of bulk NRS and the heat sensitivity of the killing factor. Thus the effect of other purified PLA2, that are commercially available, was tested. Recombinant human PLA2 was purchased from Abcam, whilst honey bee (*Apis mellifera*) and spitting cobra (*Naja mossambica mossambica*) venom PLA2 were purchased from Sigma-Aldrich.

The protein sequences of the rabbit, human and honey bee PLA2 were found in the NCBI Protein Database, but as no sequence for spitting cobra was available the sequence for PLA2 from Indian cobra (*Naja naja*) was used instead. Sequence analysis showed the domain corresponding to the PLA2 superfamily in all of them. All of the rabbit PLA2 catalytic residues were conserved in the human and cobra proteins, while only three of the five residues were conserved in the honey bee phospholipase (Figure 5.1). The pairwise sequence alignment using the EMBOSS Neddle program showed that the rabbit PLA2 shares more identity with the human protein (77.1%) than with the Indian cobra (48.7%) or the honey bee (18.6%) phospholipases.

The bactericidal activity of the recombinant human PLA2 (rhPLA2) and of the purified spitting cobra and honey bee venom PLA2 against *S. aureus* was tested first in buffer. It has been reported that the activity of PLA2 can be tested when bacteria are suspended in Purified Phospholipase A2 Buffer (Pernet et al, 2014). For these experiments a range of different concentrations of PLA2 were inoculated with *S. aureus* SH1000, after which killing assays were carried out as explained previously (Chapter 2.23).

Figure 5.1 Multiple sequence alignment between PLA2 from rabbit, human, Indian cobra and honey bee.

Alignment of the sequences for phospholipase A2 from rabbit (accession number XP_002719839.1), human (NP_000919.1), Indian cobra (AAA66029.1) and honey bee (NP_001011614.1) venom was carried out using the Multiple Sequence Analysis from Clustal Omega (previously ClustalW) using the EBLOSUM 62 matrix. Residues marked with an asterisk (*) are fully conserved; colon (:) indicates strongly conserved substitutions and full stop (.) indicates weakly conserved residue substitutions. The red squares show the catalytic residues of the rabbit phospholipase A2

Recombinant human PLA2 (Figure 5.2A) and honey bee PLA2 (Figure 5.2B) had no effect on bacterial numbers. Spitting cobra PLA2 led to bacterial death under the detection limit after 4 hours of incubation when 65 or 100 units of the enzyme were added to the PLA2 Buffer (Figure 5.2C). More detailed analysis was carried out to study the kinetics of bacterial death, revealing that 65 units/L of cobra PLA2 (final concentration) caused bacterial death after 2 hours (Figure 5.3).

5.2.2 Effect of purified spitting cobra venom (*Naja mossambica mossambica*) PLA2 (cvPLA2) on the bactericidal activity of NRS

The effect of the addition of this purified enzyme to the NRS was studied. For these experiments 65 units/L of cvPLA2 were added to aliquots of rabbit serum and the bactericidal activity of these mixtures against *S. aureus* was tested. Counterintuitively, when cvPLA2 was added to NRS, growth was observed rather than enhanced bacterial killing (Figure 5.4). This suggested that the added enzyme was interacting with the PLA2 or with another component of the serum, inactivating its bactericidal activity.

As explained in the previous chapter, the 100-fold increase in the bacterial numbers observed between 3 and 4 hours in figure 5.4 could be due to an error in the dilution process.

The same enhanced bacterial growth was observed when cvPLA2 was added to heat inactivated NRS when compared to the heated serum (Figure 5.5A). This suggests that the added cvPLA2 interacts with components of the serum that caused the bacteriostatic effect observed in the heated NRS. However, when cvPLA2 was mixed with human serum no significant differences were observed when compared to the effect of the human serum on *S. aureus* (Figure 5.5B).

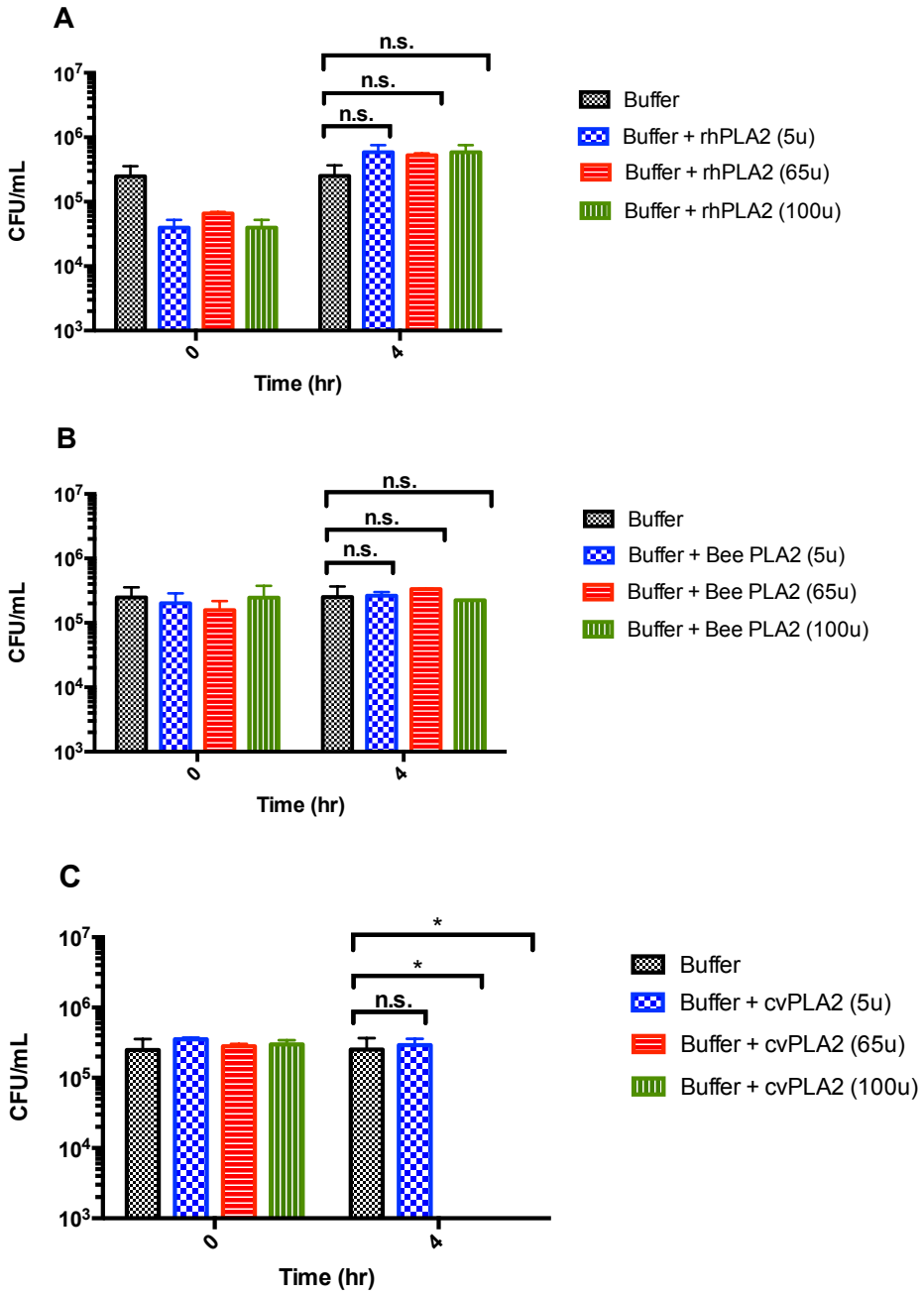


Figure 5.2 Effect of purified PLA2 on *S. aureus*

The effect of **A)** recombinant human PLA2, **B)** purified honey bee PLA2, or **C)** spitting cobra PLA2 (cvPLA2) on *S. aureus* SH1000 viability was tested (Chapter 2.9). Bacterial survival in buffer is also shown (black bars). The experiments were carried out in triplicate (i.e. biological triplicates) and the error bars represent the standard deviation of the means. Student's t-tests were carried out and the significant differences are marked with asterisks. n.s.: non-significant differences.

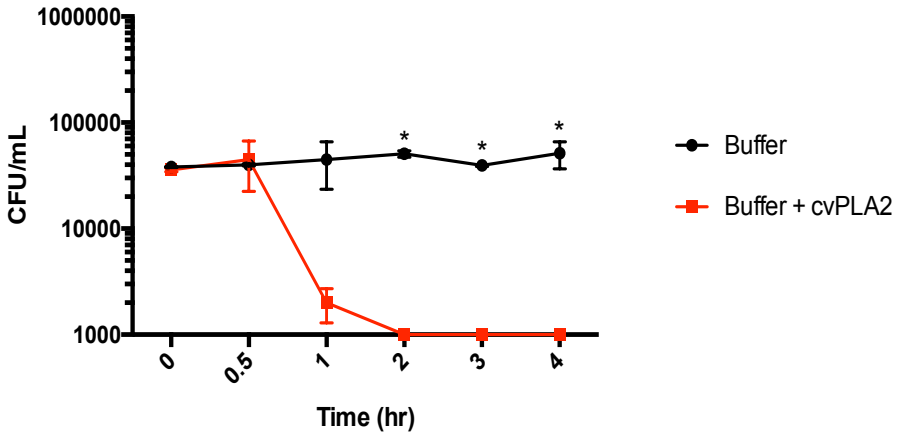


Figure 5.3 Effect of cvPLA2 on *S. aureus*

The effect of 65 units/L (final concentration) of cvPLA2 on 10^5 cells/ml of *S. aureus* SH1000 survival was tested and compared to PLA2 Buffer (black line). The experiments were carried out in triplicate (i.e. biological triplicates) and the error bars represent the standard deviation of the means. Student's t-tests were carried out and the significant differences are marked with asterisks.

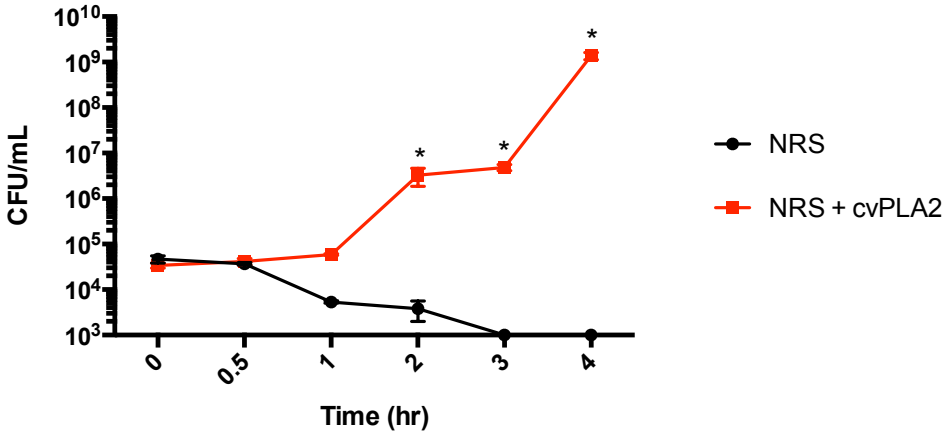


Figure 5.4 Effect of the addition of cvPLA2 on the bactericidal activity of NRS

The bactericidal effect of rabbit serum (black line) on 10^5 cells/ml of SH1000 was compared to the effect of the rabbit serum with 65 units/L of cvPLA2 (final concentration) added. These experiments were carried out in triplicate (i.e. biological triplicates). The error bars represent the standard deviation of the means. Student's t-tests were carried out and the significant differences are marked with asterisks.

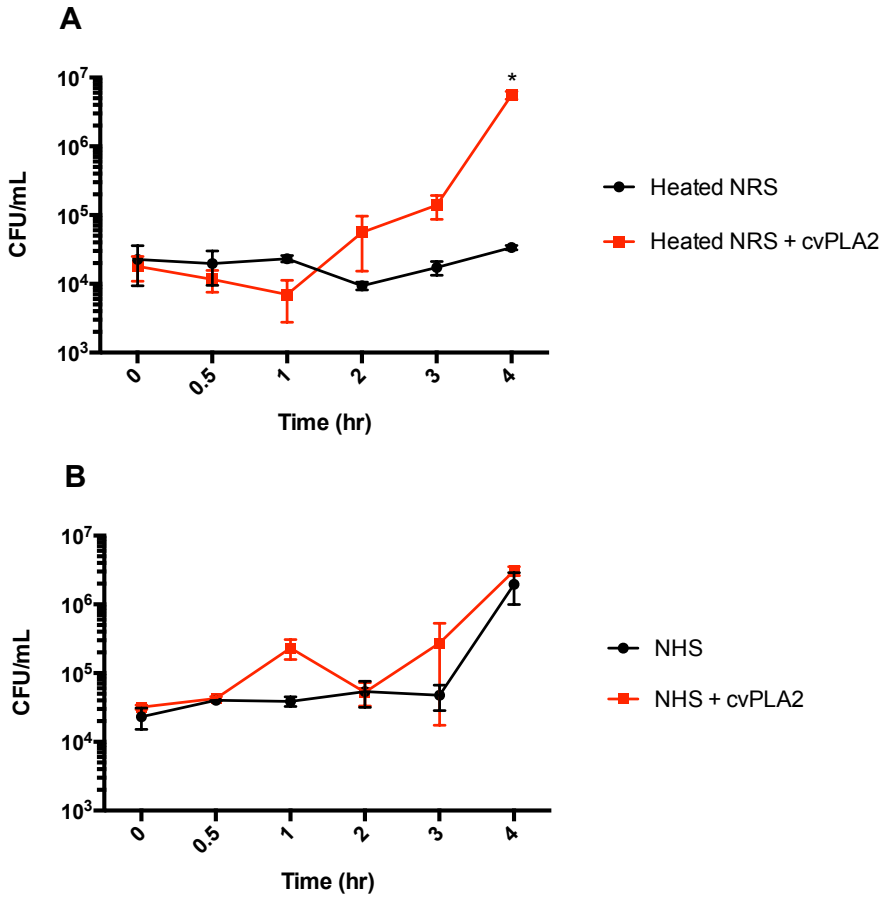


Figure 5.5 Effect of the addition of cvPLA2 on the bactericidal activity of heated NRS and human serum

65 units/L of cvPLA2 were added to **A)** Heated rabbit serum (30 minutes at 56ºC), and **B)** Human serum, prior to a *S. aureus* killing study using an initial inoculum of 10^4-10^5 cells/ml (Chapter 2.9). These experiments were carried out in triplicate (i.e. biological triplicates) and the error bars correspond to the standard deviation of the means. Student's t-tests were carried out and the significant differences are marked with asterisks.

5.2.3 Analysis of the bactericidal activity of heat-treated cvPLA2 when added to rabbit serum

In order to explain the enhanced bacterial growth observed when cvPLA2 was added to NRS and to heated NRS, experiments testing the activity of the enzyme were carried out. To test if this effect was caused by interactions between components of the rabbit serum and the active PLA2, the enzyme was inactivated before the start of the experiments. The enzyme was inactivated using the heat treatment protocol used to inactivate NRS: 56 ºC for 30 minutes.

Heat-treated cvPLA2 loses its bactericidal activity (Figure 5.6A). However, the enhanced bacterial growth was still present when the heat-treated cvPLA2 was added to both NRS and to heated NRS (Figures 5.6B and C, respectively). Thus cvPLA2 does not have to be bactericidally active in buffer to inactivate NRS.

5.2.4 Effect of boiled cvPLA2 on *S. aureus*

As cvPLA2 activity does not appear to be required for its inhibitory properties on NRS, the role of its overall structure was determined. cvPLA2 was boiled (100ºC) for 30 minutes to denature it.

Denaturated cvPLA2 had no bactericidal activity (Figure 5.7A). The mixture of NRS with the boiled enzyme was able to kill the bacteria to less than the detection limit (10^3 cfu/ml) after two hours of incubation (Figure 5.7B). No significant differences were observed when the boiled enzyme was added to heated rabbit serum or human serum compared to serum alone (Figure 5.7C and D, respectively). Thus the structure of the cvPLA2 is important for the interaction with rabbit components, not its activity.

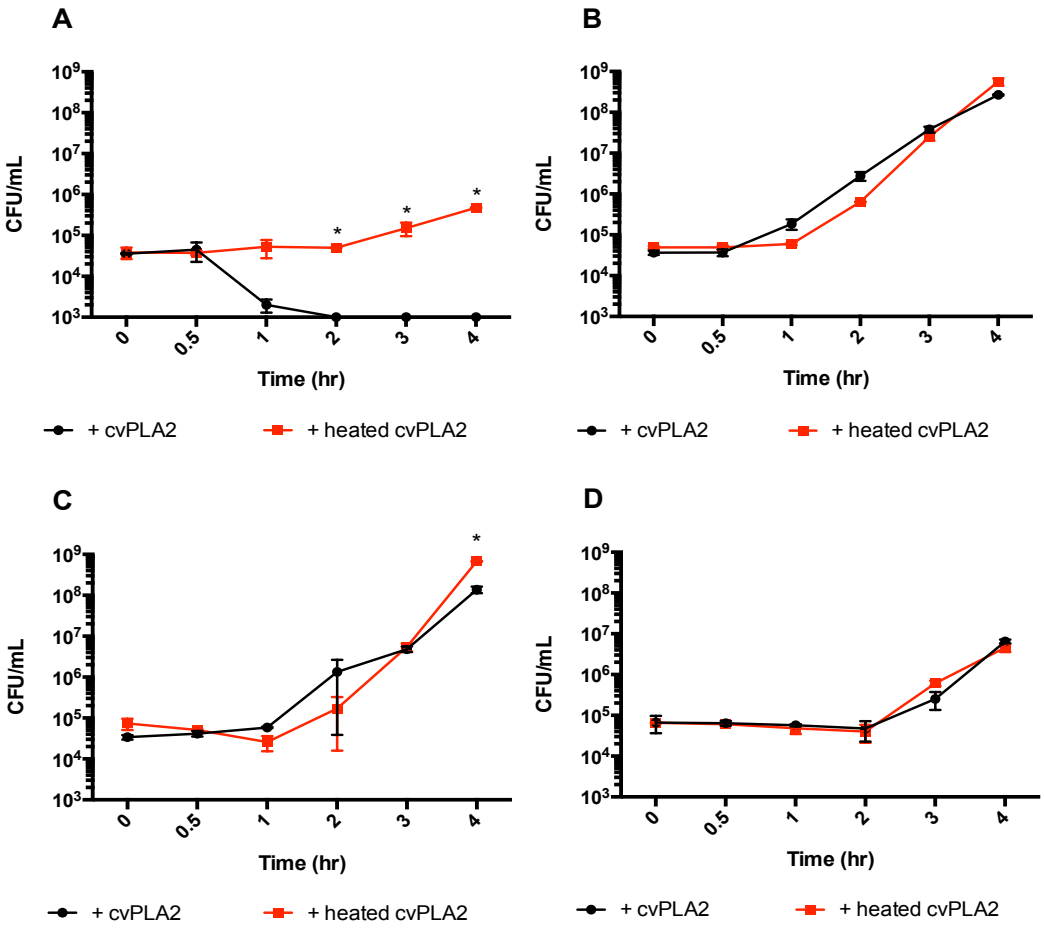


Figure 5.6 Effect heat-treated cvPLA2 on *S. aureus*

65 units/L of untreated (black line) or heat-treated (56ºC for 30 minutes) (red line) cvPLA2 was added to **A)** PLA2 Buffer, **B)** NRS, **C)** Heated NRS, **D)** Human serum. *S. aureus* SH1000 killing assays were carried out using an initial inoculum of 10^5 cells/ml (Chapter 2.9). The experiments were carried out in triplicate (i.e. biological triplicates). The error bars represent the standard deviation of the means. Student's t-tests were carried out and significant differences are shown with asterisks.

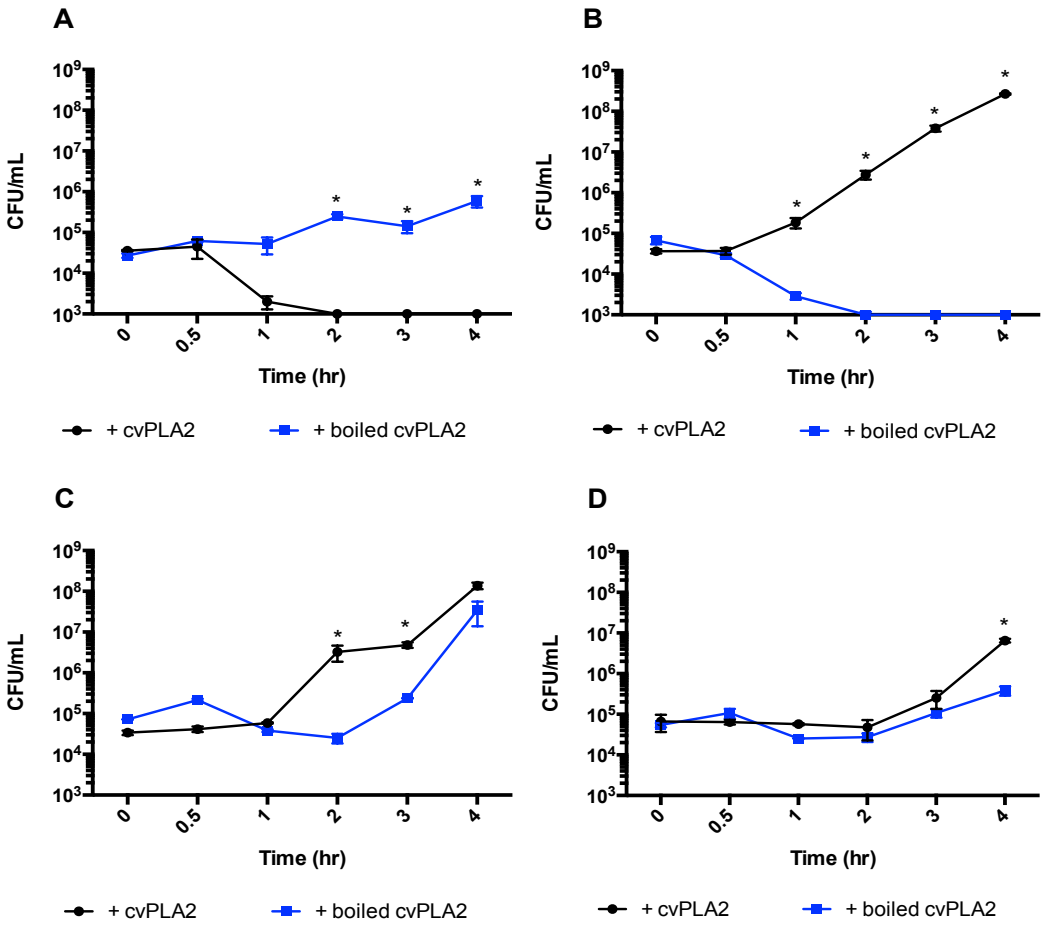


Figure 5.7 Effect boiled cvPLA2 on *S. aureus*

65 units/L of untreated (black line) or boiled (100ºC for 30 minutes) (blue line) cvPLA2 were added to **A)** PLA2 Buffer, **B)** NRS, **C)** Heated NRS, **D)** Human serum. S*. aureus* SH1000 killing assays were carried out using an initial inoculum of 10^5 cells/ml (Chapter 2.9). The experiments were carried out in triplicate (i.e. biological triplicates) and the error bars represent the standard deviation of the means. Student's t-tests were carried out and significant differences are shown with asterisks.

5.2.5 Effect of pre-incubation with cell wall material on the bactericidal activity of cvPLA2

The previous chapter (Chapter 4.2.6) demonstrated that other elements present in the rabbit serum affected the bactericidal activity of the phospholipase A2. Experiments using cvPLA2 confirmed these results, as the addition of the purified enzyme into the rabbit serum inactivates the bactericidal activity of both the NRS and the cvPLA2. This interaction occurs whether the purified PLA2 is active or not, but the structure of the PLA2 is necessary for the inactivation of the NRS. This led to the study of the role of the modulating factors present in the rabbit serum in order to confirm the hypothesis that these components might be involved in the interaction between the PLA2 and the bacterial surface.

Previously I have shown that purified cell wall material can be used as an affinity matrix to absorb PLA2 from NRS, and that cvPLA2 inhibits NRS bactericidal activity. In order to test if cvPLA2 interacts with cell wall it was pre-incubated with different purified cell wall fractions (Chapter 2.24), after which the mixture was centrifuged and the supernatant was used for killing assays in PLA2 Buffer. In all cases cvPLA2 activity was lost after pre-incubation with all types of cell wall material (Figure 5.8). The supernatant from Δ*dltA* cell wall absorbed material showed significantly less killing activity compared to other cell wall preparations ($p < 0.01$, Figure 5.8).

The effect of cvPLA2 absorbed with cell wall material on the bactericidal activity of NRS was also studied. The addition of the supernatant from all the pre-incubations led to partial inactivation of the activity of the rabbit serum and to bacterial growth after 4 hours of incubation (Figure 5.9). This was significantly less ($p<0.05$) than the inactivation caused by the untreated cvPLA2. Thus purified cvPLA2 binds to the different components of the bacterial cell wall, but not entirely in the conditions used.

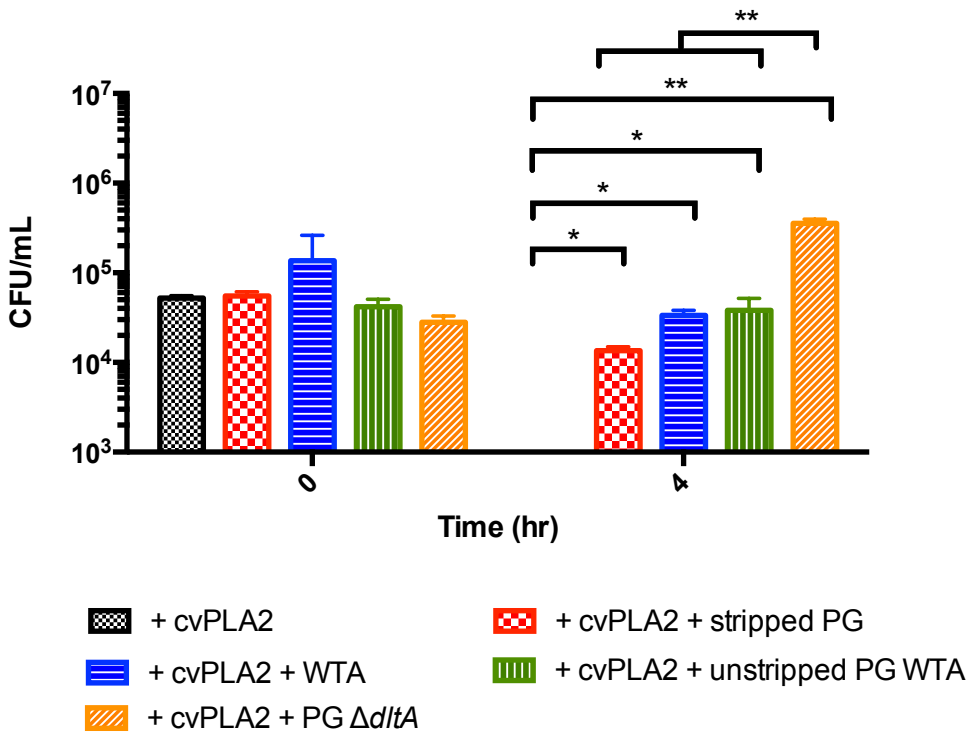


Figure 5.8 Effect of pre-incubation of cvPLA2 with cell wall material on its bactericidal activity.

65 units/L cvPLA2 (final concentration) was pre-incubated with 1mg/ml of stripped peptidoglycan (PG, red bars), pure wall teichoic acid (WTA, blue bars), unstripped cell wall (PG WTA, green bars) from SH1000 parental strain, and with unstripped cell wall from Δ*dltA* strain (PG Δ*dltA*, orange bars) for 30 minutes at 4ºC. Killing assays were carried out on supernatants after absorption and centrifugation as explained, using an initial inoculum of 10^5 cells/ml of SH1000 (Chapter 2.9). The experiments were carried out in triplicate (i.e. biological triplicates) and the error bars represent the standard deviation of the means. Student's t-tests were carried out and significant differences are marked with asterisks.

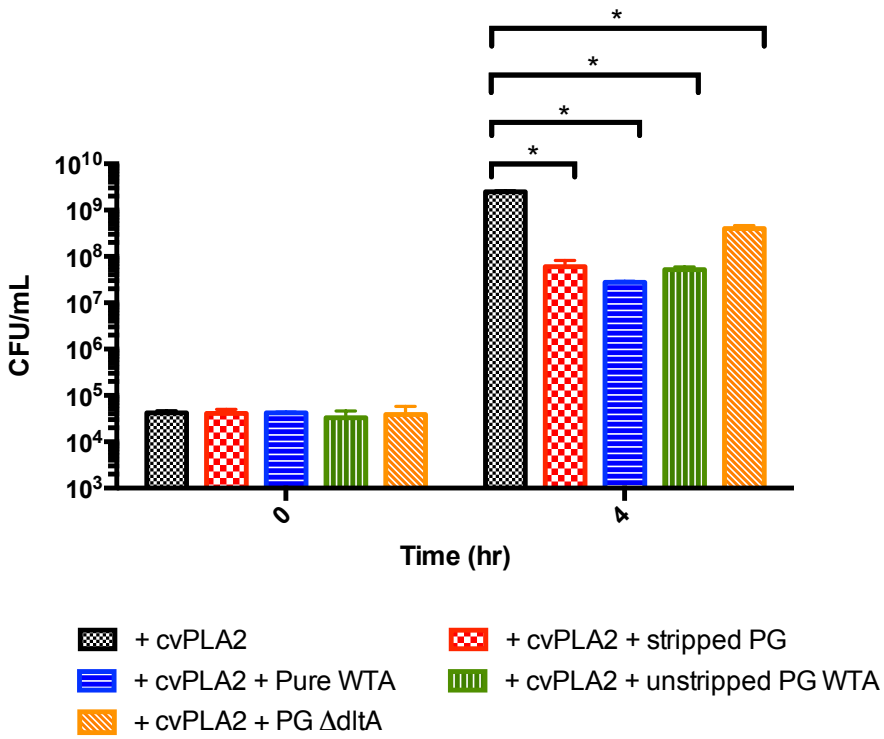


Figure 5.9 Effect of pre-incubation of cvPLA2 with cell wall material on killing activity in rabbit serum

65 units/L (43µg/ml) of cvPLA2 was pre-incubated with stripped peptidoglycan (PG, red bars), pure wall teichoic acid (WTA, blue bars), unstripped cell wall (PG WTA, green bars) from the SH1000 parental strain, and with unstripped cell wall from a Δ*dltA* strain (PG Δ*dltA*, orange bars) as previously explained (Chapter 2.24). Killing assays were carried out on supernatants after centrifugation, using an initial inoculum of 10^5 cells/ml of SH1000 (Chapter 2.9). The experiments were carried out in triplicate (i.e. biological triplicates). The error bars represent the standard deviation of the means, Student's t-tests were carried out and significant differences are marked with asterisks.

5.2.6 Fluorescence microplate assay for functional characterization of PLA2 activity

An explanation for the bactericidal activity of rabbit serum against *S. aureus* can be found in the difference in concentration and the catalytic activity of PLA2 from rabbits when compared to other mammals. As stated previously, it has been reported that rabbits have higher levels of this enzyme in comparison to humans (Kudo et al, 1993; Weinrauch et al, 1996; Weinrauch et al, 1998; Nevalainen et al, 2008), and that the rabbit PLA2 is also more catalytically active than the human protein (Aufenanger et al, 1993; Zhang et al, 2005; Nevalainen et al, 2008).

Results presented in Chapter 5.2.1 showed that cvPLA2 was able to kill *S. aureus* when the concentration used was similar to that found in rabbit serum (65 units/L), but could not when a human equivalent concentration was used (5 units/L). Human and rabbit serum phospholipase A2 activity was measured using fluorogenic substrates. Colorimetric and fluorometric assays have been used to study phospholipase activity (Huang et al, 2006; Price, 2007), including the activity of PLA2 present in alligator serum using fluorometric assays and BODIPY incorporated into *E. coli* (Merchant et al, 2009). This led to the study of the activity of the phospholipase A2 present in rabbit and in human sera via fatty acid digestion using dye labelled PLA2 substrate incorporated into liposomes (Rahman et al, 2010; Tsao et al, 2015).

For the determination of phospholipase activity, liposomes were synthesised using 1,2-Dioleoyl-sn-glycero-3-phosphocholine (DOPC), 1,2-Dioleoyl-sn-glycero-3-rac-(1-glycerol) (DOPG) and glycerophosphocholines labelled in the sn-2 position with BODIPY (boron-dipyrromethene) (Figure 5.10) following the instructions explained in Chapter 2.23. These liposomes were then mixed with aliquots of rabbit and human serum and incubated at 37ºC. The fluorescence of each mixture was measured using an excitation wavelength of 485 nm and an emission wavelength of 535 nm in a Victor^{TM} X3 2030 Multilabel Reader

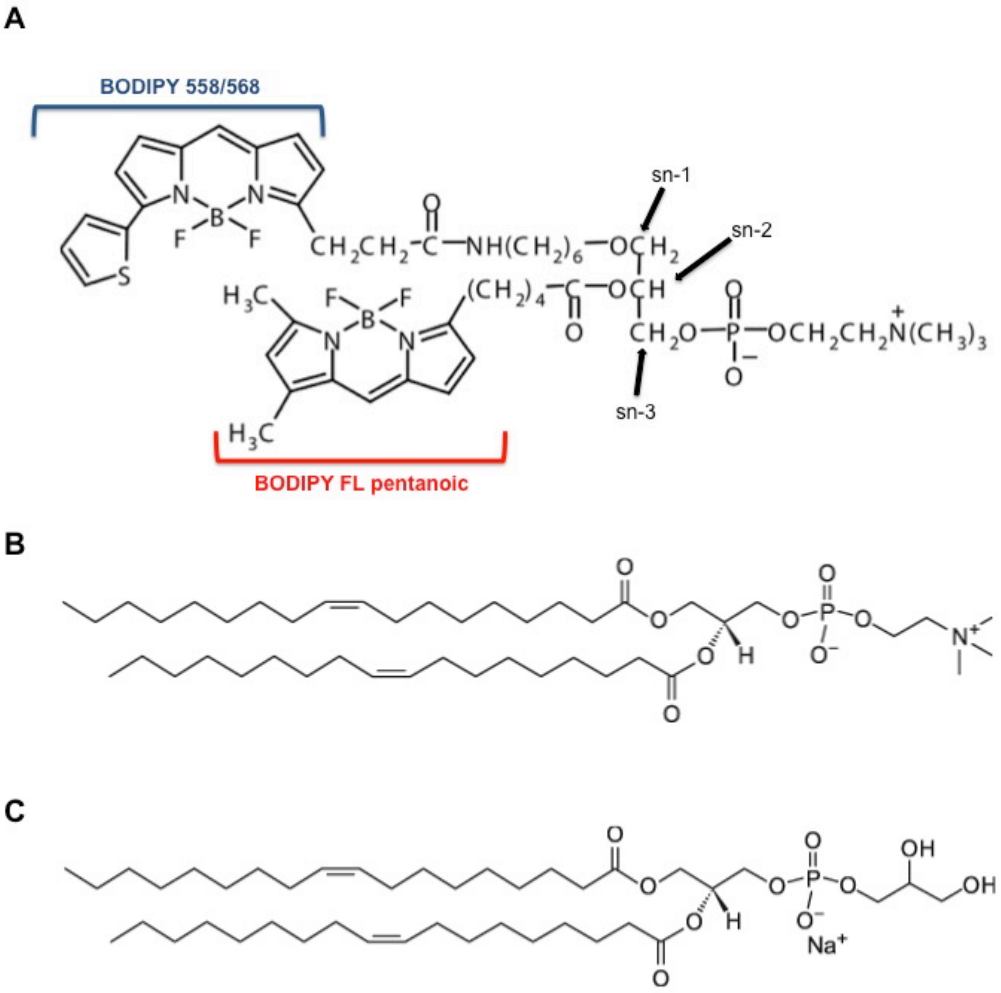


Figure 5.10 Structure of the BODIPY substrate and both lipids used for liposome synthesis

A. Structure of the BODIPY labelled glycerophosphocoline. The BODIPY® 558/568 attached at the sn-1 position and the BODIPY® FL pentanoic are shown. Adapted from 'Fluorogenic Phospholipase A Substrates' manual from Invitrogen (Thermo Fisher Scientific).

B. 1,2-Dioleoyl-sn-glycero-3-phosphocholine (DOPC)

C. 1,2-Dioleoyl-sn-glycero-3-rac-(1-glycerol) (DOPG)

Lipid structures modified from the Avanti® Lipids webpage (http://www.avantilipids.com/)

(Perkin Elmer). The cleavage of the labelled glycerophosphocholine in sn-2 position by PLA2 results in an increase in the BODIPY® FL fluorescence emission detected at 535 nm due to a decrease in the quenching by fluorescence resonance energy transfer (FRET) to the BODIPY ® 558/568 present in the sn-1 position (Figure 5.10A). PLA2 specific activity was expressed as the change in the fluorescence intensity per minute per µl of the PLA2 containing sample (Cai et al, 2013). Even though the fluorescence was measured at 30 minutes intervals for 4 hours, the specific activity was calculated using the change in the fluorescence intensity in the first 60 minutes as this corresponds to the initial rate period.

Significant differences in the fluorescence intensity were observed between the rabbit and human serum from 30 minutes of incubation until the end of the experiment ($p<0.001$, Figure 5.11A), and comparison of the specific activities of the PLA2 present in the serum also showed significant differences ($p<0.001$, Figure 5.11B).

The catalytic activity of inactivated rabbit serum was also examined. Aliquots of rabbit serum were inactivated using heat treatment and by adding a calcium chelating agent (EGTA) to the heated serum as explained previously (Chapter 2.10.1 and 2.10.2, respectively), and the fluorescence intensity was measured (Chapter 2.23). Inactivation of the NRS using heat led to a 66% decrease in the specific PLA2 activity, whereas heat followed by EGTA pre-incubation caused a 92% decrease in the specific phospholipase activity (Figure 5.12).

It has been reported that the bactericidal activity of rabbit serum is lost when diluted 4-fold in sterile PBS (Bozakouk, 2011). The phospholipase activity of the diluted serum was examined and no significant difference between the undiluted NRS and the diluted samples was found until the rabbit serum was diluted 10^{-2}-fold ($p<0.01$, Figure 5.13). Thus the loss of killing activity does not correlate directly with PLA2 activity hinting that other dilution dependent factors are found in the serum.

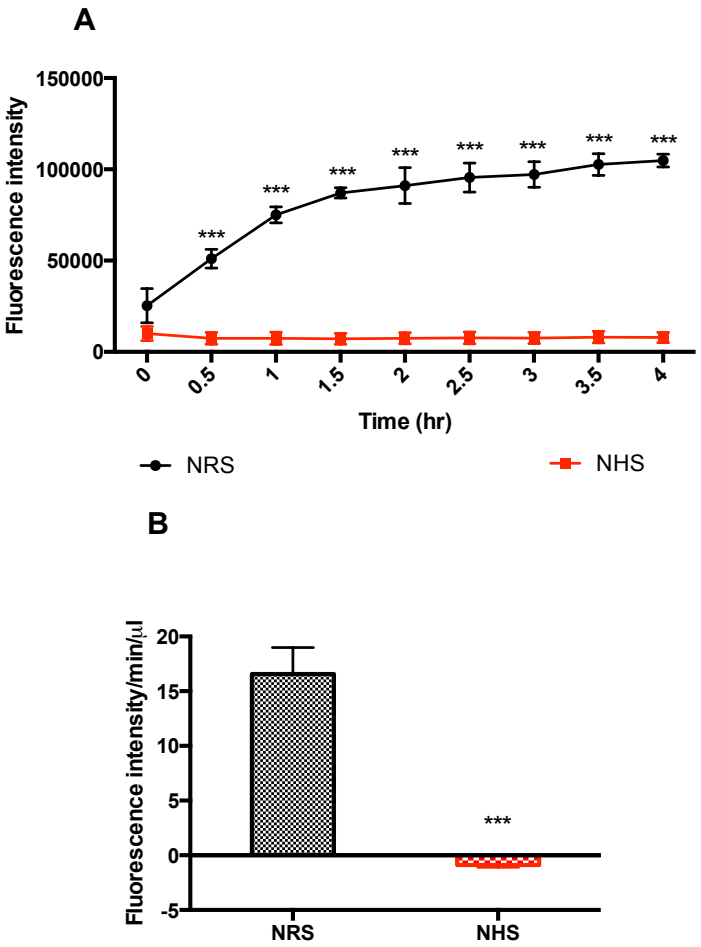


Figure 5.11 Comparison of the phospholipase activity of rabbit and human serum **A)** Progress curves, and **B)** Specific activity for PLA2 rabbit and human serum. 50 µl of BODIPY® labelled liposomes were mixed with 50 µl of rabbit (black) or human (red) serum, and the fluorescence intensity of the mixtures was measured every 30 minutes at 535 nm as explained (Chapter 2.23). The curves represent the mean of three independent experiments. The error bars correspond to the standard deviation of the means and significant differences are marked with asterisks ($p<0.001$, Student's t-test).

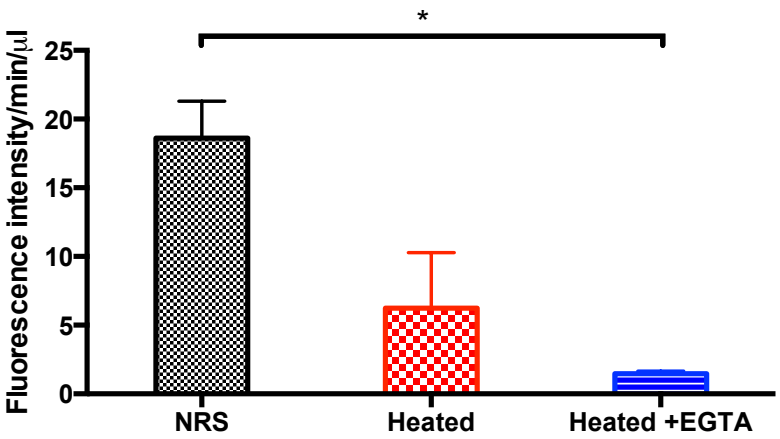
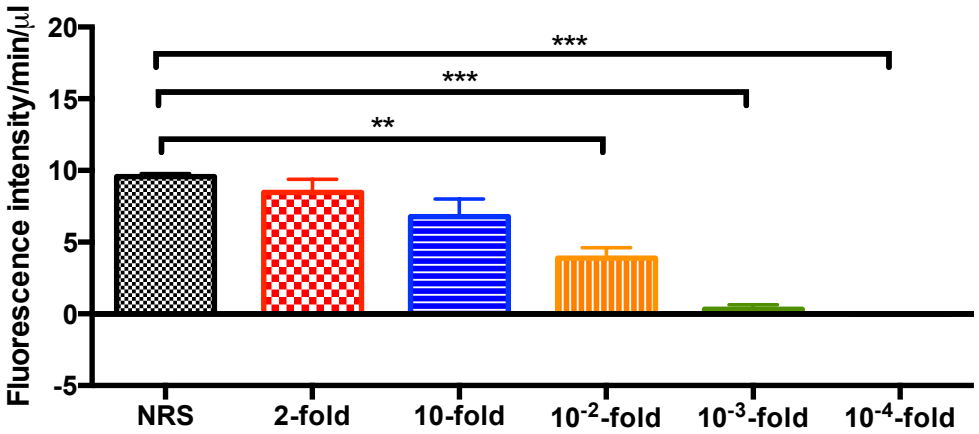


Figure 5.12 Specific phospholipase activity of inactivated rabbit serum

Heat inactivated NRS (red bar) and heat inactivated NRS pre-incubated with EGTA (blue bar) were tested for PLA2 activity (Chapter 2.23). The fluorescence intensity registered was used for the specific activity calculations, and these values were compared to untreated NRS (black bar). Significant differences are shown with asterisks ($p<0.05$, Student's t-test). These experiments were carried out in triplicate and the error bars represent the standard deviation of the means.



	NRS	2-fold	10-fold	10^{-2}-fold	10^{-3}-fold	10^{-4}-fold
Specific activity (%)	100	88.3	70.7	40.5	3.5	0.0

Figure 5.13 Specific phospholipase activity of diluted NRS

Aliquots of dye labelled liposomes were mixed with 50 µl of samples containing different dilutions of rabbit serum. The fluorescence intensity of the mixture was measured every 30 minutes at 535nm as explained (Chapter 2.23). The specific activity of the diluted samples was compared to NRS (black bars) and significant differences are shown with asterisks ($p<0.01$ and $p<0.001$, Student's t-test). These experiments were carried out in triplicate and the error bars represent the standard deviation of the means.

Next, the activity of the different purified PLA2s was measured. For these purified PLA2s, the specific activity was calculated as the fluorescence intensity per minute per µg of protein. Human recombinant PLA2 showed no activity, whereas both the honey bee and spitting cobra PLA2 had significantly higher activity than rabbit serum ($p<0.001$, Figure 5.14). By comparing the specific activities of NRS and cvPLA2 it was possible to determine that the NRS has 12.04 µg/L of sPLA2. This value is within the range of sPLA2 in serum measured for mammals (Nevalainen et al, 1985; Dorsam et al, 1995).

5.2.7 Role of annexin A1 in the bactericidal activity of PLA2

Experiments using fractionated rabbit serum suggested that sPLA2 was not the only serum component involved in the bactericidal mechanism against *S. aureus* (Figures 4.13 and 4.14). This hypothesis was supported by the study of phospholipase activity of diluted rabbit serum. Whilst bactericidal activity of NRS is lost when the serum is diluted 4-fold in sterile PBS, PLA2 activity was only lost by >100-fold dilution.

Due to the large number of proteins present in serum (Pieper et al, 2003; Chiaradia et al, 2012), the search for this extra factor involved in the antibacterial activity of NRS started with the list of proteins identified by mass spectrometry analysis of the serum proteins bound to affinity matrices (Chapter 4.2.7). As PLA2 was present in the bound proteins analysed by mass spectrometry, it was speculated that the new factor capable of modulating PLA2 could also be found in these fractions. Amongst these proteins identified by mass spectrometry was annexin A1.

The annexins are a family of proteins that are capable of binding to phospholipids in a calcium-dependent manner (Parente and Solito, 2004; Rescher and Gerke, 2004). This family contains hundreds of gene products expressed in most species including insects, plants, protozoa and vertebrates

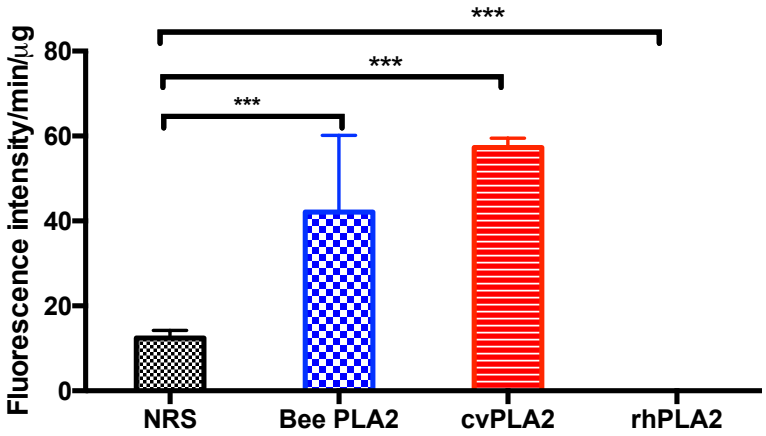


Figure 5.14 Comparison of the specific phospholipase activity of NRS and purified PLA2

The fluorescence intensity for samples containing honey bee, spitting cobra and recombinant human PLA2 was determined (Chapter 2.23). The specific phospholipase activity of these samples was compared to the equivalent specific activity of NRS (12.04µg/L, black bar) and significant differences are shown with asterisks ($p<0.001$, Student's t-test). These experiments were carried out in triplicate and the error bars correspond to the standard deviation of the means.

(Morgan and Fernandez, 1997; Rand, 2000; Rescher and Gerke, 2004). Twelve different annexins have been described in humans (I-XI and XIII) (Gerke and Moss, 2002).

These proteins have two main domains: an N-terminal region, diverse in sequence and length, responsible for the regulation of the interaction with other proteins and the membrane (Raynal and Pollard, 1994; Gerke and Moss, 2002; Rescher and Gerke, 2004); and the conserved C-terminal region, also called 'annexin core domain', which possesses the calcium and the membrane binding sites (Gerke and Moss, 2002; Rescher and Gerke, 2004). The annexins are involved in several intracellular and extracellular processes, including cell division, apotosis, regulation of the activity of ion channels, vesicle trafficking, phagocytosis, growth regulation, calcium signalling, binding of molecules to the cell surface, coagulation and control of inflammatory responses (Gerke and Moss, 2002; Hayes and Moss, 2004; Gerke et al, 2005).

Annexin A1 (Anx-A1) is capable of binding to negatively charged phospholipids in a pH- and calcium- dependent manner (Rosengarth et al, 2001; D'Acquisto et al, 2008), and it is involved in the regulation of the innate and adaptive immune responses. This protein inhibits phagocytosis (D'Acquisto et al, 2008), mast cells degranulation (Bandeira-Melo et al, 2005) and polymorphonuclear leukocyte trafficking and adherence (Chatterjee et al, 2005; D'Acquisto et al, 2008). It also prevents the adhesion of monocytes to endothelial cells (Solito et al, 2000). Annexin A1 can promote T-cell proliferation, inflammatory responses and Th1 differentiation, but can also inhibit Th2 differentiation (D'Acquisto et al, 2007; D'Acquisto et al 2008).

Annexin A1 is also involved in the modulation of the inflammatory responses through the regulation of the activity of PLA2 (Parente and Solito, 2004; D'Acquisto et al, 2008). It has been reported that annexin A1 is able to inhibit the activity of cytosolic PLA2, but the mechanism involved in this is not clear. While some studies showed that the inhibition occurs through substrate depletion (Davidson et al, 1987), others reported that annexin A1 interacts directly with this phospholipase (Kim et al, 1994; Lim and Pervaiz, 2007). Due

to the known regulatory role of annexin A1 on cPLA2, this protein was chosen as the first serum element to test for a role in the bactericidal mechanism of sPLA2 present in NRS against *S. aureus*.

To elucidate the role of the annexin A1 in the activity of sPLA2, experiments were carried out using purified human protein (Abcam). First, it was confirmed that annexin A1 had no bactericidal activity against *S. aureus* on its own as no significant difference was found between PLA2 Buffer alone and PLA2 Buffer with the protein (Figure 5.15).

Next, the effect of annexin A1 on the bactericidal activity of cvPLA2 was tested. Aliquots of PLA2 buffer were inoculated with 65 units/L and with 32.5 units/L of cvPLA2 and the bactericidal activity of these solutions was tested. As shown previously, 65 units/L of this PLA2 was enough to decrease the bacterial count lower than the detection limit, whilst 32.5/L units had no bactericidal effect (Figure 5.16A and B, respectively).

No effect was observed when the protein was added to the aliquot of PLA2 Buffer containing 65 units/L of cvPLA2 (Figure 5.16A). However addition of annexin to 32.5 units/L of cvPLA2 gave a significant increase in the bactericidal activity against *S. aureus* ($p<0.01$, Figure 5.16B). These results, along with those showed in Figure 5.2C, suggested that there is a critical concentration of cvPLA2 that needs to be present in order to kill *S. aureus*. A 2-fold dilution of cvPLA2 allows bacterial survival, but the presence of annexin A1 increases the bactericidal activity by lowering the threshold needed to kill the bacteria.

Native polyacrylamide gels electrophoresis was used to examine any direct interaction between annexin A1 with cvPLA2 using antibodies against annexin A1. Secreted PLA2 has a molecular mass of 14-16 kDa (Schaloske and Dennis, 2006; Dennis et al, 2011) and annexin A1 has a molecular mass of 37 kDa (Parente and Solito, 2004; Lim and Pervaiz, 2007). cvPLA2 can be found as dimers and other oligomers in the presence of calcium (Plückthun and Dennis, 1985; Jabeen et al, 2005; Dennis et al, 2011). It has been proposed that

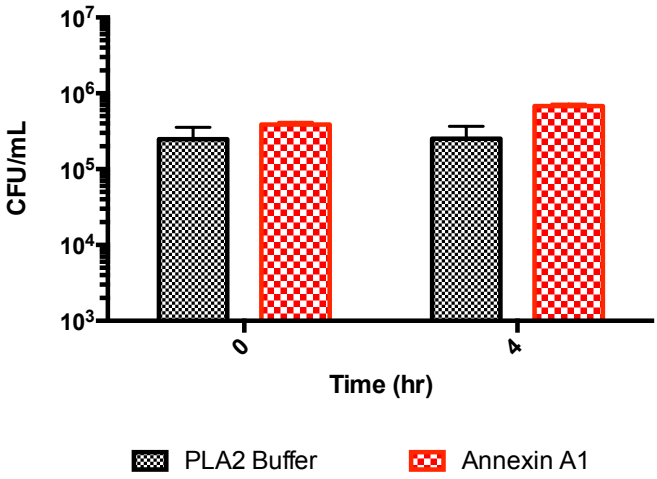


Figure 5.15 Effect of annexin A1 on *S. aureus* viability

The effect of 1.5µl of human annexin A1 (50µg/ml final concentration) on 10^5 cells/ml of *S. aureus* SH1000 viability was measured. The experiments were carried out in triplicate (i.e. biological triplicates) and the error bars represent the standard deviation of the means. Student's t-tests were carried out and no significant differences were found.

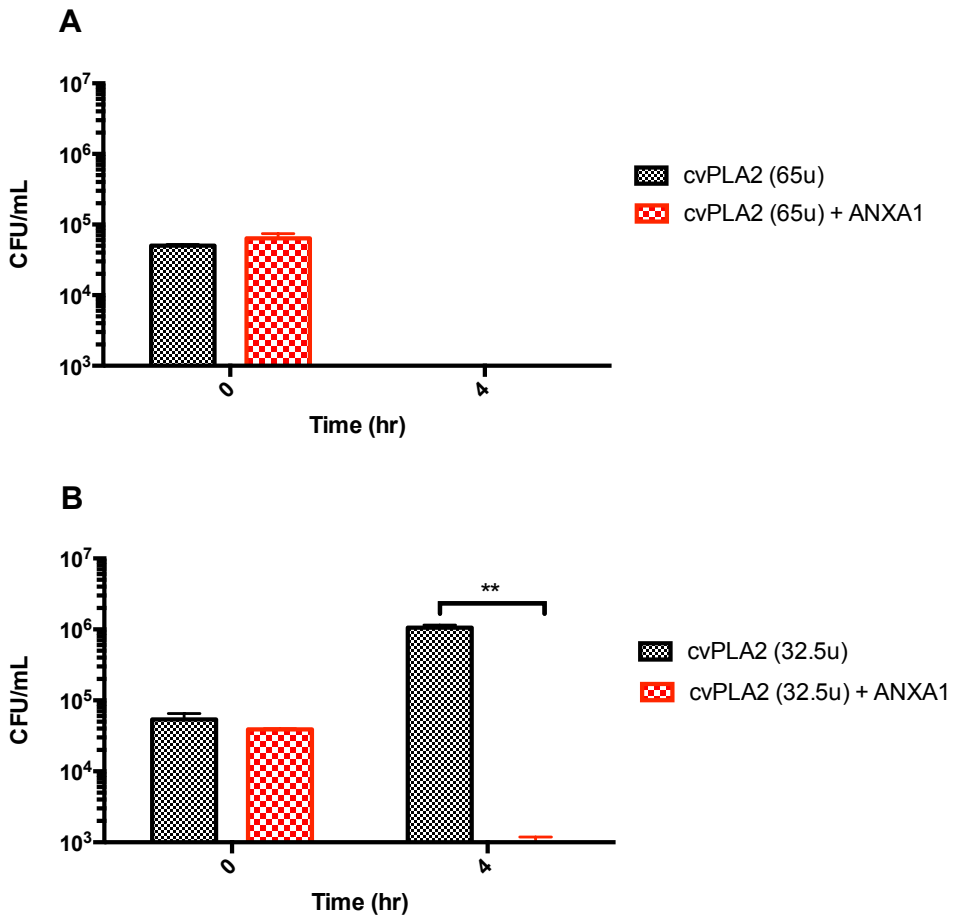


Figure 5.16 Effect of purified annexin A1 on cvPLA2 bactericidal activity on *S. aureus*

A. 65 units (43µg/ml) and **B.** 32.5 units (21.5µg/ml) of cvPLA2 were added to PLA2 Buffer in presence or absence of 1.5µl of annexin A1 (50µg/ml final concentration) prior to an *S. aureus* SH1000 killing assay using an initial inoculum of 10^5 cells/ml. These experiments were carried out in triplicate (i.e. biological triplicates) and significant differences are shown with asterisks (Student's t-test, $p<0.01$). The error bars represent the standard deviation of the means.

annexin A1 forms a dimer causing membrane fusion between cells (Rescher and Gerke, 2004; Donato et al, 2013; Lizarbe et al, 2013). 50 µg/ml of annexin A1 and 50 µg/ml of PLA2 were mixed and incubated at 37ºC for 30 minutes before native separation. In order to achieve protein migration in native gels, the isoelectric point of each protein (4.5-5.0 for cvPLA2 and 6.5 for annexin A1) was taken into consideration. After electrophoresis, the gels were silver stained (Chapter 2.21.5) and a Western blot with anti-annexin A1 antibodies performed (Chapter 2.21.7). Silver staining confirmed the presence of the proteins (data not shown), whilst the western blot showed a single band for annexin A1 (Figure 5.17). This band was shifted in the presence of PLA2 indicating an interaction between the two proteins.

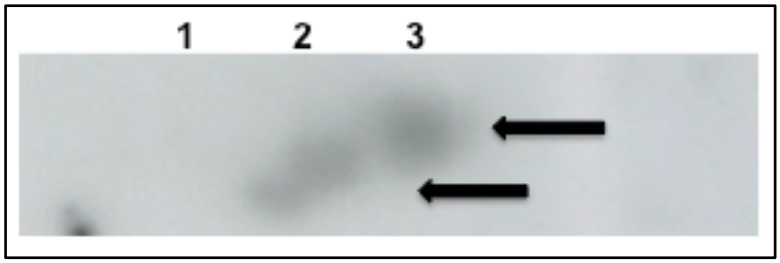


Figure 5.17 Native gel electrophoresis of annexin A1 and cvPLA2

Aliquots of 50 µg of cvPLA2 (lane 1), human annexin A1 (lane 2) and a mixture of both proteins (lane 3) were analysed by 8% (w/v) native polyacrylamide gel electrophoresis and Western blot with anti-annexin A1 antibodies. The arrows show the location of each band.

5.3 Discussion

In the previous chapter the role of the secreted phospholipase A2 (sPLA2) in the bactericidal activity of NRS against *S. aureus* was identified. However, as these results were obtained within the context of complete rabbit serum, experiments were carried out using purified PLA2 to determine its specific bactericidal activity.

As rabbit phospholipase A2 is not commercially available, recombinant human phospholipase A2 and sPLA2 purified from spitting cobra and honey bee venom were used. Due to differences in both the concentration and catalytic activity of the PLA2 present in human and rabbit serum (Aufenanger et al, 1993; Kudo et al, 1993; Zhang et al, 2005; Nevalainen et al, 2008), experiments were carried out using the three types of PLA2 at different concentrations. This showed that whilst human and bee PLA2 had no effect on the bacteria, 65 units (43 µg/ml) of spitting cobra PLA2 (cvPLA2) was able to kill *S. aureus* with similar kinetics to NRS, causing a decrease in the bacterial count lower than the detection limit of the experiment after 2 hours of incubation (Figure 5.3).

As the specific phospholipase activities of honey bee and cvPLA2 obtained were similar (Figure 5.14), this suggests that the lack of bactericidal activity against *S. aureus* observed for the honey bee PLA2 (Chapter 5.2, figure 5.2B) is not due to the absence of catalytic activity but might be caused by differences in affinity of the spitting cobra and the honey bee enzymes for the bacterial cell wall.

It seems plausible that the bactericidal activity of the rabbit serum against *S. aureus* could be attributed to the activity of phospholipase A2. Even though this enzyme is present in the serum of both humans and rabbits, the difference in the activity of the PLA2 present in NRS may be the key. This hypothesis was supported when the specific phospholipase activity of rabbit and human serum was compared, as the human serum had significantly less specific PLA2 activity than the rabbit serum ($p<0.001$; Figure 5.11B).

However, the addition of cvPLA2 to NRS led to bacterial growth instead of the expected enhanced bacterial killing, suggesting that the cobra enzyme interacts with some component of the rabbit serum and inactivates not only its own activity but also the bactericidal activity of the NRS. As the cvPLA2 used was purified from spitting cobra venom, it is also possible that the purification not only contains cvPLA2 but also contaminants or peptides that might influence the activity of the components of the NRS responsible for the killing of *S. aureus* (Zhao and Kinnunen, 2003; Samy et al, 2007). Further experiments showed that the structure of the cvPLA2 was important for the interaction with the rabbit serum components, not its activity.

This interaction was specific for rabbit serum, as the addition of cvPLA2 to human serum had no effect on the serum. This supported the hypothesis that species-specific components of the rabbit serum are able to interact with the PLA2 and affect its activity, which might explain why rabbit serum is the only serum with bactericidal activity against *S. aureus*.

The importance of the interaction of the PLA2 with other factors present in the rabbit serum was also supported by the study of phospholipase activity of diluted rabbit serum. The bactericidal activity of the NRS disappears when the serum is diluted 4-fold, whereas significant differences in the specific phospholipase activity are found only when undiluted serum is compared to 100-fold diluted NRS ($p<0.01$; Figure 5.13). The loss of bactericidal activity by 4-fold dilution may therefore be caused by a dilution of other components present in the serum that are necessary for PLA2 killing.

In the previous chapter the importance of the interaction of the PLA2 with the bacterial cell wall for its bactericidal activity was shown extensively by the use of different cell wall fractions as affinity matrices. It has been widely reported that cell wall interaction corresponds to the first step of activity of PLA2 (Foreman-Wykert et al, 1999; Koprivnjak et al, 2008). Experiments were carried out to study the ability of the purified cvPLA2 to bind to different cell wall components, as this could shed light on the requirements for other factor in PLA2 activity. This showed that whilst the unidentified components of the rabbit serum are

involved in PLA2 activity, they are not required for cell wall binding (Figures 5.8 and 5.9).

The work also highlighted potential differences in the affinity of cvPLA2 and sPLA2 in rabbit serum for the cell wall components. Whilst the pre-incubation of cvPLA2 with pure peptidoglycan, pure WTA and peptidoglycan with WTA from *S. aureus* SH1000 caused a similar level of inactivation of the bactericidal activity of this purified PLA2 (Figure 5.8), pre-incubation of NRS with pure peptidoglycan and pure WTA caused partial inactivation (Figure 3.17A and 3.18). Total inactivation was caused by treatment with peptidoglycan with WTA (Figure 3.17B).

Other factors interacting with PLA2 activity are also likely to show affinity for cell wall fractions. The mass spectrometry analysis of the serum proteins bound to the different types of cell walls provided a list of proteins as candidates for PLA2 modulators.

One of the proteins was annexin A1, a known regulator of the activity of phospholipase A2 (Parente and Solito, 2004; D'Acquisto et al, 2008). It has been reported that annexin A1 is able to inhibit the activity of cytosolic PLA2 through either substrate depletion (Davidson et al, 1987) or direct interaction with this phospholipase (Kim et al, 1994; Lim and Pervaiz, 2007). However, even though annexin A1 plays an inhibitory role against against the cytosolic PLA2 and modulates the inflammatory response (Parente and Solito, 2004), the presence of annexin A1 in the cell wall material purified from the Δ*dltA* strain suggested a different role. As explained previously, the Δ*dltA* mutant is much more sensitive to the bactericidal activity of NRS than its parent and pre-incubation of the serum with cell wall material purified from this mutant completely inactivates the serum. This led to the hypothesis that the bactericidal elements present in the rabbit serum have more affinity for the cell wall of this mutant than for the parent strain, which is supported by the literature (Koprivnjak et al, 2002; Hunt et al, 2006). If the annexin A1 inhibited the activity of sPLA2, its presence on the cell surface of the Δ*dltA* strain should cause an inhibition of the bactericidal activity, but that is not the case.

Whilst the inhibitory role of annexin A1 for the activity of cPLA2 has been studied and reported (Kim et al, 1994; Parente and Solito, 2004; Lim and Pervaiz, 2007; D'Acquisto et al, 2008), and the role of annexin V on different types of sPLa2 has been reported (Buckland and Wilton, 1998), the effect of annexin A1 on the secreted PLA2 has not been demonstrated.

As annexin A1 had no bactericidal activity by itself, this protein was chosen as a serum factor to test for its modulating role on the bactericidal activity of sPLA2 against *S. aureus*. To differentiate positive or negative roles for annexin A1, it was added to aliquots of cvPLA2. The addition of annexin A1 to 32.5 units (21.5 µg/ml) of cvPLA2 led to bacterial death after 4 hours of incubation, whereas cvPLA2 alone did not kill ($p<0.01$; Figure 5.16B). Thus annexin A1 interacts with cvPLA2 and enhances its bactericidal activity against the bacteria. This interaction is also direct as shown by native polyacrylamide gel electrophoresis and Western blot with anti-annexin A1 antibodies (Figure 5.17). The picture of how NRS mediates its bactericidal effect on *S. aureus* has now become more complex with the identification of annexin A1 as a likely regulating factor.

6 Chapter 6:

General Discussion

S. aureus is an important pathogen with the ability to cause a wide range of diseases (Archer, 1998; Lowy, 1998; Foster, 2005; Lowy 2010; Hennekinne et al, 2012) and can rapidly gain resistance against antibiotics (Demerec, 1945; Lowy, 2003; Gordon and Lowy, 2008; Chambers and DeLeo, 2009). Against the backdrop of an increasing need to control *S. aureus* the novel bactericidal mechanism in rabbit serum was studied. Previously the antibacterial activity was characterized and the importance of wall teichoic acids (WTA) was identified (Bozakouk, 2011). However the mechanism involved in the bacterial killing remained unknown.

Evidence gathered during this project led to the identification of wall teichoic acid as the bacterial receptor for the 'killing factor' present in the rabbit serum. WTA is also important due of the presence of D-alanine esters, molecules that affect the interaction of the 'killing factor' with the bacterial surface and its bactericidal activity.

The secreted phospholipase A2 (sPLA2) was identified as the bactericidal component responsible for the antibacterial activity of the rabbit serum. It has been reported that sPLA2 has potent antibacterial activity against *S. aureus* (Weinrauch et al, 1996; Qu and Lehrer, 1998; Koprivnjak et al, 2002; Merchant et al, 2011). The role of sPLA2 as the 'killing factor' corroborates the importance of WTA and of D-alanylation on the survival of *S. aureus* on NRS (Koprivnjak et al, 2002; Hunt et al, 2006; Koprivnjak et al, 2008).

The species specificity of the sPLA2 bactericidal mechanism and the differences between the bactericidal and the phospholipase activity of diluted NRS suggested that other components present in the rabbit serum could affect the activity of sPLA2. Annexin A1, a known regulator of the activity of PLA2 (Parente and Solito, 2004; D'Acquisto et al, 2008), was found bound to a cell

wall affinity matrix along with sPLA2. Experiments carried out showed that annexin A1, without bactericidal activity by itself, was able to interact with the PLA2 and support its bactericidal activity against *S. aureus*.

All the evidence gathered during this project leads to a model for the bactericidal mechanism of the NRS against *S. aureus*. The sPLA2 present in the NRS is able to recognize and bind to the WTA in the cell wall of *S. aureus*. The protein then crosses the peptidoglycan with the help of the WTA until it reaches the bacterial membrane, where the enzyme hydrolyses phospholipids and cause bacterial death (Figure 6.1A).

The role of WTA in both the initial interaction between sPLA2 and the bacterial cell (Koprivnjak et al, 2002) and its involvement in the movement of sPLA2 through the peptidoglycan (Koprivnjak et al, 2008) has been demonstrated. However, questions about these processes remain.

As stated previously (Chapter 4.3), the initial interaction between sPLA2 and the bacteria is based upon electrostatic interactions due to the highly cationic nature of sPLA2 and the overall anionic charge of the bacterial surface (Foreman-Wykert et al, 1999; Beers et al, 2002; Koprivnjak et al, 2002). However, there are no reports about the identity of the component of the bacterial cell wall that interacts with sPLA2 and allows the entry of the protein into the peptidoglycan. Whilst the role of teichoic acids has been speculated (Foreman-Wykert et al, 1999), this has not been demonstrated. We showed that pre-incubation of NRS with purified peptidoglycan containing wall teichoic acids completely inactivates its bactericidal activity (Figure 3.17B) due to the binding of sPLA2 to this affinity matrix (Table 4.2). This suggests that WTA directly interacts with sPLA2 and that it might act as the entry point on the outer surface, allowing the penetration of sPLA2 through the peptidoglycan layer.

Once sPLA2 is bound to the *S. aureus* cell surface, the protein has to cross the peptidoglycan in order to reach the cell membrane. Therefore, the permeability of the cell wall is an important factor in the bactericidal activity of sPLA2. It has been suggested that the level of cross-linking of the cell wall plays a role in the

susceptibility of different bacteria to sPLA2 (Qu and Leher, 1998; Buckland and Wilton, 2000). It has also been reported that the growth phase also affects the susceptibility of *S. aureus* to sPLA2 (Foreman-Wykert et al, 1999). Bacteria in logarithmic growth phase are more susceptible to sPLA2 as it is less difficult for the protein to reach the membrane through the dividing cell (Foreman-Wykert et al, 1999; Dennis et al, 2011).

As teichoic acids form a negatively charged network that connects the bacterial cell wall and the membrane (Neuhaus and Baddiley, 2003), it has been proposed that WTA and LTA act as a polyanionic ladder through which positively charged molecules, such as sPLA2, are able to move from the outside to the bacterial membrane (Foreman-Wykert et al, 1999; Hunt et al, 2006; Koprivnjak et al, 2008). The results obtained using the Δ*tagO* strain (Figures 3.6 and 3.19A) support this model, but only if WTA is the first step in this polyanionic ladder. These results confirm that the absence of WTA in the cell wall of *S. aureus* gives resistance to the activity of sPLA2 (Figure 6.1B).

The electrostatic nature of the initial interaction of sPLA2 and *S. aureus*, along with the use of the negatively charged teichoic acids as a ladder in order to reach the bacterial membrane, also explains why the lack of D-alanylation in the WTA increases the affinity of these interactions, helping sPLA2 to reach its target (Figure 6.1C).

After crossing the peptidoglycan, sPLA2 contacts the phospholipids present in the bacterial membrane. It has been reported that sPLA2 attaches itself to the bacterial membrane before loading a single phospholipid molecule into the active site to start the hydrolysis reaction (Jain et al, 1986; Bezzine et al, 2002), generating a fatty acid and a lysophospholipid (Schaloske and Dennis, 2006; Dennis et al, 2011). Different mechanisms have been proposed for the killing mechanism of sPLA2, such as the negative effect that the hydrolysis of the membrane phospholipids has on *S. aureus* cell division due to the accumulation of fatty acids (Ocampo et al, 2010). The important role that bacterial autolysins, activated by the activity of sPLA2, play in killing and lysis of *S. aureus* has also been reported, as autolysis-deficient strains were not lysed and were able to

repair the damage caused by sPLA2 (Foreman-Wykert et al, 1999). Further work needs to be carried out in order to demonstrate which of these mechanisms is directly responsible for the death of *S. aureus*.

Results obtained in this project (Chapter 5.2.7) showed that annexin A1 contributes to the interaction of the sPLA2 with the bacteria (Figure 6.1D). Even though there are no reports about the enhancement of sPLA2 activity by annexin A1, it has been suggested that not all the members of the annexin family are able to inhibit the activity of cPLA2 (Kim et al, 2001).

It has been reported that annexin I (or A1), IV and V are capable of binding to the lipopolysaccharide and the lipoteichoic acid present in the surface of Gram-negative and Gram-positive bacteria, respectively (Gotoh et al, 2005; Rand et al, 2012). As the binding of annexin V to a surface results in the displacement of sPLA2 (Buckland and Wilton, 1998), it is possible that the binding of annexin A1 to the LTA present in *S. aureus* encourages sPLA2 to bind to the WTA also present in the cell wall, helping in the first step of the bactericidal activity of sPLA2. This, however, needs to be studied further.

Whilst the direct interaction between annexin A1 and sPLA2 was shown in this project (Figure 5.18) and the interaction between cPLA2 and annexin A1 has been reported (Kim et al, 2001; Parente and Solito, 2004), the relevance of this to NRS bactericidal activity is unknown.

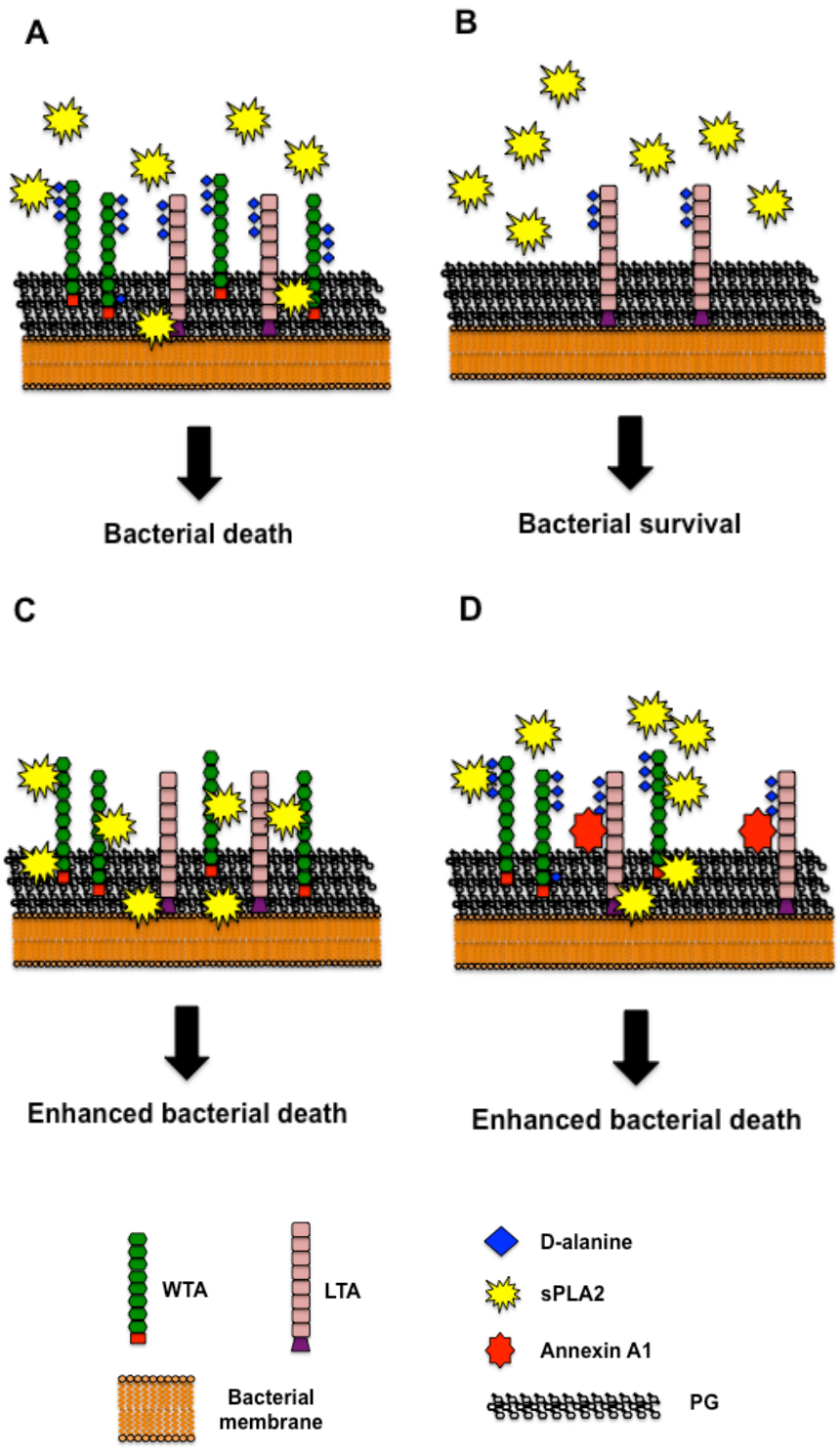

A
B
Bacterial death
Bacterial survival
C
D
Enhanced bacterial death
Enhanced bacterial death
WTA
LTA
Bacterial membrane
D-alanine
sPLA2
Annexin A1
PG

Figure 6.1 Model of the bactericidal mechanism of NRS against *S. aureus*

A) The presence of WTA in the cell surface allows the binding and translocation of the sPLA2 through the peptidoglycan to the bacterial membrane, causing bacterial death.

B) The absence of WTA gives the bacteria resistance to the bactericidal activity of sPLA2 due to the inability of sPLA2 to interact with the bacteria and to reach the phospholipids in the bacterial membrane.

C) Absence of D-alanine increases the affinity of sPLA2 for the bacterial cell wall, causing enhanced bacterial death.

D) Annexin A1 contributes to the interaction of the sPLA2 with the bacteria, leading to enhanced bacterial death.

LTA: lipoteichoic acid; PG: peptidoglycan; sPLA2: secreted phospholipase A2; WTA: wall teichoic acid.

6.1 Future work

This project has identified sPLA2 as the component of the NRS responsible for the bacterial killing. However, more information about how it interacts with the cell surface and crosses the peptidoglycan layer is needed. The interaction and translocation of the protein could be explored using labelled sPLA2 and fluorescence microscopy. Rabbit recombinant sPLA2 could be expressed in either in *E. coli* or Chinese hamster ovary (CHO) cells as for the human protein (Levine et al, 1992; Stadel et al, 1992; Weiss et al, 1994; Huang et al, 2006), with a fluorescent protein fused to it or using the SNAP-tag technique (Sun et al, 2011). Another option could be to purify the sPLA2 from rabbit serum (Gijón et al, 1995; Grönroos et al, 2002) and then bind a fluorescent dye to the protein (Kim et al, 2008; Izquierdo-Alvarez and Martínez-Ruiz, 2011). It is important to test that the fluorescent probe does not affect the initial binding of the sPLA2 to the bacteria, its translocation through the peptidoglycan, or its enzymatic activity in the phospholipids present in the bacterial membrane.

Even though the bactericidal mechanism of the NRS against *S. aureus* was uncovered in this project, identifying WTA as the bacterial receptor for the bactericidal activity of the sPLA2 present in the rabbit serum, the modulating role of annexin A1 on this activity needs to be further explored. The presence of the annexin A1-sPLA2 complex could be verified by gel filtration chromatography, as this technique is commonly used to identify protein complexes. Immunoprecipitation of sPLA2 present in NRS could be used to examine this interaction and to identify any other serum components that able to bind to sPLA2, along with confirm sPLA2 as the bactericidal component of the rabbit serum.

Current treatments against *S. aureus* are expensive and difficult not only because of multi-antibiotic resistance, but also due to high number of virulence factors produced by the bacteria that damage the host. Along with new antibiotics (Ling et al, 2015) and vaccines (Spellberg and Daum, 2012; Fowler and Proctor, 2014), the use of elements of the immune system (Hancock and

Sahl, 2006; Seo et al, 2012; Faccone et al, 2014) is also being studied in order to fight this bacterium.

Mastitis corresponds to the inflammation of the mammary gland due to a bacterial infection commonly caused by *S. aureus* (Ostermar and Rahm, 2000; Jahanfar at al, 2013; Ju et al, 2015). This infection leads to significant economic loss of diary livestock, such as cows, sheep and goats (Da Silva et al, 2005; Virdis et al, 2010; Ju et al, 2015). It also affects approximately 20% of breastfeeding women (Cullinane et al, 2015) and treatment normally involves pain medication and oral antibiotics that do not interfere with the breastfeeding process (Amir et al, 2006; Jahanfar et al, 2013; Cullinane et al, 2015). Rabbit sPLA2 could be produced in the milk of transgenic animals and help inhibit the growth of *E. coli* and *S. aureus* responsible for mastitis, as it has been reported that human lysozyme production in transgenic goat's milk inhibits bacterial growth (Maga et al, 2006).

It has also been reported that the consumption of this milk with human lysozyme helps young pigs to recover faster from diarrhea caused by enterotoxigenic *E. coli* (Cooper et al, 2013), and rabbit sPLA2 in milk could also be used for this type of treatment. This infection kills a significant number of children in developing countries and current treatment includes the administration of fluids and rest in order to prevent dehydration and fatigue (Gohar et al, 2016), along with the use of antibiotics (García et al, 2011).

As MRSA and other antibiotic resistant *S. aureus* strains have been found in livestock (Weese, 2010; Fitzgerald, 2012; McCarthy et al, 2012), rabbit sPLA2 could be used to fight *S. aureus* infection and stop spreading from animal to animal and from animal to human. Enhanced versions of sPLA2 could be synthesized, resulting in a more potent protein that could be tested in local skin infections.

After testing the ability of the enhanced rabbit sPLA2 in livestock, it could be used to treat *S. aureus* infections in humans, especially as a prophylactic treatment for implants and catheters. It has been reported that *S. aureus* is

responsible for most of the bacterial infections related to catheters and are currently controlled by constant use of antibiotics followed by catheter removal or surgery (Fysh et al, 2013; Lui et al, 2016). The urgent need for alternative antibiotics necessitate new approaches to control such a difficult adversary.

REFERENCES

- **Abraham, S.N., St John, A.L. (2010)** Mast cell-orchestrated immunity to pathogens. *Nature Reviews Immunology*, 10, p. 440-452.
- **Achila, D., Liu, A., Banerjee, R., Li, Y., Martinez-Hackert, E., Zhang, J., Yan, H. (2015)** Structural determinants of host specificity of complement Factor H recruitment by *Streptococcus pneumoniae*. *Biochemical Journal*, 465 (2), p. 325-335.
- **Ackermann, E.J., Conde-Frieboes, K., Dennis, E.A. (1995)** Inhibition of macrophage Ca^{2+}independent phospholipase A2 by bromoenol lactone and trifluoromethyl ketones. *Journal of Biological Chemistry*, 270, p. 445-450.
- **Adkins, J.N., Varnum, S.M., Auberry, K.J., Moore, R.J., Angell, N.H., Smith, R.D. Springer, D.L., Pounds, J.L. (2002)** Toward a Human Blood Serum Proteome: analysis by multidimensional separation couples with mass spectrometry. *Mollecular and Cellular Proteomics*, 1 (12), p. 947-955.
- **Akira, S., Uematsu, S., Takeuchi, O. (2006)** Pathogen recognition and innate immunity. *Cell*, 124, p. 783-801.
- **Amdahl, H., Jongerius, I., Meri, T., Pasanen, T., Hyvärinen, S., Haapasalo, K., van Stripj, J., Rooijakkers, S.H., Jokiranta (2013)** Staphylococcal Ecb Protein and Host Complement Regulator Factor H Enhance Functions of Each Other in Bacterial Immune Evasion. *Journal of Immunology*, 191 (4), p. 1775-1784.
- **Amir, L.H., Garland, S.M., Lumley, J. (2006)** A case-control study of mastitis: nasal carriage of *Staphylococcus aureus*. *BMC Family Practice*, 7, p. 75.
- **Anisimov, A.P., Lindler, L.E., Pier, G.B. (2004)** Intraspecific Diversity of *Yersinia pestis*. *Clinical Microbiology Reviews*, 17 (2), p. 434-464.
- **Ansaloni, L., Catena, F., Pinna, A.D. (2009)** What is the Function of the Human Vermiform Appendix? *European Surgical Research*, 43, p. 67-71.
- **Ansari, M.J., Sayegh, M.H. (2008)** Complement dances with T cells. *Blood*, 112, p. 1551-1552.
- **Archer, G.L. (1998)** *Staphylococcus aureus*: a well-armed pathogen. *Clinical Infectious Diseases*, 26, p.179-1181.
- **Ardrey, R.E. (2003)** Liquid Chromatography-Mass Spectrometry: an introduction. John Wiley & Sons, Ltd. ISBNs: 0-471-49799-1 (HB); 0-471-49801-7 (PB).
- **Atilano, M.L., Yates, J., Glittenberg, M., Filipe, S.R., Ligoxygakis, P. (2011)** Wall Teichoic Acids of *Staphylococcus aureus* Limit Recognition by the Drosophila Peptidoglycan Recognition Protein-SA to Promote Pathogenicity. *PLoS Pathogen*, 7(12): e1002421.
- **Atkins, K.L., Burman, J.D., Chamberlain, E.S., Cooper, J.E., Poutrel, B., Bagby, S., Jenkins, T.A., Feil, E.C., van den Elsen, J.M.H. (2008)** *S. aureus* IgG-binding proteins SpA and Sbi: Host specificity and mechanisms of immune complex formation. *Molecular*

Immunology, 45, p. 1600-1611.

- **Aufenanger, J., Zimmer, W., Kattermann, R. (1993)** Characteristics and clinical application of a radiometric *Escherichia coli*-based phospholipase A2 assay modified for serum analysis. *Clinical Chemistry*, 39, p. 605-613.
- **Ausubel, F.D. (2005)** Are innate immune signalling pathways in plants and animals conserved? *Nature Immunology*, 6 (10), p. 973-979.
- **Avison, M., Bennet, P., Howe, R., Walsh, T. (2002)** Preliminary analysis of the genetic basis for vancomycin resistance in *S. aureus* strain Mu50. *Journal of Antimicrobial Chemotheraphy.* 49, p. 255–260.
- **Baddiley, J., Buchanan, J.G., Rajbhandary, U.L., Sanderson, A.R. (1962)** Teichoic acid from *Staphylococcus aureus* H. Structure of the N-acetilglucosaminylribitol residues. *Biochemical Journal*, 82 (3), p. 439-448.
- **Balsinde, J., Balboa, M.A., Insel, P.A., Dennis, E.A. (1999)** Regulation and inhibition of phospholipase A2. *Annual Review of Pharmacology and Toxycology*, 39, p. 175-189.
- **Balsinde, J., Winstead M.V., Dennis, E.A. (2002)** Phospholipase A2 regulation of arachidonic acid mobilization. *FEBS Letters*, 531, p. 2-6.
- **Bandeira-Melo, C., Bonavita, A.G., Diaz, B.L., e Silva, P.M., Carvalho, V.F., Jose, P.J., Flower, R.J., Perretti, M., Martins, M.A. (2005)** A novel effect for annexin 1-derived peptide ac2-26: reduction of allergic inflammation in the rat. *Journal of Pharmacologics and Experimental Therapeutics*, 313, p. 1416-1422.
- **Barber, M., Rozwadowska-Dowzenko, M. (1948)** Infection by penicillin-resistant *staphylococci. Lancet,* 2**,** 641-644.
- **Barrington, R., Zhang, M., Fischer, M., Carrol, M.M. (2001)** The role of complement in inflammation and adaptive immunity. *Immunological Reviews*, 180 (1), p. 5-15.
- **Becker, C.H., Bern, M. (2011)** Recent Developments in quantitative proteomics. *Mutation Research*, 722, p. 171-182.
- **Beckerdite-Quagliata, S., Simberkoff, M., Elsback, P. (1975)** Effects of Human and Rabbit Serum on Viability, Permeability, and Envelope Lipids of *Serratia marcescens*. *Infection and Immunity*, 11 (4), p. 758-766.
- **Beers, S.A., Buckland, A.G., Koduri, R.S., Cho, W., Gelb, M.H., Wilton, D.C. (2002)** The antibacterial properties of secreted phospholipase A2: a major physiological role for the group II enzyme that depends on the very high pI of the enzyme to allow penetration of the bacterial cell wall. *Journal of Biological Chemistry*, 277 (3), p. 1788-1793.
- **Beischer, A.D., Bhathlal, P., de Steiger, R., Penn, D., Stylli, S. (2002)** Synovial ablation in a rabbit rheumatoid arthritis model using photodynamic therapy. *ANZ Journal of Surgery*, 72 (7), p. 517-522.
- **Bera, A., Herbert, S., Jakob, A., Vollmer, W., Gotz, F. (2005)** Why are pathogenic staphylococci so lysozime resistant? The peptidoglycan O-acetyltransferase OatA is the major determinant for lysozime resistance of *Staphylococcus aureus*. *Molecular Microbiology*, 55 (3), p. 778-787.

• **Bera, A., Biswas, R., Herbert, S., Kulauzovic, E., Weidenmaier, C., Peschel, A., Götz, F. (2007)** Influence of Wall Teichoic Acid on Lysozyme Resistance in Staphylococcus aureus. *Journal of Bacteriology*, 189 (1), p. 280-283.

• **Berends, E., Dekkers, J., Nijland, R., Kuipers, A., Soppe, J.A., van Strijp, J., Rooijakkers, S.H.M. (2013)** Distinct localization of the complement C5b-9 complex on Gram-positive bacteria. *Cell Microbiology*, 15 (12), p. 1955-1968.

• **Berg, O.G., Gelb, M.H., Tsai, M.D., Jain, M.K. (2001)** Interfacial enzymology: the secreted phospholipase A(2)-paradigm. *Chemical Reviews*, 1001 (9), p. 2613-2654.

• **Bergdoll, M.S. (1990)** Analytical methods for *Staphylococcus aureus*. *International Journal of Food Microbiology*, 10 (2), p.91-99.

• **Bernal, P., Zloh, M., Taylor, P.W. (2009)** Disruption of D-alanyl esterification of *Staphylococcus aureus* cell wall teichoic acid by the b-lactam resistance modifier (-)-epicatechin gallate. *Journal of Antimicrobial Chemotherapy*, 63, p. 1156-1162.

• **Bertsche, U., Yang, S. Kuehner, D., Wanner, S., Mishra, N.N., Roth, T., Nega, M., Schneider, A., Mayer, C., Grau. T., Bayer, A.S., Weidenmaier, C. (2013)** Increased Cell Wall Teichoic Acid Production and D-alanylation Are Common Phenotypes among Daptomycin-Resistant Methicillin Resistant *Staphylococcus aureus* (MRSA) Clinical Isolates. *Plos ONE*, DOI:10.1371/journal.pone.0067398.

• **Bestebroer, J., Poppelier, M., Ulfman, L., Lenting, P., Denis, C., van Kessel, K., van Stripj, J., de Haas, C. (2007)** Staphylococcal superantigen-like 5 binds PSGL-1and inhibits P-selectin-mediated neutrophil rolling. *Blood*, 109 (7), p. 2936-2943.

• **Bestebroer, J., Aerts, P., Rooijakkers, S., Pandey, M., Kohl, J., van Strijp, J., de Hass, C. (2010)** Functional basis for complement evasion by staphylococcal superantigen-like 7. *Cell Microbiology*, 12 (10), p. 1506-1516.

• **Beutler, B. (2004)** Innate immunity: an overview. *Molecular Immunology*, 40, p. 845-859.

• **Bezzine, S., Bollinger, J.G., Singer, A.G., Veatch, S.L., Keller, S.L., Gelb, M.H. (2002)** On the Binding Preference of Human Groups IIA and X Phospholipases A2 for Membranes with Anionic Phospholipids. *Journal of Biological Chemistry*, 277 (50), p. 48523-48534.

• **Bhakdi, S., Tranum-Jensen, J. (1991)** Alpha-Toxin of *Staphylococcus aureus*. *Microbiological Reviews*, 55 (4), p.733-751.

• **Bhavsar, A.P., Beveridge, T.J., Brown, E.D. (2001)** Precise deletion of *tagD* and controlled depletion of its product, glycerol 3-phosphate cytidylyltransferase, leads to irregular morphology and lysis of *Bacillus subtilis* grown at physiological temperature. *Journal of Bacteriology*, 183, p. 6688-6693.

• **Biswas, R., Martinez, R.E., Gohring, N., Schlag, M., Josten, M., Quoging, X., Hegler, F., Gekeler, C., Gleske, A., Götz, F., Sahl, H., Kappler, A., Peschel, A. (2012)** Proton binding capacity of *Staphylococcus aureus* wall teichoic acid and its role in controlling autolysin activity. *PLoS ONE*, 7:e41415.

• **Boehm, T., Iwanami, N., Hess, I (2012)** Evolution of the Immune System in the Lower Vertebrates. *Annual Reviews of Genomics and Human Genetics*, 12, p. 127-149.

• **Boneca, I.G., Huang, Z.H., Gage, D.A., Tomasz, A. (2000)** Characterization of *Staphylococcus aureus* cell wall glycan strands, evidence for a new beta-N-acetylglucosaminidase activity. *The Journal of Biological Chemistry*, 275 (14), p. 9910–9918.

• **Boneca, I.G., Dussurget, O., Cabanes, D., Nahori, M., Sousa, S., Lecuit, M., Psylinakis, E., Bouriotis, V., Hugot, J., Giovannini, M., Coyle, A., Bertin, J., Namane, A., Rousselle, J., Cayet, N., Prévost, M., Balloy, V., Chignard, M., Philpott, D.J., Cossart, P., Girardin, S.E. (2007)** A critical role for peptidoglycan N-deacetylation in *Listeria* evasion from the host innate immune system. *PNAS*, 104 (3), p. 997-1002.

• **Bozakouk, I.H. (2011)** Characterization of the Effect of Mammalian Serum on *Staphylococcus aureus*. PhD Thesis, University of Sheffield.

• **Brennan, P.J., Nikaido, H. (1995)** The envelope of mycobacteria. *Annual Reviews of Biochemistry*, 64, p.29-63.

• **Brodeur, S.R., Angelini, F., Bacharier, L.B., Blom, A.M., Mizoguchi, E., Fujiwara, H., Plebani, A., Notarangelo, L.D., Dahlback, B., Tsitsikov, E., Geha, R.S. (2003)** C4b-binding protein (C4BP) activates B cells through the CD40 receptor. *Immunity*, 18, p. 837-848.

• **Brodgen, K.A. (2005)** Antimicrobial peptides: pore formers or metabolic inhibitors in bacteria? *Nature Reviews Microbiology*, 3, p. 238-250.

• **Brown, A.F., Leech, J.M., Rogers, T.R., McLoughlin, R.M. (2014)** *Staphylococcus aureus* colonization: modulation of host immune response and impact on human vaccine design. *Frontiers in Immunology*, 4, p.1-20.

• **Brown, J.S., Hussell, T., Gilliland, S.M., Holden, D.W., Paton, J.C., Ehrenstein, M.R., Walport, M.J., Botto, M (2002)** The classical pathway is the dominant complement pathway required for innate immunity to *Streptococcus pneumoniae* infection in mice. *PNAS*, 99 (26), p. 16969-16974.

• **Brown, S., Zhang, Y., Walker, S (2008)** A Revised Pathway Proposed for Staphylococcus aureus Wall Teichoic Acid Biosynthesis Based on In Vitro Reconstitution of Intracellular Steps. *Chemistry & Biology*, 15 (1), p.12-21.

• **Brown, S., Xia. G., Lubachack, L.G., Campbell, J., Meredith, T.C., Winstel, V., Gekeler, C., Irazoqui, J.E., Peschel, A., Walker, S. (2012)** Methicillin resistance in *Staphylococcus aureus* requires glycosylated wall teichoic acids. *PNAS*, 109 (46), p. 18909-18914.

• **Brown, S., Santa Maria Jr., J.P., Walker, S. (2013)** Wall teichoic acids of Gram-positive bacteria. *Annual Review of Microbiology*, 67, p. 313-336.

• **Brown, W.H. (1928)** Calcium and Inorganic Phosphorus in the Blood of Rabbits: Results of repeated and prolonged observations on normal rabbits. *Journal of Experimental Medicine*, 47 (4), p. 539-559.

• **Bryan, C.S. (1974)** Sensitation of *E. coli* to the Serum Bactericidal System and to Lysozyme by Ethyleneglycoltetraacetic Acid. *Proceedings of the Society for Experimental*

Biology and Medicine, 145 (4), p. 1431-1433.

- **Buckland, A.G., Wilton, D.C. (1998)** Inhibition of secreted phospholipases A2 by annexin V. Competition for anionic phospholipid interfaces allows an assessment of the relative interfacial affinities of secreted phospholipases A2. *Biochimica et Biophysica Acta*, 1391 (3), p. 367-376.
- **Buckland, A.G., Heeley, E.L., Wilton, D.C. (2000)** Bacterial cell membrane hydrolysis by secreted phospholipases A (2): a major physiological role of human group IIa sPLA(2) involving both bacterial cell wall penetration and interfacial catalysis. *Biochimica et Biophysica Acta*, 1484, p. 195-206.
- **Buckland, A.G., Wilton, D.C. (2000)** The antibacterial properties of secreted phospholipases A2. *Biochimica et Biophysica Acta*, 1488, p. 71-82.
- **Bullen, J.J. (1981)** The significance of iron in infection. *Reviews of Infectious Diseases*, 3 (6), p. 1127-1138.
- **Bullen, J.J., Rogers, H.J., Spalding, P.B., Ward, C.G. (2005)** Iron and Infection: the heart of the matter. *FEMS Immunology and Medical Microbiology*, 43 (3), p. 325-330.
- **Burdett, V. (1991)** Purification and characterization of Tet (M), a protein that renders ribosomes resistant to tetracycline. *Journal of Biological Chemistry*, 266 (5), p. 2872-2877.
- **Burke, J.E., Dennis, E.A. (2009)** Phospholipase A2 structure/function, mechanism, and signalling. *Journal of Lipid Research*, Supplement, p. S237-242.
- **Burman, J.D., Leung, E., Atkins, K.L., O'Seaghdha, M.N., Lango, L., Bernardo, P., Bagby, S., Svergun, D.I., Foster, T.J., Isenman, D.E., van den Elsen, J.M. (2008)** Interaction of human complement with Sbi, a staphylococcal immunoglobulin-binding protein: indications of a novel mechanism of complement evasion by *Staphylococcus aureus*. *Journal of Biological Chemistry*, 283, p. 17579–17593.
- **Cai, H., Chiorean, E.G., Chiorean, M.V., Rex, D.K., Robb, B.W., Hahn, N.M., Liu, Z., Loehrer, P.J., Harrison, M.L., Xu, Y. (2013)** Elevated Phospholipase A2 Activities in Plasma Samples from Multiple Cancers. *PLoS One*, 8 (2): e57081. doi: 10.1371/journal.pone.0057081.
- **Campbell, J., Singh, A.K., Santa Maria Jr., J.P., Kim, Y., Brown, S. (2011)** Synthetic lethal compound combinations reveal a fundamental connection between wall teichoic acid and peptidoglycan biosynthesis in *Staphylococcus aureus*. *ACS Chemical Biology*, 6, p.106-116.
- **Cappadona, S., Baker, P.R., Cutillas, P.R., Heck, A., van Breukelen, B. (2012)** Current challenges in software solutions for mass spectrometry-based quatitative proteomics. *Amino Acids*, 43 p. 1087-1108.
- **Carroll, M.C. (2004)** The complement system in regulation of adaptive immunity. *Nature Immunology*, 4 (10), p. 981-986.
- **Cartron, M.L., England, S.R., Chiriac, A.I., Josten, M., Turner, R., Rauter, Y., Hurd, A., Sahl, H.G., Jones, S., Foster, S.J. (2014)** Bactericidal activity of the human skin fatty acid cis-6-hexadecanoic acid on *Staphylococcus aureus*. *Antimicrobial Agents and Chemotherapy*, 58 (7), p. 3599-3609.
- **Carvalho, F., Atilano, M.L., Pombinho, R., Covas, G., Gallo, R.L., Filipe, S.R.,**

Sousa, S., Cabanes, D. (2015) L-Rhamnosylation of *Listeria monocytogenes* Wall Teichoic Acids Promotes Resistance to Antimicrobial Peptides by Delaying Interaction with the Membrane. *PLoS Pathogen*, 11 (5), e1004919.

- **Castle, A.M., Castle, D.J. (1981)** The purification and partial characterization of Phospholipase A2, a secretory protein of rabbit parorid gland. *Biochimica et Biophysica Acta*, 666, p. 259-274.
- **Chakraborti, S. (2003)** Phospholipase A2 isoforms: a perspective. *Cellular Signalling*, 15, p. 637-665.
- **Chambers, H.F. (2003)** Solving staphylococcal resistance to beta-lactams. *Trends in Microbiology*, 11, p. 145-148.
- **Chambers, H.F., DeLeo, F.R. (2009)** Waves of Resistance: *Staphylococcus aureus* in the Antibiotic Era. *Nature Reviews Microbiology*, 7 (9), p. 629-641.
- **Chan, K.G., Mayer, M., Davis, E.M., Halperin, S.A., Lin, T., Lee, S.F. (2007)** Role of D-alanylation of *Streptococcus gordonii* Lipoteichoic Acid in Innate and Adaptive Immunity. *Infection and Immunity*, 75 (6), p. 3033-3042.
- **Chaplin, D.D. (2010)** Overview of the immune response. *Journal of Allergy and Clinical Immunology*, 125 (2 Suppl. 2), p. S3-S23.
- **Chatterjee, A.N. (1969)** Use of Bacteriophage-resistant Mutants to Study the Nature of the Bacteriophage Receptor Site of *Staphylococcus aureus*. *Journal of Bacteriology*, 98 (2), p. 519- 527.
- **Chatterjee, B.E., Yona, S., Rosignoli, G., Young, R.E., Nourshargh, S., Flower, R.J., Perretti, M. (2005)** Annexin 1-deficient neutrophils exhibit enhanced transmigration in vivo and increased responsiveness in vitro. *Journal of Leukocyte Biology*, 78, p. 639-646.
- **Chavakis, T., Hussain, M., Kanse, S.M., Peters, G., Bretzel, R.G., Flock, J.I., Herrmann, M., Preissner, K.T. (2002)** *Staphylococcus aureus* extracellular adherence protein serves as anti-inflammatory factor by inhibiting the recruitment of host leukocytes. *Nature Medicine*, 8 (7), p. 687-693.
- **Chavakis, T., Wiechmann, K., Preissner, K.T., Herrmann, M. (2005)** *Staphylococcus aureus* interactions with the endothelium: The role of bacterial “Secretable Expanded Repertoire Adhesive Molecules“ (SERAM) in disturbing host defense systems. *Thrombosis and Haemostasis*, 94, p. 278-285.
- **Chiaradia, E., Avellini, L., Tartaglia, M., Gaiti, A., Just, I., Scoppetta, F., Czentnar, Z., Pich, A. (2012)** Proteomic evaluation of sheep serum proteins. *BMC Veterinary Research*, 8, p. 66-78.
- **Clark, G., Angel, N., Kato, M., López, J.A., MacDonald, K., Vuckovic, S., Hart, D.N. (2000)** The role of dendritic cells in the innate immune system. *Microbes and Infection*, 2, p. 257-272.
- **Clarke, S.R., Foster, S.J. (2006)** Surface Adhesins of *Staphylococcus aureus*. *Advances in Microbial Physiology*, 51, p. 187-224.
- **Clemans, D., Kolenbrander, P., Debabov, D., Zhang, Q., Lunsford, R.D., Sakone,**

H., Whittaker, C., Heaton, M., Neuhaus, F. (1999) Insertional Inactivation of Genes Responsible for the D-Alanylation of Lipoteichoic Acid in *Streptococcus gordonii* DL1 (Challis) Affects Intrageneric Coaggregations. *Infection and Immunity*, 67 (5), p. 2464-2474.

- **Clements, M.O., Foster, S.J., (1999)** Stress resistance in *Staphylococcus aureus*. *Trends in Microbiology*, 7 (11), p. 458-462.
- **Cleveland, M.G., Gorham, J.D. Murphy, T.L., Tuomanen, E., Murphy, K.M. (1996**) Lipoteichoic acid preparations of Gram-positive bacteria induce interleukin-12 through a CD14-dependent pathway. *Infection and Immunity*, 64, p.1906-1912.
- **Collins, L.V., Kristian, S.A., Weidenmaier, C., Faigle, M., van Kessel, K., van Strijo, J., Götz, F., Neumeister, B., Peschel, A. (2002)** *Staphylococcus aureus* Strains Lacking D-alanine Modifications of Teichoic Acids are Highly susceptible to Human Neutrophil Killing and are Virulence Attenuated in Mice. *Journal of Infectious Diseases*, 186, p. 214-219.
- **Cooper, M.D, Alder, M.N. (2006)** The evolution of Adaptive Immune Systems. *Cell*, 124, p. 815-822.
- **Cooper, C.A., Garas Klobas, L.C., Maga, E.A., Murray, J. (2013)** Consuming Transgenic Goats' Milk Containing the Antimicrobial Protein Lysozyme Helps Resolve Diarrhea in Young Pigs. *PLOS One*, 8 (3), DOI: 10.1371/journal.pone.0058409.
- **Courvalin, P., Fiandt, M. (1980)** Aminoglycoside-modifying enzymes of *Staphylococcus aureus*; expression in *Escherichia coli*. *Gene*, 9 (3-4), p.247-269.
- **Courvalin, P. (2006)** Vancomycin resistance in gram-positive cocci. *Clinical Infectious Diseases,* 42 (Suppl 1)**,** p.S25-34.
- **Crokaert, F., Lismont, M.J., van der Linden, M.P., Yourassowski, E. (1988)** Determination of serum bactericidal activity against *Escherichia coli* by an automated photometric method. *Journal of Clinical Microbiology*, 26 (10), 2069-2076.
- **Cui, L., Murakami, H., Kuwahara-Arai, K., Hanaki, H., Hiramatsu, K. (2000)** Contribution of a thickened cell wall and its glutamine nonamidated component to the vancomycin resistance expressed by *Staphylococcus aureus* Mu50. *Antimicrobial Agents and Chemotherapy,* 44**,** p.2276-2285.
- **Cui, L., Iwamoto, A., Lian, J. Q., Neoh, H. M., Maruyama, T., Horikawa, Y., Hiramatsu, K. (2006)** Novel mechanism of antibiotic resistance originating in vancomycin-intermediate *Staphylococcus aureus*. *Antimicrobial Agents and Chemotherapy,* 50**,** p.428-438.
- **Cullinane, M., Amir, L.H., Donath, S.M., Garland, S.M., Tabrizi, S.N., Payne, M.S., Bennet, C.M. (2015)** Determinants of mastitis in women in the CASTLE study: a cohort study. *BMC Family Practice*, 16, p. 181.
- **D'Acquisto, F., Merghani, A., Lecona, E., Rosignoli, G., Raza, K., Buckley, C.D., Flower, R.J., Perretti, M. (2007)** Annexin-1 modulates T-cell activation and differentiation. *Blood*, 109, p. 1095-1102.
- **D'Acquisto, F., Perretti, M., Flower, R.J. (2008)** Annexin-A1: a pivotal regulator of the innate and adaptive immune system. *British Journal of Pharmacology*, 155, p. 152-169.
- **Da Silva, E.R., do Carmo, L.S., da Silva, N. (2005)** Detection of the enterotoxins A, B,

and C genes in *Staphylococcus aureus* from goat and bovine mastitis in Brazilian diary herds. *Veterinary Microbiology*, 106 (1-2), p. 103-107.

- **Dancer, S. J. (2008)** The effect of antibiotics on methicillin-resistant *Staphylococcus aureus*. *Journal of Antimicrobial Chemotherapy,* 61**, p.** 246-253.
- **Dassy, B., Hogan, T., Foster, T.J., Fournier, J.M. (1993)** Involvement of the accessory gene regulator (*agr*) in expression of type 5 capsular polysaccharide by *Staphylococcus aureus*. *Journal of General Microbiology*, 139, p.1301-1306.
- **Daum, G., Lees, N.D, Bard, M., Dickson, R. (1998)** Biochemistry, Cell Biology and Molecular Biology of Lipids of *Saccharomyces cerevisiae. Yeast*, 14, p. 1471–1510.
- **Davidson, F.F., Dennis, E.A., Powell, M., Glenney, J.R. Jr (1987)** Inhibition of phospholipase A2 by 'lipocortins' and calpactins. An effect of binding to substrate phospholipids. *Journal of Biological Chemistry*, 262 (4), p. 1698-1705.
- **Davie, J.M., Paul, W.E., Mage, R.G., Goldman, M.B. (1971)** Membrane-Associated Immunoglobulin of Rabbit Peripheral Blood Lymphocytes: Allelic Exclusion at the b Locus. *PNAS*, 68 (2), p. 430-434.
- **Dawicki, W., Marshall, J.S. (2007)** New and emerging roles for mast cells in host defence. *Current Opinion in Immunology*, 19, p.31-38.
- **Debabov, D.V., Kiriukhin, M.Y., Neuhaus, F.C. (2000)** Biosynthesis of Lipoteichoic Acid in *Lactobacillus rhamnosus*: Role of DltD in D-Alanylation. *Journal of Bacteriology*, 182 (10), p. 2855-2864.
- **De Bock, M., De Seny, D., Meuwis, M.A., Chapelle, J.P, Louis, E., Malaise, M., Merville, M.P., Fillet, M. (2010)** Challenges for biomarker discovery in body fluids using SELDI-TOF-MS. *Journal of Biomedicine and Biotechnology,* Vol. 2010, Article ID 906082.
- **D'Elia, M.A., Henderson, J.A., Beveridge, T.J., Heinrichs, D.E., Brown, E.D. (2009).** The N-acetylmannosamine transferase catalyzes the first commited step of teichoic acid assembly in *Bacillus subtilis* and *Staphylococcus aureus*. *Journal of Bacteriology*, 191, p. 4030-4034.
- **de Haas, C.J., Veldkamp, K.E., Peschel, A., Weerkamp, F., Van Wamel, W.J., Heezius, E.C., Poppelier, M.J., Van Kessel, K.P., van Strijp, J.A. (2004)** Chemotaxis inhibitory protein of *Staphylococcus aureus*, a bacterial antiinflamatory agent. *The Journal of Experimental Medicine*, 199 (5), p. 687-695.
- **De Leo, F.R. (2004)** Modulation of phagocyte apoptosis by bacterial pathogens. *Apoptosis*, 9 (4), p. 399-413.
- **Demerec, M. (1945)** Production of *Staphylococcus* strains resistant to various concentrations of penicillin. *PNAS*, 31 (1), p. 16-24.
- **Demerec, M. (1948)** Origin of bacterial resistance to antibiotics. *Journal of Bacteriology*, 46 (1), p. 63-74.
- **Dennis, E.A., Cao, J., Hsu, Y.H., Magrioti, V., Kokotos, G. (2011)** Phospholipase A2 enzymes: physical structure, biological function, disease implication, chemical inhibition, and therapeutic intervention. *Chemical Reviews*, 111 (10), p. 6130-6185.

• **Des Prez, R.M., Bryan, C.S., Hawiger, J., Colley, D.G. (1975)** Function of the Classical and Alternate Pathways of Human Complement in Serum Treated with Ethylene Glycol Tetraacetic Acid and $MgCl_2$-Ethylene Glycol Tetraacetic Acid. *Infection and Immunity*, 11 (6), p. 1235-1243.

• **DiLillo, D.J., Pawluczkowycz, A.W., Peng, W., Kennedy, A.D., Beum, P.V., Lindorfer, M.A., Taylor, R.P. (2006)** Selective and efficient inhibition of the alternative pathway of complement by mAB that recognizes C3b/iC3b. *Molecular Immunology*, 43 (7), p. 1010-1019.

• **Dominiecki, M.E., Weiss, J. (1999)** Antibacterial Action of Extracellular Group IIA Phospholipase A2 against Grossly Clumped *Staphylococcus aureus*. *Infection and Immunity*, 67 (5), p. 2299-2305.

• **Donato, R., Cannon, B.R., Sorci, G., Riuzzi, F., Hsu, K., Weber, D.J., Geczy, C.L. (2013)** Functions of S100 Proteins. *Current Molecular Medicine*, 13 (1), p. 24-57.

• **Dorsam, G., Harris, L., Payne, M., Fry, M., Franson, R. (1995)** Development and Use of ELISA to Quantify Type II Phospholipase A2 in Normal and Uremic Serum. *Clinical Chemistry*, 41 (6), p. 862-866.

• **Dowzicky, M., Talbot, G. H., Feger, C., Prokocimer, P., Etienne, J., Leclercq, R. (2000)** Characterization of isolates associated with emerging resistance to quinupristin/dalfopristin (synercid) during a worldwide clinical program. *Diagnostic Microbiology and Infectious Diseases*, 37 (1), p. 57-62.

• **Dramsi, S., Magnet, S., Davison, S., Arthur, M. (2008)** Covalent attachment of proteins to peptidoglycan. *FEMS Microbiology Reviews*, 32 (2), p. 307–320.

• **Drouet-Viard, F., Fortun-Lamothe, L. (2002)** The organization and functioning of the immune system: particular features of the rabbit. *World Rabbit Science*, 10 (1), p. 15-23.

• **Dziarski, R., Tapping, R.I., Tobias, P. (1998)** Binding of bacterial peptidoglycan to CD14. *Journal of Biological Chemistry*, 273, p. 8680–8690.

• **Dziarski, R., Gupta, D. (2005)** Peptidoglycan recognition in innate immunity. *Journal of Endotoxin Research*, 11 (5), p. 304-310.

• **Dziarski, R., Gupta, D. (2006)** Mammalian PGRPs: novel antibacterial proteins. *Cellular Microbiology*, 8 (7), p. 1059-1069.

• **Ecker, E.E., Pillemer, L., Seifter, S., Dozois, T.F., San Clemente, C.L. (1943a)** Human Complement. *Science*, 98 (2532), p. 43-44.

• **Ecker, E. E., Pillemer, L., Seifter, S. (1943b)** Immunochemieal studies on human serum. 1. Human complement and its components. *Journal of Immunology*, 47, 181-193.

• **Ehlers, N., Ahrons, S. (1971)** Corneal Transplantation and Histocompatibility. *Acta Ophthalmologica*, 49 (4), p.513-527.

• **Elek, S. D. (1956)** Experimental staphylococcal infection in the skin of man. *Annals of the New York Academy of Sciences*, 65 (3), p.85-90.

• **Elhelu, M.A. (1983)** The Role of Macrophages in Immunology. *Journal of the National Medical Association*, 75 (3), p. 314-317.

• **Elsbach, P., Weiss, J., Franson, R.C., Beckerdite-Quangliata, S., Schneider, A.,**

Harris, L. (1979) Separation and purification of a potent bactercidal/permeability-increasing protein and a closely associated phosphoLipase A_2 from rabbit polymorphonuclear leukocytes: observations on their relationship. *Journal of Biological Chemistry*, 254, p. 11000-11009.

- **Ember, J.A., Jagels, M.A., Hugli, T.E. (1998)** Characterization of complement anaphylatoxins and their biological responses. In: Volanakis, J.E., Frank, M.M. (Eds.), The human complement system in health and disease. Marcel Dekker Inc., New York, p. 241–284.
- **Enright, M.C., Spratt, B.G. (1999)** Multilocus sequence typing. *Trends in Microbiology*, 7, p. 482-487.
- **Errington, J., Jones, L., Carballido-Lopez, R. (2001)** Control of Cell Shape in Bacteria: Helical, Actin- like Filaments in *Bacillus subtilis. Cell*, 104, p. 913–922.
- **Ellwood, D.C., Tempest, D.W. (1972)** Effects of environment on bacterial wall content and composition. *Advances in Microbial Physiology*, 7, p. 83-116.
- **Fabretti, F., Theilacker, C., Baldassarri, L., Kaczynski, Z., Kropec, A., Holst, O., Huebner, J. (2006)** Alanine Esters of Enterococcal Lipoteichoic Acid Play a Role in Biofilm Formation and Resistance to Antimicrobial Peptides. *Infection and Immunity*, 74 (7), p. 4164-4171.
- **Faccone, D., Veliz, O., Corso, A., Noguera, M., Martínez, M., Payes, C., Liliana, S., Maffía, P.C. (2014)** Antimicrobial activity of de novo designed cationic peptides against multi-resistant clinical isolates. *European Journal of Medicinal Chemistry*, 71, p. 31-35.
- **Faure, G. (2000)** Natural inhibitors of toxic phospholipase A2. *Biochimie*, 82, p. 833-840.
- **Fearon, D.T., Austen, K.F. (1975)** Properdin: binding to C3b and stabilization of the C3b-dependent C3 convertase. *Journal of Experimental Medicine*, 142, p. 856-863.
- **Fearon D.T., Austen, K.F. (1977)** Activation of the alternative complement pathway due to resistance of zymosan-bound amplification convertase to endogenous regulatory mechanisms. *PNAS*, 74 (4), p. 1683-1687.
- **Fedtke, I., Götz, F., Peschel, A. (2004)** Bacterial evasion of innate host defenses – the *Staphylococcus aureus* lesson. *International Journal of Medical Microbiology*, 294, p. 189-194.
- **Fedtke, I., Mader, D., Kohler, T., Moll, H., Nicholson, G., Biswas, R., Henseler, K., Gotz, F., Zahringer, U., Peschel, A. (2007)** A *Staphylococcus aureus ypfP* mutant with strongly reduced lipoteichoic acid (LTA) content: LTA governs bacterial surface properties and autolysin activity. *Molecular Microbiology*, 65 (4), p. 1078-1091.
- **Femling, J.K., Nauseef, W.M., Weiss, J.P. (2005)** Synergy between extracelullar group IIA phospholipase A2 and phagocyte NADPH oxidase in digestion of phospholipids of *Staphylococcus aureus* ingested by human neutrophils. *Journal of Immunology*, 175 (7), p. 4653-4661.
- **Fernández Falcon, M.F., Echague, C.G., Hair, P.S., Nyalwidhe, J.O., Cunnion, K.M. (2011)** Protease inhibitors decrease IgG shedding from *Staphylococcus aureus*, increasing complement activation and phagocytosis efficiency. *Journal of Medical Microbiology*, 60, p. 1415-1422.

• **Ferrero, L., Cameron, B., Crouzet, J. (1995)** Analysis of *gyrA* and *grlA* mutations in stepwise- selected ciprofloxacin-resistant mutants of *Staphylococcus aureus*. *Antimicrobial Agents and Chemotherapy,* 39, p.1554-1558.

• **Fine, D.P. (1977)** Comparison of Ethyleneglycoltetraacetic Acid and its Magnesium Salt as Reagents for Studying Alternative Complement Pathway Function. *Infection and Immunity*, 16 (1), p. 124-128.

• **Fischer, W. (1993)** Molecular analysis of lipid macroamphiphiles by hydrobopic interaction chromatography, exemplified with lipoteichoic acids. *Analytical Biochemistry*, 208, p. 49-56.

• **Fischer, W. (1994)** Lipoteichoic acids and lipoglycans. *New Comprehensie Biochemistry*, 27, p. 199-215.

• **Fischer, W.H., Hugli, T.E. (1997)** Regulation of B cell functions by C3a and C3a(desArg): suppression of TNF-alpha, IL-6, and the polyclonal immune response. *Journal of Immunology*, 159, p. 4279–4286.

• **Fischer, W.H., Jagels, M.A., Hugli, T.E. (1999)** Regulation of IL-6 synthesis in human peripheral blood mononuclear cells by C3a and C3a(desArg). *Journal of Immu*nology, 162, p. 453–459.

• **Fitzgerald, J.R. (2012)** Livestock-associated *Staphylococcus aureus*: origin, evolution and public health threat. *Trends in Microbiology*, 20 (4), p. 192-198.

• **Flajnik, M.F., Du Pasquier, L. (2004)** Evolution of innate and adaptive immunity: can we draw a line? *TRENDS in Immunology*, 25 (12), p. 640-644.

• **Fluckinger, M., Haas, H., Merschak, P., Glasgow, B.J., Redl, B. (2004)** Human Tear Lipocalin Exhibits Antimirobial Activity by Scavenging Microbial Siderophores. *Antimicrobial Agents and Chemotheraphy*, 48 (9), p. 3367-3372.

• **Foreman-Wykert, A.K., Weinrauch, Y., Elsbach, P., Weiss. J (1999)** Cell-wall determinants of the bactericidal action of group IIA phospholipase A2 against Gram-positive bacteria. *Journal of Clinical Investigation*, 103, p. 715-721.

• **Foster, S.J., Popham, D.L. (2002)** Structure and synthesis of cell wall, spore cortex, teichoic acids, S-layers, and capsules. In: Bacillus subtilis *and its closest relatives: from genes to cells*. p. 21-41. Edited by Sonenshein, A.L., Hoch, J.A., Losick, R. ASM Press, Washington, D.C.

• **Foster, T.J., Höök, M. (1998)** Surface protein adhesins of *Staphylococcus aureus*. *Trends in Microbiology*, 6 (12), p. 484-488.

• **Foster, T.J. (2004)** The *Staphylococcus aureus* 'superbug'. *Journal of Clinical Investigation*, 114 (12), p. 1693-1696.

• **Foster, T.J. (2005)** Immune evasion by Staphylococci. *Nature Reviews Microbiology*, 3 (12), p. 948-958.

• **Fournier, B., Philpott, D.J. (2005)** Recognition of *Staphylococcus aureus* by the innate immune system. *Clinical Microbiology Reviews*, 18 (3), p. 521-540.

• **Fowler, V.G. Jr, Proctor, R.A. (2014)** Where does a *Staphylococcus aureus* vaccine

stand? *Clinical Microbiology and Infection*, 20 (Suppl.5), p. 66-75.

- **Fox, J.W., Bjarnason, J.B., (1998)** Metalloproteinase inhibitors. In: Bailey, G.S., (Ed.), Enzymes from Snake Venoms, Alaken Inc, Fort Collins, CO, p. 599–632.
- **Frank, M.M., Joiner, K., Hammer, C. (1987)** The Function of Antibody and Complement in the Lysis of Bacteria. *Reviews of Infectious Diseases,* 9 (Suppl. 5), p. S537-545.
- **Fritzinger, D.C., Hew, B.E., Thorne, M., Pangburn, M.K., Janssen, B., Gros, P., Vogel, C. (2009)** Functional characterization of human C3-cobra venom fector hybrid proteins for therapeutic complement depletion. *Developmental and Comparative Immunology*, 33, p. 105-116.
- **Froberg, M.K., Fitzgerald, T.J., Hamilton, T.R., Hamilton, B., Zrabi, M (1993)** Pathology of congenital syphilis in rabbits. *Infection and Immunity*, 61 (11), p. 4743-4749.
- **Fysh, E.T., Tremblay, A., Feller-Kopman, D., Mishra, E.K., Slade, M., Garske, L., Clive, A.O., Lamb, C., Boshuizen, R., Ng, B.J., Rosenstengel, A.W., Yarmus, L., Rahman, N.M., Maskell, N.A., Lee, Y.C. (2013**) Clinical outcomes of indwelling pleural catheter-related pleural, infections: an international multicentre study. *Chest*, 144 (5), p. 1597-1602.
- **Gál, P., Barna, L., Kocsis, A., Zavodsky, P. (2007)** Serine proteases of the classical and lectin pathways: Similarities and differences. *Immunobiology*, 212, p. 267-277.
- **Gál, P., Dobó, J., Závodszky, P., Sim, R.B.M. (2009)** Early complement proteases: C1r, C1s and MASPs. A structural insight into activation and functions. *Molecular Immunology*, 46, p. 2745-2752.
- **García, P.G., Silva, V.L., Diniz, C.G. (2011)** Occurrence and antimicrobial drug susceptibility patterns of commensal and diarrheagenic *Escherichia coli* in fecal microbiota from children with and without acute diarrhea. *Journal of Microbiology*, 49 (1), p. 46-52.
- **Gasque, P. (2004)** Complement: a unique innate immune sensor for danger signals. *Molecular Immunology*, 41, p. 1089-1098.
- **Georgel, P., Crozat, K., Lauth, X., Makrantonaki, E., Seltmann, H., Sovath, S., Hoebe, K., Du, X., Rutshmann, S., Jiang, Z., Bigby, T., Nizet, V., Zouboulis, C., Beutler, B. (2005)** A Toll-like Receptor 2- Receptive Lipid Effector Pathway Protects Mammals against Skin Infections with Gram-Positive Bacteria. *Infection and Immunity*, 73 (8), p. 4512-4521.
- **Gerke, V., Moss, S.E. (2002)** Annexins: From Structure to Function. *Physiologycal Reviews*, 82 (2), p. 331-371.
- **Gerke, V., Creutz, C.E., Moss, S.E. (2005)** Annexins: linking Ca^{2+} signalling to membrane dynamics. *Nature Reviews Mollecular Cell Biology*, 6 (6), p. 449-461.
- **Giaouris, E., Briandet, R., Meyrand, M., Courtin, P., Chapot-Chartier, M. (2008)** Variations in the Degree of D-Alanylation of Teichoic Acids in *Lactococcus lactis* Alter Resistance to Cationic Antimicrobials but Have No Effect on Bacterial Surface Hydrophobicity and Charge. *Applied and Environmental Microbiology*, 74 (15), p. 4764-4767.
- **Gibb, J. A. (1990)** The European Rabbit *Oryctolagus cuniculus*. In: J. A. Chapman and J. E. C. Flux (eds), Rabbits, hares and pikas: Status survey and conservation action plan, p. 116-120. IUCN, Oxford, United Kingdom.

• **Gijon, M.A., Perez, C., Mendez, E., Sanchez Crespo, M. (1995)** Phospholipase A2 from plasma of patients with septic shock is associated with high-density lipoproteins and C3 anaphylotoxin: some implications for its functional role. *Biochemical Journal*, 306, p. 167-175.

• **Girgis, D.O., Dajcs, J.J., O'Callaghan, R.J. (2003)** Phospholipase A2 Activity in Normal and *Staphylococcus aureus*-infected Rabbit Eyes. *Investigative Ophthalmology & Visual Science*, 44 (1), p. 197-202.

• **Glaser, L., Lindsay, B. (1974)** The synthesis of lipoteichoic acid *carrier. Biochemical and Biophysical Research Communications*. 59, p. 1131-1136.

• **Glish, G.L., Vachet, R.W. (2003)** The Basics of Mass Spectrometry in the twenty-first century. *Nature Reviews Drug Discovery*, 2, p. 140-150.

• **Glover, S., de Carvalho, M.S., Bayburt, T., Jonas, M., Chi, E., Leslie, C.C., Gelb, M.H. (1995)** Translocation of the 85-kDa phospholipase A2 from cytosol to the nuclear envelope in rat basophilic leukemia cells stimulated with calcium ionophore or IgE/antigen. *Journal of Biological Chemistry*, 270, p. 15259-15267.

• **Gohar, A., Abdeltawab, N.F., Fahmy, A., Amin, M.A. (2016)** Development of safe, effective and immunogenic vaccine candidate for diarrheagenic *Escherichia coli* main pathotypes in a mouse model. *BMC Research Notes*, 9, p. 80.

• **Goldstein, B.H., Tissot, R.G., Laskin, D.M., Cohen, C. (1975)** Histocompatibility and tooth transplantation in the rabbit. *Oral Surgery, Oral Medicine, Oral Pathology and Oral Radiology*, 39 (6), p. 929-933.

• **Gomez, M.I., Lee, A., Reddy, B., Muir, A., Soong, G., Pitt, A., Cheung, A., Prince, A. (2004)** *Staphylococcus aureus* protein A induces airway epithelial inflammatory responses by activating TNFR1. *Nature Medicine*, 10, p.842-848.

• **Gordon, J., Whitehead, H. R., & Wormall, A. (1926)** The Action of Ammonia on Complement. The Fourth Component. *Biochemical Journal*, 20 (5), 1028–1035.

• **Gordon, R.J., Lowy, F.D. (2008)** Pathogenesis of Methicillin-Resistant *Staphylococcus aureus* Infection. *Clinical Infectious Diseases*, 46 (Suppl.5), p. 350-359.

• **Gotoh, M., Takamoto, Y., Kurosaka, K., Masuda, J., Ida, M., Satoh, A., Takayama, E., Kojima-Aikawa, K., Kobayashi, Y., Matsumoto, I. (2005)** Annexins I and IV inhibits *Staphylococcus aureus* attachment to human macrophages. *Immunology Letters*, 98, p. 297-302.

• **Grönroos, J.O., Laine, V.J.O., Nevalainen, T.J. (2002)** Bactericidal Group IIA Phospholipase A2 in Serum of Patients with Bacterial Infections. *Journal of Infectious Diseases*, 185, p. 1767-1772.

• **Gross, M., Cramton, S., Gotz, F., Peschel, A. (2001)** Key Role of Teichoic Acid Net Charge in *Staphylococcus aureus* Colonization of Artificial Surfaces. *Infection and Immunity*, 69 (5), p. 3423-3426.

• **Grundling, A., Schneewind, O. (2007a)** Synthesis of glycerol phosphate lipoteichoic acid in *Staphylococcus aureus*. *PNAS*, 104 (20), p. 8478-8483.

• **Grundling, A., Schneewind, O. (2007b)** Genes Required for Glycolipid Synthesis and

Lipoteichoic Acid Anchoring in *Staphylococcus aureus*. *Journal of Bacteriology*, 189 (6), p. 2521-2530.

- **Guan, R., Mariuzza, R.A. (2007)** Peptidoglycan recognition proteins of the immune system. *TRENDS in Microbiology*, 15 (3), p. 127-134.
- **Guay, G.G., Khan, S.A., Rothstein, D.M. (1993)** The tet(K) gene of plasmid pT181 of *Staphylococcus aureus* encodes an efflux protein that contains 14 transmembrane helices, *Plasmid*, 30 (2), p.163-166.
- **Guinane, C.M., Ben Zakour, N.L., Tormo-Mas, M.A., Weinert, L.A., Lowder, B.V., Cartwright, R.A., Smyth, D.S., Smyth, C.J., Lindsay, J.A., Gould, K.A., Witney, A., Hinds, J., Bollback, J.P., Rambaut, A., Penadés, J., Fitzgerald, J.R. (2010)** Evolutionary Genomics of *Staphylococcus aureus* Reveals Insights into the Origin and Molecular Basis of Ruminant Host Adaptation. *Genome Biology and Evolution*, 2, p. 454-466.
- **Gupta, D., Kirkland, T.N., Viriyakosol, S., Dziarski, R. (1996)** CD14 is a cell-activating receptor for bacterial peptidoglycan. *Journal of Biological Chemistry*, 271, p. 23310-23316.
- **Gupta-Bansal, R., Parent, J.B., Brunden, K.R. (2000)** Inhibition of complement alternative pathway function with anti-properdin monoclonal antibodies. *Molecular Immunology*, 37 (5), p. 191-201.
- **Gutsmann, T., Müller, M., Carroll, S.F., MacKenzie, R.C., Wiese, A., Seydel, U. (2001)** Dual Role of Lipopolysaccharide (LPS)-Binding Protein in Neutralization of LPS and Enhancement of LPS-Induced Activation of Mononuclear Cells. *Infection and Immunity*, 69 (11), p. 6942-6950.
- **Haas, R., Koch, H.U., Fischer, W. (1984)** Alanyl turnover from lipoteichoic acid to teichoic acid in *Staphylococcus aureus*. *FEMS Microbiology Letters*, 21 (1), p. 37-31.
- **Haas, P. and van Strijp, J. (2007)** Anaphylatoxins: Their Role in Bacterial Infection and Inflammation. *Immunological Research*, 37 (3), p. 161-175.
- **Hageman, J. C., Pegues, D. A., Jepson, C., Bell, R. L., Guinan, M., Ward, K. W., Cohen, M. D., Hindler, J. A., Tenover, F. C., Mcallister, S. K., Kellum, M. E., Fridkin, S. K. (2001)** Vancomycin- intermediate *Staphylococcus aureus* in a home health-care patient. *Emerging Infectious Diseases,* 7**,** p.1023-1025.
- **Hague, B.F., Kindt, T.J. (1998)** Rabbit Immune System. *Encyclopedia of Immunology*, second edition, Roitt, I.M. and Delves, P.J. (Eds.), p.2046-2049.
- **Hair, P.S., Echague, C.G., Sholl, A.M., Watkins, J.A., Geoghegan, J.A., Foster, T.J., Cunnion, K.M. (2010).** Clumping factor A interaction with complement factor I increases C3b cleavage on the bacterial surface of *Staphylococcus aureus* and decreases complement-mediated phagocytosis. *Infection and Immunity*, 78, p. 1717-1727.
- **Hakansson, K., Reid, K.B.M. (2000)** Collectin structure: A review. *Protein Science*, 9, p. 1607-1617.
- **Hammel, M., Nemecek, D., Keightley, J.A., Thomas, G.J. Jr, Geisbrecht, B.V (2007a)** The *Staphylococcus aureus* extracellular adherence protein (Eap) adopts an elongated

but structured conformation in solution. *Protein Science*, 16, p. 2605-2617.

- **Hammel, M., Sfyroera, G., Pyrpassopoulus, S., Ricklin, D., Ramyar, K.X., Pop, M., Jin, Z., Lambris, J.D., Geisbrecht, B.V. (2007b)** Characterization of Ehp, a Secreted Complement Inhibitory Protein from *Staphylococcus aureus*. *Journal of Biological Chemistry*, 282 (41), p. 30051-30061.
- **Hamon, M., Bierne, H., Cossart, P. (2006)** *Listeria monocytogenes*: a multifaced model. *Nature Reviews Microbiology*, 4, p. 423-434.
- **Hancock, R., Sahl, H. (2006)** Antimicrobial and host-defense peptides as new anti-infective therapeutic strategies. *Nature Biotechnology*, 24 (12), p. 1551-1557.
- **Hanly, W.C., Artwohl, J.E., Bennet, B.T. (1995)** Review of polyclonal antibody production procedures in mammals and poultry. *ILAR Journal*, 37, p. 93-118.
- **Hanson, B.R., Neely, M.N. (2011)** Coordinate regulation of Gram-positive cell surface components. *Current Opinion in Microbiology*, 15, p. 1–7.
- **Harboe, M., Mollnes, T.E. (2008)** The alternative complement pathway revisited. *Journal of Cellular and Molecular Medicine*, 12 (4), p. 1074-1084.
- **Harboe, M., Garred, P., Lindstad, J.K., Pharo, A., Müller, F., Stahl, G.L., Lambris, J.D., Mollnes, T.E. (2012)** The role of properdin in zymosan- and *Escherichia coli*-induced complement activation. *Journal of Immunology*, 189 (5), p. 2606-2613.
- **Harris, L. G., Foster, S. J. & Richards, R. G. (2002)** An introduction to *Staphylococcus aureus*, and techniques for identifying and quantifying *S. aureus* adhesins in relation to adhesion to biomaterials: review. *European Cells Materials,* 4**,** p. 39-60.
- **Harris, J.B., Scott-Davey, T. (2013)** Secreted Phospholipases A2 of Snake Venoms: Effects on the Peripheral Neuromuscular System with Comments on the Role of Phospholipases A2 in Disorders of the CNS and Their Uses in Industry. *Toxins*, 5, p. 2533-2571.
- **Hartmann, K., Henz, B.M., Kruger-Krasagakes, S., Köhl, J., Burger, R., Guhl, S., Haase, I., Lippert, U., Zuberbier, T. (1997)** C3a and C5a stimulate chemotaxis of human mast cells. *Blood*, 89, p. 2863–2870.
- **Harwig, S.S., Tan, L., Qu, X.D., Cho, Y., Eisenhauer, P.B., Lehrer, R.I. (1995)** Bactericidal properties of murine intestinal phospholipase A2. *Journal of Clinical Investigation*, 95 (2), p. 603-610.
- **Hayes, M.J., Moss, S.E. (2004)** Annexins and disease. *Biochemical and Biophysical Research Communications*, 322, p. 1166-1170.
- **Hayden, M. K., Rezai, K., Hayes, R. A., Lolans, K., Quinn, J. P., Weinstein, R. A. (2005)** Development of Daptomycin resistance *in vivo* in methicillin-resistant *Staphylococcus aureus*. *Journal of Clinical Microbiology,* 43**,** p. 5285-5287.
- **Hayhurst, E.J., Kailas, L., Hobbs, J.K., Foster, S.J. (2008)** Cell wall peptidoglycan architecture in *Bacillus subtilis*. *PNAS*, 105 (38), 14603-14608.
- **Hedrick, S.M (2004)** The Acquired Immune System: A Vantage from Beneath. *Immunity*, 21 (5), p.607-615.

• **Hennekinne, J. A., De Buyser, M. L. & Dragacci, S. (2012)** *Staphylococcus aureus* and its food poisoning toxins: characterization and outbreak investigation. *FEMS Microbiology Reviews,* 36, p.815-836.

• **Hermans, S.J., Baker, H.M., Sequeira, R.P., Langley, R., Baker, E.N., Frasera, J.D. (2012)** Structural and Functional Properties of Staphylococcal Superantigen-Like Protein 4. *Infection and Immunity*, 80 (11), p. 4004-4013.

• **Herron-Olson, L., Fitzgerald, J.R., Musser, J.M., Kapur, V. (2007)** Molecular correlates of host specialization in *Staphylococcus aureus*. *PLoS One*, 2 (10): e1120. doi:10.1371/journal.pone.0001120

• **Heyman, B. (2000)** Regulation of antibody responses via antibodies, complement, and Fc receptors. *Annual Review of Immunology*, 18, p. 709-737.

• **Higgins, J., Loughman, A., van Kessel, K., van Strijp, J., Foster, T.J. (2006)** Clumping factor A of *Staphylococcus aureus* inhibits phagocytosis by human polymorphonuclear leucocytes. *FEMS Microbiology Letters*, 258, p. 290-296.

• **Hill, D.J., Griffiths, N.J., Borodina, E., Virji, M. (2010)** Cellular and molecular biology of *Neisseria meningitidis* colonization and invasive disease. *Clinical Science (London, England: 1979)*, 118 (Pt 9), p. 547-564.

• **Hillebrandt, S., Wasmuth, H.E., Weiskirchen, R., Hellerbrand, C., Keppeler, H., Werth, A., Schirin-Sokhan, R., Wilkens, G., Geier, A., Lorenzen, J., Köhl, J., Gressner, A.M., Matern, S., Lammert, F. (2005)** Complement factor 5 is a quantitative trait gene that modifies liver fibrogenesis in mice and humans. *Nature Genetics*, 37, p. 835-843.

• **Hiramatsu, K., Aritaka, N., Hanaki, H., Kawasaki, S., Hosoda, Y., Hori, S., Fukuchi, Y., Kobayashi, I. (1997)** Dissemination in Japanese hospitals of strains of *Staphylococcus aureus* heterogeneously resistant to vancomycin. *Lancet,* 350**, p.**1670-1673.

• **Holweg, A., Schnare, M., Gessner, A. (2011)** The bactericidal/permeability-increasing protein (BPI) in the innate defence of the lower airways. *Biochemical Society Transactions*, 39, p. 1045-1050.

• **Hope, R., Mushtaq, S., James, D., Pllana, T., Warner, M., Livermore, D. M. (2010)** Tigecycline activity: low resistance rates but problematic disc breakpoints revealed by a multicentre sentinel survey in the UK. *Journal of Antimicrobial Chemotherapy,* 65**,** p.2602-2609.

• **Horsburgh, M. J., Aish, J. L., White, I. J., Shaw, L., Lithgow, J. K., and Foster, S. J. (2002)** σ^B modulates virulence determinant expression and stress resistance: characterization of a functional *rsbU* strain derived from *Staphylococcus aureus* 8325-4. *Journal of Bacteriology*, 184 (19), 5457-5467.

• **Huang, C., Zhou, L., Liu, Y., Lai, L. (2006)** A continuous fluoresce assay for phospholipase A2 with nontagged lipid. *Analytical Biochemistry*, 351, p. 11-17.

• **Hunt, C.L., Nauseef, W.M., Weiss, J.P. (2006)** Effect of d-Alanylation of (Lipo)Teichoic Acids of *Staphylococcus aureus* on Host Secretory Phospholipase A2 Action before and after Phagocytosis by Human Neutrophils. *Journal of Immunology*, 76, p. 4987-4994.

• **Inal, J.M., Hui, K.M., Miot, S., Lange, S., Ramirez, M.I., Schneider, B., Krueger, G.,**

Schifferli, J.A. (2005) Complement C2 receptor inhibitor trispanning: a novel human complement inhibitor receptor. *Journal of Immunology*, 174, p. 356-366.

- **Iordanescu, S., Surdeanu, M. (1976)** Two restriction and modification system in *Staphylococcus aureus* NCTC8325. *Microbiology*, 69 (2), p. 277-281.
- **Isenmen, D.E., Kells, D.I., Cooper, N.R., Müller-Eberhard, H.J., Pangburn, M.K. (1981)** Nucleophilic modification of human complement protein C3: correlation of conformational changes with acquisition of C3b-like functional properties. *Biochemistry*, 20 (15), p. 4458-5567.
- **Iwasaki, A, Medzhitov, R. (2004)** Toll-like receptor control of the adaptive immune responses. *Nature Immunology*, 5, p. 987–995.
- **Iwasaki, H., Shimada, A., E. Ito, E. (1986)** Comparative studies of lipoteichoic acids from several *Bacillus* strains. *Journal of Bacteriology*, 167, p. 508-516.
- **Izquierdo-Álvarez, A., Martínez-Ruiz, A. (2011)** Thiol redox proteomics seen with fluorescent eyes: The detection of cysteine oxidative modifications by fluorescence derivatization and 2-DE. *Journal of Proteomics*, 75, p. 329-338.
- **Jabeen, T., Sharma, S., Singh, N., Kaur, P., Perbandt, M., Betzel, Ch., Srinivasan, A., Singh, T.P. (2005)** Crystal Structure of a Calcium-Induced Dimer of Two Isoforms of Cobra Phospholipase A2 at 1.6 Å Resolution. *PROTEINS: Structure, Function, and Bioinformatics*, 59, p. 856-863.
- **Jacquier, N., Viollier, P.H., Greub, G. (2015)** The role of peptidoglycan in chlamydial cell division: towards resolving the chlamydial anomaly. *FEMS Microbiology Reviews*, 39, p. 262-275.
- **Jahanfar, S., Ng, C.J., Teng, C.L. (2013)** Antibiotics for mastitis in breastfeeding women. *The Cochrane Database of Systematic Reviews*, doi: 10.1002/14651858.CD005458.pub3.
- **Jain, M.H., Rogers, J., Maredeck, J.F., Ramirez, F., Eibl, H. (1986)** Effect of the structure of phospholipid on the kinetics of intravesicle scooting of phospholipase A2. *Biochimica et Biophysica Acta*, 860, p. 462-474.
- **Janeway, C.A. Jr, Travers, P., Walport, M., Walport, M., Schomchick, M.J. (2001)** Immunobiology: The Immune System in Health and Disease. 5th edition. New York: Garland Science.
- **Janeway, C.A. Jr, Medzhitov, R. (2002)** Innate Immune Recognition. *Annual Review of Microbiology*, 20, p. 197-216.
- **Jaros, J.A., Guest, P.C., Bahn, S., Martins-de-Souza, D. (2013)** Affinity depletion of plasma and serum for mass spectrometry based proteome analysis. *Methods in Molecular Biology*, 1002, p. 1-11.
- **Jeklova, E., Leva, L., Kudlackova, H., Faldyna, M. (2007)** Functional development of immune response in rabbits. *Veterinary Immunology and Immunopathology*, 118, p. 221-228.
- **Jenni, R., Berger-Bächi, B. (1998)** Teichoic acid content in different lineages of *Staphylococcus aureus* NCTC8325. *Archives of Microbiology*, 170, p. 171-178.
- **Jevons, M.P, Rolinson, G.N., Knox, R. (1961)** Celbenin-Resistant Stapylococci,

British Medical Journal, 1, 124-125.

- **Jin, T., Bokarewa, M., Foster, T., Mitchell, J., Higgins, J., Tarkowski, A. (2004)** *Staphylococcus aureus* Resists Human Defensins by Production of Staphylokinase, a Novel Bacterial Evasion Mechanism. *The Journal of Immunology*, 172 (2), p. 1169-1176.
- **Johannessen, M., Sollid, J.E., Hanssen, A. (2012)** Host and microbe determinants that may influence the success of *S. aureus* colonization. *Frontiers in Cellular and Infection Microbiology*, 2, p.1-14.
- **Joiner, K., Brown, E., Hammer, C., Warren, K., Frank, M. (1983**) Studies on the mechanism of bacterial resistance to complement-mediated killing. III. C5b-9 deposits stably on rough and type 7 *S. pneumoniae* without causing bacterial killing. *Journal of Immunology*, 130, p. 845-849.
- **Jongerius, I., Kohl, J., Pandey, M.K., Ruyken, M., van Kessel, K.P.M., van Strijp, J.A.G., Rooijakkers, S.H.M. (2007)** Staphylococcal complement evasion by various convertase-blocking molecules. *The Journal of Experimental Medicine*, 204 (10), p. 2461-2471.
- **Jongerius, I., vonKöckritz-Blickwede, M., Horsburgh, M.J., Ruyken, M., Nizet, V., Rooijakkers, S.H.M. (2012)** *Staphylococcus aureus* Virulence is Enhanced by Secreted Factors that Block Innate Immune Defences. *Journal of Innate Immunity*, 4, p. 301-311.
- **Jorasch, P., Warnecke, D.C., Lindner, B., Zähringer, U., Heinz, E. (2000)** Novel processive and non-processive glycosyltransferases from *Staphylococcus aureus* and *Arabidopsis thaliana* synthesize glycoglycerolipids, glycophospholipids, glycosphingolipids and glycosylsterols. *European Journal of Biochemistry,* 267, p. 3770-3783.
- **Jozni, M., Zipfel, P.F. (2008)** Factor H family proteins and human diseases. *Trends in Immunology*, 29 (8), p. 380-387.
- **Ju, Z., Wang, C., Wang, X., Yang, C., Sun, Y., J., Q., Wang, E., Li, M., Zhong, J., Huang, J. (2015)** Role of an SNP in Alternative Splicing of Bovine NCF4 and Mastitis Susceptibility. *PLoS One*, 10 (11), e0143705.
- **Juergens, W. G., Sanderson, A., Strominger, J.L. (1963)** Chemical basis for an immunological specificity of a strain of *Staphylococcus aureus*. *Journal of Experimental Medicine*, 117, p. 925-935.
- **Kabli, A.F. (2013)** Identification and Characterization of Cell Division Proteins in *Staphylococcus aureus.* PhD Thesis, University of Sheffield.
- **Karavolos, M.H., Horsburgh, M., Ingham, E., Foster, S.J. (2003)** Role and regulation of the superoxide dismutases of *Staphylococcus aureus*. *Microbiology*, 149(Pt. 10), p. 2749-2758.
- **Kemper, C., Atkinson, J.P., Hourcade, D.E. (2010)** Properdin: Emerging Roles of a Pettern-Recognition Molecule. *Annual Review of Immunology*, 28, p. 131-155.
- **Kerr, M.A., Porter, R.R. (1978)** The purification and properties of the second component of human complement. *Biochemical Journal*, 171 (1), p. 99-107.
- **Kim, D.K., Bonventre, J.V. (1993)** Purification of a 100kDa phospholipase A2 from spleen, lung and kidney: antiserum raised to pig spleen phospholipase A2 recognizes a similar

form in bovine lung, kidney and platelets, and immunoprecipitates phospholipase A2 activity. *Biochemical Journal*, 294, p. 261-270.

- **Kim, D., Song, W. (2001)** Membrane complement regulatory proteins. *Clinical Immunology*, 118 (2-3), p. 127-136.
- **Kim, H.K., Thammavongsa, V., Schneewind, O., Missiakas, D. (2012)** Recurrent infections and immune evasion strategies of *Staphylococcus aureus*. *Current Opinion in Microbiology*, 15, p. 92-99.
- **Kim, K.M., Kim, D.K., Park, Y.M., Kim, C., Na, D.S. (1994)** Annexin-I inhibits phospholipase A2 by specific interaction, not by substrate depletion. *FEBS Letters*, 343, p. 251-255.
- **Kim, S-W., Ko, J., Kim, J.H., Choi, E.C., Na, D.S. (2001)** Differential effects of annexins I, II, III and V on cytosolic phospholipase A2 activity: specific interaction model. *FEBS Letters*, 489, p. 243-248.
- **Kim, Y., Ho, S.O., Gassman, N.R., Korlan, Y., Landorf, E.V., Collart, F.R., Weiss, S (2008)** Efficient Site-Specific Labeling of Proteins via Cysteines. *Bioconjugate Chemistry*, 19 (3), p. 786-791.
- **Kimura, Y., Madhavan, M., Call, M.K., Santiago, W., Tsonis, P.A., Lambris, J.D., Del Rio-Tsonis, K. (2003)** Expression of complement 3 and complement 5 in newt limb and lens regeneration. *Journal of Immunology*, 170, p. 2331–2339.
- **Kiriukhin, M.Y., Debabov, D.V., Shinabarger, D.L., Neuhaus, F.C. (2001)** Biosynthesis of the Glycolipid Anchor in Lipoteichoic Acid of *Staphylococcus aureus* RN4220: Role of YpfP, the Diglucosyldiacylglycerol Synthase. *Journal of Bacteriology*, 183 (11), p. 3506-3514.
- **Kloos W.E., Schleifer KH (1986)** Genus IV – Staphylococcus Rosenbach 1884. In: Sneath P.H.A., Mair N.S., Sharpe M.E., (eds). Bergey's Manual of Systemic Bacteriology, Vol 2. Williams and Wilkins, Baltimore.
- **Kloos, W. E., and D. W. Lambe, Jr (1991)** *Staphylococcus*. In: Balows, A., W. J. Hausler, W.J., Herrmann, K.L., Isenberg H.D., Shadomy H.J., (Eds.) Manual of clinical microbiology, (5th Edition). American Society for Microbiology, Washington: p.222-237.
- **Klos, A., Tenner, A.J., Johswich, K., Ager, R.R., Reis, E.S., Köhl, J. (2009).** The role of the anaphylatoxins in health and disease. *Molecular Immunology*, 46, p. 2753-2766.
- **Koduri, R.S., Baker, S.F., Snitko, Y., Han, S.Y., Cho, W., Wilton, D.C., Belb, M.H. (1998)** Action of Human Group IIa Secreted Phospholipase A2 on Cell Membranes: Vesicle but not heparinoid binding determines rate of fatty acid release by exogenously added enzyme. *Journal of Biological Chemistry*, 273 (48), p. 32142–32153.
- **Koduri, R.S., Grönroos, J.O., Laine, V.J.O., Le Calvez, C., Lambeau, G., Nevalainen, T.J., Gelb, M.H. (2002)** Bactericidal Properties of Human and Murine Groups I, II, V, X and XII Secreted Phospholipases. *Journal of Biological Chemistry*, 277 (8), p. 5849-5857.
- **Kohler, T., Weidenmaier, C., Peschel, A. (2009)** Wall Teichoic Acid Protects *Staphylococcus aureus* against Antimicrobial Fatty Acids from Human Skin. *Journal of*

Bacteriology, 191 (13), p. 4482-4484.

- **Kono, K., Arakana, K. (1995)** Methicillin-resistant *Staphylococcus aureus* (MRSA) isolated in clinics and hospitals in the Fukuoka city area. *Journal of Hospital Infection*, 29, p. 265-273

- **Koprivnjak, T., Peschel, A., Gelb, M.H., Liang, N.S., Weiss, J.P. (2002)** Role of Charge Properties of Bacterial Envelope in Bactericidal Action of Human Group IIA Phospholipase A2 against *Staphylococcus aureus. Journal of Biological Chemistry*, 277 (49), p. 47636-47644.

- **Koprivnjak, T., Mlakar, V., Swanson, L., Fournier, B., Peschel, A., Weiss, J.P. (2006)** Cation-Induced Transcriptional Regulation of the *dlt* Operon of *Staphylococcus aureus*. *Journal of Bacteriology*, 188 (10), p. 3622-3630.

- **Koprivnjak, T., Weidenmaier, C., Peschel, A., Weiss, J.P. (2008)** Wall Teichoic Acid Deficiency in Staphylococcus aureus Confers Selective Resistance to Mammalian Group IIA Phospholipase A2 and Human–Defensin. *Infection and Immunity*, 76 (5), p. 2169-2176.

- **Kovacs, M., Halfman, A., Fedtke, I., Heintz, M., Peschel, A., Vollmer, W., Hakenbeck, R., Brücknr, R. (2006)** A Functional *dlt* Operon, Encoding Proteins Required for Incorporation of D-Alanine in Teichoic Acids in Gram-Positive Bacteria, Confers Resistance to Cationic Antimicrobial Peptides in *Streptococcus pneumoniae*. *Journal of Bacteriology*, 188 (6), p. 5797-5805.

- **Kotb M. (1995)** Bacterial pyrogenic exotoxins as superantigens. *Clinical Microbiology Reviews*, 8, p.411-426.

- **Kreis, C. A., Raschke, M. J., Rosslenbroich, S. B., Tholema-Hans, N., Loffler, B., Fuchs, T. (2013)** Therapy of intracellular *Staphylococcus aureus* by tigecyclin. *BMC Infectious Diseases,* 13, 267.

- **Kreiswirth, B.N., Lofdahl, S., Betley, M.J., O'Reilly, M., Schlievert, P.M., Bergdoll, M.S., Novick, R.P., (1983)** The toxic shock syndrome exotoxin structural gene is not detectably transmitted by a prophage*. Nature,* 305, p. 709–712.

- **Kudo, I., Murakami, M., Hara, S., Inoue, K. (1993)** Mammalian non-pancreatic phospholipases A2. *Biochimica et Biophysica Acta*, 1171, p. 217-231.

- **Kulaga, H., Folks, T., Rutledge, R., Truckenmiller, M.E., Gugel, E., Kindt, J. (1989)** Infection of rabbits with Human Immunodeficiency Virus 1: a Small Animal Model for Acquired Immunodeficiency Syndrome. *The Journal of Experimental Medicine*, 169, p. 321-326.

- **Kumar, V., Sharma, A. (2010)** Neutrophils: Cinderella of innate immune system. *International Immunopharmacology*, 10, p 1325–1334.

- **Kunze, H., Bohn, E. (1978)** Phospholipase A_2 and prostaglandins in human seminal plasma. *Advances in Prostaglandin, Thromboxane and Leukotriene Research*, 3, p. 159-165.

- **Kuroda, M., Ohta, T., Uchiyama, I., Baba, T., Yuzawa, H., Kobayashi, I., Cui, L., Oguchi, A., Aoki, K., Nagai, Y., Lian, J., Ito, T., Kanamori, M., Matsumaru, H., Maruyama, A., Murakami, H., Hosoyama, A., Mizutani-Ui, Y., Takahashi, N.K., Sawano, T., Inoue, R., Kaito, C., Sekimizu, K., Hirakawa, H., Kuhara, S., Goto, S., Yabuzaki, J., Kanehisa, M.,**

Yamashita, A., Oshima, K., Furuya, K., Yoshino, C., Shiba, T., Hattori, M., Ogasawara, N., Hayashi, H., Hiramatsu, K. (2001) Whole genome sequencing of methicillin-resistant *Staphylococcus aureus*. *Lancet*, 357, p.1225-1240.

- **Laarman, A.J., Ruyken, M., Malone, C.L., van Strijp, J., Horswill, A.R., Rooijakkers, S.H.M. (2011)** Staphylococcus aureus Metalloprotease Aureolysin Cleaves Complement C3 To Mediate Immune Evasion. *Journal of Immunology*, 186, p. 6445-6453.
- **Lacey, R. W. (1975)** Antibiotic resistance plasmids of *Staphylococcus aureus* and their clinical importance. *Bacteriological Reviews*, 39 (1), p.1-32.
- **Lacey, R. W., Chopra, I. (1972)** Evidence for mutation to streptomycin resistance in clinical strains of *Staphylococcus aureus*. *Journal of General Microbiology*, 73 (1), p.175-180.
- **Laine, V.J.O., Grass, D.S., Nevalainen, T.J. (1999)** Protection by Group II Phospholipase A2 Against *Staphylococcus aureus*. *Journal of Immunology*, 162, p. 7402-7408.
- **Lambeau, G., Gelb, M.H. (2008)** Biochemistry and Physiology of Mammalian Secreted Phospholipase A2. *Annual Review of Biochemistry*, 77, p. 495-520.
- **Langley, R., Wines, B., Willoughby, N., Basu, I., Proft, T., Fraser, J.D. (2005)** The staphylococcal superantigen-like protein 7 binds IgA and complement C5 and inhibits IgA-Fc alpha RI binding and serum killing of bacteria. *Journal of Immunology*, 174 (5), p. 2926-2933.
- **Langley, R., Patel, D., Jackson, N., Clow, F., Fraser, J.D. (2010)** Staphylococcal superantigen super-domains in immune evasion. *Critical Reviews™ in Immunology*, 30 (2), p. 149-165.
- **Law, S.K., Lichtenberg, N.A., Levine, R.P. (1980)** Covalent binding and hemolytic activity of complement proteins. *PNAS*, 77 (12), p. 7194-7198.
- **Law, S.K., Dodds, A.W. (1997)** The internal thioester and the covalent binding properties of the complement proteins C3 and C4. *Protein Science*, 6, p. 263-274.
- **Lecuit, M. (2005)** Understanding how *Listeria monocytogenes* targets and crosses host barriers. *Clinical Microbiology and Infection*, 11 (6), p. 430-436.
- **Lee, L.Y., Liang, X., Hook, M., Brown, E.L. (2004a)** Identification and characterization of the C3 binding domain of the *Staphylococcus aureus* extracellular fibrinogen binding protein (Efb). *Journal of Biological Chemistry*, 279 (49), p. 50710-50716.
- **Lee, L.Y., Hook, M., Haviland, D., Wetsel, R.A., Yonter, E.O., Syribeys, P., Vernachio, J., Brown, E.L. (2004b)** Inhibition of Complement Activation by a Secreted *Staphylococcus aureus* Protein. *The Journal of Infectious Diseases*, 190, p. 571–579.
- **Leenaars, M., Hendriksen, C. (2005)** Critical Steps in the Production of Polyclonal and Monoclonal Antibodies: Evaluation and Recommendations. *ILAR Journal*, 46 (3), p. 269-279.
- **Lehrer, R.I., Ganz, T. (1996)** Endogenous Vertebrate Antibiotics: Defensins, Protegrins and Other Cysteine-Rich Antimicrobial Peptides. *Annals of the New York Academy of Sciences*, 797, p. 228-239.
- **Lenting, H.B.M., Neys, F.W., van den Bosch, H. (1988)** Regulatory aspects of mitochondrial phospholipase A2 from rat liver: effects of proteins, phospholipids and calcium ions. *Biochimica et Biophysics Acta*, 961, p. 129-138.

• **Leulier, F., Parquet, C., Pili-Floury, S., Ryu, J., Caroff, M., Lee, W., Mengin-Lecreulx, Lemaitre, B. (2003)** The *Drosophila* immune system detects bacteria through specific peptidoglycan recognition. *Nature Immunology*, 4, p. 478-484.

• **Lévesque, C.M., Voronejskaia, E., Huang, Y.K., Mair, R.W., Ellen, R.P., Cvitkovitch, D.G. (2005)** Involvement of Sortase Anchoring of Cell Wall Proteins in Biofilm Formation by *Streptococcus mutans*. *Infection and Immunity*, 73 (6), p. 3773-3777.

• **Levin, W., Daniel, R.F., Stoner, C.R., Stoller, T.J., Wardwell-Swanson, J.A., Angelillo, Y.M., Familletti, P.C., Crowl, R.M. (1992)** Purification of recombinant human secretory phospholipase A2 (Group II) produced in long-term immobilized cell culture. *Protein Expression and Purification*, 3 (1), 27-35.

• **Liechti, G.W., Kuru, E., Hall, E., Kalinda, A., Brun, Y.V., Van Nieuwenhze, M., Maurelli, A.T. (2014)** A new metabolic cell-wall labelling method reveals peptidoglycan in *Chlamydia trachomatis*. *Nature*, 506, p. 507–510.

• **Lim, D., Strynadka, N. C. (2002)** Structural basis for the beta lactam resistance of PBP2a from methicillin-resistant *Staphylococcus aureus*. *Nature Structural Biology,* 9**,** p.870-876.

• **Lim, L.H.K., Pervaiz, S. (2007)** Annexin 1: the new face of an old molecule. *FASEB Journal*, 21, p. 968-975.

• **Lin, L.L., Wartmann, M., Lin, A.Y., Knopf, J.L., Seth, A., Davis, R.J. (1993)** cPLA2 is phosphorylated and activated by MAP kinase. *Cell*, 72, p. 269-278.

• **Ling, J.M., Schryvers, A.B. (2006)** Perspectives on interactions between lactoferrin and bacteria. *Biochemistry and Cell Biology*, 84 (3), p. 275-281.

• **Ling, L.L., Schneider, T., Peoples, A.J., Spoering, A.L., Engels, I., Conlon, B.P., Mueller, A., Schäberle, T.F., Hughes, D.E., Epstein, S., Jones, M., Lazarides, L., Steadman, V.A., Cohen, D.R., Felix, C.R., Fetterman, K.A., Millett, W.P., Nitti, A.G., Zullo, A.M., Chen, C., Lewis, K. (2015)** A new antibiotic kills pathogens without detectable resistance. *Nature*, 517, p. 455-459.

• **Litman, G.W., Ras, J.P., Fugmann, S.D. (2010)** The origins of vertebrate adaptive immunity. *Nature Reviews Immunology*, 10 (8), p. 543-553.

• **Liu, G.Y., Essex, A., Buchanan, J.T., Datta, V., Hoffman, H.M., Bastian, J.F., Fierer, J., Nizet, V. (2005)** *Staphylococcus aureus* golden pigment impairs neutrophil killing and promotes virulence through its antioxidant activity. *Current Opinion in Microbiology*, 15, p. 92-99.

• **Livermore, D.M. (2000)** Antibiotic resistance in staphylococci. *International Journal of Antimicrobial Agents*, 16 (Suppl.), p. S3–S10.

• **Lizano, S., Domont, G., Perales, J. (2003)** Natural phospholipase A2 myotoxin inhibitor proteins from snakes, mammals and plants. *Toxicon*, 42, p. 963-977.

• **Lizarbe, M.A., Barrasa, J.I., Olmo, N., Gavilanes, F., Turnay, J. (2013)** Annexin-Phospholipid Interactions. Functional Implications. *International Journal of Molecular Sciences*, 14, p. 2652-2683.

• **Llewelyn, M. and Cohen, J. (2002)** Superantigens: microbial agents that corrupt immunity, *Lancet Infectious Diseases*, 2 (3), p.156-62.

• **Loll, P.J., Axelsen, P.H. (2000)** The structural biology of molecular recognition by vancomycin. *Annual Review of Biophysics and Biomolecular Structure*, 29, p. 265-289.

• **Loweth, A.C., Scarpello, J.H.B., Morgan, N.G. (1996)** A Specific Inhibitor of Cytosolic Phospholipase A2 Activity, $AACOCF_3$, Inhibits Glucose-Induced Insulin Secretion from Isolated Rat Islets. *Biochemical and Biophysical Research Communications*, 218, p. 423-427.

• **Lowy, F.D. (1998)** *Staphylococcus aureus* infections. *The New England Journal of Medicine*, 339 (8), p.520-532.

• **Lowy, F.D. (2003)** Antimicrobial resistance: the example of *Staphylococcus aureus*. *The Journal of Clinical Investigation*, 111, p. 1265-1273.

• **Lowy, F.D., (2010)** Staphylococcal Infections. In: Kasper, D.L., Fauci, A.S. eds. 2010. *Harrison's Infectious Diseases*. McGraw-Hill Professional, Chapter 35.

• **Lu, L., Ma, Z., Jokiranta, T.S., Whitney, A.R., DeLeo, F.R., Zhang, J. (2005)** Species-Specific Interaction of *Streptococcus pneumoniae* with Human Complement Factor H1. *Journal of Immunology*, 181, p. 7138-7146.

• **Lui, M.M.S., Thomas, R., Lee, Y.C.G. (2016)** Complications of indwelling pleural catheter use and their management. *BMJ Open Respiratory Research*, 3 (1), e000123.

• **Lynch, N.J., Roscher, S., Hartung, T., Morath, S., Matsushita, M., Maeneel, D.N., Kuraya, M., Fujita, T., Schwaeble, W.J. (2004)** L-Ficolin Specifically Binds to Lipoteichoic Acid, a Cell Wall Constituent of Gram-Positive Bacteria, and Activates the Lectin Pathway of Complement. *Journal of Immunology*, 172, p. 1198-1202.

• **Ma, Y.G., Cho, M., Zhao, M., Park, J.W., Matsushita, M., Fujita, T., Lee, B.L. (2004)** Human Mannose- binding Lectin and L-Ficolin Function as Specific Pattern Recognition Proteins in the Lectin Activation Pathway of Complement. *The Journal of Biological Chemistry*, 279 (24), p. 25307-25312.

• **Machiah, D.K., Gowda, T.V. (2006)** Purification of a post-synaptic neurotoxic phospholipase A2 from *Naja naja* venom and its inhibition by a glycoprotein from *Withania somnifera*. *Biochimie*, 88, p. 701-710.

• **Madigan, M. T., Martinko, J. M., Parker, J. (2006)** *Brock Biology of Microorganism.* United States of America, Pearson Prentice Hall. Pearson Education Inc.

• **Maga, E.A., Cullor, J.S., Smith, W., Anderson, G.B., Murray, J.D. (2006)** Human Lysozyme Expressed in the Mammary Gland of Transgenic Dairy Goats Can Inhibit the Growth of Bacteria That Cause Mastitis and the Cold-Spoilage of Milk. *Foodborne Pathogens and Disease*, 3 (4), p. 384-392.

• **Malla, B.K. (2003)** A study on 'Vermiform Appendix'-a caecal appendage in common laboratory mammals. *Kathmandu University Medical Journal*, 1 (4), Issue 4, p. 272-275.

• **Mannion, B.A., Weiss, J., Elsbach, P. (1990)** Separation of Sublethal and Lethal Effects of the Bactericidal/Permeability Increasing Protein on *Escherichia coli*. *Journal of Clinical Investigation*, 85, p. 853-860.

• **Mantovani, A., Cassatella, M.A., Costantini, C., Jaillon, S. (2011)** Neutrophils in the activation and regulation of innate and adaptive immunity. *Nature Review Immunology*, 11(8), p. 519-531.

• **Marshall, J., Krump, E., Lindsay, T., Downey, G., Ford, D.A., Zhu, P., Walker, P., Rubin, B. (2000)** Involvement of Cytosolic Phospholipase A2 and Secretory Phospholipase A2 in Arachidonic Acid Release from Human Neutrophils. *Journal of Immunology*, 164, p. 2084-2091.

• **Mastellos, D., Papadimitriou, J.C., Franchini, S., Tsonis, P.A., Lambris, J.D. (2001)** A novel role of complement: mice deficient in the fifth component of complement (C5) exhibit impaired liver regeneration. *Journal of Immunology*, 166, p. 2479-2486.

• **Mastellos, D., Prechl, J., László, G., Papp, K., Oláh, E., Argyropoulos, E., Franchini, S., Tudoran, R., Markiewski, M., Lambris, J.D., Erdei, A. (2004)** Novel monoclonal antibodies against mouse C3 interfering with complement activation: description of fine specificity and applications to various immunoassays. *Molecular Immunology*, 40, p. 1213-1221.

• **Matsushita, M., Fujita, T. (1992)** Activation of the classical complement pathway by mannose-binding protein in association with a novel C1s-like serine protease. *Journal of Experimental Medicine*, 176, p. 1497-1502.

• **Matsushita, M., Fujita, T. (1995)** Cleavage of the third component of complement (C3) by mannose-binding protein-associated serine protease (MASP) with subsequent complement activation. *Immunobiology*, 194, p. 443-448.

• **Matsushita, M., Thiel, S., Jensenius, J.C., Terai, I., Fujita, T. (2000a)** Proteolytic Activities of Two Types of Mannose-Binding Lection-Asspciated Serine Proteases. *The Journal of Immunology*, 165 (5), p. 2637-2642.

• **Matsushita, M., Endo, Y., Fujita, T. (2000b)** Cutting Edge: Complement-Activating Complex of Ficolin and Mannose-Binding Lectin-Associated Serine Protease. *The Journal of Immunology*, 164 (5), p. 2281-2284.

• **Mauël, C., Bauduret, A., Chervet, C., Beggah, S., Karamata, D. (1995)** In *Bacillus subtilis* 168, teichoic acid of the cross-wall may be different from that of the cylinder: a hypothesis based on transcription analysis of *tag* genes. *Microbiology*, 141, p. 2379-2389.

• **May, J.J, Finking, R., Wiegeshoff, F., Weber, T.T., Bandur, N., Koert, U., Marahiel, M.A. (2005)** Inhibition of the D-alanine:D-alanyl carrier protein ligase from *Bacillus subtilis* increases the bacterium's susceptibility to antibiotics that target the cell wall. *FEBS Journal*, 272 (12), p. 2993–3003.

• **Mayer, R.J., Marshall, L.A. (1993)** New insights on mammalian phospholipase A2(s); comparison of arachidonoyl-selective and -nonselective enzymes. *FASEB Journal*, 7, p. 339-348.

• **McCarthy, A.J., Witney, A.A., Gould, K.A., Moodley, A., Guardabassi, L., Voss, A., Denis, O., Broens, E.M., Hinds, J., Lindsay, J.A. (2011)** The Distribution of Mobile Genetic Elements (MGEs) in MRSA CC398 Is Associated with Both Host and Country. *Genome Biology*

Evolution, 3, p. 1164-1174.

- **McCarthy, A.J., Lindsay, J.A., Loefflert, A. (2012)** Are all meticillin-resistant *Staphylococcus aureus* (MRSA) equal in all hosts? Epidemiological and genetic comparison between animal and human MRSA. *Veterinary Dermatology*, 23, p. 267-e57.
- **McCormick, J.K., Yarwood, J.M., Schlievert, P.M. (2001)** Toxic Shock Syndrome and Bacterial Superantigens: an update. *Annual Review Microbiology*, 55, p.77-104.
- **McDermott, A.M. (2013)** Antimicrobial Compounds in Tears. Experimental Eye Research, 117, doi:10.1016/j.exer.2013.07.014.
- **McDevitt, D., Nanavaty, T., House-Pompeo, K., Bell, E., Turner, N., McIntire, L., Foster, T., Höök, M. (1997)** Characterization of the interaction between the *Staphylococcus aureus* clumping factor (ClfA) and fibrinogen. *European Journal of Biochemistry*, 247, p. 416-424.
- **McGavin, M.J., Arsic, B., Nickerson, N.N. (2012)** Evolutionary blueprint for host- and niche- adaptation in *Staphylococcus aureus* clonal complex CC30. *Frontiers in Cellular and Infection Immunology*, DOI=10.3389/fcimb.2012.00048
- **McKeegan, K.S., Borges-Walmsey M.I., Walmsley, A.R. (2002)** Microbial and viral drug resistance mechanisms. *Trends in Microbiology*, 10 (10), Suppl., p. 8-14.
- **McLoughlin, R.M., Solinga, R.M., Rich, J., Zaleski, K.J., Cocchiaro, J.L., Risley, A., Tzianabos, A.O., Lee, J.C. (2006)** CD4+ T cells and CXC chemokines modulate the pathogenesis of *Staphylococcus aureus* wound infections. *PNAS*, 103(27), p. 10408-10413.
- **Medzhitov, R., Janeway, C. Jr (2000)** Innate immune recognition: mechanisms and pathways. *Immunological Reviews*, 173, p. 89-97.
- **Medzhitov, R., Janeway, C. Jr. (2002)** Decoding the Patterns of Self and Nonself by the Innate Immune System. *Science*, 296 (5566), p. 298-300.
- **Mengin-Lecreulx, D., Lemaitre, B. (2005)** Structure and metabolism of peptidoglycan and molecular requirements allowing its detection by the *Drosophila* innate immune system. *Journal of Endotoxin Research,* 11 (2), p. 105-111.
- **Menschikowski, M., Hagelgans, A., Siegert, G. (2006)** Secretory phospholipase A2 of group IIA:Is it an offensive or a defensive player during atherosclerosis and other inflammatory diseases? *Prostaglandins & other Lipid Mediators*, 79, p. 1–33.
- **Merchant, M., Small, J., Falconi, R., Crookshank, B.J. (2009)** A rapid fluorometric assay for the determination of serum phospholipase A2 activity. *Microchemical Journal*, 91, p. 82-84.
- **Merchant, M., Juneau, K., Gemillion, J., Falconi, R., Doucet, A., Shirley, M.H. (2011)** Characterization of Serum Phospholipase A2 Activity in Three Diverse Species of West African Crocodiles. *Biochemistry Research International*, vol. 2011, Article ID 925012, doi:10.1155/2011/925012
- **Meredith, T.C., Swoboda, J.G., Walker, S. (2008)** Late-stage polyribitol phosphate wall teichoic acid biosynthesis in *Staphylococcus aureus*. *Journal of Bacteriology*, 190, p. 3046–3056.

• **Merrel, K., Southwick, K., Graves, S.W., Esplin, M.S., Lewis, N.E., Thulin, C.D. (2004)** Analysis of Low-Abundance, Low-Molecular-Weight Serum Proteins Using Mass Spectrometry. *Journal of Biomolecular Techniques*, 15 (4), p. 238-248.

• **Michel, T., Reichhart, .M. Hoffmann, J.A., Royet, J. (2001)** Drosophila Toll is activated by Gram-positive bacteria through a circulating peptidoglycan recognition protein. *Nature*, 414, p. 756-759.

• **Miller, S.J., Aly, R., Shinefeld, H.R., Elias, P.M. (1988)** In vitro and in vivo antistaphylococcal activity of human stratum corneum lipids. *Archives of Dermatology*, 124 (2), p. 209-215.

• **Mishra, R.S., Carnevale, K.A., Cathcart, M.K. (2008)** iPLA2β: front and center in human monocyte chemotaxis to MCP-1. *Journal of Experimental Medicine*, 205, p. 347-359.

• **Miwa, T., Song, W. (2006)** Membrane complement regulatory proteins: insight from animal studies and relevance to human diseases. *International Immunopharmacology*, 1 (3), p.445-459.

• **Mizushima, H., Kudo, I., Horigome, K., Murakami, M., Hayakawa, M., Kim, D.K., Kondo, E., Tomita, M., Inoue, K. (1989)** Purification of rabbit platelet secretory phospholipase A2 and its characteristics. *Journal of Biochemistry*, 105 (4), p. 520-525.

• **Moerman, L., Bosteels, S., Noppe, W., Willems, J., Clynen, E., Schoofs, L., Thevissen, K., Tytgat, J., van Eldere, J., van der Walt, J., Verdonck, F. (2002)** Antibacterial and antifungal properties of a-helical, cationic peptides in the venom of scorpions from the South Africa. *European Journal of Biochemistry*, 269, p. 4799-4810.

• **Mook, D., Knauer, M., Bueno-De-Mesquita, J.M., Retel, V.P., Wesseling, J., Linn, S.C., Van'T Veer, L.J., Rutgers, E.J. (2010)** Metastatic potential of T1 breast cancer can be predicted by the 70-gene MammaPrint signature. *Annals of Surgical Oncology*, 17 (5), 1406-1413.

• **Moreira, L.A., Ito, J., Ghosh, A., Devenport, M., Zieler, H., Abraham, E.G., Crisanti, A., Nolan, T., Catteruccia, F., Jacobs-Lorena, M. (2002)** Bee Venom Phospholipase Inhibits Malaria Parasite Development in Transgenic Mosquitoes. *Journal of Biological Chemistry*, 277 (43), p. 40839-40843.

• **Morera, D., MacKenzie, S.A. (2011)** Is there a direct role for erythrocytes in the immune response? *Veterinary Research*, 42, p. 89.

• **Morgan, B.P. (1995)** Complement regulatory molecules: application to therapy and transplantation. *Immunology Today*, 16 (6), p. 257-259.

• **Morgan, B.P., Gasque, P. (1997)** Extrahepatic complement biosynthesis: where, when and why? *Clinical and Experimental Immunology*, 107 (1), p. 1–7.

• **Morgan, B.P. (Editor) (2000)** Complement Methods and Protocols. Methods in Molecular Biology, Volume 150. Humana Press Inc., Totowa, NJ.

• **Morgan, R.O., Fernandez, M.P. (1997)** Annexin gene structure and molecular evolution genetics. *Cellular and Molecular Life Sciences*, 53, p. 508-515.

• **Mosior, M., Six, D.A., Dennis, E.A. (1998)** Group IV cytosolic phospholipase A2 binds

with high affinity and specificity to phosphatidylinositol 4,5-bisphosphate resulting in dramatic increases in activity. *Journal of Biological Chemistry,* 273, p. 2184-2191.

- **Müller-Eberhard, H.J. (1986)** The Membrane Attack Complex of complement. *Annual Reviews Immunology*, 4, p. 503-528.
- **Mullineaux, C.W., Nenninger, A., Ray, N., Robinson, C. (2006)** Diffusion of green fluorescent protein in three cell environments in *Escherichia coli*. *Journal of Bacteriology*, 188, p. 3442-3448.
- **Muñoz-Bellido, J. L., Alonzo Manzanares, M., Martínez Andres, J. A., Gutiérrez Zufiaurre, M. N., Ortiz, G., Segovia Hernández, M., García-Rodríguez, J. A. (1999)** Efflux pump-mediated quinolone resistance in *Staphylococcus aureus* strains wild type for *gyrA*, *gyrB*, *grlA*, and *norA*. *Antimicrobial Agents and Chemotherapy,* 43, p. 354-356.
- **Murakami, M., Kobayashi, T., Umeda, M., Kudo, I., Inoue, K. (1988)** Monoclonal Antibodies against Rat Platelet Phospholipase A2. *Journal of Biochemistry*, 104, p. 884-888.
- **Murakami, M., Taketomi, Y., Girard, C., Yamamoto, K., Lambeau, G. (2010)** Emerging roles of secreted phospholipase A2 enzymes: Lessons from transgenic and knockout mice. *Biochimie*, 92, p. 561-582.
- **Murakami, M., Taketomi, Y., Sato, H., Yamamoto, K. (2011)** Secreted phospholipase A2 revisited. *Journal of Biochemistry*, 150 (3), p. 233-255.
- **Murakami, M., Lambeau, G. (2013)** Emerging roles of secreted phospholipase A_2 enzymes: An update. *Biochimie*, 95, p. 43-50.
- **Murray, I. A., Shaw, W. V. (1997)** O-Acetyltransferases for chloramphenicol and other natural products. *Antimicrobial Agents and Chemotherapy*, 41 (1), p. 1-6.
- **Naidoo, J., Noble, W. C. (1978)** Transfer of gentamicin resistance between strains of *Staphylococcus aureus* on skin. *Journal of General Microbiology,* 107**,** 391-393.
- **Navarre, W.W., Schneewind, O. (1999)** Surface Proteins of Gram-Positive Bacteria and Mechanisms of Their Targeting to the Cell Wall Envelope. *Microbiology and Molecular Biology Reviews*, 63 (1), p. 174-229.
- **Neth, O., Jack, D.L., Dodds, A.W., Holzel, H., Klein, N.J., Turner, M.W. (2000)** Mannose-Binding Lectin Binds to a Range of Clinically Relevant Microorganisms and Promotes Complement Deposition. *Infection and Immunity*, 68 (2), p. 688-693.
- **Neuhaus, C., Baddiley, J. (2003)** A Continuum of Anionic Charge: Structures and Functions of D- Alanyl-Teichoic Acids in Gram-Positive Bacteria. *Microbiology and Molecular Biology Reviews*, 67(4), p. 686-732.
- **Neumann, Y., Ohlsen, K., Donat, S., Engelmann, S., Kusch, H., Albrecht, D., Cartron, M., Hurd, A., Foster, S.J. (2015)** The effect of skin fatty acids on *Staphylococcus aureus. Archives of Microbiology*, 197 (2), p. 254-267.
- **Nevalainen, T.J., Eskola, J.U., Aho, A.J., Hapia, V.T., Lövgren, T.N-E., Näntö, V (1985)** Immunoreactive Phospholipase A2 in Serum in Acute Pancreatitis and Pancreatic Cancer. *Clinical Chemistry*, 31 (7), p.1116-1120.
- **Nevalainen, T.J., Meri, K.M., Niemi, M. (1993)** Synovial type (group II) phospholipase

A_2 in human seminal plasma. *Andrologia*, 25, p. 355-358.

- **Nevalainen, T.J., Graham, G.G., Scott, K.F. (2008)** Antibacterial actions of secreted phospholipases A2: Review. *Biochimica et Biophysica Acta*, 1781, p. 1-9.
- **Neves-Ferreira, A.G.C., Cardinale, N., Rocha, S.L.G., Perales, J., Domont, G.B. (2000)** Isolation and characterization of DM40 and DM43, two snake venom metalloproteinase inhibitors from *Didelphis marsupialis* serum. *Biochimica et Biophysica Acta*, 1474, p. 309-320.
- **Ng, E. Y., Trucksis, M., Hooper, D. C. (1994)** Quinolone resistance mediated by norA: physiologic characterization and relationship to flqB, a quinolone resistance locus on the *Staphylococcus aureus* chromosome. *Antimicrobial Agents and Chemotherapy,* 38**,** p.1345-1355.
- **Nizet, V. (2007)** Understanding how leading bacterial pathogens subvert innate immunity to reveal novel therapeutic targets. *Journal of Allergy and Clinical Immunology*, 120 (1), p. 13-22.
- **Nordahl, E.A., Rydengard, V., Nyberg, P., Nitsche, D.P., Morgelin, M., Malmsten, M., Bjorck, L., Schmidtchen, A. (2004)** Activation of the complement system generates antibacterial peptides. *PNAS*, 101, p. 16879-16884.
- **Noore, J., Noore, A., Li, B. (2013)** Cationic Antimicrobial Peptide LL-37 Is Effective against both Extra- and Intracellular *Staphylococcus aureus. Antimicrobial Agents and Chemotherapy*, 57 (3), p. 1283-1290.
- **Nurden, A.T., Butcher, P.D., Hawkey, C.M. (1977)** Comparative studies on the glycoprotein composition of mammalian platelets. *Comparative Biochemistry and Physiology*, 56 (B), p. 407-413.
- **O'Brien, L., Kerrigan, S.W., Kaw, G., Hogan, M., Penadés, J., Litt, D., Fitzgerald, D.J., Foster, T.J., Cox, D. (2002)** Multiple mechanisms for the activation of human platelet aggregation by *Staphylococcus aureus*: roles for the clumping factors ClfA and ClfB, the serine–aspartate repeat protein SdrE and protein A. *Molecular Microbiology*, 44 (4), p. 1033-1044.
- **Ocampo, J., Afanor, N., Vives, M.J., Moreno, J.C., Leidy, C. (2010)** The antibacterial activity of phospholipase A2 type IIA is regulated by the cooperative lipid chain melting behavior in *Staphylococcus aureus*. *Biochimica et Biophysica Acta*, 1798, p. 1021-1028.
- **Okerman, L. (1989)** General background. In: Diseases of Domestic Rabbits. Oxford: Blackwell Scientific Publications, p. 4-8.
- **Olling, S. (1977)** Sensitivity of gram-negative bacilli to the serum bactericidal activity: a marker of the host-parasite relationship in acute and persisting infections. *Scandinavian Journal of Infectious Diseases* (Suppl), 10, p. 1-40.
- **O'Neill, L.A.J, Greene, C (1998)** Signal transduction pathways activated by the IL-1 receptor family: ancient signalling machinery in mammals, insects, and plants. *Journal of Leukocyte Biology*, 63, p. 650-657.
- **Ong, P.Y., Ohtake, T., Brandt, C., Strickland, I., Boguniewicz, M., Ganz, T., Gallo, R.L., Leung, D.Y. (2002)** Endogenous antimicrobial peptides and skin infections in atopic dermatitis. *The New England Journal of Medicine*, 347 (15), p. 1151-1160.

• **O'Riordan, K., Lee, J.C. (2004)** *Staphylococcus aureus* capsular polysaccharides. *Clinical Microbiology Reviews*, 17, p. 218-234.

• **Osborn, M.J., Rothfield, L. (2007)** Cell shape determination in *Escherichia coli*. *Current Opinion in Microbiology*, 10, p. 606–610.

• **Osterman, K.L., Rahm, V.A. (2000)** Lactation mastitis: bacterial cultivation of breast milk, symptoms, treatment, and outcome. *Journal of Human Lactation*, 16 (4), p. 297-302.

• **Ouyang, Y., Kaminski, N.E. (1999)** Phospholipase A2 inhibitors p-bromophenacyl bromide and arachidonyl trifluoromethyl ketone suppressed interleukin-2 (IL-2) expression in murine primary splenocytes. *Archives Toxicology*, 73, p. 1-6.

• **Paliyath, G., Pinhero, R.G., Yada, R.Y., Murr, D.P. (1999)** Effect of Processing Conditions on Phospholipase D Activity of Corn Kernel Subcellular Fractions. *Journal of Agricultural and Food Chemistry*, 47, p. 2579−2588.

• **Palm, N., Medzhitov, R. (2009)** Pattern recognition receptors and control of adaptive immunity. *Immunological Reviews*, 227 (1), p. 221–233.

• **Palmqvist, N., Patti, J.M., Tarkowski, A., Josefsson, E. (2004)** Expression of staphylococcal clumping factor A impedes macrophage phagocytosis. *Microbes and Infection*, 6, p. 188-195.

• **Pan, X., Yang, Y., Zhang, J. (2014)** Molecular basis of host specificity in human pathogenic bacteria. *Emerging Microbes and Infections*, 3, e23; doi:10.1038/emi.2014.23

• **Pangburn, M.K. (1992)** Spontaneous Reformation of the Intramolecular Thioester in Complement Protein C3 and Low Temperature Capture of a Conformational Intermediate Capable of Reformation. *The Journal of Biological Chemistry*, 267 (12), p. 8584-8590.

• **Pantosti, A. (2012)** Methicillin-resistant *Staphylococcus aureus* associated with animals and its relevance to human health. *Frontiers in Microbiology*, 3(127), 1-12.

• **Parente, L., Solito, E. (2004)** Annexin 1: more than an anti-phospholipase protein. *Inflammation Research*, 53, p. 125-132.

• **Park, K., Kurokawa, K., Zheng, L., Jung, D., Tateishi, K., Jin, J., Ha, N., Kang, H.J., Matsushita, M., Kwak, J., Takahashi, K., Lee, B.L. (2010)** Human Serum Mannose-binding Lectin Senses Wall Teichoic Acid Glycopolymer of *Staphylococcus aureus*, Which Is Restricted in Infancy. *The Journal of Biological Chemistry*, 285 (35), p. 27167-27175.

• **Parkin, J., Cohen, B. (2001)** An overview of the immune system. *Lancet*, 357, p.1777-1789.

• **Pasare, C, Medzhitov, R. (2004)** Toll-dependent control mechanisms of CD4 T-cell activation. *Immunity*, 21, p. 733-741.

• **Patel, D., Wines, B.D., Langley, R.J., Fraser, J.D. (2010)** Specificity of staphylococcal superantigen-like protein 10 toward the human IgG1 Fc domain. *Journal of Immunology*, 184, p. 6283-6292.

• **Patton, N.M. (1994)** Colony husbendry. In: Manning, P.J., Ringler, D.H., Newcomer, C.E. (Eds.) The biology of the laboratory rabbit. Second ed. Academic Press, San Diego, p. 367–376.

• **Peacock, S. J., de Silva, I., and Lowy, F. D. (2001).** What determines nasal carriage of *Staphylococcus aureus*? *Trends Microbiology*, 9 (12), p. 605-610.

• **Pepys, M.B. (1974)** Role of complement in induction of antibody production in vivo. Effect of cobra factor and other C3-reactive agents on thymus-dependent and thymus-independent antibody responses. *Journal of Experimental Medicine*, 140, p. 126-145.

• **Perales, J., Amorim, C.Z., Rocha, S.L.G., Domont, G.B., Moussatché, H. (1992)** Neutralization of the oedematogenic activity of *Bothrops jaraca* venom on the mouse paw by an antibothropic fraction isolated from opossum (*Didelphis marsupialis*) serum. *Agents and actions*, 37 (3-4), p. 250-259.

• **Percy, M.G., Gründling, A. (2014)** Lipoteichoic Acid Synthesis and Function in Gram-Positive Bacteria. *Annual Review of Microbiology*, 68, p. 81-100.

• **Perego, M., Glaser, P., Minutellot, A., Strauch, M.A., Leopold, K., Fischer, W. (1995)** Incorporation of D-alanine into Lipoteichoic Acid and Wall Teichoic Acid in *Bacillus subtilis*: Identification of genes and regulation. *The Journal of Biological Chemistry*, 270 (26), p. 15598-15606.

• **Pereira, M.P., D'Elia, M.A., Troczynska, J., Brown, E.D. (2008)** Duplication of Teichoic Acid Biosynthetic Genes in *Staphylococcus aureus* Leads to Functionally Redundant Poly-(Ribitol Phosphate) Polymerases. *Journal of Bacteriology*, 190 (16), p. 5642-5649.

• **Pérez, J.C., Sánchez, E.E. (1999)** Natural protease inhibitors to hemorrhagins in snake venoms and their potential use in medicine. *Toxicon*, 37, p. 703–728.

• **Pernet, E., Guillemot, L., Burgel, P., Martin, C., Lambeau, G., Sermet-Gaudelus, I., Sands, D., Leduc, D., Morand, P.C., Jeammet, L., Chignard, M., Wu, Y., Touqui, L. (2014)** *Pseudomonas aeruginosa* eradicates *Staphylococcus aureus* by manipulating the host immunity. *Nature Communications*, 5:5105, doi: 10.1038/ncomms6105.

• **Perry, R.D., Fetherston, J.D. (1997)** *Yersinia pestis*- Etiologic Agent of Plague. *Clinical Microbiology Reviews*, 10 (1), p. 35-66.

• **Peschel, A., Otto, M., Jack, R.W., Kalbacher, H., Jung, G., Gotz, F. (1999)** Inactivation of the *dlt* operon in *Staphylococcus aureus* confers sensitivity to defensins, protegrins and other antimicrobial peptides. *Journal of Biological Chemistry*, 274, p. 8405–8410.

• **Peschel, A., Vuong, C., Otto, M., Gotz, F. (2000)** The D-alanine residues of *Staphylococcus aureus* teichoic acids alter the susceptibility to vancomycin and the activity of autolytic enzymes. *Antimicrobial Agents and Chemotheraphy*, 44, p. 2845-2847.

• **Peschel, A., Jack, R.W., Otto, M., Collins, L.V., Staubitz, P., Nicholson, G., Kalbacher, H., Nieuwenhuizen, W.F., Jung, G., Trakowski, A., vanKessel, K., van Strijp, J. (2001)** *Staphylococcus aureus* Resistance to Human Defensins and Evasion of Neutrophil Killing via the Novel Virulence Factor MprF Is Based on Modification of Membrane Lipids with L-Lysine. *Journal of Experimental Medicine*, 193 (9), p. 1067-1076.

• **Peschel, A., Sahl, H. (2006)** The co-evolution of host cationic antimicrobial peptides and microbial resistance. *Nature Reviews Microbiology*, 4, p. 529-536.

• **Petersen, S., Thiel, S., Jensen, L., Vorup-Jensen, T., Koch, C., Jensenius, J.C.**

(2000) Control of the classical and the MBL pathway of complement activation. *Molecular Immunology*, 37, p. 803- 811.

- **Petras, D., Sanz, L., Segura, A., Herrera, M., Villalta, M., Solano, D., Vargas, M., Leon, G., Warrell, D.A., Theakston, R.D.G., Harrison, R.A., Durfa, N., Nasidi, A., Guitiérrez, J.M., Calvete, J.J. (2010)** Snake Venomics of African Spitting Cobras: Toxin Composition and Assessment of Congeneric Cross-Reactivity of the Pan-African EchiTAb-Plus-ICP Antivenom by Antivenomics and Neutralization Approaches. *Journal of Proteome Research*, 10, p. 1266-1280.
- **Pichoff, S., Lutkenhaus, J. (2007)** Overview of cell shape: cytoeskeletons shape cells. *Current Opinion in Microbiology,* 10 (6), p. 601–605.
- **Pieper, R., Gatlin, C.L., Makusky, A.J., Russo, P.S., Schatz, C.R., Miller, S.S., Su. Q., McGrath, A.M., Estock, M.A., Parmar, P.P., Zhao, M., Huang, S., Zhou, J., Wang, F., Erquer-Blasco, R., Anderson, N.L., Taylor, J., Steiner, S. (2003)** The human serum proteome: Display of nearly 3700 chromatographically separated protein spots on two-dimensional electrophoresis gels and identification of 325 distinct proteins. *Proteomics*, 3, p. 1345-1364.
- **Pishchany, G., McCoy, A.L., Torres, V.J., Krause, J.C., Crowe, J.E., Fabry, M.E., Skaar, E.P. (2010)** Specificity for Human Hemoglobin Enhances *Staphylococcus aureus* Infection. *Cell Host & Microbe*, 8, p. 544-550.
- **Phinikaridou, A., Hallock, K.J., Qiao, Y., Hamilton, J.A. (2009)** A robust rabbit model of human atherosclerosis and atherothrombosis. *Journal of Lipid Research*, 59 (5), p.787-797.
- **Platts-Mills, T., Ishizaka, K. (1974)** Activation of the Alternate Pathway of Human Complement by Rabbit Cells. *Journal of Immunology*, 113 (1), p. 348-358.
- **Pospisil, R., Mage, R.G. (1998)** Rabbit appendix: a site of development and selection of the B cell repertoire. *Current Topics in Microbiology and Immunology*, 229, p. 59-70.
- **Postma, B., Poppelier, M.J., van Galen, J.C., Prossnitz, E.R., van Strijp, J., de Haas, C., van Kessel, K. (2004)** Chemotaxis Inhibitory Protein of *Staphylococcus aureus* Binds Specifically to the C5a and Formylated Peptide Receptor. *Journal of Immunology*, 172, p. 6994-7001.
- **Powell, D. A., Duckworth, M., Baddiley, J. (1975)** A membrane-associated lipomannan in micrococci. *Biochemical Journal*, 151, p.387–397.
- **Poyart, C., Pellegrini, E., Marceau, M., Baptista, M., Jaubert, F., Lamy, M.C., Trieu-Cuot, P. (2003)** Attenuated virulence of *Streptococcus agalactiae* deficient in D-alanyl-lipoteichoic acid is due to an increased susceptibility to defensins and phagocytic cells. *Molecular Microbiology*, 49, p. 1615-1625.
- **Price, J.A. III (2007)** A colorimetric assay for measuring phospholipase A2 degradation of phosphatidylcholine at physiological pH. *Journal of Biochemical and Biophysical Methods*, 70, 441-444.
- **Projan, S.J., Novick, R.P. (1997)** The molecular basis of pathogenicity. In: Crossley K.B., Archer G.L (eds). The Staphylococci in human disease. *Churchill Livingstone New York*, pp. 55-81.

• **Plückthun, A., Dennis, E.A. (1985)** Activation, Aggregation, and Product Inhibitionof Cobra Venom Phospholipase A2 and Comparison with Other Phospholipase. *Journal of Biological Chemistry*, 260 (20), p. 11099-11106.

• **Qi, Z.Q., Yonaha, K., Tomihara, Y., Toyama, S. (1994)** Characterization of the antihemorrhagic factors of mongoose (*Herpestes edwardsii*). *Toxicon*, 32, p. 1459-1469.

• **Qian, Z., Yin, Y., Zhang, Y., Lu, L., Li, Y., Jiang, Y. (2006)** Genomic characterization of ribitol teichoic acid synthesis in *Staphylococcus aureus*: genes, genomic organization and gene duplication. *BMC Genomics*, 7, 74.

• **Qiu, Z.H., de Carvalho, M.S., Leslie, C.C. (1993)** Regulation of phospholipase A2 activation by phosphorylation in mouse peritoneal macrophages. *Journal of Biological Chemistry,* 268, p. 24506-24013.

• **Qiu, Z.H., Gijon, M.A., de Carvalho, M.S., Spencer, D.M., Leslie, C.C. (1998)** The role of calcium and phosphorylation of cytosolic phospholipase A2 in regulating arachidonic acid release in macrophages. *Journal of Biological Chemistry,* 273, p. 8203-8211.

• **Qu, X., Lehrer, R. (1998)** Secretory Phospholipase A2 Is the Principal Bactericide for Staphylococci and Other Gram-Positive Bacteria in Human Tears. *Infection and Immunity*, 66(6), p. 2791-2797.

• **Rahman, M.S., Ammerman, N.C., Sears, K.T., Ceraul, S.M., Azad, A.F. (2010)** Functional Characterization of a Phospholipase A2 Homolog from *Rickettsia typhi*. *Journal of Bacteriology*, 192 (3), p. 3294-3303.

• **Rand, J.H. (2000)** The annexinopathies: a new category of diseases. *Biochimica et Biophysica Acta*, 1498, p. 169-173.

• **Rand, J.H., Wu, X., Lin, E.Y., Griffel, A., Gialanella, P., McKitricka, J.C. (2012)** Annexin A5 Binds to Lipopolysaccharide and Reduces Its Endotoxin Activity. *mBio*, 3 (2), e00292-11.

• **Rawal, N., Pangburn, M. (2001)** Structure/function of C5 convertases of complement. *International Immunopharmacology*, 1, p. 415-422.

• **Raynal, P., Pollard, H.B. (1994)** Annexins: the problem of assessing the biological role for a gene family of multifunctional calcium- and phospholipid-binding proteins. *Biochimica et Biophysica Acta- Molecular Cell Research*, 1197, p. 63-93.

• **Redelman, D., Russell, R.A. (1980)** In vitro studies of the rabbit immune system. VII. The generation of rabbit anti-mouse cytotoxic T lymphocytes. *Journal of Immunological Methods*, 35, p. 69-81.

• **Redrobe, S. (2002)** Calcium metabolism in rabbits. *Seminars in avian and exotic pet medicine*, 11 (2), p. 94-101.

• **Reichmann, N.T., Picarra Cassona, C., Gründling, A. (2013)** Revised mechanism of D-alanine incorporation into cell wall polymers in Gram-positive bacteria. *Microbiology*, 159, p. 1868-1877.

• **Resch, G., Francois, P., Morisset, D., Stojanov, M., Bonetti, E.J., Schrenzel, J., Sakwinska, O., Moreillon, P. (2013)** Human-to-Bovine Jump of *Staphylococcus aureus* CC8 Is

Associated with the Loss of a β-Hemolysin Converting Prophage and the Acquisition of a New Staphylococcal Cassette Chromosome. *PLOS One*, 8 (3), e58187.

- **Rescher, U., Gerke, V. (2004)** Annexins- unique membrane binding proteins with diverse functions. *Journal of Cell Science*, 117, p. 2631-2639.
- **Rice, L. B. (2006)** Antimicrobial resistance in gram-positive bacteria. *American Journal of Infection Control,* 34**,** S11-9; discussion S64-73.
- **Richter, S.G., Elli, D., Kim, H.K., Hendrickx, A.P.A., Sorg, J.A., Schneewind, O., Missiakas, D. (2013)** Small molecule inhibitor of lipoteichoic acid synthesis is an antibiotic for Gram-positive bacteria. *PNAS*, 110 (9), p. 3531-3536.
- **Ricklin, D., Tzekou, A., Garcia, B.L., Hammel, M., McWhorter, W.J, Sfyroera, G., Wu, Y., Holers, M., Herbert, A.P., Barlow, P.N., Geisbrecht, B.V., Lambris, J.D. (2009)** A Molecular Insight into Complement Evasion by the Staphylococcal Complement Inhibitor Protein Family. *Journal of Immunology*, 183 (4), p. 2565-2574.
- **Ricklin, D., Hajishengallis, G., Yang, K., Lambris, J.D. (2010)** Complement: a key system for immune surveillance and homeostasis. *Nature Immunology*, 11 (9), p. 785-797.
- **Rigby, K.M., DeLeo, F.R. (2012)** Neutrophils in innate host defense against *Staphylococcus aureus* infections. *Seminars in Immunopathology*, 34, p. 237-259.
- **Roberts, M.F., Sutcliffe, J., Courvalin, P., Jensen, L.B., Rood, J., Seppala, H. (1977)** Chemical modification of the histidine residue in phospholipase A2 (Naja naja naja). A case of half-site reactivity. *Journal of Biological Chemistry*, 252 (7), 2405-2411.
- **Roberts, M. C., Sutcliffe, J., Courvalin, P., Jensen, L. B., Rood, J., Seppala, H. (1999)** Nomenclature for macrolide and macrolide-lincosamide-streptogramin B resistance determinants. *Antimicrobial Agents and Chemotherapy*, 43 (12), p.2823-2830.
- **Roethlisberger, P., Iida-Tanaka, N., Hollemeyer, K., Heinzle, E., Ishizuka, I., Fischer, W. (2000)** Unique poly(glycerophosphate) lipoteichoic acid and the glycolipids of *Streptococcus* sp closely related to *Streptococcus pneumoniae. European Journal of Biochemistry*, 267, p. 5520-5530.
- **Rollins, S.A., Matis, L.A., Springhorn, J.P., Setter, E., Wolff, D.W. (1995)** Monoclonal antibodies directed against human C5 and C8 block complement-mediated damage of xenogeneic cells and organs. *Transplantation*, 60 (11), p. 1284-1292.
- **Rooijakkers, S., van Kessel, K., van Strijp, J. (2005a)** Staphylococcal innate immune evasion. *TRENDS in Microbiology*, 13 (12), p. 596-601.
- **Rooijakkers, S., van Wamel, W., Ruyken, M., van Kessel, K., van Strijp, J. (2005b)** Anti-opsonic properties of staphylokinase. *Microbes and Infection*, 7, p. 476-484.
- **Rooijakkers, S., Ruyken, M., Roos, A., Daha, M.R., Presanis, J.S., Sim, R.B., van Wamel, W.J.B., van Kessel, K.P.M., van Strijp, J. (2005c)** Immune evasion by a staphylococcal complement inhibitor that acts on C3 convertases. *Nature Immunology*, 6 (9), p. 920-927.
- **Rooijakkers, S., Ruyken, M., van Roon, J., van Kessel, K., van Strijp, J., van Wamel, W. (2006)** Early expression of SCIN and CHIPS drives instant immune evasion by

Staphylococcus aureus. *Cellular Microbiology*, 8 (8), p. 1282–1293.

- **Rooijakkers, S., van Strijp, J. (2007)** Bacterial complement evasion. *Molecular Immunology*, 44, p. 23-32.
- **Rooijakkers, S., Milder, F.J. Bardoel, B.W., Ruyken, M., van Stripj, J., Grost, P. (2007)** Staphylococcal Complement Inhibitor: Structure and Active Sites. *Journal of Immunology*, 179 (5), p. 2989-2998.
- **Roos, A., Bouwman, L.H., van Gijlswijk-Janssen, D.J., Faber-Krol, M.C., Stahl, G.L., Daha, M.R. (2001)** Human IgA Activates the Complement System Via the Mannan-Binding Lectin Pathway. *Journal of Immunology*, 167 (5), p. 2861-2868.
- **Roos, A., Bouwman, L.H., Munoz, J., Zuiverloon, T., Faber-Krol, M.C., Fallaux-van den Houten, F.C., Klar-Mohamada, N., Hack, C.E., Tilanus, M.G., Daha, M.R. (2003)** Functional characterization of the lectin pathway of complement in human serum. *Molecular Immunology*, 39, p. 655–668.
- **Rosengarth, A., Rosgen, J., Hinz, H.J., Gerke, V. (2001)** Folding energetics of ligand binding proteins II. Cooperative binding of Ca^{2+} to annexin I. *Journal of Molecular Biology*, 306, p. 825-835.
- **Rosenthal, K.L. (2006)** Calcium metabolism in rabbits: what is new? Proceedings of the North American Veterinary Conference, January 7-11 (2006), Orlando, Florida, US, Volume 20, p. 1764-1765.
- **Rother, R.P., Rollins, S.A., Mojcik, C.F., Brodsky, R.A., Bell, L. (2007)** Discovery and development of the complement inhibitor eculizumab for the treatment of paroxysmal nocturnal hemoglobinuria. *Nature Biotechnology*, 25, p. 1256-1264.
- **Ryu, S., Song, P., Seo, C., Cheong, H., Park, Y. (2014)** Colonization and Infection of the Skin by *S. aureus*: Immune System Evasion and the Response to Cationic Antimicrobial Peptides. *International Journal of Molecular Sciences*, 15, p. 8753-8772.
- **Samy, R.P., Gopalakrishnakone, P., Thwin, M.M., Chow, T.K.V., Bow, H., Yap, E.H., Thong, T.W.J. (2007)** Antibacterial activity of snake, scorpion and bee venoms: a comparison with purified venom phospholipase A2 enzymes. *Journal of Applied Microbiology*, 102, p. 650-659.
- **Santangelo, R.T, Nouri-Sorkhabi, M.H., Sorrell, T.C., Cagney, M., Chen, S.C.A., Kuchel, P.W., Wright, L.C. (1999)** Biochemical and functional characterisation of secreted phospholipase activities from *Cryptococcus neoformans* in their naturally occurring state. *Journal of Medical Microbiol*ogy, 48, p. 731-740.
- **Schaefler, S. (1989)** Methicillin-resistant strains of *Staphylococcus aureus* resistant to quinolones. *Journal of Clinical Microbiology*, 27 (2), p. 335-336.
- **Schaloske, R.H., Dennis, E.A. (2006)** The phospholipase A2 superfamily and its group numbering system. *Biochimica et Biophysica Acta*, 1761(11), p. 1246-1259.
- **Scheffers, D., Pinho, M.G. (2005)** Bacterial Cell Wall Synthesis: New Insights from Localization Studies. *Microbiology and Molecular Biology Reviews*, 69 (4), p.585-607.
- **Scheuer, W. (1998)** Phospholipase As- Regulation and Inhibition. *Klin Wochenschr*, 67,

p. 153-159.

- **Schirner, K., Marles-Wright, J., Lewis, R.J., Errington, J. (2009)** Distinct and essential morphogenic functions for wall- and lipo-teichoic acids in *Bacillus subtilis*. *EMBO Journal*, 28, p. 830-842.
- **Schlaf, G., Demberg, T., Beisel, N., Schieferdecker, H.L., Götze, O. (2001)** Expression and regulation of complement factors H and I in rat and human cells: some critical notes. *Molecular Immunology*, 38, p. 231-239.
- **Schlievert, P.M., Case, L.C., Strandberg, K.L., Abrams, B.B., Leung, D.Y.M. (2008)** Superantigen Profile of *Staphylococcus aureus* Isolates from Patients with Steroid-Resistant Atopic Dermatitis. *Clinical Infectious Diseases*, 46 (10), p.1562-1567.
- **Schlitt, M., Lakeman, A.D., Wilson, E.R., To, A., Acoff, R.W., Harsh III, G.R., Whitley, R.J. (1986)** A Rabbit Model of Focal Herpes simplex Encephalitis. *Journal of Infectious Diseases*, 153 (4), p. 732-735.
- **Schneewind, O., Missiakas, D.M. (2012)** Protein secretion and surface display in Gram-positive bacteria. *Philosophical Transactions of the Royal Society B: Biological Sciences*, 367, p. 1123- 1139.
- **Schneewind, O., Missiakas, D. (2014)** Lipoteichoic Acids, Phosphate-Containing Polymers in the Envelope of Gram-Positive Bacteria. *Journal of Bacteriology*, 196 (6), p. 1133-1142.
- **Schumann, R.R., Rietschel, E.T., Loppnow, H. (1994)** The role of CD14 and lipopolysaccharide-binding protein (LBP) in the activation of different cell types by endotoxin. *Medical Microbiology Immunology* (Berlin), 183, p. 279-297.
- **Schwaeble, W., Dahl, M.R., Thiel, S., Stover, C., Jensenius, J.C. (2002)** The Mannan-Binding Lectin-Associated Serine Proteases (MASPs) and MAp19: Four Components of the Lectin Pathway Activation Complex Encoded by Two Genes. *Immunobiology*. 205, p. 455-466.
- **Seelen, M.A., Roos, A., Wieslander, J., Mollnes, T.E., Sjfholm, A.G., Wurzner, R., Loos, M., Tedesco, F., Sim, R., Garred, P., Alexopoulos, E., Turnet, M., Daha, M.R. (2005)** Functional analysis of classical, alternative and MBL pathways of the complement system: standardization and validation of simple ELISA. *Journal of Immunological Methods*, 296, p. 187-198.
- **Sell, S. (1979)** The rabbit immune system: Characterization of cell surface markers and functional properties of rabbit lymphocytes. *Molecular Immunology*, 16, p. 1045-1058.
- **Seo, M., Won, H., Kim, J., Mishigh-Ochir, T., Lee, B. (2012)** Antimicrobial Peptides for Therapeutics Applications. A Review. *Molecules*, 17, p. 12276-12286.
- **Servais, G., Walmagh, J., Duchateau, J. (1991)** Simple quantitative haemolytic microassay for determination of complement alternative pathway activation (AP50). *Journal of Immunological Methods*, 140, p. 93-100.
- **Sewell, E.W.C., Brown, E.D. (2014)** Taking aim at wall teichoic acid synthesis: new biology and new leads for antibiotics. *The Journal of Antibiotics*, 67, p. 43–51.

• **Sharp, J.A., Echague C.G., Hair P.S., Ward M.D., Nyalwidhe J.O., Geoghegan, J.A., Foster, T.J., Cunnion, K.M. (2012)** *Staphylococcus aureus* Surface Protein SdrE Binds Complement Regulator Factor H as an Immune Evasion Tactic. *PLoS ONE* 7(5): e38407. doi:10.1371/journal.pone.0038407.

• **Shaw, L., Golonka, E., Potempa, J., Foster, S.J. (2004)** The role and regulation of the extracellular proteases of *Staphylococcus aureus*. *Microbiology*, 150, p. 217-228.

• **Shen, Y., Kim, J., Strittmatter, E.F., Jacobs, J.M., Camp, D.G., Fang, R., Tolié, N., Moore, R.J., Smith, R.D. (2005)** Characterization of the human blood plasma proteome. *Proteomics*, 5, p. 4034-4045.

• **Shepheard, M.A., Fleming, V.M., Connor, T.R., Corander, J., Feil, E.J., Fraser, C., Hanage, W.P. (2013)** Historical Zoonoses and Other Changes in Host Tropism of *Staphylococcus aureus*, Identified by Phylogenetic Analysis of a Population Dataset. *PLOS One*, 8 (5), e62369

• **Shi, L., Takahashi, K., Dundee, J., Shahroor-Karni, S., Thiel, S., Jensenius, J.C., Gad, F., Hamblin, M.R., Sastry, K.N., Ezekowitz, A.B. (2004)** Mannose-binding Lectin-deficient Mice Are Susceptible to Infection with *Staphylococcus aureus*. *Journal of Experimental Medicine*, 199 (10), p. 1379-1390.

• **Shimada, T., Park, B., Wolf, A., Brikos, C., Goodridge, H., Becker, C., Reyes, C.N., Maio, E.A., Aderem, A., Götz, F., Liu, G.Y., Underhill, D.M. (2010)** *Staphylococcus aureus* evades the lysozyme-based digestion of peptidoglycan that links phagocytosis and macrophage IL-1β secretion. *Cell Host Microbe*, 21; 7(1): p. 38.

• **Shishido, S.N., Varahan, S., Yuan, K., Li, X., Flemming, S.D. (2012)** Humoral innate immune response and disease. *Clinical Immunology*, 144, p. 142-158.

• **Sieprawska-Lupa, M., Mydel, P., Krawczyk, K., Wojcik, K., Puklo, M., Lupa, B., Suder, P., Silberring, J., Reed, M., Pohl, J., Shafer, W., McAleese, F., Foster, T., Travis, J., Potempa, J. (2004)** Degradation of human antimicrobial peptide LL-37 by *Staphylococcus aureus*-derived proteinases. *Antimicrobial Agents and Chemotherapy*, 48, p. 4673-4679.

• **Silhavy, T.J., Kahne, D., Walker, S. (2010)** The Bacterial Cell Envelope. *Cold Spring Harbor Perspectives Biology,* 2 (5).

• **Sim, R.B., Arlaud, G.J., Colomb, M.G. (1979)** C1 inhibitor-dependent dissociation of human complement component C1 bound to immune complexes. *Biochemical Journal*, 179, p. 449-457.

• **Simpson, R.M., Zhao, T.M., Hubbard, B.S., Sawasdikosol, S., Kindt, T.J. (1996)** Experimental acute adult T cell leukemia-lymphoma is associated with thymic atrophy in human T cell leukemia virus type I infection. *Laboratory Investigation*, 74 (3), p. 696-710.

• **Singer, A.G., Ghomashchi, F., Le Calvez, C., Bollinger, J., Bezzine, S., Rouault, M., Sadilek, M., Nguyen, E., Lazdunski, M., Lambeau, G., Gelb, M.H. (2002)** Interfacial Kinetic and Binding Properties of the Complete Set of Human and Mouse Groups I, II, V, X, and XII Secreted Phospholipases A2. *Journal of Biological Chemistry*, 277 (50), p. 48535-48549.

• **Singh, P.K., Parsek, M.R., Greenberg, E.P., Welsh, M.J. (2002)** A component of

innate immunity prevents bacterial biofilm development. *Nature*, 417, p. 552–555.

- **Six, D.A., Dennis, E.A. (2000)** The expanding superfamily of phospholipase A2 enzymes: classification and characterization. *Biochimica et Biophysica Acta*, 1488, p. 1-19.
- **Smith, M.C., Pensky, J., Naff, G.B. (1982)** Inhibition of Zymosan-Induced Alternative Complement Pathway Activation by Concanavalin A. *Infection and Immunity*, 38 (3), p. 1279-1284.
- **Smyth, D.S., Feil, E.J., Meaney, W.J., Hartigan, P.J., Tollersrud, T., Fitzgerald, J.R., Enright, M.C., Smyth, C.J. (2009)** Molecular genetic typing reveals further insights into the diversity of animal-associated *Staphylococcus aureus*. *Journal of Medical Microbiology*, 58, p. 1343-1353.
- **Sobhanifar, S., Worrall, L.J., Gruninger, R.J., Wasney, G.A., Blaukopf, M., Baumann, L., Lameignere, E., Somononson, M., Brown, E.D., Withers, S.G., Strynadka, N.C.J. (2015)** Structure and mechanism of *Staphylococcus aureus* TarM, the wall teichoic acid α-glycosyltransferase. *PNAS*, 112 (6), E576-585, doi: 10.1073/pnas.1418084112.
- **Solito, E., Romero, I.A., Marullo, S., Russo-Marie, F., Weksler, B.B. (2000)** Annexin 1 binds to U937 monocytic cells and inhibits their adhesion to microvascular endothelium: involvement of the alpha 4 beta 1 integrin. *Journal of Immunology*, 165, p. 1573-1581.
- **Solomon, J.B. (1970)** Unification of foetal and neonatal immunology. *Nature*, 227, p. 895–897.
- **Spellberg, B., Daum, R. (2012)** Development of a vaccine against *Staphylococcus aureus*. *Seminars in Immunopathology*, 34, p., 335-348.
- **Stábeli, R.G., Simoes-Silva, R., Kayano, A.M., Gimenez, G.S., Moura, A.A., Caldeira, C.A.S, Coutinho-Neto, A., Zaqueo, K.D., Zuliani, J.P., Calderon, L.A., Soares, A.M. (2012)** Purification of Phospholipases A2 from American Snake Venoms. 'Chromatography - The Most Versatile Method of Chemical Analysis', Dr. Leonardo Calderon (Ed.), ISBN: 978-953-51-0813-9, InTech, DOI: 10.5772/53052.
- **Stadel, J.M., Jones, C., Livi, G.P., Hoyle, K., Kurdyla, J., Roshak, A., McLaughlin, M.M., Pfarr, D.A., Comer, S., Strickler, J. (1992)** Recombinant human secretory phospholipase A2: purification and characterization of the enzyme for active site studies. *Journal of Molecular Recognition*, 5 (4), p. 145-153.
- **Standiford, T.J., Arenberg, D.A., Danforth, J.M., Kunkel, S.L., Van Otteren, G.M., Strieter, R.M. (1994)** Lipoteichoic acid induces secretion of interleukin-8 from human blood monocytes: a cellular and molecular analysis. *Infection and Immunity*, 62(1), p. 119-125.
- **Stefani, S., Chung, D.R., Lindsay, J.A., Friedrich, A.W., Kearns, A.M., Westh, H., MacKensie, F.M. (2012)** Review: Methicillin-Resistant *Staphylococcus aureus* (MRSA): Global epidemiology and harmonization of typing methods. *International Journal of Antimicrobial Agents*, 39, p. 273-282.
- **Steinberg, D.A., Hurst, M.A., Fujii, C.A., Kung, A.H.C., Ho, J.F., Cheng, F.C., Loury, D.J., Fiddes, J.C. (1997)** Protegrin-1: A broad-spectrum, rapidly microbicidal peptide with in vivo activity. *Antimicrobial Agents and Chemotherapy*, 41 (8), p. 1738-1742.

• **Steinman, R.M., Hemmi, H. (2006)** Dendritic Cells: Translating Innate to Adaptive Immunity. *Current Topics in Microbiology and Immunology*, 311, p. 17-58.

• **Street, I.P., Lin, H.K., Laliberte, F., Ghomashchi, F., Wang, Z., Perrier, H., Tremblay, N.M., Huang, Z., Weech, P.K., Gelb, M.H. (1993)** Slow- and tight- binding inhibitors of the 85-kDa human phospholipase A2. *Biochemistry,* 32, p. 5935-5940.

• **Sun, X., Zhang, A., Baker, B., Sun, L., Howard, A., Buswell, J., Maurel, D., Masharina, A., Johnsson, K., Noren, C.J., Xu, M.Q., Correa, I.R. Jr. (2011)** Development of SNAP-Tag Fluorogenic Probes for WashFree Fluorescence Imaging. *ChemBioChem*, 12, p. 2217-2226.

• **Sung, J. M., Lloyd, D. H., Lindsay, J. A. (2008)** *Staphylococcus aureus* host specificity: comparative genomics of human versus animal isolates by multi-strain microarray. *Microbiology,* 154, p. 1949-1959.

• **Sutcliffe, I.C. (1998)** Cell envelope composition and organisation in the genus *Rhodococcus*. *Antonie Van Leeuwenhoek*. 74, p.49–58.

• **Swoboda, J.G., Campbell, J., Meredith, T.C., Walker, S. (2010)** Wall Teichoic Acid Function, Biosynthesis, and Inhibition. *ChemBioChem*, 11 (1), p. 35-45.

• **Takahashi, M., Endo, Y., Fujita, T., Matsushita, M. (1999)** A truncated form of mannose-binding lectin-associated serine protease (MASP)-2 expressed by alter- native polyadenylation is a component of the lectin complement pathway. *International Immunology*, 11, p.859-863.

• **Takahashi, K., Eddie Ip, W.K., Michelow, I.C., Ezekowitz, R.A.B. (2006)** The mannose-binding lectin: a prototypic pattern recognition molecule. *Current Opinion in Microbiology*, 18, p. 16-23.

• **Takayama, K., Hara, S., Kudo, I., Inoue, K (1991)** Detection of 14-kDa group II phospholipase A2 in human seminal plasma. *Biochemical and Biophysical Research Communications*, 178, p. 1505-1511.

• **Taylor, P.W. (1983)** Bactericidal and Bacteriolytic Activity of Serum Against Gram-Negative Bacteria. *Microbiological Reviews*, 47 (1), p. 46-83.

• **Tenner, A.J., Volkin, D.B. (1986)** Complement subcomponent C1q secreted by cultured human monocytes has subunit structure identical with that of serum C1q. *Biochemical Journal*, 233, p. 451-458.

• **Tessitore, A., Gaggiano, A., Cicciarelli, G., Verzella, D., Capece, D., Fischietti, M., Zazzeroni, F., Alesse, E. (2013)** Serum Biomarkers Identification by Mass Spectrometry in High-Mortality Tumors. *International Journal of Proteomics*, Volume 2013, Article ID 125858.

• **Thakker, M., Park, J., Carey, V., Lee, J.C. (1998)** *Staphylococcus aureus* Serotype 5 Capsular Polysaccharide is Antiphagocytic and Enhances Bacterial Virulence in a Murine Bacteremia Model. *Infection and Immunity*, 66 (11), p. 5183-5189.

• **Thiel, S., Vorup-Jensen, T., Stover, C.M., Schwaeble, W., Laursen, S.B., Poulsen, K., Willis, A.C., Eggleton, P., Hansen, S., Holmskov, U., Reid, K.B., Jensenius, J.C. (1997)** A second serine protease associated with mannan-binding lectin that activates complement.

Nature, 386, p. 506-510.

- **Thomas, K.J, Rice, C.V. (2014)** Revised model of calcium and magnesium binding to the bacterial cell wall. *Biometals*, 27, p. 1361-1370.
- **Thomas, S., Karnik, S., Barai, R.S., Jayaraman, V.K., Idicula-Thomas, S. (2010)** CAMP: a useful resource for research on microbial peptides. *Nucleic Acids Research*, 38 (Database issue), p. D774-D780.
- **Thompson, H.V., King, C.M. (1994)** The European Rabbit: The History and Biology of a Successful Colonizer. Oxford University Press, Oxford, UK, New York, USA and Tokyo, Japan.
- **Timms, J.F., Menon, U., Devetyarov, D., Tiss, A., Camuzeaux, S., McCurrie, K., Nouretdinov, I., Buford, B., Smith, C., Gentry-Maharaj, A., Hallett, R., Ford, J., Luo, Z., Vovk, V., Gammerman, A., Cramer, R., Jacobs, I. (2011)** Early Detection of Ovarian Cancer in Samples Pre-Diagnosis Using CA125 and MALDI-MS Peaks. *Cancer Genomics & Proteomics*, 8, p. 289-306.
- **Tjoelker, L.W., Eberhardt, C., Unger, J., Trong, H.L., Zimmerman, G.A., McIntyre, T.M., Stafforini, D.M., Prescott, S.M., Gray, P.W. (1995)** Plasma platelet-activating factor acetylhydrolase is a secreted phospholipase A2 with a catalytic triad. *Journal of Biological Chemistry*, 270, p. 25481-25487.
- **Tompkins, D.C., Hatcher, V.B., Patel, D., Orr, G.A., Higgins, L.L., Lowy, F.D. (1990)** A Human Endothelial Cell Membrane Protein that Binds *Staphylococcus aureus* In Vitro. *Journal of Clinical Investigation*, 85, p.1248-1254.
- **Topham, N.*J.,* Hewitt, E.W. (2009)** *Natural killer cell cytotoxicity: how do they pull the trigger? Immunology*, 128 (1), p. 7-15.
- **Torii, M., Kabat, E.A., Bezer, A.E. (1964)** Separation of teichoic acid of *Staphylococcus aureus* into two immunologically distinct specific polysaccharides with alpha- and beta-n-acetylglucosaminyl linkages respectively. Antigenicity of teichoic acids in man. *Journal of Experimental Medicine*, 120, p. 13-29.
- **Torres, V.J., Pishchany, G., Humayun, M., Schneewind, O., Skaar, E.P. (2006)** *Staphylococcus aureus* IsdB Is a Hemoglobin Receptor Required for Heme Iron Utilization. *Journal of Bacteriology*, 188 (24), p. 8421-8429.
- **Trauger, S.A., Webb, W., Siuzdak, G. (2002)** Peptide and protein analysis with mass spectrometry. *Spectroscopy*, 16 (1), p. 15-28.
- **Tsao, F.H.C., Xiang, Z., Meyer, K.C. (2015)** Fluorescent Determination of Secretory Phospholipase A2 (sPLA2)- Mediated Human Serum Albumin Binding Activity with Membrane Phospholipids and Fatty Acids. *Translational Medicine*, 5: 150. doi: 10.4172/2161-1025.1000150.
- **Tsiodras, S., Gold, H. S., Sakoulas, G., Eliopoulos, G. M., Wennersten, C., Venkataraman, L., Moellering, R. C., Ferraro, M. J. (2001).** Linezolid resistance in a clinical isolate of *Staphylococcus aureus*. *Lancet*, 358 (9277), p.207-208.
- **Turner, R.D., Vollmer, W., Foster, S.J. (2014)** Different walls for rods and balls: the

diversity of peptidoglycan. *Molecular Microbiology*, 91 (5), p. 862-874.

- **Tzagoloff, H. and Novick, R. (1977)** Geometry of cell division in *Staphylococcus aureus*. *Journal of Bacteriology*, 129 (1), p.343-350.
- **Upadhyay, A., Burman, J.D., Clark, E.A., Leung, E., Isenman, D.E., den Elsen, J.M.H., Bagby, S. (2008)** Structure-Function Analysis of the C3 Binding Region of *Staphylococcus aureus* Immune Subversion Protein Sbi. *Journal of Biological Chemistry*, 283 (32), p. 22113-22120.
- **Urb, M., Sheppard, D.C. (2012)** The Role of Mast Cells in the Defence against Pathogens. *PLoS Pathogen*, 8(4), e1002619.
- **Usharauli, D. (2010)** Chronic infection and the origin of adaptive immune system. *Medical Hypotheses*, 75 (2), p. 241–243.
- **Valanne, S., Wang, J., Rämet, M. (2011)** The Drosophila Toll Signalling Pathway. *Journal of Immunology*, 186, p. 649-656.
- **Valentin, E., Lambeau, G. (2000)** Increasing molecular diversity of secreted phospholipases A2 and their receptors and binding proteins. *Biochimica et Biophysica Acta*, 1488, p. 59-70.
- **van Belkum, A., Melles, D.C., Nouwen, J., van Leeuwen, W.B., van Wamel, W., Vos, M.C., Weirtheim, H.F.L., Verbrugh, H.A. (2009)** Co-evolutionary aspects of human colonisation and infection by *Staphylococcus aureus*. *Infection, Genetics and Evolution*, 9, p. 32–47.
- **van Loo, I., Huijsdens, X., Tiemersma, E., de Neeling, A., van de Sande-Bruinsma, N., Beauhean, D., Voss, A., Kluytmas, J. (2007)** Emergence of Methicillin-Resistant *Staphylococcus aureus* of Animal Origin in Humans. *Emerging Infectious Diseases*, 13 (12), p. 1834-1839.
- **Vancraeynest, D., Haesebrouck, F., Deplano, A., Denis, O., Godard, C., Wildemauwe, C., Hermans, K. (2006)** International Dissemination of a High Virulence Rabbit *Staphylococcus aureus* Clone. *Journal of Veterinary Medicine*, B 53, p. 418-422.
- **Vergara-Irigaray, M., Maira-Litran, T., Merino, N., Pier, G. B., Penades, J. R., and Lasa, I. (2008)** Wall teichoic acids are dispensable for anchoring the PNAG exopolysaccharide to the *Staphylococcus aureus* cell surface. *Microbiology*, 154 (Pt 3), p. 865-877.
- **Viana, D., Selva, J., Segura, P., Penades, J.R., Corpa, J.M. (2007)** Genotypic characterization of *Staphylococcus aureus* strains isolated from rabbit lesions. *Veterinary Microbiology*, 121, p. 288-298.
- **Viana, D., Blanco, J., Tormo-Más, M.A., Selva, L., Guinane, C.M., Baselga, R., Corpa, J.M., Lasa, I., Novick, R.P., Fitzgerald, J.R., Penadés, J.R. (2010)** Adaptation of *Staphylococcus aureus* to ruminant and equine hosts involves SaPI-carried variants of von Willebrand factor-binding protein. *Molecular Microbiology*, 77 (6), p. 1583-1594.
- **Viana, D., Comos, M., McAdam, P.R., Ward, M.J., Selva, L., Guinane, C.M., González-Muñoz B.M., Tristan, A., Foster, S.J., Fitzgerald, J.R., Penadés, J.R. (2015)** A single nucleotide mutation alters bacterial pathogen host tropism. *Nature Genetics*, 47, p.361-

366.

- **Vinogradov, E., Deschatelets, L, Lamoureux, M., Patel, G.B., Tremblay, T., Robotham, A., Goneau, M., Cummings-Lorbetskie, C., Watson, D.C., Brisson, J., Kelly, J.F., Gilbert, M. (2012)** Cell surface glycoproteins from *Thermoplasma acidophilum* are modified with an N-linked glycan containing 6-*C*-sulfofucose**.** *Glycobiology*, 22 (9), p. 1256-1267.
- **Virdis, S., Scarano, C., Cossu, F., Spanu, V., Spanu, C., De Santis, E.P.L. (2010)** Antibiotic Resistance in *Staphylococcus aureus* and Coagulose Negative Staphylococci Isolated from Goats with Subclinical Mastitis. *Veterinary Medicine International*, vol.2010, article ID 517060.
- **Vivier, E., Malissen, B. (2005)** Innate and adaptive immunity: specificities and signalling hierarchies revisited. *Nature Immunology*, 6 (1), p. 17-21.
- **Vollmer, W., Tomasz, A. (2000)** The *pgdA* gene encodes for a peptidogly- can *N*-acetylglucosamine deacetylase in *Streptococcus pneumoniae*. *Journal of Biological Chemistry*, 275, p. 20496-20501.
- **Vollmer, W., Tomasz, A. (2002)** Peptidoglycan N-Acetylglucosamine Deacetylase, a Putative Virulence Factor in *Streptococcus pneumoniae*. *Infection and Immunity*, 70 (12), p. 7176-7178.
- **Vollmer, W. (2008)** Structural variation in the glycan strands of bacterial peptidoglycan. *FEMS Microbiology Reviews*, 32, p. 287-306.
- **Vollmer, W., Blanot, D., de Pedro, M.A. (2008)** Peptidoglycan structure and architecture. *FEMS Microbiology Reviews*, 32, p.149-167.
- **Vollmer, W., Seligman, S.J. (2010)** Architecture of peptidoglycan: more data and more models. *Trends in Microbiology*, 18 (2), p. 59-66.
- **Volwerk, J.J., Jost, P.C., de Haas, G.H., Griffith, O.H. (1984)** Evidence That The Zymogen Of Phospholipase A2 Binds To A Negatively Charged Lipid-Water Interface. *Chemistry and Physics of Lipids*, 36, p. 101-110.
- **von Zabern, I., Nolte, R., Vogt, W. (1981)** Treatment of Human Complement Components C4 and C3 with Amines or Chaotropic Ions. *Scandinavian Journal of Immunology*, 13 (5), p. 413-431.
- **Vuong, C., Kocianova, S., Voyich, J.M., Yao, Y., Fischer, E.R., DeLeo, F.R., Otto, M. (2004)** A Crucial Role for Exopolysaccharide Modification in Bacterial Biofilm Formation, Immune Evasion, and Virulence. *Journal of Biological Chemistry*, 279 (52), p. 54881-54886.
- **Waisbren, B.A., Brown, I. (1961)** The bactericidal activity of human serum against *Escherichia coli*. *Journal of Immunology*, 88 (2), p. 249-256.
- **Wang, Y., Rollins, S.A., Madri, J.A., Matis, L.A. (1995)** Anti-C5 monoclonal antibody therapy prevents collagen-induced arthritis and ameliorates established disease. *PNAS*, 92 (19), p. 8955-8959.
- **Waymouth, C. (1970)** Osmolality of Mammalian Blood and of Media for Culture of Mammalian Cells. *In Vitro*, 6 (2), p. 109-127.

• **Webb, N.R. (2005)** Secretory phospholipase A2 enzymes in atherogenesis. *Current Opinion in Lipidology*, 16, p. 341-344.

• **Weidenmaier, C., Kokai-Kun, J., Kristian, S. Chanturiya, T., Kalbacher, H., Gross, M., Nicholson, G., Neumeister, B., Mond, J., Peschel, A. (2004)** Role of teichoic acids in *Staphylococcus aureus* nasal colonization, a major risk factor in nosocomial infections. *Nature Medicine*, 10, p. 243-245.

• **Weidenmaier, C., Peschel, A., Kempf, V.A.J., Lucindo, N., Yeaman, M., Bayer, A.S. (2005a)** DltABCD- and MprF-mediated cell envelope modifications of *Staphylococcus aureus* confer resistance to platelet microbial proteins and contribute to virulence in a rabbit endocarditis model. *Infection and Immunity*, 73 (12), p. 8033-8038.

• **Weidenmaier, C., Peschel, A., Xiong, Y.Q., Kristian,S.A., Dietz, K., Yeaman, M.R., Bayer, A.S. (2005b)** Lack of wall teichoic acids in *Staphylococcus aureus* leads to reduced interactions with endothelial cells and to attenuated virulence in a rabbit model of endocarditis. *Journal of Infectious Diseases*, 191, p. 1771-1777.

• **Weidenmaier, C., Peschel, A. (2008)** Teichoic acids and related cell-wall glycopolymers in Gram-positive physiology and host interactions. *Nature Reviews Microbiology*, 6, p. 276-287.

• **Weidenmaier, C., Kokai-Kun, J.F., Kulauzovic, E., Kohler, T., Thumm, G., Stoll, H., Götz, F., Peschel, A. (2008)** Differential roles of sortase-anchored surface proteins and wall teichoic acid in *Staphylococcus aureus* nasal colonization. *International Journal of Medical Microbiology*, 298, p. 505-513.

• **Weinrauch, Y., Elsbach, P., Madsen, L.M., Foreman, A., Weiss, J. (1996)** The potent anti-*Staphylococcus aureus* activity of a sterile rabbit inflammatory fluid is due to a 14-kD phospholipase A2. *Journal of Clinical Investigation*, 97, p. 250-257.

• **Weinrauch, Y., Abad, C., Liang, N.S., Lowry, S.F., J. Weiss, J. (1998)** Mobilization of potent plasma bactericidal activity during systemic bacterial challenge: role of group IIA phospholipase A2. *Journal of Clinical Investigation*, 102, p. 633-638.

• **Weiss. J., Inada, M., Elsbach, P., Crowl, R.M. (1994)** Structural Determinants of the Action against *Escherichia coli* of a Human Inflammatory Fluid Phospholipase A2 in Concert with Polymorphonuclear Leukocyte. *Journal of Biological Chemistry*, 269 (42), p. 26331-26337.

• **Werner, G., Cuny, C., Schmitz, F. J., Witte, W. (2001)** Methicillin-resistant, quinupristin- dalfopristin-resistant *Staphylococcus aureus* with reduced sensitivity to glycopeptides. *Journal of Clinical Microbiology,* 39**,** p. 3586-3590.

• **Weese, J.S. (2010)** Methicillin-resistant *Staphylococcus aureus* in animals. ILAR *Journal*, 51 (3), p. 233-244.

• **White, R.T., Damn, D., Hancock, N., Rosen, B.S., Lowell, B.B., Usher, P., Flier, J.S., Spiegelman, B.M. (1992)** Human adipsin is identical to complement component factor D and is expressed at high levels in adipose tissue. *Journal of Biological Chemistry*, 267 (13), p. 9210-9213.

• **Whitehead, A.S., Sim, R.B., Bodmer, W.F. (1981)** A monoclonal antibody against

human complement component C3: the production of C3 by human cells in vitro. *European Journal of Immunology*, 11 (2), p. 140-146.

- **Wikoff, W.R., Anfora, A.T., Liu, J., Schultz, P.G., Lesley, S.A., Peters, E.C., Siuzdak, G. (2009)** Metabolomics analysis reveals large effects of gut microflora on mammalian blood metabolites. *PNAS*, 106 (10), p. 3698-3703.
- **Wilkinson, B. J. (1997)** In the staphylococci in human disease. Crossley, K. B., Archer, G.L., (eds.) Churchill Livingstone, New York: p.1-38.
- **Wille, J.J., Kydonieus, A. (2003)** Palmitoleic Acid Isomer (C16:1Δ6) in Human Skin Sebum Is Effective against Gram-Positive Bacteria. *Skin Pharmacology* and *Applied Skin Physiology,* 16, p. 176- 187.
- **Wilson, I., Vogel, J., Sommerville, S. (1997)** Signalling pathways: A common theme in plants and animals? *Current Biology*, 7, p. R175–R178.
- **Winstel, V., Xia, Q., Peschel, A. (2014)** Pathways and roles of wall teichoic acid glycosylation in *Staphylococcus aureus*. *International Journal of Medical Microbiology*, 304 (3-4), p. 215-221.
- **Witte, W., Braulke, C., Strommenger, B. (2008)** Community-associated methicillin-resistant *Staphylococcus aureus* ST8 ("USA300") in an HIV-positive patient in Cologne, Germany, February 2008. *Euro Surveillance,* 13.
- **Wouters, D., Wiessenberg, H.D., Hart, M., Bruins, P., Voskuyl, A., Daha, M.R., Hack, C.E. (2005)** Complexes between C1q and C3 or C4: Novel and specific markers for classical complement pathway activation. *Journal of Immunological Methods*, 298, p. 35-45.
- **Wu, Y., Raymond, B., Goossens, P.L., Njamkepo, E., Guiso, N. , Paya, M., Touqui, L., (2010)** Type-IIA secreted phospholipase A2 is an endogenous antibiotic-like protein of the host. *Biochimie*, 92(6), p. 583-587.
- **Würzner, R., Joysey, V.C., Lachman, P.J. (1994)** Complement Component C7: Assessment of In Vivo Synthesis After liver Transplantation Reveals That Hepatocytes do Not Synthesize the Majority of Human C7. *Journal of Immunology*, 152 (9), p. 4624-4629.
- **Wysocki, V.H., Resing, K.A., Zhang, Q., Cheng, G. (2005)** Mass spectrometry of peptides and proteins. *Methods*, 35, p. 211-222.
- **Xia, Q., Kohler, T., Peschel, A. (2010a)** The Wall Teichoic Acid and Lipoteichoic Acid Polymers of *Staphylococcus aureus*. *International Journal of Medical Microbiology*, 300, p. 148-154.
- **Xia, Q., Maier, L., Sanchez-Carballo, P., Li, M., Otto, M., Holst, O., Peschel, A. (2010b)** Glycosylation of Wall Teichoic Acid in *Staphylococcus aureus* by TarM. *The Journal of Biological Chemistry*, 285 (18), p. 13405-13415.
- **Yang, D., Biragyn, A., Kwak, L.W., Oppenheim, J.J. (2002)** Mammalian defensins in immunity: more than just microbicidal. *Trends Immunology,* 23, p. 291-296.
- **Yang, S., Mishra, N.N., Rubio, A., Baver, A.S. (2013)** Causal Role of Single Nucleotide Polymorphisms within the *mprF* Gene of *Staphylococcus aureus* in Daptomycin Resistance. *Antimicrobial Agents and Chemotherapy*, 57(11), p. 5658-5664.

• **Yanni, A.E. (2004)** The laboratory rabbit: an animal model of atherosclerosis research. *Laboratory Animals*, 38, p. 246-256.

• **Yao, L., Lowy, F.D., Berman, J.W. (1996)** Interleukin-8 gene expression in *Staphylococcus aureus*-infected endothelial cells. *Infection and Immunity*, 64(8), p. 3407-3409.

• **Yeaman, M.R., Yount, N.Y. (2003)** Mechanisms of antimicrobial peptide action and resistance. *Pharmacological Reviews*, 55 (1), p. 27-55.

• **Yongquing T., Drentin, N., Duncan, R., Wijeyewickrema, L., Pike, R. (2012)** Mannose-binding lectin serine proteases and associated proteins of the lectin pathway of complement: Two genes, five proteins and many functions? *Biochimica et Biophysica Acta*, 1824, p. 253–262.

• **Yoshimura, A., Lien, E., Ingalls, R.R., Tuomanen, E., Dziarski, R., Golenbock, D. (1999)** Cutting Edge: Recognition of Gram-Positive *Bacterial Cell Wall Components by the Innate Immune System Occurs Via Toll-Like Receptor 2. Journal of Immunology*, 163 (1), p. 1-5.

• **Zasloff, M (2002)** Antimicrobial peptides of multicellular organisms. *Nature*, 415, p. 389-395.

• **Zhang, L., Jacobson, K., Vasi, Y., Lindberg, M., Frykberg, L (1998)** A second IgG-binding protein in *Staphylococcus aureus*. *Microbiology*, 144, p. 985-991.

• **Zhang, S., Wang, C., Wang, Y., Wei, R., Jiang, G., Ju, H. (2003)** Presence and Characterization of Complement-like Activity in the Amphioxus *Branchiostoma belcheri tsingtauense*. *Zoological Science*, 20, p. 1207-1214.

• **Zhang, Z., Bast, R.C.Jr., Yu, Y., Li, J., Sokoll, L.J., Rai, A.J., Rosenzweig, J.M., Cameron, B., Wang, Y.Y., Meng, X., Berchuck, A., van Haaften-Day, C., Hacker, N.F., de Bruijn, H., van der Zee, A., Jacobs, I.J., Fung, E.T., Chan, D.W. (2004)** Three Biomarkers Identified from Serum Proteomic Analysis for the Detection of Early Stage Ovarian Cancer. *Cancer Research*, 64, p. 5882-5890.

• **Zhang, J.X., Dang, S.C., Qu, J.G., Wang, X.Q., Chen, G.Z. (2005)** Changes of gastric and intestinal blood flow, serum phospholipase A2 and interleukin-1β in rats with acute necrotizing pancreatitis. *World Journal of Gastroenterology*, 11, p. 3578-3581.

• **Zhao, H., Kinnunen, P.K.J. (2003)** Modulation of the Activity of Secretory Phospholipase A2 by Antimicrobial Peptides. *Antimicrobial Agents and Chemotherapy*, 47 (3), p. 965-971.

• **Ziccardi, R.J., Cooper, N.R. (1979)** Active disassembly of the first complement component, C1, by C1 inactivator. *Journal of Immunology*, 123, p. 788-792.