# Impact of *S. aureus* colonisation in human & mouse populations and relationship to vaccination




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Amy Louise Flaxman

St. Hugh's College

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“If it doesn’t challenge you,

it doesn’t change you”

Fred DeVito

## Abstract

*Staphylococcus aureus* (*S. aureus*) is both a commensal and a pathogenic Gram-positive bacterium. Approximately one third of the adult population are asymptomatic nasal carriers of *S. aureus*. Clinical staphylococcal disease can range from skin and soft tissue infections to bacteraemia, often caused by the carriage strain. For this reason, interventions which reduce colonisation should reduce the mortality and morbidity of disease, something which has been proved using antimicrobial decolonisation. An alternative approach to doing this is through vaccination. However, to date, vaccines against *S. aureus* in clinical trials have been unsuccessful, their failure highlighting our lack of understanding of the interactions between the bacterium and the host in both carriage and infection situations. While human carriage studies and animal experimental models are both useful for dissecting out such interactions, the latter represent a platform in which to test pre-clinical vaccine candidates against colonisation.

In this thesis I have investigated the relationship between *S. aureus* colonisation and immune responses against the organism. I have done this both in a human cohort study in a unique confined population, and in a murine model. I present evidence that interactions between *S. aureus* and other species within the microbiome can influence magnitude of immune responses in humans in a confined environment. I have established a novel, long-term murine *S. aureus* gastrointestinal (GI) tract model which is needle-free and does not require antibiotics. I used this model to investigate immune phenotype of colonisation and to assess efficacy of *S. aureus* vaccine candidates in

reducing colonisation. I identified a vaccine candidate which, when administered prophylactically, can reduce subsequent experimental *S. aureus* colonisation in mice.

Through studies of both human and murine colonisation, this thesis increases our understanding of the host-bacterium relationship during colonisation. A new colonisation model is now available for testing vaccines and one vaccine candidate deserves further investigation.

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## List of Abbreviations

| | |
|---|---|
| 4E3 | an anti-IgG1 hinge antibody |
| ACK | Ammonium-Chloride-Potassium |
| AdHu5 | Human Adenovirus 5 |
| AdHu5 | Human Adenovirus 5 |
| ALP | Alkaline Phosphatase |
| BCA | Clf**B**, Bit**C**, Sp**A**, a potential combination *S. aureus* vaccine |
| BCG | Bacillus Calmette-Guérin, a vaccine against tuberculosis |
| BitC | Brachyspira iron transport C homologue, a *S. aureus* antigen |
| BSA | Bovine Serum Albumin |
| CC | Clonal Complex |
| CD | Cluster of Differentiation |
| cfu | Colony forming units |
| ChAdOx | Chimpanzee Adenovirus Oxford |
| ChAdOx1 | Chimpanzee Adenovirus Oxford 1 |
| ClfA | Clumping Factor A, a *S. aureus* antigen |
| ClfB | Clumping Factor B, a *S. aureus* antigen |
| COP | Colonising Opportunistic Pathogens |
| CPS | Capsular polysaccharide(s) |
| CST | Community State Type |
| DMEM | Dulbecco's Modified Essential Medium |
| *E. coli* | *Escherichia coli* |
| EapH | Extracellular adhesion protein homologue, *S. aureus* antigens |

| | |
|---|---|
| EDTA | Ethylenediaminetetraacetic acid |
| EIA | Enzyme ImmunoAssay |
| ELA2 | neutrophil elastase-2 |
| ELISA | Enzyme Linked Immunosorbent Assay |
| ELISpot | Enzyme Linked Immunospot |
| Fc | Fragment crystallisable, of an antibody |
| FCS | Foetal Calf Serum |
| FMO | Fluorescence minus one |
| GALT | Gut-Associated Lymphoid Tissue |
| GFP | Green fluorescent protein |
| GI | Gastrointestinal |
| GSK | GlaxoSmithKline |
| *H. influenzae* | *Haemophilus influenza* |
| HEK293 | Human embryonic kidney 293 cell line |
| HRP | Horseradish Peroxidase |
| IEC | Immune Evasion Cluster |
| IFN | Interferon |
| Ig | Immunoglobulin |
| IL | Interleukin |
| ILC | Innate Lymphoid Cells |
| IM | Intramuscular |
| IN | Intranasal |
| IsdB | Iron-regulated surface determinant protein B, a *S. aureus* antigen |

| | |
|---|---|
| IV | Intravenous |
| LIPS | Luciferase Immuno-Precipitation System |
| LTCB | Long Term Carriage Burden |
| MCMC | Markov chain Monte Carlo |
| MLST | Multi locus sequence typing |
| MntC | Manganese transporter protein C, a *S. aureus* antigen |
| MODREC | Ministry of Defence Research Ethics Committee |
| MRSA | Methicillin resistant *S. aureus* |
| MVA | Modified Vaccinia Ankara |
| MVA | Modified Vaccinia Virus |
| *N. meningitidis* | *Neisseria meningitides* |
| NC3Rs | National Centre for the Replacement, Refinement and Reduction of Animals in Research |
| NGAL | lipocalin-2 |
| OTU | Operational Taxonomic Unit |
| OVA | Ovalbumin |
| *P. acnes* | *Propionibacterium acnes* |
| PBMC | Peripheral Blood Mononuclear Cell |
| PBS | Phospho Buffered Saline |
| PCA | Principal Component Analysis |
| PFA | Paraformaldehyde |
| pNPP | p-Nitrophenyl phosphate |
| *rsp* | Repressor of surface proteins, a *S. aureus* gene |

| | |
|---|---|
| *S. aureus* | *Staphylococcus aureus* |
| *S. epidermidis* | *Staphylococcus epidermidis* |
| *S. pneumoniae* | *Streptococcus pneumoniae* |
| SaF | *S. aureus* Flaxman, clade isolated from mice |
| SaF_1 | *S. aureus* Flaxman, isolate from one mouse used for experimental colonisation |
| sfb | segmented filamentous bacteria |
| SNV | Single nucleotide variant |
| SOPF | Specific and Opportunistic Pathogen Free |
| *spa* | gene encoding Protein A, a *S. aureus* antigen |
| SPSS | Statistics package for social sciences |
| SSTI | Skin and Soft Tissue Infection |
| ST | Sequence type |
| TB | Tuberculosis |
| TCR | T-cell receptor |
| Th | T-helper |
| TMB | Tetramethylbenzidine |
| TPA | Tissue plasminogen activator |
| VISA | Vancomycin intermediate *S. aureus* |
| VRSA | Vancomycin resistant *S. aureus* |
| WGS | Whole Genome Sequencing |

# 1. Introduction

## 1.1 *S. aureus* causes disease

*S. aureus* is a Gram positive bacterium which can cause a range of diseases in humans and other mammals. It has a number of clinical manifestations. At one end of the spectrum, *S. aureus* causes mild skin and soft tissue infections (SSTIs), such as boils. At the other end of the spectrum it is one of the leading causes of bacteraemia and infective endocarditis[1]. Bacteraemia associated mortality ranges from 20 to 40%, depending on age and comorbidities[2]. Treatment involves prolonged intravenous therapy and, if possible, removal of any deep source of infection[1]. *S. aureus* is often associated with wound infection and can cause infections post-surgery in hospital settings. Since it is capable of biofilm formation, *S. aureus* is also often responsible for infection caused by medical devices such as catheters[1]. It is also an important cause of community infection[3]. *S. aureus* causes pneumonia, as it can take advantage of a weakened host after viral infections of the lower respiratory tract, such as influenza[4]. Due to the range of infections caused by *S. aureus*, this bacterium is has a large impact on healthcare in terms of morbidity and mortality.

*S. aureus* can effectively avoid several host defences which allows it to disseminate and cause numerous different clinical outcomes. Staphylococcal Protein A is expressed on the surface of the bacterium and binds immunoglobulins (Ig) by their Fc region. This allows it to deplete B cells and also, along with other proteins, to evade complement-mediated opsonophagocytosis[5]. *S. aureus* is also very efficient at evading neutrophil phagocytosis[4].

## 1.2 *S. aureus* colonises asymptomatically

As well as being a pathogen, causing various types of infection, *S. aureus* is also a commensal and can colonise asymptomatically[6]. In humans, the nares are the most commonly reported carriage site, although carriage in the gut and skin are also well described[7 8]. It is possible that nasal carriage is the source of colonisation for other body sites; higher bacterial load in the nares is associated with increased chance of other areas being colonised[9].

Estimates of nasal colonisation rates in populations vary from 20-50%[10]. However recent studies have shown that adults can be viewed as persistent carriers and non-persistent carriers[11]. This classification revised previously classifications including an 'intermittent carrier' category[10]. Persistent carriers are detectably colonised most of the time in longitudinal studies, and such individuals can be operationally defined as those that have 2 or more positive swabs upon repeat sampling, and those with one or no positive swabs fall into the non-persistent carrier category[12]. Persistent carriers are commonly colonised with the same strain for long periods, but modelling suggests that ongoing acquisition occurs in all humans, with loss rates differentiating individuals with persistent versus non-persistent carriage[13].

Each individual can have different probabilities of colonisation throughout their life. Carriage rates are highest within the first year of life, and then peak again during adolescence, before settling to around 20-30% in adults[10]. Since the first carriage monitoring studies in the 1930s, carriage rates have also fallen, from around 40% to around 25% in 2010[10]. This could be due to the advent of antibiotics and due to increased personal hygiene.

*S. aureus* colonisation is thought be a pre-requisite for infection[14]. Risk of infection is two to 12 times higher in nasal carriers than non-carriers[10]. Persistent carriers tend to get infected with their own nasal strain[15]; studies have shown that nasal and infectious isolates from each patient are commonly almost identical[16].

The mechanisms involved in staphylococcal carriage and disease differ. Clumping factor B (ClfB) is required for *S. aureus* binding to host protein loricrin in squamous epithelium during colonisation[17]. Dissemination of *S. aureus* from colonisation sites to areas in which it causes infection may occur in a number of ways. Cross-contamination from the nares or skin to open wounds may occur. Alternatively, *S. aureus* evades host immune responses to cross epithelial barriers and enter the bloodstream[4]. *S. aureus* can survive inside macrophages and neutrophils and use these as 'Trojan Horses' to gain access to other injured sites, effectively metastasising[18].

The immunological impact of colonisation in human populations has been studied, especially in the context of anti-*S. aureus* antibody titres[19]. Antibodies against various *S. aureus* antigens can be detected across the human population[11], although higher levels of both serum IgG and IgA against certain *S. aureus* antigens have been reported in carriers versus non-carriers[20-22]. Despite induction of these humoral responses, the ability of prior exposure to *S. aureus* to provide protection from subsequent infection is weak at best[19].

A number of human colonisation studies were performed in the 1960s. Neonates were colonised with a low virulence *S. aureus* strain (502A) in the nasal mucosa. This intervention interfered with subsequent acquisition of a second, potentially more

pathogenic strain. It showed promise in protecting children in a nursery setting from colonisation and disease with virulent *S. aureus* strains, which had previously been causing outbreaks in nurseries[23]. In a more recent artificial colonisation study, both carriers and non-carriers were inoculated with a mixture of strains. Persistent carriers selected their original strain whereas non-persistent carriers eliminated the inoculated strains[24]. Another study found that during hospital stays, new strains were more readily acquired in persistent carriers compared to non-carriers[25].

Therefore, both host immunological factors and bacterial factors have a potential role in the establishment and maintenance of carriage. Despite numerous studies investigating *S. aureus* carriage in humans and other mammals, the determinants of carriage are still not defined. It is likely that a number of host, bacterial and environmental factors are involved[19].

## 1.3 Current treatment for *S. aureus* infections

Antibiotics are the major treatment for *S. aureus* infections. However, antibiotic resistance is a major socio-economic problem. In 1928 penicillin was first discovered through its ability to kill *S. aureus*. Today most circulating *S. aureus* strains are penicillin resistant. The rise of methicillin resistant *S. aureus* (MRSA) in the past 30 years means that daptomycin and vancomycin are the antibiotics of choice in the treatment of MRSA[26]. However, since 2000, vancomycin intermediate *S. aureus* (VISA) and increasingly vancomycin resistant *S. aureus* (VRSA) strains are on the rise[27]. Cost, limited efficacy, development of resistance, and a requirement for intravenous therapy all complicate therapy. The lack of new antibiotics coming to market, together with serial acquisitions of high-level antimicrobial resistance[28] means that antibiotic resistance to all available antibiotics is a possible future outcome.

In the UK, patients are often swabbed for MRSA upon hospital admission. If a patient is a carrier, this allows decolonisation procedures to be undertaken, if required, for example, prior to major surgery. These interventions can reduce infection risk in some settings[29]. Despite preventative measures such as these, alternative solutions for the prevention and treatment of *S. aureus* infection are required. This is paramount due to the rise of anti-microbial resistance[27].

Recently, several strategies to improve and enhance current antibiotics have been described. Coupling of vancomycin to an anti-*S. aureus* antibody allows delivery of the antibiotic into the phagolysosome, where it becomes activated and kills intracellular *S. aureus*[30]. Enhancement of vancomycin which allows it to act via

three antimicrobial mechanisms has recently been described, although this has not yet been tested *in vivo*[31].

Various non-antibiotic drugs have been tried and tested for their ability to combat *S. aureus*. These include natural products such as tenuigenin from root of a Chinese herb[32], Manuka honey[33] and date syrup[34]. Even tea has been trialled for reducing MRSA nasal carriage[35]. Green tea in combination with gentamycin has been shown to enhance anti-microbial effect over gentamycin alone for some *S. aureus* strains[36]. The anti-psychotic drug, Thioridazine, has been tested *in vivo* and shown to have anti-staphylococcal ability in a skin infection model[37]. Lysostaphin has shown some efficacy in treating MRSA induced endophthalmitis in a rabbit model[38]. One strategy which builds on one of the natural host defences against *S. aureus* is to use anti-microbial peptides as therapeutics. These peptides are termed "lantibiotics" and a number of them acting in tandem have been shown to have potency against MRSA[39].

Monoclonal antibodies have also been developed for the treatment of *S. aureus*. A monoclonal against mutated Protein A was able to neutralise immunoglobulin binding properties of Protein A and protect mice in a *S. aureus* sepsis model[40]. Further monoclonal antibodies against other *S. aureus* antigens have also been tested in murine models and shown to protect against bacteraemia[41 42]. Arsanis, a biotechnology company, are currently testing a monoclonal antibody treatment which targets multiple virulence factors in Phase II clinical trials for *S. aureus* pneumonia[43].

## 1.4 Rationale for a *S. aureus* vaccine

Despite these advances to improve current treatment strategies, a prophylactic such as an efficacious vaccine against *S. aureus* could prevent numerous cases of MRSA, and therefore be beneficial to society as a whole. Vaccines against other bacterial infections have been very successful in recent years; including vaccines against *Streptococcus pneumoniae* (*S. pneumoniae*) and *Neisseria meningitidis* (*N. meningitidis*)[44].

### 1.4.1 Vaccines in Clinical Trials to date

Despite numerous clinical trials testing vaccines against *S. aureus*, as yet there is no licenced vaccine. Two vaccines have reached Phase III clinical trials. StaphVAX is an unadjuvanted, bivalent vaccine composed of capsular polysaccharides (CPS) types 5 and 8 bound to the mutant non-toxic recombinant *Pseudomonas aeruginosa* exotoxin A. StaphVAX protected mice against bacterial challenge[45] before being tested in human clinical trials. Efficacy was assessed in renal disease patients receiving dialysis; as these patients have decreased resistance to bacterial infection, thus representing a susceptible population. StaphVAX induced partial immunity (approximately 60%) against *S. aureus* bacteraemia for up to 10 months[46]. However, a further FDA-mandated trial of an identical product, which is as yet unpublished, showed no efficacy[44].

More recently, a vaccine targeting Iron-regulated surface determinant protein B (IsdB) has been developed. Pre-clinical tests showed efficacy of this vaccine in a murine *S. aureus* sepsis model[47]. Therefore it moved forward into clinical trials, culminating in a Phase III study in patients undergoing cardiothoracic surgery.

Compared with placebo, the vaccine did not reduce the rate of serious postoperative *S. aureus* infections. In fact, the vaccine was associated with increased mortality among patients who developed *S. aureus* infections[48]. A follow-up study found that low basal IL-2 levels and undetectable IL-17A in patients receiving V710 who went on to develop *S. aureus* disease were associated with increased mortality[49]. The authors hypothesise that since IL-2 can regulate Th17 cells and as IL-17A has been implicated in clearing *S. aureus*, low levels of both cytokines lead to predisposition to fatal *S. aureus* infection[49]. The observation that baseline cytokine levels could affect outcome in a clinical trial should be taken into consideration when planning future trials of *S. aureus* vaccine efficacy. Animal data and Phase I and II clinical data from V710 did not highlight any safety signals.

The failure of V710, together with the huge cost of the ~ 8000 patient multicentre Phase III trial, has led the field of *S. aureus* vaccine research to reflect carefully. As yet, no radical changes in direction are evident. However, transition of the next *S. aureus* vaccine candidate from pre-clinical animal testing into human clinical trials could be complemented by observational cohort studies. Such studies might be able to identify individuals with particular baseline cytokine levels which may make them more susceptible to *S. aureus* infection.

A multivalent vaccine developed by Pfizer has recently been trialled in a first-in-human Phase I/II study. The vaccine, SA4Ag, consists of CP5 and CP8 conjugated to non-toxic diphtheria toxin with recombinant Clumping factor A (ClfA) and recombinant manganese transporter protein C (MntC). A single dose of SA4Ag induced rapid and robust bacteria-killing antibody responses in healthy adults[50 51].

Consequently, a multinational phase IIb efficacy study of this product in spinal surgery patients is ongoing[52].

Numerous pre-clinical *S. aureus* vaccine studies are on-going. GlaxoSmithKline (GSK) have a multivalent vaccine candidate, 4C-staph, which protects mice from *S. aureus* disease in two challenge models[53]. A cell lysate vaccine has recently been shown to induce B and T cells responses and induce protection in three different mouse models[54].

The lack of success in human clinical trials when vaccine candidates perform well in terms of efficacy in murine infection models is concerning and requires further investigation[44]. Unlike other bacterial pathogens, such as *N. meningitidis*[55], there is no correlate of protection for *S. aureus*. Therefore when designing both clinical and pre-clinical trials, both immunogenicity and efficacy are monitored. However, the immune responses which lead to efficacy are not well understood in *S. aureus* disease, and may well be different between mouse and man. One approach is to use multivalent vaccines, to target multiple arms of *S. aureus'* repertoire of mechanisms to avoid attack by the host. This approach is used in some of the examples of vaccines above[50 53].

### 1.4.2 Reducing the carriage burden

The concept that reducing *S. aureus* carriage burden will reduce *S. aureus* disease burden is not new. Studies have shown that decolonisation practices such as bathing in or topically applying chlorhexidine and intranasal delivery of mupirocin results in reduced *S. aureus* colonisation on the skin and in the nares[56 57]. *S. aureus*

decolonisation programs are frequently used as preventative mechanisms for avoiding surgical site infection post-surgery[58]. Decolonisation methods have recently been shown to successfully reduce *S. aureus* burden whilst maintaining other skin and nasal commensals[59]. However, a recent study showed that risk of infection after decolonisation for previously *S. aureus* colonised patients was increased over that in non-colonised patients[60]. Thus alternatives to chemical decolonisation strategies would be welcomed.

One such alternative which could have potential against bacterial pathogens, especially those which are becoming increasingly antibiotic resistant, is to use genetically engineered bacteriophages, which are specific to one bacterial species[61]. Ultimately, a vaccine would present the best solution to successfully decolonise the population of *S. aureus*. This is because it would be specific for *S. aureus* and induce immune responses which protect against subsequent colonisation or infection.

### 1.4.3 Vaccines against colonisation

Vaccines against *S. aureus* tested in clinical phase II/III trials to date, as described in above section (1.4.1), have all been focused on preventing invasive disease; with prevention of disease or reduction in disease severity being the primary endpoint. However, the relationship of V710 efficacy with *S. aureus* colonisation was also considered. In nasal carriers, *S. aureus* infections developed in 3.3% receiving V710 and 5.5% receiving placebo. In non-carriers, *S. aureus* infections developed in 1.6% receiving V710 and 1.8% receiving placebo[48]. In the V710 trial, therefore, infection rates were higher in *S. aureus* nasal carriers compared with non-carriers. Since infection rate was lower in vaccinated carriers compared to placebo-receiving carriers, vaccine-induced immunogenicity may vary depending on whether the patient is a carrier or non-carrier.

Thus, reducing the carriage burden within the population through vaccination against colonisation is a valid strategy for reducing invasive disease caused by *S. aureus*. The ability of StaphVAX to reduce colonisation rates in healthy adults was investigated in a pilot study. However, colonisation rates did not change significantly after vaccination, despite induction of specific antibody[62]. In one mouse study, vaccination with ClfB has been reported to be efficacious in reducing nasal colonisation[63], and the protein's expression impacts clearance rate in man[64].

Vaccines against other bacterial pathogens have resulted in reduction of carriage of vaccine-targeted strains. Conjugate vaccines against *S. pneumoniae*, *N. meningitidis* and *Haemophilus influenzae* (*H. influenzae*) type B protect against carriage of, and disease caused by, their respective bacterial strains[65]. There is a growing body of

evidence that vaccination against one disease or pathogen can impact upon immunogenicity to other commensals or pathogens. Carriage levels of both *S. pneumoniae* and *H. influenzae* were reduced after measles-yellow fever vaccination[66]. Since the introduction of the pneumococcal conjugate vaccine, nasal carriage levels of vaccine strains of *S. pneumoniae* have fallen. However, at the same time *H. influenzae* and *S. aureus* carriage rates have increased. Elimination of certain *S. pneumoniae* strains seems to have opened a niche for other nasal commensals[67 68]. The negative association between *S. aureus* and *S. pneumoniae* has been further investigated in both mice and man. Mice colonised with *S. pneumoniae* resisted colonisation with *S. aureus* through development of a cross-reactive antibody response[69]. Human experimental colonisation with *S. pneumoniae* serotype 6B resulted in lower incidence of *S. aureus* carriage 14 days after colonisation compared to non-colonised controls[70].

Vaccines against other diseases, not only bacterial, but also viral could affect colonisation with bacterial commensals. For example, the live attenuated influenza vaccine enhances colonisation of both *S. pneumoniae* and *S. aureus* in mice. This parallels the increased susceptibility to bacterial infection from *S. pneumoniae* and *S. aureus* after influenza virus infection[71]. Thus, investigation of impact of vaccines against other pathogens on *S. aureus* colonisation would be an interesting area of study. Ideally, this would be done in a small animal *S. aureus* colonisation model.

## 1.5 Models for *S. aureus* colonisation

As well as asymptomatically colonising humans, *S. aureus* also colonises various other mammals; including wild, farmed and companion animals[72]. MRSA outbreaks in pigs and cattle are especially problematic for farmers in Europe. Colonisation in laboratory mice has only been observed within the last 10 years[73] and only within the last few years has the colonisation status of mice used in *S. aureus* research been seriously considered a potential confounder in experimental results[74]. Rodent models of *S. aureus* colonisation existed prior to the discovery of 'natural' colonisation of laboratory mice. However, experimental nasal colonisation of mice with *S. aureus* is normally short-lived (<21 days) and requires antibiotics for success[75 76]. Mice are normally anaesthetised before *S. aureus* is administered into the nares by pipette, much the same as for intra-nasal infection, albeit using a lower inoculum dose. Recently described GI tract colonisation models use starvation and oral gavage[18] or intranasal administration[77], as well as antibiotics prior to *S. aureus* administration.

A murine colonisation model which better represents the human *S. aureus* carriage is desirable. Whilst dynamics of *S. aureus* carriage can be determined through observational carriage studies in human cohorts, a number of limitations exist, particularly the inability to intervene in a timely and cost-effective way. A reliable small animal model would assist with down-selection of vaccine or drug therapies for the much more expensive process of human clinical trials. A model which does not require antibiotics, involves minimum stress to the animal to induce and is long lived (>21 days) is desirable. Establishment of such a model would allow characterisation

of the model to better understand the mechanism, impact and prevention of colonisation.

## 1.6 Host-bacterium interplay

Clinical trials testing vaccines monitor immunological responses induced by the vaccine of interest. This is because vaccine-induced immunity should lead to efficacy in a successfully designed vaccine program. However, in situations where vaccine immunogenicity is easily induced but efficacy is not seen, as is the case for most *S. aureus* vaccines, this highlights a defective understanding of mechanisms of protection. In particular, since *S. aureus* is a commensal to which all humans are exposed, the host immune response to colonisation is of interest. If the interactions between host and pathogen/commensal are better understood, better vaccine design should follow.

Table 1-1 shows different factors which are associated with *S. aureus* nasal colonisation in humans. Host genetics are likely to influence *S. aureus* carriage. Polymorphisms in a number of genes are associated with persistent carriage[78]. The microbiome is a useful study area to help understand interactions between *S. aureus* and other microbiota[79]. As described above in section 1.2, humoral antibody responses against *S. aureus* are easily detectable. Innate immune factors such as antimicrobial peptides may also play a role in controlling colonisation[80]. Adaptation of *S. aureus* to the nasal cavity is also an interesting area for study[16 81]. Environmental factors such as male sex have been shown to be associated with *S. aureus* carriage[82].

**Table 1-1: Factors associated with nasal carriage of *S. aureus*.** Adapted from Mulcahy *et al,* 2016, who comprehensively review the evidence for the observations summarised here[19].

| Factor | Examples | Influence on carriage |
|---|---|---|
| Genetic Factors | SNPs in various genes, such as those coding for IL-4, C-Reactive Protein, Mannose-Binding Lectin | Polymorphisms in these genes have been associated with persistent carriage |
| Bacterial Interference | *S. epidermidis*<br>*S. pneumoniae*<br>Corynebacterium | These species have an adverse effect on *S. aureus* nasal carriage |
| Host Immunity | Innate – Toll Like Receptor2, antimicrobial peptides<br>Adaptive – T cells<br>– Antibodies | Inhibiting TLR2 expression and AMP production reduces colonisation<br>T-cell mediated clearance of nasal colonisation in mouse<br>Patterns differ in carriers & non-carriers |
| Niche Adaptation | Stress tolerance genes | Role in adapting to host colonisation environment |
| Environmental Factors | Age<br>Gender<br>Smoking | Carriage rates differ across age ranges<br>Male sex associated with carriage<br>Smoking has been inversely correlated with carriage |

Human cohort studies are important to study the impact of host-*S. aureus* relationships. Comprehensive human cohort studies could investigate multiple known risk factors as shown in Table 1-1[19]. Bacterial interference, host immunity and environmental factors are investigated in the small human cohort study described in Chapter 3 of this thesis. Genetic factors could be investigated through whole genome sequencing (WGS) of human cohort volunteers and niche adaptation could be investigated through assays to investigate the difference in virulence and other

phenotypes between nasal isolates from human cohort volunteers. However these studies are outside the scope of this thesis.

As described above, host immunology and bacterial components can influence 'colonisability'. Evidence has shown that different bacterial factors (ClfB, wall teichoic acid) are required for colonisation in mouse models[17 77]. To further study bacterial genetic requirements for colonisation, an experimental murine *S. aureus* colonisation model would be useful. Ideally, this model would be established in genetically identical mice to reduce the differences in immune phenotype between hosts.

## 1.7 Aims and Objectives

### 1.7.1 Research Questions

I have two key research questions which I will address in this thesis:

1. What are the characteristics of *S. aureus* carriage in human and murine populations?
   (covered in Chapters 3 to 5)

2. Can vaccination reduce *S. aureus* carriage in a novel *S. aureus* colonisation model?
   (covered in Chapter 6)

### 1.7.2 Research Objectives

Within these two research questions I have a number of defined research objectives which direct the experimental work performed:

1. Investigate *S. aureus* colonisation in a unique human cohort of submariners and its association with skin health, immune markers and nasal microbiome
2. Characterise natural murine *S. aureus* carriage
3. Establish murine *S. aureus* colonisation experimentally
4. Characterise murine *S. aureus* experimental colonisation model
5. Determine bacterial genetic requirements for carriage
6. Assess vaccine efficacy using murine colonisation models
7. Investigate impact of murine experimental colonisation on:
    a. faecal microbiome
    b. immune response to other vaccinations

# 2. Materials & Methods

## 2.1 Materials

**Table 2-1: Details of Materials and Reagents used**

| Material/Reagent | Supplier | Catalogue No. |
|---|---|---|
| **NK1.1-PE (PK136)** | BioLegend | 108708 |
| **2N $H_2SO_4$** | Scientific Laboratory Supplies | CHR3688 |
| **2N NaOH** | Scientific Laboratory Supplies | CHE3368 |
| **5% Salt Broth** | Oxoid (Thermo Fisher Scientific) | EB1296E |
| **ALP-conjugated anti-human IgA** | Sigma-Aldrich | A9669 |
| **ALP-conjugated anti-human IgG** | Sigma-Aldrich | A3187 |
| **ALP-conjugated anti-human IgG1 [4E3]** | Abcam | ab99773 |
| **ALP-conjugated anti-human IgG2** | Abcam | ab99783 |
| **ALP-conjugated anti-human IgG3** | Abcam | ab99828 |
| **ALP-conjugated anti-human IgG4** | Abcam | ab99822 |
| **ALP-conjugated anti-human IgM** | Sigma-Aldrich | A3275 |
| **ALP-conjugated anti-mouse IgA α chain** | Abcam | ab98681 |
| **ALP-conjugated anti-mouse IgG1 heavy chain** | Abcam | ab98690 |

| | | |
|---|---|---|
| **ALP-conjugated anti-mouse IgG2a heavy chain** | Abcam | ab98695 |
| **ALP-conjugated anti-mouse IgG2b heavy chain** | Abcam | ab98700 |
| **ALP-conjugated anti-mouse IgG3 heavy chain** | Abcam | ab98705 |
| **ALP-conjugated anti-mouse IgM µ chain** | Abcam | ab98672 |
| **Ammomium Chloride (NH4Cl)** | Sigma-Aldrich | A-4514 |
| **AP Conjugate substrate kit** | BioRad | 1706432 |
| **Bovine Serum Albumin (BSA)** | Sigma-Aldrich | A3059 |
| **BS24 Agar plates** | Oxoid (Thermo Fisher Scientific) | PO1186A |
| **Carbonate-Bicarbonate Buffer** | Sigma-Aldrich | C-3041 |
| **CD11b-BV650 (M1/70)** | BioLegend | 101259 |
| **CD11c-APC (N418)** | BioLegend | 117310 |
| **CD19-FITC (6D5)** | BioLegend | 115506 |
| **CD28-PE/Cy7 (E18)** | BioLegend | 122014 |
| **CD3-BV605 (17A2)** | BioLegend | 100237 |
| **CD4-PerCP-Cy5.5 (RM4-5)** | BioLegend | 100540 |
| **CD8α-AF700 (53-6.7)** | BioLegend | 100730 |
| **DMEM (Dulbecco's Modified Essential Media)** | Sigma-Aldrich | M-4526 |

| | | |
|---|---|---|
| **EDTA** | Sigma-Aldrich | E7889-100ml |
| **Ethylenediaminetetraacetic acid disodium salt dihydrate ($Na_2EDTA$)** | Sigma-Aldrich | ED2SS |
| **Foetal Calf Serum (FCS)** | Sigma-Aldrich | F7524 |
| **Filter plates for anti-*S. aureus* ELISA** | Merck Millipore | MAGVS2210 |
| **Flat bottom 96 well plates** | Thermo Scientific | 10547781 |
| **gentleMACS M tubes** | Miltenyi Biotech | 130-096-335 |
| **Glycerol** | Sigma-Aldrich | G7757-1L |
| **Halt protease inhibitor kit** | ThermoScientific | 78430 |
| **Horse Blood Agar plates** | Oxoid (Thermo Fisher Scientific) | PB0114A |
| **HRP-conjugated anti-human IgG1 [HP6070]** | Life Technologies | MH1715 |
| **IFN-γ ELISpot ALP Kit** | Mabtech | 3321-2A |
| **L-Glutamine** | Sigma-Aldrich | G-7513 |
| **Lipofectamine 2000** | Invitrogen | 11668-050 |
| **LIVE/DEAD® Fixable Aqua Dead Cell Stain Kit** | Invitrogen | L34966 |
| **Luminex™ Cytokine 20-Plex Mouse Panel** | ThermoFisher Scientific | LMC0006M |
| **Magnesium Chloride (MgCl2 - 6 H2O)** | Fluka Analytical | 63068 |

| | | |
|---|---|---|
| **Milliplex® Map Human Cytokine detection kit** | Millipore | HCYTOMAG-60K |
| **Milliplex® Map Human Cytokine detection kit** | Millipore | HSP3MAG-63K |
| **Milliplex® Map Mouse Cytokine detection kit** | Merck Millipore | MCYTOMAG-70K |
| **Mouse IFN-g/IL-17A FluoroSpot Kit** | Mabtech | FS-4144-10 |
| **NaCl** | Sigma-Aldrich | 204439 |
| **Opti-MEM** | Gibco | 31985-062 |
| **Pen-Strep** | Sigma-Aldrich | P-0781 |
| **Peptide pools for ELISpot** | Mimotopes | Individual orders |
| **Plates for ELISpot** | Merck Millipore | MAIPS4510 |
| **Plates for LIPS assay** | Merck Millipore | MSHVN4B50 |
| **p-Nitrophenyl phosphate (pNPP)** | Sigma-Aldrich | N2770-50SET |
| **Potassium Bicarbonate ($KHCO_3$)** | Sigma-Aldrich | P-9144) |
| **Protein A/G beads** | Thermo scientific | 53132 |
| **Renilla luciferase assay system** | Promega | E2820 |
| **RPMI** | Sigma-Aldrich | R0883-500ml |
| **Staphylase test kit** | Oxoid (Thermo Fisher Scientific) | DR0595 |

| | | |
|---|---|---|
| **Sterile PBS** | Sigma-Aldrich | D8862 |
| **Tetramethylbenzidine (TMB)** | Sigma-Aldrich | T0440 |
| **Tris** | Fluka | 93313-1L |
| **Triton X-100** | MP Biomedicals | 807423 |
| **TruStain fcX™ anti-mouse CD16/32 Antibody** | BioLegend | 101320 |
| **Tween 20** | Fisher BioReagents | BP337-1100ml |
| **V-bottom 96 well plates** | Thermo Scientific | 11897403 |
| **Viscose breakpoint swabs** | Techinical Service Consultants | TS/19-S |
| **γδTCR-BV421 (GL3)** | BioLegend | 118120 |

## 2.2 Methods for Human studies

### 2.2.1 Ethics

The submarine study was approved by the UK Ministry of Defence Research Ethics Committee (MODREC) protocol reference number 0903/228.

### 2.2.2 Study participants and study design

Samples used in this study were collected during a prospective cohort study in UK submariners, undertaken as part of the Royal Navy's occupational health programme. A total of 50 serving Royal Navy submariners provided written consent; a nasal swab and blood sample were taken and a pre-patrol skin health questionnaire (Figure S1), including a baseline skin health diary (Figure S2), was completed between 24 and 72 hours later. While on patrol, submariners completed a weekly skin health diary. A second nasal swab and blood sample were obtained from submariners, by the medical officer on board, before disembarking. Blood samples were separated within 4 hours of sampling and serum was stored initially at -20°C, then at -80°C.

### 2.2.3 *S. aureus* detection by nasal swabbing

Nasal swabbing was performed as previously described[83 84] using viscose breakpoint swabs. Each swab was then snapped off into 2 mL sterile PBS (Sigma-Aldrich, D8862) in bijoux, which were stored and transported at 4°C. All swabs were processed within 4 days and 10 hours of being taken. Each bijoux was vortexed, and 50 µL of sample was spirally plated onto horse blood agar and Brilliance Staph 24 agar plates using Autoplate Automated Spiral Plater (Advanced Instruments, Inc). Plates were read using QCount Automated Colony Counter (Advanced Instruments, Inc) after 24 and 48 hours. A total of 400 µL of sample from each bijoux was also

enriched for 24 hours at 37°C in 5% salt broth before being plated onto Brilliance Staph 24 agar plates as described above. Suspected positive *S. aureus* colonies on Brilliance Staph 24 plates were confirmed using Staphylase Test Kit. All data show bacterial recovery in cfu/mL.

### 2.2.4 Microbiome Analysis

200 µL of sample from each bijou containing a nasal swab (obtained as described above) was stored at -80°C. These samples were shipped to collaborators, led by Robert Skov and Paal Skytt Andersen at the Statens Serum Institut in Copenhagen, Denmark for further analysis. Briefly, our collaborators isolated DNA from each sample and performed MiSeq analysis on the variable V3 and V4 regions of the 16S rRNA as previously described[85]. This provided 30,000 to 40,000 read depth which was subsequently refined to ~10,000. Reads were mapped to operational taxonomic units (OTUs) using BION software. This analysis returned 1475 OTUs and the dataset was further refined as follows: <5 reads were set to 0, everything that could not be annotated to genus level was removed, *S. epidermidis*, *S. aureus*, *P. acnes* and *E. coli* were kept on species level, and all other species were merged to genus level.

The final dataset generated by my collaborators, Paal Skytt Andersen and Berit Lilje, consisted of number of reads for a total of 432 OTUs for each submarine sample. This dataset was used for further analysis.

### 2.2.5 Enzyme Linked Immunosorbent Assay (ELISA) for *S.aureus*

The methodology described here was carried out on the Submarine samples by Julia Whitehouse; a Research Assistant within the *S. aureus* vaccine development group.

All bacterial strains used in this thesis are detailed in Supplementary Table S1.

The assay was performed as described previously[86]. Briefly, 2 x $10^7$ CFU/well paraformaldehyde (PFA)-fixed *S. aureus* (ΔSpA Newman strain) were immobilized on filter plates. Plates were blocked for at least 1hr with 1% BSA/10% FBS/0.1% Tween 20 and incubated overnight at 4°C with different serum concentrations. Plates were washed with PBS with 0.1% Tween 20 using MultiScreenHTS Vacuum Manifold (Merck Millipore, MSVMHTS00) and incubated with various alkaline phosphatase (ALP) or horseradish peroxidase (HRP) conjugated secondary antibodies for 1hr at room temperature (anti-human IgG1 [HP6070], anti-human IgG1 [4E3], anti-human IgG2, anti-human IgG3, anti-human IgG4, anti-human IgG, anti-human IgA & anti-human IgM). After further washing, the relevant substrate was added; (tetramethylbenzidine (TMB) or p-Nitrophenyl phosphate (pNPP). The reaction was stopped by addition of 2N H2SO4 or 2N NaOH, and two thirds of the reaction was transferred into clean 96 well plates. Absorbance was read at 450nm or 405nm, depending on substrate (CLARIOstar®, BMG Labtech). The 4 Parameter Logistic (4PL) nonlinear regression model was used for all ELISA assays for the interpolation from a reference serum, the concentration of which was assigned 100, 000 units per mL (U $mL^{-1}$). Further analysis was performed on $\log_{10}$ transformed data.

### 2.2.6 Cytokine analysis

The methodology described here was carried out on the Submarine samples by Yuko Yamaguchi; a Post-Doctoral Scientist within the *S. aureus* vaccine development group.

Milliplex® Map Kits were used according to manufacturer's protocol, to detect following analytes: IL-1β, IL-4, IL-6, IL-8, IL-10, IL-12p40, IL-12p70, IL-17A, IFNα2,

IFNγ, TNFα, MCP-1, MIP-1α, lipocalin-2 (NGAL), lactoferrin, resistin, thrombospondin, and neutrophil elastase-2 (ELA2). Luminex MAGPIX system with xPONENT software was used for data acquisition and data analysis.

### 2.2.7 Statistical Analysis

Analysis of submarine samples as described above yielded a large dataset including *S. aureus* detection by culture data, microbiome data obtained by 16S sequencing, demographic and skin health data obtained from questionnaires, cytokine data and anti-*S.aureus* isotyping data obtained from serum samples. All of this data was collated and managed using IBM SPSS Statistics version 22.

Subsequent statistical analysis to investigate relatiohships between these datasets was performed using IBM SPSS Statistics version 22, RStudio version 0.99.903 for Windows and GraphPad Prism version 6.03.

To direct my investigation of the relationship between the nasal microbiome profile in submariners and demographic and immunological data, I followed a pre-defined analysis plan:

#### *Pre-defined analyses*

**Objective:** conduct association studies to investigate relationship of *S. aureus* and other nasal microbiome taxa on skin health, serum cytokine levels and serological responses (serum antibody titres against *S. aureus* cell surface).

*S. aureus* culture data from nasal swabs was assessed previously. Isolation by culture is the gold standard method of *S. aureus* detection. Therefore, a sensible primary

analysis was to validate quantitatively whether detection of *S. aureus* by 16S isolation was proportional to isolation by culture.

Next, a description of diversity of the microbiome dataset was planned [87]. To allow comparison of the microbiome profiles from different individuals I planned to assign the microbiome profiles to Community State Types (CSTs), as described previously in similar studies conducted by my Danish collaborators[85]. Further analysis would include investigating differences in microbiome profiles in *S. aureus* carriers, intermittent carriers and non-carriers and allow comparison of microbiome profiles pre and post voyage.

Once the microbiome profiles of each submariner were defined, association studies between microbiome profiles and skin health, serum cytokine profiles and anti-*S. aureus* isotyping were planned

### *Post-hoc analyses*

A number of analyses performed in relation to the submariner cohort were performed after initial exploratory data analysis of the microbiome data. Assignment of a metrics which were used for subsequent analysis was data driven, namely CST match and mis-match groupings and IgG 4E3 high and low. Combination of those metrics with *S. aureus* carrier status was also data driven. Grouping of *S. aureus* intermittent and non-carriers together as 'non-carriers' for comparison purposes when comparing microbiome profiles with skin health, cytokine and isotyping datasets was data driven.

Selection of statistical tests was prespecified to use the most powerful test available given the distribution of the data; the latter was unknown when the plan was written.

Non-parametric tests were planned when deviation from normality was suspected, and parametric tests were planned otherwise. For cytokine fold change where multiple data sets were analysed, not all datasets followed a normal distribution, so non-parametric tests (Mann-Whitney and Kruskal-Wallis) were used (Table 3-8, Table 3-9, Table 3-10, Table 3-12 and Table 3-13). Bonferroni correction was applied for these multiple comparisons, resulting in an altered *p* value for determining statistical significance.

We performed hierarchical clustering of a correlation matrix of cytokine concentrations pre-patrol using the R hclust function and a Euclidean distance metric, and defined three subgroups (section 3.2.5). In subsequent analyses, which examined $\log_{10}$ fold change in cytokines, we analysed properties of the members of these three subgroups ('gene sets') using non-parametric tests, which is an established approach[88]. We prespecified a comparison between carriers and non-carriers of *S. aureus* in our analysis plan. To effect this, we computed for each individual cytokine a metric reflecting the ranks of cytokine levels between carrier and non-carrier individuals; we used the p-value derived from a two-sided Mann-Whitney test with the null hypothesis that it is equally likely that a randomly selected sample from carriers is less or greater than a randomly selected sample from non-carriers. Having done so, we compared the distribution of p-values in the three groups of cytokines identified using a Kruskal-Wallis test with Dunn's correction for multiple comparisons.

When individual cytokines were further assessed (Figure 3-13), and those data sets were normally distributed, parametric analyses were used.

## 2.3 Methods for Murine studies

### 2.3.1 Ethics

All mouse procedures were conducted in accordance with the Animal (Scientific Procedures) Act 1986 (UK Home Office Project licences 30/2825 & 30/3385) and were approved by the University of Oxford Animal Care and Ethical Review Committee.

### 2.3.2 Animals

All mouse strains used in this thesis are detailed in Supplementary Table S2.

6 week old female BALB/c mice (both certified *S. aureus*-free and colonised) were obtained from Harlan (Blackthorn, UK). Certified *S. aureus*-free 6 week old specific and opportunistic pathogen free (SOPF) female BALB/c mice were obtained from Taconic (Ejby, Denmark). 6 week old female C57BL/6 mice were obtained from Harlan. 6 week old CD1 mice were obtained from both Envigo (formerly Harlan) and Charles River (Harlow, UK). On arrival, animals were randomly distributed into individually ventilated cages, housed in groups of 3, 4, 5 or 6 and fed and watered ad libitum. For neonatal experiments, pregnant C57BL/6 mice were obtained from Harlan; 6 week old BALB/c (*S. aureus*-free) male and female mice from Harlan were mated and bred in house. An in-house breeding colony of BALB/c mice originating from Jackson Laboratories, USA prior to 2006 was also investigated.

### 2.3.3 *S. aureus* identification and carriage monitoring

Stool samples were collected from individual animals to isolate *S. aureus* and monitor carriage levels. In all experiments, each mouse was transferred from its cage to a clean container until it produced a fresh stool sample. This minimised potential

contamination from other mice or the environment. Stools were weighed, homogenised in 500µl sterile PBS and plated onto Brilliance Staph 24 (Oxoid Ltd, Basingstoke, UK) agar plates using an Autoplate® Automated Spiral Plater (Advanced Instruments, Inc., Norwood, MA, USA) or by hand. Plates were read using QCount Automated Colony Counter (Advanced Instruments, Inc) or manually after 24 hour incubation at 37°C. In parallel, homogenised stools were enriched for 24 hours at 37°C in 5% salt broth (Oxoid Ltd) before being plated onto Brilliance Staph 24 agar plates as above. Suspected positive *S. aureus* colonies on Brilliance Staph 24 plates were confirmed using Staphylase Test Kit (Oxoid Ltd).

Cheek and nasal swabbing in mice were performed with a viscose breakpoint swab (Technical Service Consultants Ltd, Heywood, UK) pre-wetted with sterile PBS and rubbed inside each cheek or across the nares. Swabs were streaked onto Brilliance Staph 24 agar and enrichment of swabs was carried out as described above. Nasal washes were performed post mortem in a small number of animals using 1ml PBS and *S. aureus* recovery determined as above. Distribution of *S. aureus* throughout the GI tract and in nasal tissue was quantified in some animals by homogenising tissues in PBS using a gentleMACS Dissociator (Miltenyi Biotech, Germany). Further processing (plating and enrichment) was performed as described above for stool samples.

*S. aureus* bacterial recovery from stools and organs was quantified as colony forming units (cfu)/g, from nasal washes as cfu/ml, and from cheek swabs as cfu/swab. The detection limit for stools was estimated as 380cfu/g (1cfu/50µl of average stool mass 0.0263g in 500µl PBS). Detection limit for cheek swabs was 5cfu/swab, nasal washes

5cfu/ml and organs 380cfu/g. For the purposes of depiction, culture negative samples were plotted below the detection limit (dashed line), at 100cfu/g, while samples positive upon enrichment only were plotted at the detection limit.

### 2.3.4 Genotyping of *S. aureus* isolates

#### *spa typing and resistotyping*

spa typing of single *S. aureus* isolates was performed as previously described[89], except that PCR products were purified using QIAquick PCR Purification Kit (Qiagen, Manchester, UK) and sequencing was performed by Source Bioscience (Oxford, UK). Resistotyping was performed according to EUCAST recommendations[90] by Nicola Gordon.

#### *Whole genome sequencing*

This was performed by Nicola Gordon. DNA was extracted from four isolates, derived from the stools of different mice, which were sequenced using Illumina technology as previously described[91].

#### *MLST typing from Whole genomic sequencing*

This was performed by David Wyllie. De novo assemblies were performed using Velvet[92]. Consensus sequence corresponding to *S. aureus* MLST loci were extracted using tblastn[93] from Velvet assemblies and compared with sequences present in PubMLST (http://pubmlst.org) in order to assign a multilocus sequence type (MLST) to the newly identified strain.

#### *Phylogeny construction from single nucleotide variants (SNVs)*

This was performed by David Wyllie and Jessica Hedge. SNVs were identified across all mapped non-repetitive sites using a previously described approach[94] involving

SAMtools[95]. Mpileup with a consensus of at least 75% was required to support an SNV, and calls were required to be homozygous under a diploid model. Only SNVs supported by at least five reads, including one in each direction, which did not occur at sites with unusual depth, were accepted. Maximum likelihood trees were estimated from the mapped whole genomes, together with a collection of ST15 *S. aureus* isolates from a previously described collection representing global *S. aureus* diversity[96] using PhyML[97] with a Jukes–Cantor model. Evidence of recombination was identified using ClonalFrameML[98], and likely times of divergence between the 4 isolates from mouse stool and human ST15 strains were estimated using BEAST. Sampling dates were incorporated and the clock rate fixed to 2.72x10-6, as reported previously[16], since from the small sample of data available there was limited clock signal, as estimated by root-to-tip regression using Tempest (http://tree.bio.ed.ac.uk/software/tempest/). MCMC traces with effective sample size (ESS) >200 were obtained in each of 4 replicate runs, which all converged to the same distribution.

### *Investigation of gene content*

This was performed by David Wyllie. To assess presence/absence of a series of genes, single reads were mapped to selected accessory and core genome gene sequences (Table S3) using Bowtie 2 with the very-sensitive option. We also mapped to control sets comprising RecA and MLST loci (yqil, tpi, pta, gmk, glpf, aroe, arc). Numbers of reads mapping were counted using Samtools[95]. An estimated coverage metric (reads mapped/gene length) was calculated for each gene, and genes were regarded as absent if gene coverage was less than 10% of the median coverage for the control set of genes, using custom R scripts (R version 3.1.1 for Windows).

### 2.3.5 Co-housing

Certified *S. aureus*-free and colonised BALB/c mice from Harlan were obtained and stool samples were taken as described above to determine baseline carriage levels. Animals were then co-housed; in four cages two animals in each cage were initially *S. aureus*-free and two animals were colonised. Two control cages containing only *S. aureus*-free or only colonised animals were also included. Stool samples were taken throughout to monitor carriage levels.

### 2.3.6 Experimental colonisation by Oral gavage

BALB/c female mice (Taconic Farms, Denmark) were experimentally colonised with *S. aureus* strain termed 'SaF_1'; an isolate obtained from a stool sample of one of the 4 BALB/c mice (Harlan, UK),which were sequenced as described above. Three to four individual colonies of SaF_1 were picked and cultured overnight in TSB (Tryptic Soy Broth, Oxoid Ltd) at 37°C with shaking at 130rpm followed by 1:100 dilution into fresh TSB to culture for 2.5 hours at 37°C, without shaking. Bacteria were washed and resuspended in PBS. Mice received either SaF ($10^8$ cfu in 100µl PBS) or PBS via an oral feeding tube (Instech, Plymouth, PA, USA) and stool samples were subsequently taken as described above to monitor carriage levels.

### 2.3.7 Experimental colonisation by environmental contamination

Certified *S. aureus*-free BALB/c mice from Harlan, C57BL/6 from Harlan and CD1 mice from Envigo and Charles River were experimentally colonised with *S. aureus*. SaF_1 or USA300 was prepared by overnight culture in TSB (Oxoid) at 37°C, 130rpm, washed and resuspended in PBS (Sigma). Contamination of cages was performed by spraying this inoculum onto bedding using a 100ml plastic spray bottle with a hand

pumped vaporiser (product 215-3092, VWR International). Each cage received 5-10ml of *S. aureus* culture at ~5 x $10^9$ cfu/ml. Mice were not sprayed directly, and cages were not cleaned until at least 7 days after spraying.

### 2.3.8 Anti-*S. aureus* antibody detection by ELISA

Serum was obtained from venous blood samples by centrifugation and stored at -20°C. Stool and nasal tissue homogenate and nasal washes were prepared as described above (*S. aureus* identification and carriage monitoring).

Anti-*S. aureus* Ig antibodies were detected from the 4 sample sources listed above. The assay was carried out as described above (Enzyme Linked Immunosorbent Assay (ELISA) for *S.aureus*). Paraformaldehyde fixed *S. aureus* ΔSpa Newman strain was used as a ΔSpa SaF strain was not available. ALP conjugated polyclonal goat anti-mouse secondary antibodies to IgA α chain, IgM μ chain, IgG1 heavy chain, IgG2a heavy chain, IgG2b heavy chain, IgG3 heavy chain (AbCam) were used. Optical density was measured at 405nm. All data shown is for 1:50 serum dilution minus background (mean + 2SD of no serum).

### 2.3.9 Luciferase Immuno-Precipitation System

Luciferase Immuno-Precipitation System (LIPS)[99] was used to measure serological responses to individual *S. aureus* antigens after colonisation or vaccination (or both).

Briefly, plasmids expressing individual *S. aureus* antigens fused to renilla luciferase were generated, transfected into HEK293 cells and cell lysates stored at -80°C. Mouse serum was serially diluted in Buffer A (50mM Tris, 100mM NaCl, 5mM MgCl2, 1% Triton X-100) and incubated with relevant cell lysate for at least 1 hour. Serum-lysate mixture was then added to Protein G UltraLink Resin beads in filter plates. After a

further 1 hour incubation and subsequent washing, assay substrate was added (Renilla luciferase assay system). Chemoluminescence was measured in a Luminometer (CLARIOstar®, BMG Labtech). Luminescence data were log transformed prior to analysis. Background luminescence (no sera) was subtracted from luminescence values with sera added to give specific luminescence.

### 2.3.10 Flow Cytometry to determine cellular response to *S. aureus* carriage

Percentages of leukocyte populations from peripheral blood samples were determined by flow cytometry. Lateral tail vein bleeds were taken into 10mM EDTA. Leukocytes were isolated from mouse blood using ACK buffer (0.15M $NH_4Cl$ , 1mM $KHCO_3$ & 0.1mM $Na_2EDTA$ in molecular biology grade water) to lyse red blood cells. For surface staining, cells were stained with LIVE/DEAD® Fixable Aqua Dead Cell Stain Kit, blocked using TruStain fcX™ anti-mouse CD16/32 antibody, stained with monoclonal antibodies against mouse CD19-FITC (6D5), NK1.1-PE (PK136), CD4-PerCP-Cy5.5 (RM4-5), CD28-PE/Cy7 (E18), γδTCR-BV421 (GL3), CD3-BV605 (17A2), CD11b-BV650 (M1/70), CD11c-APC (N418) and CD8α-AF700 (53-6.7). Data acquisition was performed using an LSRII (BD Biosciences) and data were analysed with FCS Express version 5 (De Novo, CA, USA). Full details of the panel used are shown in Figure S4A. Analysis gates were defined using fluorescence minus one (FMO) controls, as shown in Figure S4B. Cellular populations investigated shown in Figure S4C. Comparisons between *S. aureus* colonised and non-colonised animals performed using Mann-Whitney test with Bonferroni correction (25 analyses performed; 3 shown in Figure 5-8 and Figure 5-9).

### 2.3.11 Cytokine Analysis of *S.aureus* carriage

Serum samples and nasal tissue homogenate were prepared as above (Enzyme Linked Immunosorbent Assay (ELISA) for *S.aureus*). Luminex™ Cytokine 20-Plex Mouse Panel LMC0006M was used according to manufacturer's protocol, to detect cytokines present in nasal tissue homogenate. Milliplex® Map Kit MCYTOMAG-70K was used according to manufacturer's protocol, to detect cytokines present in serum. Comparison of analytes present in the 2 different kits used is found in Table S4. Different kits were used due to discounts available from manufacturers at the time of completion of each assay. Luminex MAGPIX system with xPONENT software was used for data acquisition and data analysis.

### 2.3.12 *S. aureus* infectious challenge models

To determine the protective effect of colonisation or vaccines I used 2 murine infectious challenge models.

#### *Intravenous infection model*

This model is based on that established by Cheng *et al*[100]. Mice were challenged with ~$10^7$ CFU *S. aureus* bacterial suspension in 0.1 mL PBS injected into the lateral tail vein. Mice were weighed and monitored daily for signs of illness using a scoring system adapted from Langford *et al*[101] Bremell *et al*[102] and shown in Table S5. Three days post infection mice were sacrificed and kidneys were harvested.

#### *Intranasal infection model*

This model is based on that described by Lijek *et al*[69]. Anaesthetised mice were challenged with ~$10^7$ CFU *S. aureus* bacterial suspension in 0.1 mL PBS administered dropwise into both nostrils. Mice were monitored at 4 timepoints in the 24 hours

following intranasal challenge for signs of illness using the scoring system described above (Table S5). 24 hours post infection mice were sacrificed and lungs harvested.

### 2.3.13 Bacterial recovery post infection

Organs harvested at post mortem were homogenised in PBS using GentleMACS dissociator. The homogenate was serially diluted in PBS and spirally plated onto horse blood agar (HBA) plates using using an Autoplate® Automated Spiral Plater. Plates were incubated overnight at 37°C and colonies were counted using QCount Automated Colony Counter. Data is presented as cfu/g for each organ.

### 2.3.14 Generation of vaccines

Viral vectored vaccines were designed to express *S. aureus* antigens ClfB, BitC, SpaM, EapH1 and EapH2 by David Wyllie. In Chapter 6 of this thesis, viral vectors expressing ClfB alone; ClfB, BitC & SpaM; EapH1 & EapH2; BitC & ClfB; and BitC, ClfB, EapH1 & EapH2 were used. Table S6 details *S. aureus* antigens used in viral vector vaccines and Table S7 details which viral vectors were used in each vaccination study in Chapter 6.

The methodology described in this paragraph was performed by Pauline van Diemen; a Post-Doctoral Scientist within the *S. aureus* vaccine development group, and Elizabeth Allen; a Research Assistant within the *S. aureus* vaccine development group. During the course of my DPhil studies, I have generated viral vectored vaccines expressing other individual *S. aureus* antigens which have not yet been tested in mouse models, see Limitations. Briefly, DNA sequences encoding vaccine constructs were human codon optimized, synthesised (ThermoFisher) with in-frame 5' Human Tissue Plasminogen Activator (TPA) leader signal sequence and a 3' V5

epitope tag and IMX313 oligomerization domain[103] and subcloned using restriction enzymes into a pMono2 mammalian expression vector (patent WO2014053861A2).

The methodology carried out as described in this paragraph was performed by the Viral Vector Core Facility, Jenner Institute, University of Oxford. For Adenovirus (AdHu5) vaccines antigen constructs or pMono2 backbone (for control vaccine) were subcloned from pMono2 into pAdH5-PL-pDEST shuttle vector using Gateway technology (Life Technologies, UK), linearized by PacI and transfected into replication-deficient adenovirus human serotype 5 (AdHu5) as described elsewhere[104 105]. For ChAdOx vaccines, antigen constructs or pMono2 backbone (for control vaccine) were subcloned from pMono2 into pAdH5-PL-pDEST shuttle vector using Gateway technology (Life Technologies, UK), linearized and transfected into replication-deficient chimpansee adenovirus (ChAdOx1) as described elsewhere[104 105]. For Modified Vaccinia Ankara (MVA) vaccines, antigens or pMono2 backbone (for control vaccine) were transferred into an MVA shuttle vector by restriction digestion, linearized with AatII and transfected into MVA as described[104 105]. All inserts were confirmed by Sanger sequencing.

### 2.3.15 Administration of vaccines

#### *Intramuscular vaccination*

Anaesthetized mice received 50µl dose of PBS alone or PBS containing $10^9$ IU AdHu5 expressing *S. aureus* antigen or no antigen via an intramuscular injection. 8-10 weeks later mice received a boost vaccination comprising 50µl dose PBS alone or PBS

containing $10^7$ PFU MVA expressing *S. aureus* antigen or no antigen via an intramuscular injection.

#### *Intranasal vaccination*

Anaesthetized mice received 100µl intranasal dose of PBS alone or PBS containing $10^9$ IU AdHu5 or $10^7$ PFU MVA expressing *S. aureus* antigen or no antigen. This administration was performed dropwise into both nostrils and appeared well tolerated, adverse events (raised respiratory rate, ruffling, and limitation of movement) were not observed in 24 hours close observation. Depending on the individual experimental design, as detailed in results and figures, mice received either a single vaccination, or two vaccinations consisting of AdHu5 prime followed 28 days later by an MVA boost.

### 2.3.16 ELISpot

Murine IFN-γ producing PBMCs were assessed by ELISpot assay after vaccination with EapH1_H2. High Protein Binding Immobilon-P membrane plates were coated overnight with 5 mg/mL anti-mouse IFN-γ, washed and blocked. Individual mouse peripheral blood samples were treated with ACK lysis buffer to remove RBCs and resuspended in a minimum required volume of DMEM supplemented with 10% heat-inactivated FCS, 100 U penicillin, 100µg streptomycin, 4mM L-Glutamine. PBMCs numbers were counted and back calculated for the analysis. PBMCs were stimulated with 5µg/mL of peptides in the presence of splenocyte suspension ($5x10^6$ cells/mL). Peptides consisted of overlapping strings of 20 amino acids spanning EapH1 and EapH2 portions of the immunogen (ordered from Mimitopes, UK). Plates were incubated overnight at 37°C, 5% $CO_2$. IFN-γ spot forming cells were visualised by 2h

incubation with rat anti-mouse IFN-γ biotin (1μg/ml) followed by 1h incubation with streptavidin-Alkaline Phosphatase (1μg/ml) and developing with AP conjugate substrate kit with washing steps between each incubation. Number of spot forming cells was counted with an ELISpot reader (AID, Germany). For graphical presentation, number of IFN-γ producing cells was calculated as number of spot forming cells in the presence of peptides minus number of spot forming cells without peptides. Any values of IFN-γ producing cells <1 were set to 1 for graphical representation. $Log_{10}$ IFN-γ producing cells was used in statistical analysis because of the approximate log-normal distribution of ELISpot counts in the mice.

### 2.3.17 Statistics

Statistical analyses were carried out using IBM SPSS Statistics version 22, RStudio version 0.99.903 for Windows or R version 3.1.1 for Windows and GraphPad Prism version 6.03.

Grouped data are presented as means with SEM, and scatterplot data presented with linear regression and 95% confidence intervals (CIs), unless otherwise indicated. Statistical significance of variations in continuous variables by group was analysed by Mann-Whitney or Kruskal-Wallis tests (for skewed data) or t-tests or ANOVA (for normally distributed data) as stated in results. For comparisons across multiple groups, Dunn's multiple comparisons test was used for skewed data and Holm-Sidak multiple comparisons test was used for normally distributed data. These corrections for multiple comparisons were recommended by GraphPad Prism. Comparison of time to endpoint used log-rank (Mantel-Cox) tests.

# 3. *S. aureus* colonisation and microbiome profiling in submariners: an observational cohort study

## 3.1 Introduction

Numerous observational cohort studies investigating *S. aureus* nasal colonisation have been performed. These range from cross-sectional studies, typically performed in healthy populations[106] to long-term follow up studies in particular at risk or disease associated cohorts[13 107 108]. Taken together, these studies show that *S. aureus* is a component of human nasal microbiome in about 1/3 individuals. Consequently, *S. aureus* falls into a class of bacteria which, while commonly part of the nasopharyngeal flora, less commonly lead to severe illness, a group recently described as Colonising Opportunistic Pathogens (COPs)[6].

In recent years microbiome studies have illuminated the importance of our resident microbes as an additional organ which can influence immune responses and susceptibility to invading pathogens[109]. Studies typically focus on gut microbiome, but the nares represent a diverse microenvironment of which sampling is much easier[110]. The number of taxa present in nares is lower than that of the gut but greater than that of the skin[87]. Whilst *S. aureus* is known to colonise the gut in human populations[8], *S. aureus* carriage in the nares is much more commonly studied. The nares are thought to be the primary colonisation site in humans[111].

Several studies have been performed which investigated the microbiome in relation to *S. aureus* carriage. Burnham and colleagues studied the effect of decolonisation in both community and hospitalised subjects. They show that while decolonisation reduced *S. aureus* levels in the nares, other components of the nasal microbiome remained unchanged[59]. A Danish twin study published recently investigated the nasal microbiome in monozygotic and dizygotic twins[85]. This study concluded that

nasal microbiome composition is determined by the environment, but that genetics can influence bacterial density. Based on the patterns of taxa observed, it was proposed that *S. aureus* colonisation can be determined by specific taxa, such as lactic acid bacteria. However, for other species, a certain threshold of absolute abundance is associated with *S. aureus* presence or absence. For example, absolute reads of *Dolosigranulum* greater than $1.2 \times 10^6$ was a predictor of *S. aureus* absence.

Taken together, these microbiome studies offer important insights into the composition of the nasal microbiome in the context of *S. aureus* carriage, but leave many questions unanswered. Although longitudinal cohort studies have investigated stability of *S. aureus* isolation over long periods[13], much less is known about the rest of the nasal microbiome; we do not know whether that too is relatively stable over time. Alternatively, as with other ecological settings, 'invasive species' or communities of commensal or pathogenic bacteria could exist, which, given adequate exposure to other human nasal flora, would establish colonisation. This seems likely, as study of *N. meningitidis*, another COP[112], has clearly demonstrated strain acquisition and disease given appropriate exposure[113]. We do not know whether *S. aureus* gain/loss events observed in longitudinal cohort studies[107] are associated with disruption of the nasal microbiome, nor whether particular patterns of microbes are stable and others are not. Finally, we do not understand the contribution of immune selection to the unidentified environmental factors controlling the nasal microbiome[85]. Thus, longitudinal monitoring of the whole nasal microbiome is an important area for study.

The confines of a submarine represent a unique environment in which to investigate some of these issues. The interior of a submarine is a confined space within which a crew resides, often at very close quarters, for a prolonged period. This environment offers the opportunity for transfer of potentially 'invasive flora' from one human nasopharynx to another. I have previously investigated whether *S. aureus* colonisation was associated with dermatitis in a submarine population[84], through assessment of questionnaires and culture of nasal samples. In this study, blood and nasal culture samples were taken immediately before the voyage and at redocking, but before emergence of personnel from the submarine. Thus, changes in microbiome, serological and other immune parameters could be evaluated before and after a six week patrol. Other members of my research group have investigated anti-*S. aureus* isotypes in serum samples in this cohort[114].

This prospective cohort study provided me with the opportunity to analyse a number of parameters, including the nasal microbiome, in submariners during a 6-week submerged submarine patrol. My rationale for collating various data sets relating to the same cohort is to investigate the overall nasal environment and immune phenotype in relation to *S. aureus* colonisation. Rather than focusing only on the organism of interest, *S. aureus*, I believed that assessment of total nasal microbiome, serology and serum cytokine responses might allow me to draw conclusions about the relationship between *S. aureus* and other nasal micro-organisms and its association with the immune system.

The study presented in this chapter is, to my knowledge, the first investigation into nasal microbiome and cytokines in a confined cohort.

Data presented here was performed under Research Objective 1:

- Investigate *S. aureus* colonisation in a unique human cohort of submariners and its association with skin health, immune markers and nasal microbiome

In order to perform such an analysis, I collated various datasets associated with the submarine cohort obtained through my own work prior to my DPhil studies (*S. aureus* carriage, demographics and skin health data[84]), work of other *S. aureus* vaccine development group members (cytokine and anti-*S. aureus* isotyping) and through collaboration with colleagues at the Statens Serum Institut, Copenhagen, Denmark (nasal microbiome sequencing). I conducted association studies to investigate risk of *S. aureus* and other nasal microbiome taxa carriage on skin health, cytokines and serological responses.

My specific research questions for this chapter are:

1. Are two methods of *S. aureus* isolation comparable: culture and microbiome?
2. Does the microbiome profile differ across different *S. aureus* isolation statuses? How does the microbiome of pre and post voyage samples compare?
3. Does the microbiome profile associate with changes in skin health?
4. Does microbiome profile associate with particular cytokine profile?
5. Does microbiome profile associate with particular anti-*S. aureus* isotyping?

I answered these questions by following my pre-defined analysis plan (2.2.7). This allowed me to draw conclusions relating to human *S. aureus* colonisation which need

to be considered when moving onto studying and developing mouse models of *S. aureus* colonisation.

## 3.2 Results

### 3.2.1 Submarine cohort and datasets analysed

A prospective observational cohort study was performed, in collaboration with the Royal Navy, to assess *S. aureus* carriage in submariners. Results have been published[84]. Briefly, submariners embarking on a submerged patrol on a Royal Navy Trafalgar Class Submarine in May 2014 were recruited to the study. 50 submariners were initially recruited to the study, representing 1/3 of the total submariners on board the ship. There were no exclusion criteria for this study and all participants were therefore eligible for inclusion. Submariners answered questionnaires, provided nasal swabs and serum samples in the 48 hours before boarding the submarine. 46/50 submariners provided nasal swabs and serum samples 40 days later at redocking, before they disembarked the submarine. Flow chart showing study design is shown in Figure 3-1.

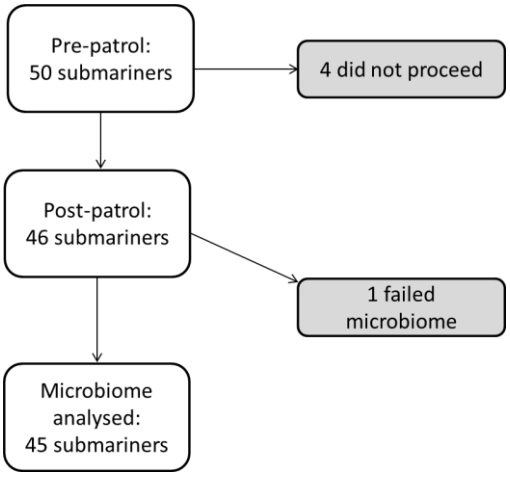



**Figure 3-1: Flow Chart showing study design for microbiome analysis**

A baseline questionnaire was completed by each recruit (Figure S1) and observational data relating to skin health were collected via weekly questionnaires throughout the

voyage (Figure S2). Nasal swabs in PBS and serum samples were batched at each timepoint and sent to Oxford for processing, as described in Materials and Methods. Briefly, an aliquot from the nasal sample was frozen at -80°C and subsequently sent to collaborators in Denmark for microbiome sequencing. Total aerobic count and *S. aureus* specific counts were determined from nasal swabs. Anti-*S. aureus* isotyping in serum was performed by Julia Whitehouse, a Research Assistant within the *S. aureus* vaccine development group. Cytokine analysis in serum was performed by Dr Yuko Yamaguchi, a Post-Doctoral Scientist within the *S. aureus* vaccine development group. I collated the data from the datasets described above. To answer my first research question for this chapter, I compared *S. aureus* bacterial recovery from nasal swabs by culture and microbiome methods.

### 3.2.2 *S. aureus* detection using culture and microbiome methods

As shown in Figure 3-1, a total of 45 pre and post microbiome samples were available which were suitable for analysis. The microbiome dataset generated by my collaborators consisted of number of reads detected for a total of 432 Operational Taxonomic Units (OTUs) for each submarine sample. An OTU is a proxy for species, and is typically used in microbiome studies as a unit to describe microbial diversity. Across all 90 samples analysed, mean number of reads was $9.7 \times 10^4$, with the minimum being $1.1 \times 10^4$ and the maximum at $9.9 \times 10^5$. Mean number of OTUs detected was 52, with a minimum of 25 and maximum of 123.

A

| | | *S. aureus* Microbiome | | | | Total |
|---|---|---|---|---|---|---|
| | | Never | Persistent | Pre only | Post only | |
| *S. aureus* Culture | Never | 22 | 0 | 2 | 0 | 24 |
| | Persistent | 3 | 8 | 1 | 3 | 15 |
| | Pre Only | 0 | 1 | 0 | 0 | 1 |
| | Post Only | 0 | 1 | 0 | 4 | 5 |
| Total | | 25 | 10 | 3 | 7 | 45 |

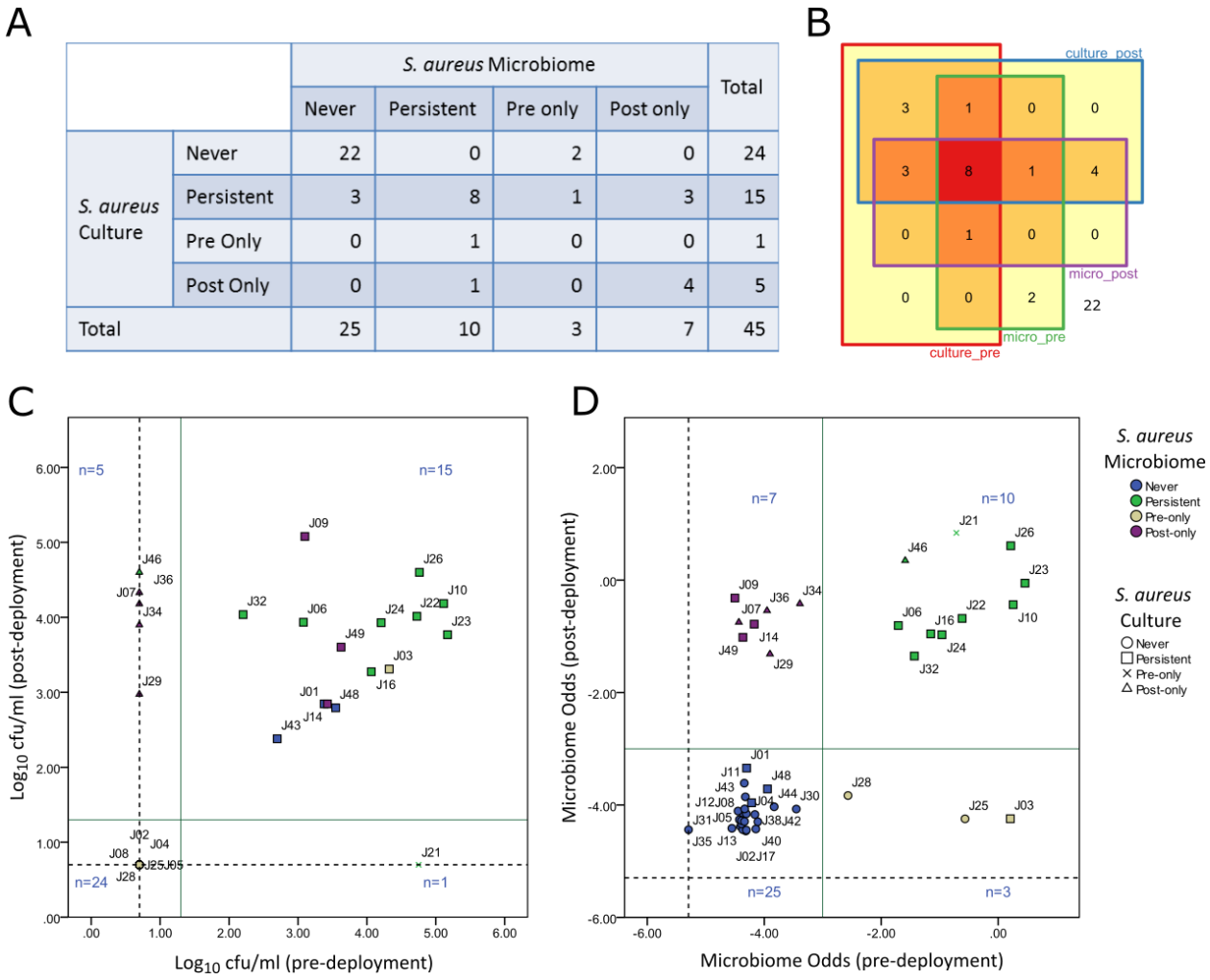



**Figure 3-2: Comparison of *S. aureus* isolation by culture and microbiome methods pre and post deployment.** Cross-tabulation of *S. aureus* isolation by microbiome and culture methods in each of 45 submariners (A). Venn diagram to show overlap of *S. aureus* positive samples pre and post using both sampling methods (B). Quantitative comparison of *S. aureus* isolation by culture (C) and microbiome (16S sequencing) methods (D).

The same 45 pre and post samples had also been previously assessed for viable *S. aureus* counts[84]. Assessment of *S. aureus* colonisation status using culture and microbiome methods was performed (Figure 3-2). Firstly, each sample was assessed

qualitatively; determined to be either positive or negative for *S. aureus*. A positive culture sample was any sample with *S. aureus* bacterial recovery above the limit of detection of 5 cfu/ml. A positive microbiome sample was considered to be any sample with more than 5 reads assigned to *S. aureus* OTU. Persistent carriage was determined as positive *S. aureus* recovery on both pre and post swabs. In 22/45 submariners *S. aureus* was never recovered by either method. 8/45 were determined to be persistent carriers by both methods. 3/45 submariners were persistent carriers by culture but microbiome sampling did not recover *S. aureus* at either timepoint(Figure 3-2A). The overlap between *S. aureus* positive samples by timepoint and sampling method is shown in the Venn diagram in Figure 3-2B. The numbers of submariners in the four groups (Never, Persistent, Pre-Only or Post-Only) determined by the two sampling methods (culture and microbiome) was found to be strongly associated ($\chi^2$=44.9, $p$ <0.001).

To further visualise whether the two methods used correlate with each other, quantitative assessment of *S. aureus* bacterial recovery by culture and microbiome was performed. For quantitation of culture, $Log_{10}$ cfu/ml was used, and relationship between pre and post samples for each submariner are shown in Figure 3-2C. For quantitation of microbiome, the $log_{10}$ odds that a read is an *S. aureus* read, 'Microbiome odds' were calculated:

$$\text{Microbiome odds} = \text{Log}_{10}\left(\frac{S.aureus \text{ reads}}{\text{total reads} - S.aureus \text{ reads}}\right)$$

Any samples with no *S. aureus* reads were considered to have 5 reads for the purposes of calculation of microbiome odds, a value chosen because samples with more than 5 *S. aureus* reads were considered to be *S. aureus* positive. The relationship between pre and post microbiome odds for each submariner is shown in Figure 3-2D. In Figure 3-2C & D, symbol shapes represent *S. aureus* isolation status by culture and symbol colours represent isolation state by microbiome, allowing comparisons between the two isolation methods to be made for each submariner. I hypothesised that a strong correlation would exist between microbiome derived odds and *S. aureus* colony forming units (cfu)/ml. When considering all 90 samples (both pre and post), significant correlation between $Log_{10}$ cfu/ml and microbiome odds exists (Spearman's $\rho = 0.68$, $p<0.001$). Therefore, assessment of *S. aureus* colonisation by nasal microbiome is comparable with the gold-standard method of culture, which provides external validation of the results obtained by microbiome analysis, albeit for a single organism.

### 3.2.3 Assessment of total microbiome during submarine voyage

As shown in the section above, individual submariners can be categorised according to whether they are carriers of *S. aureus*, as determined by presence of *S. aureus* reads in the microbiome analysis of their 2 nasal swabs. Those with 2 positive swabs are Persistent carriers and those with no positive swabs are termed 'Never'. Those submariners which had only one positive swab were grouped together and termed 'Intermittent' carriers. The next question I wanted to address was whether any differences in the microbiome profile existed between the different *S. aureus* carriage statuses. To do this, I needed a metric which represents the diversity and landscape of

the microbiome from each nasal sample. Typically in microbiome studies, approaches from ecological biodiversity studies are used[87 115]. R. H. Whittaker described the use of α and β diversity in his 1960 study of vegetation[116]. Alpha diversity provides a metric of species diversity within an individual habitat or sample. Beta diversity measures differences between habitats or samples. Therefore, I calculated alpha diversity to assess the diversity within each individual sample and beta diversity to investigate the change in diversity of species between pre to post samples from each submariner.

I calculated both Shannon and Simpson diversity indices[87] to assess alpha diversity.

Shannon's diversity index is calculated:

$$H' = \sum_{i=1}^{s} (p_i)(\ln p_i)$$

$p_i$ is the relative abundance of taxon I (i.e. number of reads of that OTU/total number of reads).

Simpson's diversity index is calculated:

$$D = \sum_{i=1}^{s} \frac{n_i(n_i - 1)}{N(N-1)}$$

N = total number of reads (all OTUs). n = number of reads within an OTU. Simpson's index can take values from 0 to 1.

I calculated beta diversity and Bray-Curtis dissimilarity to assess beta diversity:

$$\beta = (S_i - C) + (S_j - C)$$

Where $S_i$ and $S_j$ are the number of species in populations i and j, (pre and post). C = number of shared taxa[87].

Bray-Curtis dissimilarity is calculated:

$$BC_{ij} = \frac{S_i + S_j - 2C_{ij}}{S_i + S_j}$$

Where $S_i$ and $S_j$ are the numbers of species in populations i and j, and $C_{ij}$ is the total number of species at the location with the fewest species[87].

$BC_{ij}$ ranges from 0 to 1, so that if $BC_{ij}$ = 0, pre and post samples have same composition and if $BC_{ij}$ = 1, pre and post samples have no species in common.

**Table 3-1: Comparison of diversity by *S. aureus* microbiome status (per volunteer).** Testing the null hypothesis that Never, Persistent and Intermittent *S. aureus* carriers differ in the diversity metric studied. *p* values determined by Kruskal Wallis Test.

| | Never (25) | | Persistent (10) | | Intermittent (10) | | |
|---|---|---|---|---|---|---|---|
| Index | Mean | Median, range | Mean | Median, range | Mean | Median, range | *p* value |
| Shannon (Pre) | 1.67 | 1.70, 0.67 – 2.39 | 1.83 | 1.75, 0.83 – 3.03 | 1.96 | 2.00, 1.10 – 2.72 | 0.512 |
| Shannon (Post) | 1.78 | 1.67, 0.96 – 2.80 | 1.75 | 1.75, 0.68 – 2.65 | 2.12 | 2.12, 1.60 – 2.66 | 0.113 |
| Simpson (Pre) | 0.68 | 0.71, 0.24 – 0.85 | 0.69 | 0.71, 0.41 – 0.93 | 0.73 | 0.77, 0.54-0.89 | 0.512 |
| Simpson (Post) | 0.69 | 0.68, 0.43 – 0.91 | 0.64 | 0.69, 0.23 – 0.88 | 0.79 | 0.79, 0.60 - 0.89 | 0.578 |
| Beta | 45.64 | 42, 24 - 101 | 47.60 | 48, 30-62 | 44.50 | 42, 31 - 57 | 0.610 |
| Bray-Curtis | 0.15 | 0.14, 0.00 – 0.46 | 0.09 | 0.08, 0.00 – 0.31 | 0.15 | 0.15, 0.01 – 0.27 | 0.098 |

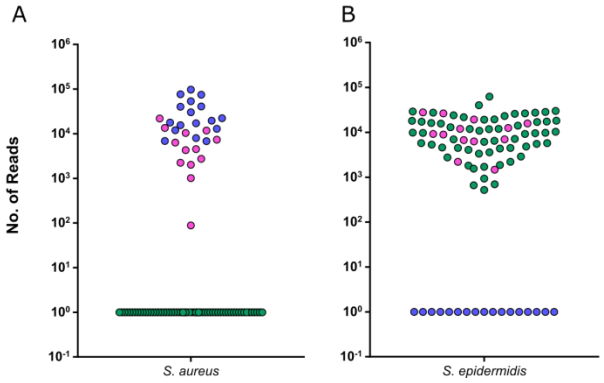
Diversity indices are summarised in Table 3-1. No differences were observed across the three groups of *S. aureus* status for any of the diversity indices described. For Shannon and Simpson diversity indices, I compared pre versus post values, using a paired t-test; however differences were not significant ($p$ = 0.47, $p$ = 0.193 for Shannon and Simpson indices respectively). To conclude, *S. aureus* carrier status is not significantly associated with the overall diversity of the nasal microbiome, and neither is spending six weeks in the close confines of a submarine.

Next, I considered whether the composition of the microbiome, as opposed to its diversity, might alter during the course of the submarine voyage. Percentage reads for each OTU is the most straightforward metric to use for comparing different OTUs in different samples. For 57/90 samples, >80% of reads and for 86/90 >60% of reads are made up from seven OTUs: *S. aureus*, *S. epidermidis*, *Corynebacterium*, *Moraxella*, *Brevibacterium*, *Dolosigranulum* and *P. acnes*.

**Table 3-2: Comparison of % reads of Top 6 OTUs by *S. aureus* microbiome status (per sample).** *p* values calculated using Mann Whitney test.

| OTU | Negative(60) | | Positive (30) | | |
|---|---|---|---|---|---|
| | Mean | Median, range | Mean | Median, range | *p* value |
| *S. epidermidis* | 13.71 | 11.49, 0.48 – 58.79 | 6.44 | 0.00, 0.00 – 24.31 | <0.001 |
| Corynebacterium | 40.42 | 37.38, 4.70 – 87.30 | 19.39 | 13.30, 1.78 – 67.12 | <0.001 |
| Moraxella | 6.16 | 0.01, 0.00 – 54.82 | 7.39 | 0.03, 0.00 – 63.21 | 0.342 |
| Brevibacterium | 0.40 | 0.05, 0.00 – 3.83 | 1.67 | 0.06, 0.00 – 44.19 | 0.631 |
| Dolosigranulum | 10.83 | 6.79, 0.00 – 55.84 | 2.91 | 0.03, 0.00 – 31.72 | <0.001 |
| *P. acnes* | 10.23 | 7.83, 0.11 – 34.71 | 9.11 | 5.41, 0.38 – 41.40 | 0.289 |

I assessed whether the proportion of the microbiome (as assessed by percentage of reads) accounted for by any of these top six OTUs (excluding *S. aureus*) were different depending on whether the sample was *S. aureus* positive or negative (Table 3-2). Proportions of *S. epidermidis*, Corynebacterium and Dolosigranulum were significantly higher in *S. aureus* negative compared to *S. aureus* positive samples. As already shown above, samples can be easily dichotomised into *S. aureus* positive and negative (Figure 3-3A). Samples can also be easily dichotomised into *S. epidermidis* positive and negative (Figure 3-3B). 17/90 samples are positive for *S. aureus* only; 60/90 are positive for *S. epidermidis* only.




C

| | | *S. epidermidis* Microbiome | | | Total |
|---|---|---|---|---|---|
| | | Never | Persistent | Intermittent | |
| *S. aureus* Microbiome | Never | 0 | 25 | 0 | 25 |
| | Persistent | 5 | 3 | 2 | 10 |
| | Intermittent | 0 | 5 | 5 | 10 |
| Total | | 5 | 33 | 7 | 45 |

**Figure 3-3: *S. aureus* and *S. epidermidis* isolation by microbiome.** Number of reads from each nasal microbiome sample (pre and post, 90 samples) assigned to each of *S. aureus* (A) and *S. epidermidis* (B) OTUs. Pink symbols represent samples positive for both *S. aureus* and *S. epidermidis*. Blue symbols represent samples positive for *S. aureus* only. Green symbols represent samples positive for *S. epidermidis* only. Cross-tabulation of *S. epidermidis* versus *S. aureus* nasal carrier status in each of 45 submariners (C).

To further investigate this, I determined whether each submariner was a persistent, intermittent or never *S. epidermidis* carrier (as done above for *S. aureus*). This analysis showed that *S. aureus* and *S. epidermidis* are inversely associated ($\chi^2$=34.9,

$p$<0.001). Whilst *S. aureus* and *S. epidermidis* can be easily dichotomised into positive and negative for each sample (Figure 3-3), the same was not true of the other five OTUs (Figure 3-4) which are not clearly dichotomous.

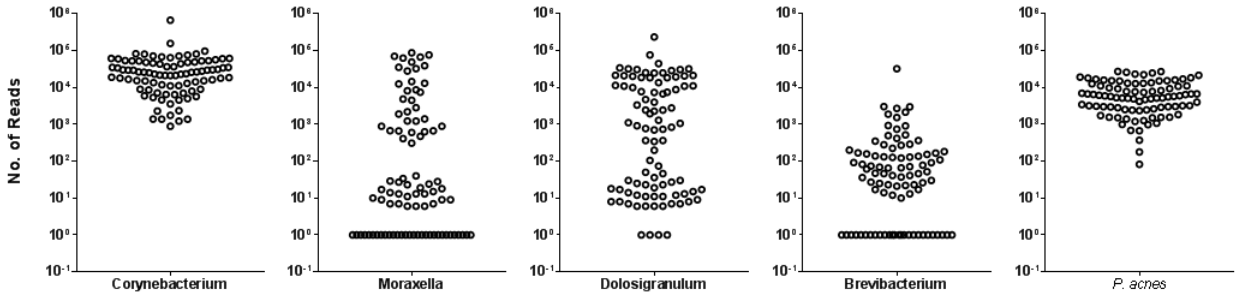



**Figure 3-4: Isolation of Corynebacterium, Moraxella, Dolosigranulum, Brevibacterium & *P. acnes* by microbiome.** Distribution of reads from each nasal microbiome sample (pre and post, 90 samples) assigned to each of the other top 5 OTUs.

To further investigate the relationships between samples in terms of their microbiome profile, I elected to construct a summary of the proportions of the various OTUs in each sample. I chose to use percent reads of each of the top seven OTUs and percent reads of all other OTUs to give 8 variables for assessment. This makes further analysis simpler, as 8 OTU groups can be much more easily visualised compared to 432 OTUs, while also reducing the potential for false discovery due to multiple testing. Bar charts capturing these 8 variables for all 90 samples tested are shown in Figure 3-5.

I next wanted to investigate the associations between microbiome profiles of the 90 samples. I expected that the pre and post samples for each individual submariner would be similar to each other. I also wanted to explore whether some microbiome profiles would cluster differently to others. To do this, I assigned Community State Types (CSTs) to each of the 90 samples. This involves using hierarchal clustering

based on all 432 OTUs using a Euclidean distance metric as described previously[85 117], followed by tree cutting.

Overlay of CSTs onto bar charts of percent reads is shown in Figure 3-5A. Six different CST were assigned and each has its own characteristics. CST 4 is *S. aureus* positive, *S. epidermidis* negative and Dolosigranulum negative. CST 3 is characterised by a greater percent reads of Moraxella (at least 17%) than other CSTs. CSTs 2, 5 and 6 are all characterised by low percent reads of *S. epidermidis* (maximum 30%) and mid to high level percent reads of Corynebacterium (16% to 87%). However, CST 6 has a greater percentage of Dolosigranulum, ranging from 16-56%, (compared to CSTs 2, maximum 28%, and 5, maximum 13%) whereas CST 2 has a greater percentage of *P. acnes*, ranging from 2-24%, (compared to CSTs 5 and 6, maximum 9% and 10%, respectively). CST 1 has the greatest percentage contribution from other OTUs.

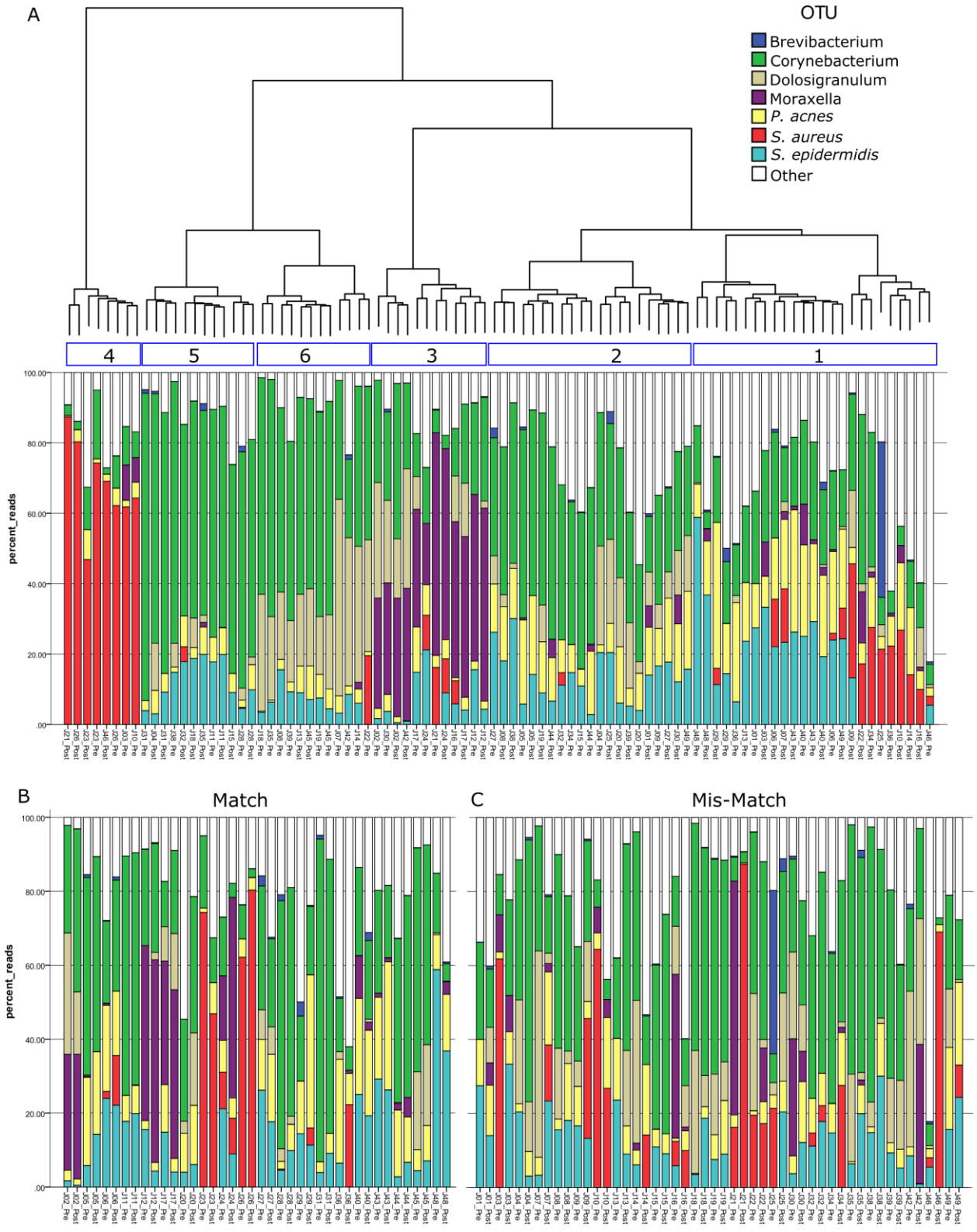



**Figure 3-5: Barplots of top 7 OTUs for each sample (pre and post).** Top 7 OTUs allow segregation of samples into six Community State Types (CSTs) (A). In (A) ordering is by

hierarchical clustering. Pre and post samples paired together according to whether the two samples had matching (B) or mis-matching (C) CSTs.

To assess whether pre and post samples from the same submariner were similar to each other, I determined whether each pair of samples fell within the same CST. 20/45 submariners were found to have 'matching' CSTs (Figure 3-5B) whilst the remaining 25/45 submariners had 'mis-matching' CSTs (Figure 3-5C). For example, submariner J02 has matching CSTs, and falls in CST 3. Submariner J07 has mis-matching CSTs, due to greater percentage Dolosigranulum and no *S. aureus* in his pre sample, which places that sample in CST 6, and presence of *S. aureus* in his post sample, placing that sample in CST 1. Interestingly, some mis-matching submariners were *S. aureus* carriers both pre and post (J10, J21 and J22); however, due to the difference in percent *S. aureus* reads between pre and post samples they are assigned to different CSTs.

**Table 3-3: Comparison of CST match/mismatch with *S. aureus* microbiome status**

| | | *S. aureus* Microbiome | | | Total |
|---|---|---|---|---|---|
| | | Never | Persistent | Intermittent | |
| CST | Mis-Match | 12 | 6 | 7 | 25 |
| | Match | 13 | 4 | 3 | 20 |
| Total | | 25 | 10 | 10 | 45 |

Since I am interested in *S. aureus* isolation status groups as a metric within which to compare other components of the microbiome, I assessed whether there was a relationship between CST matching or mis-matching and *S. aureus* isolation status

(Table 3-3). However, there was not a significant association between *S. aureus* isolation status and CST status ($\chi^2$ =1.5, $p$ =0.472).

In this section I have described the microbiome dataset and found that sample diversity is not related to *S. aureus* carriage. I have shown that *S. aureus* and *S. epidermidis* carriage are inversely related to one another, and are mutually exclusive. I have shown that pre and post samples are very similar to one another in some individuals as assessed by OTU proportions, but in other individuals, pre and post samples are very different in terms of percent reads. I assigned each sample to a CST and determined whether each individual is matching or mis-matching in terms of whether their pre and post samples belong to the same CST. I show that CST status is unrelated to *S. aureus* carriage status. However, both of these metrics can be used in further analyses to investigate the relationship between nasal microbiome and skin health, cytokines and serological responses to *S. aureus*.

### 3.2.4 Association of microbiome profile with demographics & skin health

Previously we showed that there was no association of skin health (at baseline or during the voyage) with *S. aureus* carriage as determined by culture method[84]. We also showed that carriers were significantly more likely to have previously had a cutaneous abscess incised and drained compared with non-carriers and that increased age, time spent in the Royal Navy and time as a submariner all trend towards an association with *S. aureus* carriage, but smoking, vaccination status, allergies, recent infection and naval rank were not significantly associated with carriage status in this study. Here, I used the same demographics and skin health data

to ask whether *S. aureus* carriage status (determined by microbiome sampling) and/or CST status are associated with skin health and/or demographics. A flow chart showing sample availability is given in Figure 3-6.

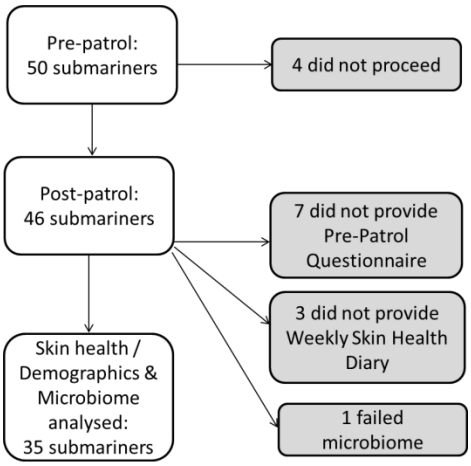



**Figure 3-6: Flow Chart showing study design to assess microbiome profile association with skin health/demographics**

Baseline and On Patrol skin scores were calculated in our previous publication[84] from a visual analogue scale which the submariners completed themselves (see Figure S1 and Figure S2). Here, I found that neither Baseline nor On Patrol Skin Scores were significantly associated with *S. aureus* microbiome status (Table 3-4), which is congruent with our findings using culture[84].

**Table 3-4: Comparison of Skin health with *S. aureus* microbiome (3 groups).** *p* values determined by Kruskal Wallis Test.

| | Never (20) | | Persistent (8) | | Intermittent (7) | | |
|---|---|---|---|---|---|---|---|
| **Index** | Mean | Median, range | Mean | Median, range | Mean | Median, range | *p* value |
| Baseline Skin Score | 1.20 | 0.52, 0-6.27 | 0.32 | 0.35, 0-0.69 | 2.43 | 1.31, 0-6.77 | 0.117 |
| On Patrol Skin Score | 0.91 | 0.48, 0-4.34 | 1.01 | 0.68, 0.15-2.81 | 1.35 | 1.16, 0-3.52 | 0.489 |

In previous studies, intermittent and non-carriers have been grouped together based on their similarities in immunological response to *S. aureus*[11]. In our previously published study, submariners were grouped into 'carriers' if they had two *S. aureus* positive swabs by culture and 'non-carriers' if they had one or no positive swabs by culture[84]. Here, I applied the same approach and grouped together the 'Never' and 'Intermittent' carriers as determined by *S. aureus* microbiome. Using *S. aureus* microbiome status as a dichotomous variable, I assessed whether differences in skin scores exist between carriers and non-carriers (Table 3-5). However, while carriers have slightly lower baseline skin score than non-carriers, this difference was not significant. No difference between carriers and non-carriers on patrol skin score exists, although in carriers skin scores increased over the course of the voyage; this was not conventionally significant ($p$ = 0.103, paired t-test).

**Table 3-5: Comparison of Skin health with *S. aureus* microbiome (2 groups).** *p* values calculated using Mann Whitney test.

| Index | Non-Carriers (27) | | Carriers (8) | | |
|---|---|---|---|---|---|
| | Mean | Median, range | Mean | Median, range | *p* value |
| Baseline Skin Score | 1.52 | 0.54, 0-6.77 | 0.32 | 0.35, 0-0.69 | 0.206 |
| On Patrol Skin Score | 1.02 | 0.54, 0-4.34 | 1.01 | 0.68, 0.15-2.81 | 0.832 |

I also assessed whether any differences in skin scores existed across groups of *S. epidermidis* status, however I found no differences here either (data not shown). Thus, I asked whether CST status was associated with changes in skin scores (Table 3-6). Whilst both baseline and on patrol skin scores were marginally higher in submariners with matching CSTs compared to those with mis-matching CSTs, they were not significantly higher.

**Table 3-6: Comparison of Skin health with CST match/mismatch.** *p* values calculated using Mann Whitney test.

| Index | Mis-Match (18) | | Match (16) | | |
|---|---|---|---|---|---|
| | Mean | Median, range | Mean | Median, range | *p* value |
| Baseline Skin Score | 1.03 | 0.58, 0-5.00 | 1.47 | 0.43, 0-6.77 | 0.684 |
| On Patrol Skin Score | 0.90 | 0.47, 0-3.52 | 1.15 | 0.78, 0-4.34 | 0.684 |

I concluded there was little evidence of an association between the nasal microbiome and skin health in this cohort.

I next analysed the demographic data obtained from submariners pre-patrol (see Figure S1) in terms of *S. aureus* isolation status and CST status. None of the demographics investigated were strongly associated with *S. aureus* microbiome status or CST status (Table 3-7). A trend exists towards matching CSTs in older submariners, however this was not significant.

**Table 3-7: Characteristics of submariners recorded in pre-patrol skin health questionnaires stratified by *S. aureus* microbiome status and CST status.** *p* values calculated using Mann-Whitney tests shown for continuous variables and Odds Ratios (OR) with 95% CIs shown for dichotomous variables. Missing data not included in statistical analyses.

| Demographics (ratio) | *S. aureus* microbiome status | | | CST status | | | Non-response rate |
|---|---|---|---|---|---|---|---|
| | Carriers (n=8) | Non-carriers (n=27) | p Value/OR and 95% CI | Mis-Match (n=18) | Match (n=17) | p Value/OR and 95% CI | |
| Male | 1 | 1 | 1 | 1 | 1 | 1 | 0.00 |
| Median age (years) | 31 | 31 | 0.65 | 29 | 34 | 0.09 | 0.03 |
| Median time in navy (years) | 8 | 11 | 0.77 | 7 | 12 | 0.27 | 0.00 |
| Median time as submariner (years) | 8 | 10 | 0.58 | 6 | 12 | 0.13 | 0.00 |
| Officer | 0.25 | 0.22 | 0.86 (0.14 to 5.40) | 0.22 | 0.24 | 1.08 (0.22 to 5.22) | 0.00 |
| Marine engineer | 0.00 | 0.37 | n/a | 0.33 | 0.24 | 0.62 (0.14 to 2.73) | 0.00 |
| Current smokers | 0.25 | 0.30 | 0.792(0.13 to 4.79) | 0.28 | 0.29 | 0.92 (0.21 to 4.00) | 0.00 |
| Asthma | 0.13 | 0.08 | 1.64 (0.13 to 20.94) | 0.06 | 0.12 | 0.50 (0.04 to 6.12) | 0.06 |
| Eczema | 0.00 | 0.04 | n/a | 0.00 | 0.06 | n/a | 0.06 |
| Hayfever | 0.00 | 0.15 | n/a | 0.18 | 0.06 | 3.43 (0.32 to 36.83) | 0.03 |
| Surgery in past month | 0.00 | 0.04 | n/a | 0.06 | 0.00 | n/a | 0.03 |
| Vaccination in past month | 0.00 | 0.00 | n/a | 0.00 | 0.00 | n/a | 0.03 |
| Flu-like illness in past month | 0.00 | 0.12 | n/a | 0.06 | 0.13 | 0.41 (0.03 to 5.03) | 0.03 |
| Cutaneous abscess on skin in past month | 0.13 | 0.08 | 1.64 (0.13 to 20.94) | 0.11 | 0.07 | 1.75 (0.14 to 21.43) | 0.06 |
| Skin infection in past month | 0.00 | 0.00 | n/a | 0.00 | 0.00 | n/a | 0.09 |
| Ever had a cutaneous abscess incised and drained | 0.25 | 0.15 | 1.83 (0.27 to 12.54) | 0.28 | 0.06 | 5.77 (0.60 to 55.95) | 0.03 |
| Antibiotic use in past 6 months | 0.00 | 0.08 | 1.08 (0.97 to 1.21) | 0.06 | 0.06 | 0.88 (0.05 to 15.37) | 0.03 |
| Received all childhood vaccines | 1.00 | 0.92 | n/a | 0.94 | 0.93 | 1.21 (0.07 to 21.22) | 0.06 |
| Ever had antibiotics post surgery | 0.50 | 0.19 | 4.20 (0.77 to 22.87) | 0.17 | 0.38 | 0.33 (0.07 to 1.65) | 0.03 |
| Previous *S. aureus* infection | 0.00 | 0.00 | n/a | 0.00 | 0.00 | n/a | 0.09 |
| Deployed within last 3 months | 1.00 | 0.89 | 0.83 (0.07 to 10.20) | 0.89 | 0.94 | 1.88 (0.15 to 22.88) | 0.03 |
| Chlorhexidine allergy | 0.00 | 0.00 | n/a | 0.00 | 0.00 | n/a | 0.03 |
| Persistent *S. aureus* carrier (microbiome determined) | n/a | n/a | n/a | 0.22 | 0.24 | 1.08 (0.22 to 5.22) | 0.00 |

In this section, I investigated whether demographics and skin health were associated with *S. aureus* isolation status and nasal microbiome profile. Neither *S. aureus* microbiome status nor CST status were associated with changes in skin score at baseline or on patrol. Likewise, no differences in any of the demographics investigated existed between *S. aureus* carriers and non-carriers or CST matching or mis-matching submariners.

### 3.2.5 Association of microbiome profile with cytokine profile and anti-*S. aureus* isotyping

The possibility that stability of the microbiome results from some form of immune selection is both intriguing and difficult to assess in humans. The final two research questions of this chapter, relating to association of microbiome profile with cytokine profile and anti-*S. aureus* isotyping in serum, pertain to this possibility. To address this, I collated cytokine and anti-*S. aureus* isotyping data from pre and post samples. Samples available and suitable for this analysis are shown in Figure 3-7.

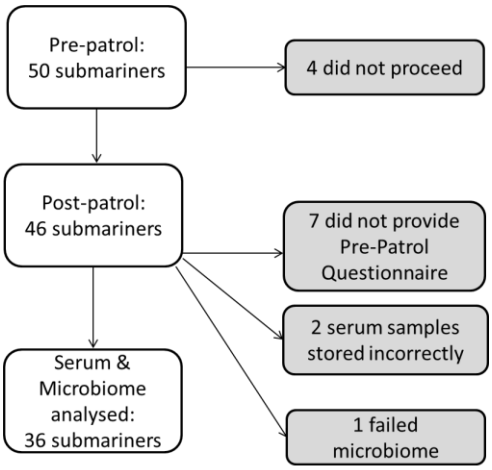



**Figure 3-7: Flow Chart showing study design to assess microbiome profile association with cytokine profile and anti-*S. aureus* isotyping**

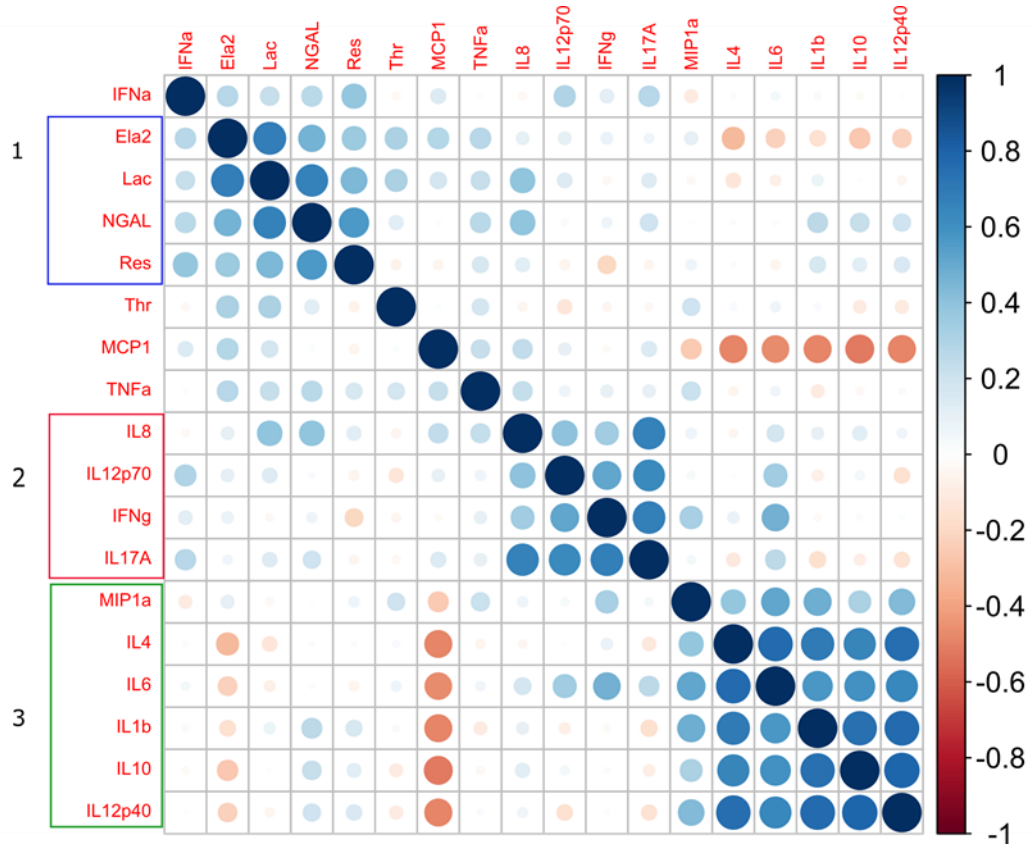



**Figure 3-8: Relationships between cytokines pre-patrol.** Correlation matrix using Spearman's rho to show relationship between cytokines measured by Luminex pre-patrol.

Firstly, I investigated the relationship between cytokines assessed in serum taken pre patrol. 18 cytokines were measured using multiplex kits. Using a correlation matrix (Figure 3-8), I discovered that some of the 18 cytokines were associated with each other. Three distinct clusters of cytokines could be defined using the correlation matrix in Figure 3-8. Cluster 1 consists of innate pro-inflammatory proteins: neutrophil elastase-2 (ELA2), lactoferrin, lipocalin-2 (NGAL) and resistin. Cluster 2 is made up from T-helper 1 (Th1) and Th17 pro-inflammatory cytokines: IL-8, IL-12p70, IFN-γ and IL17A. Cluster 3 comprises some Th2, some regulatory and some pro-inflammatory cytokines: MIP-1α, IL-4, IL-6, IL-1β, IL-10 and IL-12p40.

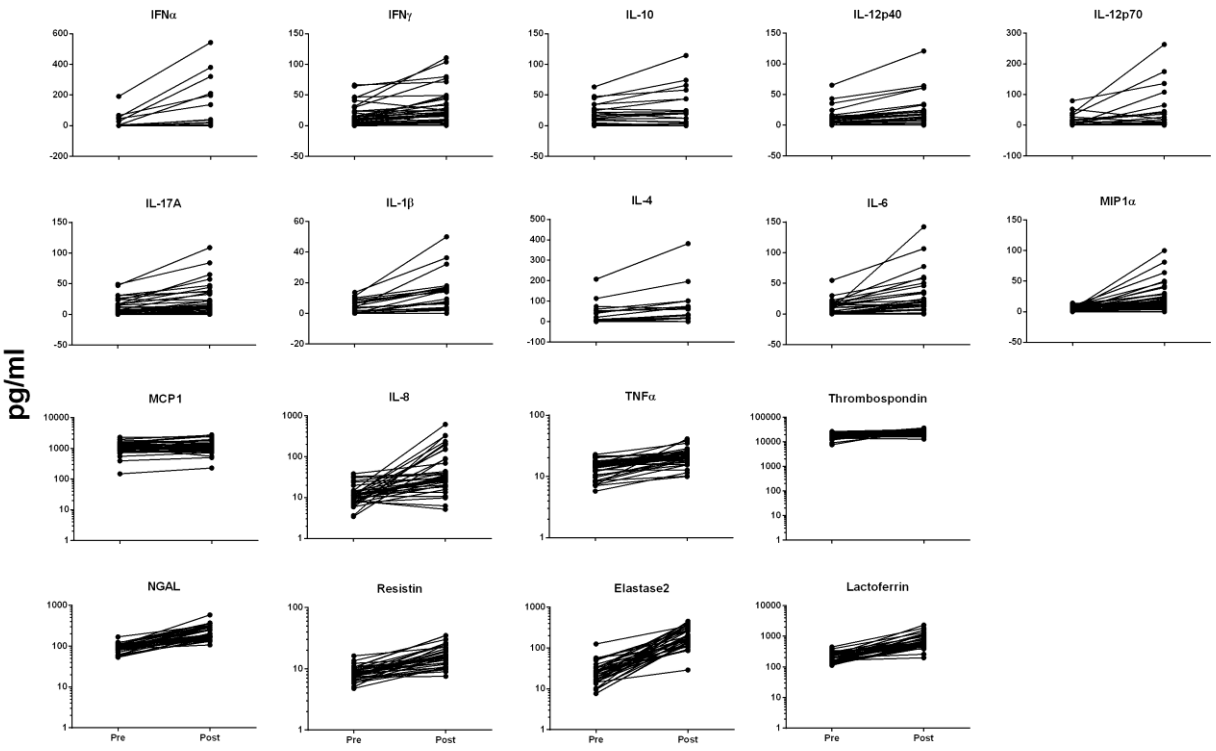



**Figure 3-9: Cytokine levels Pre and Post submarine voyage.** Relationship between levels of each cytokine per submariner pre and post patrol as measured by Luminex assays in serum.

Next I investigated the relationship between pre and post samples from each submariner to allow assessment of any changes in cytokine levels during the submarine patrol (Figure 3-9). The limit of detection of the assay was 0.8 pg/ml, and therefore any data points at or below 0.8 pg/ml were set to 0 pg/ml in this depiction. In general, Figure 3-9 shows that cytokine levels increase during patrol, indicating that a submarine voyage is a pro-inflammatory environment. For some cytokines the increases are seen in some individuals but not others, namely those shown in the top two rows of Figure 3-9 on a linear scale. For these 8 cytokines, baseline levels are around zero, and remain at zero for some submariners. In contrast, the remaining cytokines and innate pro-inflammatory proteins have baseline levels greater than zero and so are shown on a log scale. IL-8 behaves much like the cytokines in the top 2 panels, in that it increases in some individuals but not others. MCP1 and Thrombospondin appear not to change much over the course of the voyage. TNF-α, NGAL, Resistin, ELA2 and Lactoferrin increased in most individuals.

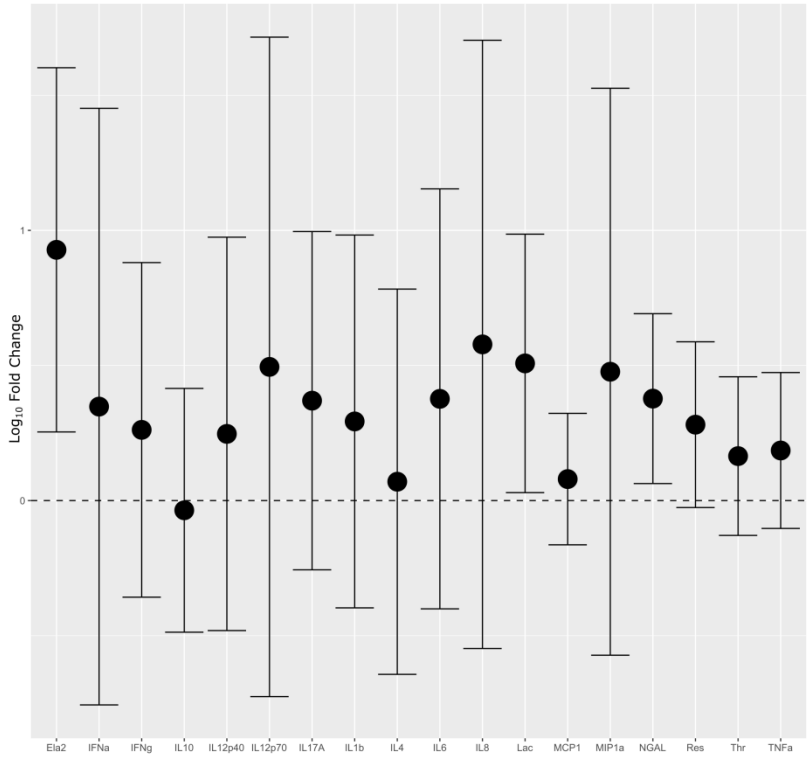



**Figure 3-10: $Log_{10}$ fold Change of each cytokine.** Values <0.8 were adjusted to 0.8 (i.e. background) to allow logs to be taken. Mean & 95% CI shown.

To investigate the impact of other variables (e.g. *S. aureus* isolation status) upon cytokine levels during the patrol, I calculated $log_{10}$ fold change for each cytokine, as $Log_{10}$ (post/pre). Figure 3-10 provides summary data for $log_{10}$ fold change for each cytokine. This allows clear visualisation of which cytokines increase the most over the course of the voyage: ELA2, Lac and NGAL, and those which change very little, namely IL-10. In this analysis, any data points at or below the detection limit of 0.8 pg/ml were set to 0.8 pg/ml, to allow $log_{10}$ fold change to be calculated.

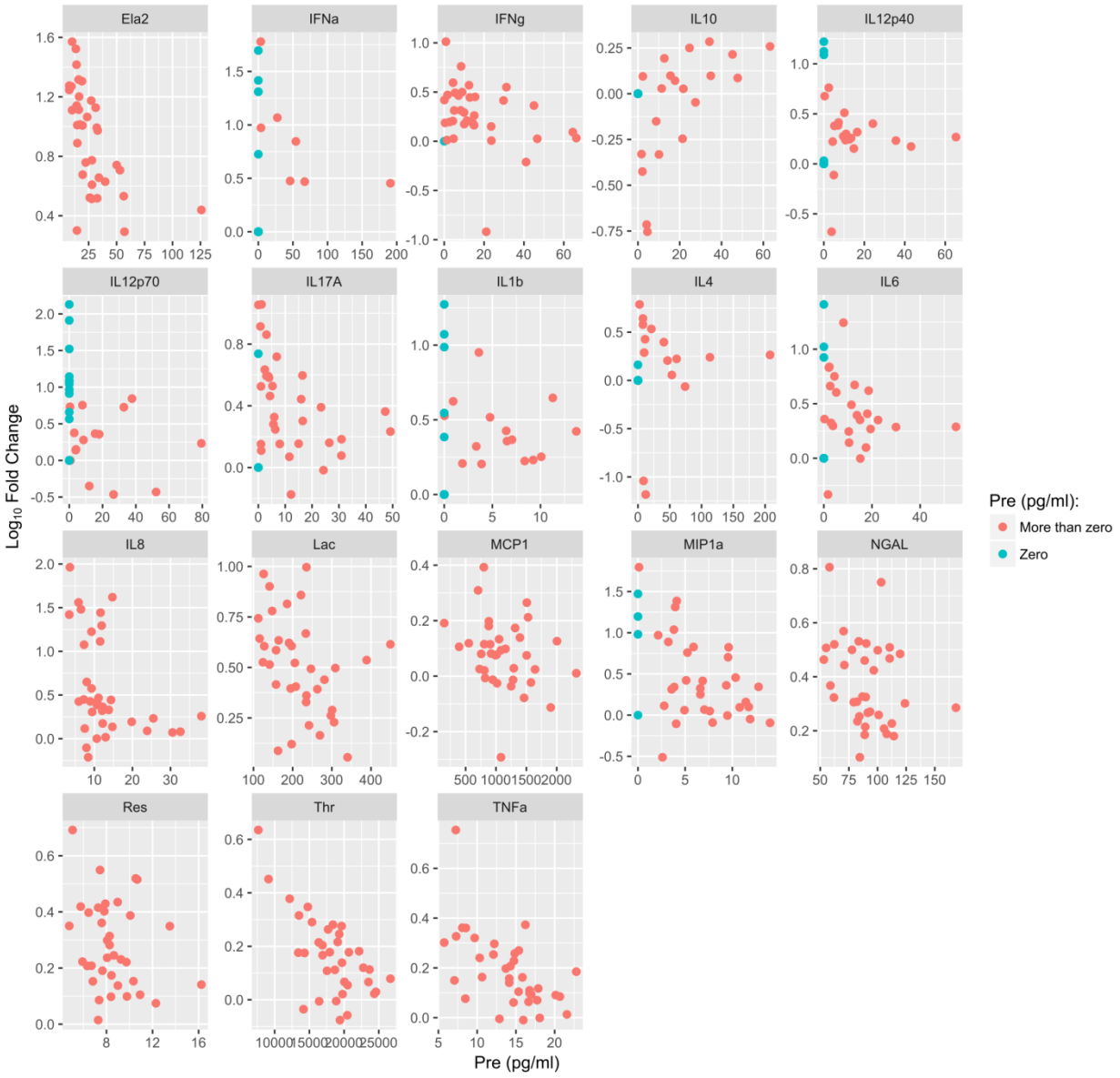



**Figure 3-11: $Log_{10}$ fold change compared to pre-patrol values for each cytokine.**

To assess whether setting the values at or below the detection limit of 0.8 pg/ml to 0.8 pg/ml produced any bias, $Log_{10}$ fold change was plotted against pre cytokine values (Figure 3-11). By assessing number of pre data points at 0 (shown in blue) for each cytokine, I can see that for most cytokines, there are few blue points and so setting <0.8 pg/ml values to 0.8 pg/ml has little impact on estimation of $log_{10}$ fold

change. IL-12p40 and IL-12p70 are exceptions and for this reason will not be included in further analyses. IFN-α values both pre and post are almost undetectable; for 24/36 individuals IFN-α levels both pre and post were <0.8 pg/ml. Therefore IFN-α will not be included in further analyses either.

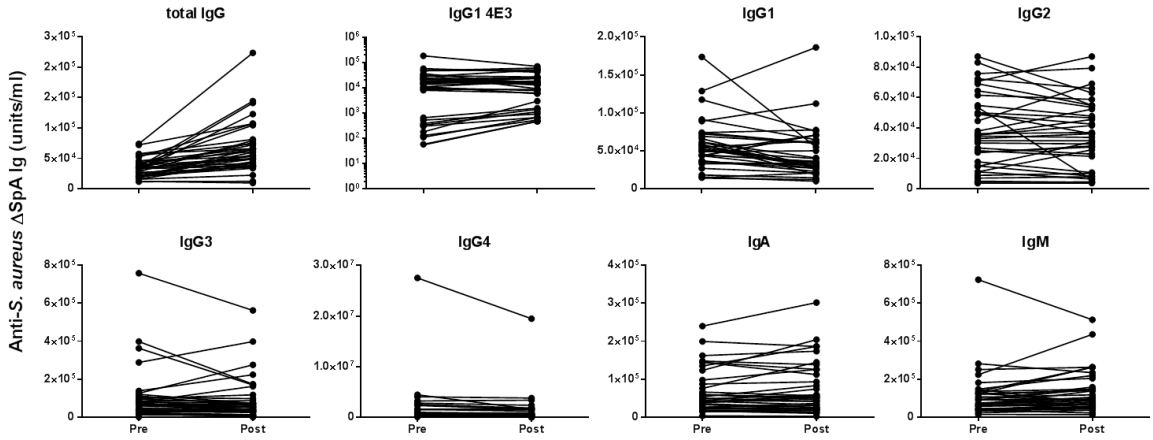



**Figure 3-12: Anti-*S. aureus* serum antibody levels Pre and Post submarine voyage.** Relationship between levels of each antibody per submariner pre and post patrol as measured by anti-*S. aureus* whole cell ELISA in serum.

I next investigated anti-*S. aureus* isotyping data for each submariner pre and post voyage (Figure 3-12). Total IgG increases over the course of the patrol in some individuals but not others. In terms of individual isotypes, no clear trends were seen in increases in titres over the course of the patrol. Two different anti-IgG1 antibodies were used: 4E3 clone is an anti-hinge IgG1 antibody and HP6070 clone is an anti-Fc IgG1 antibody. This was because my colleague, Julia Whitehouse, observed a dichotomy across three different cohorts in IgG1 titres when 4E3 was used but not when HP6070 was used. Submariners can be grouped into $4E3^{High}$ and $4E3^{Low}$ based on their pre IgG1 4E3 titre. These 2 groups can be easily seen in panel 2 of Figure

3-12. Interestingly, the levels of IgG1 in the $4E3^{Low}$ group increase over the course of the patrol whereas levels of IgG1 in the $4E3^{High}$ group stay the same over the course of the patrol. Therefore, 4E3 status (high or low) is another variable which I can explore for differences in cytokine and isotyping profiles, and this will be addressed at the end of the chapter.

I next calculated $\log_{10}$ fold change for anti-*S. aureus* isotyping data, as I did above for the cytokine data. This gave me a dataset with a total of 15 cytokines and 8 isotyping variables to investigate further in terms of *S. aureus* status and microbiome profile features.

**Table 3-8: $Log_{10}$ fold change in cytokines & anti-*S. aureus* isotyping across *S. aureus* microbiome status: 3 groups.** *p* values determined by Kruskal Wallis Tests.

| | Never (20) | | Persistent (9) | | Intermittent (7) | | |
|---|---|---|---|---|---|---|---|
| Index | Mean | Median, range | Mean | Median, range | Mean | Median, range | *p* value |
| IFNγ | 0.21 | 0.21, -0.92-0.6 | 0.46 | 0.46, 0.01-1.01 | 0.16 | 0.03, -0.21-0.5 | 0.124 |
| IL-10 | -0.06 | 0, -0.75-0.28 | -0.02 | 0, -0.33-0.26 | 0.01 | 0, -0.25-0.19 | 0.929 |
| IL17A | 0.29 | 0.29, -0.18-0.63 | 0.63 | 0.74, 0.07-1.06 | 0.26 | 0.11, -0.02-0.72 | 0.044 |
| IL-1β | 0.30 | 0.23, 0-1.27 | 0.22 | 0, 0-0.65 | 0.37 | 0, 0-1.07 | 0.900 |
| IL-4 | 0.15 | 0.11, -1.04-0.79 | -0.04 | 0, -1.18-0.58 | -0.01 | 0, -0.06-0 | 0.042 |
| IL-6 | 0.35 | 0.31, -0.33-1.41 | 0.55 | 0.67, 0-1.24 | 0.22 | 0, 0-0.84 | 0.326 |
| IL-8 | 0.50 | 0.39, -0.1-1.62 | 0.73 | 0.43, 0-1.48 | 0.62 | 0.31, -0.21-1.96 | 0.834 |
| MCP1 | 0.09 | 0.09, -0.29-0.39 | 0.09 | 0.09, -0.01-0.19 | 0.03 | 0.01, -0.11-0.21 | 0.474 |
| MIP1α | 0.41 | 0.33, -0.51-1.8 | 0.53 | 0.7, -0.11-1.31 | 0.60 | 0.34, -0.09-1.39 | 0.858 |
| TNFα | 0.16 | 0.15, -0.01-0.37 | 0.26 | 0.25, 0.06-0.75 | 0.16 | 0.11, 0.01-0.36 | 0.577 |
| Elastase2 | 0.85 | 0.83, 0.29-1.42 | 1.17 | 1.17, 0.51-1.57 | 0.85 | 0.76, 0.52-1.28 | 0.080 |
| Lactoferrin | 0.47 | 0.51, 0.06-0.96 | 0.64 | 0.63, 0.16-1 | 0.45 | 0.4, 0.12-0.74 | 0.220 |
| NGAL | 0.35 | 0.32, 0.1-0.75 | 0.47 | 0.47, 0.18-0.81 | 0.35 | 0.31, 0.19-0.51 | 0.266 |
| Resistin | 0.25 | 0.23, 0.01-0.55 | 0.36 | 0.36, 0.1-0.69 | 0.25 | 0.19, 0.09-0.43 | 0.236 |
| Thrombospondin | 0.18 | 0.18, -0.08-0.64 | 0.15 | 0.11, -0.01-0.45 | 0.15 | 0.14, -0.04-0.35 | 0.872 |
| Total IgG | 0.21 | 0.17, -0.1-0.61 | 0.39 | 0.44, 0.05-0.66 | 0.23 | 0.2, 0.06-0.45 | 0.107 |
| IgG1 4E3 | 0.09 | 0, -0.44-0.93 | 0.26 | 0.21, -0.54-1.21 | 0.16 | 0.12, -0.23-0.78 | 0.654 |
| IgG1 | -0.11 | -0.1, -0.5-0.21 | -0.01 | 0.01, -0.36-0.21 | -0.22 | -0.15, -0.5--0.1 | 0.030 |
| IgG2 | -0.06 | -0.01, -0.96-0.19 | 0.02 | 0.01, -0.08-0.09 | -0.01 | -0.07, -0.26-0.36 | 0.442 |
| IgG3 | -0.26 | -0.08, -3.08-0.34 | -0.04 | -0.07, -0.36-0.31 | -0.11 | -0.11, -0.28-0.08 | 0.785 |
| IgG4 | -0.10 | -0.07, -0.45-0.03 | -0.12 | -0.12, -0.27--0.01 | -0.09 | -0.11, -0.33-0.1 | 0.580 |
| IgA | 0.02 | 0.04, -0.21-0.28 | 0.02 | -0.03, -0.14-0.24 | 0.00 | 0, -0.21-0.18 | 0.995 |
| IgM | 0.07 | 0.06, -0.18-0.24 | 0.07 | 0.08, -0.35-0.33 | 0.05 | 0.07, -0.21-0.29 | 0.955 |

Firstly, I asked whether any of the cytokine or isotyping variables differ across the three groups of *S. aureus* microbiome status: Never, Persistent and Intermittent carriers (Table 3-8). Since 23 different comparisons have been made, using Bonferroni's correction, a *p* value of < 0.002 is considered significant. I observed a

trend towards changes between groups in levels of fold change of IL-17A, IL-4,IgG1 and ELA2 over the course of the submarine patrol.

**Table 3-9: $Log_{10}$ fold change in cytokines & anti-*S. aureus* isotyping across *S. aureus* microbiome status: 2 groups.** *p* values calculated using Mann Whitney tests.

| Index | Non-Carriers (27) | | Carriers (9) | | |
|---|---|---|---|---|---|
| | Mean | Median, range | Mean | Median, range | *p* value |
| IFNγ | 0.20 | 0.2, -0.92-0.6 | 0.46 | 0.46, 0.01-1.01 | 0.056 |
| IL-10 | -0.04 | 0, -0.75-0.28 | -0.02 | 0, -0.33-0.26 | 0.720 |
| IL17A | 0.28 | 0.28, -0.18-0.72 | 0.63 | 0.74, 0.07-1.06 | 0.014 |
| IL-1β | 0.32 | 0.22, 0-1.27 | 0.22 | 0, 0-0.65 | 0.667 |
| IL-4 | 0.11 | 0, -1.04-0.79 | -0.04 | 0, -1.18-0.58 | 0.428 |
| IL-6 | 0.32 | 0.29, -0.33-1.41 | 0.55 | 0.67, 0-1.24 | 0.279 |
| IL-8 | 0.53 | 0.36, -0.21-1.96 | 0.73 | 0.43, 0-1.48 | 0.565 |
| MCP1 | 0.08 | 0.08, -0.29-0.39 | 0.09 | 0.09, -0.01-0.19 | 0.693 |
| MIP1α | 0.46 | 0.34, -0.51-1.8 | 0.53 | 0.7, -0.11-1.31 | 0.886 |
| TNFα | 0.16 | 0.15, -0.01-0.37 | 0.26 | 0.25, 0.06-0.75 | 0.312 |
| Elastase2 | 0.85 | 0.77, 0.29-1.42 | 1.17 | 1.17, 0.51-1.57 | 0.024 |
| Lactoferrin | 0.46 | 0.5, 0.06-0.96 | 0.64 | 0.63, 0.16-1 | 0.086 |
| NGAL | 0.35 | 0.31, 0.1-0.75 | 0.47 | 0.47, 0.18-0.81 | 0.109 |
| Resistin | 0.25 | 0.22, 0.01-0.55 | 0.36 | 0.36, 0.1-0.69 | 0.093 |
| Thrombospondin | 0.17 | 0.18, -0.08-0.64 | 0.15 | 0.11, -0.01-0.45 | 0.693 |
| Total IgG | 0.22 | 0.17, -0.1-0.61 | 0.39 | 0.44, 0.05-0.66 | 0.036 |
| IgG1 4E3 | 0.11 | 0.05, -0.44-0.93 | 0.26 | 0.21, -0.54-1.21 | 0.450 |
| IgG1 | -0.14 | -0.13, -0.5-0.21 | -0.01 | 0.01, -0.36-0.21 | 0.022 |
| IgG2 | -0.05 | -0.04, -0.96-0.36 | 0.02 | 0.01, -0.08-0.09 | 0.263 |
| IgG3 | -0.22 | -0.09, -3.08-0.34 | -0.04 | -0.07, -0.36-0.31 | 0.494 |
| IgG4 | -0.10 | -0.07, -0.45-0.1 | -0.12 | -0.12, -0.27--0.01 | 0.312 |
| IgA | 0.02 | 0.03, -0.21-0.28 | 0.02 | -0.03, -0.14-0.24 | 0.971 |
| IgM | 0.07 | 0.06, -0.21-0.29 | 0.07 | 0.08, -0.35-0.33 | 0.802 |

As explained in the above section (3.2.4), *S. aureus* status can be refined into 2 groups: Carriers and Non-Carriers. Therefore I compared cytokine and isotyping variables across these 2 groups (Table 3-9). Although not significant when taking into account multiple comparisons (as assessed by Bonferroni's correction), there was a trend towards greater increase in $\log_{10}$ fold change of IFN-γ, IL-17A, ELA2, Lactoferrin, NGAL, Resistin total IgG and IgG1 in carriers compared to non-carriers. Therefore, there is a pattern of increased fold change in carriers in the related Cluster 1 cytokines (as defined in Figure 3-8). Next, I decided to assess whether the differences in fold change between carriers and non-carriers differ across the 3 cytokine clusters. Dunn's multiple comparison tests showed that there was a significant difference ($p$ = 0.013) in the $p$ values obtained from Mann-Whitney comparisons (between carriers and non-carriers) in Cluster 1 versus Cluster 3 cytokines. Therefore, Cluster 1 cytokine fold change is significantly greater in *S. aureus* carriers compared to non-carriers than Cluster 3 fold change.

**Table 3-10: $Log_{10}$ fold change in cytokines & anti-*S. aureus* isotyping across CST status.** *p* values calculated using Mann Whitney test.

| Index | Mis-Match (20) | | Match (16) | | |
|---|---|---|---|---|---|
| | Mean | Median, range | Mean | Median, range | *p* value |
| IFNγ | 0.31 | 0.31, -0.21-0.76 | 0.20 | 0.19, -0.92-1.01 | 0.223 |
| IL-10 | -0.03 | 0, -0.71-0.28 | -0.04 | 0, -0.75-0.19 | 0.937 |
| IL17A | 0.43 | 0.45, -0.02-1.06 | 0.30 | 0.21, -0.18-1.05 | 0.262 |
| IL-1β | 0.31 | 0.23, 0-1.27 | 0.28 | 0.21, 0-0.99 | 0.814 |
| IL-4 | 0.13 | 0, 0-0.79 | 0.00 | 0, -1.18-0.64 | 0.648 |
| IL-6 | 0.48 | 0.4, 0-1.41 | 0.24 | 0.27, -0.33-1.02 | 0.067 |
| IL-8 | 0.53 | 0.42, -0.21-1.56 | 0.64 | 0.38, -0.1-1.96 | 0.912 |
| MCP1 | 0.05 | 0.02, -0.29-0.39 | 0.12 | 0.12, -0.08-0.31 | 0.016 |
| MIP1α | 0.39 | 0.33, -0.51-1.31 | 0.58 | 0.4, -0.09-1.8 | 0.459 |
| TNFα | 0.17 | 0.15, -0.01-0.75 | 0.20 | 0.2, 0-0.36 | 0.236 |
| Elastase2 | 0.82 | 0.77, 0.29-1.57 | 1.07 | 1.15, 0.44-1.52 | 0.020 |
| Lactoferrin | 0.44 | 0.41, 0.06-1 | 0.59 | 0.61, 0.23-0.96 | 0.049 |
| NGAL | 0.31 | 0.29, 0.1-0.57 | 0.46 | 0.47, 0.21-0.81 | 0.004 |
| Resistin | 0.22 | 0.2, 0.01-0.43 | 0.36 | 0.37, 0.1-0.69 | 0.007 |
| Thrombospondin | 0.12 | 0.13, -0.08-0.29 | 0.22 | 0.2, -0.01-0.64 | 0.102 |
| Total IgG | 0.26 | 0.25, -0.1-0.66 | 0.27 | 0.19, 0.02-0.61 | 0.888 |
| IgG1 4E3 | 0.06 | 0.05, -0.54-1.21 | 0.26 | 0.25, -0.28-0.93 | 0.140 |
| IgG1 | -0.12 | -0.1, -0.5-0.16 | -0.08 | -0.1, -0.36-0.21 | 0.765 |
| IgG2 | -0.05 | -0.01, -0.96-0.36 | -0.01 | -0.01, -0.18-0.19 | 0.912 |
| IgG3 | -0.24 | -0.07, -3.08-0.21 | -0.10 | -0.1, -0.53-0.34 | 0.987 |
| IgG4 | -0.09 | -0.08, -0.33-0.1 | -0.12 | -0.09, -0.45-0.03 | 0.838 |
| IgA | 0.03 | 0.03, -0.21-0.24 | 0.00 | -0.04, -0.21-0.28 | 0.422 |
| IgM | 0.03 | 0.06, -0.35-0.29 | 0.12 | 0.12, -0.18-0.33 | 0.140 |

Next I compared cytokine and isotyping variables across the 2 groups of CST status (Table 3-10). I observed higher fold change in the Cluster 1 cytokines in individuals

with matching CSTs compared to mis-matchers, although these were not significant ($p<0.002$) when taking into account Bonferroni's correction. Cluster 1 cytokine fold change is significantly greater in CST matchers compared to non-matchers than both Cluster 2 and 3 fold change ($p = 0.023$, $p = 0.005$ respectively, Dunn's multiple comparison test).

As shown previously in Table 3-3, CST status was not significantly associated with *S. aureus* microbiome as assessed by Never, Persistent and Intermittent carriers. However, as described in the above section (3.2.4), *S. aureus* status can be further refined into 2 groups: Carriers and Non-Carriers. Therefore, I also assessed whether there was a relationship between CST status and *S. aureus* isolation status when split into 2 groups (Table 3-11). As for 3 groups, there was not a significant association between CST status and *S. aureus* status: 2 groups (Fisher's exact test, $p = 1$).

**Table 3-11: Comparison of CST match/mismatch with *S. aureus* microbiome status: 2 groups**

| | | *S. aureus* Microbiome | | Total |
|---|---|---|---|---|
| | | Non-Carrier | Carrier | |
| CST | Mis-Match | 15 | 5 | 20 |
| | Match | 12 | 4 | 16 |
| Total | | 27 | 9 | 36 |

Although there is not a significant association between CST status and *S. aureus* isolation status by microbiome, *S. aureus* level in some samples affects the allocation

to CST, as seen in Figure 3-5. Table 3-9 and Table 3-10 also show that increases in $Log_{10}$ fold change of some cytokines is seen in both *S. aureus* carriers compared to non-carriers and those with matching CSTs compared to mis-matching CSTs. To dissect out further any overlap in the 2 grouping variables (CSTs and *S. aureus* status), I created a variable including these 2 variables, which has 4 groups: Mis-match Non-carriers, Mis-match Carriers, Match Non-Carriers and Match Carriers.

I used this new variable (Combined CST and *S. aureus* status) to compare $Log_{10}$ fold change in cytokines and anti-*S. aureus* isotyping levels (Table 3-12). Fold change of IL-17A and Cluster 1 cytokines trend towards being different across the groups of Combined CST and *S. aureus* status, although not significantly so as assessed by Kruskal-Wallis tests with Bonferroni's correction. Cluster 1 cytokine fold change is significantly different across the groups of Combined CST and *S. aureus* status compared to Cluster 3 fold change ($p = 0.006$, Dunn's multiple comparison test).

**Table 3-12: $Log_{10}$ fold change in cytokines & anti-*S. aureus* isotyping across combined variable of CST status and *S. aureus* status.** *p* values determined by Kruskal Wallis Test.

| Index | Mis-Match Non-Carrier (15) | | Mis-Match Carrier (5) | | Match Non-Carrier (12) | | Match Carrier (4) | | |
|---|---|---|---|---|---|---|---|---|---|
| | Mean | Median, range | Mean | Median, range | Mean | Median, range | Mean | Median, range | *p* value |
| IFNγ | 0.25 | 0.26, -0.21-0.6 | 0.50 | 0.55, 0.15-0.76 | 0.13 | 0.18, -0.92-0.49 | 0.41 | 0.31, 0.01-1.01 | 0.141 |
| IL-10 | -0.06 | 0, -0.71-0.28 | 0.04 | 0, -0.15-0.26 | -0.02 | 0, -0.75-0.19 | -0.08 | 0, -0.33-0 | 0.713 |
| IL17A | 0.29 | 0.33, -0.02-0.72 | 0.83 | 0.86, 0.6-1.06 | 0.27 | 0.23, -0.18-0.63 | 0.38 | 0.19, 0.07-1.05 | 0.013 |
| IL-1β | 0.31 | 0.21, 0-1.27 | 0.29 | 0.38, 0-0.65 | 0.33 | 0.23, 0-0.99 | 0.13 | 0, 0-0.53 | 0.761 |
| IL-4 | 0.12 | 0, 0-0.79 | 0.16 | 0, 0-0.58 | 0.10 | 0.08, -1.04-0.64 | -0.30 | 0, -1.18-0 | 0.335 |
| IL-6 | 0.43 | 0.39, 0-1.41 | 0.65 | 0.75, 0-1.24 | 0.18 | 0.27, -0.33-0.6 | 0.42 | 0.34, 0-1.02 | 0.193 |
| IL-8 | 0.40 | 0.32, -0.21-1.56 | 0.91 | 1.08, 0.12-1.48 | 0.69 | 0.47, -0.1-1.96 | 0.50 | 0.29, 0-1.42 | 0.494 |
| MCP1 | 0.03 | 0.01, -0.29-0.39 | 0.08 | 0.08, -0.01-0.19 | 0.13 | 0.12, -0.08-0.31 | 0.10 | 0.1, 0.08-0.13 | 0.075 |
| MIP1α | 0.33 | 0.31, -0.51-1.2 | 0.57 | 0.7, -0.11-1.31 | 0.62 | 0.4, -0.09-1.8 | 0.48 | 0.47, 0-0.98 | 0.812 |
| TNFα | 0.13 | 0.14, -0.01-0.37 | 0.29 | 0.25, 0.08-0.75 | 0.20 | 0.2, 0-0.36 | 0.21 | 0.22, 0.06-0.36 | 0.376 |
| Elastase2 | 0.73 | 0.68, 0.29-1.14 | 1.09 | 1.06, 0.51-1.57 | 1.00 | 1.07, 0.44-1.42 | 1.27 | 1.22, 1.11-1.52 | 0.015 |
| Lactoferrin | 0.37 | 0.36, 0.06-0.74 | 0.64 | 0.63, 0.16-1 | 0.58 | 0.61, 0.23-0.96 | 0.63 | 0.66, 0.41-0.81 | 0.048 |
| NGAL | 0.28 | 0.27, 0.1-0.51 | 0.40 | 0.46, 0.18-0.57 | 0.43 | 0.44, 0.21-0.75 | 0.56 | 0.49, 0.44-0.81 | 0.014 |
| Resistin | 0.19 | 0.17, 0.01-0.43 | 0.28 | 0.3, 0.1-0.43 | 0.33 | 0.33, 0.1-0.55 | 0.47 | 0.41, 0.36-0.69 | 0.020 |
| Thrombospondin | 0.11 | 0.12, -0.08-0.29 | 0.15 | 0.18, 0-0.28 | 0.24 | 0.22, 0.03-0.64 | 0.15 | 0.08, -0.01-0.45 | 0.183 |
| Total IgG | 0.21 | 0.2, -0.1-0.48 | 0.41 | 0.42, 0.05-0.66 | 0.23 | 0.17, 0.02-0.61 | 0.38 | 0.45, 0.15-0.48 | 0.224 |
| IgG1 4E3 | 0.00 | 0.05, -0.44-0.56 | 0.25 | 0.21, -0.54-1.21 | 0.25 | 0.26, -0.28-0.93 | 0.27 | 0.16, -0.14-0.9 | 0.344 |
| IgG1 | -0.15 | -0.11, -0.5-0.15 | -0.05 | -0.06, -0.36-0.16 | -0.12 | -0.19, -0.36-0.21 | 0.04 | 0.02, -0.07-0.21 | 0.117 |
| IgG2 | -0.07 | -0.06, -0.96-0.36 | 0.02 | 0.01, -0.03-0.07 | -0.02 | -0.03, -0.18-0.19 | 0.02 | 0.03, -0.08-0.09 | 0.711 |
| IgG3 | -0.29 | -0.07, -3.08-0.14 | -0.10 | -0.13, -0.36-0.21 | -0.14 | -0.13, -0.53-0.34 | 0.04 | -0.04, -0.1-0.31 | 0.474 |
| IgG4 | -0.08 | -0.07, -0.33-0.1 | -0.15 | -0.15, -0.27--0.05 | -0.13 | -0.09, -0.45-0.03 | -0.09 | -0.09, -0.16--0.01 | 0.478 |
| IgA | 0.02 | 0.03, -0.21-0.22 | 0.06 | 0.03, -0.12-0.24 | 0.01 | 0.01, -0.21-0.28 | -0.04 | -0.07, -0.14-0.13 | 0.731 |
| IgM | 0.06 | 0.06, -0.21-0.29 | -0.09 | -0.04, -0.35-0.08 | 0.07 | 0.05, -0.18-0.24 | 0.27 | 0.31, 0.12-0.33 | 0.013 |

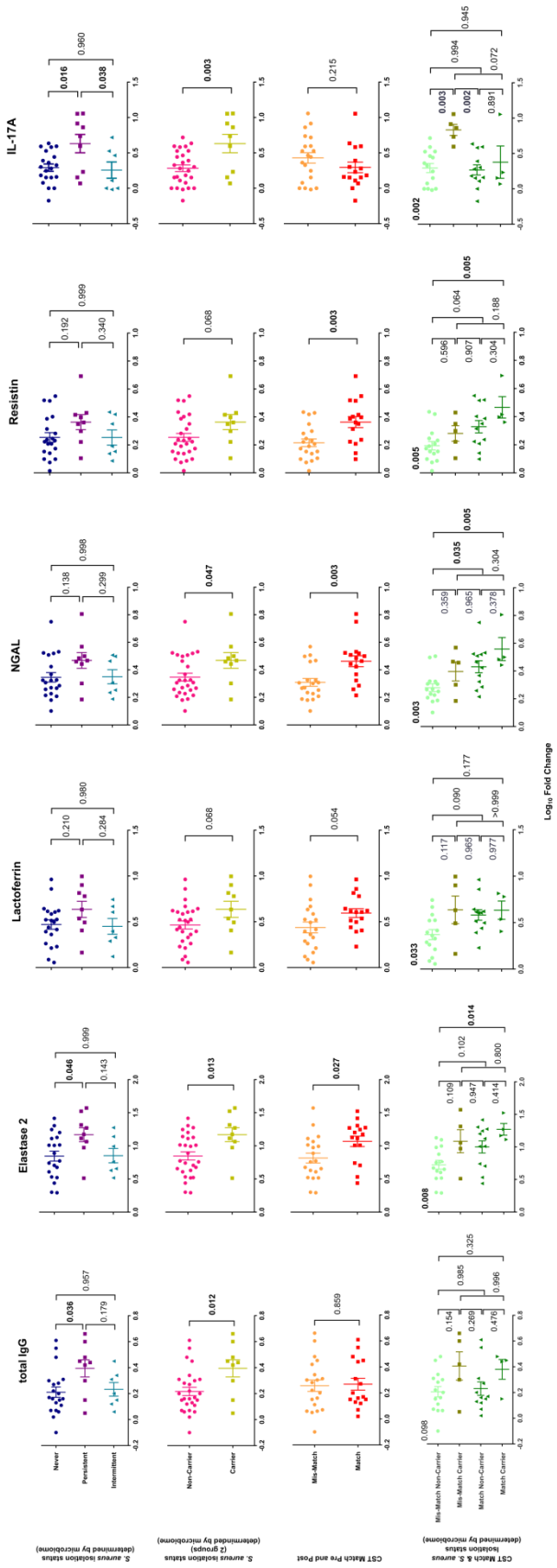



**Figure 3-13: *S. aureus* microbiome and CST match/mismatch association with cytokine levels & anti-*S. aureus* isotyping.** Log fold change for total IgG calculated from Anti-*S. aureus* ΔSpA IgG pre and post (units/ml), and for cytokines from pre and post (pg/ml). Mean and SE of mean shown. Comparisons across 3 groups calculated using multiple comparison tests with Tukey's correction. Comparisons across 2 groups calculated using unpaired 2 tailed t test (without Welch correction).

Whilst the data presented in Table 3-8, Table 3-9, Table 3-10 and Table 3-12 allows comparison of each cytokine and anti-*S. aureus* isotyping variable on a group level, it does not allow visualization of individual data points, which is of interest in those variables in which I observe changes between groups of *S. aureus* status and CST status. Therefore, I have displayed $\log_{10}$ fold change data visually for individual submariners in the variables in which I consistently saw changes between categories of *S. aureus* carriage and CST status in Figure 3-13. These variables are: total IgG, ELA2, Lactoferrin, NGAL, Resistin and IL-17A. The top panel compares data across 3 groups of *S. aureus* microbiome status, and shows that total IgG against *S. aureus*, innate pro-inflammatory proteins (Elastase 2, Lactoferrin, NGAL and Resistin) and IL-17A are all elevated in persistent carriers compared to non-carriers or intermittent carriers. (Data for all cytokines and isotypes investigated by *S. aureus* microbiome: 3 groups shown in Table 3-8). Since those who never had *S. aureus* detected by microbiome and those who had one positive *S. aureus* sample by microbiome appear similar, they were grouped together: those with 2 positive *S. aureus* microbiome detections are 'Carriers', all others are 'Non-carriers' as shown in the second panel. (Data for all cytokines and isotypes investigated by *S. aureus* microbiome: 2 groups shown in Table 3-9). The third panel shows that there is no difference in total IgG against *S. aureus* in those with matching and non-matching CSTs, however innate pro-inflammatory proteins levels increase more during the submarine voyage in individuals with matching CSTs compared to mis-matchers. A slight increase in IL-17A $\log_{10}$ fold change was seen in those with mis-matching CSTs compared to matchers (Data for all cytokines and isotypes investigated by CST status is shown in Table 3-10).

CST match/mismatch and *S. aureus* microbiome status (2 groups) are not significantly associated with each other (as shown above Table 3-11) and therefore I compared cytokine and anti-*S. aureus* isotyping $\log_{10}$ fold change in sub-groups which include *S. aureus* microbiome status and CST status, as shown in the bottom panel of Figure 3-13 (Data for all cytokines and isotypes investigated by CST status and *S. aureus* microbiome: 2 groups shown in Table 3-12). This analysis shows that differences in increase of innate pro-inflammatory proteins and IL-17A are dependent on both CST status and *S. aureus* carriage status. As would be expected, anti-*S. aureus* total IgG is dependent only on *S. aureus* carriage.

Different patterns of increased cytokine levels are seen in innate pro-inflammatory proteins compared with IL-17A. As shown in Figure 3-8, these 4 innate pro-inflammatory proteins cluster together, suggesting their expression is related to one another, and therefore concordant changes in all 4 is to be expected. For these Cluster 1 cytokines, the smallest increase in cytokine levels during the submarine patrol was seen in non-carriers with mis-matching CSTs. The largest increase in Cluster 1 cytokine levels during the submarine patrol was seen in carriers with matching CSTs. Carriers with mismatched CSTs and non-carriers with matching CSTs behaved similarly; their increase in Cluster 1 cytokines sits between the other 2 groups. These data suggest that being a *S. aureus* carrier and not being able to change CST (i.e. inflexible microbiome) is a pro-inflammatory state. For IL-17A, those with matching CSTs had similar increases in IL-17A during the submarine voyage, regardless of *S. aureus* microbiome status. Non-carriers with mis-matched CSTs also had similar increases to those with matching CSTs. However, carriers with mis-matched CSTs had significantly larger increases in IL17A production during the submarine voyage

compared to other groups. The individuals within this group have mis-matching CSTs due to changes in % reads of either S. *aureus*, and/or Moraxella, or *S. epidermidis* (see Figure 3-5). Therefore these data indicate a link between *S. aureus* carriage coupled with changing microbiome and increased IL-17A production.

As described above and shown in Figure 3-12, IgG1 4E3 levels at baseline are dichotomous. Therefore, I used 4E3 status as a metric across which to compare fold change in cytokine levels and anti-*S. aureus* isotyping. I found that $4E3^{High}$ individuals have significantly lower fold change of the Cluster 1 cytokines, ELA2 and LAC, even after Bonferroni's correction is applied. (Table 3-13). Cluster 1 cytokine fold change is significantly lower in $4E3^{High}$ individuals compared to Cluster 3 fold change ($p$ = 0.007, Dunn's multiple comparison test).

**Table 3-13: $Log_{10}$ fold change in cytokines & anti-*S. aureus* isotyping across $4E3^{High}$ and $4E3^{Low}$ groups.** *p* values determined by Mann-Whitney tests.

| Index | Low (10) | | High (26) | | |
|---|---|---|---|---|---|
| | Mean | Median, range | Mean | Median, range | *p* value |
| IFNγ | 0.31 | 0.45, -0.92-1.01 | 0.24 | 0.2, -0.21-0.76 | 0.177 |
| IL-10 | 0.03 | 0, -0.33-0.26 | -0.06 | 0, -0.75-0.28 | 0.337 |
| IL17A | 0.50 | 0.59, -0.18-1.05 | 0.32 | 0.24, -0.02-1.06 | 0.087 |
| IL-1β | 0.27 | 0.16, 0-0.95 | 0.30 | 0.22, 0-1.27 | 0.821 |
| IL-4 | 0.18 | 0.08, -0.06-0.64 | 0.03 | 0, -1.18-0.79 | 0.433 |
| IL-6 | 0.52 | 0.35, -0.33-1.41 | 0.32 | 0.29, 0-0.92 | 0.355 |
| IL-8 | 0.99 | 1.15, 0.02-1.96 | 0.42 | 0.31, -0.21-1.62 | 0.012 |
| MCP1 | 0.10 | 0.12, -0.08-0.19 | 0.07 | 0.08, -0.29-0.39 | 0.337 |
| MIP1α | 0.78 | 0.83, -0.05-1.47 | 0.36 | 0.28, -0.51-1.8 | 0.049 |
| TNFα | 0.28 | 0.28, 0.09-0.75 | 0.15 | 0.15, -0.01-0.37 | 0.041 |
| Elastase2 | 1.21 | 1.24, 0.89-1.57 | 0.82 | 0.75, 0.29-1.52 | 0.001 |
| Lactoferrin | 0.74 | 0.7, 0.51-1 | 0.42 | 0.41, 0.06-0.81 | 0.000 |
| NGAL | 0.45 | 0.48, 0.26-0.57 | 0.35 | 0.3, 0.1-0.81 | 0.015 |
| Resistin | 0.34 | 0.35, 0.22-0.55 | 0.26 | 0.21, 0.01-0.69 | 0.094 |
| Thrombospondin | 0.25 | 0.23, 0.03-0.45 | 0.13 | 0.11, -0.08-0.64 | 0.017 |
| Total IgG | 0.31 | 0.23, 0.06-0.66 | 0.24 | 0.21, -0.1-0.6 | 0.590 |
| IgG1 4E3 | 0.65 | 0.59, 0.24-1.21 | -0.04 | -0.03, -0.54-0.27 | 0.000 |
| IgG1 | -0.08 | -0.08, -0.36-0.21 | -0.11 | -0.11, -0.5-0.21 | 0.590 |
| IgG2 | 0.03 | 0.02, -0.18-0.19 | -0.05 | -0.04, -0.96-0.36 | 0.145 |
| IgG3 | -0.03 | -0.06, -0.36-0.34 | -0.23 | -0.1, -3.08-0.31 | 0.286 |
| IgG4 | -0.11 | -0.09, -0.27-0.03 | -0.10 | -0.08, -0.45-0.1 | 0.958 |
| IgA | -0.01 | -0.07, -0.21-0.28 | 0.03 | 0.03, -0.21-0.24 | 0.320 |
| IgM | 0.05 | 0.04, -0.35-0.33 | 0.07 | 0.08, -0.21-0.33 | 0.689 |

Thus far I have observed that greater fold change of Cluster 1 cytokines is seen in *S. aureus* carriers compared to non-carriers, in matching CST individuals versus mis-matching and in 4E3$^{Low}$ compared to 4E3$^{High}$ individuals. Therefore, I investigated whether the 4E3 phenotype was related to *S. aureus* microbiome status or CST status (Table 3-14).

| | | IgG1 status | | Total |
|---|---|---|---|---|
| | | Low | High | |
| *S. aureus* Microbiome | Non-Carrier | 7 | 20 | 27 |
| | Carrier | 3 | 6 | 9 |
| Total | | 10 | 26 | 36 |

| | | IgG1 status | | Total |
|---|---|---|---|---|
| | | Low | High | |
| CST | Mis-Match | 3 | 17 | 20 |
| | Match | 7 | 9 | 16 |
| Total | | 10 | 26 | 36 |

| | | IgG1 status | | Total |
|---|---|---|---|---|
| | | Low | High | |
| CST & *S. aureus* Microbiome | Mis-Match Non-Carrier | 1 | 14 | 15 |
| | Mis-Match Carrier | 2 | 3 | 5 |
| | Match Non-Carrier | 6 | 6 | 12 |
| | Match Carrier | 1 | 3 | 4 |
| Total | | 10 | 26 | 36 |

**Table 3-14: Comparison of IgG1 4E3 status with *S. aureus* microbiome status and CST status.** Top panel compares IgG1 4E3 with *S. aureus* status. Middle panel compares IgG1 4E3 with CST status. Bottom panel compares IgG1 4E3 with the combined variable of CST status and *S. aureus* status.

Although there are more *S. aureus* non-carriers in the 4E3$^{High}$ group compared to 4E3$^{Low}$ group, no significant association was observed between IgG1 4E3 status and *S. aureus* status (Fisher's exact test, $p$ =0.686).

More CST mis-matching individuals fall into the $4E3^{High}$ group compared with $4E3^{Low}$ group, but the same was not seen for CST matching individuals. The association between IgG1 4E3 status and CST status nears significance. (Fisher's exact test $p$ =0.073).

When 4E3 status is compared across the 4 groups of CST and *S. aureus* status, I observed that all but one mis-match non-carrier submariner fall into the $4E3^{High}$ group. In the other 3 groups, similar numbers of submariners fell into $4E3^{High}$ and $4E3^{Low}$ groups.

The association between IgG1 4E3 status and combined variable of CST status and *S. aureus* status nears significance ($\chi^2 = 6.7$, $p = 0.083$). When non-carriers and carriers (as determined by *S. aureus* microbiome) are separated out, $4E3^{High}$ status is associated with mis-matching CST in non-carriers (Fisher's exact test, $p$ =0.024) but not in carriers (Fisher's exact test, $p$ =1).

To summarise, my findings in this section show that aspects of the microbiome profile of submariners are related to changes in some serum cytokines and anti-*S. aureus* isotyping titres over the course of the submarine patrol. Greater increases in Cluster 1 cytokine levels occurred in *S. aureus* carriers compared to non-carriers and matching CSTs compared to mis-matchers. Despite this, there was no significant association between *S. aureus* status and CST status. When factoring in a third variable, 4E3 status (as determined by high or low response using anti-hinge IgG1 antibody), I found that $4E3^{Low}$ individuals also had greater increases in Cluster 1 cytokine levels compared to $4E3^{High}$ individuals. In non-carriers, mis-matched CSTs were associated with $4E3^{High}$ status and this group of 14 submariners had lower fold

change of Cluster 1 cytokine levels compared with the other 22 submariners analysed (data not shown).

## 3.3 Discussion

In this chapter I have shown that identification of *S. aureus* by 16S microbiome analysis correlated well with the gold standard method of culture to determine *S. aureus* colonisation. I found an inverse association of *S. aureus* with *S. epidermidis* in the nasal microbiome. I assigned the microbiome profile of each sample to a community state type (CST) and found that some individuals changed CST during the submarine patrol, whereas others did not. The change in CST was not associated with *S. aureus* colonisation. I found no association of microbiome with skin health (at baseline or on patrol) or demographic data collated prior to submarine voyage. However, persistent *S. aureus* carriage and unchanging CST were both associated with greater increase in pro-inflammatory cytokine production during the course of the submarine patrol. Finally, I observed a possible association between CST mis-match and high binding of serum to an anti-hinge IgG1 antibody (4E3 clone). This observation was significant when only *S. aureus* non-carriers were considered.

In this study, I did not observe any differences in microbial diversity between *S. aureus* carriers and non-carriers. This is in contrast to results observed by others. One study found that MRSA colonised subjects had significantly lower diversity compared with non-MRSA colonised subjects[118]. My study is much smaller, and did not assess MRSA versus MSSA status which may explain why I did not observe any differences in diversity.

I observed a clear inverse correlation between *S. aureus* and *S. epidermidis* presence in the nasal microbiota; an observation corroborated by other studies[79 119]. These data suggest that competition exists between these two staphylococcal species within

the niche of the nares. Evidence for inhibition of *S. aureus* nasal colonisation by *S. epidermidis* has been previously reported[120].

The results presented in this chapter show that a submarine patrol is a pro-inflammatory environment; increases in innate pro-inflammatory proteins (NGAL, Resistin, Lactoferrin, ELA2) are related to one another and are associated with *S. aureus* persistent carriage and unchanging CST. However, I did not find a significant association between CST matching and *S. aureus* carriage. This does not mean that such as association does not exist, it may be that numbers in the current study are too small to dissect out such an association. When a third dichotomous variable, 4E3 status, is also considered, I showed that $4E3^{High}$ status and CST mis-match are linked in *S. aureus* non-carriers. This phenotype was also associated with lower Cluster 1 cytokine levels compared with all other *S. aureus*, CST status and 4E3 status combinations. This suggests that having high titres of antibody to IgG1 hinge region, not being a *S. aureus* carrier and being able to alter CST (implying a flexible nasal microbiome over time) prevents elevation of innate pro-inflammatory protein production in pro-inflammatory situations such as a six-week submarine patrol.

A possible mechanism to explain this observation is as follows and illustrated in Figure 3-14. IgG1 can control nasal carriage of various species, evidence for this is clear from vaccination regimes against certain *S. pneumoniae* strains, after which other strains will then occupy the niche[67]. The data obtained by my colleagues that titres of a commercial antibody to IgG1 hinge region are dichotomous suggests that a polymorphism exists within the human population within the hinge region. Evidence for such a polymorphism was published recently and associated with rheumatic heart

disease[121]. The IgG1 hinge polymorphism could control or is associated with something that controls the stability of nasal microbiome. In *S. aureus* non-carriers, the polymorphism leads to high 4E3 titres, and therefore high IgG1 titres, which are sufficient to reduce abundance of or eliminate certain taxa from the nares. This then allows other taxa into the nares, resulting in the CST mis-match phenotype. In *S. aureus* carriers, the situation is different as *S. aureus* surface proteins Protein A and Sbi can bind the Fc region of immunoglobulins[122], thus stopping them eliminating other taxa, even in those with high 4E3 titres, so that they appear as those with low 4E3 titres. In this study, this is not seen, but could be due to low numbers of *S. aureus* carriers. A follow up study in a larger study group could help to investigate the hypothesis that high 4E3 titre is associated with functional killing which increases turnover of nasal microbiota; a mechanism which is abrogated by *S. aureus*.

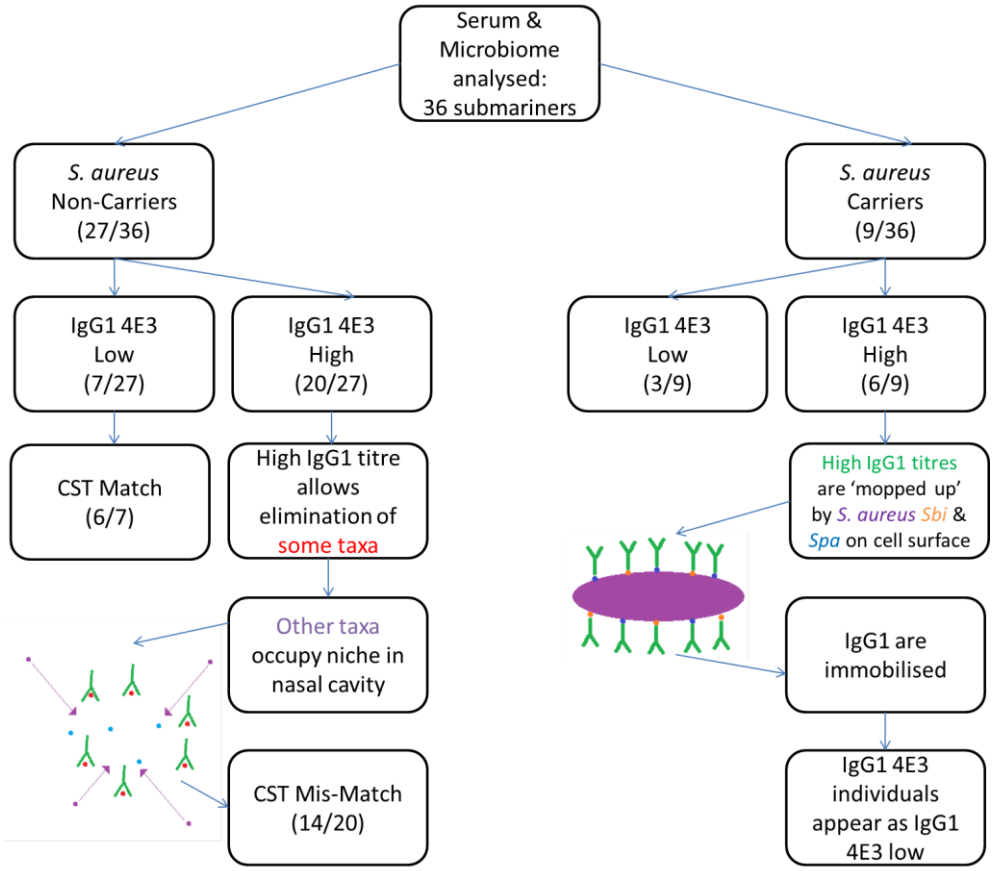



**Figure 3-14: Flow chart to illustrate possible mechanism by which IgG1 polymorphism affects nasal microbiome in *S. aureus* carriers and non-carriers.**

One limitation of this study was that it was performed retrospectively. Therefore power calculations to assess the required number of submariners needed to answer questions relating to skin health, cytokine levels and serological responses were not performed. This study could be used to direct planning of future studies to further investigate patterns seen here.

The main limitation of the study described in this chapter is that I have only investigated a submariner cohort. I have not assessed whether similar changes in cytokine profile and associations with *S. aureus* colonisation and microbiome profile

would occur in a control population. An appropriate control population could be naval recruits undergoing on shore training, from which serum and nasal swabs could be taken six weeks apart. Since these recruits also live in close quarters, another control population could be a cohort of students, which would be more representative of the general population, compared to naval cohorts. Comparisons across all 3 types of cohorts would be interesting.

One key factor relating to the submarine cohort is that cytokine levels increase during the patrol. I would hypothesise that the same would not be true in a cohort of general population sampled six weeks apart. There are a number of factors relating to a submarine patrol which could make it a pro-inflammatory inducing environment. Living in close quarters, lack of exercise and increased food intake could all play a part. The lack of vitamin D could also influence a pro-inflammatory state. This has been investigated previously, and daily supplementation during a patrol had similar effects to placebo[123].

Other studies have investigated the long term effects of being a submarine. Stress rates in submariners are higher than those of other Royal Navy service personnel[124]. Concern about submariners being at increased risk of metabolic syndrome has also been investigated[125]. In a large follow up study, the long term effect of being a submariner on mortality and cancer was found to be no different to that of other Navy personnel. Overall naval personnel had lower all-cause mortality compared to the general population[126]. However, these long term effects cannot explain the short term changes observed in this chapter in serum cytokines.

Further work could also include investigation of microbiome in relation to *S. aureus* colonisation in at risk groups, for example, infants. *S. aureus* is a common component of the microbiome in pre-term infants; one study found *S. aureus* in the gut microbiomes of all 11 preterm infants studied[127]. Further studies into the high prevalence of *S. aureus* in relation to microbiome and immune system development in premature babies may shed light on host requirements for *S. aureus* carriage.

In conclusion, I found differences in changes in cytokine levels in *S. aureus* carriers and non-carriers during a submarine patrol. However, human observational studies cannot attribute causality nor allow for interventions. To investigate the functional impact of immunological changes, animal models are required. Ideally, establishment of a *S. aureus* colonisation model in mice would allow further investigation of the immune phenotype induced by *S. aureus* colonisation. Interventions such as vaccines against *S. aureus* could also be tested in a mouse model of *S. aureus* colonisation.

# 4. *S. aureus* colonisation models in mice: Characterisation and Dynamics

## 4.1 Introduction

Whilst human colonisation by *S. aureus* is well studied[12], the requirements for establishment and maintenance of carriage are not well understood, despite numerous studies[111 128 129]. All humans are exposed at a young age[130], most likely from their mother[131], and serological responses to various *S. aureus* antigens can be detected in both carriers and non-carriers[20]. Various factors may be at play in the mechanisms for carriage establishment, including bacterial factors[132], immune phenotype of the individual[133], other components of the microbiome[134] and possibly host genetics[82 135].

Decolonisation and recolonisation studies in adult humans have been performed and show that propensity for colonisation is a persistent state, with individuals who were *S. aureus* carriers prior to decolonisation being recolonised with the same strains[11]. Despite numerous colonisation studies in children and adults, studies to investigate how or why an infant becomes colonised are very difficult to do in humans. Therefore, an animal model would provide the ideal platform on which to investigate requirements for colonisation and intervention strategies to reduce colonisation. Compared to human cohort studies, an animal model would be well-controlled, for example in terms of exposure from other *S. aureus* carriers.

As well as colonising humans, *S. aureus* can colonise farm animals such as cows, goats and sheep, where it can cause mastitis[136]. Farm animals such as pigs are frequently colonised by *S. aureus*; particular problems exist in Denmark with farmed pigs being colonised with an MRSA strain, Clonal Complex (CC) 398, which can act as a reservoir for transmission to humans[137]. Colonisation has been described in

rabbits[138 139] and other small wild mammals[140]. Thus, *S. aureus* can colonise multiple mammalian species.

However, until a few years ago, laboratory mice were not thought to be carriers of *S. aureus*. An outbreak of preputial gland infections in an animal facility in 2008 was found to be caused by a strain of *S. aureus* persistently colonising the mice[73]. More recently, numerous strains of laboratory mice from various suppliers have been identified as carriers of *S. aureus*[74].

*S. aureus* colonisation models in laboratory rodents exist; the most well-established being long term experimental colonisation in cotton rats [141]. However these animals are often aggressive and therefore difficult to handle. Nasal colonisation models in mice exist, but are short lived, and require antibiotic administration for success[75 76]. Therefore, to date, only the short term impact of *S. aureus* colonisation in mice has been investigated[75 76]. Thus, there is a requirement to investigate the longer term impact of colonisation to better model the situation in humans. This could involve establishment of a long-term colonisation model in mice, which would provide a valuable tool which is more representative of human colonisation than current short-lived models.

Data presented here was performed under Research Objectives 2, 3 & 4:

- Characterise natural murine *S. aureus* carriage
- Establish murine *S. aureus* colonisation experimentally
- Characterise murine *S. aureus* experimental colonisation model

In this chapter, I sought to identify whether *S. aureus* colonises laboratory mice in our animal unit. Following successful identification of *S. aureus* in laboratory mice, I then characterised the colonising strain and showed that some mice remain colonised while others lost their colonisation. Finally, I investigated different methods to induce experimental colonisation in *S. aureus* naïve mice. I was able to establish long term colonisation (21 days and longer) in 3 strains of mice using a needle and antibiotic free methodology. I then use the model in subsequent chapters to investigate immunological impact of colonisation and effect of vaccines on colonisation.

## 4.2 Results

### 4.2.1 Mice are naturally colonised with *S. aureus*

Given that other laboratories had reported problems associated with colonisation of mice with *S. aureus*[73], a sensible starting point for studying *S. aureus* colonisation in mice was to determine whether the mice used within the *S. aureus* vaccine development group were already colonised with *S. aureus*. Studies within the group typically use BALB/c mice, obtained from Harlan (Blackthorn, UK, now Envigo) at 6 weeks old. Initially, I took stool samples from a selection of 36 BALB/c mice aged 6 weeks old, on the day of arrival or within 7 days of arrival from the supplier. All stool samples tested were found to be *S. aureus* positive (Table 4-1, Figure 4-1A), as determined by growth of blue colonies on chromogenic selective agar plates (Brilliance Staph24 agar plates, Oxoid).

**Table 4-1: Successful recovery of *S. aureus* from stool samples, cheek swabs and nasal swabs was compared. *p* values calculated using 2 sided Fisher's Exact test.**

| Data analyzed | Positive | Negative | Total |
|---|---|---|---|
| **Stool sampling** | 36 | 0 | 36 |
| **Cheek Swabbing** | 27 | 9 | 36 |
| **Total tested** | 63 | 9 | 72 |
| ***p* value** | 0.002 | | |
| | | | |
| **Stool Sampling** | 36 | 0 | 36 |
| **Nasal Swabbing** | 1 | 4 | 5 |
| **Total tested** | 37 | 4 | 41 |
| ***p* value** | < 0.001 | | |
| | | | |
| **Cheek Swabbing** | 27 | 9 | 36 |
| **Nasal Swabbing** | 1 | 4 | 5 |
| **Total tested** | 28 | 13 | 41 |
| ***p* value** | 0.028 | | |

Stool sampling was determined to be the least invasive sampling method to detect *S. aureus* colonisation. I also took cheek swabs from the same 36 mice, and found that this method was not as sensitive as stool sampling with 27/36 positive samples, compared to 36 positive stools; a difference which was found to be statistically significant (Table 4-1).

Nasal swabbing in humans is used routinely in clinical studies to determine *S. aureus* carrier status[12 128]. Ideally, this methodology would be used in mice too; but technically is much more challenging and more invasive than taking stool samples. Since nasal colonisation and GI tract colonisation may be separate colonisation niches, I took a subset of nasal swabs from 5/36 mice. Since only 1/5 nasal swabs was positive, nasal swabbing was found to be significantly less sensitive than either cheek swabbing or stool sampling (Table 4-1).

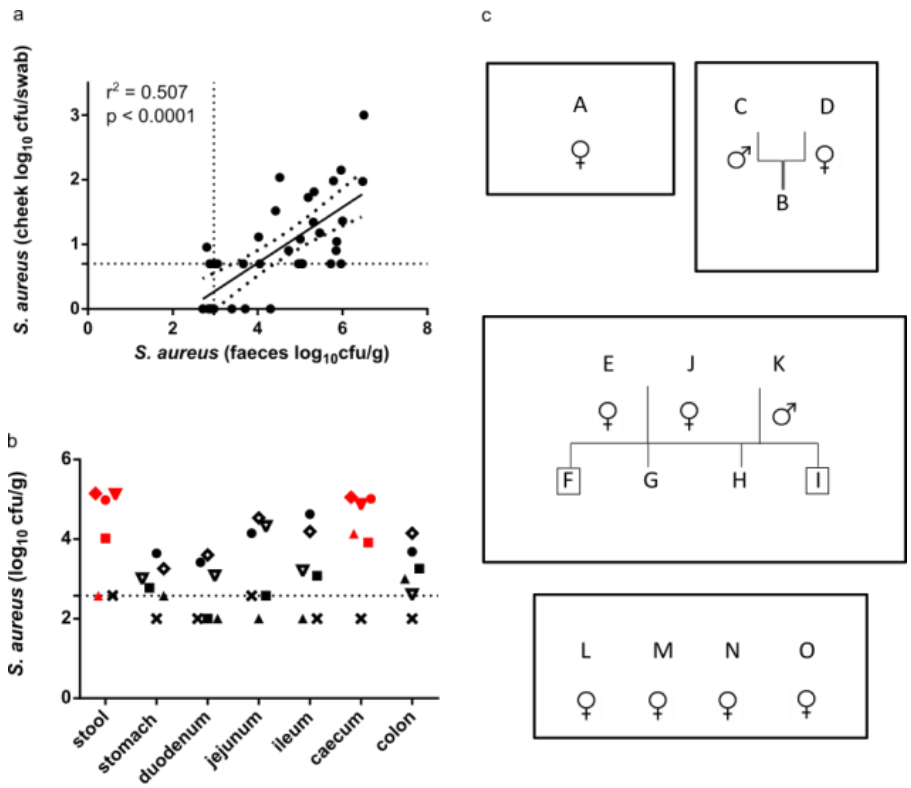



**Figure 4-1: *S. aureus* naturally colonises the GI tract of mice.** (A) *S. aureus* bacterial recovery from stools and cheek swabs in 36 animals. (B) *S. aureus* bacterial recovery from GI tract sections in 6 animals. (A, B) *S. aureus* bacterial recovery from stool samples, cheek swabs and sections of GI tract from female BALB/c mice from Harlan was determined by plating onto selective agar. Horizontal dotted lines in (A) and (B) represent the limit of detection of the culture assay. For cheek swab samples, negative samples are plotted as if 1 colony/g colony was detected. For faecal and organ samples, negative samples are plotted as if 100 colonies/g were detected. Samples coloured red were *spa* typed and found to be t084. (C) *S. aureus* is efficiently transmitted from parents to offspring. Stool samples were obtained from an in-house breeding colony of BALB/c mice. Each box represents a cage within the colony. A, C, D E, J & K are breeders, B, F, G, H, I are pre-weaned pups and L, M, N, O are weaned pups (samples from parents of these animals were not available). *S. aureus* was recovered from stools of all animals except F and I (indicated by boxed letters).

Levels of *S. aureus* colonisation from stool samples and cheek swabs were quantified by counting blue colony forming units (cfu) on the selective agar plates. Bacterial recovery from cheek and stools showed strong positive correlation ($p < 0.0001$, $r^2 = 0.507$) (Figure 4-1A). To investigate distribution of *S. aureus* throughout the GI tract, sections of GI tract and nasal wash were taken after post mortem of 6 mice. Bacterial recovery was greatest in stool and caceal samples, (Figure 4-1B) and bacterial recovery in different parts of the GI tract positively correlated with faecal bacterial recovery (Figure 4-2).

Along with other members of the research group in which I was working, I took stool samples from all BALB/c female mice arriving from Harlan for use in *S. aureus* vaccination studies over a period of 18 months. 94% BALB/c (416/433) mice aged 6-8 weeks were found to be *S. aureus* positive within 7 days of arrival. Therefore, *S. aureus* was found to be colonising the GI tract of BALB/c mice obtained from Harlan, and mice are likely to be exposed early in life. Given this, I hypothesised that other, in-house, breeding colonies could be colonised with *S. aureus*. Samples from an in house breeding colony were kindly supplied by Dr Claire Pearson. I detected faecal carriage of *S. aureus* in 3/5 pre-weaned pups, 4/4 post weaning pups and 6/6 adults (Figure 4-1C). This indicates that acquisition of carriage does indeed occur early in life, likely from the mother, and that *S. aureus* is efficiently acquired by offspring within a breeding colony.

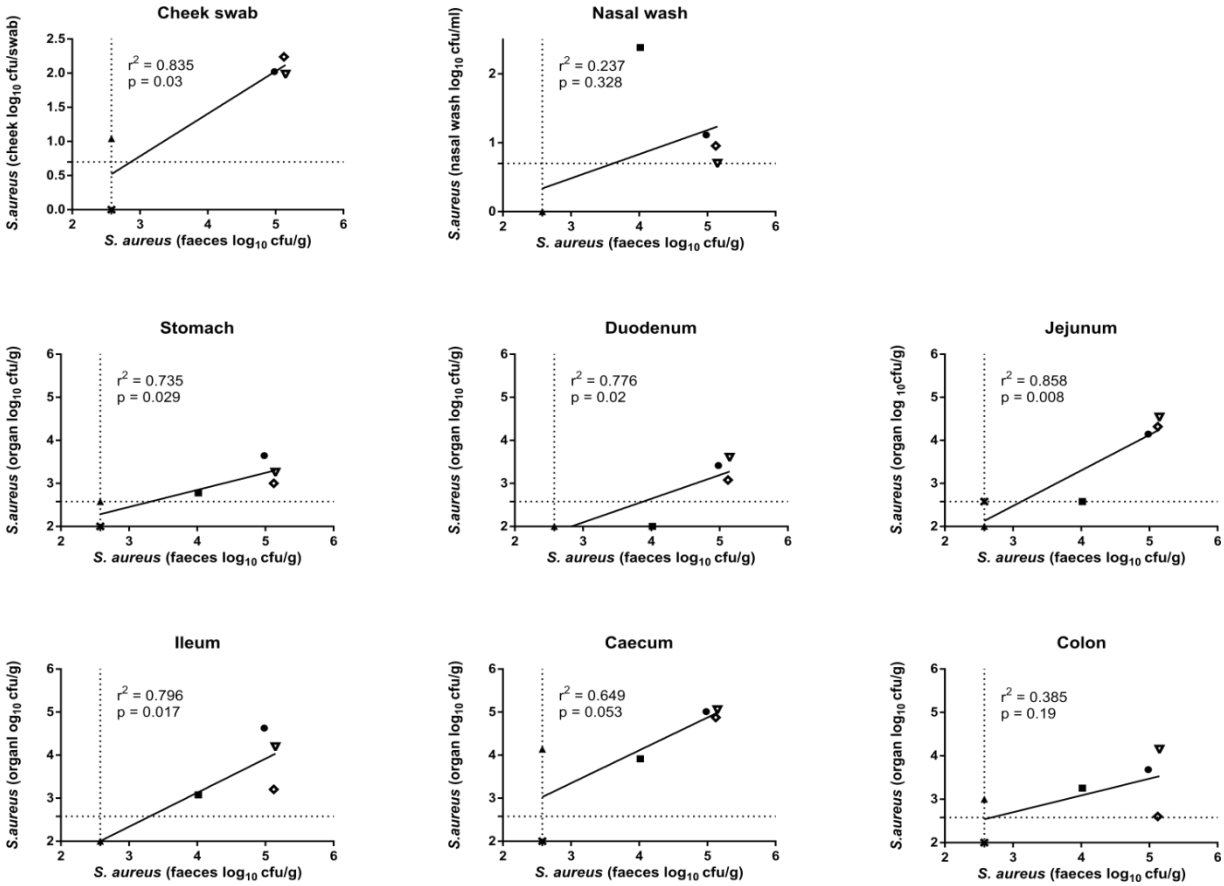



**Figure 4-2: *S. aureus* recovery from throughout GI tract positively correlates with recovery from stool samples.** 6 female BALB/c mice from Harlan were confirmed as *S. aureus* carriers on day of arrival by stool sampling. 3 days later stool samples and cheek swabs were taken, animals were culled and organs were removed and nasal wash performed. *S. aureus* recovery from cheek swab, nasal wash and organs against *S. aureus* recovery from stool, positively correlates with recovery from stool samples.

Stool sampling was selected as the measure of murine *S. aureus* carriage in live mice for the rest of the study, as it is the most sensitive and least invasive.

### 4.2.2 Characterisation of the colonising strain

DNA sequencing of the repeat region of *S. aureus* protein A gene (*spa* typing) is routinely used to type *S. aureus* isolates[142]. The number and order of the repeats determines the assigned *spa* type.

I performed *spa* typing on *S. aureus* isolates from stools of 4 pups from the in-house breeding colony and found them to be s*pa* type t2177. I also performed *spa* typing on *S. aureus* isolates from stools of mice obtained from Harlan. In total 91 isolates from 62 different BALB/c mice obtained were *spa* typed and found to be t084. Samples which were *spa* typed are shown as red symbols in Figure 4-1, Figure 4-4 and Figure 4-5. These samples were from multiple different deliveries from Harlan and include isolates from stool and caecum (Figure 4-1B). Two further isolates from stools from two different mice were found to be *spa* types t120 and t346 respectively, which each differ from t084 by only one repeat. These findings indicated that different *S. aureus* sequence types are capable of colonising mice, as t2177 and t084 are not related, although one clone may dominate particular facilities.

To further investigate characteristics of the *S. aureus* strain colonising BALB/c mice arriving at our animal facility from Harlan, 4 individual isolates were selected. Each of these 4 *S. aureus* isolates originated from one mouse of 4, all of which were housed in the same cage, and were *spa* type t084. The antibiotic resistance profile of these 4 isolates was the same (Table 4-2), including resistance to penicillin, sensitivity to methicillin (oxacillin used to test as a proxy), and sensitivity to other commonly used antibiotics.

**Table 4-2: Antibiotic resistance profile of 4 *S. aureus* isolates from mouse stool**

| Antibiotic / Sample | AF003-1A | AF003-1B | AF003-1C | AF003-1D |
|---|---|---|---|---|
| **Penicillin** | Resistant | Resistant | Resistant | Resistant |
| **Oxacillin** | Sensitive | Sensitive | Sensitive | Sensitive |
| **Ciprofloxacin** | Sensitive | Sensitive | Sensitive | Sensitive |
| **Vancomycin** | Sensitive | Sensitive | Sensitive | Sensitive |
| **Gentamicin** | Sensitive | Sensitive | Sensitive | Sensitive |
| **Erythromycin** | Sensitive | Sensitive | Sensitive | Sensitive |
| **Tetracycline** | Sensitive | Sensitive | Sensitive | Sensitive |
| **Trimethoprim** | Sensitive | Sensitive | Sensitive | Sensitive |
| **Mupirocin** | Sensitive | Sensitive | Sensitive | Sensitive |
| **Fucidin** | Sensitive | Sensitive | Sensitive | Sensitive |
| **Rifamicipin** | Sensitive | Sensitive | Sensitive | Sensitive |
| **Clindamycin** | Sensitive | Sensitive | Sensitive | Sensitive |
| **Clinda D test for inducible mechanism of resistance (Mlsb)** | Negative | Negative | Negative | Negative |

Illumina-based whole genomic sequencing (WGS) of these isolates was performed by colleagues at the John Radcliffe hospital, led by Dr Nicola Gordon. *In silico* data analysis performed in this section was performed with David Wyllie and Dr Jessica Hedge.

WGS results indicated that all four isolates investigated were identical except for one isolate (AF003-1C) which differed from the other three by 17 single nucleotide polymorphisms, scattered across the core genome. Therefore, a clade of closely related *S. aureus*, which I termed 'SaF', was colonising the GI tract of BALB/c mice arriving at the animal unit from Harlan. I selected one of the three identical isolates

(AF003-1A) to use in all further experimental colonisation work and named this strain 'SaF_1'. *In silico* multilocus sequence typing showed that the 4 SaF clade isolates belong to sequence type (ST) 15.

To assess the possible origin of the SaF clade, the 4 SaF clade isolates were compared with a set of fifteen ST15 human isolates, obtained from nasal screening at GP practices in the UK, previously described by Everitt *et al*, 2014. A maximum likelihood phylogenetic tree was constructed, and evidence of recombination between the human and mouse strains sought using ClonalFrameML. The maximum likelihood tree is shown (Figure 4-3A). There was no evidence of recombination (not shown). BEAST was therefore used to reconstruct the likely evolutionary relationship between my four isolates. In the absence of any other published estimates of an ST15 clock rate, and insufficient clock signal in the samples available to estimate this directly, a published estimate of the *S. aureus* molecular clock was used[16]. This indicated that among the four SaF isolates, AF003-1C diverged from the others approximately 1.6 years previously (95% credible interval 1.0-2.4 years). Assuming a single species jump event, this approach also indicated that the isolated murine strains may have diverged from the related human strain about 24.2 years (95% credible interval 22.2-26.2 years) prior to the isolation date.

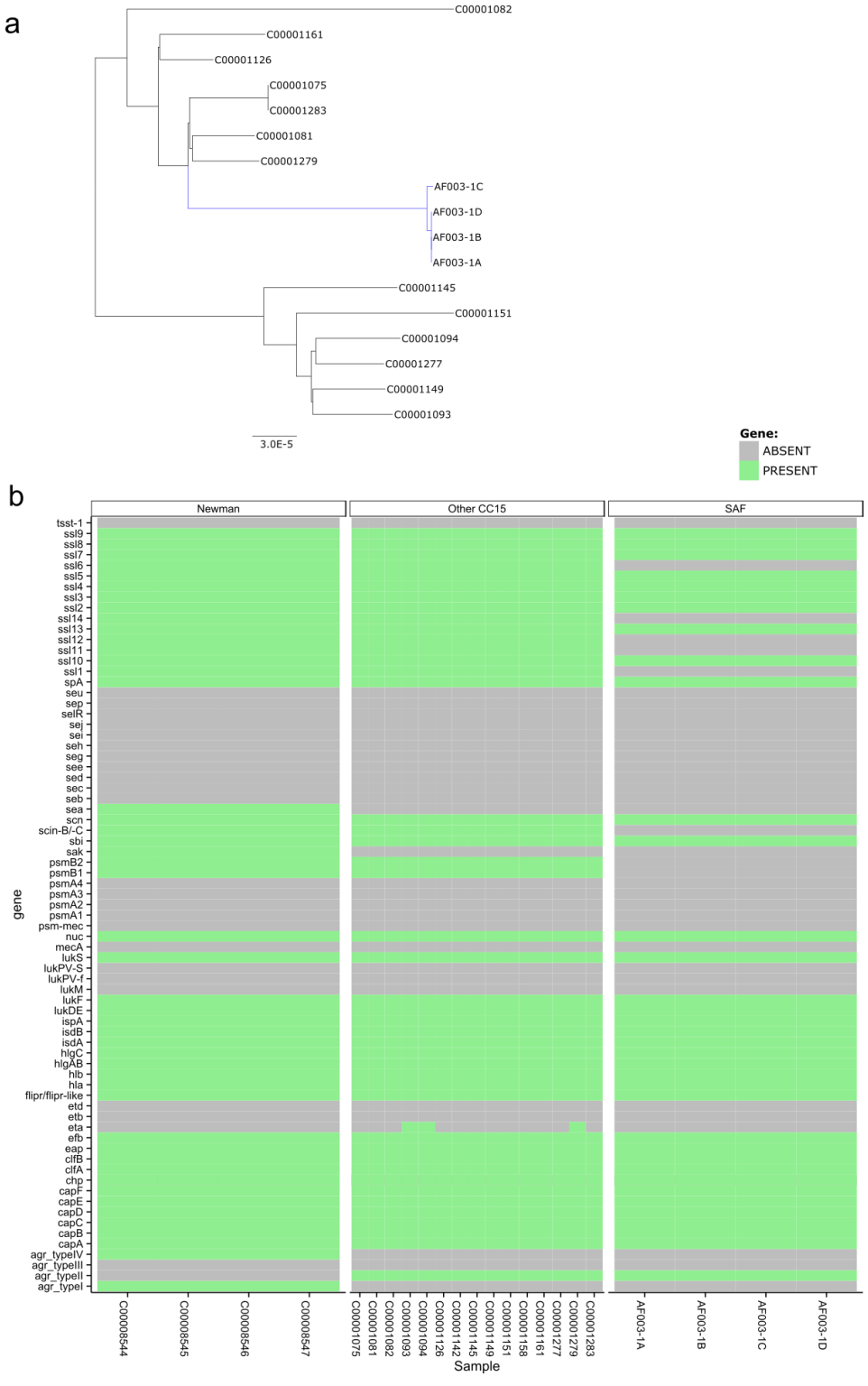



**Figure 4-3: Characteristics of SaF strain revealed from whole genome sequencing.** WGS was performed on *S. aureus* isolated from stools from 4 female BALB/c

mice, which we termed SaF. Isolates were labelled AF003-1A, -1B, -1C and 1D. (A) Maximum likelihood tree showing relationship to human ST15 isolates (SaF clade highlighted in blue). Scale bar refers to per base substitution rate. (B) Gene presence/absence table comparing 'SaF' isolates to human CC15 isolates and multiple independent sequencing of the standard laboratory strain Newman. Grey indicates absence and green presence of the gene.

The 4 SaF clade isolates were mapped to reference genome MRSA252 and compared with the documented variation in human ST15 isolates from a published collection[77 96] representative of human *S. aureus* diversity. This indicates that the SaF clade is similar to, and maps within, identified human ST15 isolates (Figure 4-3A). To assess diversity in the accessory genome, gene presence/absence estimation was performed against a 73 gene panel *in silico* (Figure 4-3B) which demonstrated that the SaF clade isolates are similar to human ST15. Sequences of the 73 genes investigated are listed in Table S3. Like human ST15 isolates, the SaF clade lacks some superantigen genes[73 143] while others are present in the ST15 human strains but absent in SaF, suggestive of gene loss. Similarly, genes associated with the immune evasion cluster (IEC) e.g.scn, were missing. I concluded that SaF was similar to human *S. aureus* ST15 clones, but lacked some genes associated with mobile elements in human derived clones. Overall, I considered the genomic analysis compatible with the strain to be of recent human origin, potentially acquired from animal care takers in the breeding facility/supplier.

### 4.2.3 *S. aureus* colonisation can persist over months

Thus far, I have shown that female BALB/c mice are colonised by an *S. aureus* strain, termed 'SaF' upon delivery from the supplier, Harlan. Next, I sought to investigate whether colonisation would be maintained in these mice whilst in our animal unit.

Therefore, I investigated the dynamics of SaF carriage over time by measuring bacterial recovery from mouse stools longitudinally.

Groups of two to four mice were randomly assigned to individually ventilated cages and *S. aureus* carriage was monitored by stool sampling for at least 90 days in three independent experiments. Data from all experiments are presented: Figure 4-4 shows Experiment 1 (24 mice) and Figure 4-5 shows Experiments 2 and 3 (12 mice in each).

Different patterns of SaF carriage were observed across different cages. For example, in Experiment 1 animals in Cage 3 had all lost SaF carriage by the end of the experiment (Figure 4-4A), whereas those in Cage 6 maintained carriage levels seen upon arrival throughout the experiment. When <4 cfus were detected per 50µl of stool homogenate, stool homogenates were enriched as described in Material and Methods. Samples which were positive upon enrichment were deemed to be at the lower limit of detection of the sampling technique. Loss of carriage was defined as the timepoint at which *S. aureus* carriage was lost or detectable by enrichment only at that timepoint and the following timepoint. Using this endpoint, time to loss of SaF carriage was assessed using survival analysis. Time to loss of carriage was significantly different across the 6 cages of the experiment shown in Figure 4-4 ($p$ = 0.004, assessed using a Log rank (Mantel-Cox) test). 6 mid-experiment isolates and 11 isolates available at the end of the experiment (from mice which had not lost carriage) were *spa* type t084 (Figure 4-4A), showing that the same *spa* type, and implying that the same strain is colonising the mice throughout the experiment.

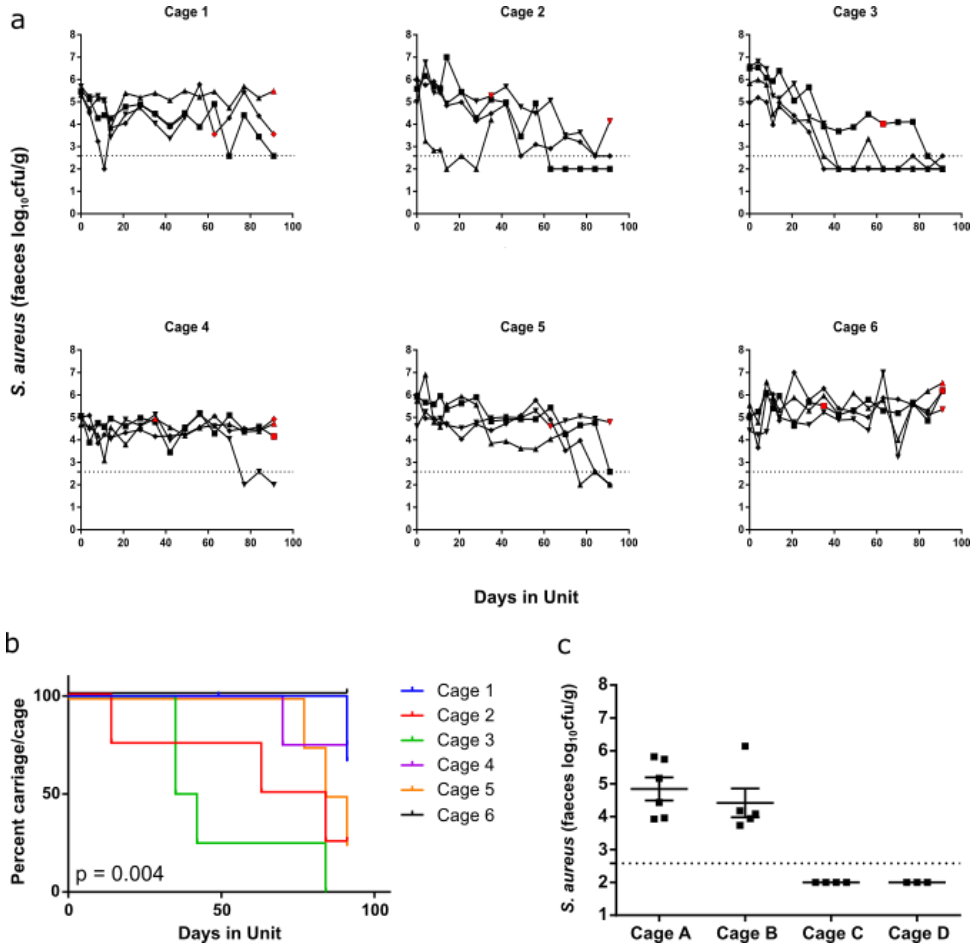
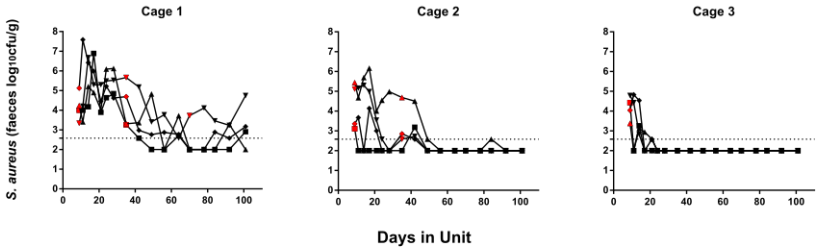


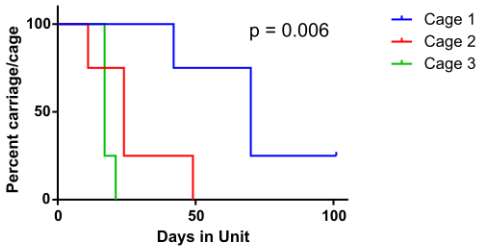

**Figure 4-4: Longitudinal monitoring of SaF carriage.** (A & B) *S. aureus* carriage levels were monitored by stool sampling in 24 female BALB/c mice, randomly allocated 4 per cage, for 3 months. (A) Carriage levels are shown per mouse within a cage. Samples coloured red were *spa* typed and found to be t084. (B) Kaplan-Meier analysis for loss of carriage per cage: *p* value given for log rank (Mantel-Cox) test. Adverse events occurred as follows – one mouse in Cage 1 had to be culled at 49 days and one mouse in Cage 2 had to be culled at 35 days due to unrelated illness. (C) Cross-section of *S. aureus* carriage in older mice. *S. aureus* carriage levels were tested by stool sampling in 18 female BALB/c mice from Harlan 13 months after arrival at the animal unit. Carriage levels are shown for individual mice at one time point only. Horizontal dotted lines in (A) and (C) represent the limit of detection of the culture assay. Negative samples are plotted as if 100 colonies/g were detected.

Differences in SaF carriage between cages and maintenance of *spa* type t084 were confirmed in two other experiments (Figure 4-5). In Experiment 2 (Figure 4-5A), all mice in Cage 3 lost their *S. aureus* carriage before day 20 after arrival from the supplier. This rate of loss is much quicker than that seen in Experiment 1 (Figure 4-4A) and in the other cages of Experiments 2 and 3 (Figure 4-5). Median time to loss of carriage across the three experiments was 77 days, with a median of 91 days for the experiment shown in Figure 3 (Experiment 1) and 24 days and 77 days for the experiments shown in Figure 4-5A (Experiment 2) and Figure 4-5C (Experiment 3) respectively. Cox regression using 'experiment' as a co-variate showed that the effect of experiment on loss of carriage was significant ($p$ = 0.019). Cox regression using 'cage' as a co-variate showed that the effect of cage on loss of carriage was significant ($p$ = 0.012) in Experiment 1, but not in Experiments 2 or 3 ($p$ = 0.229 and 0.128 respectively). Taken together, the data presented in Figure 4-4 and Figure 4-5 imply that each cage is a microenvironment within which the dynamics of SaF carriage differ. However, the same colonising strain is maintained throughout experiments.

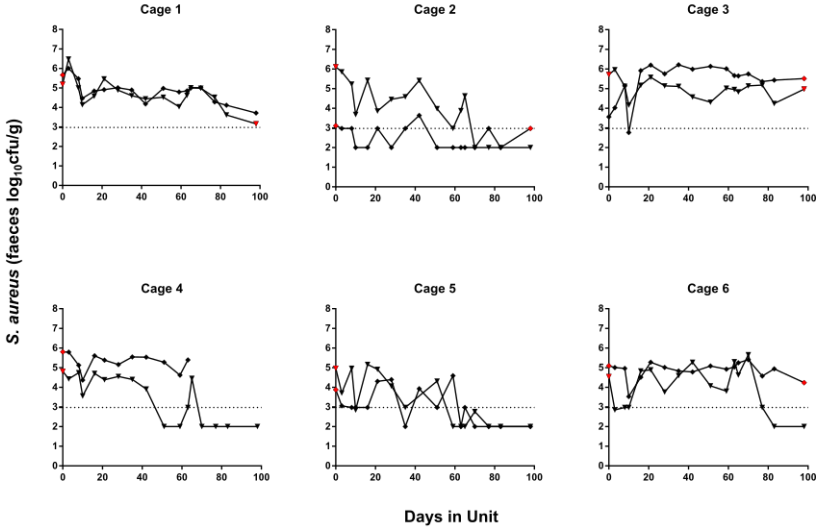
a


Cage 1
Cage 2
Cage 3
S. aureus (faeces log10cfu/g)
Days in Unit

b


p = 0.006
Cage 1
Cage 2
Cage 3
Percent carriage/cage
Days in Unit

c


Cage 1
Cage 2
Cage 3
Cage 4
Cage 5
Cage 6
S. aureus (faeces log10cfu/g)
Days in Unit

d

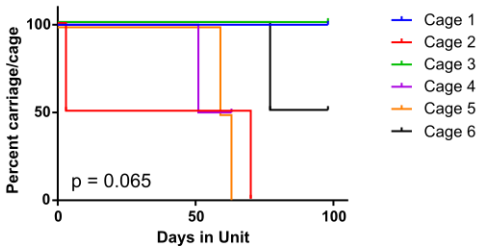

Cage 1
Cage 2
Cage 3
Cage 4
Cage 5
Cage 6
p = 0.065
Percent carriage/cage
Days in Unit

**Figure 4-5: Longitudinal monitoring of SaF carriage in a further two independent experiments.** Data shown here is independent of experiment shown in Figure 4-4. *S. aureus* carriage levels were monitored by stool sampling in 12 female BALB/c mice from Harlan, randomly allocated 4 per cage, for 3 months (A & B) and in a further 12 female BALB/c mice from Harlan, randomly allocated 2 per cage, for 3 months (C & D). (A, C) Carriage levels shown per mouse within a cage. Samples coloured red were *spa* typed and found to be t084. In (A) quantitative analysis of carriage was performed from 9 days onwards. (B, D) Kaplan-Meier analysis for loss of carriage per cage. *p* value given for Log rank (Mantel-Cox) test. *S. aureus* unrelated adverse events occurred as follows – one mouse in Cage 4 had to be culled at 63 days due to illness.

As the mice used in Experiments 1 to 3 described above (Figure 4-4A & B and Figure 4-5) were aged 6 weeks to 4-5 months, I investigated whether SaF carriage existed in an apparently dichotomous state in older mice. Dr Christina Dold (Oxford Vaccine Group) kindly allowed me to take stool samples from some of her mice, as her experiments involve mice over a year old. I performed a cross-sectional analysis of SaF carriage in 18 BALB/c mice 13 months after arrival in the animal unit, and found that SaF carriage was present in 2 out of the 4 cages tested (Figure 4-4C). This finding is compatible with the polarisation that I observed in younger animals persisting for up to 12 months and possibly longer.

### 4.2.4 Co-housed mice can transmit *S. aureus*

Since I observed cage-specific trends in loss of *S. aureus* carriage in animals naturally colonised at an early age, I considered whether transmission between mice housed in the same cage could occur. Evidence from another study showed that if *S. aureus* experimentally infected mice were co-housed with *S.aureus* free mice, the infecting

strain could be recovered from the stools of the non-infected mice from one to seven days later[144]. Therefore, I hypothesised that naturally colonised mice could transmit colonisation to *S. aureus* free mice if housed together.

Certified *S. aureus* free (SOPF) BALB/c mice were obtained from Harlan. These mice were bred inside a different barrier to those which have been used in all experiments described so far, and I confirmed that they were *S. aureus* free by stool sampling upon arrival at the animal unit. Naturally colonised mice were also obtained from Harlan and confirmed to be *S. aureus* colonised upon arrival by stool sampling. Both groups of mice were females, aged 6 weeks upon arrival.

To determine whether naturally colonised mice can transmit *S. aureus* to other mice, I designed a co-housing experiment with the *S. aureus*-free and colonised BALB/c mice (Figure 4-6). Initially, 4 mice were housed in each cage, all of which were either *S. aureus*-free (3 cages, 12 mice) or *S. aureus* colonised (3 cages, 12 mice). I took baseline stool samples from all mice to quantify colonisation levels (Figure 4-6A), and then performed a 'cage-swap'. This resulted in 4 cages of co-housed mice: 2 *S. aureus*-free animals were housed with 2 colonised animals (by swapping 2 mice from a *S. aureus*-free cage to a colonised cage and *vice versa*). One cage with only *S. aureus*-free mice and one cage with only colonised mice were used as controls throughout (no swapping) (Figure 4-6B). I quantified *S. aureus* carriage levels in all mice for 45 days (Figure 4-6C).

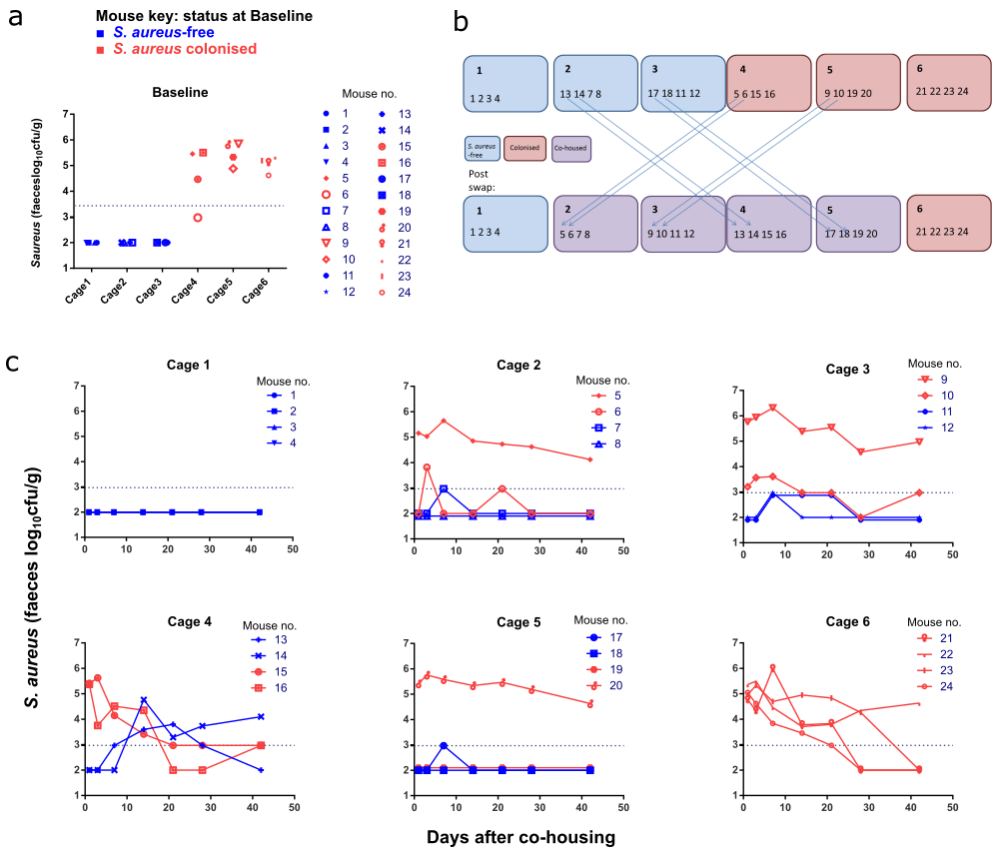



**Figure 4-6: Co-housing to investigate transmission between *S. aureus*-free and colonised mice**. 24 female BALB/c mice were monitored for GI tract colonisation by stool sampling longitudinally. Mice were housed in cages of 4 at all times. At first *S. aureus*-free mice were housed in cages 1 to 3 and *S. aureus* colonised mice in cages 4 to 6. After taking baseline stool samples, a 'cage-swap' was performed: two *S. aureus*-free mice were co-housed with two colonised mice in Cages 2-5. No swapping occurred in Cages 1 & 6; these acted as negative and positive controls, respectively. (A) Baseline carriage levels for individual mice. (B) Experimental design. (C) Carriage levels after 'cage-swap'. Individual mice labelled 1-24 throughout experiment. Horizontal dotted lines in (A) and (C) represent the limit of detection of the culture assay. Negative samples are plotted as if 100 colonies/g were detected.

As would be expected given previous results, levels of colonisation in the initially *S. aureus* colonised mice decreased over the course of the experiment. In some initially

*S. aureus*-free animals I observed *de novo* acquisition of *S. aureus* (Figure 4-6C, mice 13 & 14); however, other co-housed mice (7, 8, 11, 12, 17 & 18) demonstrated either low-level carriage, which was only transiently detected, or no acquisition of carriage. Thus, under the conditions found in the cages of this experiment, transmission of *S. aureus* GI tract colonisation to *S. aureus* naïve mice can occur, albeit infrequently. Transmissibility, as well as rate of loss of carriage, may be dependent on the cage microenvironment.

### 4.2.5 Short term experimental colonisation by oral gavage

So far in this chapter, I have identified and characterised a strain of *S. aureus* colonising nasal and GI tract of BALB/c mice. I have shown that genetically identical animals dichotomise into carrier and non-carrier populations, and have presented evidence that this is environment-specific. I next investigated whether I could induce experimental colonisation in *S. aureus* free mice using the *S. aureus* strain (SaF_1) isolated from naturally colonised mice. Establishment of such an experimental colonisation would allow me to test intervention strategies to reduce carriage such as vaccines, as well as investigating the immunological impact of colonisation in colonised mice versus naïve mice. The experiment described in this section was a collaborative effort between me and Dr Pauline van Diemen, a post-doctoral research scientist within the *S. aureus* research group. The methodology selected to colonise mice was by oral gavage, as we determined that this would be the most direct route to administer *S. aureus* to the GI tract.

37 certified *S. aureus* free BALB/c mice were obtained (Taconic Farms, Denmark) and their *S. aureus* free status was confirmed by stool sampling upon arrival. Faecal carriage was monitored after mice received oral gavages at Day 0 and at Day 35 of either PBS (Figure 4-7B bottom panels) or $10^8$ cfu of SaF_1 in the same volume of PBS (Figure 4-7B top panels). The experiment was designed such that there were 4 different treatment group; mice received either PBS twice, or SaF_1 twice or one dose of SaF_1 and one dose of PBS, according to the experimental design shown in Figure 4-7A.

Faecal carriage of very short duration was induced in mice receiving SaF_1, with all mice except two in the group receiving two doses of SaF_1 losing faecal carriage within 7 days of each gavage (Figure 4-7B). SaF_1 carriage was detectable in these two mice up to 133 days after first oral gavage, although they were housed in different cages. *S. aureus* isolates from their stool, nose and cheek from 84 days after first oral gavage were confirmed as *spa* type t084, and therefore presumed to be SaF_1. One of the mice persistently carrying SaF_1 was housed in a cage with other mice which received PBS twice (Figure 4-7C), indicating that, at least in some animals, colonisation can persist without repeated re-exposure from others in the cage. At the end of the experiment, 203 days after the first oral gavage, I did not detect *S. aureus* colonies in segments of GI tract from 3 mice which received SaF_1 twice and subsequently lost faecal carriage. This implies that loss of faecal carriage is indicative of loss of carriage throughout the GI tract. Taken together, these results demonstrate that short term exposure to *S. aureus* can result in persistent colonisation, but at a low frequency. It also supports the previous experiment (Figure 4-6) indicating that

co-housing with a colonised mouse is not sufficient to consistently initiate long-term colonisation in adults.

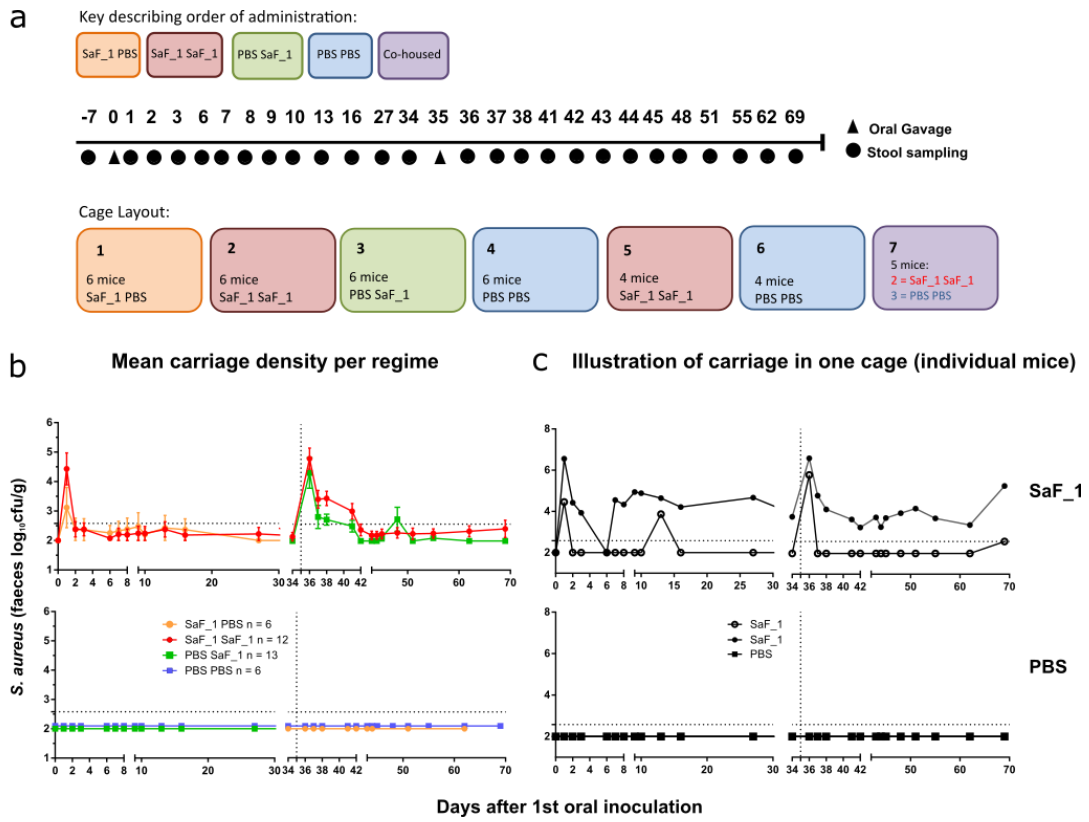



**Figure 4-7: Experimental colonisation of *S. aureus*-free mice with SaF by oral gavage.** 37 Female BALB/c mice were confirmed to be *S. aureus*-free by stool culture. Animals were given $10^8$ cfu SaF_1 or PBS by oral gavage and carriage levels were monitored by stool sampling. Each mouse received two oral gavage treatments at 0 days and 35 days (indicated by a vertical dotted line) with SaF_1 or PBS. (A) Experimental Design and Timescale (B & C) Top panels show SaF_1, bottom panels show PBS exposure. (B) Mean carriage levels shown per regime. 6 – 13 mice per regime. (C) Individual carriage levels for one cage containing 5 mice: 2 received 2 doses of SaF_1, 3 received 2 doses of PBS. Horizontal dotted lines represent the limit of detection of the culture assay. Negative samples are plotted as if 100 colonies/g were detected.

### 4.2.6 Experimental colonisation by environmental contamination

Since colonisation by oral gavage only resulted in short-lived (<7 days) *S. aureus* GI tract colonisation, I tried an alternative approach, with the aim of inducing longer term colonisation. This involved spraying the SaF_1 inoculum into the cage environment, which represents a refinement in terms of methodology recognised by the Home Office inspector. The mice were not sprayed directly; instead the bedding and sawdust were contaminated with the inoculum.

Initial experiments were performed using pregnant females, since I hypothesised that SaF_1 exposure at a very young age would better represent the human situation, in which babies are likely colonised by their mothers[131]. I successfully colonised both BALB/c and C57BL/6 mice as neonates and since the methodology was successful, I repeated in adult mice, which also proved successful, with colonisation being maintained for at least 100 days in some animals (Figure 4-8).

Figure 4-8 shows data from 6 experiments in 2 mouse strains, with 3+ non-colonised control animals in each experiment. A representative stool sample from each cage of negative control animals was tested at each timepoint. These animals remained *S. aureus* free throughout the experiments (data points in black)

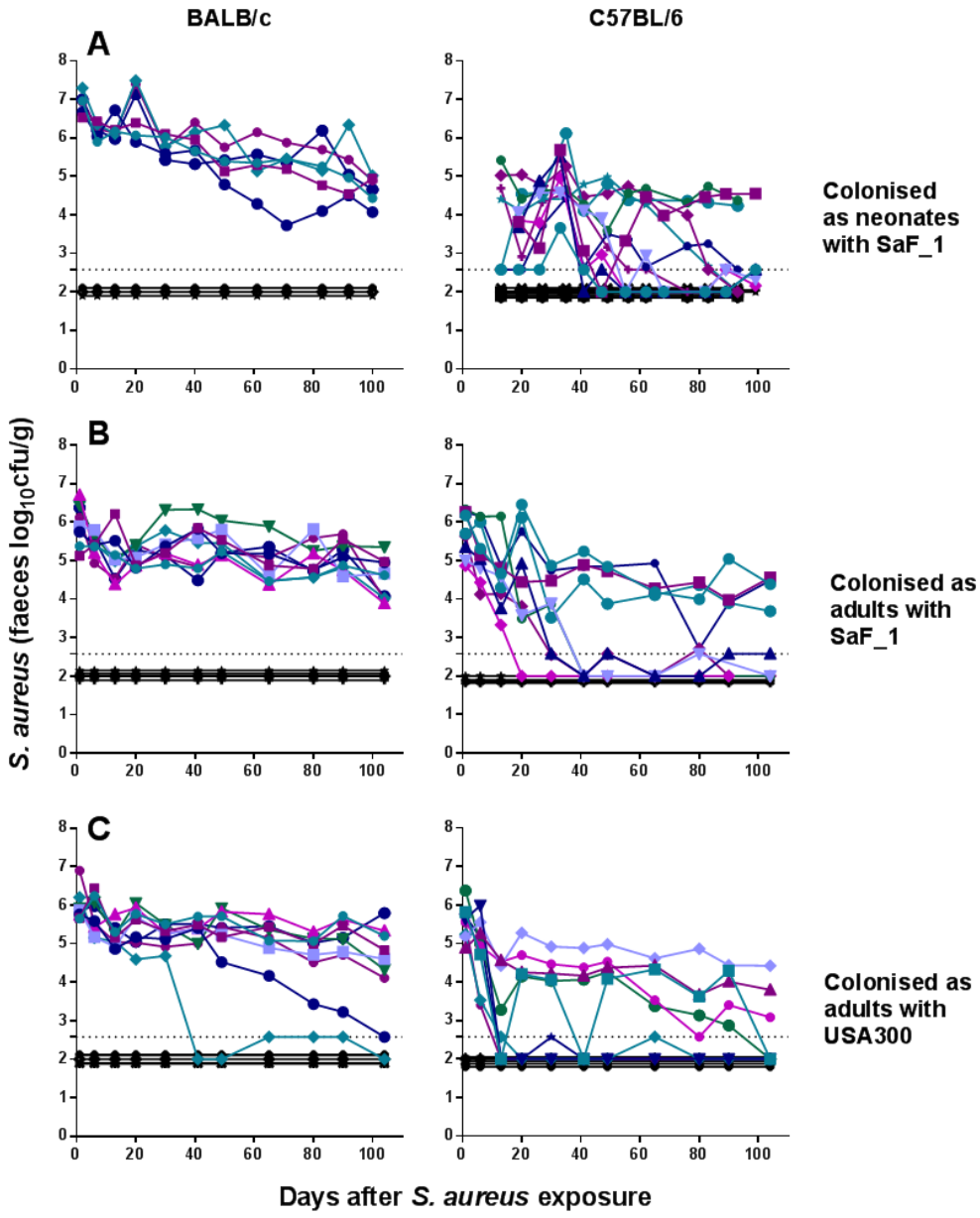



**Figure 4-8: Experimental colonisation of 2 strains of *S. aureus*-free mice by environmental contamination.** Animals were confirmed *S. aureus* free by stool sampling. Animals colonised as neonates (A) received 4 SaF_1 doses from -1 to 14 days old, whilst animals colonised as adults (B & C) received 1 dose at 6 weeks, of either SaF_1 strain (B) or USA300 (C) *S. aureus* colonisation levels were monitored by stool sampling for at least 100 days in BALB/c mice (left panels) and C57Bl/6 mice (right panels). Each coloured symbol

represents an individual mouse which received *S. aureus* treatment (n = 6-11). Black symbols represent control animals which received PBS treatment (n = 3-13). Horizontal dotted lines represent the limit of detection of the culture assay. Negative samples are plotted as if 100 colonies/g were detected.

Environmental contamination resulted in colonisation in adults and neonates in 2 mouse strains. Bacterial density was higher and colonisation was longer lived in BALB/c compared to C57BL/6 animals, whether colonised as neonates (Figure 4-8A, $p$ <0.0001) or as adults (Figure 4-8B, $p$ =0.0006) (Tukey's multiple comparisons test).

To determine whether the success of this cage contamination method was specific to the SaF-1 strain, I performed colonisation by cage contamination using a human *S. aureus* isolate, USA300; an MRSA strain. Long-term colonisation with USA300 was also achieved in adult mice (Figure 4-8C). There was no difference in bacterial density between SaF_1 and USA300 in either BALB/c ($p$ = 0.985) or C57BL/6 ($p$ = 0.719) mice (Tukey's multiple comparisons test).

As shown for mice naturally colonised with *S. aureus*, mice experimentally colonised with SaF_1 also had *S. aureus* throughout their GI tract at 100 days post treatment (Figure 4-9). I was also able to recover low levels of *S. aureus* from the nasal tissue of colonised mice. Colonisation levels of GI tract sections were higher in BALB/c mice compared to C57BL/6, but the reverse was true of nasal tissue.

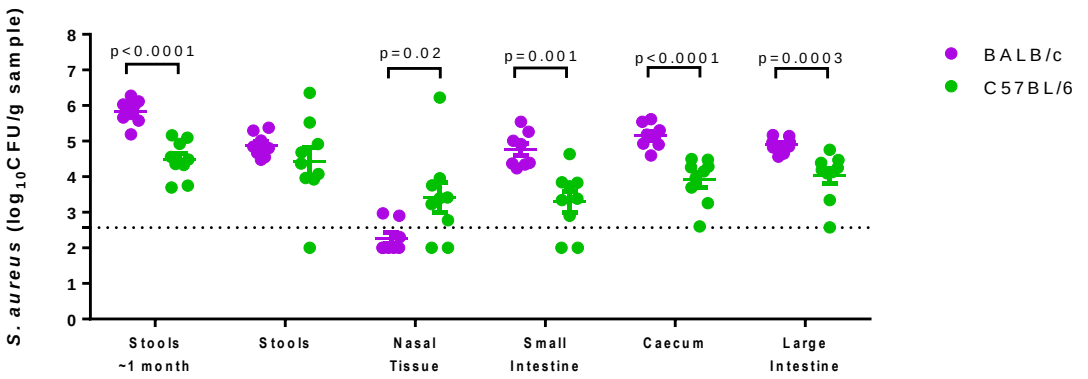



**Figure 4-9: Comparison of colonisation levels in 2 mouse strains.** *S. aureus* recovery from BALB/c and C57Bl/6 mice colonised with SaF_1 as adults at one month (stools only) and at ~100days (throughout GI tract). Data shown relates to the same mice as those shown with coloured symbols in Figure 4-8B. *p* values given calculated by Mann-Whitney test.

Due to supply issues and cost of SOPF certified *S. aureus* free BALB/c mice, I also colonised another strain of mice, CD1 mice. CD1 mice were confirmed to be *S. aureus* free by stool sampling upon arrival. CD1 mice were successfully colonised for 21 days with USA_300 (Figure 4-10). Whilst this time-frame is shorter than that performed for C57Bl/6 and BALB/c mice, this time-frame should be sufficient for testing interventions such as vaccines and measuring vaccine efficacy in reducing colonisation levels.

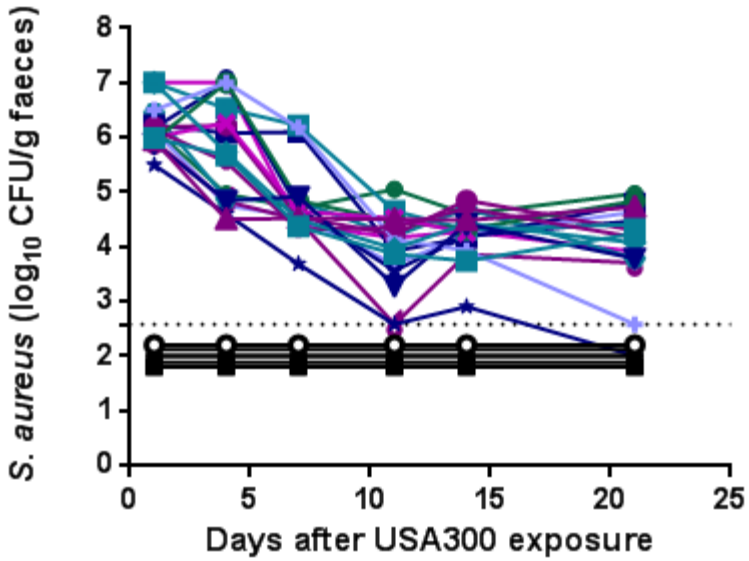



**Figure 4-10: Experimental colonisation of a third strain of *S. aureus*-free mice by environmental contamination.** CD1 mice were confirmed *S. aureus* free by stool sampling and received a single dose of USA300 at 6 weeks of age. *S. aureus* colonisation levels were monitored by stool sampling for 21 days. Each coloured symbol represents an individual mouse which received *S. aureus* treatment (n=18). Black symbols represent control animals which received PBS treatment (n=6). Horizontal dotted line represents the limit of detection of the culture assay. Negative samples are plotted as if 100 colonies/g were detected.

## 4.3 Discussion

In this chapter, I have described natural GI tract colonisation of mice with *S. aureus*. I have characterised the strain responsible and shown that natural colonisation is stable in some mice but not in others. I have established a new long term (>21 days) *S. aureus* colonisation model, which is needle free and does not require antibiotics. This model can be set up in 3 strains of mice and will allow testing of intervention strategies such as vaccines to reduce colonisation.

It seems likely that mother-to-pup transmission occurs in natural colonisation described in this chapter (Figure 4-1, Figure 4-2, Figure 4-4 & Figure 4-5). Evidence to support this hypothesis is that animals arrive from supplier already colonised at age 6 weeks and that adults, pre- and post-weaning pups in a breeding colony were colonised with the same *S. aureus* strain. In humans, colonised mothers and their babies frequently share the same strain[130], and so it seems reasonable to infer from my data that neonatal mice become colonised via their mother within the breeding facility.

My data shows that after colonisation early in life, genetically identical mice dichotomise into carrier and non-carrier populations. The situation is similar in humans, albeit humans have greater genetic diversity; they can be classed as persistent carriers and intermittent or non-carriers[11]. Human experimental colonisation studies were performed in the 1960's and showed that neonatal colonisation could be established efficiently[145]. A decolonisation and recolonisation study showed that after recolonisation, individuals that were persistent carriers originally have a much slower loss rate compared to individuals that were

intermittent or non-carriers[11]. Increased rates of community associated MRSA recurrent infections have been linked to household environmental contamination[146]. Thus loss rates and environmental factors have a role in human colonisation of *S. aureus* carriage.

Natural colonisation in mice appears to be similar to that in humans, and it seems, in mice at least, that genetic factors are not driving the dichotomisation seen. A more likely explanation for the divergence of mice into carriers and non-carriers is the microenvironment in which the mice live; this could include their own microbiome, and that of the other co-housed mice. My data shows that in some cases individual mice can persistently carry *S. aureus* without contact with other mice (Figure 4-4, Cage 3) but other times patterns of carriage in all mice within one cage are very similar (Figure 4-4, Cage 6). Therefore it is likely that a number of factors are at play in determining whether a mouse remains colonised or loses colonisation over time. This is something to be considered in the design of intervention experiments; all animals in the same cage should not receive the same treatment[147]. Acquired immunological factors could play a role here and these could also be linked to the microbiome. In recent years microbiome studies in laboratory mice have highlighted the previously underestimated impact of the microbiome on outcome of *in vivo* studies[148-150]. Furthermore, differences exist in microbiome of pigs depending on whether they are colonised with *S. aureus* or not[151].

One limitation of the study discussed so far is that I was only able to assess naturally colonised mice in one strain, BALB/c mice. We did not detect natural colonisation in

any other strains used in our facility (C57BL6 and CD1), but this does not mean to say that these strains cannot be colonised. Indeed a recent report showed the extent to which different strains of laboratory mice from different vendors are colonised with *S. aureus*[74]. Dynamics of natural colonisation may be different in other strains of mice.

After assessing dynamics of *S. aureus* GI tract colonisation in naturally colonised mice, the next step in establishing a reproducible and reliable model of colonisation was to colonise *S. aureus* naïve mice experimentally. I used 3 different approaches to colonise *S. aureus* naïve animals. Firstly, I co-housed adult *S. aureus* naïve mice with *S. aureus* colonised mice in 1:1 ratio, but this only resulted in 2/12 *S. aureus* naïve mice becoming colonised. Next, I attempted to colonise adult *S. aureus* naïve mice by oral gavage, and this was successful in all mice (24/24) for the short term (<7 days), but only in 2/24 mice for long term colonisation (>21 days). The final method, contamination of the cage environment, had a much higher success rate (9/9) than the previous two methods in terms of establishing long-term (>21 days) colonisation in *S. aureus* naïve adult BALB/c mice.

A number of reasons may explain the difference in success rates between the colonisation methods used here in *S. aureus* naïve adult BALB/c mice. Each method represents a different level of exposure; exposure from other mice in the cage provides the least exposure in terms of cfu, whereas oral gavage administration represents the most exposure, and directly to the site of colonisation. Environmental contamination, whilst using a large inoculum dose (5-10ml of *S. aureus* culture at ~5 x $10^9$ cfu/ml per cage), probably results in the mice becoming colonised over a period

of several hours. This method is also less stressful for the mice themselves, compared to oral gavage, in which the mice must be restrained and the *S. aureus* dose is given in a bolus in a matter of seconds. Therefore, level and route of exposure are likely to affect whether a mouse can be colonised. Further work could include a dose escalation study to determine the minimum dose required for *S. aureus* colonisation by environmental contamination. Cage type, spraying equipment and number of mice per cage could also be optimised. However, since the dose received by each individual mouse cannot be controlled, for the remainder of this thesis I decided to continue working with the dose and conditions already used.

In addition to previously used BALB/c mice, C57BL/6 mice were colonised by the environmental contamination method, as that is the genetic background upon which most genetic manipulations are performed, therefore it is important that this strain could be colonised for any future genetic studies. CD1 mice were also colonised using the environmental contamination method. Unlike BALB/c and C57BL/6 mice, CD1 mice are outbred, and therefore genetically more heterogeneous, and so would represent a better model of human *S. aureus* carriage. Colonisation was successful in *S. aureus* naïve adult C57BL/6 and CD1 mice. Mouse strain, therefore, is another factor in determining success of colonisation. I also colonised naïve neonatal C57BL/6 and BALB/c mice. Within each strain, patterns of colonisation in neonates were similar, although in BALB/c mice initial colonisation levels were higher in neonates compared with adults. In the neonatal experiments, mice received multiple doses, whereas the adults received only one dose, and so this may account for differences in colonisation levels. Another explanation is that mice might be more susceptible to colonisation at an early age, before development of various immune responses[152].

This is another area which could be further investigated. However, for the purposes of this thesis, further experiments were carried out in adult mice, since the methodology involved is much simpler.

Throughout this chapter stool sampling has been my method of choice for monitoring *S. aureus* carriage in mice. This method was selected as it was the least invasive and most reproducible (Table 4-1). Therefore, strictly speaking, my colonisation model represents GI tract colonisation, rather than nasal colonisation. Both colonisation methods are used in the field[63 77] and so can be considered relevant. GI tract colonisation may represent a different model to nasal colonisation. However, I have shown evidence that my environmental contamination method elicits colonisation of nasal tissue and cheeks by swabbing and by harvesting tissue post mortem (nasal tissue only). For a model of colonisation to test interventions and investigate immune responses, one reliable method of monitoring colonisation is required. For this reason, stool sampling was selected as the method of quantifying *S. aureus* colonisation in further studies in this thesis.

In this chapter I have shown that my highly efficient needle- and antibiotic-free model involving experimental exposure leads to the initiation of *S. aureus* GI carriage in mice. This model offers a less invasive methodology to induce colonisation compared to models currently used[17 63 75 141 153]. However, there are a number of limitations to the experimental colonisation model. I rely on contamination of bedding and sawdust for mice to become colonised. Contamination from other sources, such as animal handlers cannot be ruled out, but the likelihood of this is minimised by not cleaning cages for at least 7 days after environmental

contamination. This type of alternative source of *S. aureus* could also occur in thenaturally colonised mice. I performed *spa* typing of various isolates from various experiments presented in this chapter and show that the same *spa* type is present throughout both natural and experimentally induced long-term colonisation studies.

Experimental colonisation has been established with 2 strains of *S. aureus*, one MRSA and one MSSA, although no differences in colonisation rates were seen between these two strains. However, other strains could be more or less able to colonise the GI tract of one mouse strain or another, so this could be investigated in order to determine the optimal GI tract colonisation model.

The establishment of a long-term GI tract colonisation model in mice allows various further studies to be performed. These include exploration of the immunological impact of *S. aureus* colonisation (Chapter 5) and intervention strategies such as vaccines to reduce colonisation (Chapter 6). Investigation of the biology surrounding how persistent colonisation is established, including investigation of whether the microbiome plays a role in colonisation, could also be performed. Elucidation of bacterial genetic requirements for carriage could be done using my experimental colonisation model to investigate ability of SaF_1 mutants in colonising the GI tract[77].

# 5. *S. aureus* colonisation models in mice: Immunological Impact

## 5.1 Introduction

In the previous chapter I showed that 3 different strains of laboratory mice can be colonised with *S. aureus*. In this chapter I set out to determine what immunological impact this colonisation has. This will allow better characterisation of the model and allow me to compare my model to the immune impact of colonisation in man. In humans, exposure commonly occurs at birth, or shortly after, with the main source being the mother[107 130]. Nasal carriage peaks during early infancy and declines during the first year of life[107 128 154] resulting in around 20-30% of adults being persistent carriers [12].

Antibody responses to *S. aureus* have been extensively studied in the adult human population. These studies show that responses to both whole *S. aureus* and individual antigens are heterogeneous across the population[20 21 155]. In one study, carried out by my colleagues, anti-*S. aureus* IgG1 antibody titres were found to be inversely correlated with age. Additionally, increased IgG1 was associated with immunoglobulin mediated attachment to phagocytes, indicating that increased IgG1 against *S. aureus* could be beneficial in protection from *S. aureus* disease[114]. In some cases, differences exist between persistent carriers and non-persistent carriers. For example, immunoglobulin levels against certain antigens were higher in carrier versus non-carrier children[156] and adults[20]. In a recent study, antibody responses against 36/43 staphylococcal antigens were higher in carriers compared with non-carriers[157].

Whilst the antibody response to *S. aureus* colonisation has been well studied, the cellular responses are not well characterised[158]. In one study, cytokine and T-cell

proliferation responses to *S. aureus* lipoproteins were weak[159]. Adaptive T cell dependent antibody responses against *S. aureus* colonisation show that a T cell mediated Th response exists, but other aspects of T cell response and their impact require investigation.

Despite these numerous studies showing immune phenotype related to *S. aureus* exposure and colonisation, as yet there is little data to suggest which, if any, of these immune responses are linked to 'colonisability'. Presumably, differences in immune response between persistent and non-persistent carriers exist which render the persistent carriers colonised for many years. For example, nasal carriers were found to be more likely than non-carriers to acquire exogenous *S. aureus* strains during a hospital stay[25]. Additionally, antibodies against various *S. aureus* antigens protected against novel *S. aureus* acquisition in children under 2 years old[155]. If the immune responses underlying these differences can be identified, this would provide information on which protective responses should be elicited by a vaccine or other intervention strategy to reduce colonisation. This work could be performed in an animal model of colonisation, and could also provide further data on which, if any, detrimental responses would not be desired.

Limited studies have been performed to investigate immune impact of colonisation in mice. A recent study detailed induction of systemic IgG against various *S. aureus* antigens during natural colonisation in laboratory mice[74]. IL-17 is involved in clearance of nasal colonisation in an experimentally induced nasal colonisation model[153 160]. The immunological response to *S. aureus* infection ('challenge') models in mice has been well documented[161]. The challenge model used in this

chapter involves intravenous infection via the tail vein and harvest of organs for assessment of bacterial load[100]. This model has been used extensively to assess vaccine efficacy[40 86 100] and therefore I use it here to assess whether colonisation can confer protection to *S. aureus* infection.

Data presented in this chapter was performed under Research Objectives 2 & 4:

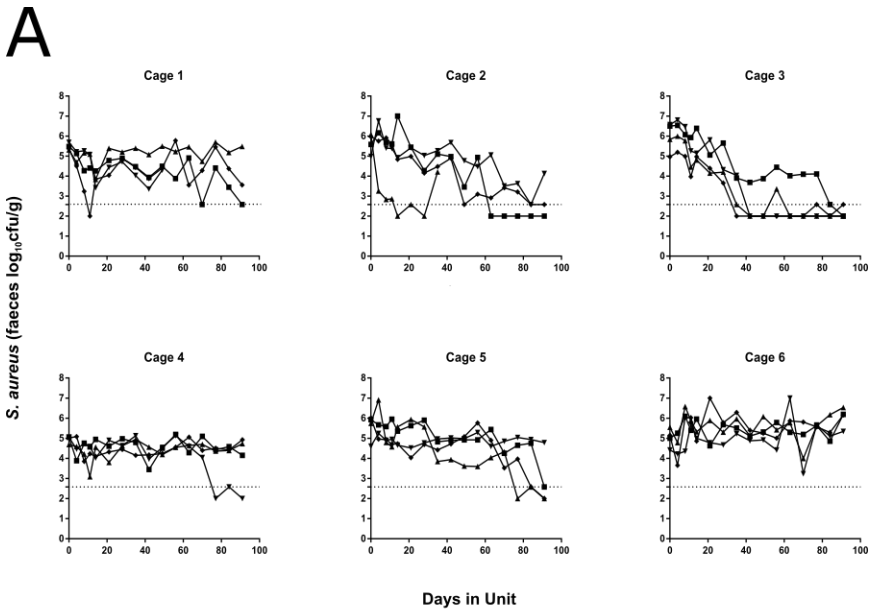
- Characterise natural murine *S. aureus* carriage
- Characterise murine *S. aureus* experimental colonisation model

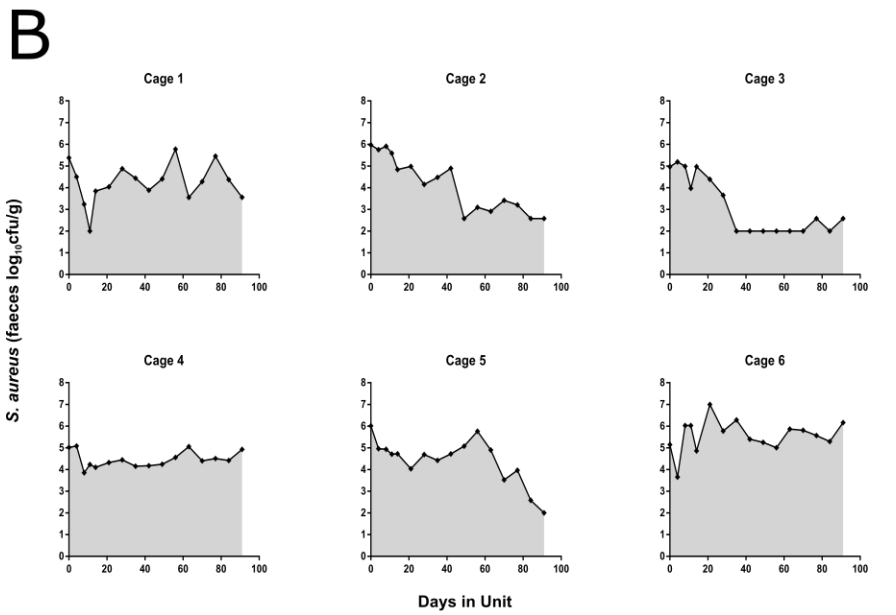
In this chapter, I set out to determine whether natural and experimentally induced *S. aureus* colonisation in mice elicited detectable antibody and cellular responses. I then investigated whether prior colonisation has a protective effect upon subsequent infectious challenge with *S. aureus*. I assessed antibody responses to individual *S. aureus* antigens in colonised and uncolonised mice. Finally, I evaluated the cytokine profiles from both serum and nasal tissue in colonised and uncolonised mice. These data further characterise the model described in Chapter 4 and provide important information to consider when selecting vaccine candidates to test for reducing colonisation in Chapter 6.

## 5.2 Results

### 5.2.1 Serological response to *S. aureus* colonisation

In Chapter 4, I described both naturally occurring and experimentally induced *S. aureus* carriage in the GI tract of BALB/c mice. I termed the clade of *S. aureus* which I obtained from naturally colonised mice 'SaF'. I selected a representative isolate, 'SaF_1' and used this isolate for experimental colonisation. To assess the immunological impact of *S. aureus* carriage, I performed three independent experiments monitoring carriage levels in naturally colonised BALB/c mice (Figure 4-4). I showed in Chapter 4 that naturally colonised mice lose colonisation at different rates, dichotomising into persistent carriers (defined as ongoing carriage at the end of follow-up, which was 90-110 days) and those that have lost carriage. Here, I developed a metric of cumulative individual *S. aureus* exposure for each mouse termed 'Long Term Carriage Burden' (LTCB). Dynamics of colonisation in one of the three independent experiments from Chapter 4 are reproduced here (Figure 5-1A). Area under the curve was calculated for each mouse; Figure 5-1B shows how this was calculated for one mouse per cage from the experiment shown in Figure 5-1A. LTCB was compared in each of the three experiments investigating dynamics of colonisation (Figure 5-1C). In all three experiments, significant differences in LTCB existed between cages. LTCB can be used as a single readout of *S. aureus* exposure for comparison with other readouts measured from these mice, such as immune phenotype.


A
Cage 1
Cage 2
Cage 3
Cage 4
Cage 5
Cage 6
S. aureus (faeces log10cfu/g)
Days in Unit


B
Cage 1
Cage 2
Cage 3
Cage 4
Cage 5
Cage 6
S. aureus (faeces log10cfu/g)
Days in Unit

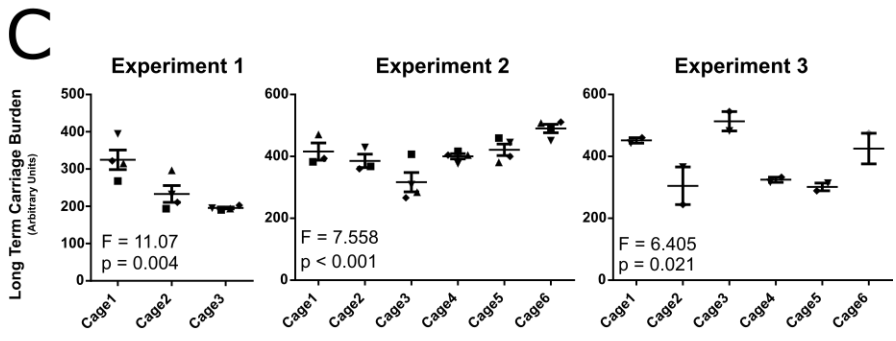

C
Experiment 1
Experiment 2
Experiment 3
Long Term Carriage Burden
(Arbitrary Units)
F = 11.07
p = 0.004
F = 7.558
p < 0.001
F = 6.405
p = 0.021
Cage1
Cage2
Cage3
Cage4
Cage5
Cage6

**Figure 5-1: Long Term Carriage Burden.** (A) *S. aureus* carriage levels per mouse within a cage (reproduced from Figure 4-4), monitored by stool sampling, data shown for Experiment 2 (of 3 independent experiments). (B) Long Term Carriage Burden (LTCB) was calculated as area under curve of carriage levels for up to 3 months; areas for one animal per cage from Experiment 2, shown in (A), are represented here. (C) LTCB for animals by cage, in each of the three independent carriage monitoring experiments. ANOVA with F statistic and *p* value are given.

Firstly, I investigated whether colonisation elicited detectable anti-*S. aureus* antibodies in nasal swabs and stool homogenates, as these are the sites of exposure. However, this attempt to evaluate specific mucosal immunoglobulin from did not detect consistent signals (Figure S3). Therefore, I elected to investigate serum antibodies, as serum can be obtained easily and without having to cull the mice.

Anti-*S. aureus* antibody phenotype was investigated in the three experiments of mice naturally colonised with *S. aureus* upon arrival from the supplier. *S. aureus* was isolated from stools and serum taken for assessment of immune phenotype on the same day. Immune phenotype was analysed according to whether mice were SaF carriers or had lost carriage. SaF carriers had higher anti-*S. aureus* IgG1 signal compared to those animals which had lost SaF carriage (Figure 5-2A). This increase was significant in Experiments 1 and 3 ($p = 0.009$ and $p = 0.017$, respectively).

I used the LTCB metric to investigate the relationship between exposure to *S. aureus* and antibody signal; I hypothesised that increased exposure would result in greater antibody production. As expected, anti-*S. aureus* IgG1 signal was positively correlated with LTCB in all three experiments (Figure 5-2B) ($p = 0.05$, $p = 0.07$, and $p < 0.0001$, respectively).

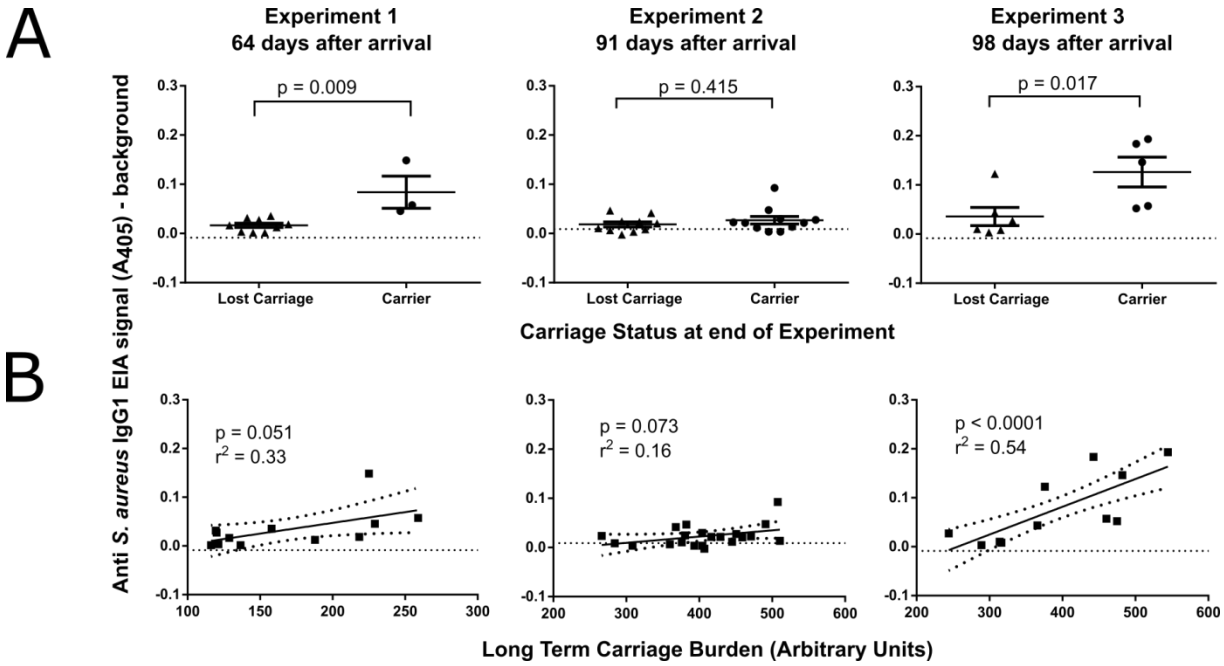



**Figure 5-2: Serological response to natural *S. aureus* carriage.** Serum IgG1 antibody responses against *S. aureus* cell surface in 3 independent carriage monitoring experiments (i.e. in 3 groups of female BALB/c mice from Harlan) stratified by (A) carriage status and (B) Long Term Carriage Burden. Levels of IgG1 against the cell surface of fixed *S. aureus* (Δspa Newman strain) are expressed as enzyme immunoassay (EIA) signal minus background. Experiment 1 was performed at 64 days, Experiment 2 at 91 days and Experiment 3 at 98 days after animals arrived at the unit. In (A) animals were dichotomised according to carriage status (carrier or lost-carriage) at the end of the experiment and EIA signal compared using Mann-Whitney test. In (B) Long Term Carriage Burden was calculated (as in Figure 5-1) for day of experiment. Horizontal dotted lines show EIA – background of control mice (*S. aureus* naïve)

Anti-*S. aureus* signal of other antibody isotypes was also investigated in one of the three experiments investigating dynamics of natural colonisation. Immunoglobulin

signal against *S. aureus* was elevated over background for IgA, IgM, IgG2a, IgG2b, and IgG3 (Figure 5-3A). However, no differences in signal between carriers and mice which had lost carriage were seen for other IgG subclasses (Figure 5-3A). There was no significant positive correlation between LTCB and any other immunoglobulin subclasses (Figure 5-3B). For this reason, only IgG1 levels were analysed in further experiments.

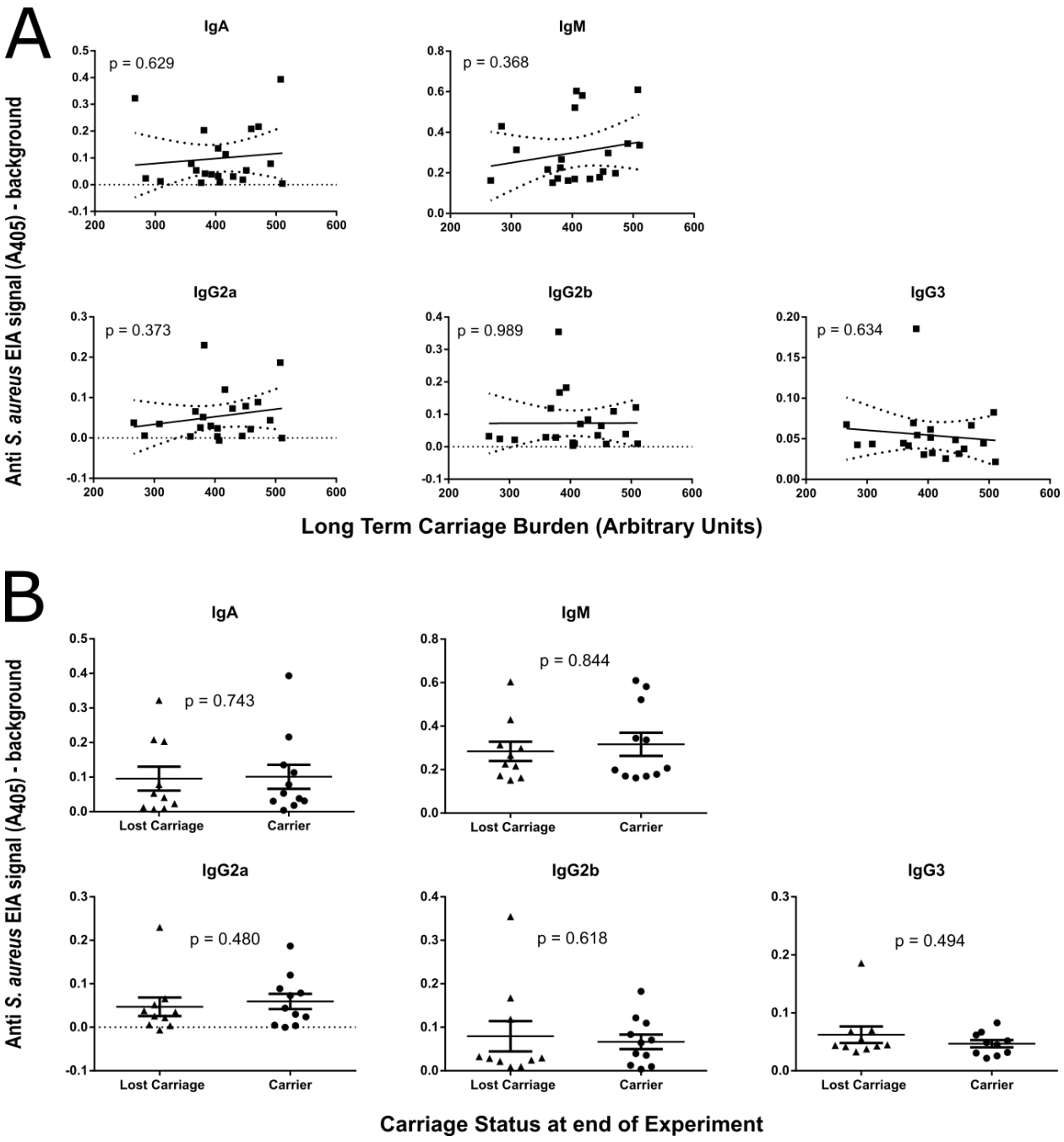



**Figure 5-3: Serum isotype antibody responses to natural *S. aureus* carriage**. Data shown for Experiment 2 shown in Figure 5-2 stratified by (A) carriage status and (B) Long Term Carriage Burden. Levels of IgA, IgM, IgG2a, IgG2b and IgG3 against the cell surface of fixed *S. aureus* (Δspa Newman strain) are expressed as signal minus background. Experiment performed at 91 days after animals arrived at the unit. Long Term Carriage Burden calculated

for 0 days to 91 days. In (B) animals were dichotomised according to carriage status (carrier or lost-carriage) on day of experiment and compared using Mann-Whitney test.

As described in Chapter 4, I attempted to colonise *S. aureus* naïve mice by three different methods: co-housing with colonised mice, oral gavage and environmental contamination. I assessed anti-*S. aureus* IgG1 responses in serum from mice involved in experiments testing all three of these colonisation methods.

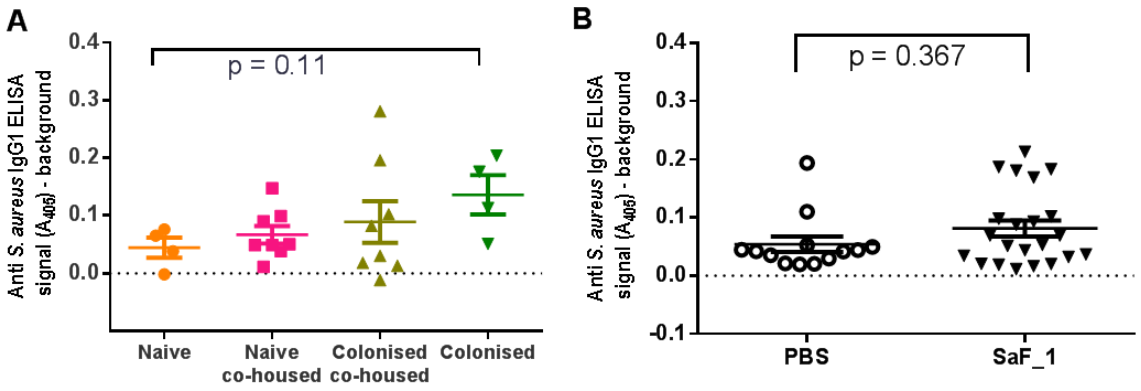



**Figure 5-4: Serological response to *S. aureus* in mice exposed by co-housing (A) and oral gavage (B).** Serum IgG1 antibody responses against *S. aureus* cell surface stratified by treatment and compared using Mann-Whitney tests. Levels of IgG1 against the cell surface of fixed *S. aureus* (Δspa Newman strain) are expressed as EIA signal minus background. In (A), experiment performed 42 days after co-housing; 4 mice per control group, 8 mice per cohoused group. In (B), experiment performed at 203 days after first oral gavage; 13 mice per control (PBS) group, 22 mice per colonised (SaF_1) group.

Figure 5-4 shows the IgG1 antibody responses in co-housed mice (Figure 5-4A) and mice receiving SaF_1 by oral gavage (Figure 5-4B). In the co-housing experiment (design shown in Figure 4-6), increasing IgG1 signal is seen across the treatment groups with increasing exposure levels, although there were no significant differences

seen between groups. In oral gavaged mice, there was no significant difference in IgG1 signal in mice gavaged with PBS or SaF_1 ($p$ = 0.367). However, in 5/22 SaF_1 exposed mice, IgG1 signal was higher. These five mice included the two which remained colonised for the duration of the experiment, compared to the other 20 mice, in which colonisation was short-lived (<7days). This indicates that in some mice, even short term exposure can elicit antibody responses to *S. aureus*.

Here I have shown that anti-*S. aureus* signal is low but detectable in mice which were exposed to *S. aureus* using the two methods which resulted in short term colonisation (co-housing and oral gavage). Given this, I hypothesised that more robust differences would exist between naïve mice treated with PBS and *S. aureus* by the third methodology: environmental contamination, which resulted in long term colonisation.

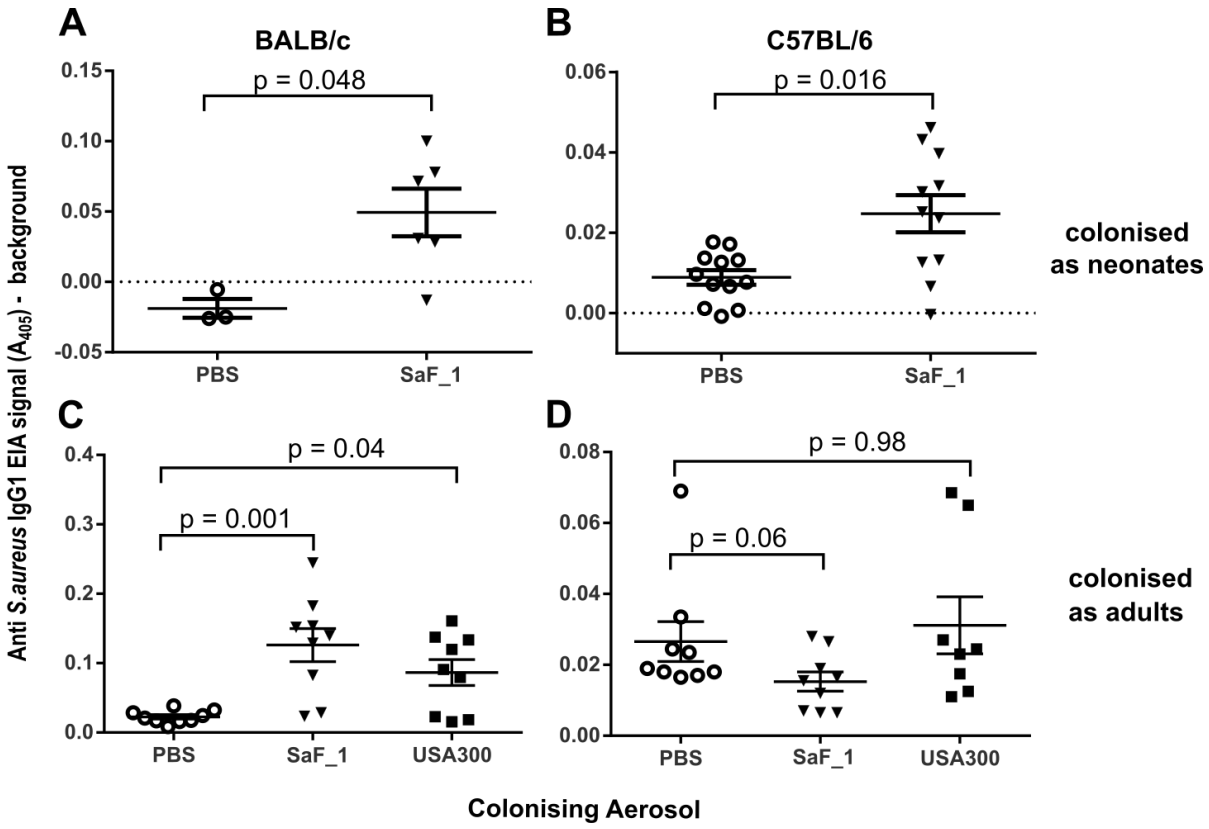



**Figure 5-5: Serological response to *S. aureus* colonisation induced experimentally by contamination of the environment.** Serum IgG1 antibody responses against *S. aureus* cell surface stratified by treatment and compared using Mann-Whitney test. Levels of IgG1 against the cell surface of fixed S. aureus (Δspa Newman strain) are expressed as EIA signal minus background, (A) in BALB/c mice colonised as neonates, (B) in C57BL/6 mice colonised as neonates, (C) in BALB/c mice colonised as adults and (D) in C57BL/6 colonised as adults. Experiments performed at (A) 78 days, (B) 93 days and (C, D) 66 days after colonisation. 3–12 mice per control (PBS) group, 6-11 mice per colonised (SaF or USA300) group.

The immune phenotype induced by *S. aureus* carriage was analysed in mice experimentally colonised by environmental contamination. I investigated anti-*S. aureus* IgG1 signal in the mice for which I presented kinetics of colonisation in

Chapter 4 (Figure 4-8); both BALB/c and C57BL/6 mice colonised as neonates and adults. I found that BALB/c mice exposed to *S. aureus* both as neonates and as adults induced significantly higher serum anti-*S. aureus* IgG1, compared to non-exposed controls ($p$ = 0.048 & $p$ = 0.0014 respectively, Figure 5-5A and C). In C57BL/6 mice, neonatal colonisation resulted in a significant induction of serum anti-*S. aureus* IgG1 ($p$ = 0.016, Figure 5-5B), however this was not seen in C57BL/6 animals colonised as adults (Figure 5-5D). As seen for kinetics of colonisation, anti-*S. aureus* IgG1 signal was similar whether SaF_1 or USA300 was used to colonise adult mice ($p$ = 0.13 in BALB/c and $p$ = 0.11 in C57BL/6).

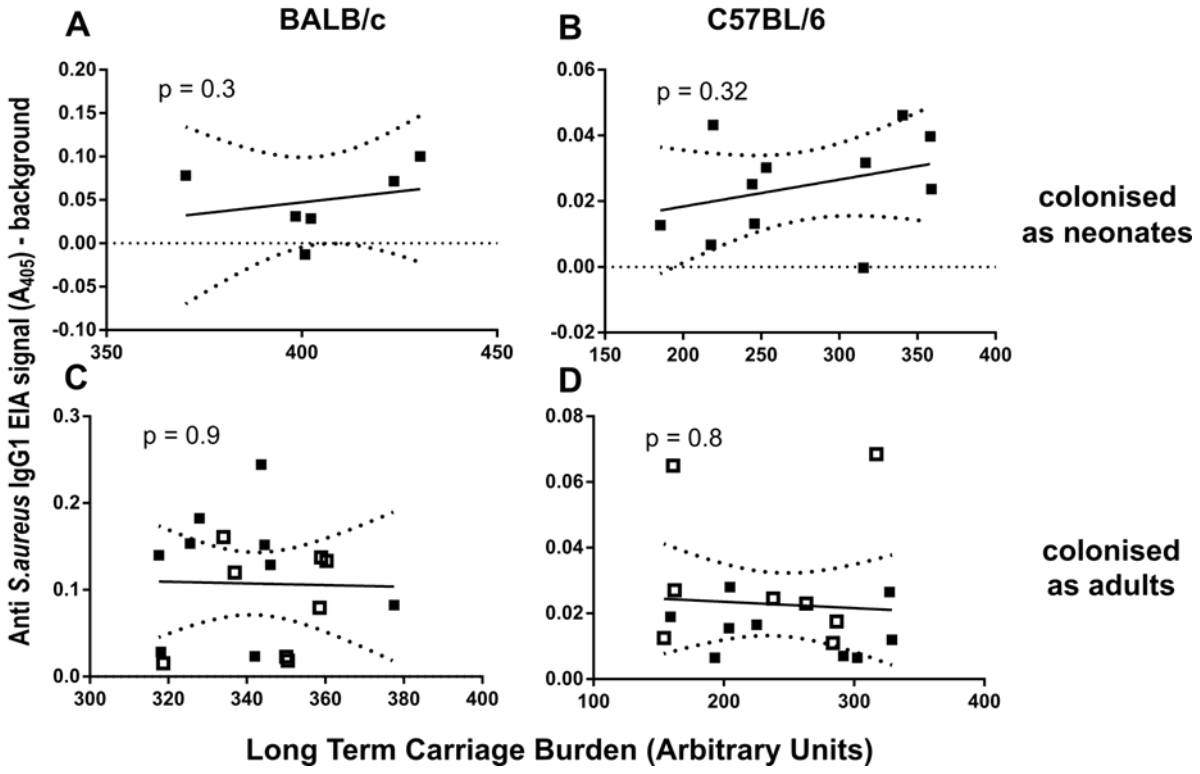



**Figure 5-6: Serum IgG1 antibody response to experimentally induced *S. aureus* colonisation stratified by Long Term Carriage Burden.** Data shown in Figure 5-5 for colonised animals is displayed here. Levels of IgG1 against the cell surface of fixed *S. aureus*

(Δspa Newman strain) are expressed as EIA signal minus background, (A) in BALB/c mice colonised as neonates, (B) in C57BL/6 mice colonised as neonates, (C) in BALB/c mice colonised as adults and (D) in C57BL/6 colonised as adults. In (C & D) animals colonised by USA300 are shown as open squares. Experiments performed at (A) 78 days, (B) 93 days and (C, D) 66 days after colonisation.

Anti-*S. aureus* IgG1 signal did not correlate with LTCB in either BALB/c or C57BL/6 mice experimentally colonised as adults or neonates (Figure 5-6). Therefore, environmental exposure of mice to *S. aureus* resulting in GI tract colonisation induces a serological response in BALB/c mice regardless of age of exposure. Conversely, in C57BL/6 mice the serological response is only seen in animals exposed as neonates.

As described in Chapter 4, CD1 mice were also experimentally colonised by the environmental method for 21 days (Figure 4-10). Anti-*S. aureus* IgG1 signal was also investigated in these mice. Here, mice were culled at day 21 and nasal tissue was harvested as well as serum (Figure 5-7). As in the other two mouse strains, CD1 mice colonised with *S. aureus* elicited significantly higher IgG1 in serum compared to PBS treated controls ($p = 0.002$). Interestingly, IgG1 from nasal tissue also trends towards being higher in exposed animals versus controls; although this was not significant ($p = 0.14$). In colonised animals, significant positive correlation existed between nasal and serum IgG1 (data not shown, Spearman's $\rho = 0.882$, $p < 0.0001$).

To summarise, this section shows that anti-*S. aureus* IgG1 antibodies can be detected in both nasal tissue and serum of mice colonised with *S. aureus* by environmental

exposure. Significant differences in the antibody signals for exposed versus control mice were seen in three different strains of mice.

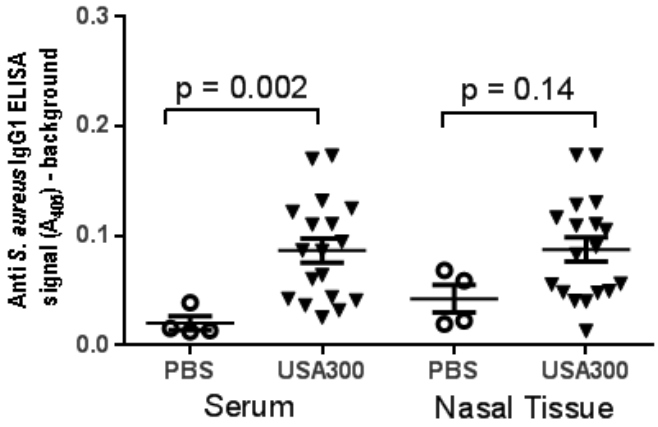



**Figure 5-7: Antibody responses to experimentally induced *S. aureus* in CD1 mice.** IgG1 antibody responses against *S. aureus* cell surface stratified by treatment and compared using Mann-Whitney test. Levels of IgG1 against the cell surface of fixed *S. aureus* (Δspa Newman strain) are expressed as EIA signal minus background in serum and nasal tissue homogenate at 21 days after colonisation. 6 mice per control (PBS) group, 18 mice per colonised (USA300) group.

### 5.2.2 Cellular response to *S. aureus* colonisation

In the previous section I described the humoral response to *S. aureus* GI tract colonisation in mice. Next I sought to investigate whether colonisation induces changes in the cellular compartment of the immune system. To do this, a panel of cell surface markers was designed to identify various immune cells present in peripheral blood by flow cytometry (Figure S4A & C). Gating strategy for flow cytometry experiments is shown in Figure S4.

Litter sizes were small in the experiment investigating colonisation of BALB/c mice as neonates. In C57BL/6 mice colonised as neonates, there were larger litter sizes and more appropriate numbers of animals in the control versus colonised groups. Therefore, I investigated cell populations in C57BL/6 mice colonised as neonates, rather than the BALB/cs. C57BL/6 mice colonised as neonates had significantly lower CD4+/CD8+ T cell ratio in blood ($p$ = 0.02) due to increased CD8+ and decreased CD4+ T cell populations (Figure 5-8A). Negative correlation between CD4+/CD8+ratio and IgG1 signal across PBS and SaF_1 exposed animals suggests a dose dependent effect of SaF_1 colonisation (Figure 5-8B). Other cellular populations were also investigated, as described in Figure S4C, however I found no differences between colonised and naïve animals in these populations.

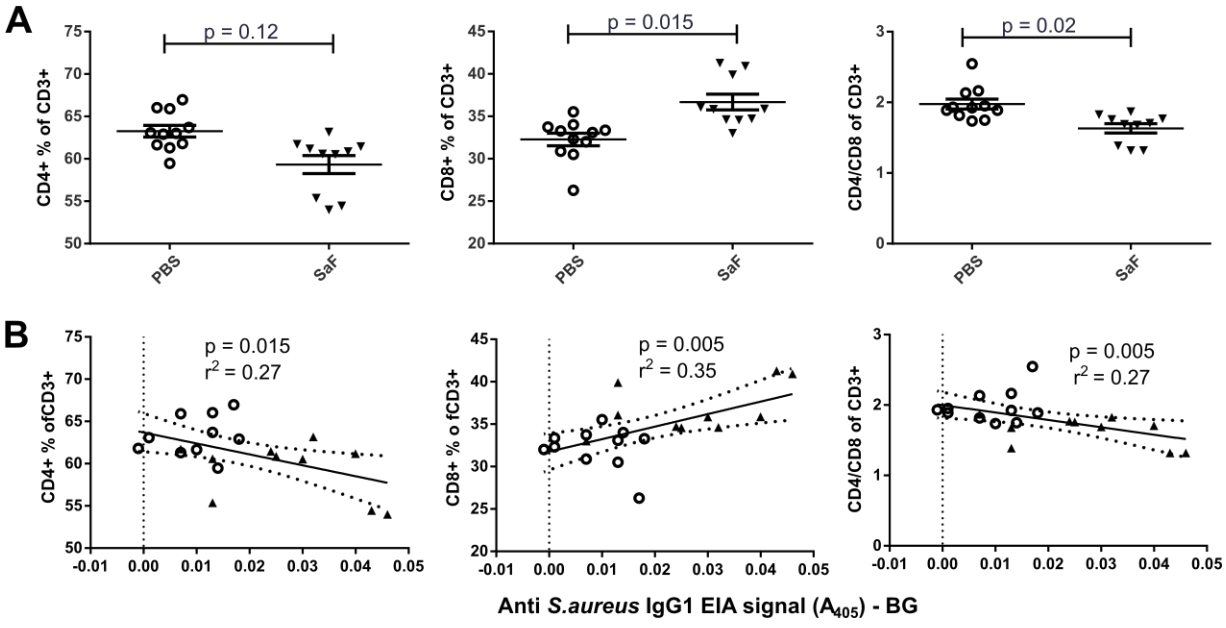



**Figure 5-8: T cell ratio in C57BL/6 mice experimentally colonised as neonates.** (A) T cell ratio in peripheral blood sample (tail bleed) determined by flow cytometry 65 days after SaF exposure, compared using Mann-Whitney with Bonferroni's correction. (B) Slope of

linear regression for T cell populations against anti-*S. aureus* serum IgG1 antibody responses (data shown in Figure 5-5B), with 95% CI. Open circles for PBS treated (12 animals), triangles for SaF_1 treated (11 animals).

I also investigated cellular populations in BALB/c and C57BL/6 mice colonised as adults (Figure 5-9). I saw no differences in T cell ratio in either BALB/c mice colonised as adults (Figure 5-9A,B) or C57BL/6 mice colonised as adults (Figure 5-9C,D).

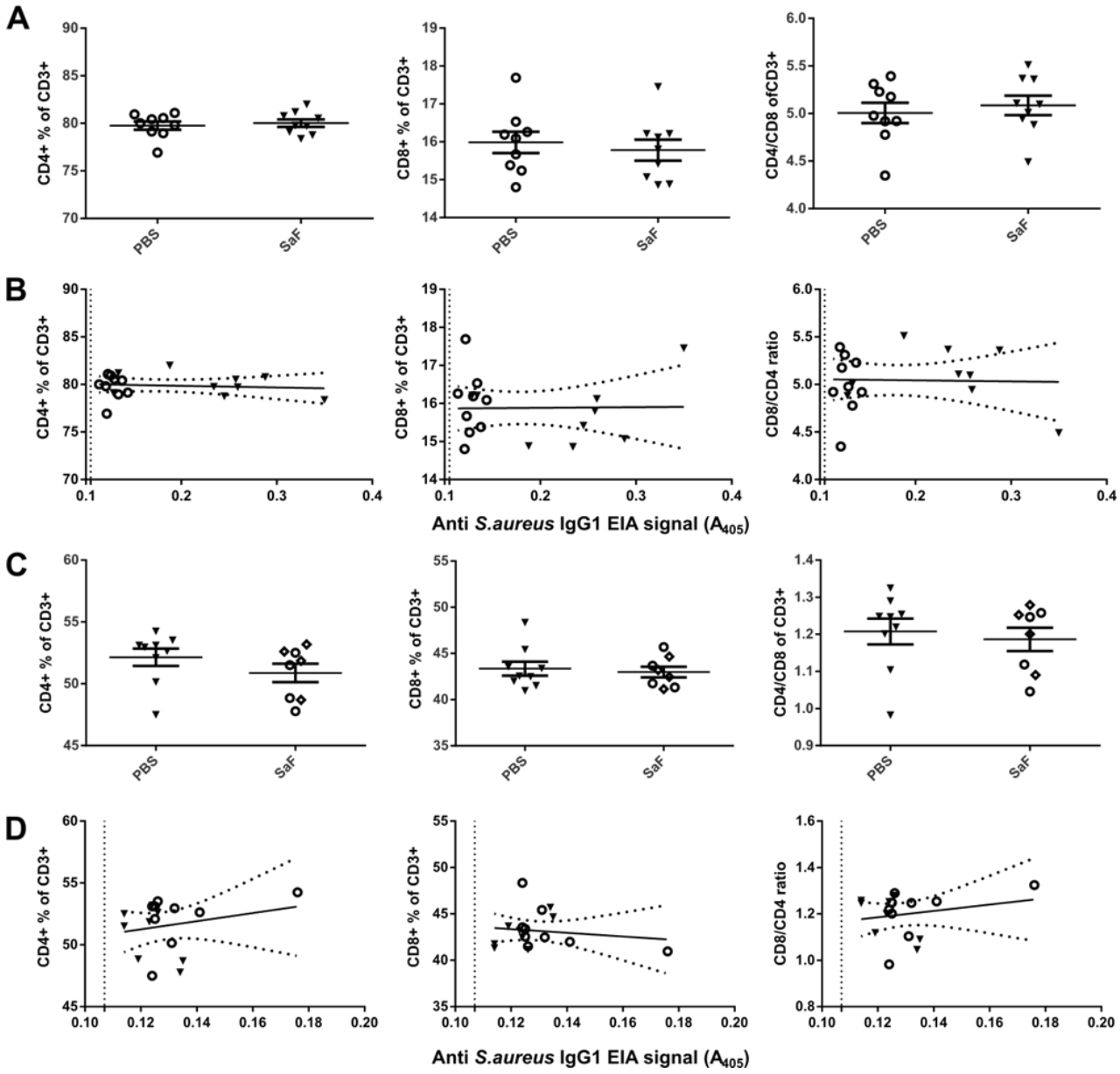



**Figure 5-9: T cell ratio in BALB/c and C57BL/6 mice experimentally colonised as adults**. (A, C) T cell ratio in peripheral blood sample (tail bleed) determined by flow cytometry 66 days after SaF exposure, compared using Mann-Whitney with Bonferroni's correction in (A) BALB/c and (B) C57BL/6 mice. (B, D) Slope of linear regression for T cell populations against anti-*S. aureus* serum IgG1 antibody responses (data shown in Figure

5-5C, D), with 95% CI in (C) BALB/c and (D) C57BL/6 mice. Open circles for PBS treated animals, triangles for SaF_1 treated animals.

Thus, I have shown that a consistent change in cellular immune profile is present in C57BL/6 mice in response to neonatal colonisation, but not to adult colonisation.

### 5.2.3 Does prior *S. aureus* colonisation protect from subsequent *S. aureus* infection?

Since immune responses induced by colonisation could have a role in protection against subsequent disease, I investigated whether SaF colonisation conferred protection to subsequent *S. aureus* intravenous (IV) challenge. I performed two experiments to investigate this; in the first, I used mice which were naturally colonised upon arrival and SOPF mice (*S. aureus* naïve) as a control group. These mice came from two different suppliers, Harlan and Taconic respectively. I asked the question: is SaF carriage associated with disease when subsequently challenged with either SaF_1 strain or a standard laboratory strain (Newman). The Harlan derived mice I term 'Carriers' and the Taconic derived mice 'SOPF'.

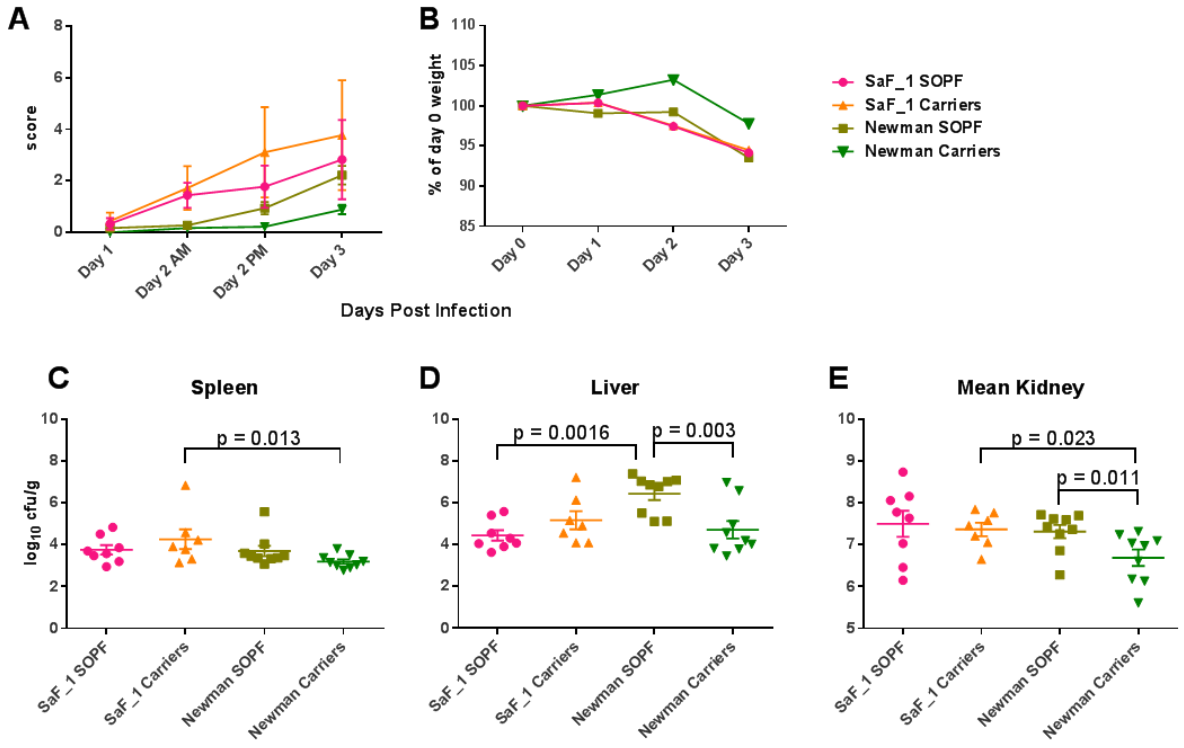



**Figure 5-10: Impact of natural *S. aureus* carriage on subsequent *S. aureus* IV challenge.** (A) Illness score (B) percent weight loss, bacterial recovery from (C)spleen, (D) liver and (E) both kidneys (mean). *p* values given calculated by Mann-Whitney test. 9 mice per group. Adverse events occurred as follows - 3 animals (2 in SaF_1 carriers and 1 in SaF_1 SOPF) reached a humane end point before day 3 were subsequently given illness scores of 15. These 3 animals were not included in (B), (C), (D) or (E).

Comparing the infecting SaF_1 and Newman strains within mouse strains, I noted that SaF_1 produced lower liver bacterial burden in SOPF animals (Figure 5-10D), but SaF_1 and Newman appeared otherwise similar in disease causation. Comparing the impact of SaF_1 or Newman strains across mouse strains, no significant differences in clinical score or weight loss were observed (Figure 5-10A,B). However, carrier mice infected with Newman had significantly reduced bacterial recovery from

organs (Figure 5-10C,D,E). Whilst SaF carriage was not associated with reduced bacterial load when challenged with SaF_1, SaF carrier animals challenged with Newman had significantly lower bacterial load in their livers and kidneys compared with SOPF animals. This implies that carriage of *S. aureus* may have some benefit upon subsequent challenge, although only when challenged with a heterologous strain. This interpretation is, however, potentially confounded by differences in mouse strain genetics between suppliers, as well as other differences in microbiota between mice.

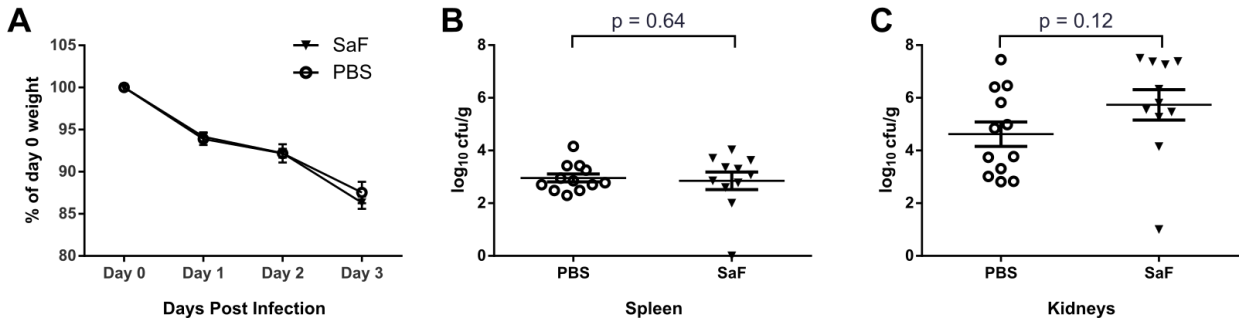



**Figure 5-11: Impact of experimental *S. aureus* carriage on subsequent *S. aureus* IV challenge.** C57BL/6 mice colonised as neonates were challenged IV with *S. aureus* Newman 93 days after SaF_1 exposure. Bacterial recovery was assessed 3 days later. 12 mice in PBS group, 11 mice in SaF_1 group. (A) percent weight loss, bacterial recovery from (B) spleen and (C) both kidneys (mean). *p* values given calculated by Mann-Whitney test.

Next, I investigated whether mice experimentally colonised with SaF_1 would be less susceptible to infectious challenge than their PBS control (uncolonised) littermates. For this, I used the C57BL/6 mice experimentally colonised with SaF_1 as neonates. These mice were challenged with intravenous infection with *S. aureus* Newman. Newman was chosen instead of SaF_1 since I had seen difference in naïve and

colonised mice upon infection with Newman but not with SaF_1 in the first experiment (Figure 5-10).

However, in mice experimentally colonised as neonates, challenge did not result in any differences in weight loss (Figure 5-11A). Bacterial recovery from spleen and kidneys (Figure 5-11B & C) was not significantly reduced in SaF_1 colonised animals compared with the non-colonised PBS controls ($p$ = 0.64 & $p$ = 0.12 respectively). No correlation was observed between bacterial recovery from kidneys and previously measured IgG1 signal (Spearman's ρ = 0.044, $p$ = 0.84). Thus, we did not observe any protective effect of experimental colonisation of C57BL/6 neonates with SaF_1 against challenge with *S.aureus* strain Newman.

Despite eliciting antibody responses and changes in T-cell ratio, experimental colonisation by environmental contamination did not result in a reduction in bacterial burden after the infectious challenges tested.

### 5.2.4 Colonisation induces detectable responses to individual *S. aureus* antigens

I have shown that colonisation induces an anti-*S. aureus* IgG1 antibody response. To further investigate this phenotype, I assessed the response to individual *S. aureus* cell surface antigens in serum. Due to supply issues with SOPF BALB/c mice, this analysis was performed in CD1 mice (confirmed to be *S. aureus* free).

I found increased antibody signal against EapH2, IsdA and IsdB in colonised mice compared to uncolonised mice (Figure 5-12A, B, C & E). Interestingly, I found that although IsdA and IsdB antibody levels both correlated with anti-SA IgG1 antibody signal, (data not shown), these two antigens do not correlate with each other (Figure 5-12D).

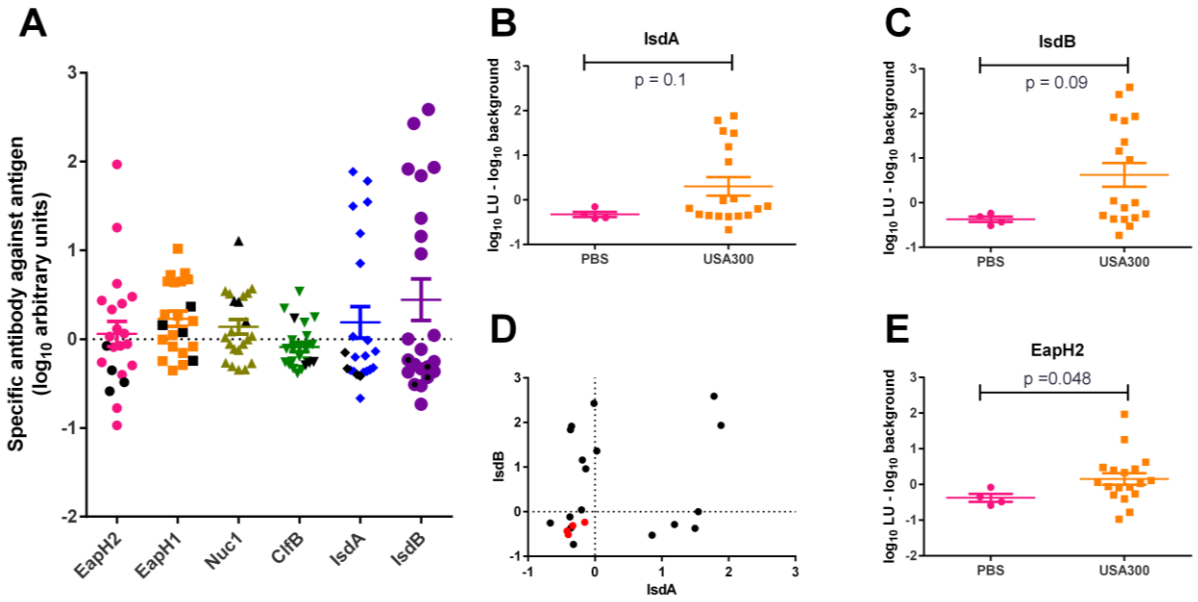



**Figure 5-12: Serological responses to individual *S. aureus* antigens in colonised mice.** Specific antibody against 6 *S. aureus* antigens was determined by LIPS in serum from CD1 mice 21 days after colonisation. Data shown for all 6 antigens (A). Data shown for individual antigens IsdA (B), IsdB(C), and EapH2 (E) in which differences in specific antibody were seen between control and colonised mice. Scatter plot showing relationship between specific antibody against IsdA and IsdB (D). Control mice (PBS treated) shown as black symbols in (A) and red symbols in (D). *p* values given calculated by Mann-Whitney test. 6 mice per control (PBS) group, 18 mice per colonised (USA300) group.

In this section, I have shown that *S. aureus* GI tract colonisation induces detectable responses to some *S. aureus* antigens but not others. Also, heterogeneity exists across the colonised mice: in some mice responses are high, whereas in others, responses are no different to baseline and uncolonised mice.

### 5.2.5 Cytokine response to *S. aureus* colonisation

To further characterise the immune response to *S. aureus* colonisation, I investigated cytokine profiles in serum and nasal tissue of CD1 mice colonised for 21 days and relevant PBS treated controls. This was done using multiplex kits; however, the kits used for nasal tissue and serum samples were different due to supply and cost issues. A comparison of the two kits used is found in Table S4.

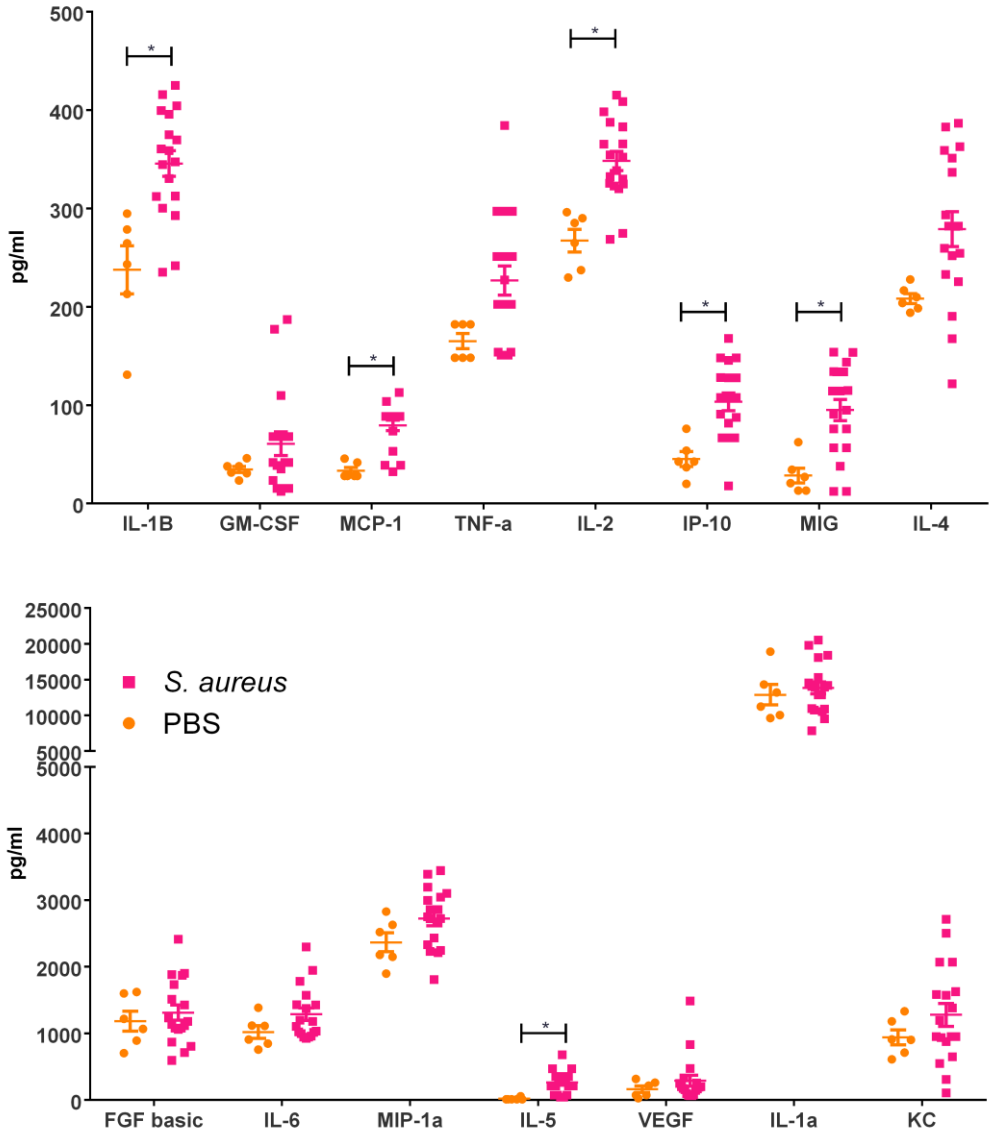



**Figure 5-13: Cytokine response in nasal tissue to *S. aureus* colonisation.** Cytokine levels in nasal tissue 21 days post colonisation were determined using 20-plex kit (Luminex). Colonisation data for these mice is shown in Figure 4-10. Top panel shows cytokines with low expression, bottom panel shows cytokines with higher expression. 6 mice in PBS group, 18 mice in *S. aureus* group. Asterisks denote $p < 0.05$ between cytokine levels in *S. aureus* colonised animals versus controls. Significance determined by multiple t tests, using Holm-Sidak method to correct for multiple comparisons.

I compared levels of 15 cytokines in nasal tissue from colonised and uncolonised animals. A further 5 cytokines were tested (IL-10, IL-13, IL-12, IL-17A & INF-γ) but removed from analysis due to low/undetectable levels. A number of cytokines were upregulated as a result of *S. aureus* colonisation; namely IL-1β, MCP-1, IL-5, TNF-α, IL-2, IP-10, MIG and IL-4 (Figure 5-13).

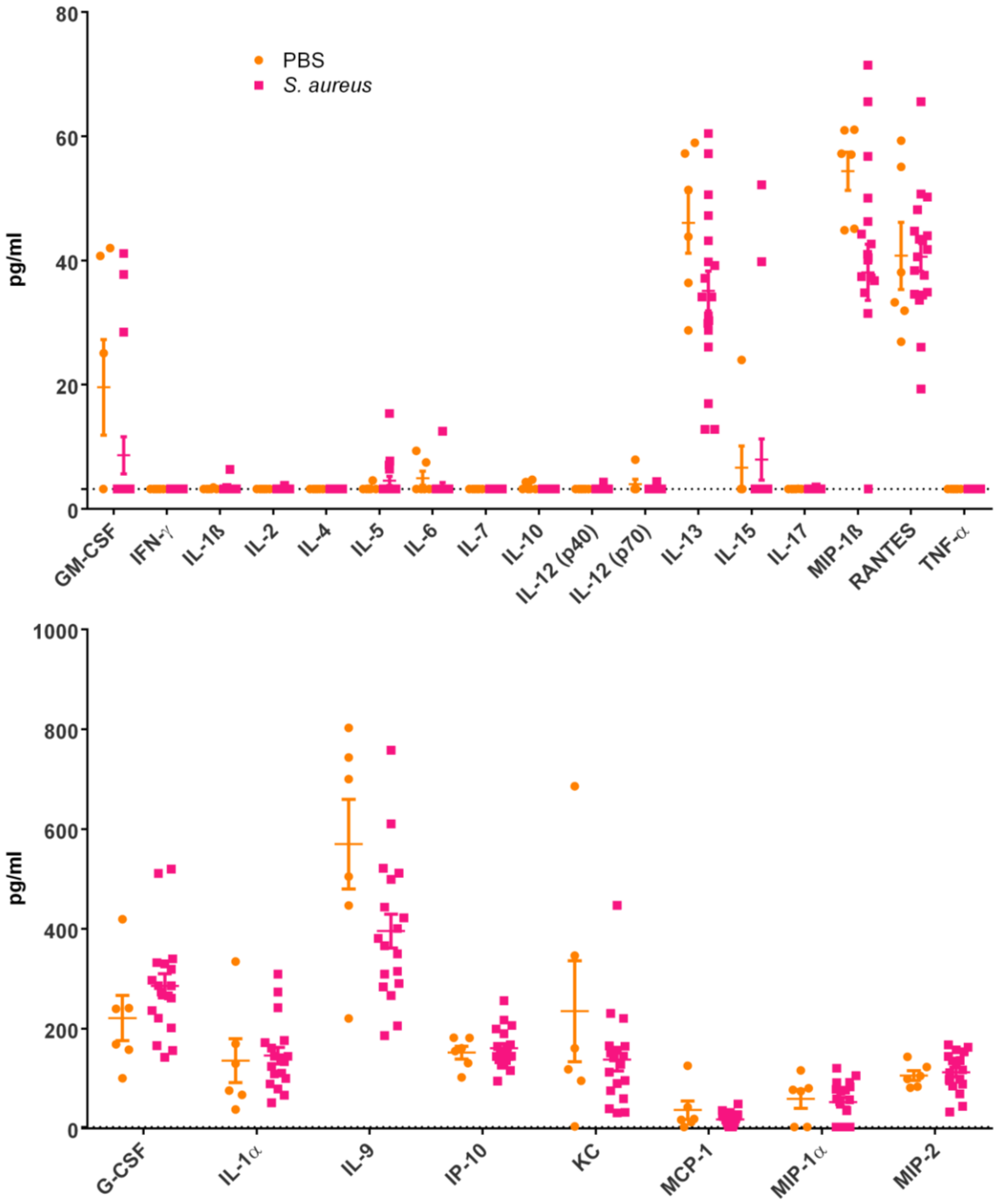



**Figure 5-14: Cytokine response in serum to *S. aureus* colonisation.** Cytokine levels in serum 21 days post colonisation were determined using 25-plex kit (Merck). Colonisation data for these mice is shown in Figure 4-10. Top panel shows cytokines with low expression, bottom panel shows cytokines with higher expression. 6 mice in PBS group, 18 mice in *S.*

*aureus* group. Significance determined by multiple t tests, using Holm-Sidak method to correct for multiple comparisons. Dashed line shows detection limit.

In contrast to nasal tissue, the cytokine profile from serum of CD1 mice 21 days post colonisation does not differ from naïve mice (Figure 5-14). Therefore, *S. aureus* colonisation induces a local but not a detectable systemic cytokine response.

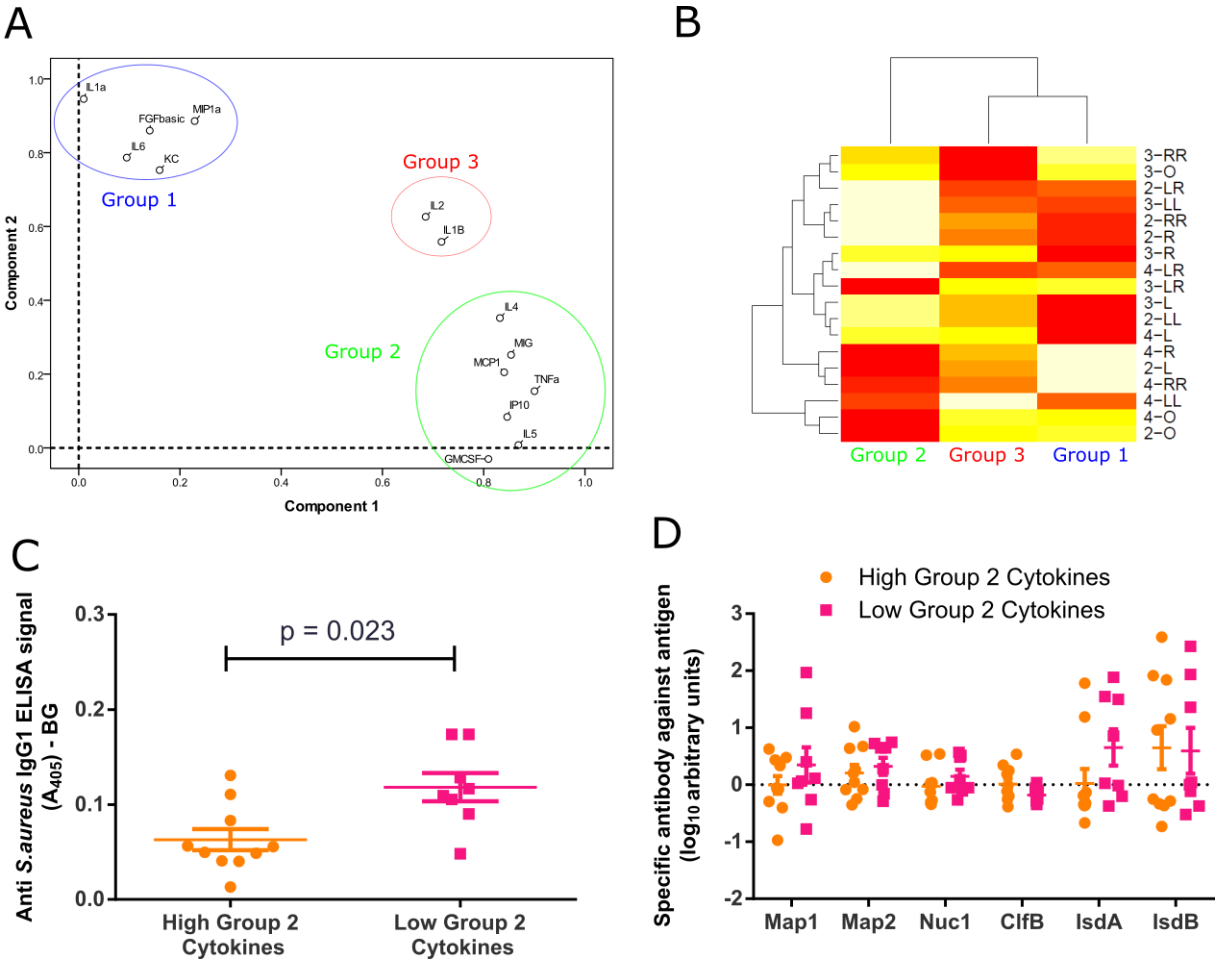



**Figure 5-15: Investigation of nasal cytokine response in colonised animals.** PCA of 14 cytokines (IL-10, IL-13, IFN-γ, VEGF, IL12 and IL17 removed) from nasal tissue in all mice (colonised and uncolonised) (A). Heat map showing relationship between three cytokine groups in colonised mice (B). Mice were further dichotomised into Group 2 Cytokine 'high'

(yellow in B) and 'low' (red in B). Anti-*S. aureus* IgG1 signal from nasal tissue of colonised mice (data shown in Figure 5-7) split according to Group 2 cytokine status (C). Specific antibody against six *S. aureus* antigens in serum of colonised mice (data shown in Figure 5-12) split according to Group 2 cytokine status (D). *p* values given calculated by Mann-Whitney test.

I further investigated the cytokine patterns in nasal tissue only. To do this I used Principal Component Analysis (PCA) to investigate associations between 14 cytokines (VEGF also removed from analysis) from both colonised and uncolonised nasal tissue. This analysis (Figure 5-15A) showed that cytokines clustered into 3 groups:

Cytokine Group 1 – IL-1α, MIP1α, IL-6, KC, FGF basic

Cytokine Group 2 – TNF-α, GM-CSF, IP-10, IL-4, MIG, MCP1, IL-5

Cytokine Group 3 – IL-2 and IL-1β

I noted that Group 2 and Group 3 cytokines were those which were significantly higher in nasal tissue of colonised mice compared with uncolonised mice (with the exception of GM-CSF). I log normalised the data set and calculated values for each Cytokine Group by summing the log normalised values for all cytokines in each group. Using these metrics, I generated a heat map (Figure 5-15B) which showed that colonised mice can be split into 2 groups – those with low level of Group 2 cytokines (red) and those with high level of Group 2 cytokines (yellow). This cytokine analysis was carried out in the same mice in which antibody signal against the whole cell surface (Figure 5-7) and six individual antigens (Figure 5-12) had been assessed. Therefore, given the unexpected heterogeneity revealed by the cytokine analysis, I investigated whether there were any differences in antibody signal in colonised mice

with low and high Group 2 cytokine status, as this readout may be an indicator of the immune response induced by colonisation.

Mice with low Group 2 cytokine status have significantly higher nasal tissue anti-*S. aureus* IgG1 compared to those with high group 2 cytokines (Figure 5-15C, $p$ = 0.023). I hypothesised that the amount of anti-*S. aureus* IgG1 is due to cumulative antibodies against different *S. aureus* antigens. Therefore, I predicted that low Group 2 cytokine mice would be those with higher responses against individual *S. aureus* antigens. As shown in Figure 5-15D, this was not the case; no significant differences in antibody signal against individual *S. aureus* antigens were seen between high and low Group 2 cytokine colonised mice.

## 5.3 Discussion

In this chapter, I have shown that colonisation induces detectable anti-*S. aureus* antibody responses both to whole cell surface and to individual antigens. The magnitude of these responses is strain and age dependent. I generated evidence that colonisation may impact T-cell ratio in animals colonised as neonates. Using the model tested thus far, colonisation does not protect from subsequent challenge. Colonisation is an inflammatory state, as judged by the elicitation of local changes in cytokine profile which may be linked with antibody responses to colonisation.

I observed differences in the relationship between LTCB (a metric of the animal's exposure to *S. aureus*) and anti-*S. aureus* IgG1 levels in natural and experimental colonisation. LTCB was positively correlated with IgG1 signal in animals naturally colonised but not in those experimentally colonised by the spraying method. This could be related to total duration of exposure, as the naturally colonised animals have presumably been exposed to *S. aureus* for six weeks prior to the start of my experiments. Human colonisation with a low virulence strain was unable to boost baseline IgG against conserved virulence factors[133], suggesting that primary exposure to *S. aureus* may be key in shaping the immune response to it. Therefore, assessing IgG1 in the manner that I have in this chapter is relevant for further informing about how immune phenotype to *S. aureus* colonisation may develop in humans.

Throughout this chapter, I observed murine strain dependent differences: in colonisation kinetics, establishment of anti-*S. aureus* IgG1 and in T cell populations. C57BL/6 and BALB/c mice are known to respond differently to severe *S. aureus*

infection[162], and similar mechanisms could exist during colonisation. Bias in the immune responses of the two inbred mouse strains used exist: C57BL/6 mice typically have a Th1 response, whereas BALB/c mice exhibit a Th2 response[163]. This could explain the differences seen between the two mouse strains but requires further investigation.

C57BL/6 mice colonised as neonates had significantly higher CD8+/CD4+ T cell ratio in peripheral blood then their non-colonised littermates, due to increased CD8+ and decreased CD4+ T cell populations. Whether this is change is also seen in human populations requires investigation. Staphylococcal superantigens have been shown to induce production of CD8+ T regulatory cells in humans[164]. My data does not allow the subset of the T-cells to be determined, but reflects *in vivo* changes, presumably due to stimulation with antigens or superantigens. Superantigen specificity might explain why the effect was seen in C57BL/6 mice, but not in BALB/c, and could also explain other differences in immune status between the mouse strains[163]. These areas could be explored further.

Although colonisation was associated with anti-*S. aureus* IgG1, it was not associated with reduced bacterial recovery following intravenous challenge, in a model commonly used to assess development of effective immunity[100]. Thus, naturally acquired immunity and heterogeneity in the microenvironment do not appear to have a large influence on protection from intravenous challenge, at least under the conditions tested. Colonisation mediated protection might develop against other diseases, such as skin and soft tissue infection[165], which is an area for further investigation.

I show that colonisation elicits responses to some *S. aureus* antigens, but not others (Figure 5-12), and that this can vary from mouse to mouse. In particular, some mice appear to respond to IsdA, and some to IsdB, while some responded to both, and some to neither. One explanation for this might involve a very low antigenic dose causing quantal effects in immune responses[166]. Another explanation might involve genetic restriction of antibody responses to particular antigens, as CD1 mice are outbred.

Interestingly, I observed dichotomy in levels of a group of cytokines in nasal tissue of *S. aureus* colonised mice (Figure 5-15). Higher cytokine levels were associated with lower levels of anti-*S. aureus* IgG1 levels. Therefore, I hypothesise that high expression of TNF-α, GM-CSF, IP-10, IL-4, MIG, MCP1, IL-5 in nasal tissue dampens production of anti-*S. aureus* antibodies. Further work should include testing of this hypothesis.

I have shown that colonisation elicits an IgG1 antibody response against whole *S. aureus* and against various specific cell surface antigens. There is a spectrum of magnitude of these responses in colonised mice, whereas uncolonised mice elicit no antibody responses. It is likely that total IgG1 antibody against *S. aureus* is a cumulative effect of antibodies generated against various cell surface antigens, which may vary from mouse to mouse, and mechanisms for which have not yet been established. I have shown that patterns of cytokine expression are different in colonised mice compared with uncolonised mice and within the colonised mice; there exists a spectrum of cytokine expression. These cytokine levels in colonised mice may also be linked with levels of antibody against specific *S. aureus* surface antigens, e.g.

IsdB, however in the experiments performed here, the results do not show this. This could be due to small numbers of mice investigated here and warrants further investigation.

Here, I show that biological differences in carriage and non-carriage exist, but as yet have no evidence as to which features permit carriage maintenance. *S. aureus* fights back against the host antibody response by using Protein A to eradicate antibodies formed against it. Protein A binds to the Fc portion of immunoglobulins and mediates B cell interactions which lead to their apoptosis[167 168]. This could explain why I observe a lower level of IgG1 in naturally colonised mice which have lost colonisation. This might also explain the fact that IgG1 against *S. aureus* in adult C57BL/6 experimentally colonised by environmental exposure was almost undetectable. Perhaps the timepoint used was too late to detect any response which had then been dampened by Protein A mediated mechanisms.

I have investigated the immune phenotype related to *S. aureus* GI tract colonisation using a number of techniques. However, one limitation is that I have not been able to use all these techniques to investigate experimentally induced colonisation in all three of the mouse strains that I have colonised. This has largely been due to supply issues with SOPF mice and cost. Ideally, I would like to assess cytokine levels in experimentally colonised BALB/c and C57BL/6 mice, however this was not possible within the scope and timeline of this thesis. Similarly, I only tested specific antibody against six *S. aureus* antigens; a bigger screen could be more useful, as would inclusion of the other two mouse strains. Functional capacity of antibodies generated has not been assessed. My colleagues have performed such assays by assessing

antibody-mediated binding of fluorescent *S. aureus* by antibodies in human serum[114]. This assay could be performed in mice and is an area for future study.

Thus far, I have investigated immune responses in peripheral blood and nasal tissue. Ideally, since my model induces GI tract colonisation, other immune tissues within the gut would be interrogated. These could include mesenteric lymph nodes to investigate DCs and gut-associated lymphoid tissue (GALT) to investigate innate lymphoid cells (ILCs). Histology of gut sections, perhaps using GFP expressing *S. aureus*, could provide more data on the precise location of *S. aureus* within the GI tract which could further inform immunological studies.

To conclude, the data presented in this chapter show that both local and systemic changes in the immune response are induced by *S. aureus* colonisation. The next step is to evaluate whether these immune responses are those that would be desired to induce a vaccine response to reduce colonisation. It is likely that both systemic antibody responses and local cellular and cytokine responses would play a part in vaccine mediated immunity, therefore the techniques used in this chapter to assess immune responses to colonisation would also be relevant in assessing immunogenicity after vaccination with the aim of reducing colonisation. Therefore, the next question is whether I can use vaccination as a strategy to reduce carriage in colonisation models.

# 6. Vaccination strategies against *S. aureus* colonisation in mice

## 6.1 Introduction

In this thesis so far I have demonstrated the importance of a human *S. aureus* carriage study for understanding the immune response and microbiome related to carriage. I have described natural colonisation in laboratory mice, established an experimental colonisation model and characterised the immune response induced in this model. In this chapter my focus is on using intervention strategies to reduce colonisation levels. One such intervention is the use of a vaccine. The rationale here is that administration of a vaccine which can reduce colonisation would also reduce burden of infection[158]. This is because, in *S. aureus* persistent carriers, infection commonly occurs with the colonising strain[169] and infection rates are higher in carriers than in non-carriers[170].

Strategies to monitor and eliminate *S. aureus* colonisation are in place in hospital settings[171]. Chlorhexidine bathing prior to surgery is routine and has some efficacy in reducing post-surgery *S. aureus* infection[58]. A recent trial showed that universal chlorhexidine and mupirocin treatment was more effective at reducing MRSA infections in intensive care units compared to screening and isolation, or screening, isolation and targeted treatment[29]. However, vaccination is an attractive alternative since it should target *S. aureus* specifically, rather than antiseptic and antibiotic usage which can eliminate a large percentage of the bacterial species of the microbiome[172]. Since I have shown the importance of the relationship between the microbiome and *S. aureus* carriage in humans in Chapter 3, an antibiotic-free intervention strategy targeting the specific microorganism which we wish to eliminate is preferred.

Previous studies have investigated the use of vaccines to reduce *S. aureus* colonisation. Ability of StaphVAX to reduce nasal colonisation rates in a human clinical trial was assessed. However, the authors concluded that there was no significant change in colonisation levels post vaccination, despite a rise in antigen-specific antibody[62]. Vaccine impact on colonisation rates is often not reported in human *S. aureus* vaccine studies. However, in mice several studies have shown vaccine efficacy in reducing colonisation. Both systemic and intranasal vaccination with recombinant ClfB resulted in lower nasal colonisation compared with control animals[63]. The staphylococcal surface protein, SasX, was able to reduce nasal colonisation through both active and passive immunisation[173].

Vaccine antigens used in this chapter were those which had been tested previously by other group members and shown some promise in reducing bacterial recovery post infectious challenge. As described above, ClfB has shown previous efficacy in reducing murine colonisation and the mechanism by which it aids colonisation of the nares has been described[17]. BitC is a well conserved predicted ABC transporter and lipoprotein[174] previously shown to elicit partial protection in mice[175]. Protein A, encoded by the gene *spa*, is an immune subversion protein which can bind immunoglobulins by the Fc portion. A mutated version of Spa, "SpaM", altered to inactivate the Fc binding, has potential as a vaccine candidate[176]. ClfB, BitC and SpaM have been investigated as a multi-component vaccine, to investigate protection in pre-clinical models of *S. aureus* disease[177]. EapH1 and EapH2 are homologues of the staphylococcal Extracellular Adhesion Protein (Eap). They are secreted serine protease inhibitors, used by staphylococci to inhibit the host response mediated by neutrophils[178]. Vaccination with EapH1 and EapH2 can impact protection in a *S.*

*aureus* challenge model[179]. Therefore, all of these antigens are valid targets for vaccination against colonisation.

Viral vectored vaccines are commonly used because of their ability to induce both humoral and T-cell responses. They also act as adjuvants by activating innate immunity[180]. Studies mentioned in the above paragraph[177 179] used viral vectored antigens.

All studies described in this chapter use viral vectored antigens, as shown in Table S6. Replication-deficient recombinant adenoviruses provide a well-studied platform for expressing vaccine antigens. More recently issues with pre-existing vector immunity in humans has been circumnavigated through the use of chimpanzee replication-deficient recombinant adenoviruses, such as ChAdOx1, used in this chapter[180]. Modified Vaccinia Ankara (MVA) was first shown to be efficacious as a vaccine delivery platform in malaria studies[181] and continues to be widely used.

Experiments performed in this chapter were done under Research Objective 6:

- Assess vaccine efficacy using murine colonisation models

In this chapter I sought to investigate whether vaccination with viral vectored vaccines (both adenoviral and MVA) expressing *S. aureus* antigens could reduce *S. aureus* GI tract colonisation in mice. I used a number of approaches which included vaccinating naturally colonised mice and those which I had experimentally colonised by the spraying method. I tested both therapeutic and prophylactic vaccination in the experimental colonisation model. I show that colonisation can induce specific antibody responses to one vaccine antigen, which can be boosted by therapeutic

vaccination. Most importantly, I show that prophylactic vaccination with adenoviral vectored EapH1_H2 accelerates loss of *S. aureus* carriage.

## 6.2 Results

### 6.2.1 Intramuscular vaccination in naturally colonised mice

I have shown previously that mice can easily be colonised with *S. aureus* (Chapter 4) and that this colonisation elicits detectable immune responses (Chapter 5). In this chapter I explore the use of vaccines to reduce or eliminate *S. aureus* carriage, as this is of interest in reducing morbidity and mortality associated with *S. aureus* disease. However, heterogeneity in carriage duration, as observed during natural carriage (see Chapter 4, Figure 4-4 & Figure 4-5 and Chapter 5, Figure 5-1), could confound estimates of vaccine impact. The importance of such confounding was assessed in experiments where naturally colonised mice were vaccinated therapeutically with a prime-boost schedule using either PBS control or adenoviral vectors followed by MVA vectors expressing no *S. aureus* antigens or the *S. aureus* antigen ClfB. I elected to use intramuscular vaccination in the first instance in order to elicit a systemic response to vaccination. Intramuscular vaccination using adenoviral vectors expressing *S. aureus* antigen(s) followed eight weeks later by boosting with MVA vectors expressing the same *S. aureus* antigen(s) has been tried and tested within the *S. aureus* research group, and consistently produces robust antibody responses to the vaccine antigens.

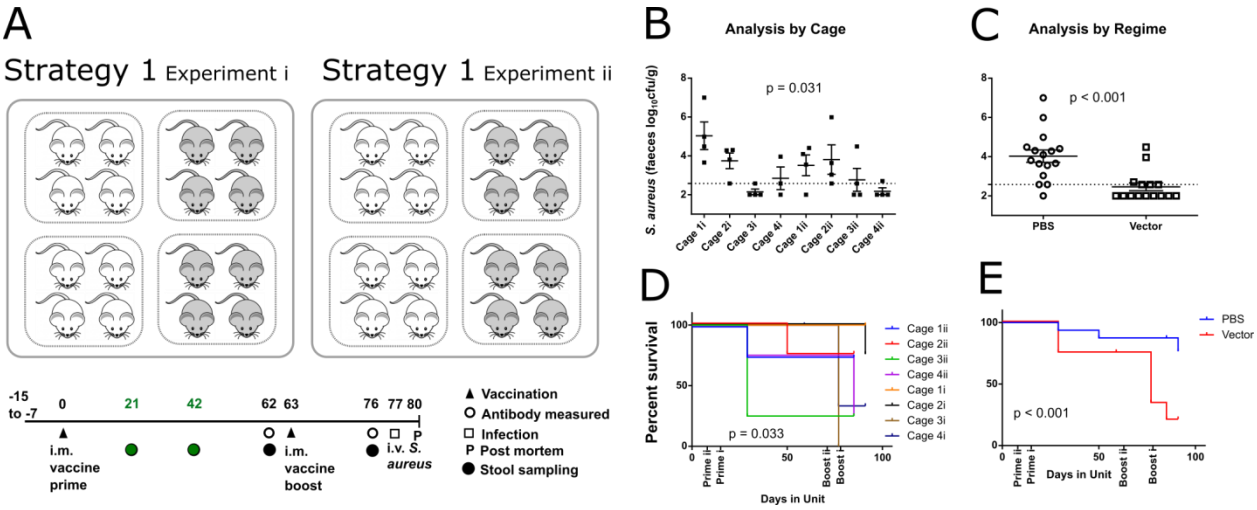



**Figure 6-1: Caging strategy 1 - Therapeutic Vaccination in Naturally Colonised mice.** Female BALB/c mice from Harlan were caged according to their vaccination regime and vaccinated (prime-boost) with PBS or empty viral vectors (AdHu5 and MVA) in 2 experiments. Experimental design and schedule (timepoints in green for Experiment ii only) (A). Effect of vaccination on carriage levels at end of experiment (d76) shown by cage, *p* value given for Kruskal-Wallis test (B) and by regime, *p* values determined by Mann-Whitney (C). Kaplan-meier analysis for loss of carriage by cage (D) and by regime (E), *p* values given for Log rank (Mantel-Cox) test.

Two experimental strategies were used; in Strategy 1 mice were caged according to vaccination regime (Figure 6-1A), while in Strategy 2 mice from all vaccination regimes were included in each cage (Figure 6-2B). In the experiment using Strategy 1, mice were vaccinated with viral vectors expressing no *S. aureus* antigens or PBS as a control. This was to assess the impact of the viral vectors themselves on *S. aureus* colonisation, as both adenovirus and MVA themselves stimulate the innate immune response[180], and so act as adjuvants. Before testing *S. aureus* antigens in viral vectors, I wanted to establish whether the vectors alone could reduce *S. aureus* colonisation.

As I have previously shown that cage to cage variation exists in loss of carriage in naturally colonised mice (Chapter 4), I assessed colonisation levels two weeks after boost vaccination by cage (Figure 6-1B, D) and by vaccination regime (Figure 6-1C, E). Whether assessing colonisation on d76 or loss of carriage across the duration of the experiment, I observed a significant association between cage and colonisation level (Figure 6-1B, $p$ = 0.031 and Figure 6-1D, $p$ = 0.033, respectively). I also observed that colonisation levels were lower on d76 and 'survival' of colonisation was lower in mice receiving viral vectors compared with those receiving PBS (Figure 6-1C, $p$ < 0.001 and Figure 6-1E, $p$ < 0.001, respectively).

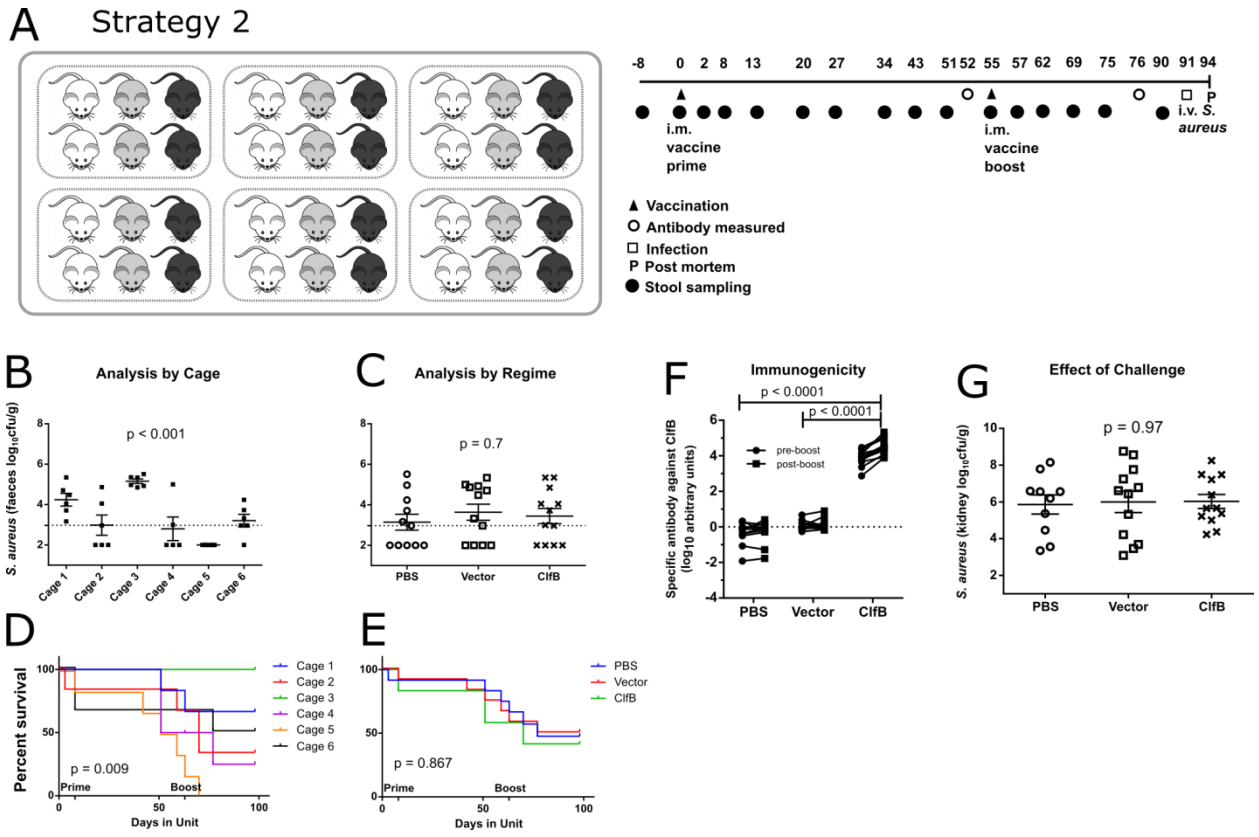



**Figure 6-2: Caging strategy 2 - Therapeutic Vaccination (ClfB) in Naturally Colonised mice.** Female BALB/c mice from Harlan were vaccinated (prime-boost) with PBS, viral vectors (AdHu5 and MVA) expressing either no *S. aureus* antigens or ClfB. Animals

within one cage received different vaccination regimes. Experimental design and schedule (A). Effect of vaccination on carriage levels at end of experiment shown by cage, *p* value given for Kruskal-Wallis test (B) and by regime, *p* values determined by Mann-Whitney (C). Kaplan-meier analysis for loss of carriage by cage (D) and by regime (E), *p* values given for Log rank (Mantel-Cox) test. Specific serum antibodies to ClfB antigen determined using luciferase immunoprecipitation (LIPS) assay in individual mice (F). *p* values given for Tukey's multiple comparisons test. Bacterial recovery from kidneys 3 days after intravenous challenge with $10^7$ *S. aureus* (G).

However, this association is potentially confounded by the influence of the microenvironment within each cage on loss of carriage. Therefore in Strategy 2, the experiment was designed to counteract this confounder. Here, the experiment included a group of mice vaccinated with viral vectors expressing the *S. aureus* antigen, ClfB. As seen in Strategy 1, there was a significant association between cage and colonisation level (Figure 6-2B, $p < 0.001$) and cage and loss of colonisation throughout the experiment (Figure 6-2D, $p = 0.009$). Unlike Strategy 1, no differences were observed in colonisation levels at the end of the experiment between vaccination groups (Figure 6-2C, $p = 0.7$), nor did loss of colonisation throughout the experiment vary (Figure 6-2E, $p = 0.867$). This highlights two important points. Firstly, cage-specific microenvironment can indeed confound estimates of vaccine efficacy, unless experimental designs to control for this (such as Strategy 2) are adopted. Secondly, neither ClfB expressed in viral vectors nor viral vectors expressing no *S. aureus* antigens were able to reduce colonisation. Despite this, the vaccination regime used is immunogenic, as antibodies against ClfB were detected (Figure 6-2F). However, this antibody response was not sufficient to protect vaccinated mice from

subsequent infectious challenge, as assessed by bacterial recovery from kidneys (Figure 6-2G).

In light of these initial experiments, all further experiments involving vaccination in the context of colonisation were designed using a schedule as in Figure 6-2A, such that equal numbers of mice from each vaccination group were included in each individually ventilated cage, to control for cage-specific effects as seen in Figure 6-1.

### 6.2.2 Intramuscular vaccination in experimentally colonised mice

I next investigated whether vaccination with *S. aureus* antigens could reduce colonisation in mice which had been experimentally colonised. The *S. aureus* antigens EapH1 and EapH2 had shown some promise in reducing bacterial load in kidneys after infectious challenge in work carried out by other members of my research group[179]. Therefore I sought to investigate whether these antigens might also be able to reduce colonisation. Again, mice were vaccinated intramuscularly in a prime-boost regime, using adenoviral and then MVA-vectors expressing EapH1 and EapH2 or expressing no *S. aureus* antigens, as per the experimental design shown in Figure 6-3A. The experiment was performed in both BALB/c (Figure 6-3) and C57BL/6 mice (Figure 6-4). For both mouse strains, 'uncolonised', or PBS-exposed mice were used as controls.

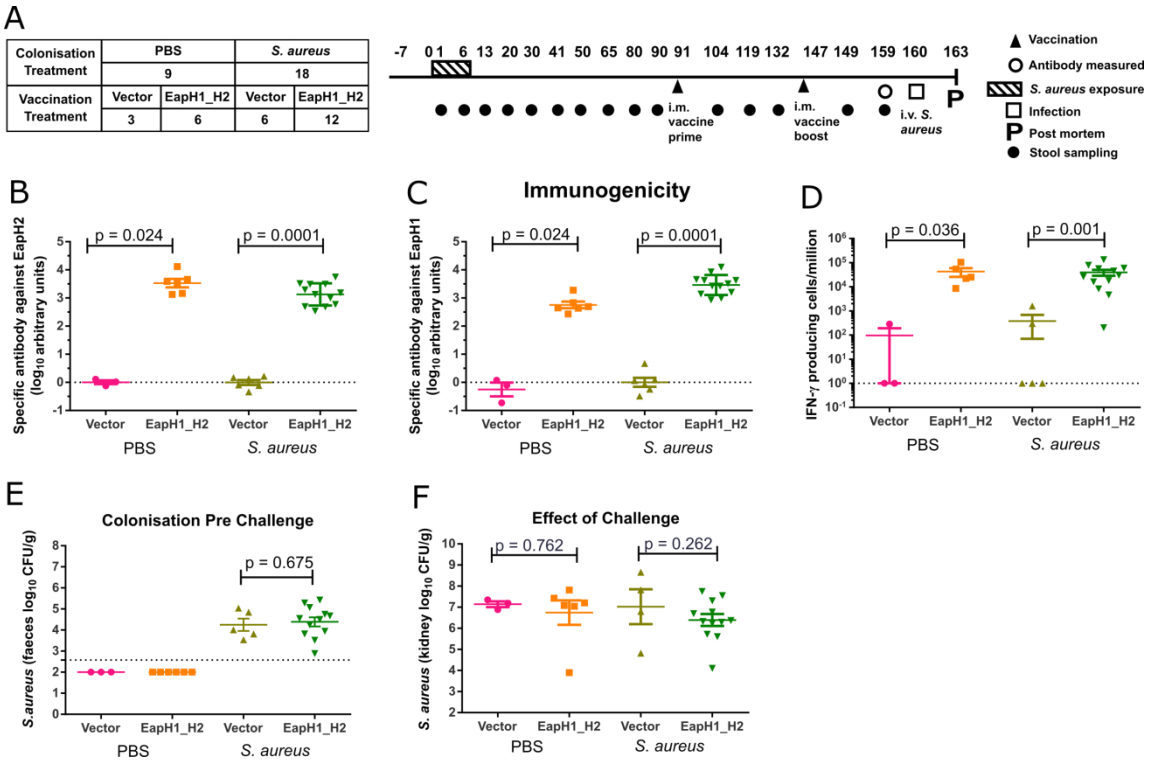



**Figure 6-3: Therapeutic vaccination (EapH1_H2) in experimentally colonised BALB/c mice.** Female BALB/c mice from Harlan were experimentally colonised with *S. aureus* by environmental exposure or received PBS via aerosol. Mice were subsequently vaccinated (prime-boost) with viral vectors (AdHu5 and MVA) expressing either no *S. aureus* antigens or EapH1_H2. Animals within each cage received different vaccination regimes. Experimental design and schedule (A). Specific serum antibodies to EapH2 (B) and EapH1 (C) antigens determined using LIPS assay. IFNγ ELISpot responses to EapH1 and EapH2 peptide pool (D). Effect of vaccination on colonisation levels prior to infectious *S. aureus* challenge (negative samples plotted as if 100 colonies/g were detected) (E). Bacterial recovery from kidney 3 days after intravenous challenge with $10^7$ *S. aureus* (F). *p* values determined by Mann-Whitney tests.

In both strains of mice, vaccination with viral vectored EapH1_H2 was immunogenic: specific antibody against both EapH2 (Figure 6-3B, Figure 6-4B) and EapH1 (Figure

6-3C, Figure 6-4C) was detected post boost vaccination. In order to assess the cellular response to vaccination, IFN-γ ELISpot was used to detect IFN-γ release from peripheral blood mononuclear cells (PBMCs) after stimulation with EapH1 and EapH2 pooled peptides. EapH1_H2 vaccinated mice had significantly more IFN-γ producing cells after peptide stimulation than control vector vaccinated mice, in both BALB/c (Figure 6-3D, $p = 0.001$) and C57BL/6 mice (Figure 6-4D, $p = 0.0001$).

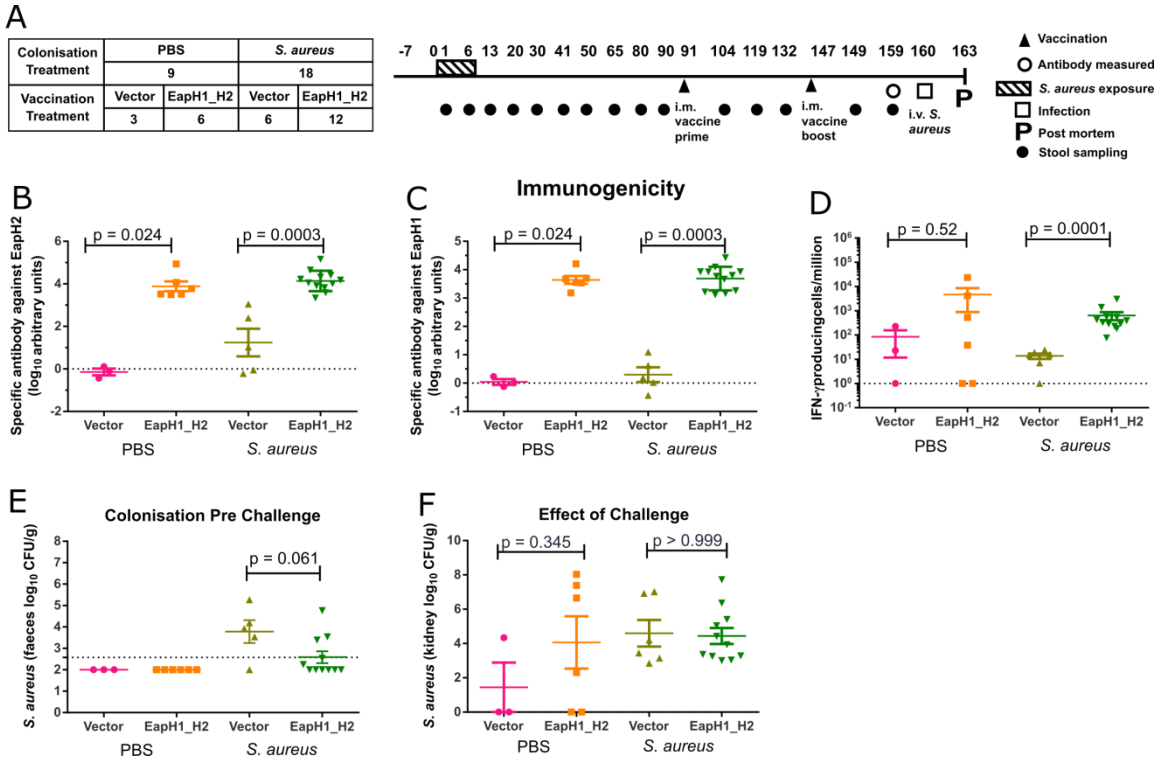



**Figure 6-4: Therapeutic vaccination (EapH1_H2) in experimentally colonised C57Bl/6 mice.** Female C57BL/6 mice from Harlan were experimentally colonised with *S. aureus* by environmental exposure or received PBS via aerosol. Mice were subsequently vaccinated (prime-boost) with viral vectors (AdHu5 and MVA) expressing either no *S. aureus* antigens or EapH1_H2. Animals within each cage received different vaccination regimes. Experimental design and schedule (A). Specific serum antibodies to EapH2 (B) and EapH1 (C) antigens determined using LIPS assay. IFNγ ELISpot responses to EapH1 and EapH2

peptide pool (D). Effect of vaccination on colonisation levels prior to infectious *S. aureus* challenge (negative samples plotted as if 100 colonies/g were detected) (E). Bacterial recovery from kidney 3 days after intravenous challenge with $10^7$ *S. aureus* (F). *p* values determined by Mann-Whitney tests.

Colonisation levels at the end of the experiment were measured by stool sampling as a measure of vaccine efficacy in reducing *S. aureus* colonisation. In colonised BALB/c mice, in this experiment, no difference was observed in colonisation levels between mice vaccinated with EapH1_H2 vectors compared to control vectors (Figure 6-3E, $p = 0.675$). In C57BL/6 mice, a reduction in colonisation was seen in the group receiving EapH1_H2, which borders on conventional statistical significance (Figure 6-4E, $p = 0.061$), indicating that the EapH1_H2 vaccine could have some efficacy in reducing *S. aureus* colonisation in this mouse strain. Mice were also challenged with *S. aureus* to assess vaccine efficacy in terms of a reduction in bacterial recovery from kidneys. In C57BL/6 mice, bacterial recovery from kidneys is lower than that from BALB/c mice (Figure 6-3F, Figure 6-4F), most likely due to the differences between the two strains. In some C57BL/6 mice in the PBS control group, no bacteria were recovered from the kidneys, which was not due to any technical error, but more likely due to the mice clearing the infection before it reached the kidneys. Despite this, no differences were observed in cfu/g from kidneys between EapH1_H2 vaccinated mice and control vector vaccinated mice in either strain (Figure 6-3F, Figure 6-4F). Therefore, intramuscular vaccination of experimentally colonised BALB/c and C57BL/6 mice with viral vectored EapH1_H2 is immunogenic but as yet has not been shown to be significantly efficacious in reducing colonisation levels.

### 6.2.3 Experimental colonisation can prime response to vaccine antigen

I have shown previously (Chapter 5) that colonisation elicits anti-*S. aureus* antibodies. As a result of this, I became interested in whether GI tract colonisation induces different responses to individual *S. aureus* antigens; particularly those which might be included in a vaccine against *S. aureus* colonisation. Since I saw no effect of EapH1_H2 vaccine when administered therapeutically and intramuscularly in reducing colonisation levels, I investigated whether prior colonisation elicits antibody responses to vaccine antigens EapH1 and EapH2.

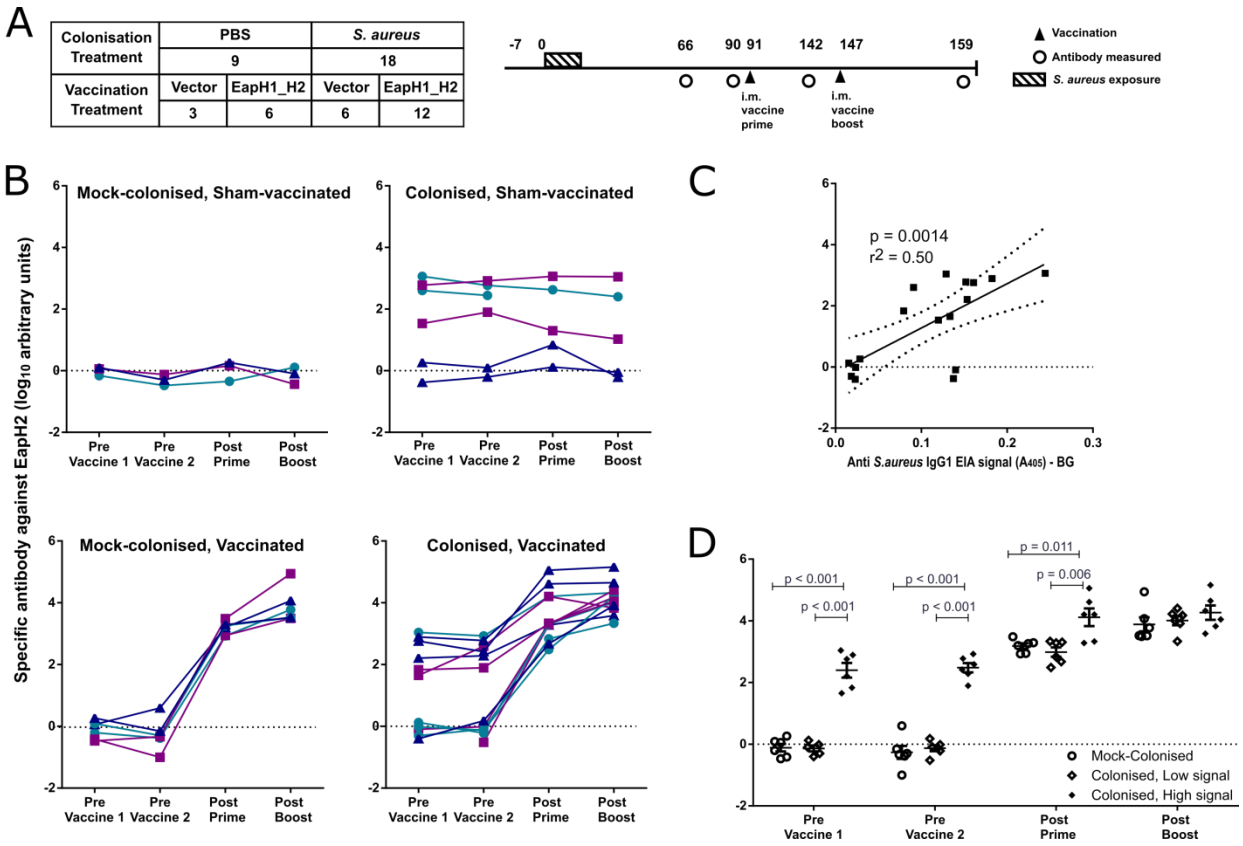



**Figure 6-5: Impact of experimental *S. aureus* carriage on vaccination with *S. aureus* antigen, EapH2.** BALB/c mice colonised as adults were subsequently vaccinated intramuscularly in a prime-boost schedule with viral-vectored vaccines containing both *S. aureus* antigens EapH1 and EapH2. (A) Experimental design and schedule. (B) EapH2 antibody response stratified by experimental group over course of experiment. (C) EapH2

antibody responses in colonised animals at Pre Vaccine 1 timepoint displayed against IgG1 against the cell surface (data plotted in Figure 5-5C) (D) Comparison of EapH2 antibody levels in uncolonised and colonised animals which received vaccination. Colonised animals which received vaccination have been dichotomised according to whether they produced a high or low EapH2 antibody response after colonisation. Pre Vaccine specific antibody against EapH2 of >1.5 log10 arbitrary units denotes high response, <1.5 denotes low response. Significant *p* values are shown for Multiple T tests, without correction for multiple comparisons.

In the experiments described in section 6.2.2, I investigated impact of EapH1_H2 vaccination in experimentally colonised and control mice in BALB/c and C57BL/6 strains. To interrogate whether colonisation induces antibody responses to *S. aureus* antigens, I used serum samples taken from these same animals at two timepoints before vaccination, as shown in the experimental schedule (Figure 6-5A). The experimental design was such that there were four different groups post-vaccination: a subset of the mice exposed to *S. aureus* by aerosol were vaccinated with EapH1_H2 while others received control vectors and the same applied to mice 'mock' colonised with PBS aerosols (Figure 6-5A).

Firstly, I investigated specific antibody to EapH2 (Figure 6-5) and EapH1 (Figure 6-6) in BALB/c mice. 66 and 90 days after colonisation, no response to EapH2 was detected in PBS exposed mice (Figure 6-5B, left panels), while specific, stable responses to EapH2 were observed in 10/18 of *S. aureus* colonised mice (Figure 6-5B, right panels). I termed these 10 mice 'immuno-responders', and the other 8 mice 'non-responders'. The basis of this phenotype does not appear to be related to exposure: Long Term Carriage Burden (LTCB) at day 66 post exposure did not differ

significantly between immuno-responders and non-responders (data not shown, $p$ = 0.8, Mann-Whitney test). Notably, in the colonised mice, there was a strong correlation between anti-*S. aureus* IgG1 signal and anti-EapH2 responses on day 66 (Figure 6-5C, $p$ = 0.0014). Immuno-response status was not associated with cage allocation, as immuno-responders and non-responders were present in all the cages in the experiment shown in Figure 6-5.

After adenoviral prime vaccination, immuno-responders had significantly increased anti-EapH2 response over non-responders (Figure 6-5D, $p$ = 0.006). This indicates that colonisation in immuno-responders has a priming effect. After MVA boost vaccination, anti-EapH2 responses were similar in both immuno-responders and non-responders (Figure 6-5, $p$ = 0.38), as anti-EapH2 responses in immuno-responders did not increase further after boost vaccination (Figure 6-5B, D).

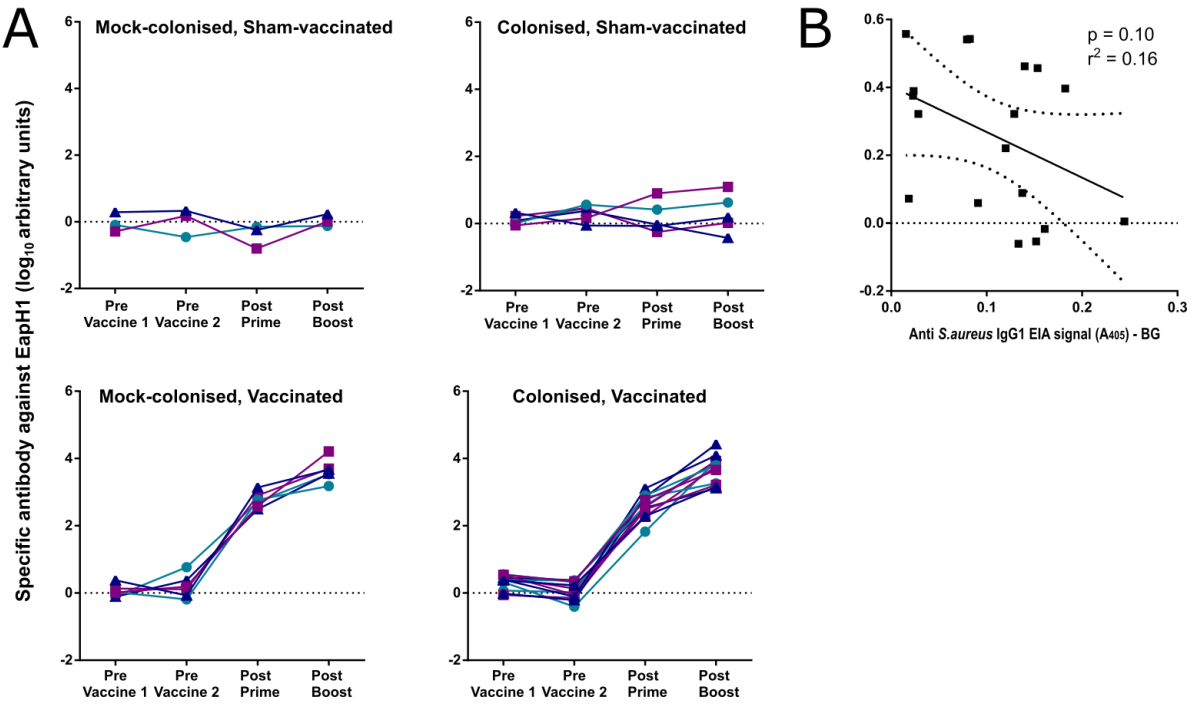



**Figure 6-6: Impact of experimental *S. aureus* carriage on vaccination with *S. aureus* antigen, EapH1.** BALB/c mice colonised as adults were subsequently vaccinated intramuscularly in a prime-boost schedule with viral-vectored vaccines containing both *S. aureus* antigens EapH1 and EapH2. (A) EapH1 antibody response stratified by experimental group over course of experiment. (B) EapH1 antibody responses in colonised animals at Pre Vaccine 1 timepoint displayed against IgG1 against the cell surface (data plotted in Figure 5-5C).

Antibody responses to the second vaccine antigen, EapH1, were observed in BALB/c mice and are shown in Figure 6-6. Unlike the response seen for EapH2, no dichotomy in the antibody response to EapH1 was observed before vaccination (Figure 6-6A). There was also no correlation between anti-*S. aureus* IgG1 signal and anti-EapH1 responses on day 66 (Figure 6-6B, $p = 0.10$). Since natural priming to the EapH1

antigen was not observed, despite high homology between EapH1 and EapH2, it is likely that the phenomenon seen for EapH2 is antigen-specific.

I next assessed the responses to EapH1 and EapH2 pre and post vaccination in colonised and uncolonised C57BL/6 mice. Although responses could be elicited in these animals by vaccination with viral vectors expressing both EapH1 and EapH2, no dichotomy in either response to EapH2 (Figure 6-7A) or EapH1 (Figure 6-7C) was observed post-prime vaccination. There was also no correlation between anti-*S. aureus* IgG1 signal and anti-EapH2 or anti-EapH1 responses on day 66 (Figure 6-7B, D).

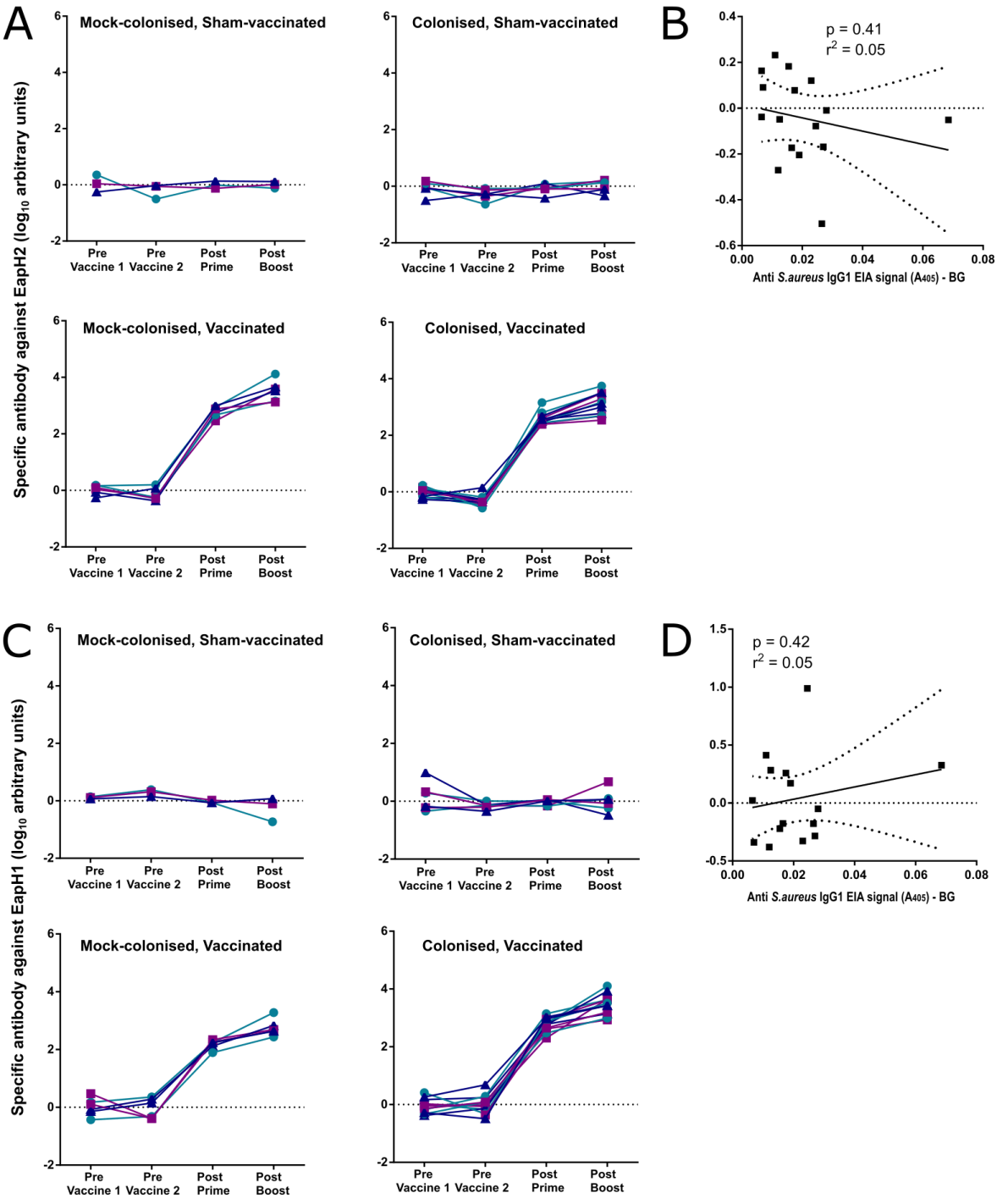



**Figure 6-7: Impact of experimental *S. aureus* carriage on vaccination with *S. aureus* antigens, EapH1 & EapH2 in C57Bl/6 mice.** C57Bl/6 mice colonised as adults

were subsequently vaccinated intramuscularly in a prime-boost schedule with viral-vectored vaccines containing both *S. aureus* antigens EapH1 and EapH2. (A) EapH2 antibody response stratified by experimental group over course of experiment. (B) EapH2 antibody responses in colonised animals at Pre Vaccine 1 timepoint displayed against IgG1 against the cell surface (data plotted in Figure 5-5D). (C) EapH1 antibody response stratified by experimental group over course of experiment. (D) EapH1 antibody responses in colonised animals at Pre Vaccine 1 timepoint displayed against IgG1 against the cell surface (data plotted in Figure 5-5D).

Given the results seen in BALB/c mice colonised as adults (Figure 6-5 & Figure 6-6) indicating 50% immuno-responder rate in EapH2 but not EapH1, I tested whether the factors determining this pattern were present in early life, before the microbiota becomes well established. I measured specific anti-EapH1 and EapH2 antibody in C57BL/6 and BALB/c mice experimentally colonised by spraying as neonates, and their PBS exposed control littermates. Serum was taken from both mouse strains at least 2 months post colonisation. The responses in BALB/c and C57BL/6 mice colonised as neonates were similar to those observed in animals colonised as adults (Figure 6-8). Although numbers are small, immuno-responders (3/6) and non-responders (3/6) can be seen in the colonised BALB/c neonates responses to EapH2 (Figure 6-8A), and strong positive correlation exists between anti-*S. aureus* IgG1 and anti-EapH2 titres (Figure 6-8B, $p = 0.01$). No such differences were observed for EapH1 in BALB/c colonised as neonates (Figure 6-8C, D) or for either EapH2 (Figure 6-8E, F) or EapH1 (Figure 6-8G, H) in C57BL/6 colonised as neonates.

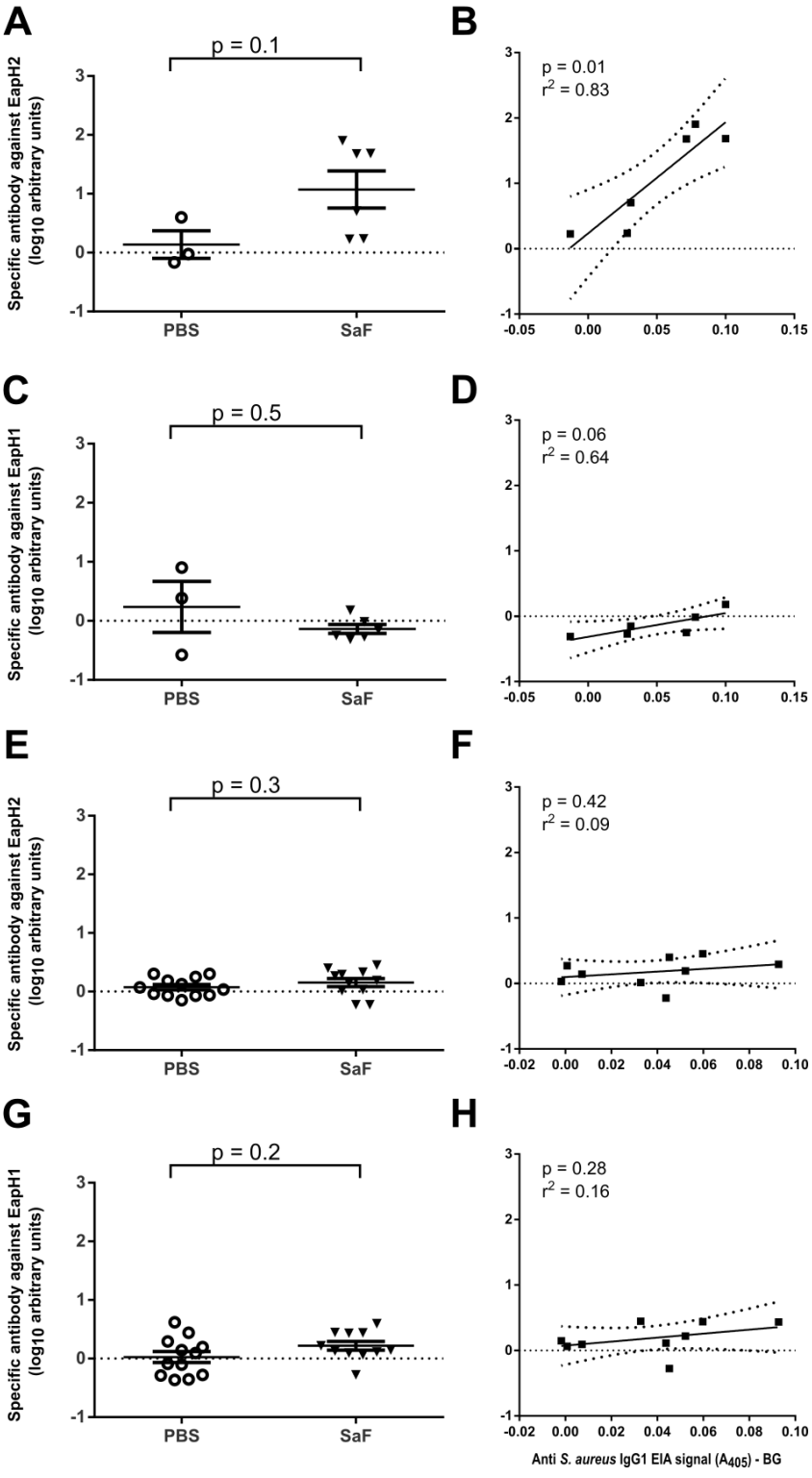



**Figure 6-8: Responses to *S. aureus* antigens, EapH1 & EapH2 in mice colonised as neonates.** (A) to (D) in BALB/c at day 78 post colonisation. (E) to (H) in C57Bl/6 at day

65 post colonisation. EapH2 (A), (E) and EapH1 (C), (G) antibody responses in colonised and uncolonised mice. EapH2 (B), (F) and EapH1 (D), (H) antibody responses in colonised animals displayed against IgG1 against the cell surface (data plotted in Figure 5-5A & B).

Since I saw differences between BALB/c and C57BL/6 in their responses to EapH2 after *S. aureus* colonisation, I investigated responses to both EapH2 and EapH1 in a third strain of mice, CD1. Here, I did not have any uncolonised control mice available and so assessed anti-EapH1 and EapH2 levels before and after colonisation in the same mice (Figure 6-9) across three independent experiments. As seen in BALB/c mice, dichotomy in the antibody response to EapH2 exists (Figure 6-9A): 8/19 mice are immuno-responders and 11/19 are non-responders, and the difference in responses between these two groups is significant ($p < 0.0001$). No response to EapH1 after colonisation was observed in CD1 mice (Figure 6-9B).

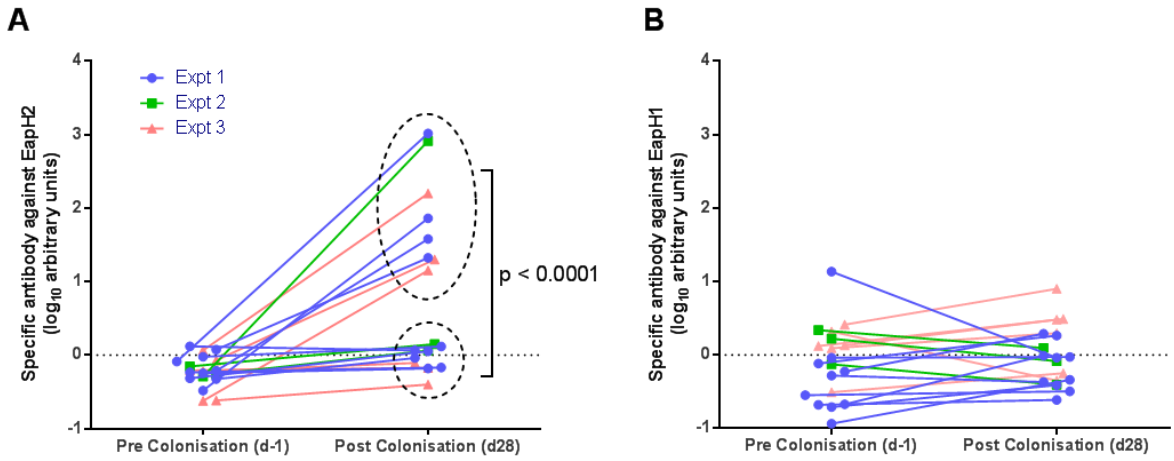



**Figure 6-9: Responses to *S. aureus* antigens, EapH1 & EapH2 in CD1 mice colonised as adults.** EapH2 (A) and EapH1 (B) antibody responses in CD1 mice pre and post colonisation, across three independent experiments.

To summarise, GI tract colonisation of mice with *S. aureus* induces immune responses. Here I have shown that variation exists between mouse strains in that some generate immune responses (CD1 and BALB/c) while another does not (C57BL/6) to EapH2; a secreted *S. aureus* antigen. Striking heterogeneity exists in responses to EapH2 in *S. aureus* colonised BALB/c and CD1 mice; these responses appear as all-or-nothing responses despite the genetic identity of the mice (for BALB/c) and *S. aureus* strains administered.

### 6.2.4 Intranasal vaccination in naturally colonised mice

Thus far in this chapter I have shown that 2 different *S. aureus* vaccines, viral vectored ClfB and EapH1_H2 were not able to reduce GI tract *S. aureus* colonisation when administered intramuscularly. The next approach I took was to investigate whether administration of vaccines via an alternative route might be more efficacious in reducing colonisation. Intranasal vaccination can induce both systemic and mucosal immune responses[182], and appears to be a safe route of delivery in humans[183]. Therefore, delivery of vaccine to one of the sites of *S. aureus* colonisation is a potential approach for reducing colonisation.

Previous data generated by Dr Claire Powers, a Post-Doctoral Scientist within the *S. aureus* vaccine development group, showed that intranasal vaccination followed by intranasal challenge is a useful alternative experimental regime in which to assess efficacy of *S. aureus* vaccine candidates, compared to intramuscular vaccination followed by intravenous challenge. Another Post-Doctoral Scientist within the *S. aureus* vaccine development group, Pauline van Diemen, had designed and tested a multivalent viral vectored vaccine. This vaccine, 'BCA', consisted of three *S. aureus*

antigens: Clf**B**, Bit**C** and mutated Sp**A** behind a TPA leader sequence in ChAdOx and MVA viral vectors. These vectors were tested by Dr van Diemen by intramuscular vaccination in a prime-boost schedule (ChAdOx followed by MVA). A reduction in bacterial load recovered from kidneys after intravenous challenge was observed with this vaccine.

Therefore, I investigated whether viral vectored BCA administered intranasally could reduce colonisation levels in naturally colonised mice. Data presented in this section was generated by me and Claire Powers. Claire designed the experiments, performed vaccinations and tail bleeds and performed the post mortem experiments. I took stool samples to assess *S. aureus* colonisation and performed the LIPS assays.

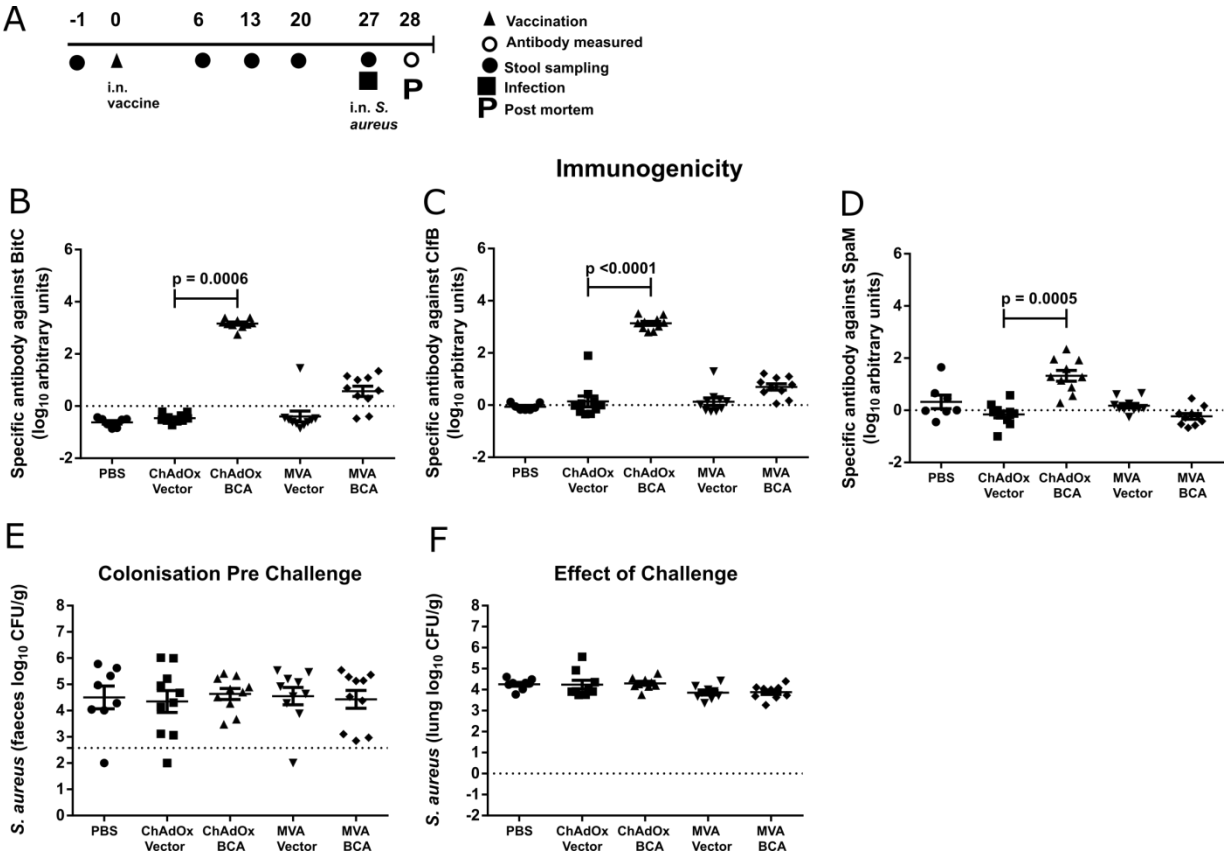



**Figure 6-10: Therapeutic vaccination (BCA) in naturally colonised mice.** Female BALB/c mice from Harlan were vaccinated intranasally (prime only) with PBS or viral vectors (ChAdOx or MVA) expressing either no *S. aureus* antigens or BCA. Animals within each cage received different vaccination regimes. Experimental design and schedule (A). Specific serum antibodies to BitC (B), ClfB (C) and SpaM (D) antigens determined using LIPS assay (*p* values determined by by Dunn's multiple comparison tests). Effect of vaccination on natural colonisation levels prior to infectious *S. aureus* challenge (negative samples plotted as if 100 colonies/g were detected) (E). Bacterial recovery from lung 24 hours after intranasal challenge with ~$10^7$ *S. aureus* (F).

In the first experiment we tested whether a single vaccination with either ChaAdOx or MVA expressing BCA could reduce GI tract colonisation in naturally colonised mice. Mice were vaccinated intranasally with either PBS, control ChAdOx, ChAdOx

expressing BCA, control MVA or MVA expressing BCA. Colonisation was monitored by stool sampling for 28 days. Mice were then challenged intranasally with *S. aureus* to measure bacterial recovery from lungs, to assess efficacy in terms of reducing bacterial burden of infection as well as reducing colonisation. Experimental design is shown in Figure 6-10A.

Single intranasal vaccination with adenoviral vectored BCA is immunogenic, for all three antigens, although levels were slightly lower for SpaM (Figure 6-10D) compared to BitC and ClfB (Figure 6-10B, C). Single MVA vectored BCA was not able to induce specific antibodies against SpaM (Figure 6-10D). However, low levels of antibodies against ClfB (Figure 6-10C) and BitC were detected (Figure 6-10B). Despite the induction of specific antibody against the vaccine antigens, no reduction in *S. aureus* colonisation was observed 27 days post vaccination, compared to control groups (Figure 6-10E). Prior vaccination with viral vectored BCA was unable to protect mice against intranasal challenge with *S. aureus*, as assessed by bacterial recovery from lungs 24hours post infection (Figure 6-10F).

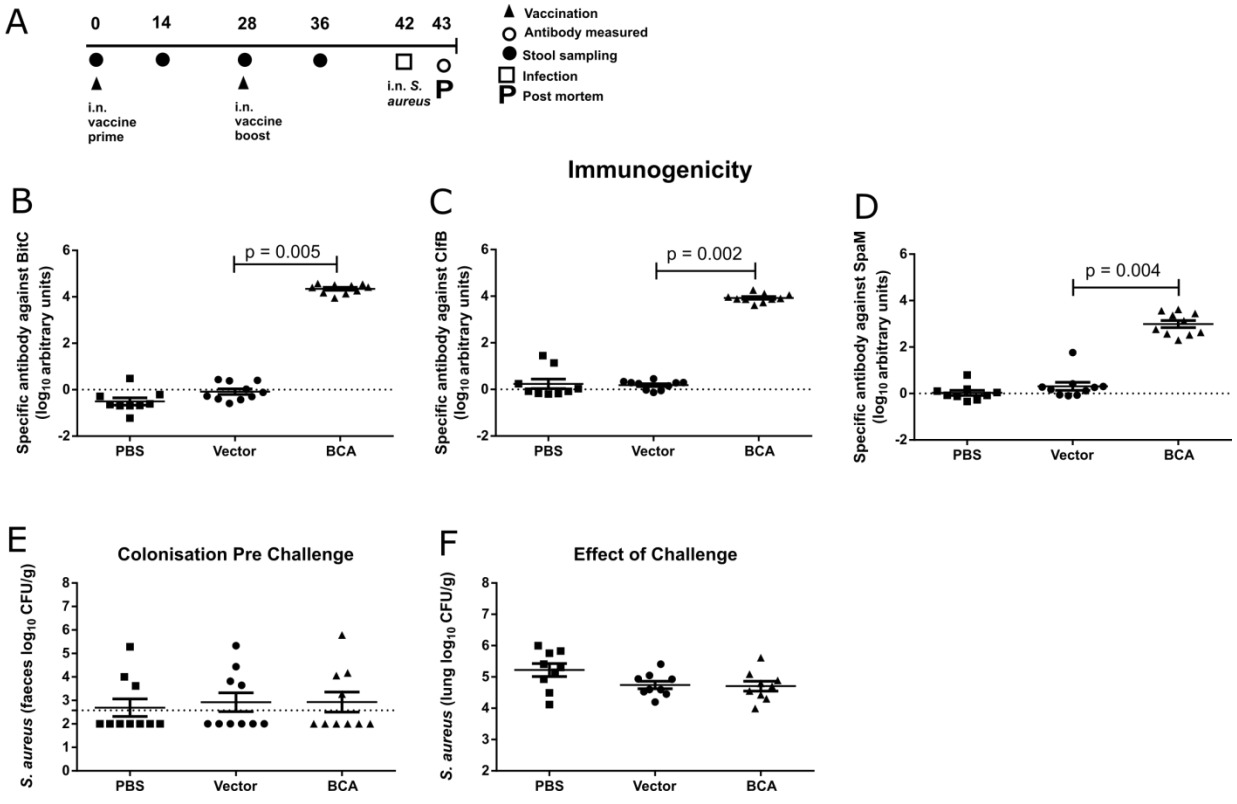



**Figure 6-11: Therapeutic vaccination (BCA) in naturally colonised mice.** Female BALB/c mice from Harlan were vaccinated intranasally (prime-boost) with PBS or viral vectors (ChAdOx and MVA) expressing either no *S. aureus* antigens or BCA. Animals within each cage received different vaccination regimes. Experimental design and schedule (A). Specific serum antibodies to BitC (B), ClfB (C) and SpaM (D) antigens determined using LIPS assay (*p* values determined by Dunn's multiple comparison tests). Effect of vaccination on natural colonisation levels prior to infectious *S. aureus* challenge (negative samples plotted as if 100 colonies/g were detected) (E). Bacterial recovery from lung 24 hours after intranasal challenge with ~$10^7$ *S. aureus* (F).

In the second experiment we tested whether prime-boost vaccination with ChAdOx-BCA followed by MVA-BCA 28 days later could reduce GI tract colonisation in naturally colonised mice. Again, mice were vaccinated intranasally and colonisation was monitored by stool sampling 14 days after boost vaccination. As above, mice were

challenged intranasally with *S. aureus* to measure bacterial recovery from lungs. Experimental design is shown in Figure 6-11A. The prime-boost approach proved to be immunogenic for all three antigens (Figure 6-11B, C, D); and antibody levels were higher than when a single vaccine dose was used (Figure 6-10B, C, D). However, the BCA prime-boost regime was not able to reduce *S. aureus* colonisation (Figure 6-11E) nor reduce bacterial burden after infectious challenge (Figure 6-11F).

In this section I have shown that intranasal vaccination with viral vectored *S. aureus* antigens is immunogenic. However, thus far, the immune responses generated to the antigens used, BCA, are unable to reduce colonisation in BALB/c animals naturally colonised by *S. aureus*.

### 6.2.5 Can prophylactic vaccination reduce subsequent colonisation levels?

Since intranasal vaccination with BCA viral vectored antigens was immunogenic but not efficacious in reducing *S. aureus* colonisation, I decided to try another approach. Here, I use prophylactic vaccination with *S. aureus* antigens in viral vectors, administered intranasally. I hypothesised that responses generated by vaccination would prevent or reduce colonisation when mice were subsequently experimentally colonised by the spraying method. For the experiments presented in this section, outbred CD1 mice were used, rather than BALB/c mice which I have used earlier in the chapter for vaccination studies. This was due to difficulty in obtaining *S. aureus* free BALB/c mice. Since I have shown that CD1 mice can also be experimentally colonised (Chapter 4), and due to these mice being outbred, thus representing a more heterogeneous population, like humans, I used them in these experiments.

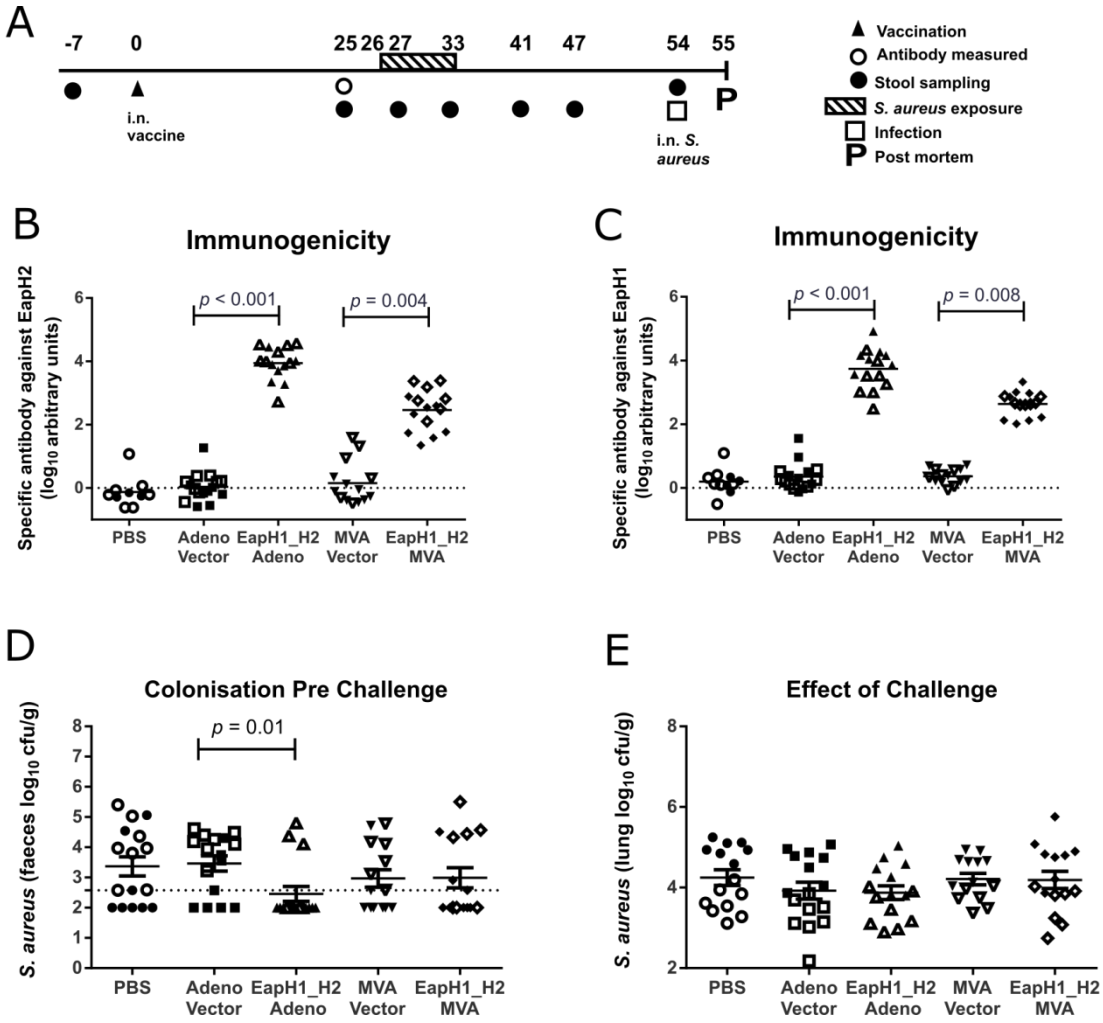



**Figure 6-12: Prophylactic vaccination (EapH1_H2) against experimental *S. aureus* colonisation.** Female CD1 mice were vaccinated intranasally (prime only) with PBS or viral vectors (AdHu5 or MVA) expressing either no *S. aureus* antigens or EapH1_H2. Animals within each cage received each of the different vaccination regimes. Experimental timescale (A). Specific serum antibodies to EapH2 (B) and EapH1 (C) antigens determined using LIPS assay (*p* values determined by Dunn's multiple comparison tests). 26 days after vaccination, mice were exposed to *S. aureus* by environmental contamination, and GI carriage of *S. aureus* was monitored. Effect of vaccination on colonisation levels prior to infectious *S. aureus* challenge (D) (negative samples plotted as if 100 colonies/g were detected, *p* value determined by Mann Whitney test) (E). Bacterial recovery from lung 24

hours after intranasal challenge with ~$10^7$ *S. aureus* (E). Data from two repeat experiments are shown; in (B) to (E) open shapes and closed shapes distinguish data points from the two experiments. Mice in one experiment (open shapes) obtained from Envigo (formerly Harlan), mice in the other experiment (closed shapes) obtained from Charles River.

Firstly, I tested whether vaccination with a single dose of viral vectored EAPH1_H2 might be able to reduce colonisation in this model. Two identical experiments were performed, except that the mice were obtained from two different suppliers, Envigo (formally Harlan) and Charles River (both were confirmed to be *S. aureus* free). Mice received PBS control, Adeno control, Adeno EapH1_H2, MVA control or MVA EapH1_H2. 26 days later mice were colonised by spraying with *S. aureus* and colonisation levels monitored by stool sampling for 28 days. Intranasal challenge and bacterial recovery from lungs followed, as per the experimental design shown in Figure 6-12A.

Intranasal vaccination with both adenoviral and MVA vectored vaccines induced specific antibody to both EapH2 (Figure 6-12B) and EapH1 (Figure 6-12C). This is in contrast to BCA, to which antibody responses after MVA vectored vaccine were very low (Figure 6-10B, C, D). Adenoviral vectored EapH1_H2 was able to accelerate the rate of loss of colonisation compared to adenovirus control (Figure 6-12D, $p = 0.01$). The same was not seen for MVA vectored EapH1_H2 (Figure 6-12D). Despite this, no reduction in bacterial recovery from lungs was seen in either adenoviral or MVA EapH1_H2 vaccine groups (Figure 6-12E).

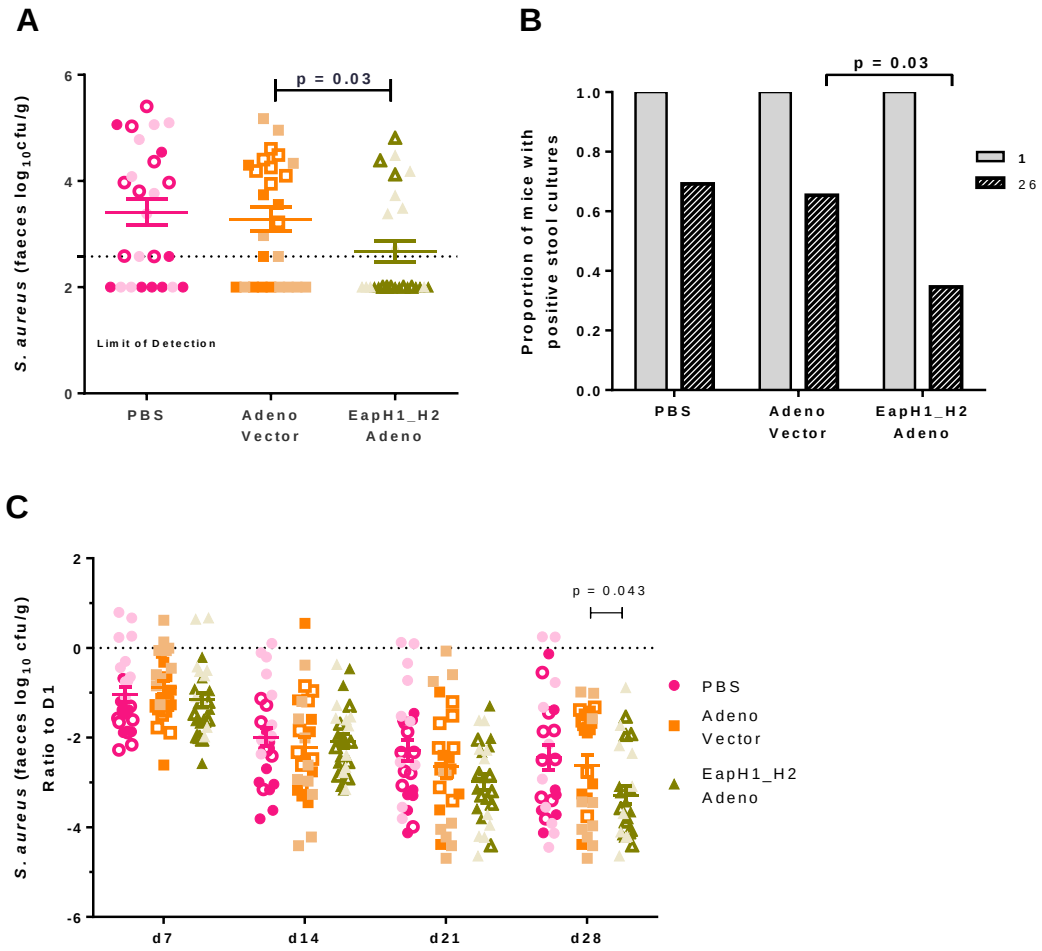



**Figure 6-13: Summary of Prophylactic vaccination (EapH1_H2) against experimental *S. aureus* colonisation.** Female CD1 mice were vaccinated intranasally (prime only) with PBS, AdHu5 viral vector expressing either no *S. aureus* antigens or EapH1_H2 in three independent experiments. Animals within each cage received each of the different vaccination regimes. Four weeks after vaccination, mice were exposed to *S. aureus* by environmental contamination, and GI carriage of *S. aureus* was monitored. Effect of vaccination on colonisation levels at day 26 after exposure (A) (negative samples plotted as if 100 colonies/g were detected, *p* value determined by Mann Whitney test). Proportion of mice with *S. aureus* positive stool samples on day 1 and day 26 (B) (combined data from all three experiments, *p* value determined by one-tailed Fisher's exact test). Effect of vaccination on

colonisation levels relative to day 1 over the 4 weeks after colonisation (C) ($p$ value determined by multiple t tests). Open shapes, closed shapes and pale coloured shapes distinguish data points from the three experiments. Mice in one experiment (open shapes) obtained from Envigo (formerly Harlan), mice in the other two experiments obtained from Charles River (closed shapes and pale shapes).

To confirm results shown in Figure 6-12, I performed another repeat experiment to investigate the effect of adenoviral vector EapH1_H2 vaccination on experimental *S. aureus* colonisation. In this experiment, three experimental groups were used: PBS, Adeno vector and EapH1_H2 Adeno. MVA vectored EapH1_H2 vaccine was not used as it had not shown efficacy in reducing carriage in either of the two previous experiments. In this experiment, only vaccination and colonisation were performed; mice were not challenged as in the two previous experiments, since I saw no differences in bacterial recovery from lungs after intranasal challenge in the two previous experiments (Figure 6-12E). Immunogenicity data for this experiment is not shown; however results were comparable with those shown in Figure 6-12B & C. Combined results for all 3 experiments are shown in Figure 6-13. Across all three experiments, *S. aureus* levels in faeces in mice receiving adenoviral EapH1_H2 were significantly lower on day 26 post colonisation than in mice receiving control adenovirus (Figure 6-13A, $p = 0.03$). An alternative to assessing quantitative bacterial recovery from stool is to score mice as *S. aureus* carriers or non-carriers based on whether *S. aureus* was recovered from their stool sample or not. On day 26 post-colonisation, the proportion of carrier mice was significantly lower in adenovirus EapH1_H2 vaccinated mice than in adenoviral control vaccinated mice (Figure 6-13B, $p = 0.03$). *S. aureus* levels in faeces throughout the colonisation period relative to Day

1 post colonisation are shown in Figure 6-13C. Whilst colonisation levels in all 3 experimental groups fall over the 26 days of colonisation, the effect of EapH1_H2 vaccine compared to empty Adeno is only seen at day 26 ($p$ = 0.043).

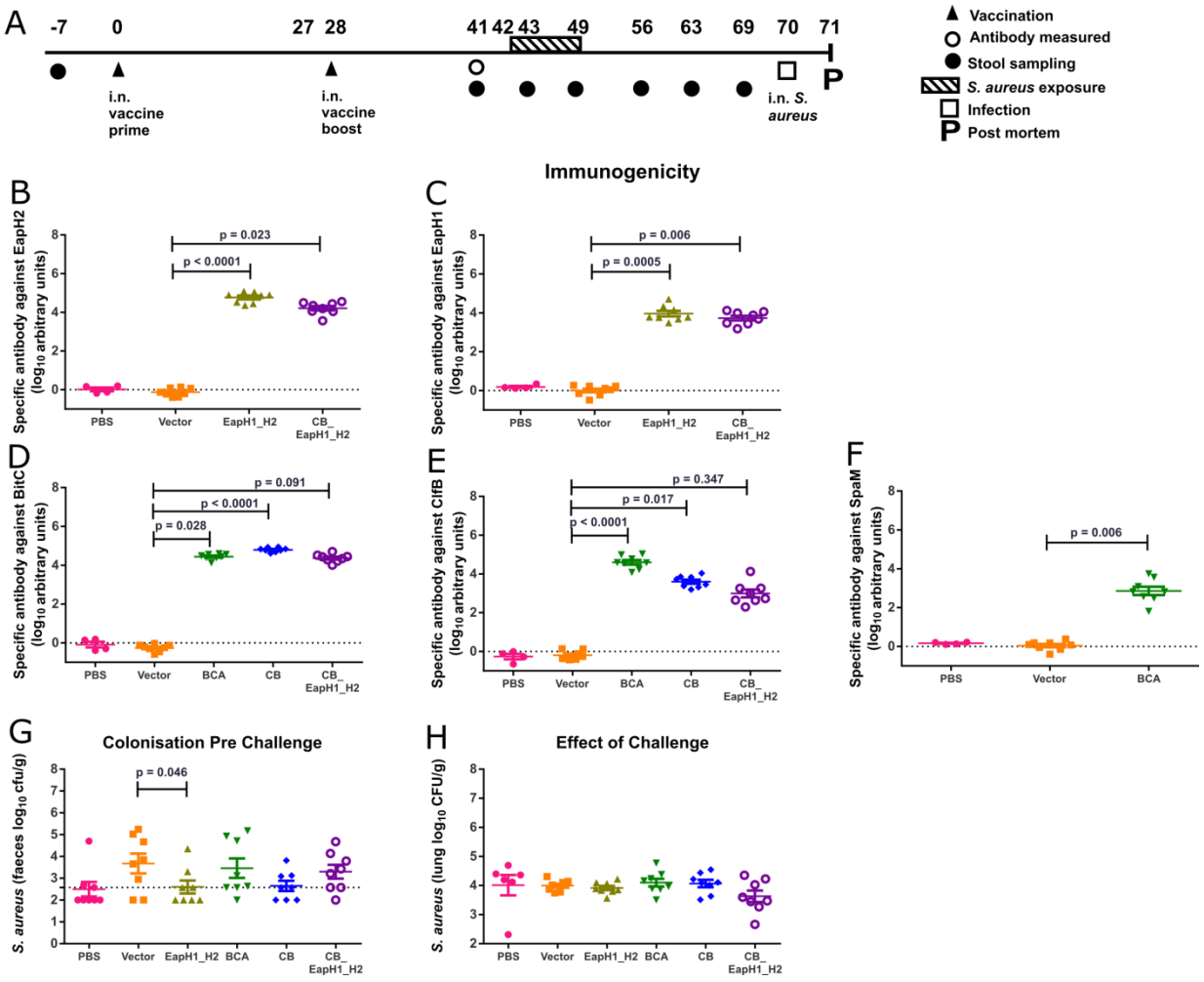



**Figure 6-14: Prophylactic vaccination (BCA & EapH1_H2) against experimental *S. aureus* colonisation.** Female CD1 mice from Charles River were vaccinated intranasally (prime-boost) with PBS or viral vectors (AdHu5, ChAdOx or MVA) expressing either no *S.aureus* antigens or BCA, EapH1_H2, or a combination of both. Animals within each cage received different vaccination regimes. Experimental timescale (A). Specific serum antibodies to EapH2 (B), EapH2 (C), BitC (D), ClfB (E) and SpaM (F) antigens determined using LIPS assay ($p$ values determined by Dunn's multiple comparison tests). 14 days after boost

vaccination, mice were exposed to *S. aureus* by environmental contamination, and GI carriage of *S. aureus* was monitored. Effect of vaccination on colonisation levels prior to infectious *S. aureus* challenge (negative samples plotted as if 100 colonies/g were detected, *p* value determined by one-tailed Mann Whitney test) (G). Bacterial recovery from lung 24 hours after intranasal challenge with ~$10^7$ *S. aureus* (F).

Since prophylactic vaccination with a single dose of EapH1_H2 showed efficacy in reducing colonisation, I investigated whether a prime-boost strategy could deliver further efficacy in terms of reducing colonisation. I also tested other *S. aureus* antigens; previously used BCA, 'CB' (developed from BCA, with SpaM removed and BitC followed by ClfB) and CB_EapH1_H2 (consisting of BitC, ClfB, EapH1 and EapH2). CB and CB_EapH1_H2 vaccines were developed by Pauline van Diemen in order to investigate whether antigens in BCA vaccine might be able to reduce colonisation and bacterial recovery after infectious challenge, if they were in a different design within the viral vectors.

Mice received a prime adenovirus vaccination followed by MVA boost four weeks later. 14 days after boost, mice were experimentally colonised by the spraying method and colonisation was monitored by stool sampling for 28 days. Mice were then challenged intranasally with *S. aureus* to measure bacterial recovery from lungs. Full experimental design is shown in Figure 6-14A. Specific antibody against each of the component antigens of each viral vectored vaccine was elicited as expected (Figure 6-14B-F). EapH1_H2 was able to significantly reduce colonisation levels on day 28, compared with control vectors (Figure 6-14G, $p = 0.046$), but the other vaccine regimes did not. None of the vaccine regimes could significantly reduce bacterial

recovery from lungs after infection (Figure 6-14H). However, bacterial load from lungs trended towards being lower in the CB_EapH1_H2 vaccine group.

Therefore, in this section I have presented evidence that a prophylactic, intranasally-administered viral vectored EapH1_H2 vaccine can accelerate loss of colonisation in a murine model of *S. aureus* colonisation.

## 6.3 Discussion

In this chapter I have presented data which supports the use of viral vectors expressing *S. aureus* antigens as vaccine candidates to reduce GI tract colonisation. I explored the use of therapeutic vaccination via the intramuscular route in both naturally and experimentally colonised animals. Whilst this strategy did not result in reduction of colonisation with any of the pre-clinical vaccine candidates, it revealed important information about experimental design in terms of cage allocation. Exposure to *S. aureus* by colonisation prior to vaccination can induce specific antibody responses to vaccine antigens which can be boosted upon vaccination. Next, I took the approach of targeting mucosal surfaces through intranasal vaccination. In naturally colonised mice, with the antigens tested, this new methodology was immunogenic but not efficacious in reducing GI tract colonisation. Therefore, I used prophylactic vaccination to assess whether immune responses to the vaccine would be able to reduce or prevent subsequent colonisation. A single dose of adenoviral vectored EapH1_H2 significantly reduced colonisation levels 26 days after colonisation. Therefore, EapH1_H2 presents a valid vaccine candidate to investigate further.

The first key finding of this chapter is that prior exposure to *S. aureus* primes the immune response to the vaccine antigen EapH2 in approximately 50% BALB/c mice, whether colonised as adults or neonates and CD1 mice colonised as adults. 'Immuno-responders' demonstrate natural priming with colonisation and reach maximal antibody response with a single dose of adenoviral vector expressing the antigen and those which are 'non-responders' require prime and boost (with MVA vector

expressing the antigen) to reach maximal antibody responses. The determinants of this dichotomy are unknown, but must be acquired antenatally or in very early life since essentially identical patterns of immuno-response are observed in adults and neonatal mice exposed to *S. aureus*. In colonised mice, I observed strong correlation between anti-*S. aureus* IgG1 EIA signal and anti-EapH2 responses indicating that 'immuno-response' may be a phenotype which applies to multiple antigens, both secreted (like EapH2) and cell surface determinants.

The observed dichotomy may reflect poorly understood but fundamental aspects of mucosal immunity and as such deserves further investigation. Mechanisms behind stochasticity in immunogenicity of priming by colonisation could be linked to environmental or microbiome factors. One hypothesis is that the 'non-responders' do not develop an immune response to *S. aureus* due to interactions with other gut flora or mucus[184]. Perhaps these interactions render the *S. aureus* hidden from innate immune system sampling by Peyer's patches. Figure 6-15 shows how this could occur in the context of mucosal checkpoints; in the 'Non-responders' the mechanical and antimicrobial barriers are sufficient to stop *S. aureus* from crossing the epithelial layer and inducing an antibody response to EapH2. However these two barriers are insufficient to remove *S. aureus* from the intestinal lumen altogether, and so GI tract colonisation persists. 'Immuno-responders', on the other hand, could develop an immune response as *S. aureus* is encountered by sampling DCs[185] potentially in Peyer's Patches[186], resulting in development of antibody responses against certain bacterial antigens. This is also shown in Figure 6-15; in 'immuno-responders' the first two barriers are unable to stop some *S. aureus* passing through the epithelial layer

and so they effectively cross the immunological barrier and elicit an antibody response through innate and adaptive immune responses.

Therefore, the cellular immune response to *S. aureus* colonisation should be investigated, with a focus on changes at the site of colonisation. Anti-*S. aureus* IgA levels in the intestine would be of particular interest.

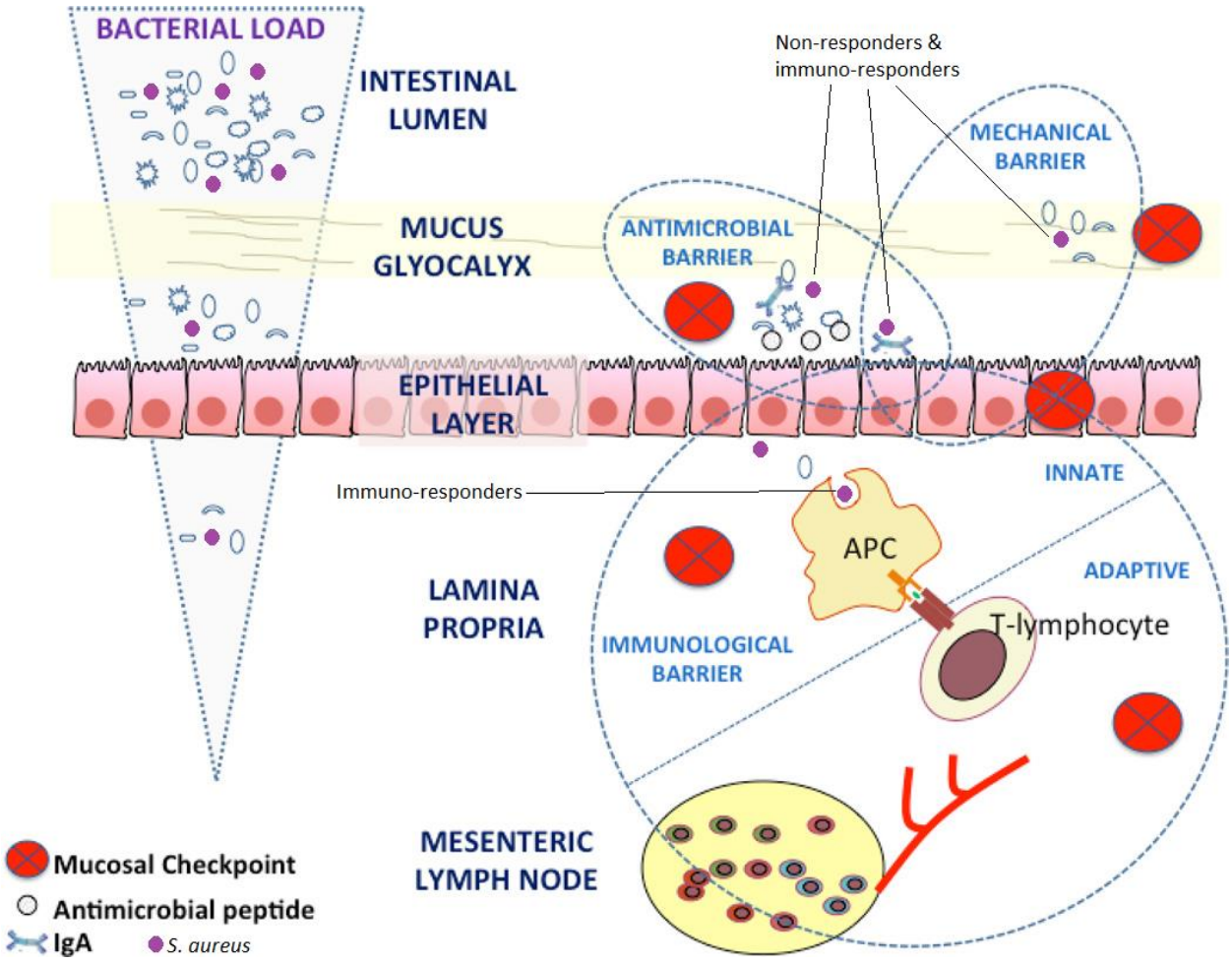



**Figure 6-15: *S. aureus* GI tract colonisation in the context of mucosal checkpoints in intestinal homeostasis.** GI tract bacteria are controlled by 3 mucosal barriers: mechanical, antimicrobial and immunological. Hypothesis for dichotomous response to *S. aureus* EapH2 seen in mice is that non-responders are able to control GI tract

*S. aureus* at the mechanical and antimicrobial barrier whereas immuno-responders control *S. aureus* at the immunological barrier, resulting in generation of systemic antibodies against EapH2 in immuno-responders, but not in non-responders. Figure adapted from Bamias *et al,* 2012[187]

The fact that C57BL/6 mice do not show this phenotype is also interesting; this also deserves further investigation. Bias in the immune responses of the two inbred mouse strains used exists: C57BL/6 mice typically have a Th1 response, whereas BALB/c mice exhibit a Th2 response[163].

The lack of priming responses to EapH1, which has high sequence homology with EapH2 is currently unexplained and deserves further investigation. In a human cohort study, EapH2 antibody levels relative to response to comparator antigens was significantly increased in non-carriers versus carriers. The same was not true of EapH1[179]. Therefore my observation in mice may be reflected in humans, although since all humans have been exposed to *S. aureus*, responses to individual antigens will be observed throughout the population. This finding also has implications in design of vaccine experiments as baseline immunogenicity is not always considered in pre-clinical studies. In a recent study, systemic IgG against numerous *S. aureus* proteins was detected in mice naturally colonised with *S. aureus*[74].

The second key finding of this chapter is that intranasal delivery of EapH1_H2 presents a promising prophylactic vaccination strategy. In experiments using only a single dose of adenovirus and in prime-boost lower GI tract colonisation was observed, indicating that this intervention increases the rate of *S. aureus* loss from the bowel. Of note, the experiments with a single dose of vaccine were carried out in

mice obtained from two different suppliers. Therefore, loss of colonisation cannot be attributed to differences in microbiome of these two groups of mice, strengthening the evidence for EapH1_H2 vaccine being able to reduce GI tract carriage.

Mucosal vaccination with EapH1_H2 is interesting as it may represent an infection control intervention, but the clinical relevance of this effect requires confirmation in humans and/or other relevant mammalian target species. I can speculate that EapH1 and/or EapH2 may play a role, perhaps with other cell surface molecules, in adherence of *S. aureus* to mucosal surfaces, and/or in defence against clearance of *S. aureus* from mucosal surfaces. This latter possibility represents a novel route to address the spread of multi-resistant *S. aureus* in humans[188], and deserves further investigation.

An ideal vaccine candidate to take to clinical trial would have efficacy in both reducing colonisation and reducing bacterial load after infection. In the final experiment shown in this chapter, I show that the 4 antigen combination, CB_EapH1_H2 resulted in a trend towards reduction in bacterial recovery (Figure 6-14H) and in colonisation (Figure 6-14G), although the latter was not to the extent seen for EapH1_H2. Therefore, a combination vaccine with all 4 antigens could be investigated further. However, immunodominant responses arise to the first antigen expressed in the viral vectors (Figure 6-14B-D). Therefore, re-engineering the vaccine construct to EapH1_H2_CB may increase the efficacy in terms of reduction in colonisation.

Data in this chapter shows that intranasal vaccination is a useful alternative to intramuscular vaccination. Other *S. aureus* research groups have reported success in

intranasal vaccination studies: Stegmiller *et al* demonstrate that intranasal vaccination with a whole *S. aureus* antigen preparation (SaAg) can reduce bacterial load in a sepsis infection model[189]. Nanoparticles loaded with *S. aureus* antigens have also been administered intranasally and can protect against infection[190]. Importantly, I have shown that intranasal vaccination induces systemic antibody to levels comparable to intramuscular vaccination (Figure 6-3 versus Figure 6-14). As yet, the cellular response to intranasal vaccination has not been assessed. This is an area for future research and could involve investigating cytokine responses in tissues from vaccinated mice after stimulation with relevant vaccine antigen peptides.

Rationale for further investigating the cellular response to vaccination against nasal colonisation is based on a number of studies which have elucidated the role of the immune response during *S. aureus* colonisation in mice. Elimination of carriage in a murine nasal colonisation model has been shown to be T-cell dependent[153] with a role for IL-17A in antimicrobial peptide production and IL-17F in clearance of colonisation[160]. IL-22 has also been shown to control *S. aureus* nasal colonisation in a mouse model, through induction of antimicrobial peptides[76]. Therefore, any cytokine analysis assessing vaccine efficacy should include measurement of IL-17 and IL-22.

Future research for a vaccine against *S. aureus* colonisation needs to be focused on further study of viral vectored vaccines incorporating EapH1, EapH2 and possibly ClfB and BitC. This would involve more extensive immunogenicity testing and with a view to moving forward into clinical trial. One of the benefits of using intranasal vaccination, as well as inducing mucosal and systemic immune responses, is that it is

needle-free which is desirable in terms of reducing pain and fear associated with vaccination[74]. This would be advantageous should an *S. aureus* vaccine which reduces colonisation be approved for use in humans. Since prophylactic vaccination would be desired, the target population would be infants of just a few months old, for whom intranasal vaccination would be preferred over intramuscular.

# 7. Discussion

## 7.1 Summary

In this thesis I set out to answer two main research questions:

1. What are the characteristics of *S. aureus* carriage in human and murine populations?
2. Can vaccination reduce *S. aureus* carriage in human and murine populations?

I have addressed the first question in results Chapters 3, 4 and 5 and the second question in Chapter 6.

I set out seven specific research objectives at the start of my project and progress towards completing each of them is as follows:

In Chapter 3 I completed my first research objective by investigating the relationship between *S. aureus* carriage, nasal microbiome, immune phenotype and skin health in a human cohort of submariners. Through this observational study in a unique human population, I have shown that an inverse relationship between *S. aureus* and *S. epidermidis* nasal carriage exists. I showed that in some submariners, the microbiome changes over the course of the submarine voyage whereas in others it does not. In those with unchanging microbiome and in *S. aureus* carriers, pro-inflammatory cytokine production was increased more than in other submariners. In *S. aureus* non-carriers, changing microbiome was associated with high titres of antibody to IgG1 hinge region.

I completed my second research objective by characterising natural murine *S. aureus* carriage. In Chapter 4 I described the dynamics of natural *S. aureus* GI tract colonisation in mice which are already colonised when obtained from the supplier. I

show that carriage is lost in a cage-specific manner and characterised the carriage strain, showing that it is similar to human carriage isolates. In Chapter 5 I further characterised natural colonisation through investigation of immune phenotype and show that anti-*S. aureus* IgG levels are higher in animals which remain colonised compared to those that lose carriage.

I achieved my third research objective in Chapter 4 by successfully colonising three strains of *S. aureus* naïve mice with the strain isolated from the naturally colonised mice. Attempts at colonising mice by co-housing and oral gavage were short-lived whereas environmental contamination resulted in GI tract colonisation for at least 21 days.

Having established an experimental *S. aureus* GI tract colonisation model, I characterised this model to complete my fourth research objective. In Chapter 4, I showed that experimental colonisation levels vary depending on mouse strain and age at which mice are colonised. In Chapter 5, I show that anti-*S. aureus* IgG1 antibodies are induced in some colonised mice and not in control mice, but this depends on mouse strain and age of exposure. I also show that colonisation may impact T-cell phenotype and that it induces local changes in cytokine production. However, thus far, colonisation was not able to protect mice from subsequent *S. aureus* infectious challenge.

My fifth research objective aimed to determine bacterial genetic requirements for carriage. Whilst some preliminary work was carried out under this research objective, it was not possible to fulfil this objective in this thesis. This is discussed further in section 7.3.2 below.

In Chapter 6 I undertook my sixth research objective and assessed vaccine efficacy in reducing colonisation using natural and experimental colonisation models. I assessed both therapeutic and prophylactic vaccination approaches. I showed that colonisation prior to vaccination can lead to antibody responses against one vaccine antigen in 50% mice and that this response is boostable by administration of the vaccine. Using prophylactic vaccination, I identified a vaccine candidate, EapH1_H2, which was able to reduce colonisation compared to the control vaccine when animals were subsequently colonised.

My seventh research objective involved investigation of the impact of experimental *S. aureus* colonisation on the gut microbiome and on the immune response to vaccines against other, non-staphylococcal, targets. Whilst some progress was made to investigate both of these areas, ultimately these objectives were outside the scope of this thesis. They are further discussed below in sections 7.3.3 and 7.3.4 respectively.

## 7.2 Conclusions

### 7.2.1 *S. aureus* carriage status and inability to change microbiome composition are linked to increased pro-inflammatory cytokine response in a confined human population

My work on the submarine cohort has shown that multi-faceted human cohort studies can provide insight into the relationship between *S. aureus* colonisation and other aspects of the microbiome and immune phenotype. Most importantly, *S. aureus* carriage was associated with increased pro-inflammatory cytokine response during the submarine voyage, as did unchanging microbiome composition during the voyage. Whilst being a *S. aureus* carrier and having unchanging microbiome were not significantly associated in this small study, the greatest increase in pro-inflammatory cytokines was seen in carriers with unchanging microbiome profiles. When investigating non-carriers only, a changing microbiome profile is associated with high levels of binding of an anti-IgG1 hinge antibody (4E3). High or low binding of this antibody is thought to be due to an as-yet unidentified polymorphism within the human population. The lowest increase in serum pro-inflammatory cytokine levels during the voyage was in non-carriers with changing microbiome profiles and high binding to 4E3. One model to explain these data would involve host genetics (perhaps involving an IgG1 polymorphism) influencing changes in the nasal microbiome in individuals without *S. aureus* carriage, but not in *S. aureus* carriers in whom the impact of immunoglobulins on the microbiota are attenuated by Protein A and other secreted immune subversion proteins.

### 7.2.2 A novel experimental *S. aureus* GI tract colonisation model has been established in mice

The experimental colonisation model I established provides a setting in which to test vaccines and investigate other intervention strategies with advantages over currently used colonisation models. The model is needle-free, does not require antibiotics and is maintained for at least 21 days depending on mouse strain used. The method of monitoring colonisation (stool sampling) is non-invasive. Establishment of colonisation by spraying the inoculum into the cage environment presents a refinement in terms of the NC3Rs (National Centre for the Replacement, Refinement and Reduction of Animals in Research) and so presents an advancement for animal welfare over currently used models.

### 7.2.3 Both natural and experimental colonisation of mice with *S. aureus* induce an immunological phenotype.

Anti-*S. aureus* IgG1 levels are higher in naturally colonised mice which maintain colonisation compared to those that lose colonisation. Overall, in experimentally colonised mice, anti-*S. aureus* IgG1 was induced, however this was not seen in control animals. Colonisation at an early age can lead to changes in circulating T-cell ratio but this may be mouse strain dependent. Changes in local but not systemic cytokine profiles indicate that some changes occur at the mucosal surfaces where the immune system first encounters the colonising bacterium, but that these responses are limited to the locality. Overall, however, *S. aureus* colonisation induces both local and systemic changes in immune parameters.

### 7.2.4 Viral Vectored EapH1_H2 is a promising vaccine candidate for reduction of *S. aureus* carriage

A single dose of intranasal adenoviral vectored EapH1_H2 is sufficient, when administered prophylactically, to significantly reduce *S. aureus* GI tract colonisation levels approximately a month after exposure. A similar effect was seen using a prime-boost regimen, providing evidence that this vaccine regime could be considered for a human clinical trial investigating its efficacy in reducing colonisation in *S. aureus* carriers. However, my vaccine studies in mice have also shown that prior colonisation with *S. aureus* can have an impact on immune responses to vaccine antigens. This is something that would need to be considered in a human clinical trial.

## 7.3 Limitations

Within this thesis I have investigated human and mouse colonisation with *S. aureus*, immune responses related to colonisation and efficacy of *S. aureus* vaccines on reducing colonisation. However, there are a number of areas of research which have not been covered. Some of these are mentioned in section 7.1 above and all are explored in more detail below.

### 7.3.1 Testing alternative vaccine candidates

Given there are upwards of 2,800 *S. aureus* genes, and that antigen screening is inherently low- throughput, challenges highlighted by this work include:

i) specific and sufficiently sensitive methods of screening for new antigens: The single-dose intranasal antigen delivery format appears one feasible option.

ii) Selection of potential candidates for vaccines against *S. aureus* colonisation.

Although a number of individual *S. aureus* antigens expressed in viral vectors and some multi-valent viral-vectored vaccines have been investigated in this thesis, there are other vaccine candidates which have not been tested due to time limitations. Irrespective of this, a strategy for downselecting candidates *in silico* or *in vitro* would be very welcome. One method used by Clarke *et al* involves screening serum from infected and uninfected individuals to identify *in vivo* expressed antigens which could be used as vaccine candidates[191]. A commercial company, Antigen Discovery Inc, provide a screening service which could identify *S. aureus* proteins in the serum of carriers and non-carriers. The identified proteins could then be investigated further

as potential vaccine candidates for the reduction and elimination of *S. aureus* colonisation[192]. However, the sensitivity and specificity of this approach is unclear.

### 7.3.2 Bacterial genetic requirements for carriage

My fifth research objective involved determining bacterial genetic requirements for carriage. I performed some preliminary work towards this objective. The rationale for determining which genes are required by the bacterium for carriage establishment is that those genes would present sensible vaccine candidates. This has been reported previously for ClfB[17 63]. I considered strategies to evaluate colonisation efficiency by different *S. aureus* strains, since I have observed cage to cage variations in maintenance of colonisation (Chapter 4). One methodology is to use competitive co-colonisation as performed previously in a murine GI tract colonisation model of *S. aureus*[77]. In this study the authors co-colonised with various mutants and wild type *S. aureus*; they produced mutant strains which were antibiotic resistant, allowing differentiation of the wild type and mutant on recovery from stool samples. I proposed to use a similar methodology; however rather than using antibiotic resistance, I would use green fluorescent protein (GFP) expressing and non-GFP expressing strains. Identification of both GFP and non-GFP strains could be performed on *S. aureus* specific agar plates by visualising them using a fluorescence filter. This was tested in a small mouse experiment, which involved colonising three cages, each housing six mice, by the spraying method used throughout this thesis. One cage received GFP-expressing USA300, one cage received USA300 and one cage received a mix of the two strains. My proposed methodology to count colonies depending on GFP presence was successful. However, after 11 days post colonisation, GFP expression started to be lost from colonies recovered from mice colonised with

only the GFP USA300 strain. This is likely due to the fact that GFP is expressed on a plasmid in this strain, as plasmids can be lost during *in vivo* colonisation[193]. *S. aureus* expressing GFP within their chromosome would therefore be required to proceed with this methodology.

Pursuing this idea would present another interesting follow on project. I would suggest testing co-colonisation between chromosomal GFP expressing USA300 (wild type) and mutants lacking a range of *S. aureus* genes, such as master regulators and cell surface proteins.

### 7.3.3 Impact of experimental colonisation on faecal microbiome

Research objective 7a involved investigation of the impact of *S. aureus* colonisation on the gut microbiome. This could have been performed using murine stool samples from experimental colonisation experiments. In light of the data presented regarding the human nasal microbiome in Chapter 3 this would have been an interesting avenue to explore. Questions regarding the murine gut microbiome in relation to *S. aureus* colonisation would include: What is the relationship between *S. aureus* and other staphylococcal species? Can CST types be assigned as was the case in human nasal microbiome? Do CST types change after experimental colonisation? How does the microbiome change over the course of colonisation? How does the microbiome of colonised mice compare with control mice? Does the microbiome of control mice change over time too? Since I observed differences in the rate of loss of carriage from different cages (Chapter 4), are there differences between the microbiome of cages which might explain this? What differences exist between mouse strains? I observed that experimental colonisation levels are higher in BALB/c mice than C57BL/6

(Chapter 4). Evidence exists that these two mouse strains differ in their IgA abundance which leads to differences in microbiome diversity[194]. This may explain why differences in colonisation are observed between the two mouse strains, but deserves further investigation.

Another interesting area to investigate would be whether the presence or absence of other gut microbial species influences ability of *S. aureus* to colonise the GI tract. The presence of segmented filamentous bacteria (sfb) resulted in less severe pneumonia in a *S. aureus* lung infection model compared to absence of sfb[134]. Therefore, it would be interesting to investigate the impact of sfb on *S. aureus* colonisation. Affymetrix offer a kit and service to do this, Axiom Microbiome Array, which would make this project quite straightforward. However, due to the costs involved, it was decided not to pursue investigation of murine gut microbiome for this thesis.

### 7.3.4 Impact of experimental colonisation on immune response to other vaccines

My final proposed research objective (7b) involved investigating impact of *S. aureus* colonisation in response to other vaccines. The proposed methodology here would be to experimentally colonise mice and then vaccinate with non-staphylococcal antigens to test the null hypothesis that there would be no difference in the immunogenicity induced by the vaccination between colonised and control animals. A sensible starting point for this investigation would be to investigate vaccination with ovalbumin (OVA), as this protein is commonly used as a model antigen. Other vaccines to investigate could be those already in use in man against other bacterial pathogens, e.g. BCG (for tuberculosis (TB)) or Bexsero (for meningitis B). This is of particular interest as

carriage status of various bacterial species is not considered when vaccines are administered in humans.

An alternative strategy would be to vaccinate first and then consider the impact of immune response to that vaccine on subsequent *S. aureus* colonisation. Lijek and colleagues showed that colonisation of mice with *S. pneumoniae* protected mice against subsequent *S. aureus* nasal colonisation[69]. It would be interesting to see if the same were true of *S. pneumoniae* vaccine. Conversely, influenza vaccination has been shown to enhance colonisation with *S. aureus* in mice[71]. This should be further tested, as if the same is true in humans, this could have repercussions after the annual influenza vaccinations.

### 7.3.5 Impact of experimental colonisation on immune response to other pathogenic organisms

Following on from the above proposal to investigate *S. aureus* colonisation in the context of vaccination against other pathogens, the impact of colonisation on immune responses to other pathogens could also be investigated. For example, does experimental colonisation with *S. aureus* alter the immune response to infection with TB or *S. pneumoniae*? This could be investigated using my experimental colonisation model. However, the use of other infectious organisms would require changes to the Project Licence under which the animal work is carried out. Therefore, this might represent a follow-on project; perhaps dependent on results from a project investigating relationship between *S. aureus* colonisation and vaccinations against other pathogens.

## 7.4 Future Directions

From the discussion of limitations in the above section (7.3), there are some areas of research which I believe should be pursued next. I believe these ideas could be easily pursued in the near future:

- Test further viral-vectored *S. aureus* antigens as vaccine candidates through prophylactic administration, followed by experimental colonisation to assess efficacy of the vaccines in reducing colonisation.
- Develop further methodology to investigate bacterial requirements for carriage in mice using co-colonisation experiments
- Investigate changes in murine gut microbiome induced by experimental *S. aureus* colonisation.
- Test impact of non-staphylococcal vaccines on *S. aureus* carriage; delivering vaccines both therapeutically and prophylactically.

## 7.5 Final Remarks

In this thesis I have presented data investigating the relationship between *S. aureus* colonisation and other nasal microbial species in relation to cytokine response in a confined environment. Human studies such as this can help inform and direct animal studies in *S. aureus* colonisation. Approaches such as establishing a novel experimental *S. aureus* GI tract model, as I have done here, help explore the impact of the commensal and pathogenic bacterium *S. aureus* in a carefully controlled manner. Observations on the immunological impact of colonisation, including antibody and cytokine changes were characterised and allow further understanding of host-bacterial interactions. For example, the fact that an antibody response can be induced against a certain *S. aureus* protein in 50% mice but not in the other 50% of genetically identical mice, helps to direct and design vaccine strategies against colonisation. If mice are vaccinated therapeutically, their existing immune response to vaccine antigens may impact vaccine immunogenicity. Therefore, in terms of a vaccine to reduce colonisation, prophylactic vaccination may be more appropriate, to prevent colonisation occurring in the first place. I present pre-clinical data supporting the immunogenicity and efficacy of a viral vectored adenovirus expressing *S. aureus* antigens EapH1 and EapH2 in reducing *S. aureus* colonisation in a murine model. The ultimate aim moving on from the work presented in this thesis would be to perform a clinical trial of a vaccine against *S. aureus* colonisation, probably using EapH1_H2 antigens and perhaps others in a multivalent viral-vectored strategy.

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# Appendices

## I. Supplementary Figures

**Figure S1: Template of Pre-Patrol Skin Health Questionnaire filled in by submariners recruited to the study**

**Pre-Patrol Skin Health Questionnaire**

**Name:** _____

**Date:** _____

**Submarine:** _____

**1. Date of baseline swab:** **Day** **Month** **Year**

| | | |
|---|---|---|
| | | |

**1. Date of Birth** / /

**2. Sex** Male ☐ Female ☐

**3. How long have you been in the Royal Navy?** _____

**4. How long have you been a submariner?** _____

**5. What is your rank or rate?** _____

**6. What is your branch and/or specialty?** _____

**7. While alongside, do you smoke?**

| | | |
|---|---|---|
| Yes | | |
| | *If so, number per day* | |
| No | | |

**8. Do you have any allergies? If so, to what?**

**9. Did you have any of the following conditions diagnosed before joining the Royal Navy?**

| ***Condition*** | **Yes/No** | **If yes, for how long?** | **Do you require treatment and if so what medication(s) and how often?** |
|---|---|---|---|
| **Asthma** | | | |
| **Eczema (Dermatitis)** | | | |
| **Hayfever** | | | |

**10. Have you undergone either of the following in the past month?**

| ***Procedure*** | **Yes/No** | |
|---|---|---|
| **Surgery** | | *If Yes, complete Appendix A* |
| **Vaccination** | | *If Yes, complete Appendix B* |

**Continued Overleaf /**

**11. Have you suffered either of the following in the past month?**

| *Procedure* | Yes/No | Date |
|---|---|---|
| **'Flu like illness** | | |
| **Boils or spots on the skin** | | |
| **Other kinds of skin infection** | | |

**12. Have you ever developed a spot or boil on the skin, which you or other people had to squeeze, open with a needle or cut open?**

Yes ☐ No ☐ Don't know ☐

If yes: approximately how long ago?

_____

**13. Have you ever received any antibiotics by mouth in the past 6 months?**

Yes ☐ No ☐ Can't remember ☐

**14.** If yes: Please give name of last antimicrobial:_____

Please give approximate date of last antimicrobial prescription:
/ /

**15. Did you receive the recommended childhood vaccines?**

Yes ☐ No ☐ Can't remember ☐

**16. Have you ever had to take antibiotics to control an infection after surgery?**

Yes ☐ No ☐ Don't know ☐

If yes: approximately how long ago?

_____

**17. Do you know whether you have had an infection due to *Staphylococcus aureus* previously?**

Yes ☐ No ☐ Don't know ☐

If yes: approximately how long ago?

_____

**18. Have you deployed on a submarine before this planned patrol in the last**

Week Yes ☐ No ☐ Don't know ☐
Month Yes ☐ No ☐ Don't know ☐
3 months Yes ☐ No ☐ Don't know ☐

**19. Have you ever been diagnosed with an allergy to chlorhexidine (a chemical used for skin cleaning)**

Yes ☐ No ☐ Don't know ☐

**Continued /**

**20. Now, please complete the below skin condition diary entry. You will be asked to fill this in weekly during the patrol.**

**BASELINE SKIN HEALTH QUESTIONNAIRE**

In the last week, did you…

| | Answer | Example answer |
|---|---|---|
| have pain, irritation or discomfort in your hands or other skin areas ? | | Yes or no |
| have worse pain, irritation or discomfort in your hands or other skin while doing your work? | | Yes or no |
| have pain, irritation or discomfort in your hands or other skin which prevented you doing your work? | | Yes or no |
| | | |
| have to see the medical team (MO, MA) about the skin problem? | | Yes or no |
| Put creams or steroids on your skin? | | Yes or no |
| Have to take any kind of antibiotic? | | Yes or no |
| Have to take any kind of antibiotic for your skin? | | Yes or no |

On a typical day in the last week,

| | Answer | Example answer |
|---|---|---|
| How many showers did you have per day? | | A number |
| Did you use a towel used by more than one person | | Yes, or no |
| Did you clean a compartment or equipment with cleaning chemicals? | | Yes, or no |
| If so, did you always wear gloves when handling | | Yes, or no |

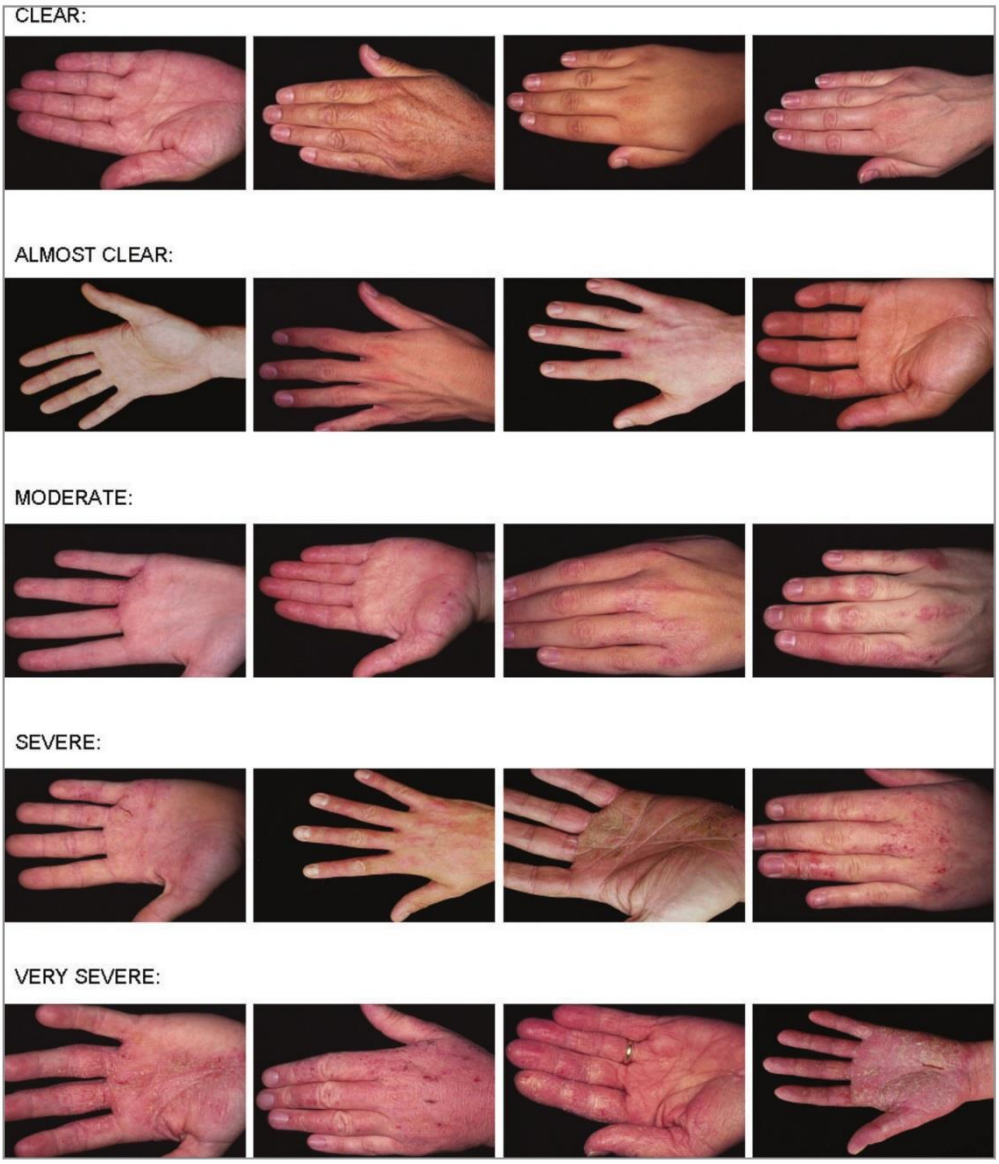
| | | |
|---|---|---|
| these chemicals? | | |

Today:

| | Answer | Example answer |
|---|---|---|
| How bad is your skin generally today? | Normal Very bad<br>0 _____<br>10 | Please put a X on the line |
| How bad is the skin of your hands today? | Normal Very bad<br>0 _____<br>10 | Please put a X on the line |
| Is your hand skin normal today? | | Yes or no |

**Continued Overleaf /**

| | | |
|---|---|---|
| If no, please look at the pictures below. Which picture does the worst affected hand most resemble? | | Clear,<br>Almost clear,<br>Moderate,<br>Severe,<br>Very severe |




**End**

**Appendix A – Details of surgery in past month**

| *Date of surgery* | *Surgical procedure* |
|---|---|
| | |
| | |
| | |

**Appendix B – Details of vaccination in past month**

| *Date of vaccination* | *Vaccine name* |
|---|---|
| | |
| | |
| | |
| | |
| | |

**Figure S2: Template of Weekly Skin Health diary completed by submariners recruited to the study**

**Weekly Skin Health Diary.**

**Name:** _____

**Date:** _____

**Submarine:** _____

Week of Deployment: , Ending (Date): _____

| | **Answer** | **Example answer** |
|---|---|---|
| have pain, irritation or discomfort in your hands or other skin areas ? | | Yes or no |
| have worse pain, irritation or discomfort in your hands or other skin while doing your work? | | Yes or no |
| have pain, irritation or discomfort in your hands or other skin which prevented you doing your work? | | Yes or no |
| | | |
| have to see the medical team (MO, MA) about the skin problem? | | Yes or no |
| Put creams or steroids on your skin? | | Yes or no |
| Have to take any kind of antibiotic? | | Yes or no |
| Have to take any kind of antibiotic for your skin? | | Yes or no |

On a typical day in the last week,

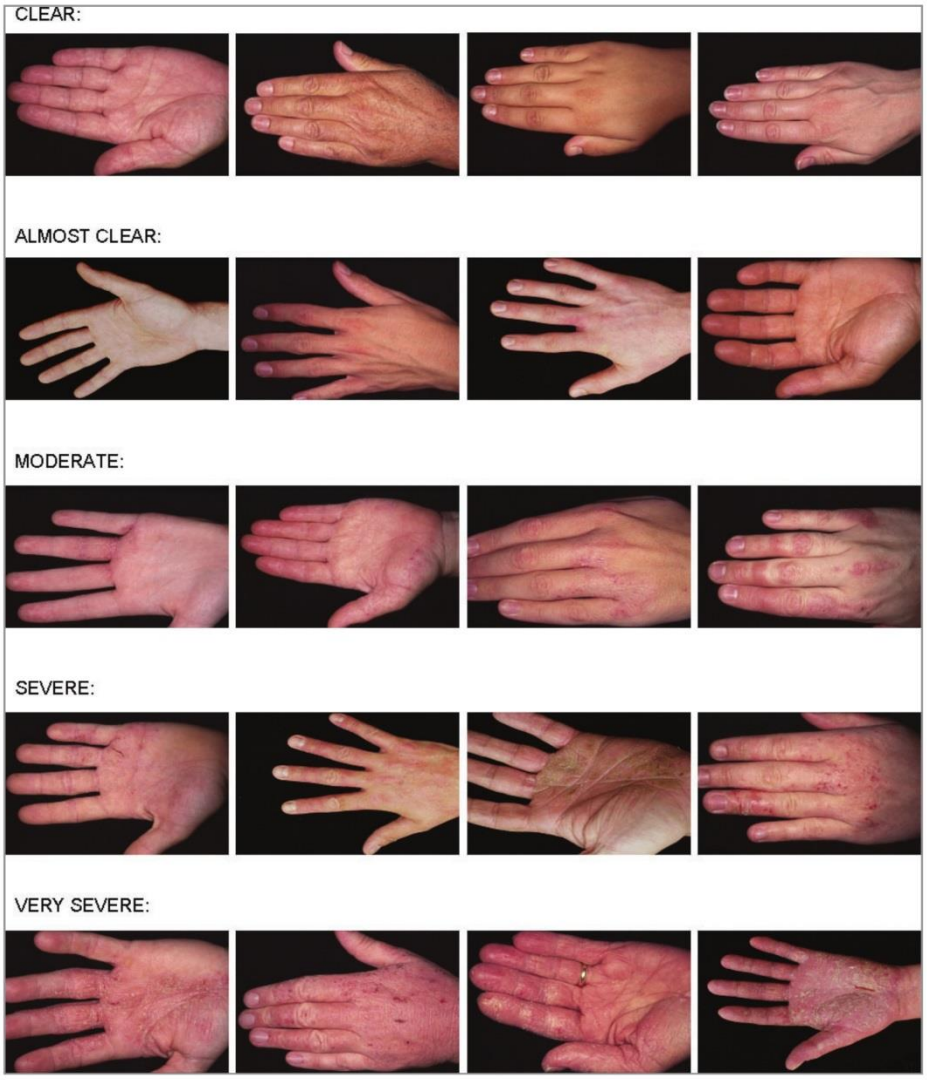
| | Answer | Example answer |
|---|---|---|
| How many showers did you have per day? | | A number |
| Did you use a towel used by more than one person | | Yes, or no |
| Did you clean a compartment or equipment with cleaning chemicals? | | Yes, or no |
| If so, did you always wear gloves when handling these chemicals? | | Yes, or no |

**Continued Overleaf /**

**Today:**

| | **Answer** | **Example answer** |
|---|---|---|
| How bad is your skin generally today? | Normal Very bad<br>0 _____<br>10 | Please put a X on the line |
| How bad is the skin of your hands today? | Normal Very bad<br>0 _____<br>10 | Please put a X on the line |
| Is your hand skin normal today? | | Yes or no |

| | | |
|---|---|---|
| If no, please look at the pictures below. Which picture does the worst affected hand most resemble? | | Clear,<br>Almost clear,<br>Moderate,<br>Severe,<br>Very severe |




**End**

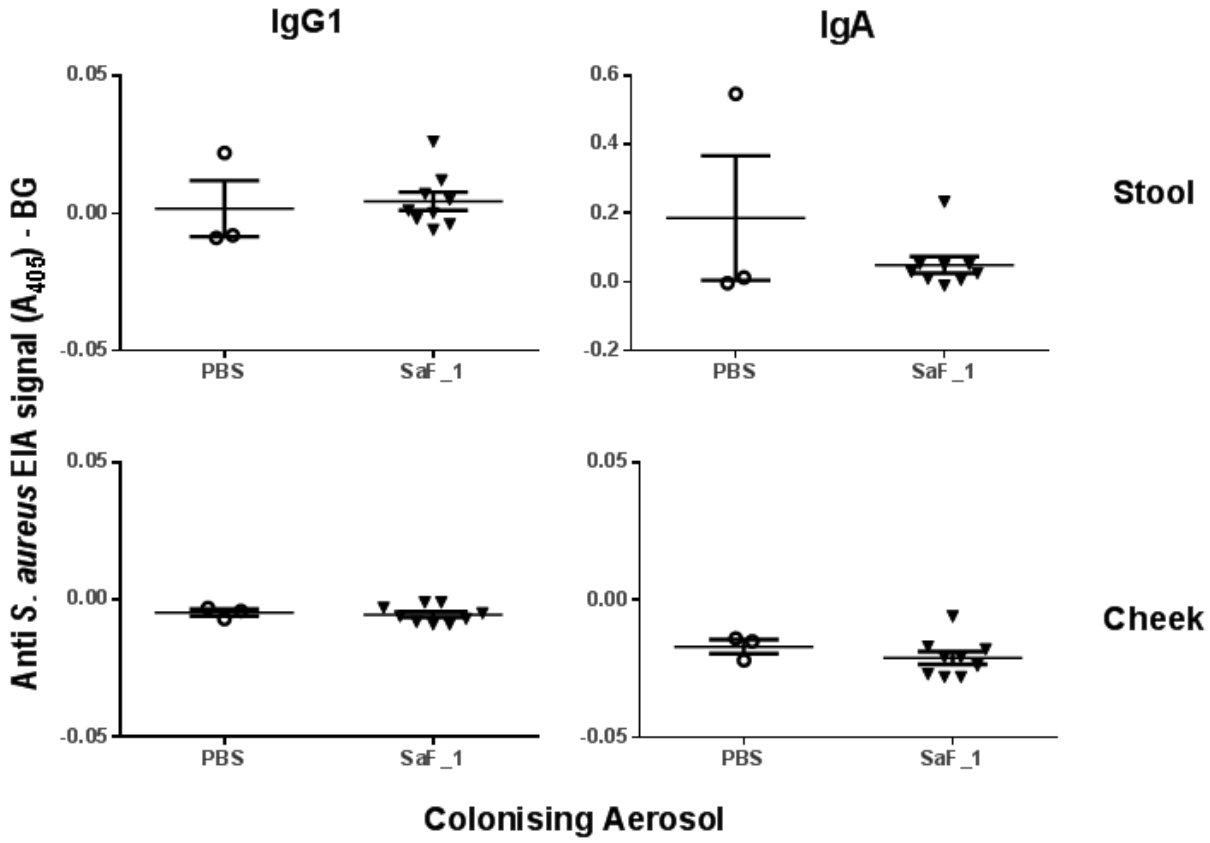



**Figure S3: Antibody response in cheek & stool to experimentally induced *S. aureus* colonisation.** IgG1 (Left) and IgA (Right) antibody responses in stool samples (top) and cheek swab samples (bottom) against *S. aureus* cell surface stratified by treatment. Levels of Ig against the cell surface of fixed *S. aureus* (Δspa Newman strain) are expressed as EIA signal minus background. Experiment performed in C57Bl/6 colonised as adults at 76 days after colonisation. 3 mice per control (PBS) group, 9 mice per colonised (SaF_1) group.

A

| Fluorophore | anti-mouse |
|---|---|
| FITC | CD19 |
| PE | NK1.1 |
| PerCP-Cy5.5 | CD4 |
| PE/CY7 | CD28 |
| BV421 | γδTCR |
| BV510 | AQUA |
| BV605 | CD3 |
| BV650 | CD11b |
| APC | CD11c |
| AF700 | CD8α |

C

| Number | Population |
|---|---|
| 1 | Lives |
| 2 | CD19+ |
| 3 | CD3+ |
| 4 | CD3-CD19- |
| 5 | CD11b_high_SSC_mid (neutrophils) |
| 6 | CD11b_mid_SSC_low (monocytes) |
| 7 | CD11b_mid_SSC_high (macrophages) |
| 8 | CD11c (DCs) |
| 9 | Mon/lymph (monocytes/CD19+CD3+) |
| 10 | γδ+ |
| 11 | NK1.1+ (NK cells) |
| 12 | CD3+CD28+ |
| 13 | CD3-CD28+ |
| 14 | DN__of_CD3 |
| 15 | DN__of_Lives |
| 16 | CD4 of lives |
| 17 | CD8 of lives |
| 18 | CD4__of_CD3 |
| 19 | CD8__of_CD3 |
| 20 | CD4/CD8_of_CD3 |
| 21 | CD3-CD28+/CD3+CD28+ |
| 22 | CD28_on_CD4s |
| 23 | CD28_on_CD8s |
| 24 | CD28_on_DN |
| 25 | CD28_on_gds |

B

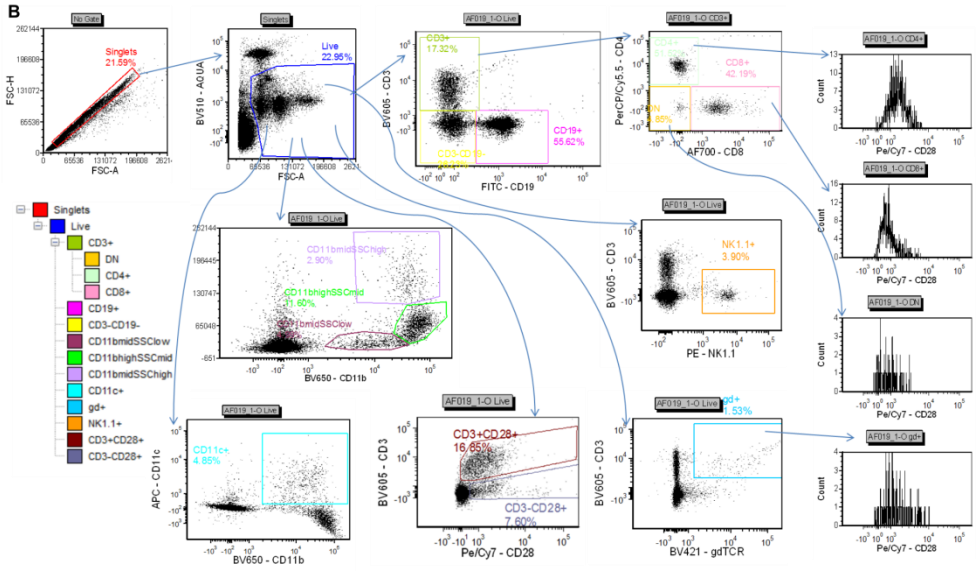



**Figure S4: Gating strategy and analysis parameters in flow cytometry experiments.** (A) Multi-colour panel used (B) Gating strategy used (C) Cellular populations investigated.

## II. Supplementary Tables

### Table S1: *S. aureus* strains used in this thesis

| Strain | Description | Source |
|---|---|---|
| **SaF_1** | Isolated from murine stool sample | This Thesis |
| **Newman** | Isolated from human infection | Kind gift of Prof. Tim Foster[195] |
| **Newman ΔSpa** | Derived from Newman (above), DU5873 spa::TcR, Protein A defective mutant | Kind gift of Prof. Tim Foster[196] |
| **USA300 JE2** | USA300, rsp+. Derivative of LAC, which was cured of three plasmids. | [197] |
| **GFP USA300** | LAC pJL75 (GFP) | Kind gift of Dr. Martin Fraunholz. |

### Table S2: Mouse strains used in this thesis

| Strain | Description | Source |
|---|---|---|
| **BALB/c** | Arrive from supplier *S. aureus* colonised | Harlan (Blackthorn, UK) |
| **BALB/c** | SOPF (certified *S. aureus* free) | Taconic Farms (Ejby, Denmark) |
| **BALB/c** | SOPF (certified *S. aureus* free) | Harlan (Blackthorn, UK) |
| **BALB/c** | In house breeding colony, found to be *S. aureus* colonised | Originated from Jackson Laboratories, USA prior to 2006 |
| **C57JBL/6** | Arrive from supplier *S. aureus* free (not certified) | Harlan (Blackthorn, UK) |
| **CD1** | Arrive from supplier *S. aureus* free (not certified) | Envigo (formerly Harlan) |
| **CD1** | Arrive from supplier *S. aureus* free (not certified) | Charles River (Harlow, UK) |

### Table S3: Targets used to determine gene presence absence in SaF and human CC15 isolates.

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|---|
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tttaggcggtgctactggttatgcaatcaacccagcacgt

>aroe
aattttaattctttaggattagatgatacttatgaagctttaaatattccaattgaagattttcatttaattaaagaaattatttcg
aaaaaagaattagatggctttaatatcacaattcctcataaagaacgtatcataccgtatttagatcatgttgatgaacaagc
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ccaaatttcattagcagatgctgaaaagtattta

>arcc
ttattaatccaacaagctaaatcgaacagtgacacaacgccggcaatgccattggatacttgtggtgcaatgtcacagggtat
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agaattacaaaaagaacagccagactcagtctttaaagaagatgcaggacgtggttatagaaaagtagttgcgtcaccact
acctcaatctatactagaacaccagttaattcgaactttagcagacggtaaaaatattgtcattgcatgcggtggtggcggtat
tccagttataaaaaaagaaaatacctatgaaggtgttgaagcg

**Table S4: Comparison of analytes present in 2 different mouse cytokine multiplex kits used.** Analytes in both kits shown on same row.

| **Luminex™ Cytokine 20-Plex Mouse Panel LMC0006M** Used for analysis of Nasal Tissue Homogenate | **Milliplex® Map Kit MCYTOMAG-70K** Used for analysis of Serum |
|---|---|
| | G-CSF |
| GM-CSF | GM-CSF |
| IFN-γ | IFN-γ |
| IL-1α | IL-1α |
| IL-1ß | IL-1β |
| IL-2 | IL-2 |
| IL-4 | IL-4 |
| IL-5 | IL-5 |
| IL-6 | IL-6 |
| | IL-7 |
| | IL-9 |
| IL-10 | IL-10 |
| IL-12 | IL-12 (p40) |
| | IL-12 (p70) |
| IL-13 | IL-13 |
| | IL-15 |
| IL-17 | IL-17 |
| IP-10 | IP-10 |
| KC | KC |
| MCP-1 | MCP-1 |
| MIP-1α | MIP-1α |
| | MIP-1β |
| | MIP-2 |
| | RANTES |
| TNF-a | TNF-α |
| FGF-Basic | |
| VEGF | |
| MIG | |

### Table S5: Scoring system used for monitoring mice after intravenous or intranasal *S. aureus* infection

| Parameter | Description | Score |
|---|---|---|
| **Appearance** | Normal | 0 |
| | General lack of grooming | 1 |
| | Coat staring, ocular and nasal discharges | 2 |
| | Piloerection, hunched up | 3 - kill |
| **Behaviour** | Normal | 0 |
| | Slight change; either less or more active than usual | 1 |
| | Agitated, alert, isolated or significantly less mobile | 2 |
| | Vocalisation, self-mutilation, unusual aggression or stillness | 3 - kill |
| **Mouse grimace score** | Orbital tightening | 0-1-2 |
| | Nose and/or cheek bulge | 0-1-2 |
| | Ear position | 0-1-2 |
| **Arthropathy** | Normal | 0 |
| | Decreased movements | 1 |
| | Decreased movements and either swelling of joints or nodose tail | 2 |
| | Decrease movements and retracted limb | 3 - kill |
| **Total score** | | 0-15 |

### Table S6: *S. aureus* antigens used in viral vector vaccines in Chapter 6

| Antigen | Description | Function | Accession Number | Reference |
|---|---|---|---|---|
| **ClfB** | Clumping Factor B, N2/N3 domains | Fibrinogen binding adhesin | YP_001333563 | [198] |
| **EapH2** | Extracellular adhesion protein homologue 2 (amino acids 24-144) | neutrophil serine protease inhibitor | WP_000769689.1 | [178 199] |
| **EapH1** | Extracellular adhesion protein homologue 1 (amino acids 24-141) | neutrophil serine protease inhibitor | WP_001549607.1 | [178 199] |
| **BitC** | Brachyspira iron transport C homologue | predicted ABC transporter and lipoprotein | NP_370749 | [174 200] |
| **SpaM** | Staphylococcus protein A with D36A mutation | an immune subversion protein | P02976 | [201] |

## Table S7: Viral Vector Vaccine constructs used in vaccination studies in Chapter 6

| Construct | *S. aureus* Antigens | Experiment strategy | Vaccination Route | *S. aureus* Colonisation | Vaccination regime |
|---|---|---|---|---|---|
| **Control AdHu5** | None | Therapeutic | Intramuscular | Natural | Prime-Boost |
| | | Therapeutic | Intramuscular | Experimental | Prime-Boost |
| | | Prophylactic | Intranasal | Experimental | Prime only |
| | | Prophylactic | Intranasal | Experimental | Prime-Boost |
| **Control ChAdOx** | None | Therapeutic | Intranasal | Natural | Prime-Boost |
| | | Therapeutic | Intranasal | Natural | Prime only |
| | | Prophylactic | Intranasal | Experimental | Prime-Boost |
| **Control MVA** | None (GFP only) | Therapeutic | Intramuscular | Natural | Prime-Boost |
| | | Therapeutic | Intramuscular | Experimental | Prime-Boost |
| | | Therapeutic | Intranasal | Natural | Prime only |
| | | Prophylactic | Intranasal | Experimental | Prime only |
| | | Prophylactic | Intranasal | Experimental | Prime-Boost |
| **AdHu5 ClfB** | ClfB | Therapeutic | Intramuscular | Natural | Prime-Boost |
| **MVA ClfB** | ClfB | Therapeutic | Intramuscular | Natural | Prime-Boost |
| **AdHu5 EapH1_H2** | EapH2, EapH1 | Therapeutic | Intramuscular | Experimental | Prime-Boost |
| | | Prophylactic | Intranasal | Experimental | Prime-only |
| | | Prophylactic | Intranasal | Experimental | Prime-Boost |
| **MVA EapH1_H2** | EapH2, EapH1 | Therapeutic | Intramuscular | Experimental | Prime-Boost |
| | | Prophylactic | Intranasal | Experimental | Prime-only |
| | | Prophylactic | Intranasal | Experimental | Prime-Boost |
| **ChAdOx BCA** | ClfB, BitC, SpaM | Therapeutic | Intranasal | Natural | Prime-Boost |
| | | Therapeutic | Intranasal | Natural | Prime only |
| | | Prophylactic | Intranasal | Experimental | Prime-Boost |
| **MVA BCA** | ClfB, BitC, SpaM | Therapeutic | Intranasal | Natural | Prime-Boost |
| | | Therapeutic | Intranasal | Natural | Prime only |
| | | Prophylactic | Intranasal | Experimental | Prime-Boost |
| **ChAdOx CB** | BitC, ClfB | Prophylactic | Intranasal | Experimental | Prime-Boost |
| **MVA CB** | BitC, ClfB | Prophylactic | Intranasal | Experimental | Prime-Boost |
| **ChAdOx CB_EapH1_H2** | BitC, ClfB, EapH2, EapH1 | Prophylactic | Intranasal | Experimental | Prime-Boost |
| **MVA CB_EapH1_H2** | BitC, ClfB, EapH2, EapH1 | Prophylactic | Intranasal | Experimental | Prime-Boost |

## III. Publications & Presentations

A. Flaxman, Y. Yamaguchi, P. van Diemen *et al*. Gastrointestinal tract colonisation with *S. aureus* induces specific antibody responses and modifies responses to a protective vaccine antigen. *Manuscript in preparation*.

E. Allen, E. Elshina, A. Flaxman *et al*. Vaccination with the Staphylococcus aureus immune evasion proteins EapH1 and EapH2 impacts both S. aureus carriage and invasive disease. *Npj Vaccines*. *Submitted manuscript*.

A. Flaxman, P. van Dieman, Y. Yamaguchi *et al*. Development of persistent gastrointestinal *S. aureus* carriage in mice. *Scientific reports*. *Accepted manuscript*.

A. Flaxman, E. Allen, C. Lindemann, *et al*. Risk factors for dermatitis in submariners during a submerged patrol: an observational cohort study. *BMJ Open* 2016;6(6).

S. Das, C. Lindemann, B.C. Young, *et al*. Natural mutations in a *Staphylococcus aureus* virulence regulator attenuate cytotoxicity but permit bacteremia and abscess formation. *Proceedings of the National Academy of Sciences* 2016;113(22):E3101-E10.

J. Whitehouse, A. Flaxman, C. Rollier *et al*. 2016. Population variation in anti-*S. aureus* IgG isotypes influences surface protein A mediated immune subversion. *Vaccine*. 2016;34(15):1792-9

Oxford D.Phil Day (11/07/17) presenting poster and receiving 2nd place poster prize, "Investigation of nasal microbiome of submariners during a submerged patrol: associations with *S. aureus* colonisation and inflammatory markers"

ISSSI 2016 - International Symposium on Staphylococci & Staphylococcal Infections (30/08-02/09/16), presenting poster "A novel experimental murine *Staphylococcus aureus* colonisation model"

Staphylococcal Diseases - Gordon Research Conference & Seminar (11-17/07/15), presenting poster "Microenvironment-specific changes in microflora and immune parameters identified during a study of murine *S. aureus* carriage"