Antibody production against *Staphylococcus aureus* CoA biosynthesis enzymes and their application in protein level quantification

by

Karli Bothma

Thesis presented in partial fulfilment of the requirements for the degree of

Master of Science (Biochemistry)

in the Faculty of Science at Stellenbosch University

Supervisor: Dr. Marianne de Villiers

Co-supervisor: Prof. Dirk Uwe Bellstedt

Department of Biochemistry

March 2021

Declaration

By submitting this thesis electronically, I declare that the entirety of the work contained therein is my own, original work, that I am the sole author thereof (save to the extent explicitly otherwise stated), that reproduction and publication thereof by Stellenbosch University will not infringe any third-party rights and that I have not previously in its entirety or in part submitted it for obtaining any qualification.

March 2021

Abstract

Antimicrobial resistance has become an increased burden worldwide as more and more human pathogens are becoming resistant to current antimicrobials. Therefore, the identification of novel drug targets and development of new antimicrobial drugs are currently of high priority. A drug target that has gained increased attention is the coenzyme A (CoA) biosynthesis pathway. CoA is an essential cofactor that is necessary for life in all organisms, including human pathogens, making it an attractive target for the development of new antimicrobial drugs. The CoA biosynthesis pathway of *Staphylococcus aureus*, which is the leading cause of hospital-associated infections, was the focus of this study. Although various studies have investigated this pathway in *S. aureus* as a possible drug target, there is still a lot that needs to be elucidated in this regard. One gap in our knowledge is that the levels of the CoA biosynthesis enzymes (PanK, CoaBC, PPAT and DPCK) under physiological conditions are currently unknown. This study therefore aimed to develop immunological techniques which could be implemented as tools to quantify the levels of these enzymes at different growth phases of *S. aureus* cultivated under physiological growth conditions.

To achieve this aim, the four CoA biosynthesis enzymes of *S. aureus* were recombinantly expressed and purified using established methods. Polyclonal antibodies were raised in rabbits by immunising the animals with the respective enzymes adsorbed to acid-treated, naked *Salmonella minnesota* R595. This method has been used to successfully produce antibodies to a wide variety of antigens, especially in cases where only small amounts of the antigen were available. With these antibodies, indirect competition enzyme-linked immunosorbent assays (ELISAs) with excellent standard curves were obtained for the quantification of each of the respective enzymes. Furthermore, cross-reactivity studies performed with ELISA and western blot revealed that the anti-*Sa*PanK, anti-*Sa*PPAT and anti-*Sa*DPCK antibodies showed limited cross-reactivity. In an attempt to quantify the amount of cross-reactivity of each antibody-antigen pair, however, it was found that only the cross-reactivity of the anti-*Sa*PPAT antibodies had an effect on the final optimised assay.

In this study an original, highly sensitive ELISA method for the detection and quantification of each of the enzymes of the CoA biosynthesis pathway of *S. aureus* was developed. These assays provide a cost-effective method for enzyme level quantification that have the potential to provide better insight on the levels of the different enzymes under physiological conditions and ultimately aid in the development of new antimicrobial drugs.

Opsomming

Antimikrobiese weerstand het ‘n toenemende las geword wêreldwyd soos meer en meer menslike patogene weerstand opgebou het teen huidige antimikrobiese teenmiddels. Die indentifisering en ontwikkeling van nuwe antimikrobiese teenmiddels en teenmiddelteikens geniet dus tans ‘n hoë prioriteit. ‘n Potensiële teenmiddelteiken wat hernude aandag ontvang is die koënsiem A (KoA) biosintese padweg. KoA is ‘n essensiële ko-faktor wat noodsaaklik is vir lewe in alle organismes, insluitend menslike patogene, en is daarom ‘n aanloklike teiken vir die ontwikkeling van nuwe antimikrobiese teenmiddels. Die KoA biosintese padweg van *Staphylococcus aureus*, wat verantwoordelik is vir menigte hospitaal-verwante infeksies, was die fokus van hierdie studie. Alhoewel verskeie studies al gefokus het op die padweg in *S. aureus* as moontlike teenmiddelteiken, is daar steeds baie ontbrekende inligting wat uitgelig moet word. Een gaping in ons kennis omtrent die KoA biosintese ensieme (PanK, CoaBC, PPAT en DPCK) is dat die ensiemvlakke onder fisiologiese kondisies tans onbekend is. Die doel van hierdie studie was dus gerig op die ontwikkeling van ‘n immunologiese tegniek wat geimplementeer kan word om die vlakke van hierdie ensieme tydens verskillende groeifases van *S. aureus*, wat opgegroei word onder fisiologiese groeitoestande, te kwantifiseer.

Die vier KoA biosintese ensieme van *S. aureus* is rekombinant uitgedruk en gesuiwer deur gebruik te maak van gevestigde metodiek. Poliklonale teenliggaampies is volgende geproduseer in konyne deur die immunisering van hierdie diere met die onderskeidelike ensieme van die KoA biosintese padweg van *S. aureus* wat geadsorbeer is aan suurbehandelde, naakte *Salmonella minnesota* R595. Hierdie tegniek is gebruik in die verlede vir die suksesvolle produksie van teenliggampies teen ‘n groot verskeidenheid antigene, veral in omstandigehede waar daar slegs klein hoeveelhede van die antigeen beskikbaar was. ‘n Kompetisie “enzyme-linked immunosorbent assay” (ELISA) met uitstekende standaardkurwes is ontwikkel vir die kwantifisering van elk van die onderskeidelike ensieme met hierdie teenliggaampies. Verder het kruisreaktiwiteitstudies, uitgevoer deur middel van ELISA en western blot analises, getoon dat die anti-*Sa*PanK, anti-*Sa*PPAT and anti-*Sa*DPCK teenliggampies geringe kruisreaktiwiteit toon. In ‘n poging om die hoeveelheid kruisreaktiwiteit van elke teenliggampie-antigeen paar te kwantifiseer, is daar egter bevind dat slegs die kruisreaktiwiteit van die anti-*Sa*PPAT teenliggaampies die finale, geoptimiseerde toets beïnvloed.

In hierdie studie is‘n oorspronklike, hoogs sensitiewe ELISA metode ontwikkel vir die deteksie en kwantifisering van die ensieme van die KoA biosintese padweg in *S. aureus*. Hierdie toetse verskaf ‘n koste-effektiewe metode vir die kwantifisering van ensiemvlakke en het dus die potensiaal om insig te lewer oor die vlakke van die verskillende ensieme onder fisiologiese toestande en uiteindelik bydrae te lewer tot die ontwikkeling van nuwe antimikrobiese teenmiddels.

~Science is what happens when you are making plans~

Acknowledgements

Pursuing a masters degree has been a tough yet very rewarding journey and I would not have been able to do it without my remarkable support system. I would therefore like to thank each and every one who has contributed to make the process easier. First and foremost, I would like to thank my supervisor, Dr Marianne de Villers, for her continuous encouragement, guidance, and support. Thank you, Dr de Villiers, for your patience (especially in the writing process) and giving me a push when I needed it the most. Secondly, I would like to thank my co-supervisor, Prof. Dirk Bellstedt for introducing me to the world of immunology and always sharing his knowledge. Your guidance has been invaluable.

Special thank you to Mrs Coral de Villiers and Dr Helba Bredell, our previous and current lab manager. Not only did you ensure that the lab is running smoothly, but also thank you for the moral support, laughs and hugs. I greatly appreciate the concern that you have taken in me.

I have been privileged to be a part of not only one, but two great groups of people. To the Bellstedt lab members, thank you for your encouragement and helping me grow as a researcher over the years. It has been a pleasure working with all of you and I will miss our conversations dearly. I would also like to thank the Strauss-de Villiers lab members for welcoming me to their lab family. Your continuous support, coffee chats and sharing the burdens of science has made this journey a memorable one. Special thank you to Dr Blake Balcomb for his patience in teaching me the daunting task of protein purification.

I would also like to thank the members of the department of Biochemistry, staff and students alike. Thank you for every encouraging smile and passing conversation.

Lastly, I would like to thank my friends and family for their unwavering support and always believing in me. To my parents, thank you for always supporting my endeavours and enabling me to embark on this journey. Although you might not always understand what I am saying, I greatly appreciate your interest in my studies. To my brother, Bertus, thank you for always encouraging me. I will always cherish our Sunday trips to Stellenbosch with the snacks and boksies Milo.

Thank you all, without your support this thesis would not have been possible.

Additional acknowledgements

- Financial assistance from Stellenbosch University Faculty of Science, Dr Marianne de Villiers and Prof. Dirk Bellstedt
- Dr Blake Balcomb, Dr Anton Hamann, Dr Konrad Mostert, Dr Koketso Mogwera and Warrick Sitzer for providing me with enzymes for my multitude of ELISAs
- Mrs Coral de Villiers for her patience and assistance with initial ELISA experiments

Table of Contents

List of Abbreviations

AAC	Antibody-antibiotic complexes
Ab	Antibody
Ab-b	Antibody conjugated to biotin
ABTS	2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)
ADP	Adenosine diphosphate
Ag	Antigen
ALP	Alkaline phosphatase
AMP	Adenosine monophosphate
APC	Antigen presenting cell
ASKHA	Acetate and sugar kinases/heat-shock protein 70/actin
ATP	Adenosine triphosphate
AVPO	Avidin peroxidase conjugate
BCIP	5-Bromo-4-chloro-3-indolyl phosphate
BSA	Bovine serum albumin
CA-MRSA	Community-associated methicillin resistant *Staphylococcus aureus*
CMP	Cytidine monophosphate
CO_2	Carbon dioxide
CoA	Coenzyme A
CoASY	CoA synthetase
CTP	Cytidine triphosphate
CV	Coefficient of variance
DAS	Double antibody sandwich
DMF	Dimethylformamide
DNA	Deoxyribonucleic acid

DPCK	Dephospho-Coenzyme A kinase
Ec	*Escherichia coli*
EDTA	Ethylenediamine tetra-acetic acid
EIA	Enzyme immunoassay
ELISA	Enzyme-linked immunosorbent assay
Fab	Antibody binding fragment
Fc	Crystallizable fragment
FCA	Freund's complete adjuvant
FMN	Flavin mononucleotide
HA-MRSA	Hospital-associated methicillin resistant *Staphylococcus aureus*
H_2O_2	Hydrogen peroxide
HPLC	High Performance Liquid Chromatography
HRP	Horseradish peroxidase
Hs	*Homo sapiens*
i.d.	Intradermal
i.m.	Intramuscular
IMAC	Immobilised metal affinity chromatography
i.p.	Intraperitoneal
IPTG	Isopropylthio-β-galactoside
i.v.	Intravenous
kDa	Kilo Dalton
K_M	Michaelis-Menten constant
LA-MRSA	Livestock-associated methicillin resistant *Staphylococcus aureus*
LB	Luria Bertani
LP	Lipoprotein
LPS	Lipopolysaccharide

$MgCl_2$	Magnesium chrloride
Mm	*Mus musculus*
MRSA	Methicillin resistant *Staphylococcus aureus*
MS	Mass spectrometry
MW	Molecular weight
NaCl	Sodium chloride
$NaHCO_3$	Sodium bicarbonate
NaN_3	Sodium azide
NB	Naked bacteria
NBT	Nitro blue tetrazolium
NFDM	Non-fat dry milk
$(NH_4)_2SO_4$	Ammonium sulfate
OD_{600}	Optical density at 600 nm
Pan	Pantothenic acid
PanK	Pantothenate kinase
$PanK_I$	Type I Pantothenate kinase
$PanK_{II}$	Type II Pantothenate kinase
$PanK_{III}$	Type III Pantothenate kinase
PantSH	Pantetheine
PBS	Phosphate buffered saline
pNPP	p-Nitrophenyl phosphate
PPAT	Phosphopantetheine adenylyltransferase
PPCDC	Phosphopantothenoylcysteine decarboxylase
PPCS	Phosphopantothenoylcysteine synthetase
PP_i	Inorganic phosphate
R^2	Coefficient of determination

RIA	Radioimmunoassay
RNA	Ribonucleic acid
Sa	*Staphylococcus aureus*
s.c.	Subcutaneous
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEM	Standard error of the mean
TNP	Trinitrophenyl
TMB	3,3′,5,5′-Tetramethylbenzidine
Tris-HCl	Tris(hydroxymethyl)aminomethane hydrochloride
WHO	World Health Organisation

List of Figures

List of Tables

Chapter 1
Introduction

1.1 The threat of Staphylococcus aureus infections

Staphylococcus aureus is a Gram-positive, commensal bacterium that can act as an opportunistic, human pathogen. As a result, it is one of the leading causes of hospital-associated infections (1–3). As a commensal bacterium, *S. aureus* forms part of the normal microbiota of the skin, nose and mucus membranes, colonizing approximately 20% - 30% of healthy individuals (2, 4, 5). However, *S. aureus* is well known for its ability to evade the immune system, causing a wide variety of infections (Fig. 1.1) ranging from less severe skin infections to bacteraemia and life-threatening septicaemia (3, 4). Furthermore, *S. aureus* is also commonly associated with the colonization of implantable medical devices, resulting in significant patient morbidity and mortality (2, 3, 6).

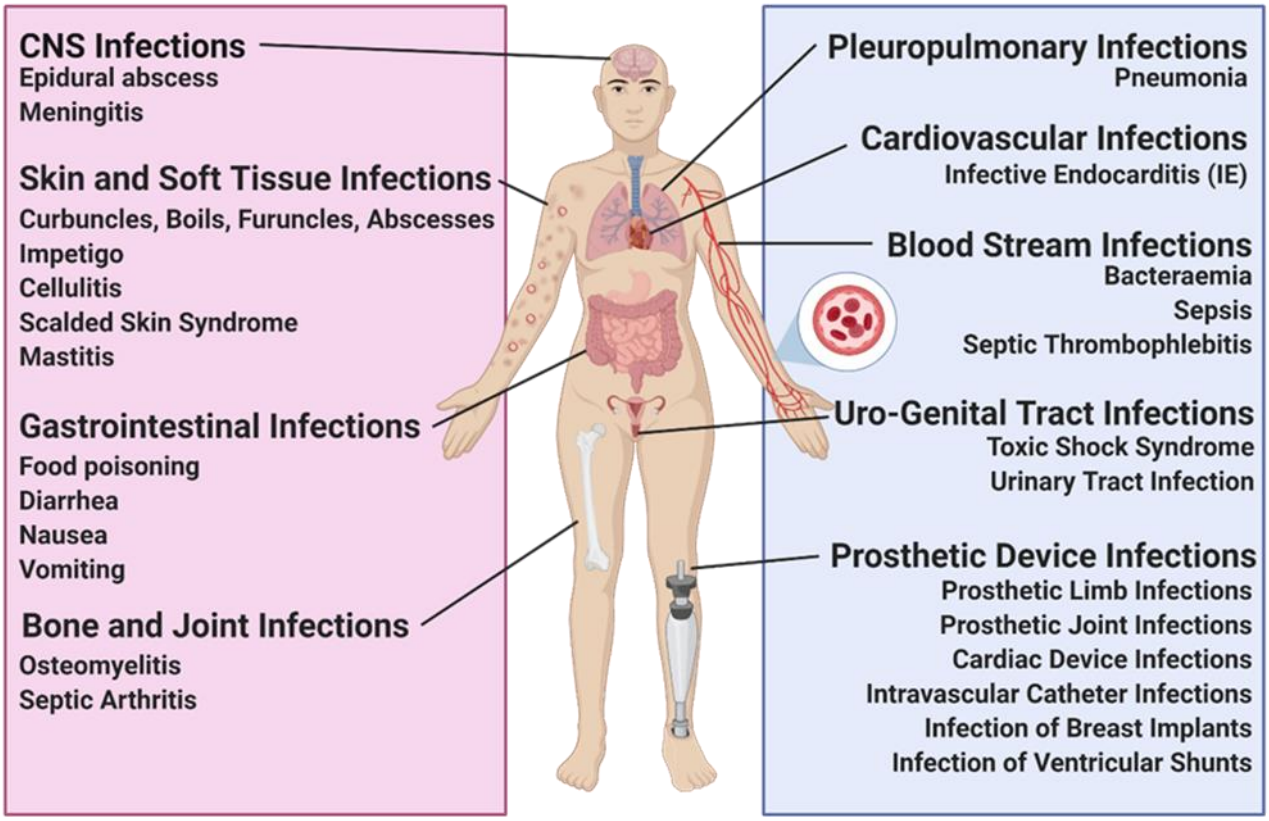

Figure 1.1: Diseases caused by *S. aureus* infections. Illustration of the common sites of infection of the human body by *S. aureus* and the subsequent states of disease that it causes. This image was created with BioRender.com.

Emerging as one of the principal public health problems of the 21st century, antimicrobial resistance threatens the effective prevention and treatment of infections caused by bacteria, viruses, fungi and parasites that are no longer susceptible to the common medicines used to treat them (7). Antimicrobial resistance in *S. aureus* emerged not long after the first introduction of penicillin in the 1940's and soon became a wide-spread problem. It was found that the resistance of this pathogen is mediated by the production of beta-lactamase, which effectively inactivates drugs such as penicillin, amoxicillin and ampicillin (8, 9). Consequently, stable beta-lactamase inhibitors were developed, which could then be used in conjunction with these antimicrobial drugs to counter this resistance. However, it became apparent that some strains of *S. aureus* also became resistant to these new penicillinase-stable antimicrobial drugs – strains which are known as methicillin-resistant *S. aureus* (MRSA) (8–10). With the increased burden of MRSA, especially in hospital settings, there has been a dramatic increase in the use of vancomycin, a glycopeptide antibiotic, as a last resort drug for the treatment of *S. aureus* infections (8, 9). Unfortunately, due to this increase in use of vancomycin, the emergence of resistance to this antimicrobial drug has also been observed (8, 10).

Initially, high levels of MRSA were only reported in a hospital setting, but in the last decade community-associated MRSA (CA-MRSA) infection has increased significantly in some parts of the world (9, 11). CA-MRSA strains were found to differ from hospital-associated strains of MRSA (HA-MRSA), commonly affecting young individuals without healthcare contact and generally resulting in pneumonia or skin infections (12). Additionally, some cases of livestock-associated (LA-MRSA) infections have also been reported, mostly in individuals working in close proximity to farm animals (5). According to the World Health Organization's (WHO) global report on antimicrobial resistance surveillance in 2014, most countries reported a proportion of MRSA exceeding 20% and, in some cases, reaching up to 80%, implying that second-line antibiotics are needed for the prophylaxis or treatment of *S. aureus* world-wide (13). Therefore, MRSA remains on the list of high-priority multidrug-resistant organisms (9) that requires renewed attention for the research and development of new antimicrobial drugs and innovative preventative approaches (14).

1.2 CoA biosynthesis as antimicrobial drug target in *Staphylococcus aureus*

1.2.1 Coenzyme A as antimicrobial drug target

Infectious diseases along with the emergence of antimicrobial resistance remains a leading cause of morbidity and mortality world-wide and therefore necessitates the development of novel antimicrobial drugs (15). One biochemical pathway that has been considered as a potential novel drug target the last decade, is coenzyme A (CoA) biosynthesis and its utilization. The potential of CoA and its biosynthesis machinery as an antimicrobial drug target will be the focus of this section.

CoA is an essential cofactor in all living organisms where it is involved in cell growth and various metabolic reactions (16). Some of these reactions include the synthesis and degradation of fatty acids, the synthesis of phospholipids as well as the tricarboxylic acid cycle (16), where it functions as an acyl-group carrier and carbonyl-activating group (17). The biosynthesis of CoA is a highly conserved process that proceeds in five enzymatic steps, starting with pantothenate or pantothenic acid, which is also more commonly known as Vitamin B5 (18). Pantothenate can either be synthesized *de novo* or obtained from extracellular sources, depending on the organism. Organisms such as most bacteria, plants and fungi produce their own pantothenate, whereas animals and some microbes must obtain it from extracellular sources (18).

The biosynthesis of CoA (Fig. 1.2) is initiated by the phosphorylation of pantothenate (Pan), which is catalysed by the enzyme pantothenate kinase (PanK), to form 4'-phosphopantothenate. This reaction is followed by the condensation of 4'-phosphopantothenate with cysteine to form 4'-phosphopantethenoylcysteine, catalysed by 4'-phosphopantethenoylcysteine synthetase (PPCS) and subsequent decarboxylation catalysed by 4'-phosphopantethenoylcysteine decarboxylase (PPCDC) to generate 4'-phosphopantetheine. Thereafter, an AMP moiety, obtained from ATP, is transferred to 4'-phosphopantetheine by 4'-phosphopantetheine adenylyltransferase (PPAT), resulting in the formation of dephospho-CoA. Lastly dephospho-CoA is phosphorylated by dephospho-Coenzyme A kinase (DPCK) to produce CoA (17, 18). Alternatively, CoA can be produced through the CoA salvage pathway which utilizes pantetheine, most likely produced from CoA degradation, instead of pantothenate. This pantothenate mimic is phosphorylated by PanK which leads to 4'-phosphopantetheine that slots into the pathway where PPAT and DPCK converts it to CoA (Fig 1.2).

The enzymes involved in the biosynthesis of CoA, have been shown to have significant diversity on a mechanistic, structural and sequence level in pathogenic micro-organisms versus humans, allowing the ability to selectively target this pathway in the pathogen without affecting the human host. This highlights the potential of this pathway as an excellent drug target for the development of selective antimicrobials (19) with the main focus currently being on *S. aureus* in this particular study.

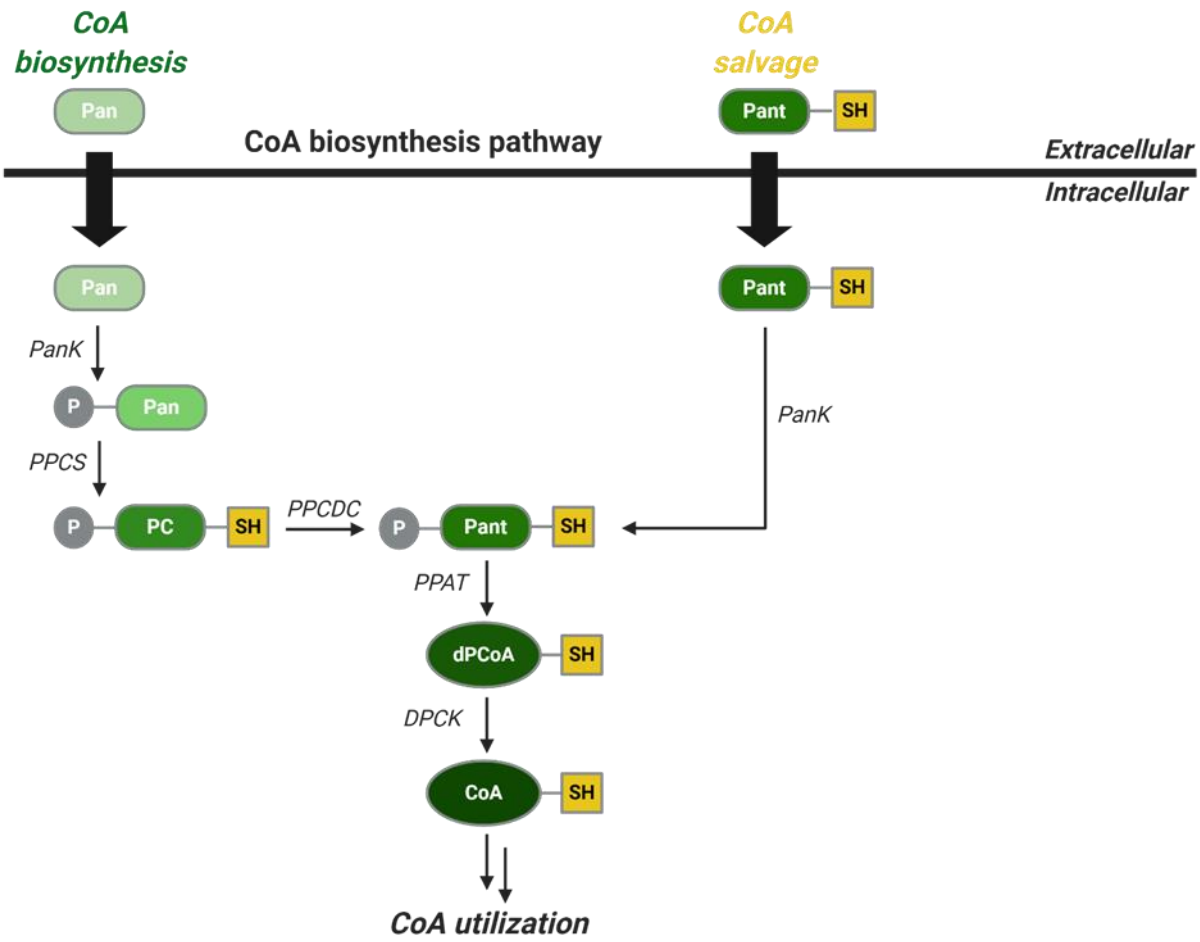

Figure 1.2: Schematic overview of the CoA biosynthesis pathway. The essential cofactor CoA can either be produced from pantothenate (Pan) in five enzymatic steps (CoA biosynthesis), as described in the text above, or from pantetheine (PantSH) in the CoA salvage pathway. The CoA salvage pathway circumvents the need for the bifunctional CoaBC enzyme and proceeds in three enzymatic steps catalysed by PanK, PPAT and DPCK. This image was created with BioRender.com.

1.2.2 CoA biosynthesis machinery in *Staphylococcus aureus*

As alluded to earlier, not only does *S. aureus* pose a great threat as an opportunistic pathogen, but it is also on the WHO's list of high-priority multidrug-resistant organisms (9). This organism has therefore been the focus of many studies in the search for novel antimicrobial drugs, including its CoA biosynthesis pathway. In this section the CoA biosynthesis machinery of *S. aureus* will be discussed.

1.2.2.1 Pantothenate kinase (PanK, CoaA)

Pantothenate kinase, or more commonly known as PanK, catalyses the ATP-dependent first step of the CoA biosynthesis pathway by phosphorylating pantothenate to form 4'-phosphopantothenate (17). PanK, which is encoded by the *coaA* gene, is the best-studied enzyme of the pathway and has received the most attention as a target for antimicrobial drug development due to its catalysis of the first committed step in the biosynthesis of CoA.

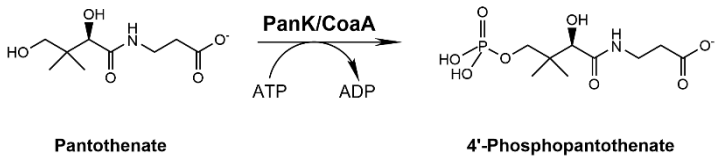

Figure 1.3: Conversion of pantothenate to 4'-phosphopantothenate. In the first enzymatic step of the CoA biosynthesis pathway, pantothenate or vitamin B5, is converted to 4'-phosphopantothenate by the transfer of a phosphate group from ATP to pantothenate by PanK.

Three types of PanKs have been discovered: type-I ($PanK_I$) and -III ($PanK_{III}$) that are associated with bacteria and type-II ($PanK_{II}$) that can be found in eukaryotes such as fungi, plants, insects and mammals (16, 20). Unlike other bacteria, *S. aureus* possesses a type-II or eukaryotic-like PanK that is moderately related to the four isoforms of eukaryotic PANKs (PANK1α, PANK1β, PANK2 and PANK3), but completely unrelated to the prototypical, bacterial $PanK_I$ found in *Escherichia coli* (16, 17, 21), indicating a similarity of merely 13% (17). Homology of *Sa*$PanK_{II}$ to mammalian PanKs has been highlighted in the alignment of *Sa*$PanK_{II}$ with human PANK2, indicating that the region of greatest similarity lies in the conserved DxGG(T,S)T(S,G)xxK(R,C) motif that is characteristic of the members of the acetate and sugar kinases/heat-shock protein 70/actin (ASKHA) family (22).

Sa$PanK_{II}$ has been expressed, purified, and characterised as a 29 kDa subunit protein, corresponding with the theoretical molecular weight predicted by its amino acid sequence (17, 23). However, characterisation of *Sa*$PanK_{II}$ by gel filtration analysis revealed a native molecular weight of 59 kDa, indicative of the existence of a homodimer (17). In contrast to the other $PanK_{II}$s, *Sa*$PanK_{II}$ has a much lower affinity for ATP with a K_M value of 34 µM and a K_M of 23 µM for pantothenate (16, 17). Importantly, *Sa*$PanK_{II}$ can further be differentiated from the other type-II PanKs, as it is not subject to feedback regulation by free CoA or CoA thioesters and the organism subsequently accumulates high concentrations of intracellular CoA, which is only regulated by the amount of pantothenate available in the medium (16, 17, 22). This occurrence could be attributed to the distinct mechanism by which this organism maintains its redox biology. Unlike most bacteria and eukaryotes, *S. aureus* lacks glutathione and therefore does not utilize glutathione and the NADPH-dependent glutathione reductase system (which makes up the primary thiol/reductase system in nature) to maintain its intracellular reducing environment (17). Instead, this organism relies on the utilization of non-esterified CoA and CoA disulfide reductase to maintain redox balance in the cell to protect against oxidative damage (16).

It is clear that these differences distinguish *Sa*$PanK_{II}$ from other organisms, especially mammalian PanK, and therefore make this enzyme an attractive target for the development of inhibitor-specific antimicrobial drugs.

1.2.2.2 Phosphopantothenoylcysteine synthetase/Phosphopantothenoylcysteine decarboxylase (PPCS/PPCDC, CoaBC)

In bacteria, and consequently *S. aureus*, the second and third enzymes of the pathway, phosphopantothenoylcysteine synthetase (PPCS) and phosphopantothenoylcysteine decarboxylase (PPCDC), are fused to form one flavin mononucleotide (FMN)-containing bifunctional enzyme, encoded by the *coaBC* gene, known as CoaBC (17). In eukaryotes as well as streptococci and enterococci, however, these enzymes remain monofunctional.

After phosphorylation of pantothenate to 4'-phosphopantothenate, 4'-phosphopantothenate is converted to 4'-phosphopantetheine in two steps. Firstly, PPCS catalyses the Mg^{2+}-dependent formation of 4'-phosphopantothenoylcysteine. In bacteria this step requires the utilization of CTP for activation, whereas ATP is favoured in eukaryotes. Lastly, PPCDC catalyses the decarboxylation of the cysteine moiety to produce the next intermediate in this pathway, 4'-phosphopantetheine (20).

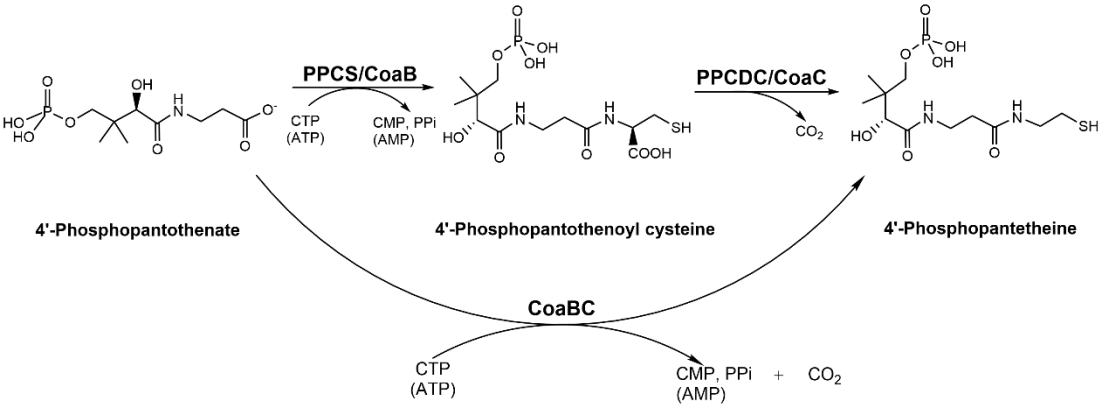

Figure 1.4: Conversion of 4'-phosphopantothenate to 4'-phosphopantetheine. The second and third steps of the CoA biosynthesis pathway is performed by the bifunctional CoaBC enzyme. These steps entail the conversion of 4'-phosphopantothenate to 4'-phosphopantothenoyl cysteine, utilizing CTP (prokaryotes) or ATP (eukaryotes) and the subsequent decarboxylation of 4'-phosphopantothenoyl cysteine to produce 4'-phosphopantetheine and CO_2.

Little information is available for *Sa*CoaBC in literature, however, the *E. coli* CoaBC protein has been found to be a homododecamer consisting of 43.8 kDa subunits (17) that has a K_M of 55 µM for 4'-phosphopantothenate and a K_M of 106 µM and 109 µM for CTP and L-cysteine, respectively (24). This enzyme is usually characterised by using a pyrophosphatase assay measuring PP_i formation. Finally, it has also been shown that the PPCDC domain of the bifunctional *E. coli* PPCS/PPCDC shares similarity with the primary structure of the human PPCDC monofunctional enzyme. However, little similarity was found between the primary structures of the PPCS domain of *E. coli* versus human PPCS (15), suggesting that it has great potential in the development of selective inhibitors which might also be applicable to this enzyme in *S. aureus*.

1.2.2.3 Phosphopantetheine adenylyltransferase (PPAT, CoaD)

In the second to last step of the pathway, phosphopantetheine adenylyltransferase (PPAT) catalyses the reversible and Mg^{2+}-dependent transfer of the AMP moiety of ATP to 4'-phosphopantetheine giving rise to dephospho-CoA (17, 20). In bacteria this enzyme is expressed as a monofunctional enzyme, which is encoded by the *coaD* gene, whereas in eukaryotes PPAT fuses with the last enzyme of the pathway, dephospho-CoA kinase (DPCK), to form a bifunctional PPAT/DPCK enzyme known as CoA synthase or CoASY.

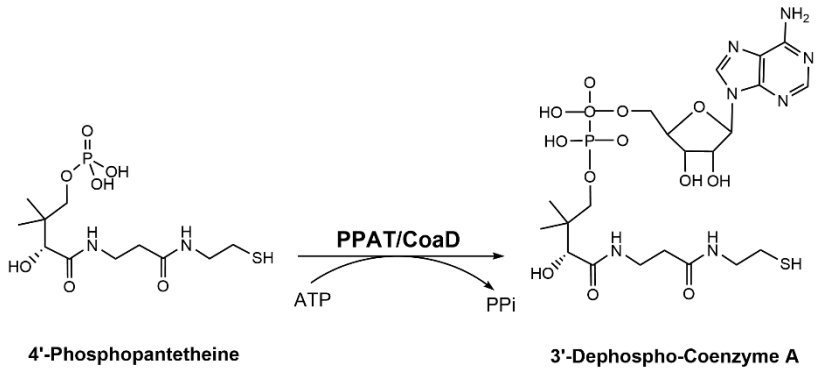

Figure 1.5: Conversion of 4'-phosphopantetheine to 3'-dephospho-Coenzyme A. In the penultimate step of the pathway, an AMP moiety is transferred to 4'-phosphopantetheine by PPAT to produce the second to last intermediate of the pathway, 3'-dephospho-CoA.

Similar to the *Sa*CoaBC enzyme, not much information is available for the PPAT enzyme of *S. aureus*, however, the PPAT of *E. coli* has been researched extensively. Overexpression, purification and characterisation of *Ec*PPAT revealed that this enzyme exists in solution as a homohexamer consisting of 17.8 kDa subunits that are arranged as a dimer of trimers (15, 17, 25). Furthermore, K_M values of 0.22 mM and 7 µM were determined for the reverse reaction for pyrophosphate and dephospho-CoA, respectively (15, 17). In humans, the PPAT domain of the bifunctional CoASY enzyme does not show any significant sequence similarity to *Ec*PPAT and its prokaryotic counterparts (15, 17).

Although not much is known about the PPAT of *S. aureus*, the crystal structure of *Sa*PPAT has been obtained (26–28) and provides a basis for further research into the workings of this enzyme. It is due to the small sequence similarity between human PPAT and the PPAT of bacteria along with the availability of the crystal structures of this enzyme that makes this enzyme an attractive target for the development of selective inhibitors in antimicrobial drug research.

1.2.2.4 Dephospho-CoA kinase (DPCK, CoaE)

The last step of the CoA biosynthesis pathway involves the Mg^{2+}-ATP-dependent phosphorylation of dephospho-CoA to yield CoA and ADP and is catalysed by dephospho-CoA kinase (DPCK), which is

encoded by the *coaE* gene. With this step, the 3'-hydroxyl moiety of the ribose is phosphorylated to produce CoA, the final product of the biosynthesis pathway (16, 20).

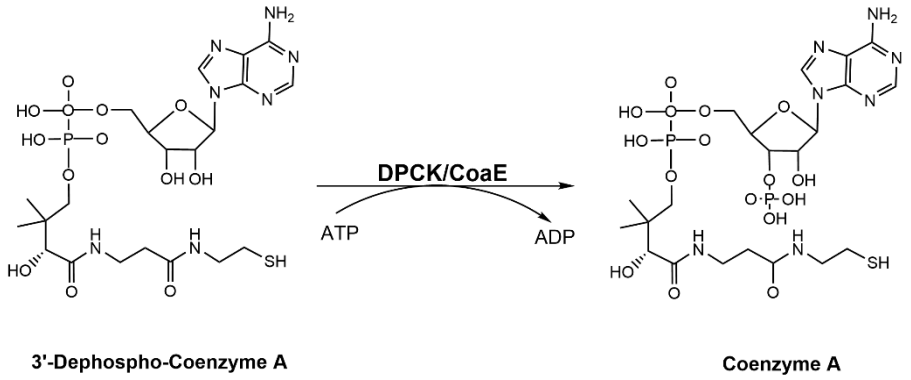

Figure 1.6: Conversion of 3'-dephospho-Coenzyme A to Coenzyme A. The transfer of a phosphate group from ATP to 3'-dephospho-CoA by DPCK concludes the CoA biosynthesis pathway to produce CoA.

As previously mentioned, this enzyme forms part of the bifunctional CoASY enzyme in eukaryotes, but unlike PPAT, the DPCK of the CoASY domain shows good sequence homology with its bacterial counterparts making it increasingly difficult for the development of selective inhibitors for this enzyme (20).

Similar to *Sa*CoaBC and *Sa*PPAT, limited information is available for the DPCK enzyme of *S. aureus* as characterisation of the enzyme has proven to be difficult. The enzyme expresses in low yields with a lot of batch-to-batch variation in activity. The activity has also proven to be too low for full characterisation (29). However, some information is available for *E. coli* DPCK. Purification and characterisation of this enzyme revealed that it is a monomer of 22.6 kDa in solution (20) but forms a trimer upon crystallization in the presence of sulfate (30). It has estimated K_M values of 0.14 mM and 0.74 mM for ATP and dephospho-CoA, respectively (17).

1.2.3 CoA biosynthesis enzyme levels under physiological conditions

Previously, we have discussed the heavy burden of antimicrobial resistance as well as the great potential of CoA biosynthesis as a drug target for the development of novel antimicrobial drugs. However, the process of drug development is no easy feat. Before the development process can start, it is important to fully understand the target of interest. The enzymes of the CoA biosynthesis pathway in bacteria and specifically *S. aureus* have been discussed and it is clear from literature that certain characteristics of each of the enzymes indicate the potential for the development of selective inhibitors of these enzymes. However, much more is still to be learned about these enzymes in different organisms for their further exploration as novel antimicrobial drugs.

One limitation in our current knowledge of the CoA biosynthesis pathway, are the levels of these enzymes under physiological conditions. This is particularly important as it could shed light on the importance of each enzyme in the pathway and its role in possible regulation of the pathway. This knowledge is key to establish what would happen to the concentration of these enzymes when subjecting specific organisms to different growth conditions as well as testing new compounds as inhibitors of these enzymes. Ultimately, it will enable us to determine the fluctuation of enzyme levels in different pathogenic organisms and disease conditions. Current methods for the determination and quantification of CoA and its derivatives have been mentioned in a mini review by Tsuchiya *et al.* (31). In this review, the authors distinguish between the use of enzymatic assays, high performance liquid chromatography (HPLC), mass spectrometry (MS) and other methods, such as the use of fluorometric assays and enzyme-linked immunosorbent assay (ELISA) kits, for measuring levels of CoA and its derivatives. However, the latter have not been evaluated for the quantification of the enzymes involved in the CoA biosynthesis pathway.

In a study done by Goosen (32), the reconstitution of the CoA salvage pathway in *E. coli* was attempted to study the biochemical control of the rate of CoA production under physiological conditions. For this study, the PaxDb4 protein abundance database (33) was consulted to determine the average abundance of PanK, PPAT and DPCK across different integrated data sets. This information was then used to establish the ratios of these enzymes in the CoA salvage pathway in an attempt to reconstitute the pathway. However, the values obtained were relative amounts of the enzymes rather than relative concentrations and made it difficult to establish the true enzyme concentrations which would be necessary for the reconstruction of this pathway. In comparison, a similar exercise can be performed for the CoA biosynthesis enzymes in *S. aureus*, which forms part of another study that is performed in our research group.

In this project, we would therefore like to establish a method for the quantification of the enzymes in the CoA biosynthesis pathway of *S. aureus* by the utilization of polyclonal antibodies in an ELISA format to not only corroborate the ratios for the CoA biosynthesis enzymes in *S. aureus*, but also determine the specific concentrations of these enzymes. The use of antibodies, specifically in protein level quantification, is therefore important to understand in order to achieve this goal.

1.3 The role and application of antibodies in the laboratory setting

1.3.1 An introduction to antibodies

It was in 1796 that Edward Jenner gave us the first glimpse into the field of immunology. Jenner's discovery that cowpox could induce protection against human smallpox broke ground for researchers such as Louis Pasteur, who devised vaccines for both cholera and rabies respectively, and Robert Koch who successfully proved that infectious diseases are caused by micro-organisms. It is these discoveries that led to the search of a mechanism of protection and subsequently the science behind our immune systems (34).

The immune system comprises of an intricate network of cells and proteins that work together to locate foreign cells, viruses, or macromolecules; to neutralize these invaders; and to ultimately remove them from the body (35, 36). These cells and proteins can be divided into two unique systems namely the innate and the adaptive immune system. Innate immunity, also known as natural or native immunity, is always present in healthy individuals and acts as the first line of defence against infection. Adaptive or acquired immunity, on the other hand, develops as an individual is exposed to different infectious micro-organisms throughout their lifetime (37).

Antibody production, which is the focus of this section, forms part of the humoral immunity portion of the adaptive immune system. In 1890 Emil von Behring and Shibasaburo Kitasato discovered that the serum of vaccinated individuals contained molecules that specifically bound to a particular pathogen, subsequently naming these molecules antibodies (34). Antibodies can be defined as proteins, more specifically immunoglobulins, that are secreted by immune cells called B-lymphocytes, and which are found in host plasma and extracellular fluid (38). These proteins have a characteristic Y-shape (Fig. 1.7) and consist of four polypeptides, two identical heavy chains and two identical light chains which are held together by interchain disulfide bonds (38, 39). Furthermore, they can be divided into two domains according to the function that each domain is associated with. The arms of the Y-shaped protein each contains an antigen binding site which forms the Fab (antibody binding fragment) portion of the protein that is specific to a certain epitope. The Fc (crystallizable fragment) portion of the antibody consists of the tail of the molecule and imparts the antibody with its biological effector function, determining whether the antibody can be bound to a cell membrane of an effector cell or whether it activates antipathogenic effector functions such as the complement system (38).

There are five types of heavy chains of antibodies or immunoglobulins which ultimately determines the class or isotype of the antibody (37). In mammals there are five different classes of antibodies namely: IgG, IgM, IgA, IgD and IgE of which in certain mammals IgG and IgA can further be divided into different subclasses or isotypes (38). Similarly, avian antibodies can be divided into three different classes, IgY (which compares to mammalian IgG), IgM and IgA (38). Each class of antibody has unique

biological and physical properties which determine the specific effector function of each of the class of antibodies (37, 38).

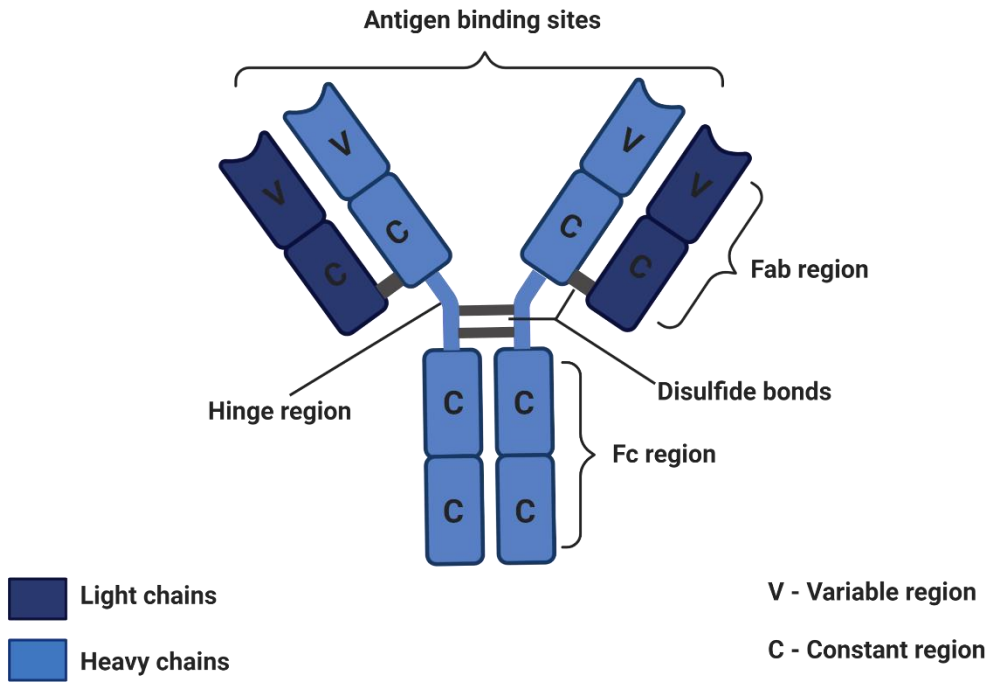

Figure 1.7: The antibody structure. The classical Y structure of the antibody consists of two identical heavy chains and two identical light chains that is held together by intra- and interchain disulfide bonds. These chains consist of constant and variable regions which contribute to either antigen binding and specificity (variable region) or the effector function of the antibody (constant region). The arms of the antibody (Fab region) house the specific antigen binding sites that allows the antibody to bind to a specific epitope and the tail of the antibody (Fc region) allows for it to either be bound to a cell membrane or result in activation of further molecular mechanisms. This image was created with BioRender.com.

Antibodies can bind with high specificity and affinity to an antigen (40) that allows them to neutralize and eliminate microbes and microbial toxins as well as prevent them from colonizing host cells and connective tissues (37). It is also this inherent specificity that allows antibodies to be used as invaluable tools in immunological research, immunohistochemistry, diagnostic testing, and vaccine quality control (40). Antibodies can further be classified as either monoclonal or polyclonal. Monoclonal antibodies are the antibodies produced by a single B-lymphocyte clone which recognises a single specific epitope on a specific antigen, whereas polyclonal antibodies are produced by a mixture of various B-lymphocyte clones, giving them the ability to bind to several different epitopes on any given antigen (40). The choice of which type of antibody to use in a specific application can be based on the characteristics of each group of antibodies with respective advantages and disadvantages, that can be observed in Figure 1.8 below. These factors need to be carefully considered when choosing a specific type of antibody for the purpose of the research to be undertaken.

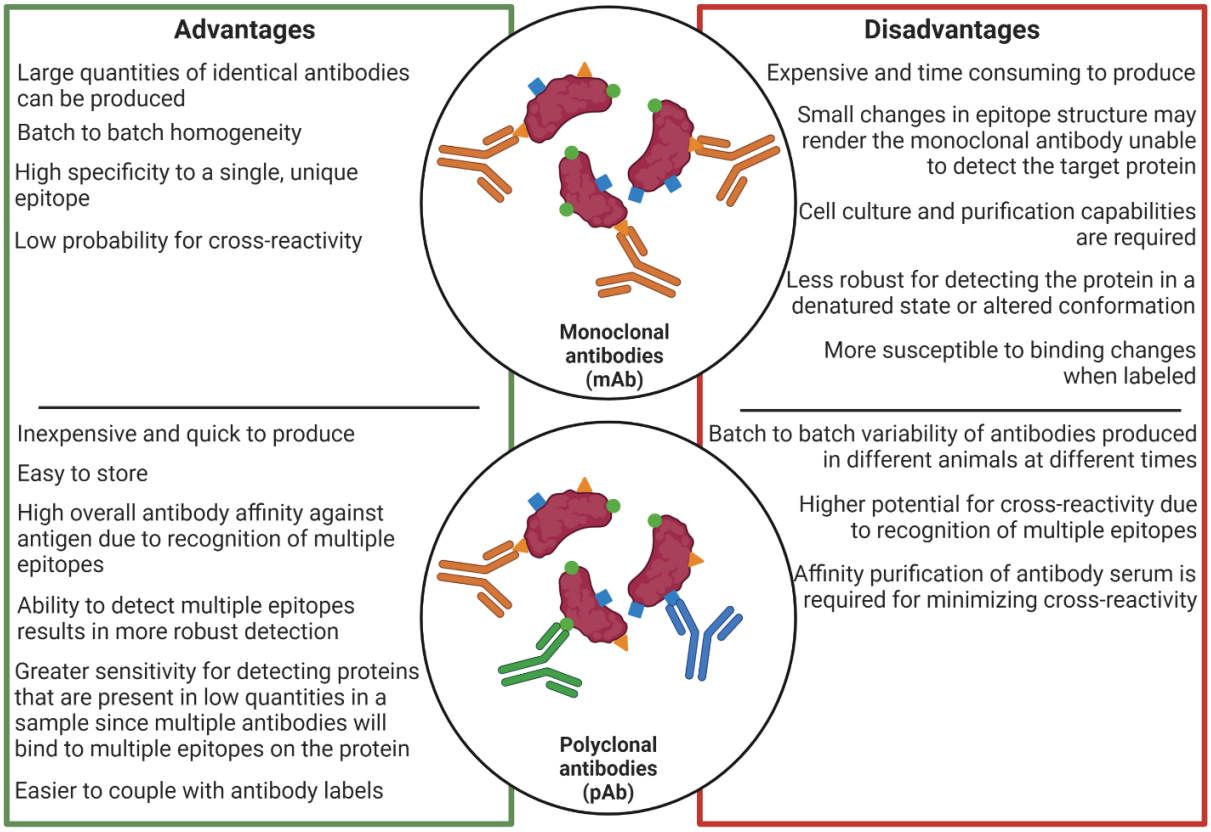

Figure 1.8: Monoclonal versus polyclonal antibodies. A figure illustrating the advantages and disadvantages of monoclonal and polyclonal antibodies. In the illustration an antigen with multiple epitopes can be seen (as indicated by the different shapes) and the corresponding antibodies that bind to their complimentary epitope. This image was created with BioRender.com.

The advantages such as inexpensive and quick production of polyclonal antibodies made these the antibodies of choice during this particular study and will be focussed on in this literature study.

1.3.2 Polyclonal antibody production strategies

Through the ages, immunological laboratory methods have become powerful and indispensable research tools in cell biology, biochemistry, and molecular biology (41). As such, antibody production in laboratory animals has become an integral part of many research and development projects and established industry practices (42). Antibodies can be produced against virtually any macromolecule (41), but before production can commence, researchers are faced with a vast variety of complex choices, which are critical for successful antibody production. Some of the most important factors to take into consideration include, the choice of host animal, immunization procedures and the design of the immunization protocol to ensure the welfare of the animals being immunized, whilst still obtaining high-titre, high-affinity antisera (42). In this section the necessary factors that need to be taken into consideration for the production of polyclonal antibodies will be explored.

1.3.2.1 Choice of antigen

An immunogen is any molecule capable of eliciting an immune response when injected into an animal. Polypeptides, polysaccharides, glycoproteins, glycolipids, and lipoproteins with molecular weights above 10 000 are typical immunogens since these are the types of molecules generally presented on the surface of pathogens (43). Antigens, however, refer to any molecule or substance that can be recognized and bound by an antibody (37). All immunogens can therefore be considered antigens, however not all antigens are immunogens, as smaller compounds, such as peptides with low molecular weights can also be recognized and bound by antibodies but are not big enough to induce an immune response when injected alone (43). These compounds may become immunogenic when bound to a larger carrier molecule (43).

Antibody production usually involves immunization with a purified or partially purified antigen, which may include proteins or peptides, nucleic acids, carbohydrates, and small organic molecules conjugated to protein carriers, to name but a few (44). When antibodies are produced, it is important to consider all characteristics of the antigen, which include the quality and quantity of the antigen as well as the antigen preparation. The specificity of the immune response obtained depends strongly on the purity of the injected antigen. Small impurities could potentially be immunodominant, resulting in more antibodies that have greater activity against the impurity than the antigen of interest and could also result in a greater potential for cross-reactivity. Another important aspect to take into consideration, is the potential toxicity of the antigen preparation due to possible contamination with endotoxins or chemical residues that are used to inactivate the microorganism, as well as antigen preparations with a very high pH level. It is very important that all diluents of antigen preparations are endotoxin free and fall within the normal physiological pH range (40).

Furthermore, the amount of antigen injected will determine the extent of the immune response elicited. Too much or too little antigen may result in suppression, sensitisation or tolerance of the immune response. The quantity of antigen utilised therefore depends on the inherent properties of the antigen, whether the antigen is purified or comprises of a mixture of antigens, the animal species to be immunized as well as the use of adjuvants and the route and frequency of the injection (43).

It is very important for the researcher to carefully consider all of these factors before commencing with immunisation, in order to produce high-titre antibodies, whilst making sure the welfare of the animal is maintained.

1.3.2.2 Choice of host animal species

When choosing a species for the production of polyclonal antibodies, there are two important aspects to consider. First, how much of the antibody containing serum will be needed for the project and

secondly, to determine the phylogenetic relationship between the recipient and the donor of the protein antigen (42). For producing polyclonal antiserum to a protein antigen, it is better to choose a donor-recipient pair that are not closely related in phylogenetic terms. A greater phylogenetic distance between the antigen recipient and donor is generally predictive of a greater number of amino acid sequence differences between the homologous proteins, which could translate to a more diverse and potentially higher-titre serum antibody response, therefore the more epitopes on a specific antigen, the more polyclonal antibodies produced (42). A large number of animal species have been used over the years for the production of polyclonal antibodies, each species offering its own advantages to a particular study (42). The animals most commonly used for antibody production (Fig. 1.9) will therefore be discussed.

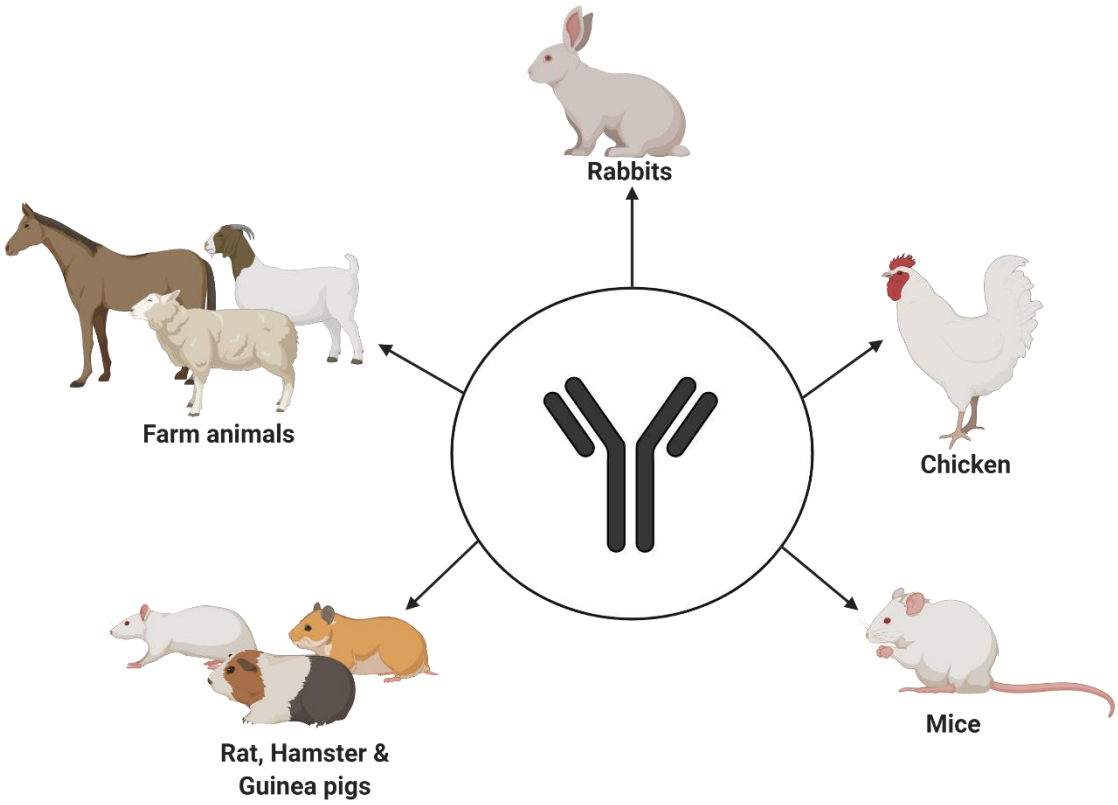

Figure 1.9: The animals most commonly used for polyclonal antibody production. This image was created with BioRender.com.

Rabbits – Rabbits are generally the most commonly used laboratory animal for the production of polyclonal antibodies (42, 43, 45). The utilisation of rabbits for the production of polyclonal antibodies has many advantages over the use of other species. Firstly, the convenient size of a rabbit makes it easy to handle and to collect large blood samples from the marginal ear vein or central auricular artery (42, 43). Furthermore, the rabbit has a relatively long lifespan (42) and due to its excellent responsiveness to a wide variety of antigens, produces adequate volumes of high-titre, high-affinity antisera (43).

Chickens – Chickens provide a unique advantage over other species to make polyclonal antibodies against mammalian antigens, due to their large phylogenetic distance from mammals (42). It is this phylogenetic distance that reduces the probability for chicken IgY to cross-react with mammalian IgG (46). Chicken IgY, also known as chicken IgG, is the functional equivalent of mammalian IgG in birds but it differs in many functional aspects to mammalian IgG. The chicken IgY is transported from the mother to the embryo via the egg yolk and the egg yolk thus contains high concentrations of chicken IgY, which can then be extracted for future use (46).

Mice – Mice are more commonly used for the production of monoclonal antibodies. In some cases, they can be advantageous for the production of polyclonal antibodies, particularly when the amount of antigen available for immunization is limited or when a small amount of antiserum is needed. However, due the small size of a mouse, it increases the difficulty of harvesting adequate amounts of polyclonal antibodies and is therefore not considered favourable (42).

Rats, Hamsters & Guinea pigs – Rats, hamsters and guinea pigs are some of the other options used for the production of polyclonal antibodies, but are, however, not favoured over rabbits. The smaller blood volumes of these animals increases the difficulty of collecting significant amounts of blood as it generally requires anaesthesia or cardiac puncture (42).

Farm animals – Farm animals such as sheep, goats and horses are generally used for the production of polyclonal antibodies when large volumes of antisera are needed. Utilisation of farm animals has its advantages, as these species generally have longer lifespans, are relatively easy to handle and blood can be collected from the jugular vein without the need of anaesthesia. The drawbacks, however, include the expense of acquiring and maintaining these animals, especially when multiple animals are needed. These animals are generally used in the commercial production of polyclonal antisera (42).

1.3.2.3 Immunisation strategies

The development of an appropriate immunisation protocol for the production of antibodies is a crucial aspect of the antibody production process to ensure the welfare of the experimental animal whilst still obtaining high-affinity antibodies with sufficient titre values that can be used for the required antibody applications. Some of the important factors that need to be taken into consideration include the dose of the immunogen to be administered, the use of adjuvants, blood volumes that can be collected from the immunized animals, frequency of immunisations and the administering of booster injections. These contributing factors will therefore be briefly discussed in the paragraphs to follow. The use of adjuvants will be discussed in more detail in the subsequent subsection and technicalities of the immunisation process will be discussed in more detail in Chapter 2 of this thesis.

Route of immunisation – The route of administering immunogen largely determines the spread and presentation of the immunogen and could have a great effect on the immune response of the immunised animal to the antigen. The ideal route for immunisation should result in the exposure of the antigen to

a large number of antigen-presenting cells (APC's) (43), such as dendritic cells or macrophages (37). Secondary to this, the site of immunisation should be chosen to minimize pain, distress and functional impairment that might be caused by the inflammatory reaction to the administered immunogen (43). Many immunisation sites have been mentioned in the production of polyclonal antibodies, each with its advantages and disadvantages. The more routinely used sites for injection include subcutaneous (s.c.), intramuscular (i.m.), intraperitoneal (i.p.), intradermal (i.d.) and intravenous (i.v.) (48). Other routes that have been mentioned, but are not favoured, nor do they provide advantages over the other routes for immunisation, include: intralymph, intrasplenic and intra-articular (43). The appropriate route of immunisation will ultimately be determined by the type and size of the animal being immunised as well as the use of adjuvants. The advantages and disadvantages of the different routes can be observed in the table below (Table 1.1). Furthermore, the recommended immunisation volumes for the respective sites of immunisation will be part of the discussion on immunogen dosages (Table 1.2).

Table 1.1: The advantages and disadvantages for the different routes of immunisation (40).

Site of injection	Advantages	Disadvantages
s.c.	Allows for immunisation with large volumes Inflammation can easily and closely be monitored	Slow absorption of immunogen
i.m.	Rapid absorption of immunogen, especially with muscular activity Allows for immunisation with large volumes in larger animals	Injections may be painful The spread of adjuvant and antigen along the interfacial planes and nerve bundles can result in damage to the sciatic nerve
i.p.	Allows for the accommodation of large volumes of inoculum	High percentage of injection failure Utilization of an oil adjuvant can result in peritonitis Risk of anaphylactic shock accompanied with booster injections
i.d.	Langerhans dendritic cells present in the dermis allows for more efficient processing of immunogen Small amounts of immunogen sufficient for eliciting an immune response	Injections may be painful Ulceration can occur with the use of oil adjuvants
i.v.	Immunogen is rapidly distributed	Does not allow for the use of oily or viscous gel adjuvants with administration High risk of anaphylactic shock accompanied with booster injection

Immunogen dosage – The dosage of the immunogen to be administered relies on many factors including the animal to be immunised, the properties of the immunogen to be administered (as

previously discussed), the route of injection (as previously discussed) and whether an adjuvant would be used to enhance an immune response (43). In the case where immunogen doses are very low, they might be insufficient to stimulate an immune response for antibody production, however, low dosages might stimulate the production of memory B-lymphocytes that could be stimulated to produce higher amounts of antibodies by a secondary immune response. In contrast to low dosages, excessive dosages of immunogen may result in immunological tolerance to the antigen or high concentrations of antibodies with low affinity for the antigen (41, 43). Therefore, determination of an optimal immunogen dosage is no easy task. With the factors mentioned above in mind, researchers have to rely on estimation of immunogen dosages according to previous experience of the researcher, guidelines and references found in literature for the antigen of interest, and the estimated immunogenicity of the antigen to be administered (43). The recommendations for the maximum volume of immunogen that may be injected at specific injections sites for different animals can be observed in Table 1.2.

Table 1.2: Maximum volume of injection per injection site recommended for immunisation of different animals (47)**.**

Animal	*Injection site				
	s.c.	**i.m.**	**i.p.**	**i.d.**	**i.v**
Mice	0.5 mL	0.05 mL	1 mL	-	0.2 mL
Hamsters	1 mL	0.1 mL	2 - 3 mL	-	0.3 mL
Guinea pigs	1 mL	0.1 mL	10 mL	-	0.5 mL
Rats	1 mL	0.1 mL	5 mL	-	0.5 mL
Rabbits	1.5 mL	0.5 mL	20 mL	0.05 mL	1 – 5 mL
Sheep & Goats	5.0 mL	-	10 mL	0.05 mL	30 mL
Chicken	4.0 mL	0.5 mL	10 mL	0.1 mL	0.5 mL

* These volumes are recommended for immunisations that does not utilize adjuvants.

Immunisation schedule – The frequency of immunisation and re-immunisation play a critical role in the successful production of polyclonal antibodies and as mentioned with regard to the previously discussed aspects, the determination of an optimal immunisation schedule relies on the type of immunogen being administered, dosage, route of injection and whether an adjuvant would be used to

enhance the immune response (42, 43). After primary immunisation, the researcher might decide to give the animal a booster immunisation according to the response of the animal after initial immunisation. The main goal of the booster immunisation is ultimately to re-stimulate the immune system of the immunised animal to increase circulating antibody levels and therefore it is very important for the researcher to get the timing of the booster immunisation right (43). To ensure that the booster immunisation is effective, it should only be administered after the immune response to primary exposure has started to decline and antibody levels have gone down (Fig.1.10). This process allows for the selection of B-lymphocytes to produce higher affinity antibodies to the antigen of interest (43). Therefore, when administering another round of immunisation too soon after initial immunisation, it might result in immune suppression rather than enhancing the secondary immune response (42, 43).

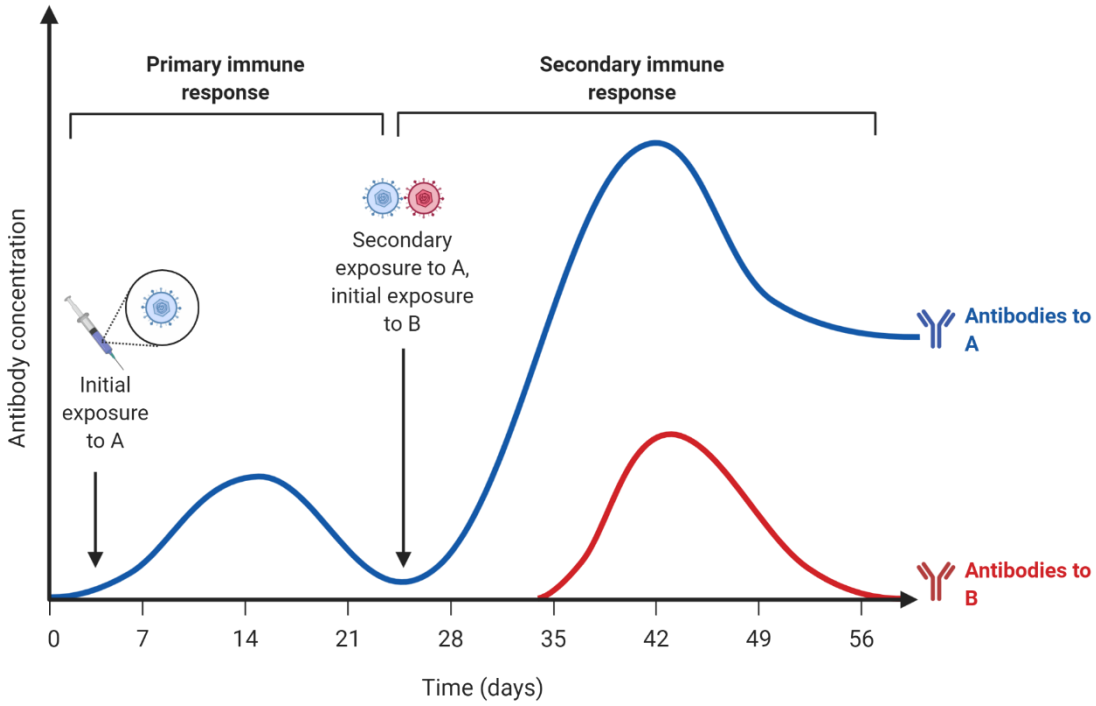

Figure 1.10: The typical antibody response. With the first encounter of an antibody on the surface of a B-cell with antigen (A), the immune system launches a primary immune response. This response may take 1 to 3 weeks after the first encounter to reach maximal antibody concentration. After second exposure to the same antigen, the immune system launches a secondary immune response, which typically takes 2 to 7 days to reach a maximal antibody concentration. Due to memory cells produced after first exposure to the antigen, the secondary immune response occurs more rapidly and to a greater extent than the primary response. After exposure to an antigen, antibody levels decline over time as infection is eradicated and the need for antibodies decline. When the immune system encounters a new antigen (B), it will launch a new primary response that will be unique to the specific antigen (34, 37). This image was created with BioRender.com.

Blood collection – During the polyclonal antibody production process, researchers have to routinely observe antibody titres and in order to do so, have to take blood samples on a regular basis. It is therefore critical to know the extent and limitations of how much blood can safely be withdrawn without causing

harm to the animal. If too much blood is drawn from an animal too rapidly or too frequently without sufficient time for replacement, it may result in hypovolemic shock and anaemia (40). It is therefore of major importance to routinely monitor the health status of animals that are frequently bled (48). Recommendations for the maximum amount of blood that can be sampled from an animal for a single sample can be observed in Table 1.3 below. As a rule of thumb, it is recommended that no more than 10-15% of the circulating blood volume of a healthy animal can be removed in a single sample (40, 45, 48). In practice, 1% of the body weight of an animal can safely be removed without causing any harm (40, 47) as roughly 5-7% of the weight of an animal can be attributed to the blood volume (45).

Table 1.3: Maximum recommended blood volume for blood collection of different animals (42).

Animal	Adult body weight (kg)	Adult blood volume (mL)	Single sample volume (mL)
Mice	0.025 - 0.040	2.5	0.3
Hamstesr	0.085 - 0.150	9	0.5
Rats	0.3 -0.5	30	2.5
Guinea pigs	0.7 - 1.2	60	5.0
Rabbits	2 - 6	150 - 400	25 - 50
Goats (45 kg)	15 - 65	3000	400[a]
Sheep (60 kg)	20 - 70	4000	600[b]
Chickens	1.5 - 2.5	240	20[c]

a Volume estimated according to a 45 kg goat

b Volume estimated according to a 60 kg sheep

c Although antibodies can be obtained from the blood of chickens, it is not recommended. As stated in section 1.3.2.2, an easier method of obtaining antibodies would be from the egg yolk of eggs lain by the immunised animal (46).

1.3.2.4 Use of adjuvants

In cases where antigens are too small or not immunogenic enough, adjuvants may be used to elicit or enhance an immune response. Adjuvants (derived from the Latin “adjuvare” which means to help) can be described as compounds or substances that are injected with the antigen of interest to elicit an enhanced immune response to antigens that are poor immunogens (43, 49, 50). The use of adjuvants generally falls within one of two categories, either in the production of prophylactic vaccines or in experimental research (50).

For the purposes of this study, however, we are more interested in the use of adjuvants in experimental research, specifically in polyclonal antibody production. For over 70 years adjuvants have been used to

enhance the immune response of host animals to antigens (50) to ensure the economical and efficient production of high-titre, high-affinity antibodies to a specific immunogen (43). More than 100 different adjuvants have been described, but only a few are routinely used (40), with one adjuvant that clearly stands out above the rest, having received the most attention.

In 1937, a researcher named Jules Freund reported that injecting guinea pigs, infected with *Mycobacterium tuberculosis*, with sheep erythrocytes resulted in elevated anti-sheep erythrocyte antibody titres (49). It was this finding that sparked Freund to examine the effect of emulsifying an aqueous solution of the antigen ovalbumin in paraffin oil along with killed tubercle bacilli with the aid of a surfactant (49). These experiments resulted into what is known today as Freund's complete adjuvant (FCA). As an important pioneer in the development of other adjuvants, FCA will be the focus of this section.

Since the first description of FCA, it has been the most effective and widely utilisedutilised adjuvant for experimental antibody production and its immunostimulatory capabilities have surpassed all other adjuvants (50). Antigens in aqueous solution are emulsified in FCA as a water-in-oil emulsion that consists of light mineral oil to which mannitane mono-oleate is added to emulsify the solution. The addition of inactivated and dried mycobacterium (*M. tuberculosis*) completes the mixture (41) and without the addition of the bacteria, the emulsifying oil is known as Freund's incomplete adjuvant. The primary principle on which FCA and other water-in-oil emulsions are based, is that the hydrophilic antigens (mostly proteins) become entrapped in small water droplets that are suspended in an oily environment (41, 49). It is this function of FCA, and other adjuvants, that allows for the slow and widespread release of the antigen of interest.

Although FCA has proven to be a good immunostimulant in the production of polyclonal antibodies, it has major drawbacks. The use of FCA has been associated with a variety of lesions and granulomas at the site of injection that cause pain and distress and are detrimental to the animal (50). It is due to these drawbacks that the use of FCA has since been limited and has resulted in numerous regulatory guidelines with regard to the use of this adjuvant to prevent harm to laboratory animals (50). With these regulations in mind, researchers have explored other avenues to achieve similar immunostimulatory effects to FCA without its great disadvantages. One such technique using acid-treated, naked bacteria, developed by Bellstedt *et al.* in 1987 (51), will be utilisedutilised in this project to produce polyclonal antibodies (Chapter 2).

1.3.3 Antibody production using acid-treated, naked bacteria

As alluded to earlier, antibodies can be produced against a wide variety of molecules but eliciting antibody production is complex. An introduced antigen can bind to an antibody receptor on a B-cell but would not necessarily elicit an effective immune response. It is usually problematic if only the antigen

is presented to the immune system. As mentioned previously, these obstacles could be overcome by the use of adjuvants such as emulsifying the antigen in FCA, but these have major constraints as explained above. To overcome these problems, Bellstedt *et al.* (51) developed an immunisation technique that involves the adsorption of a protein antigen of interest to acid-treated, naked *Salmonella minnesota* R595 bacteria, which act as an immune carrier for protein antigens to stimulate and enhance an immune response in cases where the antigen itself is not sufficiently immunogenic (51).

The first use of the term acid-treated, naked bacteria, also referred to as naked bacteria (NB), was used by Galanos *et al.* (52) to describe *S. minnesota* R595 that have been stripped of their natural antigenic determinants on the cell surface by mild acid hydrolysis. Galanos *et al*. (52) found that the NB could act as immune carriers and have excellent immune stimulating properties when antigens were adsorbed to them. In the subsections to follow, we will briefly discuss the immunogenic properties of naked bacteria as well as their use in polyclonal antibody production.

1.3.3.1 The Gram-negative cell wall

Bacteria may be divided into one of two groups according to their specific staining patterns. In 1884, Hans Christian Gram devised a method for the staining of bacteria that is based on the composition of their cell walls and can therefore either be classified as Gram-positive or Gram-negative (53). *S. minnesota* R595 is a Gram-negative bacterium and thus has a more complex cell-wall structure than Gram-positive bacteria. The cell wall of Gram-negative bacteria consists of multiple layers starting with the inner cytoplasmic membrane, which is surrounded by a peptidoglycan layer, which is finally surrounded by an outer membrane layer (54). Furthermore, the outer membrane consists of many components, such as various proteins, lipopolysaccharides (LPS), lipoprotein (LP) and phospholipids, as illustrated in Figure 1.11 A.

The LPS complex in the outer membrane of the cell wall is also known as endotoxin. Endotoxins elicit various toxic effects in Gram-negative infected mammals that result in characteristic symptoms such as fever, changes in blood pressure, headache and in severe cases could even result in death. These toxic effects do not change whether the bacterium is dead or alive (55). LPS molecules (Fig. 1.11 B) consist of mainly two components, a hydrophilic polysaccharide portion and a hydrophobic lipid portion that is also known as lipid A. The polysaccharide portion of LPS can further be divided into the core polysaccharide chain and the O-specific saccharide chain (55).

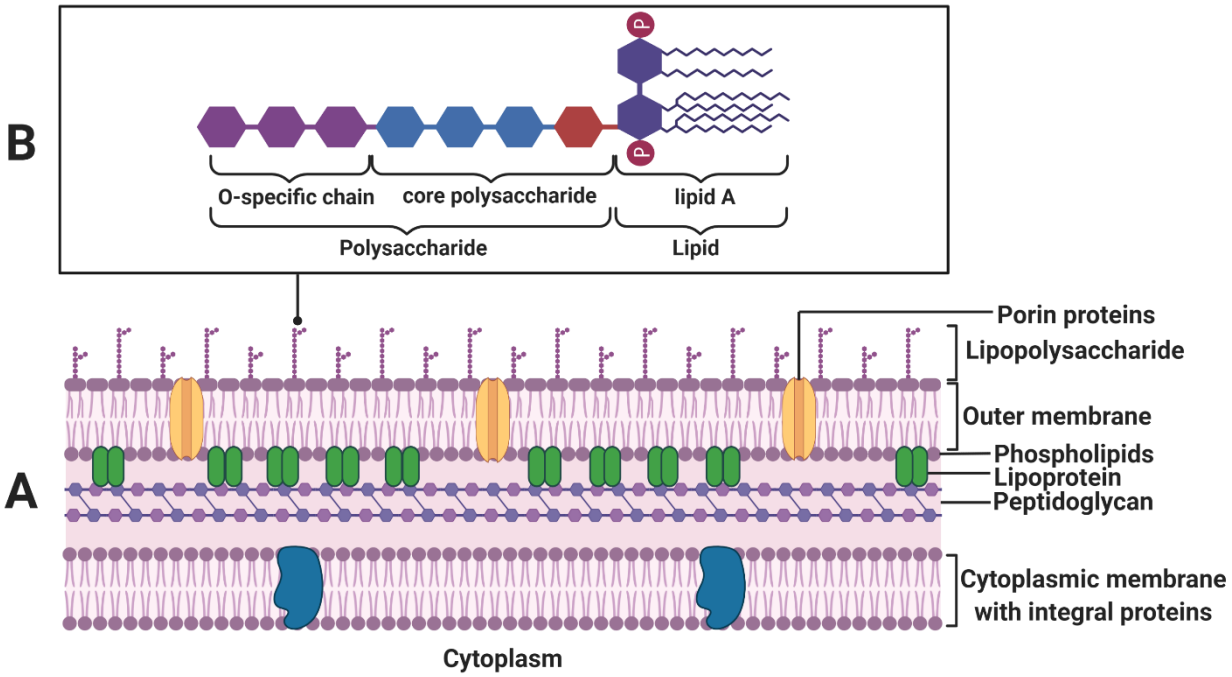

Figure 1.11: The Gram-negative cell wall. The Gram-negative cell wall (**A**) consists of many components that make it more complex than the Gram-positive cell wall. The innermost layer of the Gram-negative cell wall is the inner cytoplasmic membrane that contains integral proteins. This membrane is followed by the periplasmic space that contains the peptidoglycan layer and several proteins such as lipoproteins and phospholipids. The outermost layer of the Gram-negative cell wall consists of the outer membrane, containing porin proteins and lipopolysaccharide (LPS) on its surface. The LPS portion of the membrane (**B**) is composed of a polysaccharide portion and a lipid portion, also known as lipid A. The polysaccharide region can further be divided into the O-specific chain and the core polysaccharide. This image was created with BioRender.com.

The polysaccharide portion of LPS indirectly plays a role in the endotoxic effect of Gram-negative bacteria and is mainly responsible for the increased solubility of the hydrophobic lipid A portion, facilitating the uptake thereof in mammalian cells. Therefore, the longer the polysaccharide chain, the easier it is taken up into the mammalian cell (56). The lipid A portion on the other hand, has been identified as the key determinant of the endotoxic activity that is associated with LPS and infections with Gram-negative bacteria (57).

1.3.3.2 The preparation of acid treated naked bacteria

The lipid A portion of LPS displays endotoxic activity and elicits an immune response in infected mammals. It is this characteristic of lipid A, that when presented in a suitable form, can be used to elicit an immune response to produce antibodies in immunised animals (52). In 1967, Risse *et al*. (58) reported a method for the cleavage of the polysaccharide portion of LPS from lipid A by utilization of acid hydrolysis. In turn, this method was utilisedutilised by Galanos *et al.* (52) to produce acid-treated, naked *S. minnesota* R595 (NB), and ultimately resulted in the immunisation method developed by Bellstedt *et al*. (51) to use the NB as an immune carrier for polyclonal antibody production.

NB are prepared by growing *S. minnesota* R595 in liquid medium and subsequently killing them by the addition of a 1% phenol solution. Thereafter, the killed bacteria are washed with distilled water, followed by acetone and finally ether. The NB were obtained by treating these bacteria with a 1% acetic acid solution at 100°C for 1 hour, resulting in the cleavage of the core polysaccharide portion along with certain sugars from the LPS present on the cell surface of the bacteria. Before storage, the NB are washed with a saline solution and water, whereafter they are lyophilised (51, 52).

1.3.3.3 Acid-treated naked bacteria as immune carriers

Since the first description of acid-treated, naked bacteria by Galanos *et al.* (52), NB have been used in many other studies for antibody production. In 1971, Galanos *et al*. (52) showed that free lipid A adsorbed to NB was able to raise specific antibodies to lipid A in rabbits. Furthermore, in 1984 Galanos *et al.* (59) also showed that raising antibodies in mice utilizing the free lipid A adsorbed to NB, required the development of a special immunisation schedule, indicating that optimisation is needed for the immunisation of different animals.

In 1987, Bellstedt *et al.* (51) determined that titre values for antibodies raised to carcino-embryonic antigen as well as human apolipoprotein A1 that were adsorbed to NB, were comparable to antibody levels achieved when using FCA to enhance an immune response. This study showed that although antibody levels were comparable, when utilizing the NB method where the antigen forms a complex with the NB, and much less antigen was required for immunisation. In a second study, Bellstedt *et al.* (60) also showed that linking the trinitrophenyl (TNP) hapten to NB, resulted in a significant immune response in mice and rabbits and that NB were effective immune carriers for the TNP hapten. Therefore, whether the antigen was adsorbed or covalently linked to the NB, it was able to elicit an effective immune response in the immunised animals.

It is clear from these studies that the utilization of NB as immune carriers has proven to be very successful in the production of antibodies, but it is important to note that it could also have disadvantages. Due to the endotoxic effect of lipid A in the cell membrane of the NB, bigger dosages of the NB could have adverse effects and therefore only limited amounts as determined by Bellstedt *et al*. (60) should be used. Nevertheless, it has been determined that the lipid A present in NB is considerably less toxic than free lipid A (61).

NB have subsequently been used to raise antibodies against many protein antigens including human Serum amyloid A (62), cytochrome P450-dependent enzymes (63), ostrich immunoglobulins (64) and many others. This has shown that this technique has distinct merits as a method for raising antibodies against proteins particularly in instances where their isolation is problematic, and in low yield and also does not lead to granuloma formation as is the case when FCA is used.

1.3.4 Polyclonal antibody alternatives

Other than polyclonal antibodies, many alternative types of antibodies are frequently used in research. Previously, monoclonal antibodies and their advantages and disadvantages as opposed to polyclonal antibodies were discussed, but other options include recombinant antibodies that can be produced in non-animal systems such as plants and bacteria. These antibodies can be either polyclonal or monoclonal and eliminate the use of animals in antibody production (65). Another upcoming alternative is the use of nanobodies, or single domain antibodies. These antibodies that are produced in Camelids are devoid of light chains and the first constant region (Fig 1.7), making them much smaller and more versatile. Consequently, these antibodies have been used in many applications such as fluorescent microscopy and super-resolution microscopy as well as protein-protein interaction analysis (66).

Although these are very attractive alternatives to polyclonal antibodies, they require additional expertise in molecular biotechnology as well as special resources (such as immunisation of llamas or alpacas to produce nanobodies) and they are also more time consuming and more expensive to produce (in the case of nanobodies). Thus, they were considered to be less ideal for the purpose of this study.

1.3.5 Antibody applications in diagnostics

Antibodies have many applications, not only as research tools but they can also play a key role in diagnostic testing. Immunodiagnostic tests have made it possible for clinicians to test for a wide variety of disease markers and are the first resort for the detection and measurement of a plethora of blood products, hormones, enzymes, nutritional factors, and drugs (67). Antibody-based assays exist in many formats such as immunohistochemistry, immunosorbent assays (radio immunoassays and enzyme immunoassays), immunoprecipitation and agglutination, and immunochromatography, to name a few (68).

Immunosorbent assays and immunochromatography are two of the more commonly used immunodiagnostic tests. Immunosorbent assays, such as the enzyme-linked immunosorbent assay (ELISA), is one of the best-known and widely utilisedutilised immunoassays and relies on the adsorption of a primary reactant to a solid surface (68). Some of the first assays used were developed for the detection of large polypeptide hormones such as glucagon and insulin, but to date ELISAs have been developed to include the detection of disease markers, therapeutic agents, recreational drugs, plasma proteins and many other molecules (67). The use of these assays has also aided in the agricultural sector for the detection of certain disease conditions, caused by plant pathogens, in crops. Antibodies and their application in ELISAs will be discussed in more detail later.

Second to ELISAs, the use of immunochromatography or lateral flow assays have become increasingly popular. Unlike ELISAs, immunochromatography does not fix analytes to a surface, but rather allows

the fluid sample to be tested to flow and migrate on thin-layer chromatography paper. As the fluid moves to one direction, it takes antibody coated particles that form part of the matrix along with it, allowing the antigen to bind to its complimentary antibody on the particle surface. A coloured stripe, which can be evaluated with the naked eye, is formed in the detection zone as the antigen-carrying particles are embedded in this region. The non-antigen-carrying particles, on the other hand, moves on to a control zone (68). Some of the most common immunodiagnostic tests that involve immunochromatography are pregnancy tests, rapid tests for the detection of certain diseases such as HIV, as well as multiple drug testing (69).

1.3.6 Antibody applications in antimicrobial drug targeting

Other than its applications in protein quantification and many other techniques such as immunohistochemistry, immunocytochemistry, immunoprecipitation, and flow cytometry (70), antibodies have also played a key role in therapeutics.

In the 19th and 20th centuries, increased accounts of the use of antibodies for therapy against microbial and viral infections have been reported (71). Serum therapy involves injecting antibodies, produced in animals against different infective micro-organisms, into healthy individuals as prophylaxis, or into infected individuals for treatment purposes. In a review by Glatman-Freedman (71), they report on the early use of serum therapy in *M. tuberculosis* (TB) infection and how it evolved over the years. In a similar review, Casadevall (72) reports on the use of serum therapy in infections caused by *Corynebacterium diptheriae*, *Streptococcus pneumoniae*, *Neisseria meningitidis*, *Haemophilus influenzae* as well as *Clostridium tetani*. By the 1930's, however, the use of serum therapy was discontinued when vaccinations and antimicrobial chemotherapy were introduced (72).

In more recent studies, the use of antibody-antibiotic complexes (AAC) for combating bacterial infections have been proposed. Antibody-antibiotic conjugation combines the key characteristics of antibiotics and antibodies, resulting in a single molecule with the ability to target specific molecules while being easily adsorbed, distributed, and metabolised (73). A study by Lehar *et al.* (74) reports on one such AAC for combating *S. aureus*, and specifically MRSA, infections. MRSA is notorious for its ability to evade the immune system by essentially hiding in phagocytes, making it increasingly difficult to target. In this study, they combined *S. aureus* anti-b-GlcNAc-WTA with dmDNA31, a rifalazil analogue that forms part of the ansamycin class of antibiotics (73) and found that these conjugates were able to kill *S. aureus* in infected cells in mice more effectively than vancomycin. Furthermore, it was also able to stop the spread of the infection in the bodies of the mice (74).

Since the outbreak of the SARS-CoV-2 virus that resulted in the recent ongoing pandemic, the use of antibodies to treat infected patients has gained renewed attention (75–77). Antibody therapy, that has been replaced with new strategies to combat bacterial and viral infection in the past decades, is one of

the strategies that are being considered for the relief of the severe burden of Covid-19 in infected patients (75–77). As reported by Abraham (77), recent non-controlled studies with convalescent plasma therapy or antibody serum therapy, have shown great potential. Patients with severe Covid-19 were transfused with high-titre, neutralizing antibody serum from individuals that had recovered from the illness. Post-transfusion, it was observed that these individuals had increased levels of neutralising antibodies, no viral RNA was detected in their blood samples at the time and these individuals also improved clinically (78). Second to serum therapy, monoclonal antibody production to spike glycoproteins on the viral surface that mediates entry into cells has also been reported. The intention of these antibodies is the neutralisation of the glycoproteins to inhibit the virus from entering the host cell (75).

1.3.7 Antibody applications in protein quantification

Antibodies are important tools that play a significant role in protein quantification. Many methods exist for the determination of protein concentration, but two of these techniques namely ELISA and western blot will be discussed.

1.3.7.1 Enzyme-linked immunosorbent assay (ELISA)

Since the development of the radioimmunoassay (RIA) in the 1960s, more and more enzyme-immunoassays (EIA), have been developed. These included the enzyme-linked immunosorbent assays (ELISA), which stem from the same technique as the RIA, but differ in that antigens or antibodies are absorbed to a solid phase instead of using an antigen antibody precipitation step. Furthermore, the antigens or antibodies are linked to enzymes instead of radioactive labels (79, 80). The ELISA was developed to eliminate the use of hazardous radiolabels, which required specialised facilities and waste management systems to dispose of radioactive waste (81). Not long after the introduction of the RIA, Perlmann and Engvall (82), introduced the use of the ELISA utilizing solid absorption and antibodies coupled to alkaline phosphatase from calf intestinal mucosa (82). This method, which can be used in a wide variety of applications, has become a standard technique in research laboratories world-wide (79).

The ELISA relies on the principle that either enzyme or antibody can be adsorbed to a solid surface, whilst still participating in highly specific antigen binding (83). The technique can be divided into homogenous or heterologous assays (79). The homogenous assay relies on the principle that the solid surface does not require washing to avoid interference between antibody and antigen binding. This method is generally used for measuring substances in small quantities and is easy to use, however, it is very expensive and has low sensitivity (79). The heterogenous assay method on the other hand, is more sensitive and more commonly used. This method relies on washing the solid surface, where the antibody

or antigen is bound to, to avoid the interference of any molecule after the binding of the antibody-antigen complex to the solid surface (79).

The heterogenous ELISA can further be divided into different ELISA systems namely, the direct ELISA, the indirect ELISA, the sandwich ELISA and lastly the competitive ELISA. These different ELISAs are used in different applications and are similar but vary in key steps. A typical ELISA starts with adsorbing either antigen or antibody to the solid surface, followed by incubation with either antigen or antibody coupled to an enzyme depending on the chosen system and lastly the addition of a substrate for the visualisation of a colour reaction. The different systems as well as the process of ELISA development will be discussed in greater depth in Chapter 3.

The ELISA has many advantages, including its sensitivity, specificity, rapidity, and its low cost. Furthermore, it is also able to detect antigens at very low concentrations (in the low ng/ml range) in solutions containing many other substances including proteins, sugars, lipids and inorganic molecules.The indirect and competitive formats of this assay are consequently widely used in protein quantification (84).

1.3.7.2 Western blotting

Second to the ELISA, western blotting is commonly used for the identification, quantification, and size determination of specific proteins (85). The use of the western blot was first reported in 1979 by Renart *et al.* (86) and Towbin *et al.* (87). The western blot as we know it today, however, was reported by Burnette in 1980 (88). The western blot forms part of the blotting techniques, Southern blotting which is used for the detection of DNA and northern blotting, which is used for the detection of RNA, all of which rely on the separation of molecules by electrophoresis followed by the transfer of the molecules to paper or membranes and probing with suitable nucleic acid or antibody probes.

In western blotting proteins are first separated based on charge and size by gel electrophoresis, followed by the transfer of the separated proteins to a membrane, whereafter the membrane is incubated with antibodies for the detection of the specific proteins (89). The proteins are visualised by incubation of the membrane with a substrate solution that could either result in a colorimetric or chemiluminescent reaction and the thickness of the bands correlate with the amount of transferred protein (89). One of the great advantages of western blotting is its ability to differentiate between different forms of the same protein or antigen, which is not the case for ELISAs. ELISAs on the other hand, have proven to be a much more sensitive method for the quantification of proteins (90).

1.4 Problem statement

1.4.1 Physiological levels of CoA biosynthesis enzymes in different bacterial growth phases are currently unknown

As stated in the first section of this Chapter, the physiological levels of the CoA biosynthesis enzymes in *S. aureus* bacteria are currently unknown. It is currently believed that the physiological representative ratios between the enzymes are 1:9:6:19 for PanK:CoaBC:PPAT:DPCK (33), but the actual levels need to be determined experimentally.

Since the CoA pathway is being investigated as a novel drug target for antimicrobial drug development, it is imperative to understand the enzyme levels in order to determine which enzyme in the pathway has the most control over CoA production and availability. This could lead to more directed drug target strategies, by targeting one specific enzyme in the pathway, rather than trying to investigate the pathway as a whole. The objective of this project would be to develop tools that utilise polyclonal antibodies in an ELISA format for the detection and quantification of these CoA biosynthesis enzymes in *S. aureus* in different growth phases of the bacterium.

1.4.2 Current antibodies available for CoA research

In order to use ELISA for protein quantification, antibodies need to be generated that will recognise the enzymes involved in CoA biosynthesis in *S. aureus*. Literature reveals (Table 1.4) that the production of antibodies for use in CoA biosynthesis research primarily falls into two categories: i.e., antibodies produced against purified enzymes and antibodies produced against specific peptides (this is the most commonly used approach). Furthermore, it can be observed that antibodies were mostly produced against the PanK enzyme of different organisms, corresponding with the statement that PanK is the best researched enzyme of the CoA biosynthesis pathway. Lastly, when assessing commercial antibodies, antibodies were only available for enzymes of the mammalian CoA biosynthesis pathway, especially antibodies against human, rat and mouse CoA biosynthesis enzymes.

As can be seen the antibody options are mostly for PanK with no known antibodies raised against any of the CoA biosynthesis enzymes of *S. aureus* specifically. This led to the realisation that there is a considerable gap in the knowledge and research tools to investigate CoA biosynthesis enzyme levels in *S. aureus*.

Table 1.4: Table of published antibodies used in CoA biosynthesis research

Antibody (polyclonal)	Method	Details	Literature
anti-*Ec*PanK	Immunisation of rabbits with purified recombinant protein	Used for purification of *Ec*PanK by immuno-affinity chromatography	Song & Jackowski 1994 (91)
anti-*Mm*PanK 1	Immunisation of rabbits with synthesised peptide (KDNYKRVTGTSLGC)	Used for the detection of expression of *Mm*PanK 1 with western Blotting	Rock *et al*. 2002 (92)
anti-CoASY	Immunisation of rabbits with purified recombinant his-dCoAK	Used for determination of protein level expression of rat CoASY with immunoblotting	Zhyvoloup *et al.* 2002 (93)
anti-*Mm*PanK (1-3)	Immunisation of rabbits with synthesised peptide linked to a carrier protein (SKDNYKRVTGTSLGC)	Used for the detection of expression of *Mm*PanK isoforms (1-3) with western Blotting	Zhang *et al.* 2005 (94)
anti-*Hs*PanK (1-3).	Immunisation of rabbits with synthesised peptide linked to a carrier protein (SKDNYKRVTGTSLGC)	Used for protein quantification with western Blotting	Zhang *et al.* 2006 (95)
anti-CoASY β	Immunisation of rabbits with synthesised peptide linked to carrier protein (MRTPRLRAQPRGAVYC)	Used for validation of protein expression with western Blotting	Nemazanyy *et al.* 2006 (96)
anti-dPanK/Fbl[a] & anti-dPPCS[b]	Immunisation of rabbits with [a,b]synthesised peptides linked to carrier protein	Used for protein quantification with western Blotting	Bosveld *et al.* 2008 (97)
anti-PanK	Immunisation of rabbits with recombinant purified human PanK 1β	Used for validation of the expression of PanK isoforms with immunoblotting	Sharma *et al.* 2018 (98)

a YFEPKDITPDEQDREC and CDEPPEKAPTSKHSTR (peptide sequences)

b CDMMPTHKMQSGDGAP and CVQKHGEFISNAQQRQ (peptide sequences)

1.4.3 Study aims and objectives

The goal of this study is to produce polyclonal antibodies that can be used in the development of ELISAs for the quantification of the CoA biosynthesis enzymes in *S. aureus.* These assays would then be used to determine the enzyme levels during different growth phases of this bacterium to confirm the current ratios of enzymes produced or to establish the actual ratios between the enzymes in the pathway. Information obtained from the utilization of these ELISAs therefore has the potential to be of considerable value in the development of selective inhibitors and novel antimicrobial drugs targeting CoA biosynthesis enzymes.

Aim 1: The production, validation, and characterisation of antibodies against CoA biosynthetic enzymes from *S. aureus*.

The first aim was to produce polyclonal antibodies against the enzymes of the CoA biosynthesis pathway of *S. aureus*. In order to achieve this aim, the enzymes of the pathway need to be produced and purified to allow their adsorption to acid-treated, naked *S. minnesota* R595. Subsequently, these complexes would be injected into rabbits for the production of polyclonal antisera. These antibodies will then be fully characterised by means of ELISA and western blot analysis as well as potential cross-reactivity determined. Aim 1 can therefore be divided into the following objectives:

Objective 1: The production and purification of the enzymes present in the CoA biosynthesis pathway of S. aureus *(PanK, CoaBC, PPAT & DPCK).*

Large scale production of each of the enzymes will be performed with recombinant protein expression in *E. coli* BL21(DE3)-star cells, followed by purification with Ni^{2+}-based immobilised metal-affinity chromatography (IMAC).

Objective 2: Antibody production to the CoA biosynthesis enzymes of S. aureus.

Polyclonal antibodies to the enzymes of the CoA biosynthesis pathway of *S. aureus* will be produced by the immunisation of rabbits with each enzyme adsorbed to acid-treated, naked *S. minnesota* R595 bacteria.

Objective 3: Antibody characterisation and cross-reactivity determination with ELISA and western blot.

Antibodies produced will be subjected to ELISA analysis to determine antibody titre values as well as determine potential cross-reactivity of the antibodies with the different enzymes of the pathway. Western Blot analysis will be performed as a comparison to ELISA.

Aim 2: The development of an ELISA for the quantification of CoA biosynthesis enzymes present in *S. aureus* bacteria in different growth phases.

The second aim of the project was to develop and optimise an ELISA assay for the quantification of each of the CoA biosynthesis enzymes of *S. aureus*. The above-mentioned polyclonal antibodies will be utilisedutilised to develop and optimise ELISAs for the quantification of each of the enzymes. The ultimate goal will be to use these assays to test *S. aureus* samples at different growth stages to determine the physiological levels of these enzymes at different growth stages. The objectives for Aim 2 are thus:

Objective 1: Development and optimisation of an ELISA assay for the respective quantification of each of the CoA biosynthesis enzymes of S. aureus *(PanK, CoaBC, PPAT & DPCK).*

Purified enzymes and polyclonal antibodies from Aim 1 will be used to develop and optimise ELISAs for determining CoA biosynthesis enzyme concentrations.

Objective 2: Utilization of the optimised ELISAs for the quantification of antibody cross-reactivity.

An optimised ELISA for each respective enzyme will be used to evaluate the effect of the cross-reactivity of the antibodies with the specific antigens on the outcome of the optimised assays.

Objective 3: Determination of physiological CoA biosynthesis enzyme levels at different growth phases of S. aureus.

A protocol for the cultivation and enzyme extraction from *S. aureus* during different growth phases will have to be established and used to prepare samples for enzyme quantification. The optimised ELISA for each of the enzymes will then be used to determine enzyme concentrations from samples obtained during different growth phases of *S. aureus*.

1.5 References

1. Lowy, F. (1998) *Staphylococcus aureus* Infections. *N. Engl. J. Med.* **339**, 520–532

2. Tong, S. Y. C., Davis, J. S., Eichenberger, E., Holland, T. L., and Fowler, V. G. (2015) *Staphylococcus aureus* infections: Epidemiology, pathophysiology, clinical manifestations, and management. *Clin. Microbiol. Rev.* **28**, 603–661

3. Pollitt, E. J. G., Szkuta, P. T., Burns, N., and Foster, S. J. (2018) *Staphylococcus aureus* infection dynamics. *PLoS Pathog.* **14**, 1–27

4. Fitzgerald, J. R. (2014) Evolution of *Staphylococcus aureus* during human colonization and infection. *Infect. Genet. Evol.* **21**, 542–547

5. Strommenger, B., Layer, F., and Werner, G. (2018) *Staphylococcus aureus* and Methicillin-Resistant *Staphylococcus aureus* in Workers in the Food Industry. in *Staphylococcus aureus* (Fetsch, A. ed), pp. 163–188

6. Hogan, S., Zapotoczna, M., Stevens, N. T., Humphreys, H., O'Gara, J. P., and O'Neill, E. (2017) Potential use of targeted enzymatic agents in the treatment of *Staphylococcus aureus* biofilm-related infections. *J. Hosp. Infect.* **96**, 177–182

7. Prestinaci, F., Pezzotti, P., and Pantosti, A. (2015) Antimicrobial resistance: A global multifaceted phenomenon. *Pathog. Glob. Health*. **109**, 309–318

8. Kuehnert, M. J., Hill, H. A., Kupronis, B. A., Tokars, J. I., Solomon, S. L., and Jernigan, D. B. (2005) Hospitalizations, United States. **11**, 868–872

9. WHO (2017) *Global Antimicrobial Resistance Surveillance System*

10. Lowy, F. D. (2003) Antimicrobial resistance: the example of *Staphylococcus aureus*. *J. Clin. Inverstigation*. **111**, 1265–1273

11. Grace, D., and Fetsch, A. (2018) *Staphylococcus aureus* - A Foodborne Pathogen: Epidemiology, Detection, Characterisation, Prevention and Control: An Overview. in *Staphylococcus aureus* (Fetsch, A. ed), pp. 3–10

12. Stryjewski, M. E., and Corey, G. R. (2014) Methicillin-resistant *Staphylococcus aureus*: An evolving pathogen. *Clin. Infect. Dis.* **58**, 10–19

13. Monaco, M., Pimentel de Aurajo, F., Cruciani, M., Coccia, E. M., and Pantosti, A. (2017) Worldwide Epidemiology and Antibiotic Resistance of *Staphylococcus aureus*. in *Staphylococcus aureus Microbiology, Pathology, Immunology, Therapy and Prophylaxis* (Bagnoli, F., Rappuoli, G., and Grandi, G. eds), pp. 21–56, Springer International Publishing

14. Lee, A. S., De Lencastre, H., Garau, J., Kluytmans, J., Malhotra-Kumar, S., Peschel, A., and Harbarth, S. (2018) Methicillin-resistant *Staphylococcus aureus*. *Nat. Rev. Dis. Prim.* **4**, 1–23

15. Spry, C., Kirk, K., and Saliba, K. J. (2008) Coenzyme A biosynthesis: An antimicrobial drug target. *FEMS Microbiol. Rev.* **32**, 56–106

16. Leonardi, R., and Jackowski, S. (2007) Biosynthesis of pantothenic acid and Coenzyme A. *EcoSal Plus*. **2**, 1–28

17. Leonardi, R., Zhang, Y. M., Rock, C. O., and Jackowski, S. (2005) Coenzyme A: Back in action. *Prog. Lipid Res.* **44**, 125–153

18. Lopez Martinez, D., Tsuchiya, Y., and Gout, I. (2014) Coenzyme A biosynthetic machinery in mammalian cells. *Biochem. Soc. Trans.* **42**, 1112–1117

19. Moolman, W. J. A., De Villiers, M., and Strauss, E. (2014) Recent advances in targeting coenzyme A biosynthesis and utilization for antimicrobial drug development. *Biochem. Soc. Trans.* **42**, 1080–1086

20. Strauss, E. (2010) Coenzyme A biosynthesis and enzymology. in *Comprehensive Natural Products II: Chemistry and Biology*, 1st Ed., pp. 351–410, Elsevier Ltd, **7**, 351–410

21. Leonardi, R., Chohnan, S., Zhang, Y. M., Virga, K. G., Lee, R. E., Rock, C. O., and Jackowski, S. (2005) A pantothenate kinase from *Staphylococcus aureus* refractory to feedback regulation by coenzyme A. *J. Biol. Chem.* **280**, 3314–3322

22. Hong, B. S., Yun, M. K., Zhang, Y. M., Chohnan, S., Rock, C. O., White, S. W., Jackowski, S., Park, H. W., and Leonardi, R. (2006) Prokaryotic Type II and Type III Pantothenate Kinases: The Same Monomer Fold Creates Dimers with Distinct Catalytic Properties. *Structure*. **14**, 1251–1261

23. Choudhry, A. E., Mandichak, T. L., Broskey, J. P., Egolf, R. W., Kinsland, C., Begley, T. P., Seefeld, M. A., Ku, T. W., Brown, J. R., Zalacain, M., and Ratnam, K. (2003) Inhibitors of pantothenate kinase: Novel antibiotics for staphylococcal infections. *Antimicrob. Agents Chemother*. **47**, 2051–2055

24. Strauss, E., Kinsland, C., Ge, Y., McLafferty, F. W., and Begley, T. P. (2001) Phosphopantothenoylcysteine Synthetase from *Escherichia coli*. Identification and characterisation of the last unidentified coenzyme A biosynthetic enzyme in bacteria. *J. Biol. Chem.* **276**, 13513–13516

25. Geerlof, A., Lewendon, A., and Shaw, W. V. (1999) Purification and characterisation of phosphopantetheine adenylyltransferase from *Escherichia coli*. *J. Biol. Chem.* **274**, 27105–

27111

26. De Jonge, B. L. M., Walkup, G. K., Lahiri, S. D., Huynh, H., Neckermann, G., Utley, L., Nash, T. J., Brock, J., San Martin, M., Kutschke, A., Johnstone, M., Laganas, V., Hajec, L., Gu, R. F., Ni, H., Chen, B., Hutchings, K., Holt, E., McKinney, D., Gao, N., Livchak, S., and Thresher, J. (2013) Discovery of inhibitors of 4'-phosphopantetheine adenylyltransferase (PPAT) to validate PPAT as a target for antibacterial therapy. *Antimicrob. Agents Chemother.* **57**, 6005–6015

27. Han, S., Forman, M. D., Loulakis, P., Rosner, M. H., Xie, Z., Wang, H., Danley, D. E., Yuan, W., Schafer, J., and Xu, Z. (2006) Crystal Structure of Nicotinic Acid Mononucleotide Adenylyltransferase from *Staphyloccocus aureus*: Structural Basis for NaAD Interaction in Functional Dimer. *J. Mol. Biol.* **360**, 814–825

28. Lee, H. H., Yoon, H. J., Kang, J. Y., Park, J. H., Kim, D. J., Choi, K. H., Lee, S. K., Song, J., Kim, H. J., and Suh, S. W. (2009) The structure of *Staphylococcus aureus* phosphopantetheine adenylyltransferase in complex with 3′-phosphoadenosine 5′-phosphosulfate reveals a new ligand-binding mode. *Acta Crystallogr. Sect. F Struct. Biol. Cryst. Commun.* **65**, 987–991

29. Bouwer, W. (2018) *Investigation and Characterisation of Dephospho-Coenzyme A kinase: A Potential Drug Target*. MSc. thesis, Stellenbosch University

30. O'Toole, N. (2003) Crystal structure of a trimeric form of dephosphocoenzyme A kinase from *Escherichia coli*. *Protein Sci.* **12**, 327–336

31. Tsuchiya, Y., Pham, U., and Gout, I. (2014) Methods for measuring CoA and CoA derivatives in biological samples. *Biochem. Soc. Trans.* **42**, 1107–1111

32. Goosen, R. (2016) *A comparative analysis of CoA biosynthesis in selected organisms: a metabolite study*. PhD. thesis, Stellenbosch University

33. Wang, M., Herrmann, C. J., Simonovic, M., Szklarczyk, D., and von Mering, C. (2015) Version 4.0 of PaxDb: Protein abundance data, integrated across model organisms, tissues, and cell-lines. *Proteomics*. **15**, 3163–3168

34. Janeway, C. A., Travers, P., Walport, M. J., and Shlomchik, M. (2001) *Immunobiology: The Immune System in Health and Disease*, 5th Ed.

35. Harlow, E., and Lane, D. (1988) *Antibodies: A Laboratory Manual*, Cold Spring Harbor Laboratory

36. Crowther, J. R. (1995) ELISA Theory and Practice. in *Methods in Molecular Biology* (Walker, J. M. ed), Humana Press Inc.

37. Abbas, A. K., Lichtman, A. H., and Pillai, S. (2016) *Basic Immunology: Functions and*

Disorders of the Immune System, 5th Ed., Elsevier

38. Lipman, N. S., Jackson, L. R., Trudel, L. J., and Weis-Garcia, F. (2005) Monoclonal versus polyclonal antibodies: Distinguishing characteristics, applications, and information resources. *ILAR J.* **46**, 258–267

39. Hnasko, R. M. (ed.) (2015) ELISA Methods and Protocols. in *Methods in Molecular Biology*

40. Leenaars, M., and Hendriksen, C. F. M. (2005) Critical Steps in the Production of Polyclonal and Monoclonal Antibodies: Evaluation and Recommendations. *Ilar J.* **46**, 269–279

41. Drenckhahn, D., Jöns, T., and Schmitz, F. (1993) Production of Polyclonal Antibodies against Proteins and Peptides. *Methods Cell Biol.* **37**, 7–56

42. Hanly, W. C., Artwohl, J. E., and Bennett, B. T. (1995) Review of Polyclonal Antibody Production Procedures in Mammals and Poultry. *ILAR J.* **37**, 93–118

43. Stills, H. F. (2012) Polyclonal Antibody Production. in *The Laboratory Rabbit, Guinea Pig, Hamster, and Other Rodents*, pp. 259–274, 10.1016/B978-0-12-380920-9.00011-0

44. Cooper, H. M., and Paterson, Y. (2008) Preparation of polyclonal antisera: Production of polyclonal antisera. *Curr. Protoc. Mol. Biol.* 10.1002/0471142727.mb1112s81

45. Jackson, L. R., and Fox, J. G. (1995) Institutional Policies and Guidelines on Adjuvants and Antibody Production. *ILAR J.* **37**, 141–152

46. Goldring, J. P. D. and Coetzer, T. H. T. (2003) Isolation of Chicken Antibodies (IgY) from egg yolk. *Biochem. Mol. Biol. Ed.* **31**, 185-187.

47. Leenaars, P. P. A. M., Hendriksen, C. F. M., De Leeuw, W. A., Carat, F., Delahaut, P., Fischer, R., Halder, M., Hanly, W. C., Hartinger, J., Hau, J., Lindblad, E. B., Nicklas, W., Outschoorn, I. M., and Stewart-Tull, D. E. S. (1999) The production of polyclonal antibodies in laboratory animals. *ATLA Altern. to Lab. Anim.* **27**, 79–102

48. Leenaars, P. P. A. M., Hendriksen, C. F. M., Koedam, M. A., Claassen, I., and Claassen, E. (1995) Comparison of adjuvants for immune potentiating properties and side effects in mice. *Vet. Immunol. Immunopathol.* **48**, 123–138

49. Jennings, V. M. (1995) Review of Selected Adjuvants Used in Antibody Production. *ILAR J.* **37**, 119–125

50. Stills, H. F. (2005) Adjuvants and antibody production: Dispelling the myths associated with Freund's complete and other adjuvants. *ILAR J.* **46**, 280–293

51. Bellstedt, D. U., Human, P. A., Rowland, G. F., and Van der Merwe, K. J. (1987) Acid-treated,

naked bacteria as immune carriers for protein antigens. *J. Immunol. Methods*. **98**, 249–255

52. Galanos, C., Lüderitz, O., and Westhphal, O. (1971) Preparation and Properties of Antisera against the Lipid-A component of Bacterial Lipopolysaccharides. *Eur. J. Biochem.* **24**, 116–122

53. Coico, R. (2005) Gram Staining BASIC PROTOCOL Commonly Used Techniques. *Curr. Protoc. Microbiol.*

54. Lugtenberg, B. E. N., and Alphen, L. V. A. N. (1983) Molecular Architecture and Functioning of the Outer Membrane of *Escherichia coli* and other Gram-negative Bacteria. *Elsevier Biomed. Press*. **737**, 51–115

55. Lüderitz, O., Freudenberg, M. A., Galanos, C., Lehmann, V., Rietschel, E. T., and Shaw, D. . (1982) Lipopolysaccharides of Gram-negative Bacteria. *Curr. Top. Membr. Transp.*

56. Rietschel, E. T., Wollenweber, H. W., Zähringer, U., and Lüderitz, O. (1982) Lipid A, the lipid component of bacterial lipopolysaccharides: Relation of chemical structure to biological activity. *Klin. Wochenschr*. **60**, 705–709

57. Galanos, C., Lehmann, V., Lüderitz, O., Rietschel, E. T., Westphal, O., Brade, H., Brade, L., Freudenberg, M. A., Hansen-Hagge, T., Lüderitz, T., McKenzie, G., Schade, U., Strittmatter, W., Tanamoto, K. -I, Zähringer, U., Imoto, M., Yoshimura, H., Yamamoto, M., Shimamoto, T., Kusumoto, S., and Shiba, T. (1984) Endotoxic properties of chemically synthesized lipid A part structures: Comparison of synthetic lipid A precursor and synthetic analogues with biosynthetic lipid A precursor and free lipid A. *Eur. J. Biochem.* **140**, 221–227

58. Risse, H. J., Dröge, W., Ruschmann, E., Lüderitz, O., Westphal, O., and Schlosshardt, J. (1967) Eine neue Gruppe von *Salmonella* R-Mutanten: Serologische und biochemische Analyse des Heptosekerns von Lipopolysacchariden aus *Salmonella minnesota*- und *Salmonella ruiru*-Mutanten. *Eur. J. Biochem.* **1**, 216–232

59. Galanos, C., Freudenberg, M. A., Jay, F., Nerkar, D., Veleva, K., Brade, H., and Strittmatter, W. (1984) Immunogenic properties of lipid A. *Rev. Infect. Dis.* **6**, 546–552

60. Bellstedt, D. U., Van der Merwe, K. J., and Galanos, C. (1988) Immune carrier properties of acid-treated *Salmonella minnesota* R595 bacteria The immune response to TNP-bacterial conjugates in rabbits and mice. *J. Immunol. Methods*. **108**, 245–254

61. Greeff, T. (1987) *'n Vergelykende Studie van die Immunogenisiteite van Verskillende Naakte Bakterië-konjugate*. M. Sc. thesis, Stellenbosch University

62. Strachan, A. F., Shepherd, A. G., Bellstedt, D. U., Coetzee, G. A., Van der Westhuyzen, D. R., and De Beer, F. C. (1989) Human serum amyloid A protein: Behaviour in aqueous and urea-

containing solutions and antibody production. *Biochem. J.*

63. Bellstedt, D. U., Louw, R. P., Farringer, B. A., Weder, N., Van der Merwe, K. J., and Swart, P. (1993) Antibody production to adrenal cytochrome P450-dependent enzymes using acid-treated bacteria as immune carriers. *Biochem. Soc. Trans.* **21**, 3394

64. Blignaut, A., Burger, W. P., Morley, A. J., and Bellstedt, D. U. (2000) Antibody responses to La Sota strain vaccines of Newcastle disease virus in ostriches (*Struthio camelus*) as detected by ELISA. *Avian Dis.*

65. Haurum, J. S. (2006) Recombinant polyclonal antibodies: the next generation of antibody therapeutics? *Drug Discov. Today*. **11**, 655–660

66. Beghein, E., and Gettemans, J. (2017) Nanobody technology: A versatile toolkit for microscopic imaging, protein-protein interaction analysis, and protein function exploration. *Front. Immunol.* **8**, 1–14

67. Twyman, R. M. (2005) Immunoassays, Applications. *Encycl. Anal. Sci. Second Ed.*

68. Gubala, V., Klein, R., Templeton, D. M., and Schwenk, M. (2014) Immunodiagnostics and immunosensor design. *Pure Appl. Chem.* **86**, 1539–1571

69. Matsuda, R., Rodriguez, E., Suresh, D., and Hage, D. S. (2015) Chromatographic immunoassays: Strategies and recent developments in the analysis of drugs and biological agents. *Bioanalysis*. **7**, 2947–2966

70. Oliver, J. (2013) Antibody Applications. *Mater. Methods*

71. Glatman-Freedman, A., and Casadevall, A. (1998) Serum therapy for tuberculosis revisited: Reappraisal of the role of antibody-mediated immunity against *Mycobacterium tuberculosis*. *Clin. Microbiol. Rev.* **11**, 514–532

72. Casadevall, A. (1996) Antibody-Based Therapies for Emerging Infectious Diseases. *Emerg. Infect. Dis.* **2**, 200–208

73. Mariathasan, S., and Tan, M. W. (2017) Antibody–Antibiotic Conjugates: A Novel Therapeutic Platform against Bacterial Infections. *Trends Mol. Med.* **23**, 135–149

74. Lehar, S. M., Pillow, T., Xu, M., Staben, L., Kajihara, K. K., Vandlen, R., DePalatis, L., Raab, H., Hazenbos, W. L., Hiroshi Morisaki, J., Kim, J., Park, S., Darwish, M., Lee, B. C., Hernandez, H., Loyet, K. M., Lupardus, P., Fong, R., Yan, D., Chalouni, C., Luis, E., Khalfin, Y., Plise, E., Cheong, J., Lyssikatos, J. P., Strandh, M., Koefoed, K., Andersen, P. S., Flygare, J. A., Wah Tan, M., Brown, E. J., and Mariathasan, S. (2015) Novel antibody-antibiotic conjugate eliminates intracellular *S. aureus*. *Nature*. **527**, 323–328

75. Venkat Kumar, G., Jeyanthi, V., and Ramakrishnan, S. (2020) A short review on antibody therapy for COVID-19. *New Microbes New Infect.* **35**, 100682

76. Abraham, J. (2020) Passive antibody therapy in COVID-19. *Nat. Rev. Immunol.* **20**, 401–403

77. Shanmugaraj, B., Siriwattananon, K., Wangkanont, K., and Phoolcharoen, W. (2020) Perspectives on monoclonal antibody therapy as potential therapeutic intervention for Coronavirus disease-19 (COVID-19). *Asian Pacific J. Allergy Immunol.* **38**, 10–18

78. Duan, K., Liu, B., Li, C., Zhang, H., Yu, T., Qu, J., Zhou, M., Chen, L., Meng, S., Hu, Y., Peng, C., Yuan, M., Huang, J., Wang, Z., Yu, J., Gao, X., Wang, D., Yu, X., Li, L., Zhang, J., Wu, X., Li, B., Xu, Y., Chen, W., Peng, Y., Hu, Y., Lin, L., Liu, X., Huang, S., Zhou, Z., Zhang, L., Wang, Y., Zhang, Z., Deng, K., Xia, Z., Gong, Q., Zhang, W., Zheng, X., Liu, Y., Yang, H., Zhou, D., Yu, D., Hou, J., Shi, Z., Chen, S., Chen, Z., Zhang, X., and Yang, X. (2020) Effectiveness of convalescent plasma therapy in severe COVID-19 patients. *Proc. Natl. Acad. Sci. U. S. A.* **117**, 9490–9496

79. Aydin, S. (2015) A short history, principles, and types of ELISA, and our laboratory experience with peptide/protein analyses using ELISA. *Peptides*. **72**, 4–15

80. Sakamoto, S., Putalun, W., Vimolmangkang, S., Phoolcharoen, W., Shoyama, Y., Tanaka, H., and Morimoto, S. (2018) Enzyme-linked immunosorbent assay for the quantitative/qualitative analysis of plant secondary metabolites. *J. Nat. Med.* **72**, 32–42

81. Lequin, R. M. (2005) Enzyme immunoassay (EIA)/enzyme-linked immunosorbent assay (ELISA). *Clin. Chem.* **51**, 2415–2418

82. Engvall, E., Jonsson, K., and Perlmann, P. (1971) Antiserum , immunoglobulin fraction , and specific antibodies Iodinated proteins. *Biochim. Biophys. Acta.* **251**, 427–434

83. KPL Inc. (2005) Technical Guide for ELISA: Protocols and Troubleshooting

84. Camponi, L., and Migliorini, P. (eds.) (1999) *Antibody usage in the lab*, Springer-Verlag

85. Jensen, E. C. (2012) The Basics of Western Blotting. *Anat. Rec.* **295**, 369–371

86. Renart, J., Reiser, J., and Stark, G. R. (1979) Transfer of proteins from gels to diazobenzyloxymethyl-paper and detection with antisera: A method for studying antibody specificity and antigen structure. *Proc. Natl. Acad. Sci. U. S. A.* **76**, 3116–3120

87. Towbin, H., Staehelin, T., and Gordon, J. (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. *Proc. Natl. Acad. Sci. U. S. A.* **76**, 4350–4354

88. Burnette, N. W. (1981) Western Blotting: Electrophoretic Transfer of Proteins from Sodium Dodecyl Sulfate-Polyacrylamide Gels to Unmodified Nitrocellulose and Radiographic Detection with Antibody and Radioiodinated Protein A. *Anal. Biochem.* 10.1063/1.4897167

89. Mahmood, T., and Yang, P.-C. (2012) Western Blot: Technique, Theory and Trouble Shooting. *N. Am. J. Med. Sci.* **4**, 429–434

90. Stott, D. I. (2000) Immunoblotting, dot-blotting, and ELISPOT assays: Methods and applications. *J. Immunoassay*. **21**, 273–296

91. Song, W. J., and Jackowski, S. (1994) Kinetics and regulation of pantothenate kinase from *Escherichia coli*. *J. Biol. Chem.* **269**, 27051–27058

92. Rock, C. O., Karim, M. A., Zhang, Y. M., and Jackowski, S. (2002) The murine pantothenate kinase (Pank1) gene encodes two differentially regulated pantothenate kinase isozymes. *Gene*. **291**, 35–43

93. Zhyvoloup, A., Nemazanyy, I., Babich, A., Panasyuk, G., Pobigailo, N., Vudmaska, M., Naidenov, V., Kukharenko, O., Palchevskii, S., Savinska, L., Ovcharenko, G., Verdier, F., Valovka, T., Fenton, T., Rebholz, H., Wang, M. L., Shepherd, P., Matsuka, G., Filonenko, V., and Gout, I. T. (2002) Molecular cloning of CoA synthase. The missing link in CoA biosynthesis. *J. Biol. Chem.* **277**, 22107–22110

94. Zhang, Y. M., Rock, C. O., and Jackowski, S. (2005) Feedback regulation of murine pantothenate kinase 3 by coenzyme A and coenzyme A thioesters. *J. Biol. Chem.* **280**, 32594–32601

95. Zhang, Y. M., Rock, C. O., and Jackowski, S. (2006) Biochemical properties of human pantothenate kinase 2 isoforms and mutations linked to pantothenate kinase-associated neurodegeneration. *J. Biol. Chem.* **281**, 107–114

96. Nemazanyy, I., Panasyuk, G., Breus, O., Zhyvoloup, A., Filonenko, V., and Gout, I. T. (2006) Identification of a novel CoA synthase isoform, which is primarily expressed in the brain. *Biochem. Biophys. Res. Commun.* **341**, 995–1000

97. Bosveld, F., Rana, A., Van Der Wouden, P. E., Lemstra, W., Ritsema, M., Kampinga, H. H., and Sibon, O. C. M. (2008) De novo CoA biosynthesis is required to maintain DNA integrity during development of the *Drosophila* nervous system. *Hum. Mol. Genet.* **17**, 2058–2069

98. Sharma, L. K., Subramanian, C., Yun, M. K., Frank, M. W., White, S. W., Rock, C. O., Lee, R. E., and Jackowski, S. (2018) A therapeutic approach to pantothenate kinase associated neurodegeneration. *Nat. Commun.* 10.1038/s41467-018-06703-2

Chapter 2

The production and characterisation of polyclonal antibodies for the CoA biosynthesis enzymes of *S. aureus*

2.1 Introduction

As discussed in the previous chapter, the increased burden of antimicrobial resistance in human pathogens has necessitated the need for novel antimicrobial drug targets (1); a target of particular interest for this study being the CoA biosynthesis pathway of *S. aureus*. Although many studies have been performed on the CoA biosynthesis pathway of *S. aureus*, not much is known about the levels of these enzymes under different conditions. Previous approaches have included the use of the PaxDb4 protein abundance database (2, 3) to determine the relative abundance of these enzymes, however, these ratios do not provide information on the actual concentrations of these enzymes. One approach to determine the concentrations of these enzymes would be the use of immunological techniques for the quantification of these enzymes, however, to our knowledge commercial antibodies to these enzymes are currently unavailable. In order to overcome this obstacle, we set out to produce polyclonal antibodies against the enzymes of the CoA biosynthesis pathway of *S. aureus* (PanK, CoaBC, PPAT and DPCK) utilizing acid-treated, naked *S. minnesota* R595 as immune carrier for these antigens. This method established by Bellstedt *et al.* (4) has successfully been used in the past for the production of polyclonal antibodies against a wide variety of antigens (5-7) including antibody production against ostrich immunoglobulins for application in the agricultural sector (8).

In this project we therefore aim to develop and optimise enzyme-linked immunosorbent assays that would be able to detect and quantify CoA biosynthesis enzyme levels under physiological conditions in *S. aureus*. The development and optimisation of such assays will be described in greater detail in Chapter 3 of this thesis. However, the production of the tools needed for the development of an ELISA will be the main objective of this chapter.

2.1.1 The importance of antibody characterisation and quality control

The reproducibility and trustworthiness of data generated with antibodies, both commercial and in-house preparations, have been brought into question (9) in view of the fact that antibodies produced against the same antigen in different animals of the same species may result in variation between antibody batches. This is mainly caused by the variation in the immune response of each animal to a particular antigen. Many factors play a role in the immune response of an animal to a particular antigen,

especially in the production of polyclonal antibodies. These factors include antigen preparation, the age of the animal, the health status of the animal as well as environment and exposure of the animal to other antigens (10, 11). The environment and exposure of the animal to other antigens is particularly important as polyclonal antibody sera contain antibodies produced by different clones of B-cells. This leads to antibodies that are specific for different epitopes of the same antigen and possible non-specific antibodies to antigens present in the environment of the host animal. These factors make it important to fully characterise antibodies that are produced in-house to establish their capabilities and limitations, which will ultimately influence their application in different laboratory techniques.

2.1.2 The enzyme-linked immunosorbent assay (ELISA)

The main technique that was utilised in this study for the characterisation of the produced polyclonal antibodies is the ELISA. Although this assay will critically be discussed in Chapter 3, it is important to already mention the specific format of the ELISA that will be used in this study as this assay was already utilised in the work reported in this chapter for the characterisation of the antibodies produced. As mentioned previously in Chapter 1, different formats of the ELISA exist which are utilised for different purposes. In this chapter, the specific focus will be on the direct versus the indirect format of this assay (Fig. 2.1).

The direct ELISA can be regarded as the simplest format of the ELISA (12). In this format, antigen is adsorbed to the wells of a microtitre plate and incubated with a primary antibody that is linked to an enzyme which results in a colour reaction when substrate is added to the wells. The indirect ELISA on the other hand, is based on the same principle, but instead of incubating with a primary antibody that is directly linked to the enzyme that is responsible for the colour reaction or response, a secondary antibody which has the ability to detect the primary antibody is linked to the colour response. The substrate solution would then react with the enzyme on the secondary antibody, resulting in a colour reaction. The indirect ELISA format has the advantage that by the addition of the secondary antibody conjugate, the sensitivity of the ELISA may be increased by amplifying the signal of the label bound to the detection partner (13).

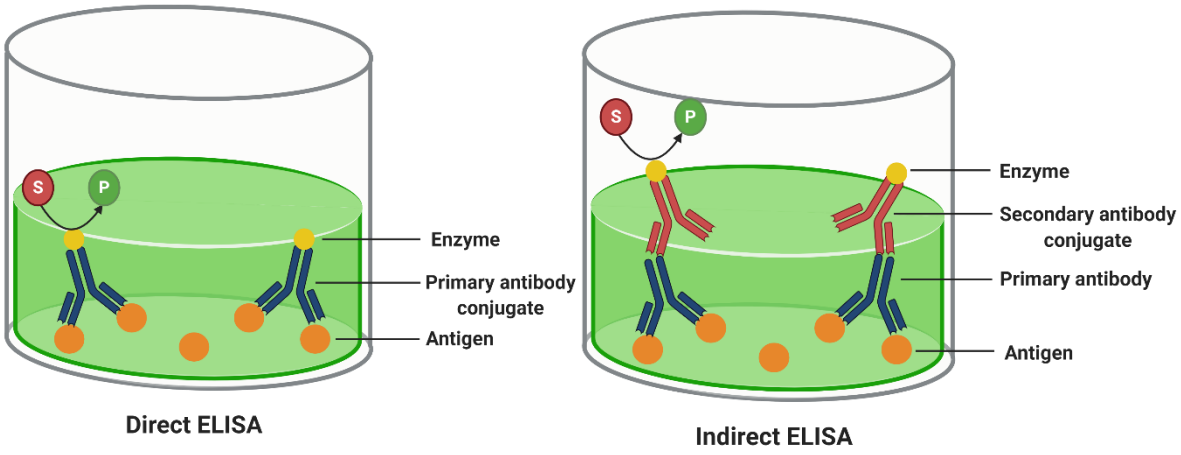

Figure 2.1: Schematic overview of the direct versus the indirect ELISA format. The difference between the direct and indirect ELISA lies in the addition of an extra secondary antibody that is conjugated to the enzyme that is responsible for a colour reaction. This additional step of the indirect ELISA is very useful to increase the signal and sensitivity of the assay. This image was created with BioRender.com.

2.1.3 Strategy used for antibody production and characterisation

In order to develop an ELISA method for the purposes of quantifying *Sa*PanK, *Sa*CoaBC, *Sa*PPAT and *Sa*DPCK levels under physiological conditions, key elements needed for the assay had to be prepared. The strategy used entailed the isolation of the *S. aureus* CoA biosynthesis enzymes by using a recombinant *E. coli* bacterial expression system, followed by the polyclonal antibody production against these enzymes in rabbits utilizing acid-treated, naked bacteria as immune carriers and stimulators. This was followed by the characterisation of these antibodies by ELISA and western blot analyses as well as the determination of potential cross-reactivity of these antibodies. The characterisation of these antibodies was a crucial part of this study for the development and optimisation of the ELISAs in the chapters to follow.

2.2 Results and discussion

2.2.1 Expression and purification of *S. aureus* CoA biosynthesis enzymes

An important part of this study was the production and purification of the proteins needed for antibody production and assay development. In order to produce antibodies to a particular antigen, it is very important that the antigen is carefully prepared to ensure that antibodies are produced against the antigen of interest and not impurities that might be present in the sample (10). The proteins of interest were overexpressed in *E. coli* BL21(DE3)-star cells using either the pET28a or pProEX expression vector as well as the introduction of an N-terminal His_6-tag. This was followed by purification of the proteins of

interest with Ni^{2+}-based IMAC using an ÄKTA prime purification system. To confirm the identity and purity of the recombinantly produced *Sa*PAnK, *Sa*CoaBC, *Sa*PPAT and *Sa*DPCK enzymes, SDS-PAGE analysis of these proteins was performed on a 10% polyacrylamide gel after each purification. The gels for each purification will not be included in this thesis as multiple cell pellets for each enzyme were purified, instead a representative gel (Fig. 2.2) with each enzyme is included to illustrate the size and purity of some samples used in antibody production and assay development.

For each sample a clear band representing each of the four proteins was observed (Fig. 2.2 B). The first sample in lane 1 contains *Sa*PanK, which is estimated to be 29 kDa in solution (14, 15). From the SDS-PAGE we observed *Sa*PanK at ~28kDa which corresponds well with sizes reported in literature. Similarly, in lanes 3 and 4, bands corresponding with bacterial PPAT (17.8 kDa) (16-18) and DPCK (22.6 kDa) (19) can be seen just below ~20 kDa (PPAT) and just above ~25 kDa (DPCK), indicative of *Sa*PPAT and *Sa*DPCK. The band representing *Sa*DPCK in lane 4 was corroborated with similar results found in a study done by Bouwer (20). All three of these proteins (*Sa*PanK, *Sa*PPAT and *Sa*DPCK) also appeared to contain no major impurities.

In lane 2, which contains the *Sa*CoaBC sample, a prominent band can be seen at ~50 kDa. We believe that this prominent band confirms the presence of *Sa*CoaBC as similar results were found in a study by van der Westhuyzen (21) where a band indicating *Sa*CoaBC was also found at ~50 kDa. However other bands are also visible in the sample: two bands at ~20 and ~30 kDa which represent minor impurities. In addition, just below ~100 kDa and ~150 kDa there are two other bands. In its native form, bacterial *Sa*CoaBC has been found to be a homododecamer consisting of 43.8 kDa subunits (15). This could explain the presence of the multiple bands in lane 2 which correspond with a monomer (~50 kDa), dimer (~100 kDa) and a trimer (~150 kDa) of the enzyme. Although these bands need to be confirmed it was decided to use *Sa*CoaBC samples for immunisation studies as multiple purifications of *Sa*CoaBC delivered similar results.

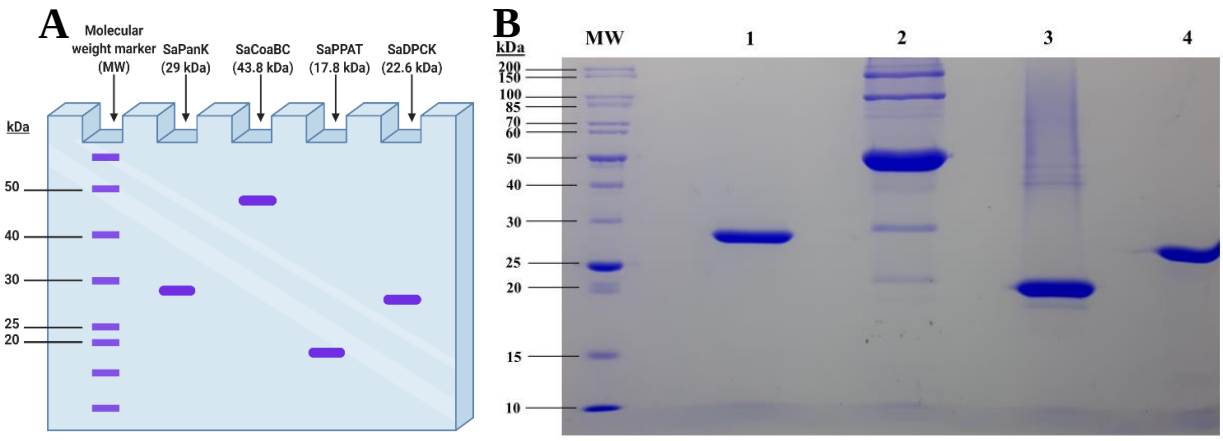

Figure 2.2: SDS-PAGE analysis of purified *S. aureus* CoA biosynthesis enzymes. Panel A: A visual representation of the estimated size and position of the enzymes on an SDS-PAGE gel. Previously established sizes of the enzymes from literature are indicated for each enzyme (14-19). This image was created with BioRender.com. **Panel B:** SDS-PAGE analysis of the purified enzymes was performed on a 10% SDS-PAGE gel and samples loaded in the following order: (MW) Unstained Protein Ladder Broad Range (New England Biosciences), (lane 1) *Sa*PanK, (lane 2) *Sa*CoaBC, (lane 3) *Sa*PPAT and (lane 4) *Sa*DPCK.

Taken together, each of the CoA biosynthesis enzymes of *S. aureus* were produced and purified to a satisfactory degree for antibody production and assay development, with the possible exception of CoaBC. Even though a denaturing and reducing gel was performed using SDS and mercapto-ethanol, it is possible that multiple oligomers of the enzyme could be present in the purified CoaBC sample (15). Western blot analysis with the *Sa*CoaBC antiserum will have to be performed to validate this observation and ensure that the enzyme sample is not contaminated with native *E. coli* proteins.

2.2.2 The immune response of rabbits to immunisation with *S. aureus* CoA biosynthesis enzymes adsorbed to acid-treated, naked bacteria

Polyclonal antibodies were produced by immunising rabbits with the respective recombinantly expressed enzymes adsorbed to acid-treated, naked *S. minnesota* R595, whereafter antibody containing sera were isolated from blood collected after immunisation (Fig. 2.3). The antibodies produced against each of the enzymes were analysed with an indirect ELISA to determine whether the rabbits responded to immunisation and to determine the titre values of the sera. Before final immune response curves could be established, the indirect ELISA first needed to be optimised by determining an optimal enzyme coating concentration for each of the four enzymes. Coating concentration of the ELISA plays an important role in the successful binding of antigen to the solid phase (ELISA plate) and can also influence binding of the antibodies to the antigen. When coating concentrations are too low, the signal to noise ratio could be too low. In contrast, when coating concentrations are too high, it could result in

stacking of the antigen which ultimately affects the stability of the binding of the reactants to follow (12).

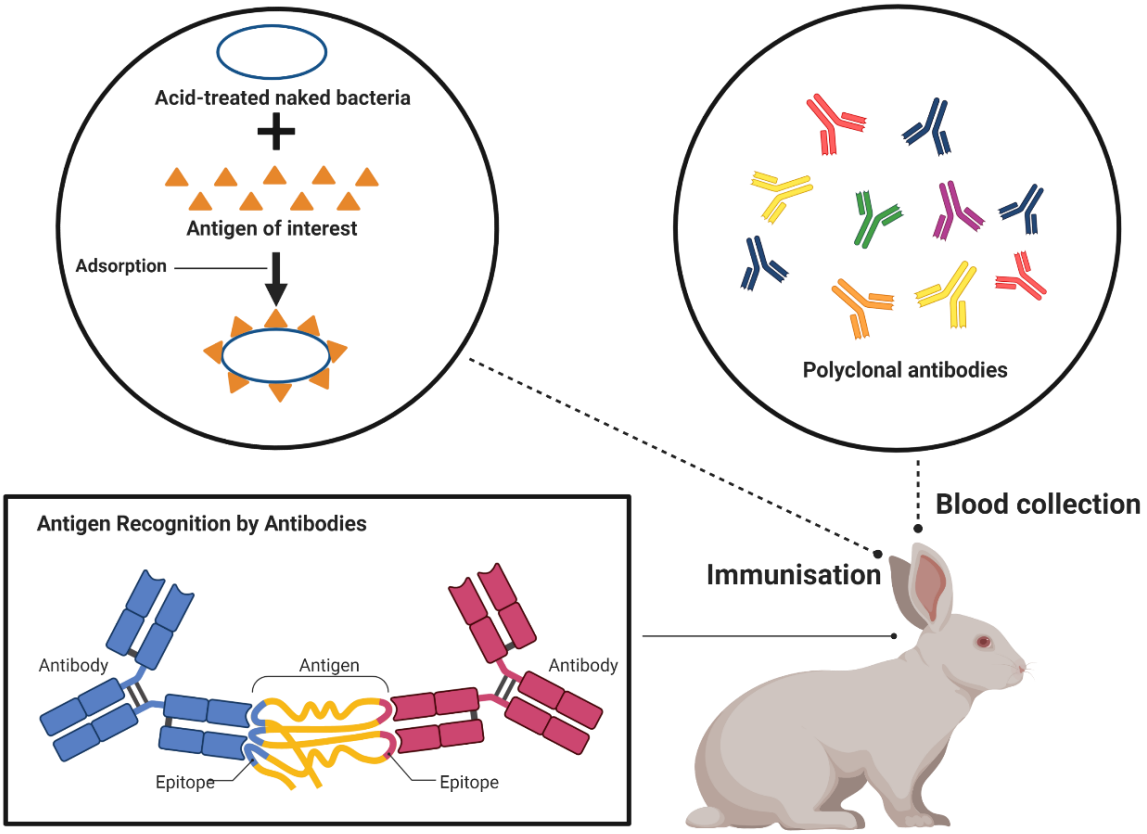

Figure 2.3: Schematic overview of the polyclonal antibody production process. In this figure, the main steps of the polyclonal antibody production process are portrayed, starting with the adsorption of the antigen of interest to acid-treated, naked bacteria, followed by the immunisation of rabbits according to a fixed immunisation schedule and ending with blood collection and antibody serum isolation. This image was created with BioRender.com.

Indirect ELISAs for each of the four enzymes were performed with different coating concentrations of the enzymes (1, 2, 5 or 10 µg/mL) and it was established that a coating concentration of 2 µg/mL was optimal for the titre determination of all of the antisera. A coating concentration of either 1 µg/mL or 10 µg/mL was too low and too high respectively, whereas coating the plate with either 2 µg/mL or 5 µg/mL appeared to be sufficient (highest absorbance value of at least 1). However, it was decided that a lower concentration of 2 µg/mL would be better as it would have a lower chance of background binding in the ELISAs. Following optimisation, antisera were analysed as blood was collected after each immunisation, titres were determined by indirect ELISA and a final immune response curve was drawn using the titres of antisera drawn on days 0, 21, 42 and 81. It was important to include a pre-immunisation bleed (day 0) to determine whether any antibodies to any of the enzymes were present in the blood of the rabbits before immunisation although this was highly unlikely.

The final immune response curves for each of the rabbits are shown in Figure 2.4. The titre values for the rabbit anti-*Sa*PanK (orange), anti-*Sa*CoaBC (green), anti-*Sa*PPAT (blue) and anti-*Sa*DPCK (red) antibodies were expressed as the reciprocal dilution of the antibody serum at an absorbance value of 0.1 and plotted against the respective days the antiserum was drawn. The immune response curves show that the rabbits responded favourably to immunisation with the respective enzymes adsorbed to the naked bacteria, as antibody titre values increase after each immunisation and plateau when maximal antibody production is reached towards day 81.

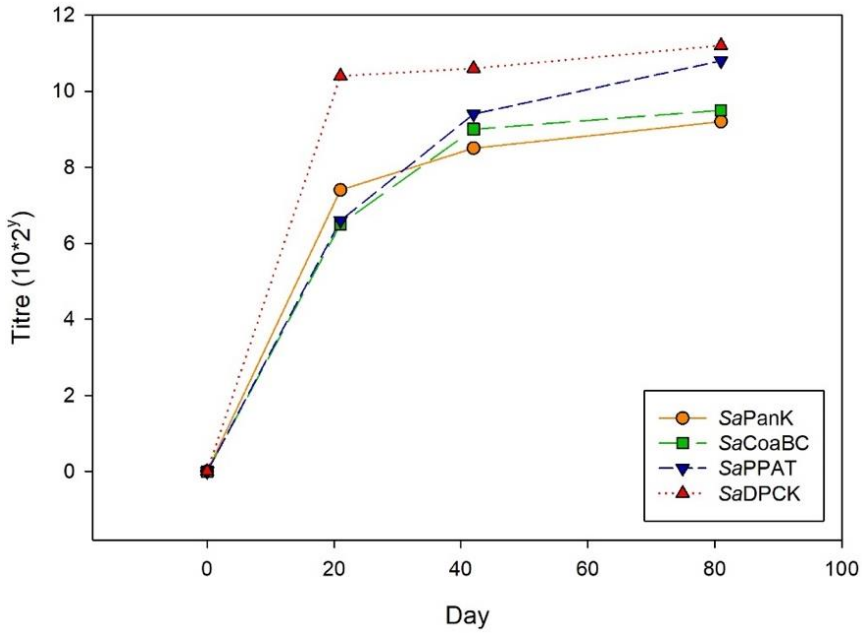

Figure 2.4: The immune response of rabbits immunised with the CoA biosynthesis enzymes of *S. aureus*. Absorbance values were obtained for antisera drawn on days 0, 21, 42 and 81 and titre values determined for serum of each day by applying an absorbance cut-off value of 0.1. Different coloured graphs represent immunisation of a rabbit with a specific antigen as indicated by the graph legend.

Typically, immunisation is continued until 6 weeks (42 days), whereafter antibody titres are examined to determine whether additional immunisation is needed. As can be seen in Figure 2.4, high antibody titre values were already observed at day 42 after the start of immunisation in all of the rabbits. Although the antibody titres obtained by day 42 were sufficient, it was decided to administer an additional boost consisting of three additional injections after a resting period of 3 weeks. A resting period after initial immunisation is important to allow for the selection of B-lymphocytes to produce higher affinity antibodies (22). Therefore, not only did the additional round of immunisation have the potential to increase the number of circulating antibodies, but to also generate antibodies with a higher affinity for the antigen of interest. It was observed that antibody titre values for the different antisera reached a plateau towards day 81.

Although it can be observed that each of the immune curves follow a similar trend, it is important to note that the immune response of each rabbit to their respective antigens is unique. It is tempting to interpret that the rabbit immunised with *Sa*DPCK showed a greater immune response, more rapidly in comparison to the others, but this may not be the case, as the presentation of the protein when it is coated to the ELISA plate, may also have an effect on the amount of antibody that binds to it. However, it does appear as though the antibody titres for the anti-*Sa*DPCK antibodies were higher and that the antibody levels obtained by day 21 of immunisation, were higher than any of the others.

From the immune response curves, it is clear that antisera with high titre values, corresponding to high antibody levels, were produced against each of the enzymes and that the use of naked bacteria as an immune carrier was successful in eliciting an immune response to each of the enzymes.

2.2.3 Cross-reactivity determination of antibodies produced to *S. aureus* CoA biosynthesis enzymes

Antibodies with high titre values were successfully produced against all four CoA biosynthesis enzymes, but before they could be used for assay development, further characterisation was needed. It is important to determine the capabilities and limitations of antibodies and also determine whether these antibodies would be suitable for the application that they are intended for. One factor that could severely affect the use of antibodies and their application in protein quantification, is the potential of these antibodies to cross-react with other antigens. It was therefore crucial to determine if each of these antibodies had any cross-reactivity especially against the other three enzymes of the CoA biosynthesis pathway of *S. aureus* respectively. In order to establish any potential cross-reactivity of the antibodies, western blot and ELISA analysis were performed.

2.2.3.1 Western blot analysis of antisera

Similar to ELISA, western blotting relies on antibodies for the detection of a specific protein, which is very useful when trying to determine the presence of a protein of interest and can also be used for the quantification of proteins. In this study western blot analysis was performed with the antisera produced against each of the enzymes. There were two goals with this experiment: firstly, to establish how the antibodies would perform in western blotting and secondly, and most importantly in the context of this study, to determine whether they might cross-react with any of the other enzymes of the CoA biosynthesis pathway of *S. aureus*.

Similar to what was previously described, enzyme samples were subjected to SDS-PAGE and after electrophoresis transferred to a nitrocellulose membrane. The membrane was incubated with each of the different day 81 antisera at a dilution of 1:5000. The analyses showed (Fig. 2.5 A) that the anti-*Sa*PanK antibodies were able to detect *Sa*PanK, represented by the thick band at ~30 kDa in lane 1.

Furthermore, anti-*Sa*PanK antibodies did not cross-react with any of the other enzymes of the pathway which were loaded in lane 2 (*Sa*CoaBC), 3 (SaPPAT) and 4 (SaDPCK). Similarly, in Figure 2.5 B, the anti-*Sa*CoaBC antibodies only detected *Sa*CoaBC at ~55kDa. Similar to what was found in the SDS-PAGE analysis to determine purity, multiple bands can again be seen in lane 2, confirming our hypothesis that the multiple bands seen for *Sa*CoaBC in Figure 2.2 B represent different oligomers of the enzyme. Prominent bands seen just below 55 kDa (monomer), at ~ 100 kDa (dimer) and ~150 kDa (trimer) correlate with the bands that were observed on the SDS-PAGE gel in Figure 2.2. In Figure 2.5 D the anti-*Sa*DPCK antibodies only detected *Sa*DPCK in lane 4, with a prominent band seen just above ~25 kDa.

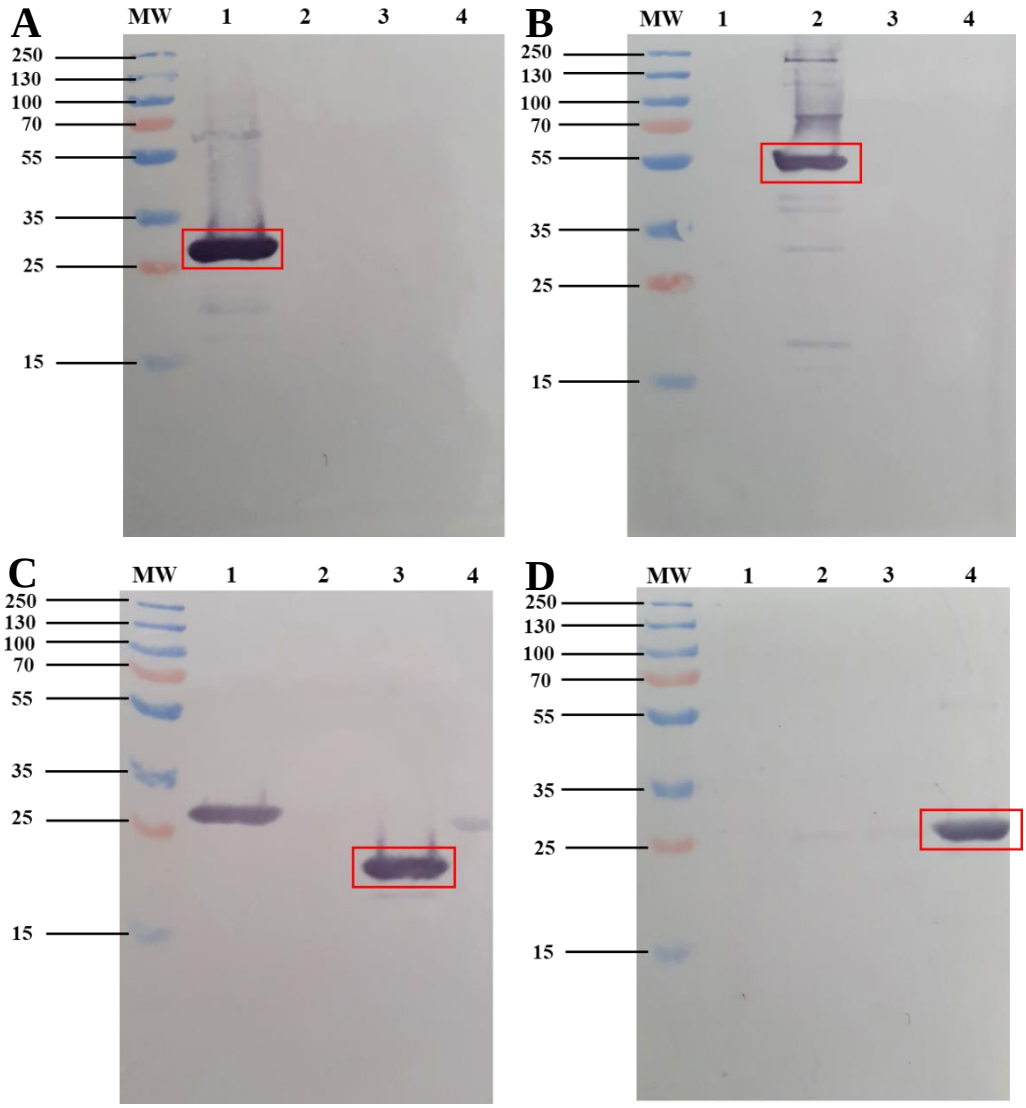

Figure 2.5: Western blot analysis of the antibody sera to the respective CoA biosynthesis enzymes. Western blot analysis was performed with the rabbit **A:** anti-*Sa*PanK, **B:** anti-*Sa*CoaBC, **C:** anti-*Sa*PPAT and **D:** anti-*Sa*DPCK antibodies at a dilution of 1:5000. Before transfer to a nitrocellulose membrane, enzyme samples were electrophoresed on a 10% gel in the following order: (MW) PageRuler Plus Prestained Protein Ladder (Thermo Fisher Scientific), *Sa*PanK (lane 1; ~30 kDa), *Sa*CoaBC (lane 2; ~55 kDa), *Sa*PPAT (lane 3; ~20 kDa) and *Sa*DPCK (lane 4; ~25 kDa).

In contrast to the other antisera, the anti-*Sa*PPAT antibodies represented in Figure 2.5 C, were able to detect *Sa*PPAT in lane 3 which is represented by a dark band at ~20 kDa, but also *Sa*PanK in lane 1, which is represented by a prominent band at ~30 kDa (similar as seen in Figure 2.5 A) and a faint band for *Sa*DPCK in lane 4 at ~25 kDa. However, these antibodies were not able to detect *Sa*CoaBC.

From the western blot data, it can be concluded that the anti-*Sa*PanK , anti-*Sa*CoaBC and anti-*Sa*DPCK antibodies had sufficient specificity to detect their respective antigens and were therefore suitable for use in western blotting systems. However, the anti-*Sa*PPAT antibodies were able to cross-react with *Sa*PanK and to a lesser extent *Sa*DPCK and were therefore not entirely specific. It must be added that this apparent cross-reactivity may have been the result of insufficient dilution, but it was decided to rather assess this cross-reactivity by ELISA. Western blot analysis using these antibodies therefore provided useful information, but it was deemed important to confirm these findings with ELISA analysis as it is a more sensitive assay.

2.2.3.2 ELISA analysis of antisera for cross-reactivity determination

With western blot analysis it was already established that the anti-*Sa*PPAT antibodies cross-react with both *Sa*PanK and to a lesser extent with *Sa*DPCK and that the other antisera do not show any cross-reactivity. To confirm these results, the cross-reactivity of the antibodies was further tested by indirect ELISA. This would give an indication of the degree of cross-reactivity of these antibodies in an ELISA. Experiments were performed with each of the different antisera to determine whether the antibodies were able to detect the other enzymes of the pathway and the extent of the cross-reactivity was assessed. The absorbance values obtained at 405 nm for each of the antigens (*S. aureus* CoA biosynthesis enzymes) and antibodies were plotted against the antibody dilution for all combinations and shown in Figure 2.6.

In Figure 2.6 A, it can be observed that the anti-*Sa*PanK antibodies were able to detect *Sa*PanK (orange) as expected, but also both *Sa*PPAT (blue) and *Sa*DPCK (red) at low antibody titre values. However, the signal decreases as antiserum dilution increases. This is in contrast to our findings in the western blot assay where no cross-reactivity was observed. This underscores the fact that ELISA is a more sensitive and appropriate technique to use for cross-reactivity experiments. Similar to the western blot results, ELISA analysis confirmed that these antibodies do not show any cross-reactivity with *Sa*CoaBC. Likewise, in Figure 2.6 D, ELISA results showed that the anti-*Sa*DPCK antibodies were also able to detect *Sa*PanK (orange) and *Sa*PPAT (blue) at low antibody titre values after which the signal decreased as dilutions were increased. It was also confirmed that the antibodies do not cross-react with *Sa*CoaBC (green).

ELISA assays supported western blot analysis for the specificity of the anti-*Sa*PPAT antibodies, as the ELISA results (Fig. 2.6 C) showed that these antibodies cross-react more strongly with both *Sa*PanK

(orange) and *Sa*DPCK (red) and that the absorbance values do not decrease to as large an extent as seen in Figure 2.6 A and D, as the antibody dilutions increase. As also seen previously, the antibodies do not cross-react with *Sa*CoaBC (green). Lastly, western blot results for anti-*Sa*CoaBC antibodies were confirmed, as Figure 2.6 B shows that the antibodies did not cross-react with any of the other three enzymes of the pathway.

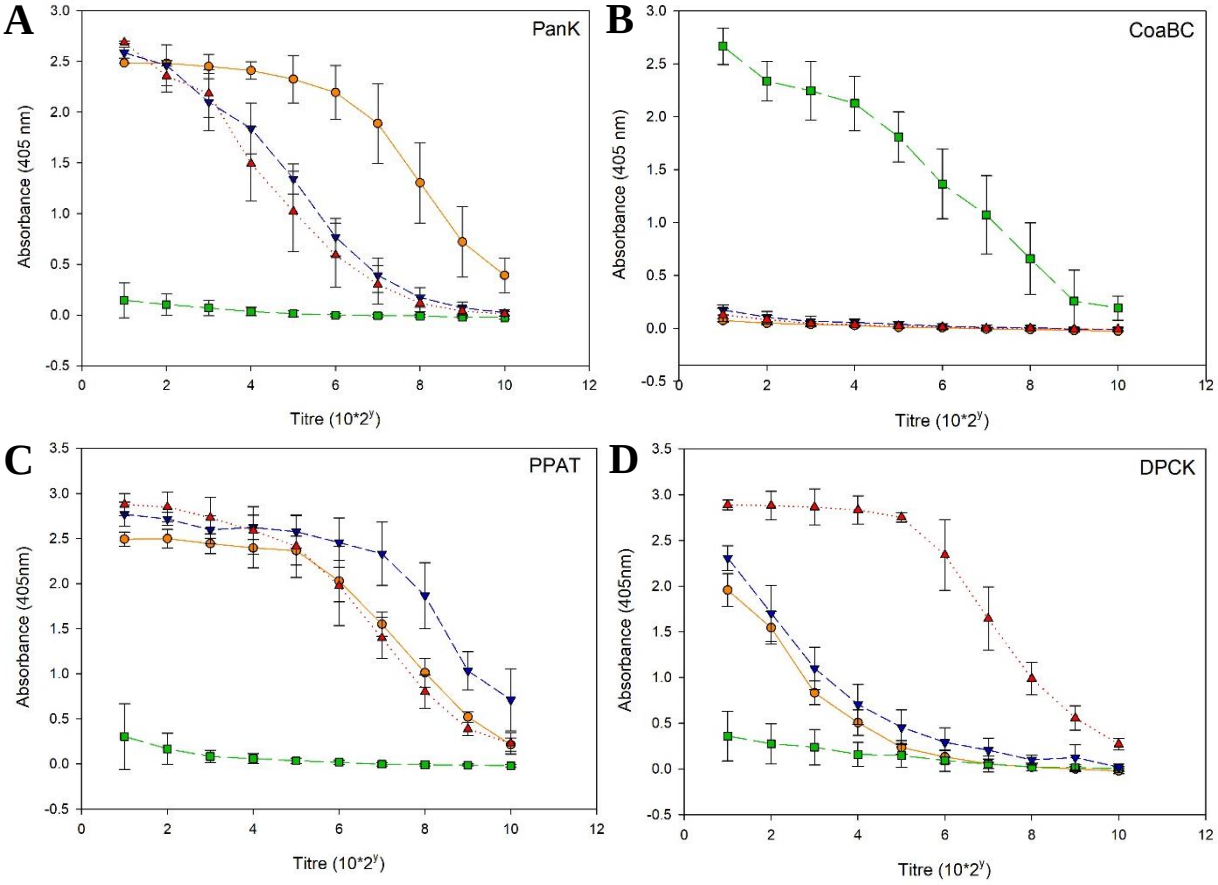

Figure 2.6: Cross-reactivity determination of antibodies produced against the CoA biosynthesis enzymes of *S. aureus* by ELISA analysis. Cross-reactivity of day 81 rabbit **A:** anti-*Sa*PanK, **B:** anti-*Sa*CoaBC, **C:** anti-*Sa*PPAT and **D:** anti-*Sa*DPCK antibodies were determined with an indirect ELISA. The graphs show the ability of the different antisera to detect the different enzymes of the CoA biosynthesis pathway of *S. aureus* as absorbance values at different antibody titre values. Different lines (*Sa*PanK – orange, *Sa*CoaBC – green, *Sa*PPAT – blue and *Sa*DPCK – red) represent the 4 respective enzymes that are detected by the respective antibody sera. Three independent experiments for each of the antibody sera were performed with different batches of enzyme. Error bars = standard deviation (Technical repeats were not performed as the assay format does not allow it).

Taken together, the ELISA results confirm that the anti-*Sa*CoaBC antibodies do not cross-react with any of the other enzymes and also that none of the other antisera were able to detect *Sa*CoaBC. The results also confirm that the anti-*Sa*PPAT antibodies cross-react with both *Sa*PanK and *Sa*DPCK. Interestingly, the results also showed that both the anti-*Sa*PanK and anti-*Sa*DPCK antibodies were able to detect the other enzymes, with the exception of *Sa*CoaBC. Although the western blot results did not indicate that these antibodies showed any cross-reactivity, the ELISA, which is a more sensitive assay,

was able to show this cross-reactivity. As the antibodies will predominantly be used in an ELISA system, observations made with the ELISA analysis need be taken into careful consideration when continuing with the development of ELISA assays for the quantification of *Sa*PanK, *Sa*CoaBC, *Sa*PPAT and *Sa*DPCK in culture conditions.

2.3 Conclusion

As described in this chapter, the tools needed for the development and optimisation of ELISA assays for the quantification of each of the enzymes of the CoA biosynthesis pathway of *S. aureus* were acquired. In an attempt to characterise the antibodies that will be used in further assay development, it was established with both western blot and ELISA analysis that the anti-*Sa*CoaBC antibodies were highly specific and did not cross-react with any of the other enzymes in the pathway. It was also observed that the anti-*Sa*PanK, anti-*Sa*PPAT and anti-*Sa*DPCK antibodies did possess certain levels of cross-reactivity with the other enzymes, with the exception of *Sa*CoaBC.

Even though ELISA results were able to provide us with insightful information on the potential cross-reactivity of the antibodies, this specific format of the ELISA used for cross-reactivity determination, does not allow for precise cross-reactivity quantification. The amount of cross-reactivity of an antibody to a specific antigen is another important factor that needs to be taken into account when trying to optimise and develop an ELISA assay, when trying to determine antigen concentrations. This aspect of the study will be further explored in Chapter 3.

Lastly, cross-reactivity studies were only performed to determine whether the different antisera were able to detect the other CoA biosynthesis enzymes of *S. aureus* specifically and did not take into account the potential cross-reactivity of these antibodies to the enzymes across different species. As describedin Chapter 1, many of these enzymes have similar sequences across certain species, such as the CoaBC, PPAT and DPCK of *E. coli* which have been fully characterised and used as the representative of these enzymes across most bacteria, including *S. aureus*. It would therefore be important going forward, to establish the potential cross-reactivity of our antibodies to the different enzymes in different species if they are to be applied to study the pathway in different microbes. Furthermore, the findings support the use of an ELISA assay for the detection of protein in culture over western blot due to it being a more sensitive technique and therefore warrants further optimisation of this technique for the purposes of the study.

2.4 Experimental procedures

2.4.1 Materials

All chemicals used in the subsequent experiments were either purchased from Sigma-Aldrich, Merck or Roche or are indicated otherwise. All expression vectors and expression strains used, were available in the lab from previous studies or previously purchased from Novagen. Purification of proteins was performed using an ÄKTA *prime* protein purification system from Amersham Biosciences with HiTrap™ Chelating HP (1 mL) and Desalting (5 mL) columns from GE Healthcare. Concentration determination of the purified proteins was performed with the Quick Start Bradford Protein Assay Kit (Bio-Rad), containing Bradford reagent and Bovine Serum Albumin standards and absorbance values were read at 595 nm with the Multiskan Sky Microplate Reader. SDS-PAGE analysis of the purified proteins was performed with a Hoefer, Inc. (USA) gel system and a Bio-Rad PowerPac HC™. 1 mL, 5 mL and 20 mL syringes for the immunisation and blood collection of rabbits were readily available in the lab along with 0.5 x 16 mm and 0.6 x 25 mm needles from Avacare™. Western blot analysis was performed with Amersham™ Protran™ 0.2 µm Nitrocellulose Blotting Membrane (GE Healthcare) and Whatman 3MM chromatography paper.

2.4.2 Protein synthesis for antibody production

2.4.2.1 Recombinant protein expression

For use in antibody production and subsequent ELISA and western blot analysis, each of the enzymes of the CoA biosynthesis pathway of *S. aureus* had to be overexpressed and purified. This was accomplished by transforming competent *E. coli* BL21(DE3)-star cells with either the pET28a(+) or pProEX plasmid vectors containing gene inserts that encoded for *Sa*PanK, *Sa*CoaBC, *Sa*PPAT and *Sa*DPCK, respectively (21,24). These plasmid vectors also encoded an *N*-terminal His_6-tag which enabled the purification of these proteins with Ni^{2+}-based IMAC. Large scale expression of these proteins was performed in Luria-Bertani (LB) media supplemented with either kanamycin (30 mg/mL) or ampicillin (100 mg/mL) according to previously established expression conditions as shown in Table 2.1 below (14, 15, 23). Cultures were grown in 500 mL LB media in 2L Erlenmeyer flasks at 37°C to an OD_{600} of 0.6 after which the cells were induced with either 0.1 mM or 0.5 mM isopropylthio-β-galactoside (IPTG) and incubated for 3 hours (*Sa*PPAT) and up to 18 hours (*Sa*PanK, *Sa*CoaBC and *Sa*DPCK) for the over-expression of these proteins. After the incubation period, bacterial cells were harvested by centrifugation in an Avanti J-26 XPI centrifuge (Beckman Coulter) for 20 minutes (48 400 x g at 4°C) and the cell pellet stored at -20°C for future purification.

Table 2.1: Conditions for the large-scale expression of *S. aureus* CoA biosynthesis proteins

Protein	Plasmid	Antibiotic	[IPTG]	Temperature	Time
***Sa*PanK**	pET28a(+)	Kanamycin	0.5 mM	37°C	overnight
***Sa*CoaBC**	pProEX	Ampicillin	0.1 mM	25°C	overnight
***Sa*PPAT**	pET28a(+)	Kanamycin	0.5 mM	30°C	3 hours
***Sa*DPCK**	pET28a(+)	Kanamycin	0.1 mM	37°C	overnight

2.4.2.2 IMAC protein purification

Proteins were purified with Ni^{+2}-based IMAC using an ÄKTA prime purification system (Amersham Biosciences) according to purification conditions as summarised in Table 2.2 (21, 24). Firstly, cell pellets were resuspended in binding buffer (Table 2.2) and sonicated to lyse the harvested cells. Centrifugation was performed with the Avanti J-26 XPI centrifuge (Beckman Coulter) at 75 600 x g for 20 min to rid of cell debris and the supernatant loaded to a previously prepared 1 mL HiTrap™ Chelating HP column (GE Healthcare). The weakly bound proteins were removed from the column by washing the column with binding buffer followed by the stepwise elution of the His-tagged protein with elution buffer containing increasing amounts of imidazole. Fractions containing the greatest amount of protein was pooled and loaded to a 5 mL HiTrap™ Desalting column (GE Healthcare). The protein (*Sa*PanK, *Sa*CoaBC and *Sa*DPCK respectively) was buffer exchanged with desalting buffer and stored in glycerol (5% v/v) at -80°C. For *Sa*PPAT, however, the buffer exchange step was performed in a VIVASPIN 20 concentrator (Sartorius Stedim Biotec) and 5% glycerol added thereafter before storage at -80°C. Concentration of the protein samples was determined using the Bradford method.

Table 2.2: Conditions for IMAC purification of *S. aureus* CoA biosynthesis enzymes with the AKTÄ prime purification system

Buffer	*Sa*PanK	*Sa*CoaBC	*Sa*PPAT	*Sa*DPCK
Binding buffer (pH 7.9)	20 mM Tris-HCl 500 mM NaCl 5 mM imidazole 0.05% NaN_3	20 mM Tris-HCl 500 mM NaCl 5 mM imidazole 0.05% NaN_3	20 mM Tris-HCl 500 mM NaCl 5 mM imidazole 0.05% NaN_3	20 mM Tris-HCl 500 mM NaCl 5 mM imidazole 0.05% NaN_3
Elution buffer (pH 7.9)	20 mM Tris-HCl 500 mM NaCl 500 mM imidazole (final) 0.05% NaN_3	20 mM Tris-HCl 500 mM NaCl 500 mM imidazole (final) 0.05% NaN_3	20 mM Tris-HCl 500 mM NaCl 500 mM imidazole (final) 0.05% NaN_3	20 mM Tris-HCl 500 mM NaCl 500 mM imidazole (final) 0.05% NaN_3
Strip buffer (pH 7.9)	20 mM Tris-HCl 100 mM EDTA 500 mM NaCl	20 mM Tris-HCl 100 mM EDTA 500 mM NaCl	20 mM Tris-HCl 100 mM EDTA 500 mM NaCl	20 mM Tris-HCl 100 mM EDTA 500 mM NaCl
Desalting buffer (pH 8.0)	25 mM Tris-HCl 5 mM $MgCl_2$	25 mM Tris-HCl 150 mM NaCl 5 mM $MgCl_2$	25 mM Tris-HCl 5 mM $MgCl_2$	25 mM Tris-HCl 5 mM $MgCl_2$

2.4.2.3 Protein concentration determination

Concentrations of the purified proteins were established with the Quick Start Bradford Protein Assay Kit (Bio-Rad) (25), using different concentrations of bovine serum albumin (BSA) to produce a standard curve from which protein concentrations could be established. Bradford reagent (250 µL) was added to both the BSA standards as well as dilutions of the protein samples (5 µL) and incubated for up to 30 min at room temperature. Absorbance values were measured at 595 nm with the Multiskan Sky Microplate Reader.

2.4.2.4 Protein identification and purity estimation

Reducing SDS-PAGE analysis of the purified proteins was performed with a Hoefer, Inc. (USA) gel system in order to establish whether protein expression and purification was successful. Before electrophoresis, samples were treated with SDS-PAGE loading buffer (0.1 M Tris (pH 6.8), 4% (w/v) SDS, 30% (v/v) glycerol, 20% (v/v) β-mercaptoethanol, 0.04% (w/v) Bromophenol blue) and incubated at 95°C for 5 min. The samples were loaded on to a 10% gel by loading 5 µg protein per well and electrophoresed at 100 V for 90 min. After electrophoresis, the gel was stained in Fairbanks A solution (25% (v/v) isopropanol, 10% (v/v) acetic acid, 0.05% Coomassie Brilliant Blue) by microwaving the gel in solution and agitating at room temperature for 30 min. Staining of the gel was completed by subsequent addition of Fairbanks B solution (10% (v/v) isopropanol, 10% (v/v) acetic acid, 0.005%

Coomassie Brilliant Blue) and Fairbanks C solution (10% (v/v) acetic acid, 0.002% Coomassie Brilliant Blue), respectively. The gel was microwaved in each solution followed by agitation at room temperature for 10 min. Lastly, the gel was destained in Fairbanks D solution (10% (v/v) acetic acid) overnight, while agitating at room temperature.

2.4.3 Polyclonal antibody production

2.4.3.1 Antigen adsorption to acid-treated naked bacteria

Before the production of the polyclonal antibodies could commence, purified *Sa*PanK, *Sa*CoaBC, *Sa*PPAT and *Sa*DPCK was adsorbed to separate batches of acid-treated, naked *S. minnesota* R595. The NB were already available in the laboratory and were previously prepared according to Bellstedt *et al.* (11) and made up to a concentration of 2 mg/mL with sterilised MilliQ water. The respective enzymes were added to 0.5 mL of the NB solution in a 1 to 5 mass ratio of 1 part antigen to 5 parts naked bacteria (200 µg enzyme to 1000 µg NB) and dried by rotary evaporation in a Savant SpeedVac™. The prepared antigen was resuspended in 2.5 mL phosphate-buffered saline (PBS) (pH 7.2) for immunisation to a final concentration of 40 µg enzyme to 200 µg NB per 0.5 ml of PBS.

2.4.3.2 Rabbit immunisation and blood collection

Prior to the start of the immunization of the rabbits for antibody production, ethical clearance was applied for and granted by the Animal Ethics Committee of the University of Stellenbosch (Principal Investigator Name: Bellstedt, Dirk U.; Protocol #: SU-ACUD16-00121; Title: Vaccination of rabbits for antibody production).

Polyclonal antibodies were prepared by immunisation of different female Flemish Giant rabbits, 1 rabbit per antigen, with the respective enzyme-NB complexes according to a fixed immunisation schedule as seen in Table 2.3 below. The rabbits were injected intravenously in the marginal ear vein with 0.5 mL of the antigen solution (i.e., 40 µg enzyme adsorbed to 200 µg NB in 0.5 ml PBS) on immunisation days and blood drawn from the central auricular ear artery every week, to determine the immune response of the rabbits to immunisation. Before immunisation and bleeding, EMLA (AstraZeneca) local anaesthetic cream was applied to the area of injection to minimize any pain and discomfort that may be caused during the immunisation and blood sampling processes. Small volumes of blood, approximately 2 mL, were drawn for determining the immune response of the rabbits to immunisation and large volumes of blood, approximately 20 mL, were drawn on days 42 and 81 of the immunisation process for antibody isolation and future use. As can be seen in the Table 2.3 below, an additional booster, consisting of 3 extra injections, was administered after day 42 to maximise antibody production.

Table 2.3: Immunisation schedule of rabbits with antigen adsorbed to acid-treated, naked bacteria.

Antigen	Days		Immunisation	Blood collection
	Immunisation	Blood collection	Dosage	volume[a]
***Sa*PanK**	0,4,7,14,18,21,28,32, 35,42,67,70,74	0,7,14,21,28,35,42,81	± 240 µg	2 mL, 20 mL
***Sa*CoaBC**	0,4,7,14,18,21,28,32, 35,42,67,70,74	0,7,14,21,28,35,42,81	± 240 µg	2 mL, 20 mL
***Sa*PPAT**	0,4,7,14,18,21,28,32, 35,42,67,70,74	0,7,14,21,28,35,42,81	± 240 µg	2 mL, 20 mL
***Sa*DPCK**	0,4,7,14,18,21,28,32, 35,42,67,70,74	0,7,14,21,28,35,42,81	± 240 µg	2 mL, 20 mL

[a]20 mL blood was collected on days 42 and 81 for future antiserum use and 2 mL was collected on other days to establish the immune response of the rabbits to the immunised antigen.

2.4.3.3 Antiserum preparation

Blood was drawn from the rabbits using a syringe and needle after application of local anaesthetic at the site of inserting of the needle into the central artery flowing into the ear. Following blood collection, the antibody containing serum was isolated from the blood by firstly allowing it to clot by incubation at 37°C for an hour. Thereafter, the test tubes with blood were incubated at 4°C overnight to allow for the contraction of the clot containing the red blood cells. The blood was then centrifuged at 800 x g for 20 min in a Hettich Universal Centrifuge and the separated antiserum was aliquoted into suitable small aliquots of 0.5 ml and stored at -20°C for future use.

2.4.4 ELISA analysis of antibody production

To determine the immune response of the rabbits to the adsorbed *Sa*PanK, *Sa*CoaBC, *Sa*PPAT and *Sa*DPCK, an indirect ELISA was performed for each of the different antisera.

2.4.4.1 ELISA coating concentration determination

Before an indirect ELISA could be used to determine the antibody titres of the rabbits after immunisation, an optimal antigen coating concentration for each antigen-antibody pair had to be established. Firstly, two separate rows on Nunc® Maxisorp 96 well microtiter plates (Thermo Scientific) respectively were coated with either 1 µg/mL, 2 µg/mL, 5 µg/mL, or 10 µg/mL of the different purified enzymes and incubated at 4ºC overnight. Column 12 on the ELISA plate did not receive coating solution to serve as a negative control. After incubation, the coating solution was decanted and the plate incubated with casein buffer (154 mM NaCl, 10 mM Tris-HCl, 0.5% (m/v)

casein, 0.02% (m/v) thiomersal, pH 7.6) (200 µL/well) at 37°C for 1 hour to prevent non-specific binding. This step was followed by the addition of a pre-diluted primary antiserum (either anti-*Sa*PanK, anti-*Sa*CoaBC, anti-*Sa*PPAT or anti-*Sa*DPCK) in one row and their respective Day 0 control bleed antisera in the second row (1:20 dilution) to wells 2, 3 and 12 (100 µL/well) and a serial dilution made in the consecutive wells with casein-Tween buffer (154 mM NaCl, 10 mM Tris-HCl, 0.5% (m/v) casein, 0.02% (m/v) thiomersal, 0.1% (v/v) Tween-20, pH 7.6). The plate was incubated at 37°C for 2 hours. Thereafter the plates were incubated with goat anti-rabbit horseradish peroxidase conjugate secondary antibody at 37º for 1 hour. A 1:2000 dilution of the secondary antibody was made with casein-Tween buffer and 100 µL of the solution added to each well. Lastly, 100 µL substrate solution (0.05% (m/v) ABTS, 0.015% (v/v) H_2O_2, 0.1 M citrate buffer, pH 5) was added to each well and absorbance values measured at 405 nm with a Labsystems Multiskan MS microtitre plate reader after 5 minutes, 15 minutes and 30 minutes. After each incubation step, the solution was decanted, and the plates washed manually 5 times with PBS-Tween wash buffer (0.1% Tween 20 (pH 7.2)). The respective day 0 and day 28 primary antibody sera were used for the determination of optimal coating concentration for future ELISAs.

2.4.4.2 Antibody titre determination

Subsequent ELISA analysis, as described in 2.4.4.1, were performed for each of the different antibody sera (day 0, 21, 42 and 81) at the established optimal antigen coating concentrations. The absorbance values obtained from these analyses were used to establish immune response curves for each of the immunised rabbits.

2.4.5 Western blot analysis of the produced antibodies

In order to characterize the polyclonal antisera produced against the CoA biosynthesis enzymes of *S. aureus*; western blots were performed. Firstly, SDS-PAGE (as described in 2.4.2.4) of the different enzymes was performed whereafter proteins were transferred to a nitrocellulose membrane in a western blotting apparatus at a constant 120 mA for 18 hours. After the transfer of the proteins to the membrane, the membrane was rinsed with MilliQ water and subsequently blocked with casein buffer (154 mM NaCl, 10 mM Tris-HCl, 0.5% (m/v) casein, 0.02% (m/v) thimerosal, pH 7.6) at 37ºC for 1 hour. Thereafter the membrane was incubated in primary antibody solution at 37ºC for 1 hour. Primary antibodies from day 81 of each of the different sera were diluted 1:5000 with casein-Tween buffer (154 mM NaCl, 10 mM Tris-HCl, 0.5% (m/v) casein, 0.02% (m/v) thimerosal, 0.1% (v/v) Tween-20, pH 7.6) for this purpose. This was followed by incubation with a goat anti-rabbit horseradish peroxidase conjugate (Sigma) secondary antibody diluted 1:2000 in casein-Tween at 37ºC for 1 hour. Each incubation step was performed whilst agitating on a Benchmark Blot Boy and after each incubation the solution was decanted, and the membrane washed 3 times for 15 min in PBS-Tween wash buffer (0.1%

Tween (pH 7.2)) whilst agitating at room temperature. Lastly, the membrane was subsequently developed in substrate solution (0.05% 4-chloro-1-naphtol, 16% MeOH, in PBS, 30% H_2O_2) at room temperature for 30 min whilst agitating, and the reaction stopped by agitating the membrane in MilliQ water for 5 min. Western blot analysis, as previously described was also utilisedutilised to determine the extent of the cross-reactivity of these antibodies by incubating a membrane on which each of the respective enzymes were blotted onto after SDS-PAGE separation, with each of the respective antisera at 1:5000 as described above.

2.4.6 Cross-reactivity determination by ELISA

Final characterisation of the antibodies included the determination of antibody cross-reactivity with the other antigens that were not complementary to each of the respective antisera. This was accomplished by performing an indirect ELISA similar to the one previously described in 2.4.4.1, but instead the 96 well plates were coated with the different enzymes as illustrated in Figure 2.4 below and incubated with the different day 81 antisera to determine whether the antibodies have the ability to also detect the other enzymes

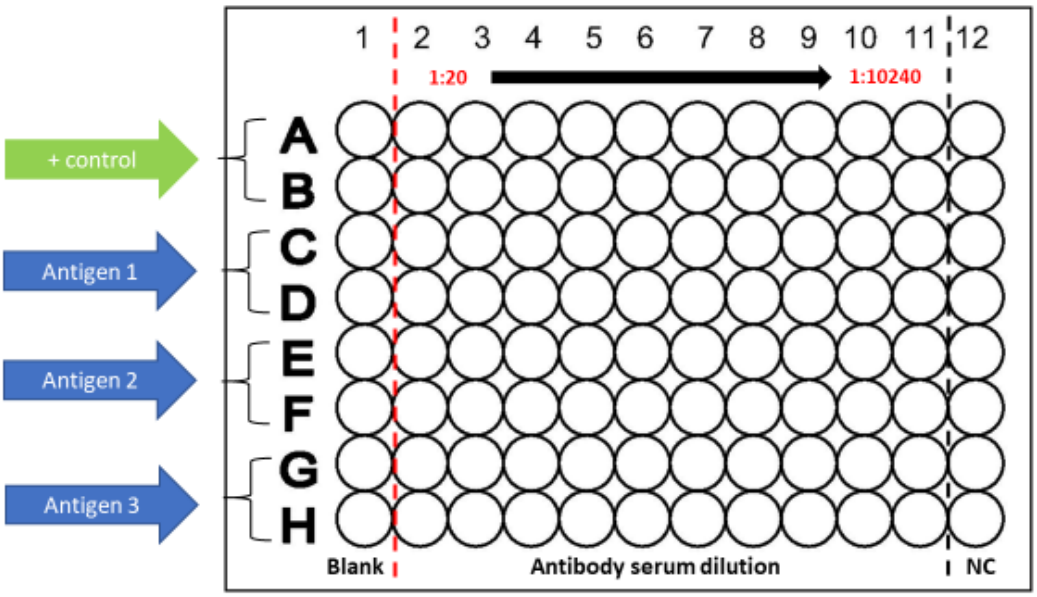

Figure 2.7: Format of the indirect ELISA used to determine potential cross-reactivity of the different antisera. Similar to the indirect ELISA format as seen in Figure 2.3, the same steps were utilisedutilised with the only difference that plates were coated firstly with the antigen (positive control) complementary to the antisera that the plate was incubated with, followed by coating subsequent rows with the remaining 3 antigens (antigen 1-3), respectively.

2.5 References

1. Prestinaci, F., Pezzotti, P., and Pantosti, A. (2015) Antimicrobial resistance: A global multifaceted phenomenon. *Pathog. Glob. Health*. **109**, 309–318

2. Wang, M., Herrmann, C. J., Simonovic, M., Szklarczyk, D., and von Mering, C. (2015) Version 4.0 of PaxDb: Protein abundance data, integrated across model organisms, tissues, and cell-lines. *Proteomics*. **15**, 3163–3168

3. Goosen, R. (2016) *A comparative analysis of CoA biosynthesis in selected organisms: a metabolite study*. Ph.D. thesis, Stellenbosch University

4. Bellstedt, D. U., Human, P. A., Rowland, G. F., and Van der Merwe, K. J. (1987) Acid-treated, naked bacteria as immune carriers for protein antigens. *J. Immunol. Methods*. **98**, 249–255

5. Bellstedt, D. U., Louw, R. P., Farringer, B. A., Weder, N., Van der Merwe, K. J., and Swart, P. (1993) Antibody production to adrenal cytochrome P450-dependent enzymes using acid-treated bacteria as immune carriers. *Biochem. Soc. Trans.* **21**, 3394

6. Bothma, K., Bellstedt, D. U., and Botes, A. (2018) *The production of antibodies to glutathione S-transferase for detecting recombinant protein expression*. Hons. thesis, Stellenbosch University

7. Le Roux, A., Bothma, K, , Bellstedt, D. U., and De Villiers, M.. (2019) *The expression, purification and antibody production of human pantetheinase*. Hons. thesis, Stellenbosch University

8. Blignaut, A., Burger, W. P., Morley, A. J., and Bellstedt, D. U. (2000) Antibody responses to La Sota strain vaccines of Newcastle disease virus in ostriches (*Struthio camelus*) as detected by ELISA. *Avian Dis.* **44**, 390–398

9. Weller, M. G. (2016) Quality Issues of research Antibodies. *Anal. Chem. Insights.* **11**, 21-27

10. Leenaars, M., and Hendriksen, C. F. M. (2005) Critical Steps in the Production of Polyclonal and Monoclonal Antibodies: Evaluation and Recommendations. *Ilar J.* **46**, 269–279

11. Hanly, W. C., Artwohl, J. E., and Bennett, B. T. (1995) Review of Polyclonal Antibody Production Procedures in Mammals and Poultry. *ILAR J.* **37**, 93–118

12. Crowther, J. R. (2009) The ELISA Guidebook. in *Methods in Molecular Biology*, 2nd Ed.

13. KPLInc. (2005) *Technical Guide for ELISA*

14. Choudhry, A. E., Mandichak, T. L., Broskey, J. P., Egolf, R. W., Kinsland, C., Begley, T. P., Seefeld, M. A., Ku, T. W., Brown, J. R., Zalacain, M., and Ratnam, K. (2003) Inhibitors of

pantothenate kinase: Novel antibiotics for staphylococcal infections. *Antimicrob. Agents Chemother.* **47**, 2051–2055

15. Leonardi, R., Zhang, Y. M., Rock, C. O., and Jackowski, S. (2005) Coenzyme A: Back in action. *Prog. Lipid Res.* **44**, 125–153

16. Spry, C., Kirk, K., and Saliba, K. J. (2008) Coenzyme A biosynthesis: An antimicrobial drug target. *FEMS Microbiol. Rev.* **32**, 56–106

17. Leonardi, R., and Jackowski, S. (2007) Biosynthesis of pantothenic acid and Coenzyme A. *EcoSal Plus.* **2**, 1–28

18. Geerlof, A., Lewendon, A., and Shaw, W. V. (1999) Purification and characterisation of phosphopantetheine adenylyltransferase from *Escherichia coli*. *J. Biol. Chem.* **274**, 27105–27111

19. Strauss, E. (2010) Coenzyme A Biosynthesis and Enzymology. in *Comprehensive Natural Products II: Chemistry and Biology*, pp. 251–410

20. Bouwer, W. (2018) *Investigation and Characterisation of Dephospho-Coenzyme A kinase: A Potential Drug Target*. MSc. thesis, Stellenbosch University

21. Van Der Westhuyzen, R. (2010) *Synthesis and evaluation of inhibitors targeting Coenzyme A biosynthesis and metabolism in Staphylococcus aureus*. Ph.D. thesis, Stellenbosch University

22. Stills, H. F. (2012) Polyclonal Antibody Production. in *The Laboratory Rabbit, Guinea Pig, Hamster, and Other Rodents*, pp. 259–274, 10.1016/B978-0-12-380920-9.00011-0

23. Van Der Westhuyzen, R., Hammons, J. C., Meier, J. L., Dahesh, S., Moolman, W. J. A., Pelly, S. C., Nizet, V., Burkart, M. D., and Strauss, E. (2012) The antibiotic CJ-15,801 is an antimetabolite that hijacks and then inhibits CoA biosynthesis. *Chem. Biol.* **19**, 559–571

24. Barnard, L. (2015) *Pantothenamides as Antibacterials: Mode of Action Studies and Improvement of their Potency by Structural Modification*. Ph.D. thesis, Stellenbosch University.

25. Bradford, M. M. (1976) A Rapid and Sensitive Method for the Quantification of Microgram Quantities of Protein Utilizing the Principle of Protein-Dye Binding. *Anal. Biochem.* **72**, 248–254

Chapter 3

The development and optimisation of ELISAs for the quantification of *S. aureus* CoA biosynthesis enzymes

3.1 Introduction

In the previous chapters the importance of the CoA biosynthesis pathway and its role in antimicrobial drug targeting was discussed, especially in pathogenic microorganisms. The main objective of this study was therefore to establish a method for the quantification of the enzymes that form part of the CoA biosynthesis pathway of *S. aureus*. Specifically, the development and optimisation of an ELISA that utilises the antigens and polyclonal rabbit antibodies that were produced as described in Chapter 2.

The ELISA that stems from the radioimmunoassay (1–3), relies on the adsorption of either antigen or antibody to a solid surface that participates in highly specific binding (4, 5). This allows for the detection and quantification of specific molecules while washing away all excess reagents, ensuring that only the antigen of interest is detected. Traditionally, the ELISA has been seen as the golden standard for targeted protein detection and quantification (6). This is due to the sensitive, specific, rapid and inexpensive nature of this method which also allows for the detection of a wide variety antigens in very low concentrations (7).

Before the ELISA development and optimisation for the quantification of the CoA biosynthesis enzymes of *S. aureus* is described, a brief overview on the factors potentially influencing this technique will be given. This will include the types of ELISA to be used, binding capabilities of the ELISA plates, prevention of non-specific binding, antigen detection, antibody detection, detection substrates and the removal of excess reagents. This will then be followed by the experimental work performed during the study and findings obtained.

3.2 ELISA development and optimisation strategies

The process of developing and optimising an ELISA is not easily achieved as many factors can influence the assay. These factors include the purpose of the ELISA as well as modifications to the different steps in a typical ELISA, are shown in Figure 3.1. With these in mind, the strategies necessary for the development and optimisation of a successful ELISA will be discussed.

3.2.1 ELISA systems

The first important factor to take into account when developing an ELISA is the type of ELISA system that would be most suitable for the quantification of the antigen of interest. Different ELISA systems are used for different types and sizes of antigens (5) and therefore the choice of ELISA system relies greatly on the properties of the antigen of interest. In the previous chapter, the direct and indirect format of the ELISA has already been described. Therefore, the focus of this section will be on the different format strategies for the quantification of the antigen of interest.

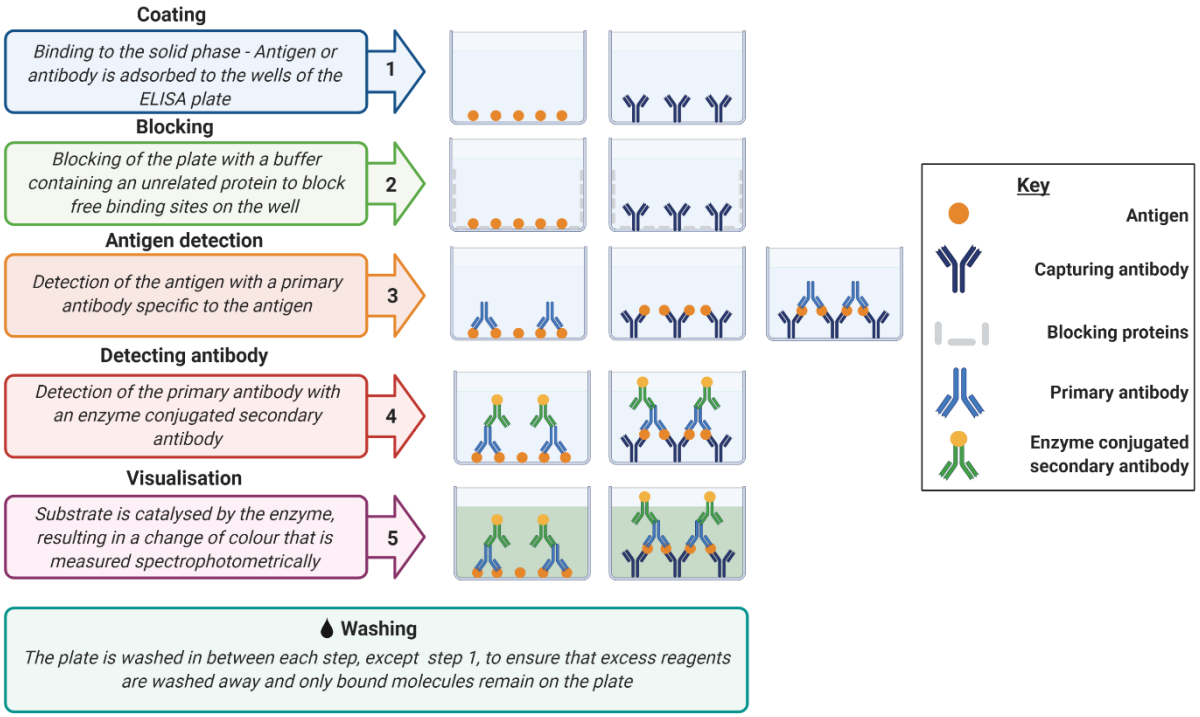

Figure 3.1: Summary of the basic steps of an ELISA. The basic steps of a typical ELISA are briefly explained and illustrated in this figure in its different configurations. This image was created with BioRender.com.

3.2.1.1 Sandwich ELISA

The sandwich ELISA is one of the standard formats for the quantification of proteins in a biological sample. Sandwich ELISAs are typically used for the quantification of macromolecules that contain multiple epitopes (8). This system therefore requires the use of matched antibody pairs, where each antibody is specific for a different, non-overlapping epitope on the antigen of interest and allows binding of the antibodies without steric hindrance (8, 9).

The first step of this ELISA system involves the binding of a capturing antibody to the ELISA plate. Antigen is then added to the plate, which binds to the capturing antibody, followed by the addition of a second antibody, creating an antigen “sandwich” (9). For this reason, it is often also referred to as a double antibody sandwich (DAS) ELISA. The protein concentration can be quantified by setting up a

calibration curve (standard curve) with a dilution series of a standard protein with known concentration. The concentration of the protein in test samples can be calculated by using the absorption value and extrapolation from the standard curve.

Factors that influence the sensitivity of the sandwich assay include the affinity of the antibody for the antigen, the ability of two antibodies to sandwich the antigen, potential experimental errors (such as pipetting errors) as well as non-specific binding of detection antibody. This assay format is favoured due to its high specificity, pure samples are not required for measurement and the assay can be used in either direct or indirect format (5).

3.2.1.2 Competition ELISA

Compared to the sandwich ELISA, the competition ELISA is used for the quantification of proteins in instances where small molecules need to be detected (5, 10). The principle of this ELISA system is based on the competition between the antigen to be tested and a reference antigen for binding to a limited number of antibody binding sites (5, 10). This assay can be performed with either antigen or antibody bound to the solid phase (5). Similar to the sandwich ELISA, the competition ELISA also relies on the generation of a standard curve for the quantification of protein in unknown samples, which is determined with each set of unknown samples to be analysed (5).

Factors that influence the sensitivity of the competition ELISA are the affinity of the antibody for the antigen as well as potential experimental errors. Additionally, it depends on having slightly fewer antibody binding sites than the antigen sites which results in increased sensitivity as it is limited by the affinity of the interaction between antibody and antigen. When saturating amounts of antibody are present, the addition of small amounts of the competitor antigen will not result in a detectable change in the activity of the assay as a result of which a specific and limited amount of antibody must be used (5).

3.2.2 Binding to the solid phase

The first crucial step of the ELISA involves the binding of either antigen or antibody to the solid phase. The most widely used solid phase is the 96 well microtitre ELISA plate that is made from polystyrene or polystyrene derivatives (5, 11). Polystyrene is composed of an aliphatic carbon chain that contains pendant benzene rings on every other carbon. This feature creates a plate with a very hydrophobic surface and plates made of polystyrene are typically referred to as either medium or low binding (5). In addition to these plates, the polystyrene surface can be altered to enhance binding by modifying the plate through irradiation. When irradiated, a certain number of the benzene rings are broken to form hydroxyl groups, carboxyl groups and even aldehydes, increasing the opportunity for hydrophilic interactions and chemical binding. These plates are typically referred to as high binding (5).

Binding of the antigen or antibody to the solid phase is achieved by passive adsorption of the molecules to the plastic surface of the plate or by direct binding. This process is typically referred to as coating (11). Most proteins adsorb to the plastic surface as a result of hydrophobic interactions between the plastic matrix and non-polar protein structures (11). The forces involved in binding includes van der Waals forces, hydrogen bonding and ionic interactions - forces that are relatively weak. In order to establish stable binding of the molecules to the plate, the molecules must make many of these weak bonds with the plate surface (5). Additionally, the aldehyde groups on the microtitre plate can react with the amino groups of proteins (including antibodies) and therefore bind chemically through the formation of Schiff bases.

Many factors influence the binding of the molecules to the plate, all of which are inherently related to each other. These factors include concentration of the substance that is adsorbed, temperature and time of adsorption (11). The polystyrene plate has the ability to bind a wide variety of proteins, but successful binding relies heavily on the concentration of the molecules in the coating solution. It is therefore important to establish an optimal coating concentration for each protein adsorbed to the plate (5, 11). A good guideline for determining optimal coating concentration, is in the range from 1–10 µg/mL, which is the typical concentration of protein needed to saturate available binding sites on the plate (11). Commonly used coating buffers such as 50 mM carbonate, pH 9.6; 10 mM PBS, pH 7.2 or 10 mM Tris-HCl, pH 8.5 are used for preparing coating solutions (5, 11). The alkaline pH of these buffers results in the deprotonation of many of the amino groups on the proteins as a result of which they can bind to the aldehyde groups on the ELISA plate surface through the formation of Schiff bases. Lastly, incubation time and temperature play an important role in successful adsorption as the amount of protein that is adsorbed relies largely on these two factors (5). In general, the rate of the hydrophobic interactions that are formed between proteins and the plate surface depend on temperature, and the higher the temperature, the greater the rate of binding (11, 12). However, incubating the plate at 4°C overnight is typically favoured as it has been found to result in the least well-to-well variation (5, 11).

3.2.3 Prevention of non-specific binding

After adsorption or binding of the antigen or antibody to the Elisa plate, the unoccupied sites in the wells must be blocked with a blocking reagent to eliminate potential non-specific binding of other molecules to the plate. Unlike the antigen-antibody interactions that are formed during the subsequent stages of the assay, binding during the adsorption phase is non-specific (11). When binding either antigen or antibody to the plate, some unoccupied binding sites remain open, thus potentially allowing for any substance to adsorb to the plate at later stages of the assay (5, 11). It is therefore very important that these empty sites must be blocked to prevent non-specific binding of reactants that could influence the sensitivity and specificity of the assay (5, 11, 13). Some of the most commonly used reagents used

for the blocking step of the ELISA are summarized in Table 3.1. These reagents can be divided into two major groups, namely protein blocking agents and detergents (5, 8, 11, 13).

Table 3.1: Commonly used reagents in the blocking step of the ELISA (5, 8, 11, 13)**.**

Protein blocking agents		
Blocking reagent	**Blocking conditions**	**Comments**
Bovine serum albumin (BSA)	Used at concentrations between 1% and 5% in PBS at pH 7	Most commonly used blocking agent as it is inexpensive and typically has low lot-to-lot variability.
Non-fat dry milk (NFDM)	Used at concentrations between 0.1% to 0.5%	Has been shown to exhibit lot-to-lot variability and a tendency to cause low signal. NDFM also contains varying concentrations of biotin which could interfere when utilizing the avidin/streptavidin – biotin system.
Casein/Caseinate	Used at concentrations between 1% to 5%	Casein is the main component of NFDM and might be a better choice as blocking reagent as it does not contain all the impurities found in NFDM. It is sometimes preferred over BSA due to its smaller size.
Normal serum	Used at dilutions between 5% and 10%	Contains a diversity of proteins, albumin being the most common. A major drawback of this blocking reagent, however, is the presence of immunoglobulin in the serum which could result in cross-reactivity.
Fish gelatin	Used at concentrations between 1% to 5%	Fish gelatin has been found to be a good blocking reagent when used in immunoblotting systems but does not perform as well in ELISA applications.

Detergents		
Blocking reagent	**Blocking conditions**	**Comments**
Tween-20	Used at concentrations between 0.05% to 0.5%	Non-ionic detergent that is the most popularly used detergent in this group of blocking reagents. It has the ability to both block non-specific binding to the solid phase and non-specific protein-protein interactions.
Tween-80		Similar to Tween-20, Tween-80 is a non-ionic detergent that has the ability to block both non-specific binding to the solid phase and non-specific protein interactions. Although its use is mentioned in some studies, not much information is available on the use of this detergent as a blocking reagent.
Triton X-100	Used at concentrations between 0.05% to 0.5%	Non-ionic detergent that prevents non-specific protein-protein interactions. Second to Tween-20, it is also a popularly used detergent for preventing non-specific binding.
Sodium dodecyl sulfate		Ionic detergent that is sometimes used in blocking reagents to remove non-specific ionic interactions between the solid phase, positively charged proteins and added reagents. Use of this reagent can influence antibody-antigen binding and should therefore be used at low concentrations.

Of the blocking agents mentioned in Table 3.1, the use of proteins is the most effective in preventing non-specific binding as they their effects seem to be permanent, and they only need to be used once (13). On the other hand, detergents do not block open binding sites in the wells of the ELISA plate, but rather reduce the non-specific interaction of subsequent reactants with the ELISA plate (13). Therefore, there is the possibility of the detergents interfering with antibody-antigen binding. In some cases, detergents are used in conjunction with blocking proteins to prevent non-specific binding (11).

3.2.4 Antigen detection

The core principle on which the ELISA is based, is the interaction between the antigen-antibody pair. As discussed in Chapter 1, antibodies are employed in biochemical techniques due to their ability to

bind with high specificity and affinity to an antigen (14). It is also these features that greatly influence the success of the ELISA.

Following the blocking step of the assay, either antigen or antibody is added to the plate, depending on the format of the ELISA. Reactions between antigen and antibody rely on the close proximity between the two molecules (11). The antigen-antibody bond is established by multiple non-covalent interactions dependent on various non-covalent forces, such as electrostatic, hydrophobic, hydrogen and Van der Waals forces (8). Specific binding between the antigenic determinant (epitope) and the specific antigen binding site on the antibody (paratope) involves only a small portion of these two molecules, comprising of just a few amino acids (16 - 21) on a small surface area (15). When the epitope and paratope comes within a proximity of several nanometres, they are attracted by long-range forces (electrostatic and hydrophobic bonds) which allows for the expulsion of water molecules, overcoming the hydration energies of the two molecules (15). This allows the epitope and paratope to move even closer at which point Van der Waals forces are responsible for binding, although some ionic groups may still play a role. At this point, the overall strength of binding of the epitope with the paratope relies on the goodness of fit between the two surfaces (15). Therefore, long-range forces are important in the rate of formation of the antigen-antibody complex at points of contact, whereas short-range forces contribute greatly to the bond strength by reducing the rate of antigen-antibody complex dissociation (8).

Binding of the antigen to the antibody is affected by many factors such as their respective concentrations, buffering conditions (especially pH), distribution on the ELISA plate as well as incubation time and temperature (11). Incubation buffers used for this step typically contain low concentrations of non-ionic detergents, such as Tween-20, which are able to disrupt non-specific hydrophobic interactions and prevent non-specific binding. Non-specific binding increases background noise, which greatly influences the sensitivity of the assay (7). Furthermore, incubation time can range from a few hours (1 – 2 hours) to a day and incubation typically occurs at either 37°C (for shorter time periods) or at 4°C (overnight). Similar to antigen binding on the solid phase, incubation for longer periods of time at a lower temperature generally results in lower well-to-well variation (7).

3.2.5 Antibody detection

In the case of an indirect ELISA format, whether it be a competition or a sandwich ELISA, a second antibody or antibody detection molecule is needed for the detection of the primary antibody. Secondary antibodies are antibodies that are produced in a different animal species and are able to bind to the primary antibody (i.e., goat anti-rabbit antibodies that are able to bind to antibodies produced in rabbits). These antibodies are covalently coupled directly to a detection molecule such as horseradish peroxidase (HRP) or alkaline phosphatase (ALP) to form antibody enzyme conjugates which are required for the final signal produced by the assay (5).

An alternative to the use of secondary antibodies is the use of the avidin-biotin system for antibody detection. Avidin, which is commonly found in egg whites, is a tetrameric biotin-binding protein that has a very high affinity for biotin (10^{15} M^{-1}). Biotin, on the other hand, is a water-soluble B-complex vitamin that can easily be conjugated to antibodies and antigen alike without any loss of activity. The basis of this system therefore relies on the high affinity of avidin, that is coupled to an enzyme, for biotin, which is coupled to the primary antibody (16). Alternatively, avidin with an even higher affinity for biotin can be obtained from *Streptococcus avidinii* bacteria, in which case it is referred to as Streptavidin. This is often used because of its higher affinity as well as ease and cost of production. Streptavidin-labelled horseradish peroxidase was used throughout in this study. With this system, antibody-biotin complexes are able to bind multiple avidin-enzyme complexes, which results in amplification of the final signal, ultimately creating a more sensitive assay (7).

3.2.6 Substrate

The last step of the ELISA involves the addition of a substrate solution that results in a coloured product by reacting with the enzyme that is coupled to either the secondary antibody or avidin (Fig. 3.2A). This colour change can then be measured spectrophotometrically and is directly proportional to the amount of antibody-enzyme conjugate or antibody-avidin conjugate and therefore antigen present in the specific well (7). Multiple chromogenic substrates/hydrogen donors exist for specific enzymes, such as HRP and ALP. Popular substrates used in ELISA analysis can be seen in Table 3.2. These chromogenic substrates/hydrogen donors and their colour reactions are displayed in Figure 3.2.

Table 3.2: Summary of typically used ELISA substrates/chromogens (5, 11)**.**

Substrate/Chromogen	Enzyme	Detection limit	Colour product	Absorbance
pNPP[a]	ALP	10^{-13} moles of ALP	Yellow	405 – 410 nm
BCIP/NBT[b]		10^{-13} moles of ALP	Blue	595 – 650 nm
H_2O_2/TMB[c]	HRP	10^{-15} moles of HRP	Blue	650 nm
H_2O_2/ABTS[d]		10^{-13} moles of HRP	Blue green	405 – 410 nm

a p-Nitrophenyl Phosphate

b 5-Bromo-4-chloro-3-indolyl phosphate/ nitro blue tetrazolium

c 3,3′,5,5′-Tetramethylbenzidine

d 2,2’-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)

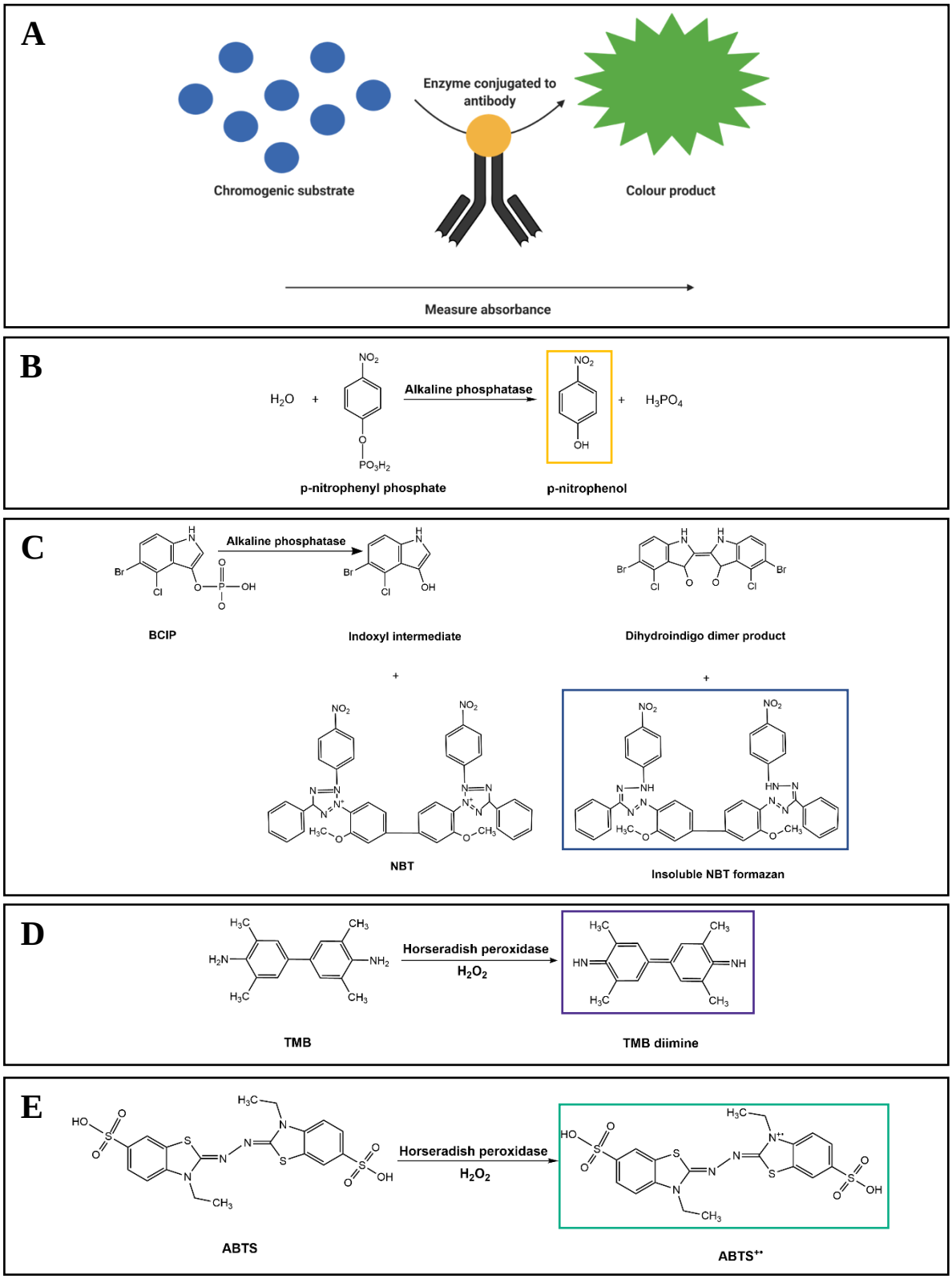

Figure 3.2: Colour reactions of the different chromogenic substrates typically used in an ELISA. **A:** Illustration of the general substrate reaction. **B:** p-NPP is converted by ALP to form a yellow p-nitrophenol colour product. **C:** BCIP is converted to an indoxyl intermediate by ALP after which is reacts with NBT to produce a blue insoluble NBT diformazan colour product and a dihydroindigo dimer product. **D:** In an oxidation reaction, TMB and H_2O_2 are converted to a blue TMB diamine colour product by HRP. **E:** In and oxidation reaction resulting in the formation of an ABTS cation radical, ABTS and H_2O_2 are converted to a blue green $ABTS^{+\bullet}$ colour product by HRP.

3.2.7 Removal of excess reagents

A crucial step in the ELISA process is the washing of the plate between incubation steps, starting after the blocking step. During the ELISA, the incubation steps result in high affinity, specific binding between reagents. However, the plate has to be washed multiple times between incubation steps to remove excess reactants that may be present as well as any loosely bound reagents as a result of low affinity interactions that might occur (5). The high affinity of the reactants for one another, including the solid phase, allows for the removal of excess reactants without removing the bound reactants during washing (7).

Typical wash buffers used to eliminate this non-specific binding usually consist of low concentrations of non-ionic detergents added to a physiological buffer at neutral pH, such as PBS. This allows for the disruption of any low affinity, non-specific interactions during washing (5). Plates are typically washed 3 – 5 times in between incubations steps, whereafter the wash buffer is decanted, and the plate tapped onto a paper towel to remove excess wash buffer from the wells. It is important, however, to ensure that the plate never dries out, which could affect the activity of some reactants (5).

3.3 Results and discussion

3.3.1 Development of a DAS ELISA

The first step in the ELISA development process was to establish an ELISA system that would be optimal for the purpose of this study. As alluded to previously, the DAS ELISA is commonly used in the quantification of proteins, therefore this was the first choice for the development of separate ELISAs that could detect and quantify the amount of each of the specific CoA biosynthesis enzymes in a sample. Figure 3.3 illustrates the basic steps of the DAS ELISA followed in this experiment.

Typically, in the DAS ELISA format, different antibodies (from different species) that are able to detect the antigen of interest are utilised, to ensure that the enzyme-labelled detection antibody only binds to the second antibody and not to the capture antibody. Alternatively, as was the case in the present project, the same rabbit antibody, used as the capturing antibody, was labelled with biotin and used as detection antibody to complete the sandwich. This also allows for the detection of the biotinylated antibody with avidin peroxidase (AVPO, in this instance Streptavidin horseradish peroxidase as explained above).

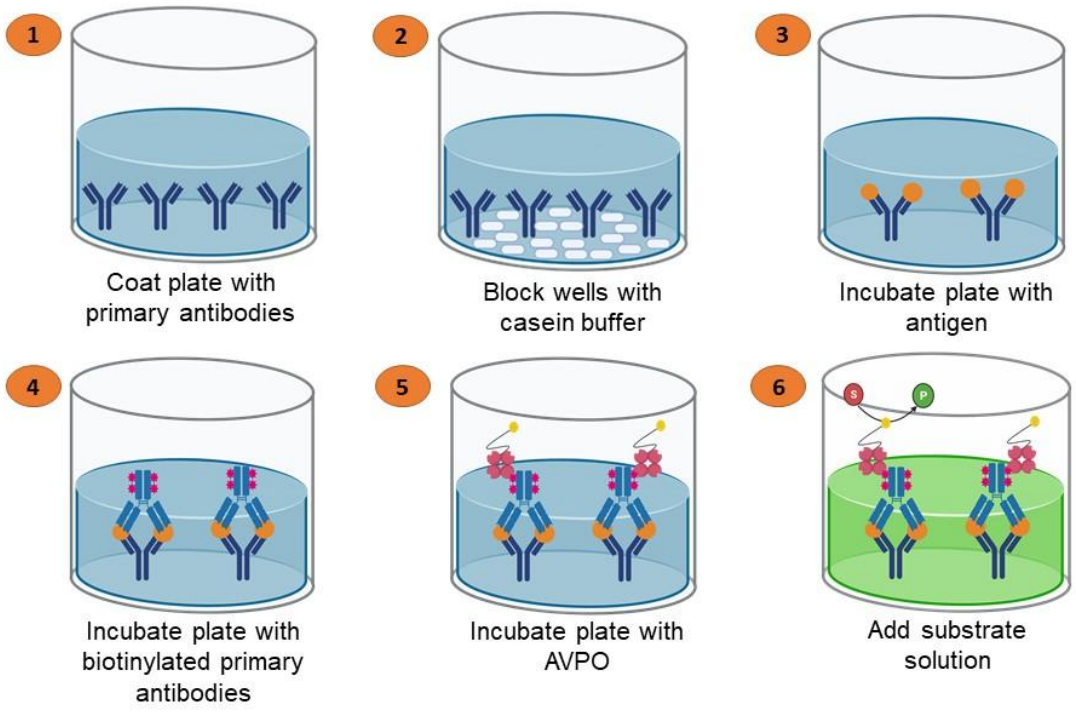

Figure 3.3: Schematic overview of the basic steps of the sandwich ELISA. In the first step of the sandwich ELISA, the plate is coated with capturing primary antibody followed by the blocking of the wells with casein buffer to eliminate any non-specific binding. After blocking, the plate is incubated with the antigen of interest, whereafter the plate is incubated with biotinylated primary antibody (the same antibodies used in step 1, but that are conjugated to biotin molecules). In step 5, the AVPO is added for the binding to and therefore detection of the biotin conjugated to the primary antibodies. Lastly, substrate solution is added to the plate to give a colour reaction that allows for the measurement of absorbance values. This image was created with BioRender.com.

This format was first attempted using *Sa*PanK as antigen with anti-*Sa*PanK antibodies but was unfortunately unsuccessful. A common problem with the use of a sandwich ELISA, is that it is better suited for larger proteins (5) and is sometimes not able to detect smaller proteins. Although *Sa*PanK would not generally be considered a small protein (approximately 29 kDa), it is relatively small in comparison to the size of an antibody (approximately 150 kDa). Among many other factors, this occurrence could influence the outcome of the sandwich ELISA. We therefore set out to detect *Sa*CoaBC, as it is a larger protein (approximately 43.8 kDa) (16). Unfortunately, this system was also not suitable for the detection of *Sa*CoaBC. As *Sa*CoaBC is the largest enzyme of the four enzymes that forms part of the CoA biosynthesis pathway, this system was not attempted for the rest of the enzymes as they are smaller in size.

3.2.2 Development of a competition ELISA with capturing antibodies

The first attempt at developing a competition ELISA, similar to the sandwich ELISA, is started off with the coating of the plate with capturing primary antibodies. The key step that differentiates this format from the rest, is the incubation of the antibody-coated plate with varying amounts of unlabelled antigen along with a fixed amount of biotin-labelled antigen. The labelled and unlabelled antigen therefore compete for binding to the capturing antibody. This allows for the quantification of unlabelled antigen in the sample. The steps of this ELISA are illustrated in Figure 3.4 below.

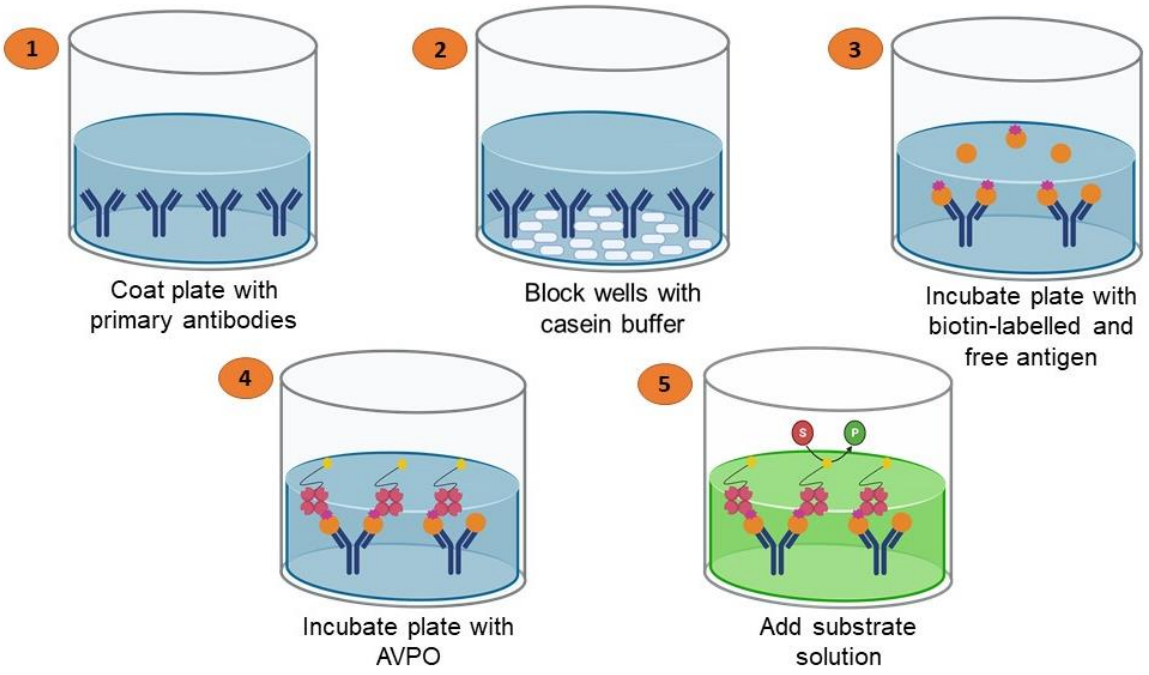

Figure 3.4: Schematic overview of the basic steps of the competition ELISA with immobilised capturing antibodies. The competition ELISA starts with the coating of the plate with capturing primary antibodies, followed by blocking with casein buffer. The plate is then incubated with varying amounts of unlabelled antigen along with a set amount of biotin-labelled antigen. Thereafter AVPO is added to the plate, which binds to the biotin molecules. Lastly, substrate/chromogen solution (H_2O_2/ABTS) is added which reacts with the peroxidase enzyme, that is conjugated to the avidin molecule, and a coloured product is observed. High absorbance values obtained would therefore indicate that a low concentration of unlabelled antigen is present in the sample, whereas the opposite is true for low absorbance values. This image was created with BioRender.com.

As opposed to the sandwich ELISA, the competition ELISA is often employed when the quantification of smaller proteins is required (5). Therefore, the competition ELISA format was the next choice for detection of the enzymes of the CoA biosynthesis pathway of *S. aureus* in this study. An attempt was made with PPAT and anti-PPAT antibodies to determine the optimal antibody dilution and labelled antigen dilution combination, but PPAT no signal was observed and therefore this format was not attempted for the other enzyme antibody combinations.

3.3.3. Development and optimisation of an indirect competition ELISA with immobilised antigen

In a last attempt to find a suitable ELISA system for the detection of the antigens of interest, a different format of the competition ELISA was considered (Figure 3.5). This format relies on the immobilisation of the antigen of interest to the ELISA plate at a specific concentration, whereafter varying concentrations of free antigen are added to the wells as well as unlabelled or biotinylated antibodies. The free antigen competes with the immobilised antigen for binding to the primary antibodies. In wells where the concentration of the free antigen is larger than the immobilised antigen, the primary antibodies favour binding to the free antigen instead, resulting in lower absorbance values after substrate addition. The opposite is true for wells where the concentration of the immobilised antigen is higher than that of the free antigen, inversely resulting in higher absorbance values. By creating a serial dilution of the free antigen, a standard curve can be established for quantifying the amount of antigen in a test sample. This ELISA is therefore known as an indirect competition ELISA.

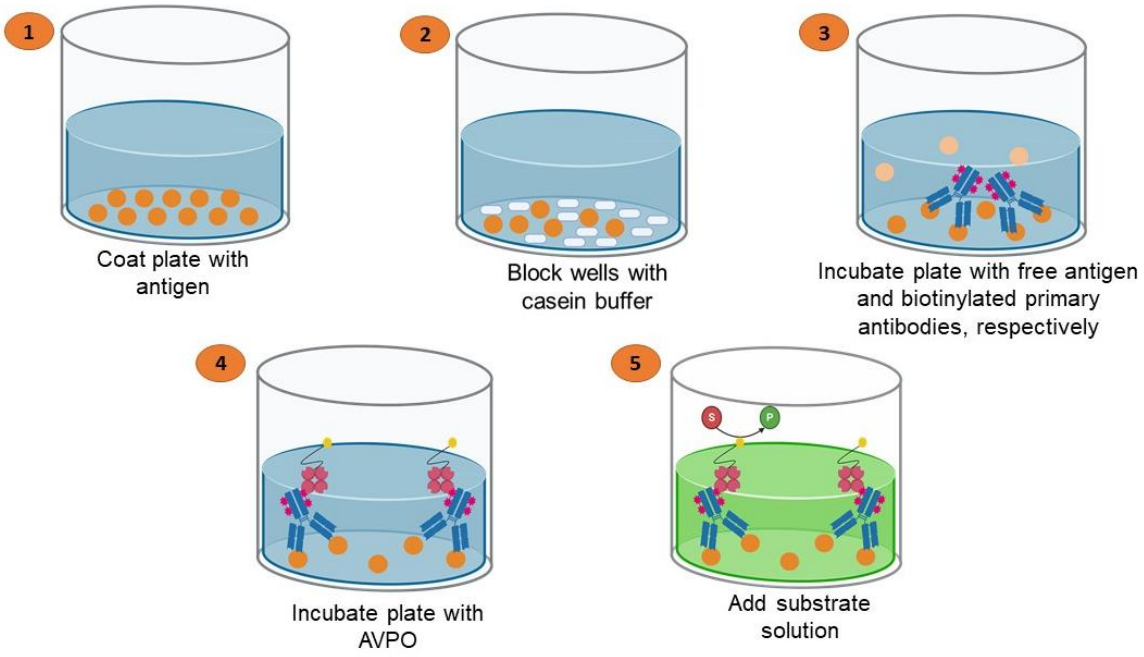

Figure 3.5: Schematic overview of the basic steps of the indirect competition ELISA. This particular competition ELISA is started with the coating of the plate with the antigen of interest, followed by blocking with casein buffer. The plate is then incubated with varying concentrations of free antigen as well as biotinylated primary antibodies. Thereafter AVPO is added to the plate, which binds to the biotin molecules. Lastly, substrate solution is added which reacts with the peroxidase enzyme, that is conjugated to the avidin molecule, and a colour reaction is observed. In contrast to the previous competition ELISA format, absorbance values obtained correlate directly with the amount of coated antigen the biotinylated primary antibodies are bound to. This image was created with BioRender.com.

As the aim of this project was to establish an ELISA for detecting and quantifying each of the four different enzymes, the development and optimisation process for each individual assay will be discussed separately. Before the optimisation for each assay will be discussed, two other important aspects that

are crucial to the optimisation process will be assessed, namely the standard curve as well as possible troubleshooting.

3.3.3.1 The standard curve and interpretation of data

The standard curve is an integral part of protein quantification with an ELISA. As mentioned previously, a standard curve is needed for both the sandwich and competition ELISA systems to enable quantification of protein in a test sample. However, this section will specifically focus on the standard curve obtained for a competition ELISA. The standard curve relies on plotting of the signal (absorbance values) on the y-axis against the concentration range of the antigen standard on the x-axis. Figure 3.6 illustrates and describes the typical sigmoidal-shaped standard curve used for the quantification of a specific antigen with either a sandwich or competition ELISA.

From the illustration (Fig. 3.6), the standard curve can be divided into three distinct parts. Firstly, the upper portion of the standard curve, marked A, forms a plateau. It is at this point of the curve where the antibody binding sites are saturated by the antigen bound to the solid phase (bound antigen) and the small changes in concentration of free antigen standard are not enough to displace antibody binding to the plate to cause a major shift between the bound and free antigen (5, 18). Secondly, the middle linear part of the curve, as indicated by B, is the part of the curve that is used for reliable protein quantification. In this part of the curve, a line of best fit can be drawn through the data points and a formula established that can be used to calculate the concentration of the antigen in a test sample (18). Lastly, the lower part of the curve, marked as C, represents the part of the curve where the free standard antigen outcompetes the bound antigen for binding to the antibodies thereby displacing the antibody binding almost completely from the solid phase.

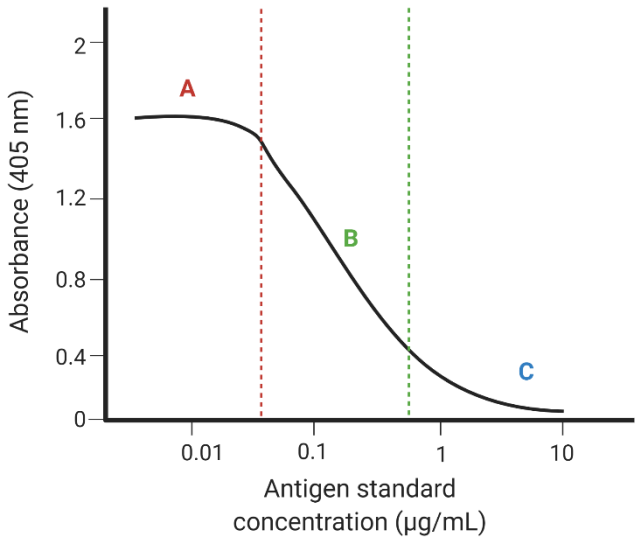

Figure 3.6: Standard curve graph used for protein quantification with ELISA. The standard curve is a semi-log curve that consists of a concentration range of unlabelled antigen standards (x-axis) plotted against their corresponding absorbance values obtained at a specific wavelength (y-axis). The graph can further be divided into sections according to the shape of the curve. As indicated by **A**, the plateau of

the standard curve indicates the portion of the curve where antibody binding sites are saturated by bound antigen (bound to the solid phase). Therefore, the small amount of free standard antigen is not enough to displace antibody binding to the plate to cause a major shift in the distribution between the bound and free antigen (5, 18). The second part of the curve indicated by **B**, represents the part of the standard curve that can be used for protein quantification. This part of the curve represents the range of concentrations of free standard antigen that is able to cause a significant shift in the distribution between the free and bound antigen (18). Lastly, the lower end of the curve, indicated by **C**, represents the part of the curve where the majority of antibodies are bound to the free antigen standard (18).

3.3.3.2 Quality control of ELISA components

Before discussing the optimisation process for the quantification of the respective enzyme antigens, it is important to discuss another important part of the development process, which is troubleshooting. ELISA development and optimisation is not an easy task and therefore requires many rounds of trial and error. An important part of this process includes troubleshooting. As illustrated in Table 3.3, it is clear that optimal antigen and antibody conditions, such as antigen concentration and antibody dilution, are crucial for developing an ELISA that is able to quantify the antigen of interest in a suitable range of concentrations. This is only possible when the antigens and antibodies that are used conform to certain standards. By performing a simple indirect ELISA, such as described in Chapter 2, quality control of these reagents could be accomplished.

Quality control of biotinylated antibodies - When stored and handled correctly, antibodies can be used for many years after production (19). There are certain conditions, however, that can influence the shelf life of antibodies such as the type of antibody (polyclonal or monoclonal), purification of antibodies, conjugated versus unconjugated antibodies, antibody concentration, pH and storage temperature, to name a few (19). For the purpose of this experiment, rabbit polyclonal antibodies, as described in Chapter 2, were subjected to ammonium sulfate precipitation and conjugated to biotin molecules (3.5.2) and are therefore more vulnerable to loss of activity as time progresses. In order to determine whether the biotinylated antibodies were conjugated to a consistent standard, an indirect ELISA as described in Chapter 2 (2.2.4) was performed for each batch of biotinylated antibodies.

Quality control of purified protein - Unlike antibodies, purified protein, and especially enzymes, are much more prone to becoming unstable when handled incorrectly. One particular problem that occurs when using a protein sample multiple times, which is common in the ELISA optimisation process, is precipitation of the protein. This occurs often due to an increased number of freeze-thaw cycles and extensive handling of the samples. In the experiments performed here, the antigens of interest are enzymes and although activity of these enzymes is not required for a successful assay, precipitation of the enzymes could result in incorrect antigen dilutions. This could greatly affect the accuracy and reproducibility of the assays and therefore necessitates careful quality control.

Similar to the quality control of the biotinylated antibodies, an indirect ELISA (2.2.4) was performed with different batches of the respective enzymes and tested with both the unlabelled rabbit primary antibodies as well as biotinylated antibodies to determine whether the enzyme samples or possibly the biotinylated antibodies conformed to a consistent standard. As it was clear from previous experiments (Chapter 2) that the unlabelled rabbit primary antibodies were functioning optimally, detection of the enzymes with the primary antibodies was chosen as a control to determine whether the enzyme samples were still intact and therefore functional. The use of the biotinylated antibodies was also included in this experiment to determine how they would perform with the different batches of enzyme in contrast to the normal rabbit primary antibodies. Using *Sa*CoaBC as an example, Figure 3.7 illustrates the layout of the indirect ELISA, described in 2.2.4 in Chapter 2, used for the quality control of the different batches of enzyme.

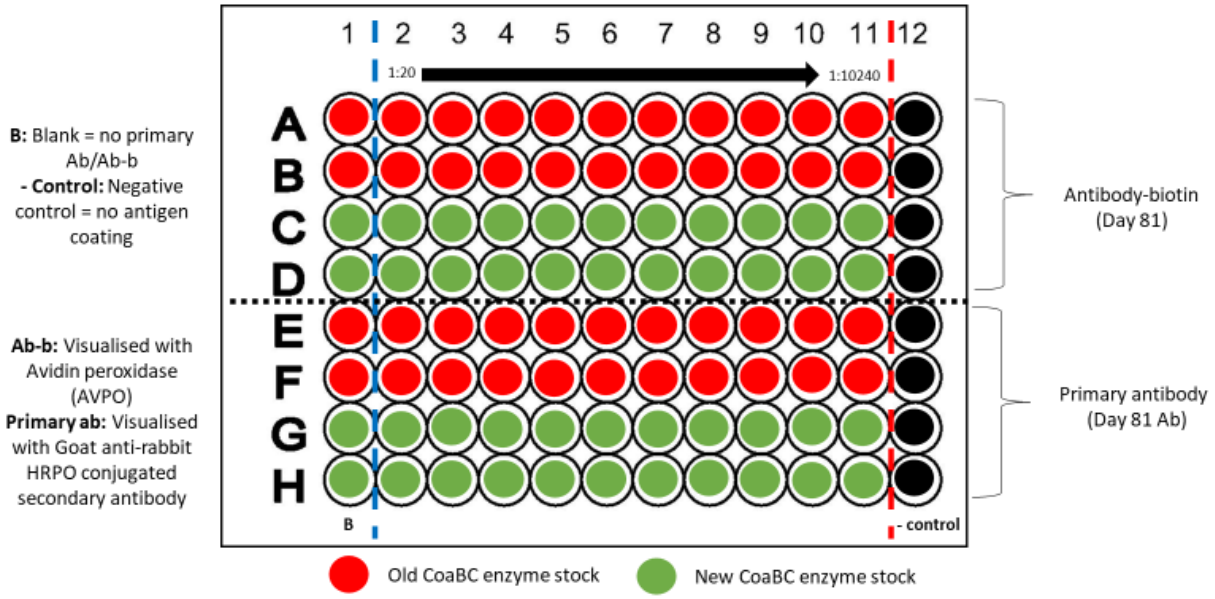

Figure 3.7: Layout of indirect ELISA for quality control of enzyme batches used in the ELISA optimisation process, utilizing *Sa*CoaBC as an example. Red dots are indicative of an older batch of enzyme whereas the green dots indicate a new batch of enzyme. Black dots indicate where the plate was not coated with antigen and represents the negative control. The plate was also separated into the parts where either biotinylated antibodies (Ab-b) or unbiotinylated antibodies (primary Ab) was used. The serial dilution made of the antibodies and conjugates is indicated with the arrow above and the letter B indicates the blank column, which did not contain any primary antibodies.

The troubleshooting of the reagents required in the development and optimisation process of each of the different assays will be discussed in conjunction with the outcomes of the optimisation process for each of the respective assays in the sections to follow.

3.3.3.3 Development and optimisation of an indirect competition ELISA for the detection and quantification of *Sa*PPAT

The first attempt to use the indirect competition ELISA format was performed with PPAT to establish a working protocol that could be applied to the rest of the enzymes. PPAT was chosen as the first candidate for this approach as larger quantities of this enzyme were available, enabling multiple rounds of optimisation. A summary of all the attempts made for the optimisation of the indirect competition ELISA is shown in Table 3.3. The standard curve obtained from a competition ELISA relies on a concentration range of free standard antigen that competes with the bound antigen (solid phase) for binding to the primary antibodies, and the signal obtained (absorbance) from this competition which could be used to determine the concentration of antigen in a test sample.

Table 3.3: Summary of ELISA conditions used for the development and optimisation of indirect competition ELISAs for the detection and quantification of *Sa*PanK, *Sa*CoaBC, *Sa*PPAT and *Sa*DPCK, respectively. The sequence of the table represents the sequence in which the optimisation process was conducted.

Antigen	[Coating]	Ab containing serum[a]	Ab dilution	Incubation Temp.[b]	Incubation time[c]	Antibody detection[d]	Attempts[e]
PPAT	2 µg/mL	Day 42 antiserum	1 in 640	37°C	2 hours	Goat anti-rabbit HRP conjugate secondary Ab	1
	0.5 µg/mL	Day 42 antiserum	1 in 5000	37°C	2 hours	Goat anti-rabbit HRP conjugate secondary Ab	2
		Day 42 antiserum	1 in 2500	37°C	2 hours	Goat anti-rabbit HRP conjugate secondary Ab	1
		Biotinylated antibodies from day 81 antiserum	1 in 200 1 in 2000	4°C	overnight	AVPO	1
		Biotinylated antibodies from day 81 antiserum	1 in 250 1 in 500	4°C	overnight	AVPO	2
		Biotinylated antibodies from Day 81 antiserum	1 in 300 1 in 450	4°C	overnight	AVPO	5
		Biotinylated antibodies from Day 81 antiserum	1 in 230 1 in 280	4°C	overnight	AVPO	2
		Biotinylated antibodies from Day 81 antiserum	1 in 330 1 in 450	4°C	overnight	AVPO	1
DPCK	0.5 µg/mL	Biotinylated antibodies from Day 81 antiserum	1 in 250 1 in 500	4°C	overnight	AVPO	3

CoaBC	0.5 µg/mL	Biotinylated antibodies from day 81 antiserum	1 in 250 1 in 500	4°c	Overnight	AVPO	4
		Biotinylated antibodies from day 81 antiserum	1 in 100 1 in 200	4°C	overnight	AVPO	1
	1 µg/mL	Biotinylated antibodies from day 81 antiserum	1 in 100	4°C	overnight	AVPO	5
	1.5 µg/mL	Biotinylated antibodies from day 81 antiserum	1 in 100	4°C	overnight	AVPO	4
	2 µg/mL	Biotinylated antibodies from day 81 antiserum	1 in 100 1 in 200	4°C	overnight	AVPO	1
PanK	0.5 µg/mL	Biotinylated antibodies from day 81 antiserum	1 in 250 1 in 500	4°C	overnight	AVPO	2
	1.5 µg/mL	Biotinylated antibodies from day 81 antiserum	1 in 100	4°C	overnight	AVPO	6
	2 µg/mL	Biotinylated antibodies from day 81 antiserum	1 in 200	4°C	overnight	AVPO	6
		Biotinylated antibodies from day 81 antiserum	1 in 250 1 in 300	4°C	overnight	AVPO	1

a – Ab (antibody)containing serum refers to the rabbit antibody containing serum that is complementary to the antigen mentioned in the first column

b – Incubation temperature refers to the incubation temperature of the ELISA plate with the respective rabbit primary antibody containing sera

c – Incubation time refers to the incubation time of the ELISA plate with the respective rabbit primary antibody containing sera

d – The detection system used to detect the rabbit primary antibody, in this case either the use of a secondary antibody or the avidin-biotin system

e – Number of attempts made under the specific conditions for the specific ELISA

In this first attempt to use this ELISA format for detecting PPAT, a starting coating concentration of 2 µg/mL was used as previously determined (Chapter 2). This was followed by the addition of day 42 polyclonal rabbit anti-*Sa*PPAT antibodies at a dilution of 1:640 (determined from immune response curves; Chapter 2) and detected with goat anti-rabbit HRP conjugate secondary antibody. Incubation of the plate with the primary antibodies was performed at 37°C for 2 hours. A semi-log standard curve was plotted with absorbance values (y-axis) against the concentration of the free antigen (x-axis) as described in Figure 3.6. This resulted in a flat standard curve with poor discrimination between the data points (absorbance values ranging from 1.392–1.63). Although the maximum absorbance values obtained were adequate, i.e., higher than 0.8 (20), the flat curve indicated that the antibody binding sites were saturated by bound antigen at all concentrations of free antigen. Therefore, the amount of free antigen was not able to compete with the amount of bound antigen for binding to the antibodies. This can be ascribed to an antigen coating concentration that was too high as well as a high primary antibody concentration (5). To obtain a better standard curve this approach was repeated with a lower PPAT coating concentration of 0.5 µg/mL as well as different primary antiserum dilutions (1:5000 and 1:2500), both attempts resulting in a better spread of the data points, but with signal values that were too low.

In an attempt to achieve a standard curve with a higher signal and better distribution of the data points, biotin molecules were linked to antibodies isolated from the day 81 antiserum and used for the detection of PPAT. Furthermore, these antibodies were detected with AVPO instead of a secondary antibody and the plate incubated at 4°C overnight, instead of the usual 37°C for 2 hours. Incubating the plate at 4°C for a longer period of time typically results in less well-to-well variation and ultimately a better distribution of data points (7), which is why this approach was considered to obtain a workable standard curve. A coating concentration of 0.5 µg/mL was used throughout the rest of the optimisation process. This new approach was attempted for different antibody dilutions, as seen in Table 3.3, until a workable standard curve was obtained with an antibody dilution between 1:250 and 1:500. Thereafter, these conditions were attempted for the rest of the enzymes.

To find an optimal primary antibody dilution for each enzyme, many more attempts were made with different antiserum dilution values ranging between 1:250 and 1:500. Quality control of the two different batches of biotinylated anti-*Sa*PPAT antibodies were also performed to determine which batch was able to detect the PPAT samples optimally. As seen in the graph in Figure 3.8, the second batch of biotinylated anti-*Sa*PPAT (orange curve) functioned better than the first batch (blue curve) and was therefore used during the rest of the optimisation process.

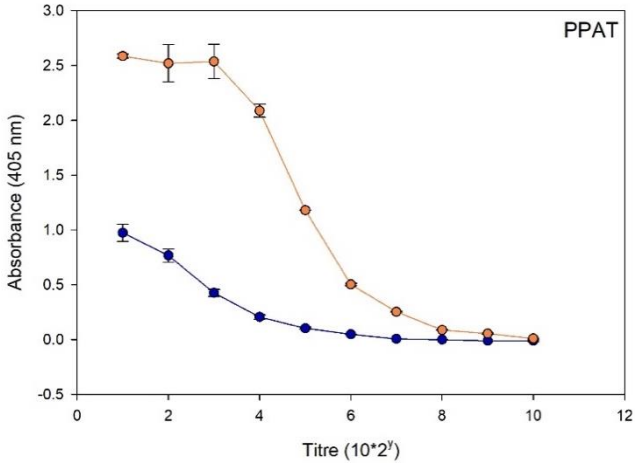

Figure 3.8: Quality control of biotinylated polyclonal rabbit anti-*Sa*PPAT antibodies. An indirect ELISA was performed with different batches of the biotinylated anti-*Sa*PPAT antibodies to determine whether the assay was functioning optimally. Absorbance values were plotted against the different antibody titres for which they were obtained. The blue graph indicates absorbance values obtained using the first batch of biotinylated antibodies and the orange graph the values obtained using the second batch of biotinylated antibodies. This assay was performed once with technical duplicates (error bars = standard deviation).

After many rounds of optimisation, it was found that a *Sa*PPAT coating concentration of 0.5 µg/mL combined with biotinylated anti-*Sa*PPAT antiserum diluted 1 in 300 and incubated at 4°C overnight, resulted in a standard curve with high signal values and a good distribution of the data points. Three biological repeats, thus three different purified batches of *Sa*PPAT, were used during this assay with each assay done in triplicate. As seen in Figure 3.9 A, the three different repeat datasets were plotted individually to show the range of the standard curve established for detection of *Sa*PPAT. The curves indicated the typical sigmoidal curve shape associated with a competition ELISA standard curve.

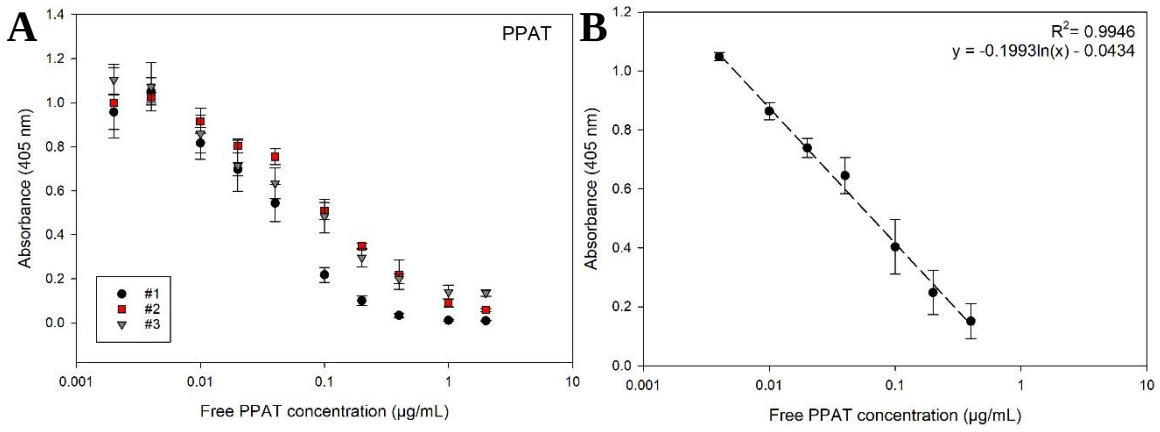

Figure 3.9: Optimisation of an indirect competition ELISA for the detection of *Sa*PPAT. A: The standard curves obtained for the optimisation of an indirect ELISA for the detection of *Sa*PPAT. Differently coloured data points were used to indicate the three different independent experiments (n=3, each plotted individually), each performed in triplicate with error bars indicating standard deviation. **B:** The average absorbance values from the three graphs in A were used to determine the part of the standard curve that can be used for enzyme quantification. A non-linear, logarithmic regression line

was established with R^2 (coefficient of determination) and formula of the line as indicated on the graph. Error bars are indicative of the SEM.

From the standard curves in Figure 3.9 A, a trendline could be fitted to the average absorbance values obtained from the repeats. It was established that the standard curve has a detection range from 0.004 – 0.4 µg/mL with a coefficient of determination (R^2) value of 0.995. In general, the closer the R^2 is to 1, the greater the fit of the trendline and the better the precision level of the curve will be (20, 21). Therefore, an excellent standard curve for the quantification of *Sa*PPAT was obtained.

3.3.3.4 Development and optimisation of an indirect competition ELISA for the detection and quantification of *Sa*DPCK

As established in the optimisation process of the indirect competition ELISA for detecting *Sa*PPAT, the following conditions were applied as a starting point for the optimisation process for the detection of *Sa*DPCK. The ELISA plate was coated with a *Sa*DPCK concentration of 0.5 µg/mL paired with polyclonal rabbit anti-*Sa*DPCK antibodies isolated from day 81 antiserum at dilutions of 1:250 and 1:500. The incubation temperature and time with the biotinylated antibodies remained at 4°C overnight. The use of optimal reagents was again found to be of importance for optimisation in order to develop a successful ELISA, especially for establishing a good standard curve. For this reason, quality control of the different batches of the biotinylated antibodies were again performed with an indirect ELISA. As seen in Figure 3.10, both batches of the biotinylated anti-*Sa*DPCK antibodies performed equally well and further experiments could be performed.

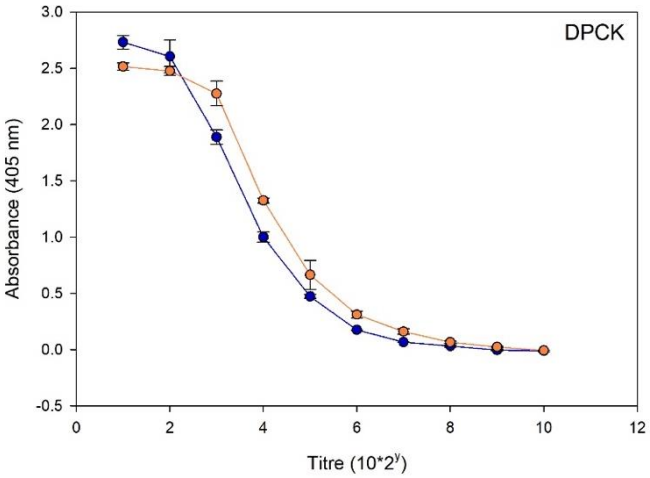

Figure 3.10: Quality control of biotinylated polyclonal rabbit anti-*Sa*DPCK antibodies. An indirect ELISA was performed with different batches of the biotinylated anti-*Sa*DPCK antibodies to determine whether they were functioning optimally. Absorbance values were plot against the different antibody dilutions from which they were obtained. The blue graph indicates absorbance values obtained for the first batch of biotinylated antibodies and the orange graph the values obtained for the second batch of biotinylated antibodies. This assay was performed once with technical duplicates (error bars = standard deviation).

Upon completion of the indirect competition ELISA for *Sa*DPCK, it was found that a DPCK coating concentration of 0.5 µg/mL functioned optimally with a biotinylated antibody dilution of 1 in 250. The standard curve obtained from these conditions had high signal values with a good dispersal of the data points, resulting in a standard curve that was almost completely linear. However, a small plateau can be observed at the upper part of the curve, as expected (Fig. 3.6). Three biological repeats of these conditions were performed by using three different batches of purified *Sa*DPCK (Fig. 3.11 A) with each experiment performed in triplicate.

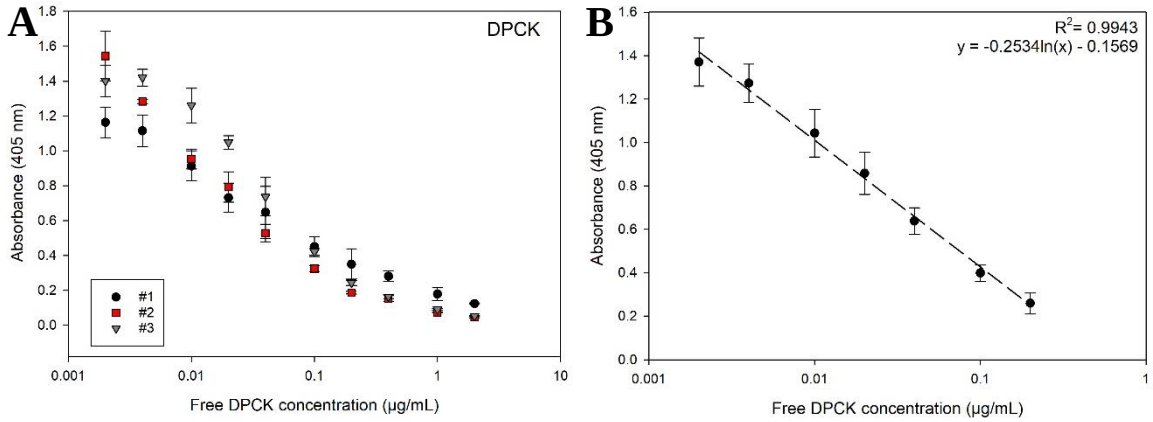

Figure 3.11: Optimisation of an indirect competition ELISA for the detection of *Sa*DPCK. A: The range of the standard curves obtained for the optimised indirect ELISA for the detection of *Sa*DPCK. Different coloured data points are indicative of the three different independent experiments (n=3, each plotted individually), each performed in triplicate with error bars indicating standard deviation. **B:** The average absorbance values from the three graphs in A were used to determine the part of the standard curve that can be used for enzyme quantification. A non-linear, logarithmic regression line was established with R^2 (coefficient of determination) and formula of the line as indicated on the graph. Error bars are indicative of the SEM.

The detection range of the standard curve for *Sa*DPCK was established to range from 0.002 – 0.2 µg/mL and a trendline fit to the average absorbance values (Fig. 3.11 B) yielded a R^2 value of 0.994 which is very close to 1. Therefore, the trendline has a very good fit to the standard curve. Furthermore, the error bars (SEM) are small, indicating little variance between the independent experiments performed. This standard curve therefore has very good potential for the quantification of *Sa*DPCK in unknown samples.

3.3.3.5 Development and optimisation of and indirect competition ELISA for the detection and quantification of *Sa*CoaBC

The attempts at optimisation ofr an indirect ELISA for the detection of *Sa*CoaBC can be seen in Table 3.3. Initial conditions of a CoaBC coating concentration of 0.5 µg/mL with biotinylated polyclonal anti-*Sa*CoaBC antibodies from the day 81 antiserum at dilutions of 1:250 and 1:500 resulted in a wave-like standard curve with data points that were scattered, not following the typical sigmoidal shape. This could be an indication that the *Sa*CoaBC standard samples were not prepared properly and therefore

required repeats of the specific assay conditions with the different *Sa*CoaBC samples. Multiple attempts at repeating these conditions were unsuccessful. Therefore, troubleshooting of both the *Sa*CoaBC samples and the anti-*Sa*CoaBC antibodies was performed. Troubleshooting of different batches *Sa*CoaBC revealed that some of the samples were precipitating, contributing to the many failed attempts. When proteins precipitate, this results in dilution errors of the protein which could have a great effect on the outcome of the assay. Furthermore, enzyme aggregates could bind to the antibody and then disaggregate with resultant disruptions of the equilibria required in the assay. As mentioned previously, a successful ELISA relies greatly on optimal reagent conditions and ultimately an optimal combination of antibody and antigen concentration. Testing of the biotinylated anti-*Sa*CoaBC antibodies with new batches of *Sa*CoaBC (Fig. 3.12) showed that both batches of biotinylated antibodies were still functioning optimally.

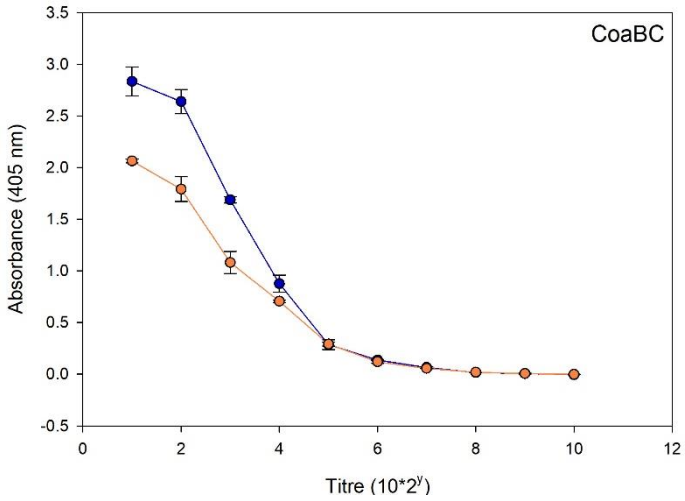

Figure 3.12: Quality control of biotinylated polyclonal rabbit anti-*Sa*CoaBC antibodies. An indirect ELISA was performed with different batches of the biotinylated anti-*Sa*CoaBC antibodies to determine whether they were functioning optimally. Absorbance values were plotted against the different antibody titres. The blue graph indicates absorbance values obtained for the first batch of biotinylated antibodies and the orange graph the values obtained for the second batch of biotinylated antibodies. This assay was performed once with technical duplicates (error bars = standard deviation).

Further optimisation attempts were therefore performed with new batches of *Sa*CoaBC enzyme. These attempts included the use of different *Sa*CoaBC coating concentrations (1 µg/mL, 1.5 µg/mL or 2 µg/mL) in conjunction with different antiserum dilutions (1:100 or 1:200), establishing an optimal coating concentration of 1 µg/mL and an antiserum dilution of 1:100. Three biological repeats of these conditions were performed using three different batches of *Sa*CoaBC enzyme. As seen in Figure 3.13 A, the last repeat of the assay conditions showed much higher absorbance values in comparison to the first two. Another repeat of the experiment with the same batch of *Sa*CoaBC was performed and similar results obtained. This occurrence could possibly be ascribed to faulty concentration determination of the specific *Sa*CoaBC sample with the Bradford method, resulting in a higher *Sa*CoaBC coating

concentration than intended. Another attempt at determining the concentration of the specific *Sa*CoaBC sample with a Bradford assay was unsuccessful as it gave inconclusive results.

Although the standard curve obtained from the last experiment was different from the previous two, it could be argued that it delivered more desirable results than the previous two. Firstly, it can be observed that the standard curves from the previous experiments yielded standard curves that have low signal (maximum absorbance values barely reaches 0.8), and the shape of the curve does not quite resemble the typical sigmoidal standard curve (Fig. 3.6) expected from a competition ELISA. In contrast, the standard curve from the last repeat somewhat resembles the sigmoidal shape with better distribution of the data points and higher signal.

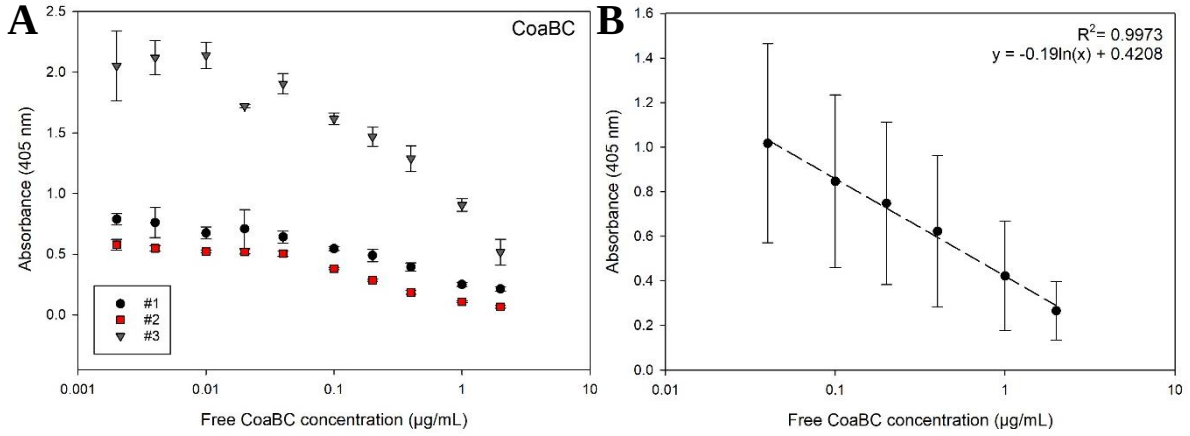

Figure 3.13: Optimisation of an indirect competition ELISA for the detection of *Sa*CoaBC. A: The range of the standard curves obtained for the optimised indirect ELISA for the detection of *Sa*CoaBC. Different coloured data points indicate the three different independent experiments (n=3, each plotted individually), each performed in triplicate with error bars indicating standard deviation. **B:** The average absorbance values from the three graphs in A were used to determine the part of the standard curve that can be used for protein quantification. A non-linear, logarithmic regression line were established with R^2 (coefficient of determination) and formula of the line as indicated on the graph. Error bars are indicative of the SEM.

Nevertheless, the trendline (Fig. 3.13 B) fitted to the average absorbance values from graphs in Figure 3.13 A, yielded a R^2 value of 0.997, again indicating that the trendline had an excellent fit to the data points. However, the large error bars (SEM), that are a result of the large variation between the three repeats, necessitates the need for further repeats of the assay conditions. This assay has a detection range of 0.04 – 2 µg/mL.

3.3.3.6 Development and optimisation of an indirect competition ELISA for the detection and quantification of *Sa*PanK

Lastly, the ELISA development process was completed for the optimisation of an indirect competition ELISA for detecting *Sa*PanK. The different conditions used in the process are shown in Table 3.3, which commenced with a *Sa*PanK coating concentration of 0.5 µg/mL and biotinylated polyclonal rabbit anti-

*Sa*PanK serum dilutions of 1:250 and 1:500. These conditions resulted in the unsuccessful detection of *Sa*PanK, even when the experiment was repeated. When troubleshooting the different batches of biotinylated antibodies (Figure 3.14), very low absorbance values were observed, which was not expected as the avidin-biotin system is typically used to achieve a greater signal. This observation prompted testing of the different batches of biotinylated antibody with alternative batches of newly purified *Sa*PanK, along with the unlabelled primary antibodies as a control for successful detection of *Sa*PanK. The second round of troubleshooting revealed much higher absorbance values for both batches of biotinylated antibodies as well as the unlabelled primary antibodies, indicating that the previously used PanK samples were not suitable for use anymore in the assay. Similarly, to what happened with several of the *Sa*CoaBC samples, it was possible that the *Sa*PanK sample that was used in the optimisation process was precipitating due to multiple freeze-thaw cycles.

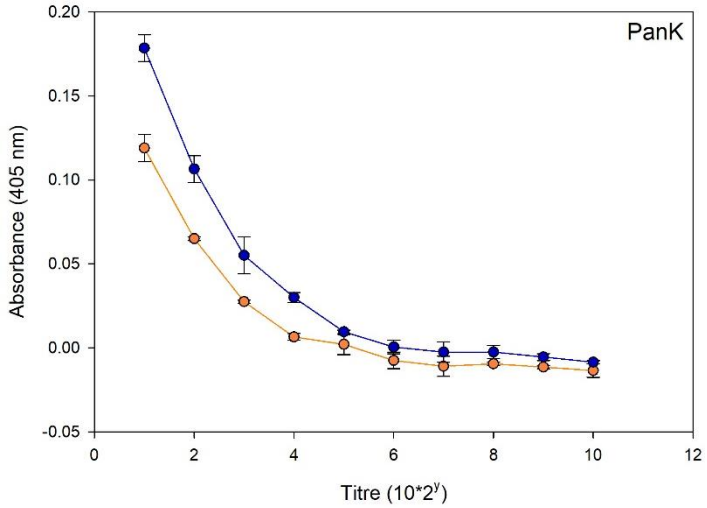

Figure 3.14: Quality control of biotinylated polyclonal rabbit anti-*Sa*PanK antibodies. An indirect ELISA was performed with different batches of the biotinylated anti-*Sa*PanK antibodies to determine whether they were functioning optimally. Absorbance values were plotted against the different antibody titres. The blue graph indicates absorbance values obtained for the first batch of biotinylated antibodies and the orange graph the values obtained for the second batch of biotinylated antibodies. This assay was performed once with technical duplicates (error bars = standard deviation).

After multiple attempts (with new batches of *Sa*PanK) with different coating concentrations (1.5 µg/mL and 2 µg/mL) and antibodies dilutions (1:100, 1:200 and 1:300) it was established that a *Sa*PanK coating concentration of 2 µg/mL and biotinylated antibodies diluted 1:200 was optimal for the detection of *Sa*PanK. Three biological repeats of these conditions were performed with three different batches of *Sa*PanK (Fig. 3.15 A). These assay conditions resulted in a standard curve with the typical sigmoidal shape, as the upper part of the curve resembles the plateau that moves into the linear part of the curve. Furthermore, the standard curve shows high signal and good distribution of the data points.

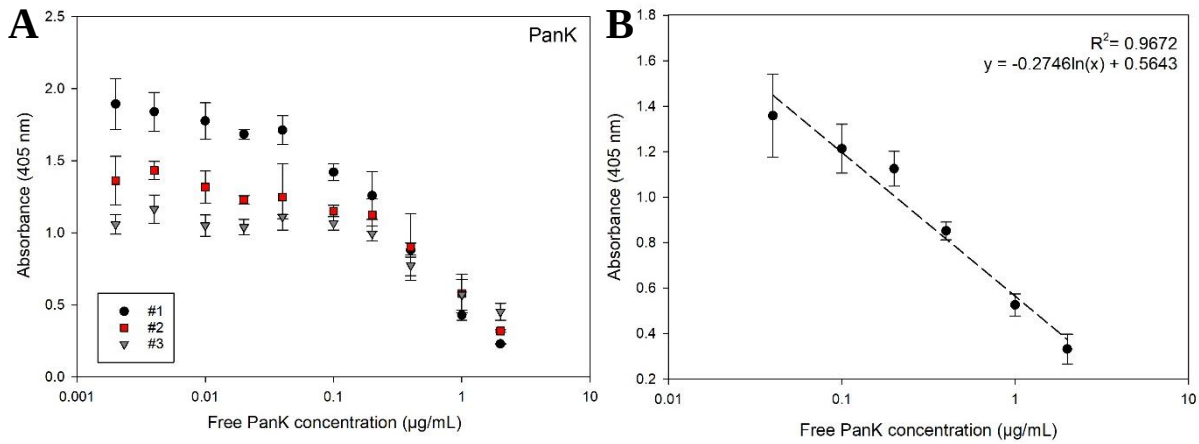

Figure 3.15: Optimisation of an indirect competition ELISA for the detection of *Sa*PanK. A: The range of the standard curves obtained for the optimised indirect ELISA for the detection of *Sa*PanK. Different coloured data points indicate the three different independent experiments (n=3, each plotted individually), each performed in triplicate with error bars indicating standard deviation. **B:** The average absorbance values from the three graphs in A were used to determine the part of the standard curve that can be used for protein quantification. A non-linear, logarithmic regression line was established with R^2 (coefficient of determination) and formula of the line as indicated on the graph. Error bars are indicative of the SEM.

The detection range of the standard curve for *Sa*PanK was established to range from 0.04 – 2 µg/mL and a trendline fit to the average absorbance values (Fig. 3.15 B) yielded a R^2 value of 0.967 which is still relatively close to 1 but is not as reliable as the standard curves obtained for the quantification of the other enzymes. However, small error bars (SEM) can be observed, indicating little variation between the independent experiments performed. Although a workable standard curve was obtained for the quantification of *Sa*PanK, some optimisation might still be needed to obtain an optimal standard curve.

3.3.3.7 Detection limit considerations

It is clear from these experiments that a good foundation for future studies in the detection and quantification of the enzymes of the CoA biosynthesis pathway of *S. aureus* has been established. However, some optimisation might still be needed. As summarised in Table 3.4, the most sensitive ELISA was obtained for the detection of *Sa*DPCK, and the least sensitive assay for *Sa*PanK. It is important, however, to keep in mind that the sensitivity of an ELISA greatly depends on the characteristics of the antibody-antigen pair and that the capabilities of a specific assay are therefore influenced by these characteristics. Furthermore, the detection range needed for the quantification of a specific antigen depends on the abundance of the antigen obtained from a specific sample. As the main purpose of this study was to establish the levels of the CoA biosynthesis enzymes of *S. aureus* in different growth phases under physiological conditions, we cannot estimate the detection range that would be required for these assays. Therefore, these assay conditions should be tested with unknown

samples before further optimisation can be performed. Future studies and prospective approaches for optimisation and use of these assays will be discussed in more detail in Chapter 4.

Table 3.4: Summary of standard curves obtained for the quantification of the enzymes from the CoA biosynthesis pathway of *S. aureus*.

Enzyme	Detection range	R^2	Comments
***Sa*Pank**	0.4 – 2 µg/mL	0.967	
***Sa*CoaBC**	0.04 – 2 µg/mL	0.997	Large error bars for all data points
***Sa*PPAT**	0.04 – 0.4µg/mL	0.995	
***Sa*DPCK**	0.002 – 0.2 µg/mL	0.994	

3.3.4 Antibody cross-reactivity quantification with optimised ELISAs

As described in Chapter 2, the potential cross-reactivity of the respective antibodies (anti-*Sa*PanK, anti-*Sa*CoaBC, anti-*Sa*PPAT and anti-*Sa*DPCK) was determined with an indirect ELISA. These results gave good insight into the possible limitations of these antibodies and whether they would be able to detect multiple enzymes in the CoA biosynthesis pathway of *S. aureus.* Although these results gave an indication of cross-reactivity, it could not give us a precise measure of the amount of cross-reactivity that a certain antibody might have to a certain antigen. Furthermore, cross-reactivity could complicate specific antigen quantification, as it could result in an overestimation of the concentration values.

In an attempt to quantify the amount of cross-reactivity of each antibody to each enzyme and how it would affect the specific ELISA method for the detection of each of the respective enzymes, the previously optimised indirect ELISAs were utilised to quantify antibody cross-reactivity. It was proposed that the amount of cross-reactivity could be plotted on a graph, such as seen in Figure 3.16, and the percentage cross-reactivity determined in relation to the positive control. This would allow for the correction of concentration values obtained from the respective indirect competition ELISAs to account for cross-reactivity.

Due to time constraints, only preliminary results were obtained to quantify the amount of cross-reactivity of each of the antibodies, and a single indirect competition ELISA performed for each of the day 81 antibodies (anti-*Sa*PanK, anti-*Sa*CoaBC, anti-*Sa*PPAT and anti-*Sa*DPCK). These results, however, do provide an indication of the cross-reactivity among the optimised indirect ELISAs for each specific antigen.

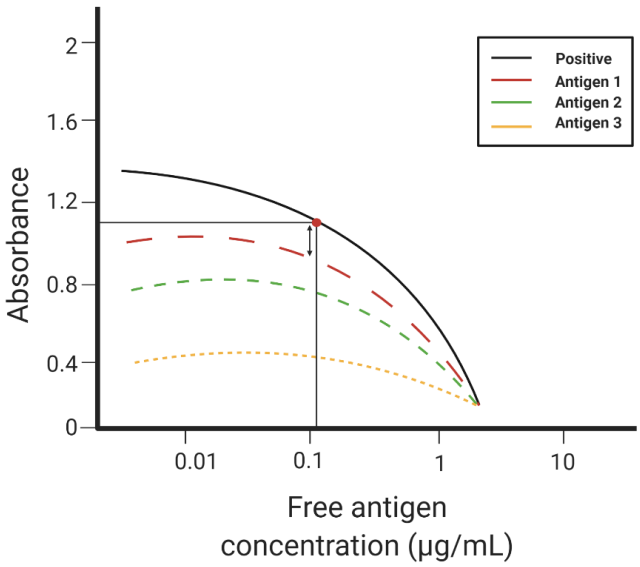

Figure 3.16: Cross-reactivity quantification graph. The cross-reactivity of each of the antibodies can be quantified by using the optimised indirect competition ELISA method established for each of the different antigen-antibody pairs. By determining the amount of cross-reactivity of each antibody with each antigen, cross-reactivity could be accounted for when quantifying a specific antigen with this ELISA format. This format includes the positive control (antigen complimentary to antibody used), which is the antigen that is quantified, followed by the antigens that cross-react with the antibody.

As seen in Figure 3.17 A, the anti-*Sa*PanK antibodies showed some cross-reactivity with both *Sa*PPAT (blue) and *Sa*DPCK (red), but to a very low extent. These antibodies, however, were not able to detect *Sa*CoaBC (green). In comparison to these results, cross-reactivity studies done as described in Chapter 2 revealed that the anti-*Sa*PanK antibodies were able to detect both *Sa*PPAT and *Sa*DPCK to a great degree at low antiserum dilutions when analysed with an indirect ELISA. Although similar antigen coating concentrations were used in both instances (2 µg/mL), the antibodies at an antiserum dilution of 1:200 did not detectthe other antigens.

From Figure 3.17 B, it is clear that the anti-*Sa*CoaBC antibodies did not cross-react with any of the other antigens. These results further corroborate the previous findings describedin Chapter 2, which indicated that the antibodies did not cross-react with any of the other antigen when tested with an indirect ELISA. The anti-*Sa*CoaBC antibodies are therefore the most specific and can be used in the indirect competition ELISA for the quantification of *Sa*CoaBC in different growth samples, as the ELISA would not experience interference from the other enzymes regardless of the assay conditions.

In comparison to the cross-reactivity results described inChapter 2, the anti-*Sa*PPAT antibodies (Fig. 3.17 C) appear to cross-react with the other antigens to a greater extent (in comparison to the other antibodies), except *Sa*CoaBC (green), even at lower antigen coating concentrations (0.5 µg/mL versus 2 µg/mL). The effect of a lower coating concentration can, however, be observed in that the previous cross-reactivity studies showed that the anti-*Sa*PPAT antibodies were able to cross-react with both

*Sa*PanK and *Sa*DPCK to some extent, even at higher antiserum dilutions. It can therefore be concluded that these antibodies are not highly specific, and this should be taken into account when using them in the quantification of *Sa*PPAT in different growth samples.

Lastly, from Figure 3.17 D it appears that the anti-*Sa*DPCK antibodies did not cross-react with any of the other antigens. This is in agreement with the western blot cross-reactivity experiment, but contrasts with the cross-reactivity results from the indirect ELISA described in Chapter 2, which indicated that the anti-*Sa*DPCK antibodies were able to detect both *Sa*PanK and *Sa*PPAT to some degree at low antibody dilutions. Again, this observation could be ascribed to a lower antigen coating concentration (0.5 µg/mL versus 2 µg/mL) in combination with an antiserum dilution of 1:250. According to these results, the use of these antibodies for the quantification of DPCK in different growth samples should not cause any interference in terms of cross-reactivity. However, repeats of these experiments should be performed to corroborate these findings.

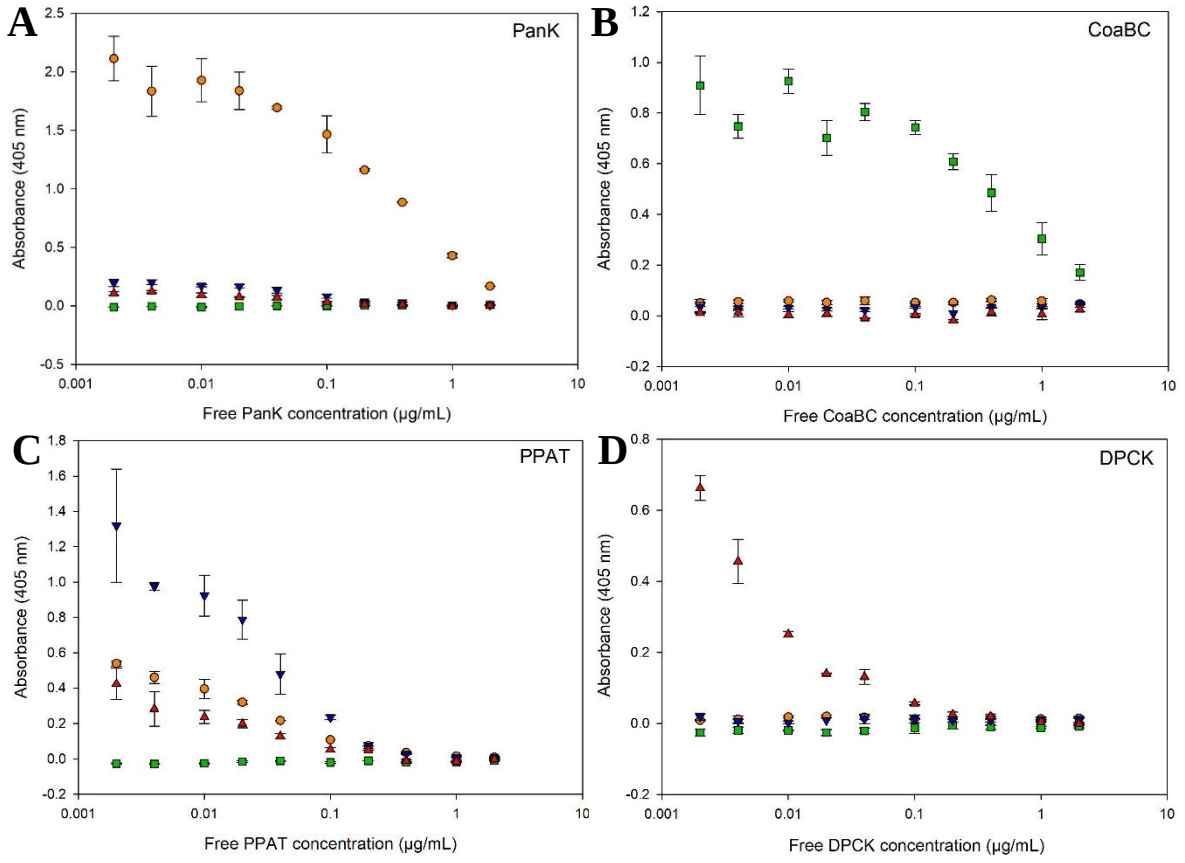

Figure 3.17: Cross-reactivity quantification of (A) anti-*Sa*PanK, (B) anti-*Sa*CoaBC, (C) anti-*Sa*PPAT and (D) anti-*Sa*DPCK antibodies. The different coloured data points are indicative of the different antigen that were detected with the respective antibodies. Orange graph: *Sa*PanK; Green graph: *Sa*CoaBC; Blue graph: *Sa*PPAT and Red graph: SaDPCK (n=1, technical duplicates, error bars = standard deviation).

Taken together, it is clear from all cross-reactivity studies performed in this project that the anti-*Sa*CoaBC antibodies do not cross-react with any of the other antigens and are therefore the most specific. This could possibly be due to CoaBC being the only enzyme of the CoA biosynthesis pathway of *S. aureus* that does not require ATP and therefore does not contain an ATP binding motif (Chapter 1). However, it is important to further investigate this and determine whether it could possibly explain the ability of the antibodies to cross-react with the other enzymes. It is also important to note that the combination of antigen coating concentration and antibody dilution plays an important role in determining whether the antibodies will show cross-reactivity in a specific ELISA format and should also be taken into consideration for future studies with these assays. Although we were not able to precisely quantify the amount of cross-reactivity of each antibody, these findings form a good basis for future studies. Future approaches to investigate the cross-reactivity of these antibodies will be discussed in Chapter 4.

3.4 Conclusion

In this chapter we were able to develop and optimise indirect competition ELISAs for the detection and quantification of each of the enzymes of the CoA biosynthesis pathway of *S. aureus*. Feasible standard curves were obtained for each of the enzymes; however, future optimisation might still be needed after testing these assays with test samples. Although these findings look promising, cross-reactivity of the anti-*Sa*PPAT antibodies is still a cause for concern as this could interfere with the accuracy and reliability of the results obtained from these assays. It is therefore important to pursue future studies to determine why these antibodies show cross-reactivity and also to establish the level of cross-reactivity for each antigen-antibody pair to allow for correction of enzyme quantitation.

Although there was not enough time to test these assays with biological samples from different growth phases of the *S. aureus* bacteria, the results obtained in this study form a good basis for future studies. These assays are completely novel, as antibodies and ELISA kits for the detection and quantification of these enzymes are not commercially available.

3.5 Experimental procedures

3.5.1 Materials

All of the chemicals used in the following experiments were either purchased from Sigma-Aldrich, Merck or Roche or as indicated otherwise. ELISAs were performed in Nunc® Maxisorp 96 well microtitre plates (Thermo Scientific) and spectrophotometric readings taken with the Labsystems Multiskan MS microtitre plate reader (Thermo Scientific).

3.5.2 Biotinylation of antibodies

An important part of the ELISA development process includes the preparation of key components of the assay. This included the biotinylation of the respective antibodies produced as described in Chapter 2 as well as their corresponding antigens.

3.5.2.1 Immunoglobulin (Ig) precipitation

Before antibodies could be biotinylated, the Ig fractions needed to be isolated from the rest of the serum. This was accomplished firstly, by the addition of 2 mL PBS to 1 mL of the day 81 antiserum, followed by the addition of 3 mL of saturated ammonium sulfate ($(NH_4)_2SO_4$) solution. The samples were incubated at 4°C for 20 min and then centrifuged at 27 000 x g for 20 min at 4°C in an Avanti J-E centrifuge (Beckman Coulter). After centrifugation, the supernatant was decanted, and the pellet redissolved in 2 mL PBS. A further 2 mL of the saturated ammonium sulfate was added to the samples and incubated at 4°C for 20 min followed by another round of centrifugation at 27 000 x g for 20 min at 4°C. Lastly, the remaining pellet was redissolved in 1 mL PBS (original serum volume) and dialysed overnight in cellulose membrane tubing (10 kDa size cut-off) at 4°C against two changes of carbonate buffer (50 mM $NaHCO_3$, pH 8.3). This was performed for all antibodies isolated from their respective day 81 rabbit antisera (anti-*Sa*PanK, anti-*Sa*CoaBC, anti-*Sa*PPAT and anti-*Sa*DPCK).

3.5.2.2 Conjugation of biotin to antibodies

After Ig precipitation, the concentration of the Ig fractions was determined spectrophotometrically at 280 nm with the NanoDrop™ Spectrophotometer (Thermo Fisher Scientific) and diluted to a concentration of 5 mg/mL with carbonate buffer. Thereafter, a 2 mg/mL solution of N-(+)-Biotinyl-6-aminocaproic acid *N*-succinimidylester in dimethylformamide (DMF) was added dropwise to the diluted Ig fraction and allowed to stir for 2 hours at room temperature. Lastly, the conjugated antibodies were dialysed in cellulose membrane tubing overnight at 4°C against two changes of PBS. Glycerol was added to the biotylated antibody preparations in a 1:1 ratio and stored at -20°C for subsequent use in all proposed ELISA systems.

3.5.3 Biotinylation of antigen

Similar to the conjugation of biotin to antibodies, the previously produced proteins, as described in Chapter 2, were biotinylated for use in a competition ELISA (3.5.5.1). Unlike biotinylation of the antibodies, the proteins were already purified, and their concentrations known. N-(+)-Biotinyl-6-aminocaproic acid *N*-succinimidylester was dissolved in DMF to produce a 2 mg/mL solution which was added dropwise to 5 mg/mL of the protein and allowed to stir for 2 hours at room temperature. Dialysis of the conjugated proteins was performed in cellulose membrane tubing overnight at 4°C against two changes of PBS. Before storage at -20°C, glycerol was added to the biotinylated protein preparations in a 1:1 ratio.

3.5.4 The DAS ELISA

Firstly, a 96-well microtitre plate was coated with the day 81 rabbit antiserum (100 µL/well) diluted 1:800 with carbonate buffer (50 mM $NaHCO_3$, pH 8.3) and incubated at 4°C overnight. All wells were coated, with the exception of column 12, which served as a negative control. Thereafter, the coating solution was decanted, and the plate blocked with casein buffer (154 mM NaCl, 10 mM Tris-HCl, 0.5% (m/v) casein, 0.02% (m/v) thimerosal, pH 7.6) (200 µL/well) and incubated at 37°C for 1 hour. The blocking step was followed by the addition of a serial dilution (Fig. 3.18) of the respective antigens (*Sa*PanK, *Sa*CoaBC, *Sa*PPAT or *Sa*DPCK) diluted with casein-Tween buffer (154 mM NaCl, 10 mM Tris-HCl, 0.5% (m/v) casein, 0.02% (m/v) thimerosal, 0.1% (v/v) Tween-20, pH 7.6). The serial dilution (100 µL/well) was added to each well in columns 2 to 11 (100 ng/mL – 0.1 ng/mL) and 12 (100 ng/mL). Column 1 did not receive any antigen as it serves as the blank column. After the addition of the antigen dilution series to the plate, it was incubated at 37°C for 2 hours.

For the next step the biotinylated primary antibodies were diluted 1:100 with casein-Tween buffer and added to the plate (100 µL/well) and incubated at 37°C for 1 hour. Thereafter, AVPO was diluted 1:100 with casein-Tween buffer and added to the plate (100 µL/well) after which it was incubated at 37°C for 1 hour. Lastly, substrate solution (0.05% (m/v) ABTS, 0.015% (v/v) H_2O_2, 0.1 M citrate buffer, pH 5) was added to the plate (100 µL/well) and the colour change observed, measuring absorbance values at 405 nm with a Labsystems Multiskan MS microtitre plate reader after 5 min, 15 min and 30 min. Between each incubation step, the previous solution was decanted, and the plate manually washed 5 times with PBS-Tween (0.1% Tween, pH 7.2) wash buffer.

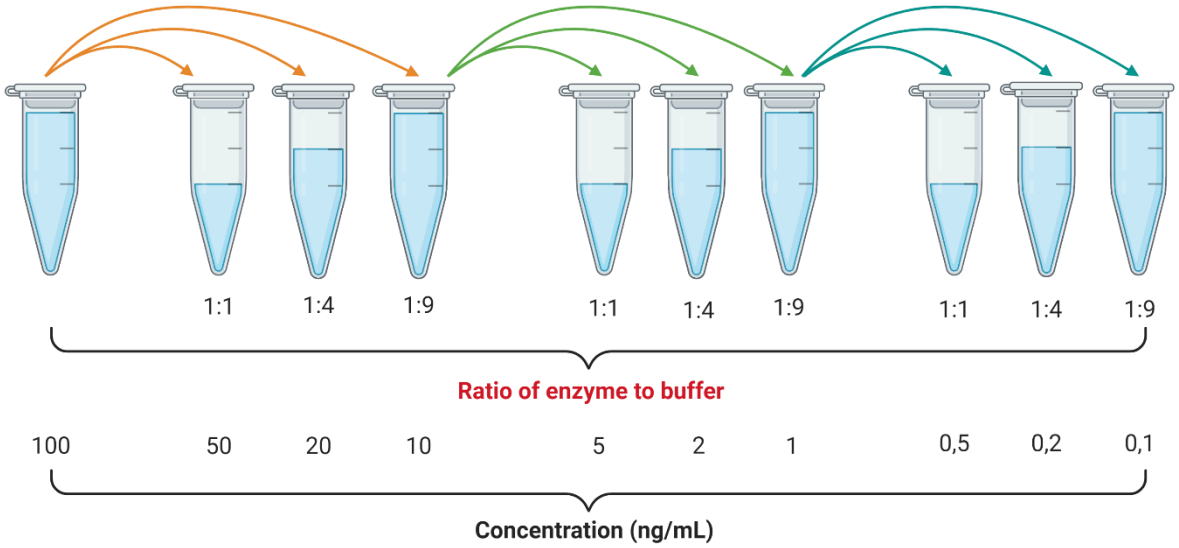

Figure 3.18: Serial dilution preparation of the antigen of interest. A 100 ng/mL stock solution of the antigen was prepared and added in equal volumes to 3 different tubes. Buffer is added to the tubes in a 1:1, 1:4 or 1:9 ratio (antigen solution:buffer). After mixing, this process is continued two more times, resulting in a dilution series ranging from 0.1 ng/mL to 100 ng/mL. This image was created with BioRender.com.

3.5.5 The competition ELISA

The second option for the quantification of proteins is the competition ELISA. In this experiment the competition ELISA was attempted in two different formats to determine which would be more suitable for the purpose of this study.

3.5.5.1 Immobilised capturing antibody

Different rows of the 96 well microtitre plate were coated with different dilutions of the respective day 81 rabbit antiserum (100 µL/well) to determine the optimal amount of capturing antibody needed for quantification. This process was started with a 1:800 dilution followed by 1:400, 1:200 and lastly 1:100, and allowed to incubate at 4°C overnight. Dilutions of the capturing primary antibodies were made with carbonate buffer (50 mM $NaHCO_3$, pH 8.3). Column 12, which served as the negative control, did not receive the diluted antibody solution. Secondly, the capturing antibody solution was decanted, and the plate blocked with 200 µL/well casein buffer (154 mM NaCl, 10 mM Tris-HCl, 0.5% (m/v) casein, 0.02% (m/v) thimerosal, pH 7.6) at 37°C for 1 hour. Thereafter, pre-diluted biotin-labelled antigen solution (1:100) was added to rows 2, 3 and 12 (100 µL/well) and a serial dilution made in the consecutive wells with casein-Tween buffer (154 mM NaCl, 10 mM Tris-HCl, 0.5% (m/v) casein, 0.02% (m/v) thimerosal, 0.1% (v/v) Tween-20, pH 7.6) to determine optimal labelled antigen dilution. Column 1 did not receive any antigen as it served as the blank column. After incubation with the labelled antigen, AVPO was added to the plate (100 µL/well) at a 1:100 dilution and incubated at 37°C for 1

hour. In the last step of the ELISA, 100 µL/well substrate solution (0.05% (m/v) ABTS, 0.015% (v/v) H_2O_2, in 0.1 M citrate buffer, pH 5) was added to the plate and absorbance values measured at 405 nm with a Labsystems Multiskan MS microtitre plate reader (Thermo Scientific) after 5 min, 15 min and 30 min. After each incubation step, the previous solution was decanted, and the plate manually washed 5 times with PBS-Tween wash buffer.

3.5.5.2 Immobilised antigen

The first step of the indirect competition ELISA involves coating the 96-well microtitre plate with the antigen of interest (100 µL/well). Dilution of the antigen samples was made with carbonate buffer (50 mM $NaHCO_3$, pH 8.3) and the plate incubated at 4°C overnight. Column 1 did not receive the antigen solution as it served as the blank column. Different antigen coating concentrations (0.5 – 2 µg/mL) were used in order to determine the optimal coating concentration for the detection of each of the respective antigens (*Sa*PanK, *Sa*CoaBC, *Sa*PPAT and *Sa*DPCK). After the plate was coated with the antigen respective antigens, the antigen solution was decanted and the plate blocked with casein buffer (154 mM NaCl, 10 mM Tris-HCl, 0.5% (m/v) casein, 0.02% (m/v) thimerosal, pH 7.6) to eliminate any non-specific binding.

Thereafter, free antigen (50 µL/well) was added to the wells followed by the addition of either primary antiserum or biotinylated primary antibody (50 µL/well). The antiserum was diluted with casein-Tween buffer (154 mM NaCl, 10 mM Tris-HCl, 0.5% (m/v) casein, 0.02% (m/v) thimerosal, 0.1% (v/v) Tween-20, pH 7.6) and a serial dilution made of the free antigen as illustrated in Figure 3.19. Both the antiserum and the free antigen were diluted to a concentration 2X greater than the intended concentration that is needed on the plate as the addition of equal volumes of both the solutions results in further dilution of both. To find the optimal antiserum dilution needed for each specific ELISA, different dilutions were attempted. The free antigen serial dilution was added to columns 1 (4 µg/mL), and 2 (4 µg/mL) to 11 (0.04 µg/mL) and the antiserum solution added to columns 2 to 12. Therefore, column 1 served as the blank column and column 12 the positive control (no competition). In the case of column 12, casein-Tween buffer was added to the antiserum solution instead of free antigen. Incubation temperature and time were also varied to determine the optimal temperature and time for each assay.

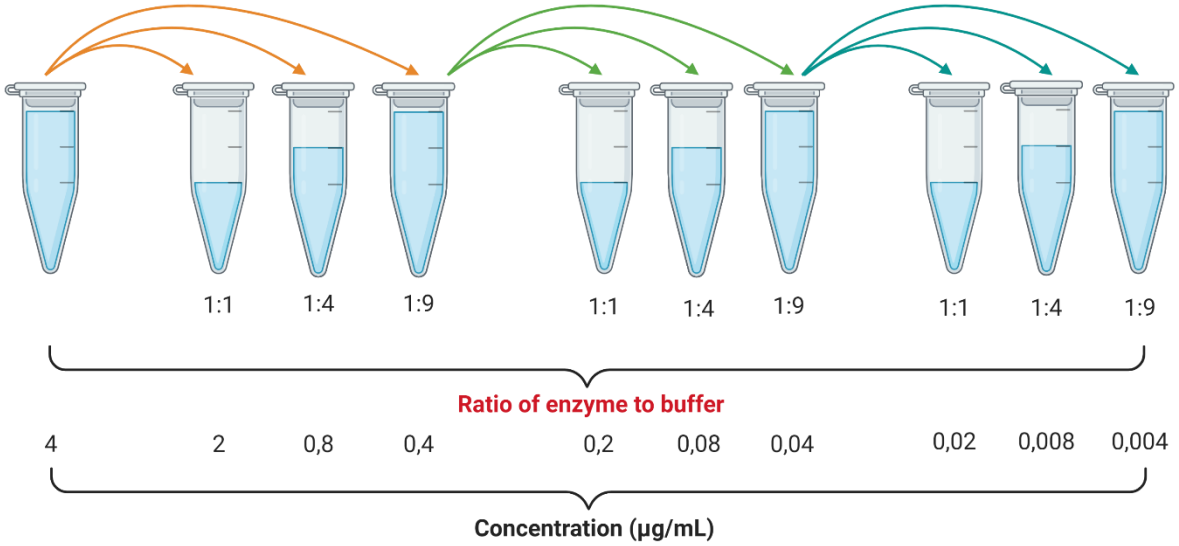

Figure 3.19: Serial dilution preparation of the antigen of interest for an indirect competition ELISA. A 4 µg/mL stock solution of the antigen was prepared and added in equal volumes to 3 different tubes. Buffer was added to the tubes in a 1:1, 1:4 or 1:9 ratio (antigen solution:buffer). After mixing, this process was continued two more times, resulting in a dilution series ranging from 0.004 µg/mL to 4 µg/mL. This image was created with BioRender.com.

Following incubation with the free antigen and the antiserum solution, the plate is incubated with either secondary antibody (1:2000 dilution) or AVPO (1:100 dilution) (100 µL/well) depending on whether the plate was previously incubated with primary antiserum or biotinylated primary antibodies. Dilutions were made with casein-Tween buffer and the plate incubated at 37°C for 1 hour. Lastly, substrate solution (0.05% (m/v) ABTS, 0.015% (v/v) H_2O_2, 0.1 M citrate buffer, pH 5) was added to the plate (100 µL/well) and absorbance values measured at 405 nm with a Labsystems Multiskan MS microtitre plate reader (Thermo Scientific) after 5 min, 15 min and 30 min. After each incubation step, the previous solution was decanted, and the plate manually washed 5 times with PBS-Tween wash buffer.

3.5.6 Antibody cross-reactivity quantification

In an attempt to quantify the amount of cross-reactivity of each antibody to each respective enzyme that is not complementary to the antibody, indirect competition ELISAs as described in section 3.5.5.2 were performed with each of the antisera.

Firstly, separate duplicate rows of the 96 well microtitre plate was coated (100 µL/well) with the four respective antigens (Fig. 3.20) at concentrations as described in Table 3.2. Antigen dilutions were made with carbonate buffer (50 mM $NaHCO_3$, pH 8.3) and the plate incubated at 4°C overnight. Following the coating step, the antigen solution was decanted, and the plate incubated with casein buffer (154 mM NaCl, 10 mM Tris-HCl, 0.5% (m/v) casein, 0.02% (m/v) thimerosal, pH 7.6) at 37°C for 1 hour.

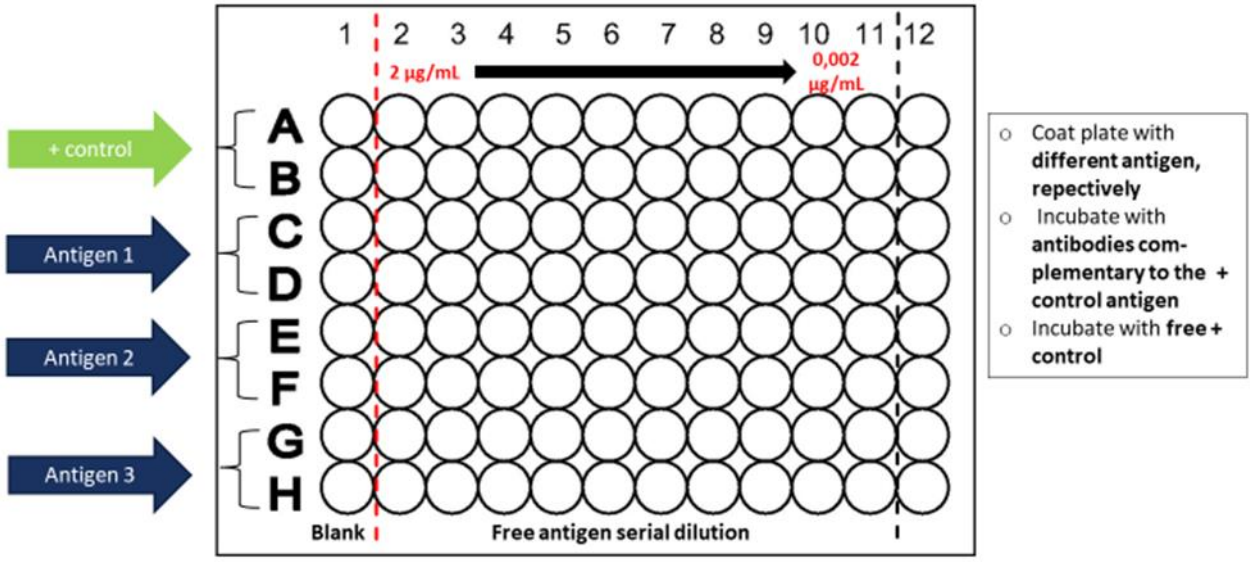

Figure 3.20: ELISA plate layout for antibody cross-reactivity quantification. The positive control indicates the enzyme that is complementary to the antibody being tested and antigen 1 to 3 refers to the remaining three enzymes of the CoA biosynthesis pathway that the specific antibody might be able to cross react with. The free antigen serial dilution range is shown above row A, and the basic steps of the ELISA indicated in the box on the right.

Table 3.5: Optimised ELISA conditions for cross-reactivity quantification of antibodies produced against the CoA biosynthesis enzymes of *S. aureus*.

Ab	Ag[a] [coating]	Ab containing serum	Ab dilution	Incubation Temp.	Incubation Time	Ab detection
Rabbit anti-*Sa*PanK	2 µg/mL	Biotinylated antibodies from day 81 antiserum	1 in 200	4°C	overnight	AVPO
Rabbit anti-*Sa*CoaBC	1 µg/mL		1 in 100			
Rabbit anti-*Sa*PPAT	0.5 µg/mL		1 in 300			
Rabbit anti-*Sa*DPCK	0.5 µg/mL		1 in 250			

a – Antigen (Ag) coating concentration

Free antigen (positive control) was added to the wells (50 µL/well) followed by the addition of day 81 biotinylated primary antibodies (50 µL/well). The biotinylated antibodies were diluted with casein-Tween buffer (154 mM NaCl, 10 mM Tris-HCl, 0.5% (m/v) casein, 0.02% (m/v) thimerosal, 0.1% (v/v) Tween-20, pH 7.6) and a serial dilution made of the free antigen as illustrated in Figure 3.18. Both the biotinylated antibodies and the free antigen were diluted to a concentration 2X greater than the intended concentration that is needed on the plate as the addition of equal volumes of both the solutions results in further dilution of both. The free antigen serial dilution was added to columns 1 (4 µg/mL), and 2 (4 µg/mL) to 11 (0.04 µg/mL) and the biotinylated antibody solution added to columns 2 to 12. Therefore column 1 served as the blank column and column 12 as the positive control (no competition). In the case of column 12, casein-Tween buffer was added to the biotinylated antibody solution instead of free antigen. The plate was then allowed to incubate at 4°C overnight.

Thereafter the plate was then incubated with a 1:100 dilution of AVPO in casein-Tween buffer (100 µL/well) at 37°C for 1 hour. Lastly, substrate solution (0.05% (m/v) ABTS, 0.015% (v/v) H_2O_2, 0.1 M citrate buffer, pH 5) was added to the plate (100 µL/well) and absorbance values measured at 405 nm with a Labsystems Multiskan MS microtitre plate reader after 5 min, 15 min and 30 min. After each incubation step, the previous solution was decanted, and the plate washed manually 5 times with PBS-Tween wash buffer.

3.6 References

1. Yalow, R. S., and Berson, S. A. (1959) Assay of Plasma Insulin in Human Subjects by Immunological Methods. *Nature*. **184**, 1648–1649

2. Aydin, S. (2015) A short history, principles, and types of ELISA, and our laboratory experience with peptide/protein analyses using ELISA. *Peptides*. **72**, 4–15

3. Sakamoto, S., Putalun, W., Vimolmangkang, S., Phoolcharoen, W., Shoyama, Y., Tanaka, H., and Morimoto, S. (2018) Enzyme-linked immunosorbent assay for the quantitative/qualitative analysis of plant secondary metabolites. *J. Nat. Med.* **72**, 32–42

4. Engvall, E., Jonsson, K., and Perlmann, P. (1971) Antiserum , immunoglobulin fraction , and specific antibodies Iodinated proteins. *Biochim. Biophys. Acta.* **251**, 427–434

5. KPL Inc. (2005) *Technical Guide for ELISA*

6. Pan, S., Aebersold, R., Chen, R., Rush, J., Goodlett, D. R., McIntosh, M. W., Zhang, J., and Brentnall, T. A. (2009) Mass spectrometry based targeted protein quantification: Methods and applications. *J. Proteome Res.* **8**, 787–797

7. Camponi, L., and Migliorini, P. (eds.) (1999) *Antibody Usage in the Lab*, Springer-Verlag

8. Diamandis, E. P., and Christopoulos, T. K. (eds.) (1996) *Immunoassay*, Academic Press Inc.

9. Boster Bio *ELISA Handbook*

10. Wilson, K., and Walker, J. (eds.) (2010) *Principles and Techniques of Biochemistry and Molecular Biology*, 7th Ed., Cambridge University Press

11. Crowther, J. R. (2009) The ELISA Guidebook. in *Springer Protocols Methods in Molecular Biology*, 2nd Ed.

12. van Dijk, E., Hoogeveen, A., and Abeln, S. (2015) The Hydrophobic Temperature Dependence of Amino Acids Directly Calculated from Protein Structures. *PLoS Comput. Biol.* **11**, 1–17

13. Deshpande, S. S. (1996) *Enzyme Immunoassays From Concept to Product Development*, 1st Ed., Chapman & Hall

14. Leenaars, M., and Hendriksen, C. F. M. (2005) Critical Steps in the Production of Polyclonal and Monoclonal Antibodies: Evaluation and Recommendations. *Ilar J.* **46**, 269–279

15. Reverberi, R., and Reverberi, L. (2007) Factors affecting the antigen-antibody reaction. *Blood Transfus.* **5**, 227–240

16. Tijssen, P. (1985) Practice and Theory of Enzyme Immunoassays. in *Laboratory Techniques in*

Biochemistry and Molecular Biology (Burdon, R. H., and van Knippenberg, P. . eds), Elsevier

17. Leonardi, R., Zhang, Y. M., Rock, C. O., and Jackowski, S. (2005) Coenzyme A: Back in action. *Prog. Lipid Res.* **44**, 125–153

18. Chard, T. (1982) Introduction to Radioimmunoassay and Related Techniques. in *Laboratory Techniques in Biochemistry and Molecular Biology* (Work, T. ., and Work, E. eds)

19. Johnson, M. (2012) Antibody Storage and Antibody Shelf-life. *Mater. Methods*

20. Abbexa (2021) ELISA Standard Curve. [online] www.abbexa.com (Accessed January 21, 2021)

21. Bio Rad (2017) ELISA Basics Guide. *Valid. Pharm. Process. Third Ed.*

Chapter 4

Conclusions and Future work

4.1 Summary of findings

As discussed in Chapter 1, the CoA biosynthesis pathway holds particular merit as a potential target for antimicrobial drugs as CoA is essential for life in all organisms (1, 2). The inherent diversity between the enzymes of the CoA biosynthesis pathway in different organisms, specifically humans and pathogenic microorganisms, enables the development of selective antimicrobials (3) especially against *S. aureus* which is the leading cause of hospital-related infections (4). Specifically, MRSA strains have been investigated due to their resistant nature to multiple antimicrobial drugs and their ability to evade the host immune system (4). Although much research has already been performed on the CoA biosynthesis of many organisms, there are still many knowledge gaps in this regard. Specifically, in this project an attempt was made to develop a method for determining each of the four CoA biosynthesis enzyme levels of *S. aureus* during different growth stages of the bacteria under physiological conditions.

4.1.1 Production of antibodies against *S. aureus* CoA biosynthesis enzymes

Key aspects of this study were the expression and purification of the four CoA biosynthesis enzymes of *S. aureus* for antibody production and assay development. It was therefore of great importance to obtain pure enzyme as it could greatly affect the outcome of both the antibody production process as well as the success of the optimised assays. Recombinant expression of these enzymes (i.e., *Sa*PanK, *Sa*CoaBC, *Sa*PPAT and *Sa*DPCK) was performed in *E. coli* expression systems followed by Ni^{2+}-based IMAC purification. Sufficient amounts of protein with good purity, as determined via Bradford concentration determination and SDS-PAGE analysis, respectively, were obtained to use for polyclonal antibody production.

Polyclonal antibodies to the CoA biosynthesis enzymes of *S. aureus* were successfully raised in rabbits utilizing the purified enzymes adsorbed to NB, which acted as immune carriers. These antibodies are not commercially available and were therefore produced in this study to be used in ELISA. To our knowledge this is the first study where such antibodies have been produced and they are therefore novel reagents for use in further applications.

4.1.2 ELISA development and optimisation for *S. aureus* CoA biosynthesis enzyme detection and quantification

The polyclonal antibodies produced to the CoA biosynthesis enzymes of *S. aureus* were used to develop and optimise ELISAs for the detection and subsequent quantification of these enzymes. After attempting a number of ELISA formats, it was established that an indirect competition ELISA was best suited for quantifying the CoA biosynthesis enzymes of *S. aureus*. Reliable standard curves with excellent reproducibility were obtained for *Sa*PanK, *Sa*PPAT and *Sa*DPCK, whereas the curves for *Sa*CoaBC might need further optimisation for future use as large error bars were observed. Therefore, more repeats should be performed of the optimised *Sa*CoaBC ELISA with newly purified batches of CoaBC to establish a more reliable standard curve. Due to time constraints the application of the ELISAs in testing enzyme levels in test samples could not be performed.

Characterisation of the antibodies was performed with both ELISA and western blot and included determinations of cross-reactivity. From the cross-reactivity studies performed with western blot analysis, only the anti-*Sa*PPAT antibodies showed some cross-reactivity and were able to detect both *Sa*PanK and *Sa*DPCK to varying degrees. These findings were corroborated with ELISA analysis of the antibodies, which revealed that the anti-*Sa*PanK and anti-*Sa*DPCK antibodies were also showing limited cross-reactivity with the *Sa*PanK, *Sa*PPAT and *Sa*DPCK enzymes of the CoA biosynthesis pathway of *S. aureus*. Both western blot and ELISA analysis confirmed that the anti-*Sa*CoaBC antibodies were highly specific as they were not able to detect any of the other enzymes of the pathway.

Further cross-reactivity studies were conducted with the optimised ELISAs in an attempt to evaluate the effect of the cross-reactivity of each antibody to a specific antigen on the optimised assays. In comparison to what was established with the previous cross-reactivity studies, these results revealed that only the cross-reactivity of the anti-*Sa*PPAT antibodies had an effect on the final optimised assay. However, more repeats of these experiments and testing of CoA biosynthesis enzyme mixtures are required before a conclusion can be made about the usefulness of these ELISAs.

4.2 Future studies

Future studies for the continuation of this project should focus on two key aspects. Firstly, cross-reactivity studies should be conducted to establish why the antibodies cross-react and to establish strategies to minimise the impact of cross-reactivity on the output of the assays. Secondly, samples obtained from *S. aureus* cultures, which would contain many potentially cross-contaminating and inhibiting compounds, need to be tested with these optimised assays and further studies should be

conducted to ensure accurate and reproducible assays. Additionally, other applications of the antibodies produced in this project will briefly be discussed.

4.2.1 Antibody cross-reactivity studies

4.2.1.1 Why do these antibodies cross-react?

Further investigations into the cross-reactivity of antibodies developed in this study are very important as they could greatly affect the outcome of results obtained from the ELISAs. With the cross-reactivity studies it has been determined that with the exception of the anti-*Sa*CoaBC antibodies, the antibodies showed cross-reactivity to varying degrees when analysed with different assays. Thus far we have speculated that the cross-reactivity of the other antibodies (anti-*Sa*PanK, anti-*Sa*PPAT and anti-*Sa*DPCK) could be due to an ATP binding motif that is required by these enzyme antigens for the utilization of ATP. However, the reasons for the cross-reactivity need to be further investigated, of which two methods will be discussed briefly in this section.

The first method in determining the possible cross-reactivity of the antibodies would include performing multiple sequence alignments of both the gene sequences encoding the respective enzymes as well as the amino acid sequences of the different *S. aureus* enzymes. These alignments may be useful for determining regions or motifs within these sequences that are conserved and therefore similar across multiple sequences which could possibly explain the cross-reactivity of these antibodies. The second method, which has gained popularity in the development of vaccines, is the use of B-cell epitope prediction programs (9). As outlined in Chapter 1, antibodies are secreted by different clones of B-cells which produce these antibodies to recognise a specific epitope on the antigen of interest. B-cell epitope prediction programs therefore set out to predict the potential of certain regions of an antigen to act as an epitope that can be recognised by an antibody. These programs could therefore highlight possible regions in the CoA biosynthesis enzymes of *S. aureus* that could potentially be B-cell epitopes. Results for each of the enzymes can be compared to see whether any of these regions might be similar in either sequence or structure which could explain cross-reactivity.

4.2.1.2 Cross-reactivity elimination strategies

In addition to establishing the precise levels of antibody cross-reactivity, there are strategies that could aid in the reduction of possible background cross-reactivity in the production of antibodies against CoA biosynthesis enzymes. As outlined in Chapter 1, it has been established that many factors played a role in successful polyclonal antibody production including the choice of antigen, immunogen dosage as well as the immunisation schedule. Therefore, the specific conditions for each of these factors can be altered to obtain more specific antibodies. Firstly, the rabbits could be immunized with lower quantities of antigens, as this could lead to a secondary immune response in which higher affinity antibodies are

produced (10, 11). Secondly, it typically takes 6 weeks to produce high titre antibodies that could be used in laboratory techniques. However, immunising the animal for longer periods of time with the addition of booster immunisations, could result in more specific antibodies with a higher affinity for the antigen of interest (11). Therefore, future antibody production studies could incorporate the use of lower dosages of antigen administered over longer periods of time to increase the probability of the production of more specific antibodies.

Another possibility would be to identify epitopes that are specific to each CoA biosynthesis enzyme. Peptides could then be synthesized and coupled to larger carrier proteins to render them immunogenic and used for vaccination. Such antibodies would potentially be specific for each of the enzymes and could then be used for the development of completely specific ELISAs for the detection of each of the enzymes of the CoA biosynthesis pathway. However, as the anti-*Sa*CoaBC antibodies showed no cross-reactivity, this approach would only have to be used for the other three enzymes

4.2.2 Additional antibody applications

An interesting prospect would be to determine the potential of these antibodies to detect the CoA biosynthesis enzymes of other organisms. In Chapter 1, it has been discussed that some of the CoA biosynthesis enzymes found in bacteria are very similar across different species. More specifically, the knowledge about the CoA biosynthesis enzymes of *E. coli* (specifically CoaBC, PPAT and DPCK) has been used to successfully identify the CoA biosynthesis enzymes found in *S. aureus* as not much information was available on the enzymes of this species. Furthermore, *S. aureus* has a type II PanK more closely related to the human type II PanK than the type I PanK found in other prokaryotes, such as *E. coli* (1, 12, 13). Therefore, studies can be performed with western blot analysis to determine whether the antibodies raised here are able to detect the enzymes of other organisms, such as *E. coli*, *M. tuberculosis*, *Plasmodium falciparum* and *H. sapiens*, to name a few that are available in our laboratory. The use of these antibodies on *P. falciparum* would be of particular use as none of the CoA biosynthesis enzymes of *P. falciparum* parasites (except PanK) have been identified and characterised. These antibodies could therefore potentially be utilised to validate the presence of these enzymes with western blot analysis in cases where it is difficult to establish whether these enzymes are present in a specific species or sample.

4.2.3 ELISA utilisation and further optimisation

The first step in testing the optimised ELISAs with samples containing potentially cross-contaminating and inhibiting compounds would be to establish a protocol for growing *S. aureus* under physiological conditions as well as establishing the best method for obtaining samples from the bacterial cells. CoA

metabolite studies in *S. aureus* and *E. coli* have been performed previously by our collaborators and therefore protocols for these experiments are available to use as a starting point (5, 6).

As illustrated in Figure 4.1 below, a typical protocol would entail cultivating *S. aureus* as described by Goosen (6) and taking samples during the different growth phases of the bacteria (Fig. 4.1 A). Subsequently, the bacterial samples would be separated from the growth media and washed rapidly by utilizing a vacuum filtration system (Fig. 4.1 B). The metabolism of the cells would be quenched by placing the filter membrane, containing the cells, in an ice-cold extraction solution. Thereafter, the cells would be flash-frozen in liquid nitrogen and disrupted mechanically with glass beads. The samples would then be freeze-dried and resuspended in an appropriate buffer before further analysis (5, 6).

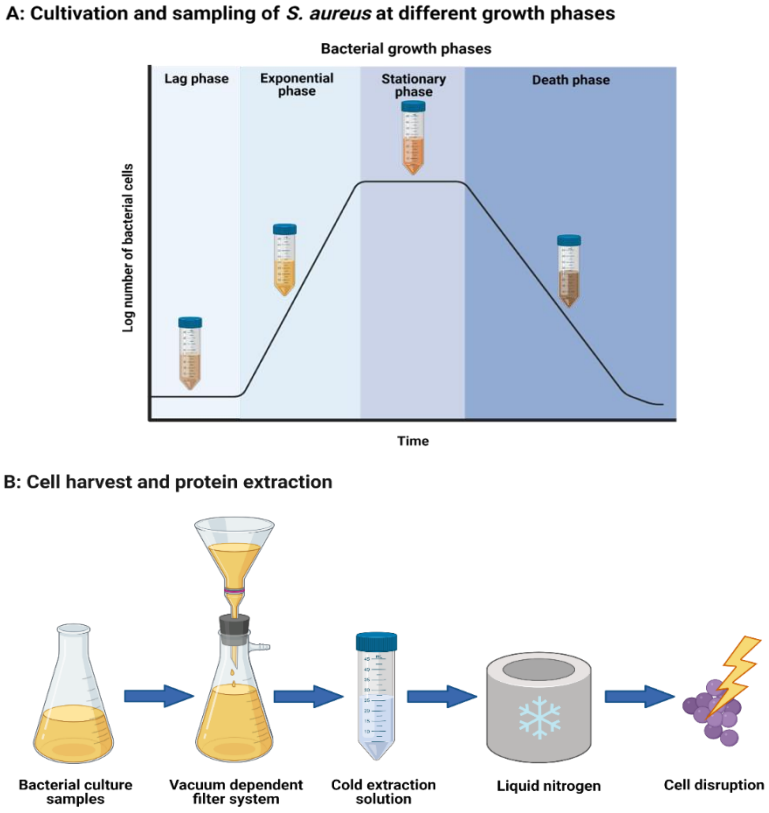

Figure 4.1: Brief overview of the sampling process of CoA biosynthesis enzymes at different growth phases of *S. aureus* cultivated under physiological conditions. A: Firstly, *S. aureus* will be cultivated under physiological conditions and samples taken at each of the different growth phases of the bacteria. **B:** The bacterial cells will then be washed and separated from media by rapid vacuum filtration followed by placing the filter membrane containing the cells in ice cold extraction solution. This step allows for quenching cellular metabolism. Lastly, the cells are flash-frozen in liquid nitrogen and mechanically disrupted whereafter it is freeze dried and stored for future analysis. This image was created with BioRender.com.

Secondly, in the developed assays, spike recovery experiments should be performed to establish whether the sample constituents might have an influence on the antibody binding to the specific antigen.

This could be accomplished by spiking biological samples with a known amount of purified target antigen, which would be subjected to ELISA analysis to determine the concentration of the spiked sample with the standard curve. The concentration obtained from the assay could then be compared to the known concentration of the purified target antigen and therefore be expressed as percentage recovery. If 100% recovery is obtained, it would mean that the concentration determined with the assay was the same as the actual concentration of the spiked protein in the sample, indicating that the other constituents of the biological sample are not interfering with antibody binding to the target antigen. However, if less than 80% recovery is obtained, further optimisation of the assay would be required (7).

Following these tests, the coefficient of variance (CV) should be determined for technical repeats of the assays (intra-assay) as well as the variance between biological repeats (inter-assay) to establish the precision and consistency of the assay (7, 8). The coefficient of variance can be calculated as established in Figure 4.2. General guidelines outline that a percentage CV of less than 15% for inter-assay and less than 10% for intra-assay are considered acceptable (7). Therefore, if these values are exceeded, further optimisation of the assay, as outlined in Chapter 3, will be required.

$$\%\text{CV} = \frac{\text{standard deviation}}{\text{mean}} \times 100$$

Figure 4.2: Calculation for the percentage coefficient of variance (CV) to determine the precision and consistency of a particular assay. This calculation describes the ratio of the standard deviation between repeats to the mean of the repeats, indicating the level of dispersion around the mean.

4.3 Concluding remarks

In this study, novel antibodies were produced and used in both ELISA and western blot analysis. ELISA was found to be a more sensitive assay than western blotting and is therefore better suited for the quantification of the CoA biosynthesis enzymes of *S. aureus* in bacterial cultures. Highly sensitive ELISA methods for the detection and quantification of all four CoA biosynthesis enzymes were developed although ways to eliminate or quantitate cross-reactivity need to be established for their specific quantification. These ELISAs may provide a cost-effective and unique method for the quantification of the enzymes of the CoA biosynthesis pathway of *S. aureus*. Further work is required to optimise the ELISAs for the quantification of these enzymes in biological samples and may provide better insight into the levels of the different enzymes under physiological conditions.

4.4 References

1. Leonardi, R., and Jackowski, S. (2007) Biosynthesis of pantothenic acid and Coenzyme A. *EcoSal Plus*. **2**, 1–28

2. Spry, C., Kirk, K., and Saliba, K. J. (2008) Coenzyme A biosynthesis: An antimicrobial drug target. *FEMS Microbiol. Rev.* **32**, 56–106

3. Moolman, W. J. A., De Villiers, M., and Strauss, E. (2014) Recent advances in targeting coenzyme A biosynthesis and utilization for antimicrobial drug development. *Biochem. Soc. Trans.* **42**, 1080–1086

4. Tong, S. Y. C., Davis, J. S., Eichenberger, E., Holland, T. L., and Fowler, V. G. (2015) *Staphylococcus aureus* infections: Epidemiology, pathophysiology, clinical manifestations, and management. *Clin. Microbiol. Rev.* **28**, 603–661

5. Meyer, H., Liebeke, M., and Lalk, M. (2010) A protocol for the investigation of the intracellular *Staphylococcus aureus* metabolome. *Anal. Biochem.* **401**, 250–259

6. Goosen, R. (2016) *A comparative analysis of CoA biosynthesis in selected organisms: a metabolite study*. Ph.D. thesis, Stellenbosch University

7. Abcam (2018) *ELISA guide: Everything you need to perform your ELISA experiments*

8. Crowther, J. R. (2009) The ELISA Guidebook. in *Springer Protocols Methods in Molecular Biology*, 2nd Ed.

9. Soria-Guerra, R. E., Nieto-Gomez, R., Govea-Alonso, D. O., and Rosales-Mendoza, S. (2015) An overview of bioinformatics tools for epitope prediction: Implications on vaccine development. *J. Biomed. Inform.* **53**, 405–414

10. Drenckhahn, D., Jöns, T., and Schmitz, F. (1993) Production of Polyclonal Antibodies against Proteins and Peptides. *Methods Cell Biol.* **37**, 7–56

11. Stills, H. F. (2012) Polyclonal Antibody Production. in *The Laboratory Rabbit, Guinea Pig, Hamster, and Other Rodents*, pp. 259–274, 10.1016/B978-0-12-380920-9.00011-0

12. Leonardi, R., Chohnan, S., Zhang, Y. M., Virga, K. G., Lee, R. E., Rock, C. O., and Jackowski, S. (2005) A pantothenate kinase from *Staphylococcus aureus* refractory to feedback regulation by coenzyme A. *J. Biol. Chem.* **280**, 3314–3322

13. Leonardi, R., Zhang, Y. M., Rock, C. O., and Jackowski, S. (2005) Coenzyme A: Back in action. *Prog. Lipid Res.* **44**, 125–153