The University of Reading

The role of *Staphylococcus aureus* FadB in resistance to bile

A thesis submitted for the degree of Doctor of Philosophy in Microbiology

by
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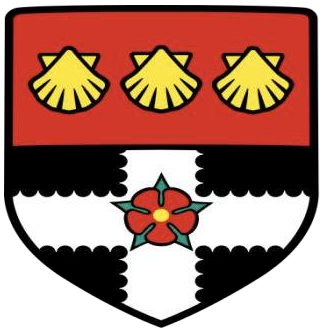

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Declaration

I confirm that this is my own work and the use of all material from other sources has been properly and fully acknowledged

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Abstract

Resistance to the bactericidal effects of bile is crucial for the survival of *Staphylococcus aureus* in the human gut. This study was conducted to identify and characterize components of the bacteria, which allow it to resist bile acids. A comparative study was used to investigate the natural protein diversity within the *Staphylococcus* in relation to bile resistance. Imaging of one-dimension gel electrophoresis showed a unique protein band in samples prepared from bile-treated *S. aureus*. Mass spectrometry and database analysis showed the protein to be FadB. which has a role in lipid metabolism in *Escherichia coli*. It is hypothesized that *fadB* was responsible for the observed bile salt resistance phenotype; to test this, a Δ*fadB* strain was created in *S. aureus* SH1000. The mutant phenotype showed a significant decrease in viability upon exposure to bile acids in comparison with the parental wild type. Furthermore, survival of *S. aureus* Δ*fadB* was attenuated in an *in vitro* human colonic model, implicating *fadB* in *S. aureus* colonization of the human intestine. Moreover, upregulated expression of *fadB* was detected upon exposure to bile salts. To further confirm the role of FadB in bile salt resistance, the gene was cloned under the control of an inducible promoter, which enabled arabinose-dose dependent expression of *fadB* in *E. coli* JW113 as a heterologous host, confirming a bile resistant phenotype. Recombinant FadB was purified and shown to have affinity for cholic acid and might possess an ability to modify bile salts.

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POSTER/ ORAL PRESENTATION

1. The annual Microbiology society conference in Liverpool, 21-24 April 2016. (poster)
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Dedication

- To my mom and my late dad for their support .
- To my Wife Dahma for her continuous support and patience.
- To my children Fatimah , Hussein and Rida
- To my beloved brothers and sisters.

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In the Name of Allah, the Most Merciful, the Most Compassionate all praise be to Allah, the Lord of the worlds; and prayers and peace be upon Mohamed His servant and messenger.

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Chapter One: Introduction

1.1 Staphylococcus aureus

S. aureus is a Gram-positive bacterium belonging to the Stapylococcaceae family (Phylum Firmicutes). It grows in pairs or grape-like clusters (Washington, 2006; Lowy, 1998). *S. aureus* colonises the nose, skin and gastrointestinal tract. Although the nose is the primary site of colonisation of *S. aureus* carriage, recent studies show that intestinal tract colonisation has an important role in terms of clinical implications (Squire *et al*., 2002; Acton *et al*., 2009). It is a commensal and pathogen for animals and humans and responsible for major health problems ranging from skin diseases to soft tissue infection and septicemia (Acton *et al*., 2009). The most common skin infection includes metastatic abscesses, wound infection, cellulitis and folliculitis. Pneumonia, septic arthritis, meningitis, endocarditis, osteomyelitis and shock syndrome represent more serious types of staphylococcal infection in humans (Sears & McCarthy, 2003). Additionally, in veterinary medicine, *S. aureus* causes a wide range of infections, which affect animal health and production and pose one of the most important sources of infection for humans (Peton & Le, 2013). Mastitis in dairy cattle and ewes is caused by many types of bacteria. However, *S. aureus* is the main etiological cause of this disease (Peton & Le, 2013).

During the decade of antibiotic treatments for bacterial infection in animals and humans, pathogenic bacteria have developed resistance to a wide range of these antibiotics (Levy, 1998). It is well documented that the cell envelope, the ribosome and nucleic acids are the main target for antibiotics in *S. aureus*. However, several studies have documented that more than 50% of *S. aureus* strains are now resistant to penicillin, methicillin, vancomycin and other antibiotics (Archer *et al*., 1994; Franklin, 2003; Hiramatsu *et al*., 1997).

Mutations that alter the drug binding sites on molecular targets and increase expression of endogenous efflux pumps are an example of the possible mechanism by which *S. aureus* resists antibiotics (Foster, 2017). The β-lactamase enzyme which is produced by *S. aureus* was originally responsible for resistance to penicillin and its derivatives; this enzyme hydrolyses the critical β-lactam ring and disrupts the drug's antibacterial activity. As an alternative to penicillin, for many years, methicillin was used to treat penicillin-resistant Gram-positive bacteria. Methicillin is a semi-synthetic β-lactam antibiotic which acts by inhibiting penicillin-binding proteins (PBPs) that are involved in the synthesis of peptidoglycan (Carretto, Visiello, & Nardini, 2018). Methicillin is no longer used in the UK after methicillin resistance was detected and isolation of Methicillin resistant *S. aureus* (MRSA) (Deurenberg & Stobberingh, 2008; Foster, 2017).

1.2 Pathogenesis of *S. aureus*

S. aureus is a commensal bacterium and pathogen in animals and humans (Wertheim *et al*., 2004). There are stages of host infection with staphylococcus, which can include colonisation, local infection, systemic dissemination, metastatic infection and toxicosis (Archer, 1998; Walter *et al*., 2004). Colonisation is the first step in staphylococcus infection and it provides shelter for bacteria until an opportunity occurs to breach the host's immune defences, thus facilitating access to the host's body and causing disease. Staphylococcus infection starts locally in the skin and mucus membrane and then bacteria access the blood flow and infects various organs (Archer, 1998). Skin traumas and surgical procedures are the main predisposing factor for *S. aureus* infection. However, *S. aureus* can infect intact skin and it can cause local cutaneous and subcutaneous infections, for instance folliculitis (inflammation of hair follicles), impetigo (inflammation of superficial skin) and skin abscesses (painful collections of pus) (Thomer *et al*., 2016).

Polymorphonuclear leukocytes (neutrophils), lysed host cells and fibrin capsules are the main components of *S. aureus* abscesses. Skin and soft tissue infection with *S. aureus* is usually accompanied by abscess formation (Kobayashi *et al*., 2015). *S. aureus* can disseminate from the skin to an internal organ. Hahn *et al*. (2009) used a mouse model to investigate the relationship between skin damage and systemic dissemination of *S. aureus* in mice. The authors found that *S. aureus* disseminates to the internal organs of rats (spleen & kidneys) after 6h of inoculation of flank skin with the organism. In the same vein, Schlecht *et al*. (2015) demonstrated a novel mechanism by which *S. aureus* disseminates to the internal organs of the human body and causes systemic infection. In order to invade host tissue, *S. aureus* adheres to the invasive hyphal elements of *Candida albicans*. *S. aureus* adhesine binds to the Als3p an adhesive cell wall of *Candida albicans.* The author concludes that *Candida albicans* enhances invasion of *S. aureus* through mucus membranes of the host which then leads to systemic infection in the host. Other routes to infection may be by production of toxins, which causes toxic shock syndrome. The ability of *S. aureus* to invade the host's body and resist its defences comes from the virulence factors. The virulence factors have various structures and different modes of action. They include staphylokinase, haemolysin, leukocidins, exfoliative toxin, toxic shock syndrome toxin and enterotoxins (Table 1). Other factors, which are involved in the establishment of infection are called the adhesive matrix molecules, which have an important role in adherence to host tissue. Fibronectin binding protein (FnBPs), collagen-binding proteins and fibrinogen-binding proteins (Clfs) are important examples of these molecules (Table 1) (Archer, 1998). In summary, three types of *S. aureus* infections include local infection: impetigo, cellulitis and folliculitis; systemic infection when *S. aureus* reaches the blood stream and spreads to the internal organ and specific types of infection when *S. aureus* locally colonises with the production of toxins (Hahn *et al*., 2009; Kobayashi *et al*., 2015).

Table 1.1 Virulence factors and adhesive matrix molecule involved in the pathogenesis of *S. aureus* (Foster *et al*., 2014).

Factor	Type	Function
Staphylokinase	Exoenzyme	Activates plasminogen; inactivates antimicrobial peptides
Haemolysin	Cytolytic toxins	α-hemolysin Induces lysis on a wide spectrum of cells, mainly platelets and monocytes β-hemolysin Hydrolysis of sphingomyelin of the plasmatic membrane of monocytes, erythrocytes, neutrophils and lymphocytes; makes cells susceptible to other lytic agents γ-hemolysin induces lysis on erythrocytes and leukocytes
Leukocidins	Cytolytic toxins	Induces lysis on leukocytes
Exfoliative toxin	Exoenzyme	Inactivates neutrophil activity; activates T cells
Toxic shock syndrome toxin	Secretory factor (Superantigens)	Massive activation of T cells and antibody presenting cells
Enterotoxins	Secretory factor (Superantigens)	Massive activation of T cells and antibody presenting cells
Fibronectin binding protein	Adhesive matrix molecule (cell surface factor)	Attachment to fibronectin and plasma clot
Collagen-binding proteins	Adhesive matrix molecule (cell surface factor)	Adherence to collagenous tissues and cartilage
Fibrinogen-binding proteins	Adhesive matrix molecule (cell surface factor)	Mediate clumping and adherence to fibrinogen in the presence of fibronectin
Staphylococcal protein A (SpA)	extracellular protein, anchored to the cell wall	Protein A interacts with IgG by binding to its Fc domain. This binding causes the surface of S. aureus to become coated with IgG molecules that cannot be recognized by the Fc receptor on neutrophils because they are in the wrong orientation
Staphylococcal superantigen-like 7 (SSL7)	member of the family of SSLs	evades the acute immune response by interacting with immunoglobulins and complement system.
Chemotaxis Inhibitory Protein of Staphylococcus aureus (CHIPS)	exoprotein	potent inhibitor of neutrophil and monocyte chemotaxis toward C5a and formylated peptides like fMLP

1.3 Colonisation of *S. aureus* in the human gastrointestinal tract

The human nose is the most common site for colonisation of *S. aureus*. However, there are many sites of colonisation including the skin, throat and gastrointestinal tract. Furthermore, 40% of people with nasal colonisation are also colonised in these sites. Several studies explain the relationship between intestinal colonisation and occurrence of infection. Patients with both nasal and intestinal colonisation are more susceptible to *S. aureus* infection at a rate of 40% as compared with an infection rate of 18% in people with nasal colonisation only (Eveillard *et al*., 2006).

The first definition of intestinal *S. aureus* carriage as one cause of antibiotic-associated diarrhoea was between 1950 and 1960. However, in 1970s, it was well established and defined that *Clostridium difficile* was a main aetiological cause of hospital acquired AAD(antibiotic associated diarrhoea) (Larson *et al*., 1978; Williams, 1963). The effect of gastrointestinal colonisation as a risk factor for *S. aureus* infection was neglected for many years then it was refocused on after the appearance and spread of methicillin resistant *S. aureus* (MRSA) and antibiotic associated diarrhoea especially among hospitalised patients and infants (Acton *et al*., 2008). In the 1990s, epidemiological increases of community-acquired (CA) MRSA were recorded among individuals in the absence of a health care-associated (HA) infection risk factors (Acton *et al*., 2008).

The relationship between the age of individuals and *S. aureus* intestinal carriage status has not been fully defined. However, several studies show that during the early stages of life (childhood) individuals are more susceptible to intestinal carriage of *S. aureus* (Acton *et al*., 2009).

Such carriage can have undesirable health effects; it is associated with food poisoning as a result of production of different enterotoxins and is associated with allergic disease in

children through CD14-related immunoregulation and inflammatory bowel disease (Lu *et al*., 2003; Dinges *et al*., 2000).

Surveys such as that conducted by Lindberg *et al*. (2000) have shown that over 75% of examined stools give positive results for the presence of *S. aureus*. This result is supported by Bjorksten *et al*. (2001) whose experiments showed that 65 % of infants have these bacteria in their stools. It is well documented that intestinal *S. aureus* carriage decreases as the individual's age increases. This might relate to microbiota protective action which is more efficient in adulthood. In healthy individuals, microbiota prevent colonisation of ingested bacteria by covering the intestinal epithelium and this mechanism is called colonisation resistance. It can be concluded that intestinal *S. aureus* carriage, increases among hospitalised patients and infants because of less development of their microbiota or disruptions in its efficiency (Björkstén *et al*., 2001; Lindberg, Nowrouzian *et al*., 2000).

Recent studies explain that colonisation of *S. aureus* in the gut may have important clinical implications such as for food poisoning, pseudomembranous intestinal infection, intestinal diseases, AAD and endocarditis (Acton *et al*., 2009; Vesterlund *et al*., 2006). Furthermore, intestinal carriage is a risk factor for dissemination of infection. Infected people with intestinal colonisation of *S.aureus* are considered an important source of infection to other people and the adjacent environment (Acton *et al*., 2008). Moreover, *S. aureus* infection of the skin and soft tissues is closely related to rectal colonisation (Squier *et al*, 2002; Misawa *et al*., 2015).

The specific mechanisms for intestinal colonisation by *S. aureus* are not completely understood. Gries *et al.* (2005) showed that the cecal mucus layer enhances intestinal colonisation by Staphylococcus. It is well established that *S. aureus* adheres to nasal mucin (Shuter *et al*., 1996). Gries *et al*.'s (2005) hypothesis is that *S. aureus* adhere to intestinal

mucus because intestinal mucin is the main competent of mucus and there is a similarity between intestinal and nasal mucin.

The first step to colonisation is adhesion. Adhesion of *S. aureus* to the human intestinal mucus and impact of a specific strain of microbiota were studied by Vesterlund *et al*. (2006). The authors found that *S. aureus* can adhere to the human intestinal mucus. However, certain lactic acid bacteria (commercial probiotics) impact on the adherence ability of *S. aureus*. Viability adherence of *S. aureus* were reduced to 39–44 % by *Propionibacterium freudenreichii* subsp, *Lactobacillus* rhamnosus GG and *Lactococcus lactis* subsp. lactis. The authors concluded that *S. aureus* has the ability to adhere to intestinal mucus *in vitro* and adherent *S. aureus* can be displaced and killed by certain *Lactococcus* species.

Misawa *et al*. (2015) found that teichoic acids(TA) which are phosphate rich sugar-based polymers attached to the Staphylococcal cell wall and have a critical involvement in the early steps to colonisation. The study was based on comparison between wild type *S. aureus* with the mutant strain lacking (wall teichoic acids) WTA. The mutant showed increased susceptibility to gastrointestinal detergent factor and poor adherence to the gastrointestinal tract as compared with the wild type. The study explains that WTA contributed to staphylococcal fitness within the GIT, providing resistance to host bactericidal factors and promoting bacterial adherence to epithelial cells.

Sortase A and clumping factors are surface proteins in the *S. aureus* wall which are defined in relation with intestinal colonisation. Sortase A is a bacterial transpeptidase which attaches protein to the *S. aureus* cell wall, while the clumping factor is the virulence factor in the bacteria that bind fibrinogen. Inactivation of sortase A and clumping factor in *S. aureus* leads to impaired ability of the bacteria to colonise in the mouse GIT (Misawa *et al*., 2015; Schaffer *et al*., 2006).

1.4 *S. aureus* infection in livestock animals and public health threat

Staphylococcus aureus is a worldwide pathogen responsible for a wide range of infections in animals and it causes severe economic losses in agriculture. In the last 50 years, studies have demonstrated that *S. aureus* has the ability to be transmitted from animals to humans and vice versa (Harrison *et al*., 2013). Studies also show that human activities such as domestication and industrialisation play important roles in the adaptation of this pathogen to infect different types of host (Fitzgerald, 2012). Recent studies show that 80% of *S. aureus* are resistant to penicillin. The increase in resistance to antibiotics and morbidity related to *S. aureus* led to investigation of the origin of the organism rather than its virulence (DeLeo *et al*., 2009).

S. aureus infects a wide range of livestock animals including cattle, sheep, horses, goats and poultry. It is one of the most important causes of mastitis in ruminants. The disease can cause severe economic losses in dairy cattle as a result of poor yield in the infected gland, treatment and milk discard. Mastitis can also be a public health problem as milk from an infected udder could contain staphylococcal enterotoxins and cause food poisoning in people who consume the milk or its products (Le Loir *et al*., 2003).

S. aureus causes septicaemia in sheep and tick pyemia in lambs. In goats, it causes morels disease, which is characterised by abscess formation in different parts of the body. *S. aureus* can also cause lymphadenitis, a disease that affects sheep and goats, characterised by enlargement and abscess formation in lymph nodes (Foster, 2012). Synovitis with lameness are the common clinical signs of *S. aureus* infection in poultry (McMamee & Smyth, 2000). In horses, the common feature of staphylococcal infection is abscess formation in wounds and surgical sites (Weese *et al*., 2004).

Intensive breeding and close contact of humans with animals give the bacteria the opportunity for transmission between the species. Juhasz and colleagues (2007) reported

transmission of *S. aureus* between Hungarian cows infected with subclinical mastitis and an agriculture worker. Molecular studies show that *S. aureus* strains have the ability to adapt to new hosts. Lack of specific host tropism results in the easy transmission of *S. aureus* from animals to humans and vice versa. This contributes to the consider of *S. aureus* infection as zoonosis and humanosis (Peton *et al*., 2013; Morgan, 2008).

1.5 The intestinal microbiota

1.5.1 Structure of intestinal microbiota

Studies of the gastrointestinal flora have been carried out for over 150 years. The development of microbiological techniques, particularly anaerobic culture, has allowed scientists to evaluate the number and species diversity of intestinal flora. Human foetuses grow in a sterile uterus and after birth, they are exposed to numerous microbes, some of which colonise the gastrointestinal tract. The density and type of flora differ at different sites in the intestine and that depends on the components of the intestinal part, for example, bacteria tend to colonise in high density in the parts of the intestinal epithelium which are extensively covered with mucus as compared with other parts of the epithelium (Moore and Holdeman, 1974; Macdonald & Gordon , 2005).

The colon is the main site of microbial colonisation; it has 1.5 kg of flora, which is estimated to be 70% of all microbes in the human body (Karlsson *et al*., 2002). The quantity and composition of the intestinal flora depends on many factors, including age, diet, environment, host genotype and disease (Simrén *et al*., 2012).

Several studies show that the human small intestine contains *Streptococcus* as a main bacterial population with variable numbers of *Clostridia* and *Veillonella* species, while the large intestine is predominantly colonised by *Bacteroides, Ruminococcus, Faecalibacterum*,

and *Eubacteria*. The mucus layer and epithelial crypts are colonised mainly by *Clostridium, Lactobacilli* and *Enterococcus* species (Zoetendal *et al*., 2012; Swidsinski *et al*., 2005).

The main changes to the composition of microbiota occur during childhood. After birth, microbes such as *Escherichia coli* and Enterococci colonise the human intestine. This microbiota is unstable, becoming more stable during breast or formula feeding. The next change in microbiota occurs during the transition to solid food. At 2 years old, a child has a microbiota similar to an adult (Zoetendal *et al*., 2001).

Bischoff *et al*. (2014) demonstrated a close relationship between microbiota composition and integrity of the intestinal mucosal barrier. Imbalance of microbiota (dysbiosis) can have an effect on intestinal epithelial function and might lead to increases in intestinal permeability and development of inflammatory disease. Dysbiosis is strongly linked to a number of body disorders which include obesity, inflammatory bowel diseases (IBD), type 2 diabetes (T2D), colorectal cancer and cardiovascular diseases. Due to the modifiable factor in the etiology of these conditions, microbiota composition represents a good indicator of individual health (Bischoff *et al*., 2014; Kang & Martin, 2017; Ley, *et al*., 2006)

Several recent studies prove that *S. aureus* is often one of the members of gut flora. In a study, Lindberg and colleagues (2000) identified *S. aureus* and superantigenic toxins (SEA, SED and TSST) in stool samples from 49 Swedish infants. They found 47 of 49 samples collected during the first year of life had *S. aureus* producing TSST-1 and enterotoxin without any gastrointestinal problems. *S. aureus* is a versatile pathogen and its emergence as a member of the normal flora in infants may be of clinical importance (Lindberg *et al*., 2000; Reddy *et al*., 2017).

1.5.2 Function of intestinal microbiota

Intestinal microbiota performs an important function inside the host; the best classification of these functions includes protective, metabolic, trophic and immunological functions (Purchiaroni *et al*., 2013).

Several studies have highlighted how the development of some of the structures of the intestine depends on luminal microbiota. These studies have found that villus capillaries in germ-free mice were less developed during the milk feed period and remained poorly developed in adults. In addition, these studies show that germ-free mice are highly susceptible to infection and have poor development of gut–associated lymphoid tissue and reduced antibody production, cytokine production, digestive enzyme activity and serum immunoglobulin levels (O'Hara & Shanahan, 2006). Interestingly, re-colonisation of germ-free animals with microbiota enhances the restoration of gut function and redevelopment of its structure. Xu & Gordon (2003) demonstrate that inoculation of free germ mice with *Bacteroides thetaiotaomicron* induced various genes in the host, which might be involved in the restoration of essential function of the gut in terms of nutrient uptake, metabolism, immunity, angiogenesis, mucosal barrier function and development of the enteric nervous system function. A recent study by Hayes *et al*. (2018) also showed that microbiota induces colonic barrier structure and function. The authors colonised germ free mice with fecal human microbiota and then investigated the colonic barrier structure and permeability through immunohistochemistry, molecular and electron microscopy techniques and, for permeability, colon tissue by using chambers, and by serum LPS and MDP detection. After 1-3 weeks of colonisation, the authors recognised increases in paracellular permeability which is important for the passage of molecules between epithelial cells. Increases in paracellular permeability were within normal levels and not representative of pathological disorder. A thin and patchy mucus layer was the main colonic barrier structure observed

after one week of colonisation and these changes allowed direct interaction between microbe and epithelial cells. Overall, adaptation of the intestinal structure and function to the presence of microbiota is crucial for maintaining intestinal haemostasis (Hayes *et al*., 2018; Xu & Gordon, 2003).

The microbiota protects the intestine from the effect of pathogens and the mechanism of this protection can be divided in to non-immune and immune-mediated pathways. In the non-immune mechanism, microbiota develop resistance to pathogens without the involvement of the host immune system. Microbiota compete with enteric pathogens in the occupation of attachment sites and consumption of nutrients. Microbiota can also produce antimicrobial substances or stimulate the host body to produce these factors (Ubeda *et al*., 2017).

In attachment competition, intestinal microbiota prevents invading pathogens from attaching to intestinal luminal cells. Species of *Lactobacillus, Bifidobacteria* and *E. coli* Nissle 1917 were well documented in terms of their role in the prevention of adhesion of enteric pathogens to the enterocyte. Interestingly, microbiota has the ability to regulate mucin production in the intestine. Mucin prohibits infection by adhering to pathogens. It has commonly been assumed that mucin inhibits the adherence of enteropathogenic *E. coli* and other pathogens to the enterocytes (Linden *et al*., 2008; Linden *et al*., 2009; Mack *et al*., 1999). Before colonisation in the gastrointestinal tract, the pathogenic bacteria have to compete for the same nutrient substance with microbiota which are adapted to the intestinal environment and very efficient at getting energy from the diet. Nutrient competition is one of the most highly efficient mechanisms by which microbiota protect the intestine from the harmful effects of pathogens (Ubeda *et al*., 2017). Maltby *et al*. (2013) demonstrate that two commensal *E.coli* strains (HS and Nissle, 1917) prevent infection produced by *E. coli* O157:H7 by preventing this pathogen from utilising sugar, which is important to grow in the gut and then affects its ability to colonise the gut. Intestinal microbiota produces

various materials which have direct actions on pathogens or stimulate expression of host antimicrobial peptides **(Figure 1.1)**, such as short chain fatty acid (SCFAs) and reactive oxygen species (ROS). These products inhibit the growth of bacteria by creating acidic pH, and also induce production of host antimicrobial peptide (Kida *et al*., 2006; Termen *et al*., 2006). Interestingly, secreted small molecules produced by certain species of microbiota have a direct effect (bacteriostatic or bactericidal) on the pathogen without involvement of the host, for instant bacteriocins, which are produced by Gram positive and microcins by Gram negative bacteria (Kommineni *et al*., 2015; Sassone-Corsi *et al*., 2016). Zipperer *et al*. (2016) demonstrated that *Staphylococcus lugdunensis*, which is a human nasal commensal produced peptide antibiotic which decreased the colonisation efficiency of *S. aureus* to the human skin or nose. In the same vein, Sassone-Corsi *et al*. (2016) found that *E.coli* strain Nissle 1917 produces microcins which reduce intestinal colonisation by *Salmonella enterica* serovar *Typhimurium*. On the other hand, certain species of microbiota have the ability to metabolise a host derivative molecule or bacterial synthesised molecule and this results in secondary metabolites which play an important role against the enteric pathogen. Bile acid is a good example of this mechanism. Bile acid is produced by the liver as primary bile acids and secreted in the intestinal tract. Some species of microbiota modify primary bile acid to secondary bile acid **(Figure 1.1)** (Buffie *et al*., 2015). Buffie *et al*. (2015) found that conversion of primary bile acid to secondary by microbiota can lead to inhibited growth of *Clostridium difficile*. This result is supported by Theriot and his colleagues (2014) who found that disruption of microbiota by using broad spectrum antibiotic leads to reductions in the level of secondary bile acid and increases the growth of *C. difficile* vegetative forms (Theriot *et al*., 2014).

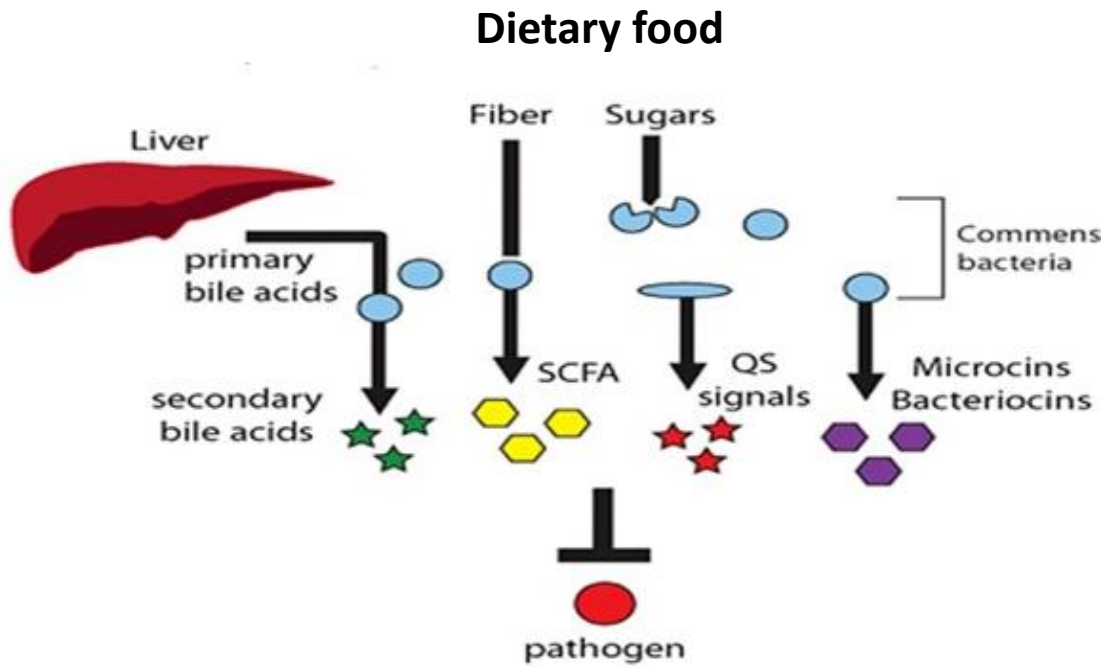


Figure 1. 1 Nonimmune protection mechanism of gut microbiota.

Gut microbiota have various mechanism to protect the human gut from the pathogenic bacteria. Microcins and bacteriocins are antibacterial peptides produce by microbiota and they have direct effect on invading pathogen. Also, microbiota stimulate host to produce antimicrobial peptide which act to inhibit growth of pathogenic bacteria. Moreover, microbiota convert primary bile acid to secondary which has also inhibit growth of pathogen. Modified from (Ubeda *et al*., 2017).

In immune-based protection, microbiota either increase the efficiency of innate immunity against the enteric pathogen or induction of the main component (B & T cells) of the immune system. Microbiota plays a role in protection of the intestine by inducing the expression of antimicrobial peptides, which act on the clearance of pathogens in the gut, for instance *B. thetaiotaomicron* promotes expression of H1F-1α, a transcriptional regulator which induces the expression of the antimicrobial peptide LL-37, which has anti-Candida activity (Fan *et al*., 2015).

The role of microbiota in specific immunity against invading pathogens in the host is not fully understood, However, microbiota induce B & T cells which play a crucial role in the defence against pathogens in the GI tract. A certain species of microbiota such as *Bacteroides fragilis* have the ability to differentiate T cells into T helper cells Th1s, Th2s, Th17s and T regulatory cells (Tregs). A lower number were found and a higher ratio of Th2/Th1 cells in the germ-free mice (GFM). Reconstitution of the GFM with *Bacteroides fragilis* restored the number of splenic CD4+cells and corrected the Th2/Th1 imbalance (Macpherson *et al*., 2000; Hand *et al*., 2012, Mazmanian *et al*., 2005).

Finally, the intestinal microbiota plays a role in harvesting energy from indigestible foods. It has the ability to produce SCFAs by fermentation of dietary fibre (complex carbohydrates) (Turnbaugh *et al*., 2006; E. Jimenez *et al*., 2008) Additionally, intestinal microbiota is also involved in the synthesis of vitamins. *Bfidobacteria* are responsible for the production of vitamin B and some digestive enzymes like lysozyme and casein phosphatase. Furthermore, intestinal flora contributes to the metabolism of drugs, toxic substances and dietary carcinogens (Debruyne *et al*., 2001; Gibson & Roberfroid, 1994).

1.6 *In vitro* human intestinal models

The human intestine is an important part of the GIT for food digestion, absorption, water and ion transportation and maintaining homeostasis with bacteria and immune cells. About ~10^{14} bacterial cells of 400-1,000 species colonise in the human gastrointestinal tract **(Figure 1.2)** (Becker *et al*., 2011; Eckburg *et al*., 2005).

The human colon microbiota represents 70% of all the microbes in the human body **(Figure 1.2)**. It is a highly diverse microbial community which contributes to the host's health through involvement in the maintenance of intestinal homeostasis and through recovery of metabolic energy from non-digested fibre and other components. The colon environment contributes to preventing pathogenic enterobacteria from surviving and establishing infection in the human gut. Short chain fatty acids are one of the important fermented products of colonic microbiota that are involved in the inhibition of invading pathogenic enteric bacteria (Hooper, Littman, & Macpherson, 2012; Pham & Mohajeri, 2018). Another critical component of the colonic environment is bile. Bile is considered to be a potent antibacterial because of its detergent action and membrane damaging properties. It is well known that bile protects the gut from invading enteric bacteria. However, certain species of microbiota have various inherent mechanisms for resisting the detergent effect of bile (Begley, Gahan, & Hill, 2005).

The ability of *S. aureus* to colonise and survive in the human gut has been documented by several studies across the world (Acton *et al*., 2009; Misawa *et al*., 2015; Schaffer *et al*., 2006; Vesterlund *et al*., 2006). For such a study, it is suitable to study the ability of *S. aureus* to resist bile in the presence of gut microbiota using a human gut model. Up to now, no suitable animal models have been found to test the carriage and survival of *S. aureus*, which have the same environment as the human gut (Adlerberth *et al*., 2000; Misawa *et al*., 2015).

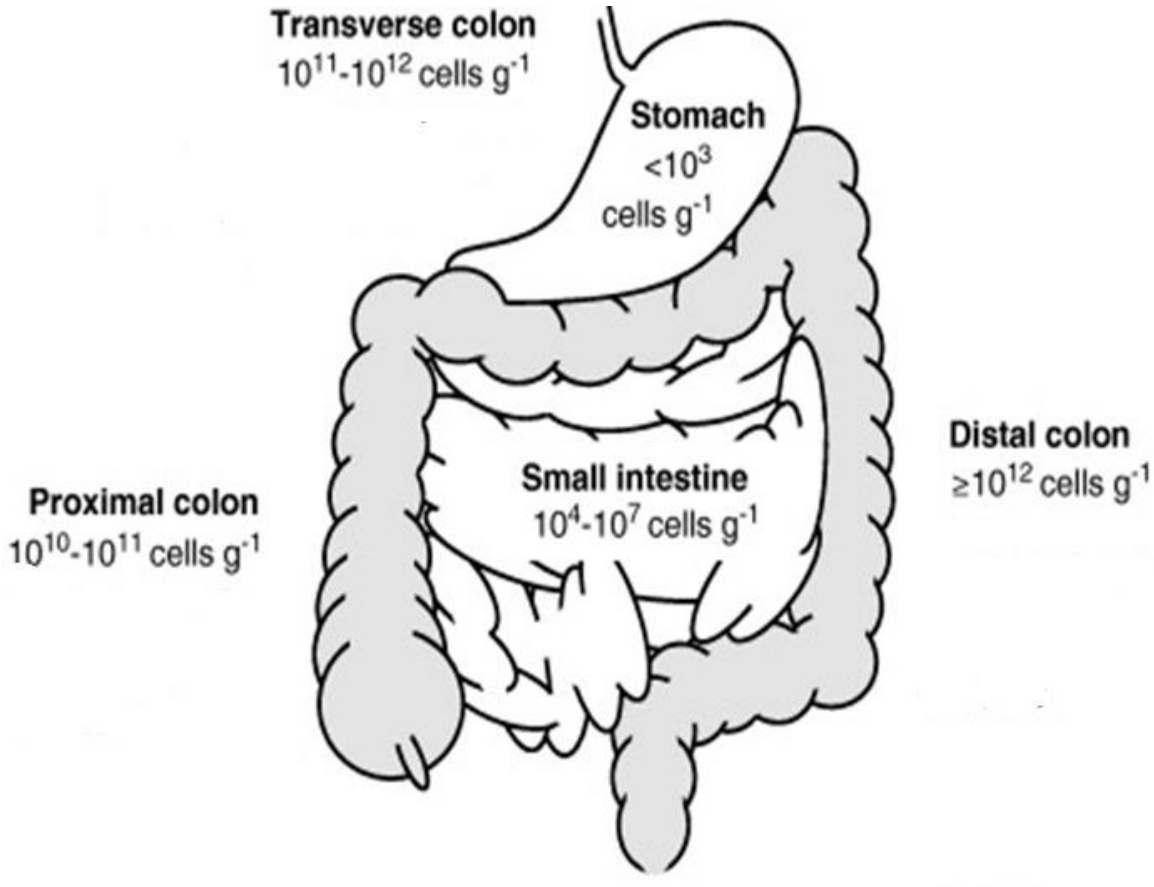


Figure 1. 2: Schematic representation of parts of the human gastrointestinal tract and bacterial population.

The majority of the human gut microbiota is in the colon so the colon is the best site in the human gastrointestinal tract to study diversity, population and function of the gut microbiota. Modified from Amanda *et al*. (2012).

Fecal sample analysis is a common method used to study microbiota composition, diversity and its impact on food digestion. A limitation of this method is that it does not provide good insights about dynamic microbial processes and functionality or digestion at their locations in the gut (Venema & Van Den Abbeele, 2013).

Alternatively, *in vitro* gut models have been extensively used for screening a large number of substances such as dietary ingredients, pathogens, drugs and toxic or radioactive compounds, to assess how they alter and are altered by gut environments and microbiota populations. An additional advantage of the model includes the fact that it provides results with high productivity, a dynamic sample over the time, there are no ethical issues for use of *in vitro* gut models and pathogens, radioactive compounds and toxic substance can be used without ethical approval (Guerra *et al*., 2012; Marzorati *et al*., 2009). It is well known that *in vitro* colonic models represent a powerful tool for studying the human gut microbiota (Hooper *et al*., 2012; Pham & Mohajeri, 2018).

In vitro gut models range from a simple batch fermentation system to sophisticated pH controlled three stage continuous culture systems, such as the three-stage continuous culture system, SHIME®, Entero Mix, Lacroix model and TIM-2. Choosing a model system depends completely on the goals of the study. It is important to consider that each system has advantages and disadvantages and these should be taken into consideration when selecting a suitable system for the experiment **(Table 1.2)** (Pham & Mohajeri, 2018).

Inoculation of a fecal sample, as a source of the gut microbiota, in nutritive media under conditions resembling the *in vivo* colon condition is the main principle of *in vitro* colonic human models. The condition of the model includes anaerobiosis, retention time, culture medium, temperature and pH. The fecal sample is collected from a healthy volunteer

Table 1. 2 Advantages and disadvantages of in vitro fermentation models (modified from Payne *et al* .,2012)

Models	Advantages	Disadvantages	References
Batch culture	Easy to set up, useful for fermentation studies and especially substrate digestion assessment	Short-term fermentation studies and weakness in microbiological control.	(Kovatcheva-Datchary *et al*., 2009; Pompei *et al*., 2008)
Continuous culture	Continuous flow mimicking conditions found *in vivo*. Environmental parameters are well controlled. No host functionality and experiments are time limited (days or weeks).	No host functionality and experiments are time limited (days or weeks).	(Duncan *et al*., 2009; Maccaferri *et al*., 2010)
Multistage continuous culture	Continuous flow into several vessels mimicking conditions found in portions of the digestive tract.	No host functionality and experiments are time limited (days or weeks).	(Maccaferri *et al*., 2010; Van Den Abbeele *et al*., 2010)
Immobilisation continuous culture	High-cell density and long-term stability of continuous fermentation system with immobilised fecal microbiota.	No host functionality.	(Le Blay *et al*., 2009; Payne *et al*., 2012)
Artificial digestive system	Continuous flow with metabolites and water exchange mimicking conditions found *in vivo*.	No immune and neuroendocrine response and experiments are limited to few days' time.	(Guerra *et al*., 2012; Kovatcheva-Datchary *et al*., 2009)

without a history to antibiotic at least a month before facal collection (Macfarlane *et al*., 2005; Payne *et al*., 2011) .

The batch fermentation model is simplest and most commonly used for studying the gut microbiome. The model consists of a closed or sealed bottle with a pH indicator probe and a pumped hot water supply adjusted to 37 °C and a source of nitrogen to provide anaerobic conditions in the model. This model is inexpensive, easy to operate, reproducible and fast. Furthermore, in this model, fermentability of various substrates can easily be determined within a short time (Allison *et al*., 1989; Macfarlane *et al*., 1994).

Furthermore, the batch fermentation model is a powerful tool for studying the effect of substrates on the biodiversity and physiology of intestinal microbiota and vice versa. The effect on microbiota is evaluated by molecular quantitative and qualitative techniques, while the impact on the metabolism is assessed by formation of metabolites such as short chain fatty acids (Aura & Maukonen, 2015).

Different responses in the individual to particular bioactive or agents or a comparison of the consequences of exposure to different sources or doses of compounds can be efficiently performed using this type of *in vitro* gut model. Additionally, it allows assessment of the type of microbial metabolites formed and helps to illustrate the pathways involved (Aura & Maukonen, 2015).

Inoculation density determines microbial growth in the model system; when a system is inoculated with low cell density, it results in a typical S-shape growth curve and the nutrient supply decreases over the time with the accumulation of toxic products which then lead to arrested growth. On the other hand, an inoculated system with a high density of cells like in the colon leads to limited growth (Gumienna, Lasik, & Czarnecki, 2011; Macfarlane & Macfarlane, 2007).

1.7 Bile salts

1.7.1 Bile salts and function

Bile is a yellowish green aqueous solution synthesised in the liver. It is secreted via the bile duct and stored in the gall bladder. Bile acids, cholesterol, phospholipids and pigments (phosphatidylcholine and biliverdin) are the main components of bile; other components which are added or excreted into bile include immunoglobulin A and mucus, endogenous substances (such as lipovitamin, water-soluble vitamins and estrogenic steroid) and exogenous substances (e.g antimicrobial agent and drugs) (Hofmann, 2001). Bile acids are synthesised from cholesterol and represent about 50% of the organic components of the bile (Monte *et al*., 2003). The concentration of bile acids in the gall bladder is 8% while in the intestine it ranges from 0.2-2% (w/v) (Cummings *et al*., 1987). There are two types of bile acids; the first type is called the primary bile acid and it is formed in the liver: cholic acid and chenodeoxycholic acid. Other types are formed in the large intestine by microflora, which act on modified bile acids by removing the hydroxyl group at carbon atom -7. The bile acids are conjugated in the liver as N-acyl amidates with either a glycine (glycoconjugated) or taurine (tauroconjugated). In humans, the conjugated free bile acid ratio of glycoconjugated to tauroconjugated is 3:1. The benefits of this conjugation are a reduction of pKa and an increase in the solubility of bile salts at physiological pH (Huijghebaert *et al*., 1986; Ridlon *et al*., 2006).

The main functions of bile are emulsification and absorption of dietary fat and lipid soluble vitamins (Bowen, 2001). Other functions include antibacterial action. Conjugated bile acid acts to stimulate the innate immune system against bacterial growth, by activation of the farnersoid-X receptor; this activation leads to increased expression of gene products, such as inducible nitric oxide synthase, angiogenin and IL

18 which forms the host immune component (Hofmann, 1999; Biet *et al*., 2002; Inagaki *et al*., 2006).

1.7.2 Antibacterial action of bile

It is well documented that bile salts have antimicrobial action. This action comes from physical and chemical characteristics and amphipathic sterol molecules of the bile salts. There are many mechanisms by which bile salt affects microbial cells. The main antimicrobial action of bile is to damage cell membranes. This action depends on several factor including concentration, the type and structure of the bile and membrane architecture and composition of the cell (Coleman *et al*., 1980; Heuman *et al*; 1996). Sannasiddappa, (2014) demonstrated that bile acids have a damaging effect on the cell membrane of *S.aureus* and this effect led to dissipation of intracellular pH and leakage of potassium.

Enzyme essay and electron microscopy have highlighted the effect of bile on mammalian cell membranes; cells show shrinkage, leakage of intracellur organelles and they might empty after exposure to bile. The electronic microscope has revealed large membrane defects when incubating rat hepatocytes as the model cell with 500 µM chenodeoxy and deoxycholic acid (Fujisawa *et al*., 1997).

Another effect includes the ability of bile to promote DNA damage and induce secondary structure production in RNA in bacterial and mammalian cells (Leverrier *et al*., 2003). Because of the detergent action of bile salts, they have the ability to affect protein by misfolding and denaturation. Bile salts also cause oxidative stress to cells by generation of oxygen free radicals. Access of bile to the cytoplasm may cause low Ph stress in cells, disturbance of ion movement and decreases of intracellular calcium and

iron (Sanyal *et al*., 1991; De Smet *et al*., 1995; Payne *et al*., 1998; Sannasiddappa, 2014).

1.8 Bile tolerance in enteric bacteria

Bile has potent antibacterial activity; however, a wide range of enteric bacteria have the ability to resist high concentrations of bile. Bile resistance is a complex phenomenon and its mechanisms are not fully understood at the molecular level. Furthermore, such resistance is affected by several factors including the bacterial strain, type of bile, growth media and experimental design (Chateau *et al*., 1994; Gunn, 2000). However, several studies have suggested that active efflux of bile salts, bile salt hydrolysis, changes in the architecture or composition of the cell membrane and cell wall, a general stress response, protection against oxidative damage and glycolytic reorganisation are the possible mechanisms for bile resistance in enteric bacteria (Begley *et al*., 2005; Ruiz *et al*., 2013; Sánchez *et al*., 2007)**.**

The bacterial cell envelope represents the physical barrier of cell which prevents bile salt uptake. However, bile salts can enter cells by diffusion or thought porins. Efflux pumps which are proteinaceous transporters found in almost enteric bacteria act to decrease concentration of bile through active efflux (Nikaido, Yamaguchi, & Nishino, 2008; Urdaneta & Casadesús, 2017). Gram-positive bacteria are more sensitive to bile as compared with Gram-negative bacteria (Dias *et al*., 2014). However, the specific mechanism by which this type of bacteria resists bile is not fully understood (Ruiz *et al*., 2014). In *S. aureus*, Sannasiddappa (2014) demonstrated that deletion of gene encoding MnhF, an efflux pump, leads to an attenuated ability of these bacteria to resist bile salts. Furthermore, *S. aureus ΔmnhF* survivability in the human colonic gut model

is significantly decreased as compared with the wild type. The author concludes that MnhF has a role in resistance to bile salts.

Listeria monocytogenes is a foodborne pathogen causing a wide range of diseases including meningitis, meningoencephalitis and gastroenteritis in human and domestic animals. These bacteria have the ability to adapt to the environment of the gastrointestinal tract. *Listeria monocytogenes* LO28 is able to resist high concentrations of bile *in vitro*; this bacterium resists bile salt inside the cell by active efflux, using the BilE efflux pump, an ABC type MDR efflux transporter. Efflux study shows a greater accumulation of radiolabelled chenodeoxycholate in the *L. monocytogenes bilE* mutant than the wild type. On the other hand, *L. monocytogenes* has a *bsh* gene which encodes a bile salt hydrolase enzyme that degrades bile acids. The enzyme activity was investigated through the oral infection of guinea pigs with bsh mutant Listeria. The results show a significant decrease in bacterial faecal carriage. It was concluded that BSH has a role in resisting the detergent effect of bile and enhancing the survival of these bacteria and their persistence in the gastrointestinal tract (Dussurget *et al*., 2002; Gahan & Hill, 2014; Sleator, Wemekamp-Kamphuiset *et al*., 2005).

Enterococcus faecalis is a Gram positive pathogen that can cause serious disease in humans including urinary tract infection, meningitis and endocarditis. It is a common inhabitant of the intestines of animals and humans and has the ability to adapt to hostile environments, for instance high salt concentrations and high temperatures. *Enterococcus faecalis* can resist high concentrations of bile which gives this bacterium the ability to survive in the gastrointestinal tract (Flores *et al*., 2003; Zhang *et al*., 2013). Zhang *et al*. (2001) identified the genes required for bile resistance in *Enterococcus faecalis* by using a transposon mutant library. The author found that the *gltK* gene

encodes the glutamate/aspartate transport system permease protein, which is involved in bile resistance.

Begley *et al*. (2005) reviewed the disrupted loci in *Enterococcus faecalis* related to bile resistance. The author classified these loci according to the function of their encoding protein to membrane composition (YvaG) or cell wall synthesis (SagA and *Streptomyces* CAC16441 homologue), DNA repair (MutS and SbcC), dGTP hydrolysis (Dgt), oxidative response (NifJ) and transcription regulation (*Bacillus halodurans*BAB04138 homolog). However, the molecular basis of bile resistance in *Enterococcus faecalis* is not fully understood.

Gram negative bacteria are inherently more resistant to bile than other bacteria to bile. Little data are available about the specific mechanism of resistance. However, *Salmonella, Escherichia, Vibrio* and *Campylobacter* are able to survive in bile at concentrations exceeding the *in vivo* physiological level. Several studies suggest that the envelope structure, purines and efflux pumps are part of the mechanism by which Enterobacteria, particularly *Escherichia coli*, *Salmonella enterica* and *Vibrio* resist bile salts (Picken & Beacham, 2009; Ramos-Morales, 2013; Nikaido *et al*., 2008; Prouty *et al*., 2002; Thanassi *et al*., 1997; Van Velkinburgh & Gunn, 1999). *Salmonella, E. coli* and *Campylobacter* are able to survive in the gall bladder. *Salmonella typhi* and *typhymurium* can resist 12% and 18% (w/v) of ox bile, respectively (Prouty *et al*., 2002; Thanassi *et al*., 1997).

The outer membrane of *Salmonella* is a strong barrier against the detergent effect of bile. It is well documented that lipopolysaccharide (LPS) has an important role in bile resistance and loss of the O-antigen results in decreases in the resistance (Lacroix *et al*., 1995; Prouty *et al*., 2002). Tol protein and efflux pump are other crucial tools for bile resistance. Tol protein is the outer membrane protein which prevents bile acid from

accumulating inside the cell; deletion of the *tol* gene encoding to this protein produces a bile-sensitive phenotype. Furthermore, S.Typhimurium has a multidrug efflux pump AcrAB which acts on pump bile salt outside the cell and decreases its toxicity. Manipulation of the gene encoding this protein leads to an accumulation of bile acid and increases the sensitivity of the bacterial cell to bile (Li & Nikaido, 2009; Prouty *et al*., 2002).

Gänzle *et al*. (1999) described how *E. coli* survives in the small intestine *in vitro* model in the presence of a high concentration of porcine bile. Thanassi *et al*. (1997) found a significant increase in the accumulation of chenodeoxycholate in the *acrAB* and *emrAB* mutant cell. The author concluded that the AcrAB transporter system is responsible for active export of bile from the *E. coli* cell and involved in bile resistance. Another important mechanism of bile resistance in *E. coli* is the physical barrier of the LPS. Several studies have found a strong relationship between changes in lipid structure and bile resistance. Phenotype screening of transposon TnphoA banks show that several bile sensitive mutants had changes in their LPS structure. These changes lead to the production of deep rough lipopolysaccharide mutants with a hyperpermeable outer membrane (Begley *et al*., 2005; Thanassi *et al*., 1997). Similarly to *E. coli*, several studies suggest that LPS plays an important role in bile resistance in *Vibrio* species. Klose *et al*. (2002) found that deletion of LPS core oligosaccharide biosynthesis genes in Vibrio cholerae increases their sensitivity to bile salts. In the same vein, Hsieh *et al*. (2003) and Hung *et al.* (2006) found that bile induces genes of an LPS structure and biofilm formation in *Vibrio parahaemolyticus* and *Vibrio cholerae*. The authors conclude that changes in the LPS structure and biofilm formation are defence mechanisms in vibrio species against the damaging effect of bile salts. On the other hand, Provenzano & Klose (2000) found that modulation of expression of OmpU and

OmpT in *Vibrio cholerae*, two outer membrane porins, have a role in bile resistance which is critical for the bacteria to bile.

The human gut has many species of microbiota. These bacteria are involved in food digestion and protect the intestines from pathogenic bacteria, induction of innate and specific immunity and several other functions. It is well documented that microbiota have an inherent ability to resist intestinal stresses such as bile toxicity, low pH and other stresses. Several genera of gut microbiota such as *Bacteroides, Lactobacillus, Clostridium, Bifidobacterium* and *Enterococcus* have bile salt hydrolase (BSH) enzymes. Several studies suggest that BSH has an important role in the survival of these bacteria in the human gut. Furthermore, this enzyme has an effect on the physiological activity of the host and microbiota (Begley *et al*., 2005; Bustos *et al*., 2018; Mertens *et al*., 2017).

De Smet *et al*. (1995) showed that *Lactobacillus plantarum* resists physiological bile concentrations because it has the ability to produce bile salt hydrolase (BSH).

Several studies have documented the hydrolysis of the amino acids of bile reduces their toxicity **(Figure 1.3)**. Deletion of *bsh* in *Actobacillus amylovorus* leads to a significant decrease in the growth rate of the mutant compare to the parent when it is grown in the presence of conjugated bile acids (Grill *et al*., 2000, Bustos *et al*., 2016; Chae, Valeriano *et al*., 2013; Jayashree *et al*., 2014).

Exopolysaccharide (EPS) represents a protective barrier against the harmful effect of bile in many types of enteric bacteria. Several studies found that EPS production in some species of probiotic bacteria induces a response to bile stress. However, the relationship between changes in EPS production and resistance to bile is not clear in Lactobacillus (Alp & Aslim, 2010; Koskenniemi *et al*., 2011; Ruiz *et al*., 2013).

Fedorová *et al*. (2019) found that *in vitro* challenging of *L. reuteri* L26 with gastric and intestinal contents leads to the highest production of EPS in this bacterium. Burns *et al*. (2010) show that production of the enzymes involved in EPS production is not affected in bile challenged in wild-type strain of *L. delbrueckii* while it is significantly overproduced in a bile-resistant strain. Active eflux pump is another mechnism by which Lactococcus strains resist the detergent effect of bile. Acording to recent studies, all transporters in Lactobacillus belong to the ATP-binding cassette.

Pfeiler & Klaenhammer (2009) described four transporters in *Lactobacillus acidophilus*: LBA0552, LBA1429, LBA1446 and LBA1679. Inactivation of these tranporters leads to increased sensitivity of *Lactobacillus acidophilus* to bile.

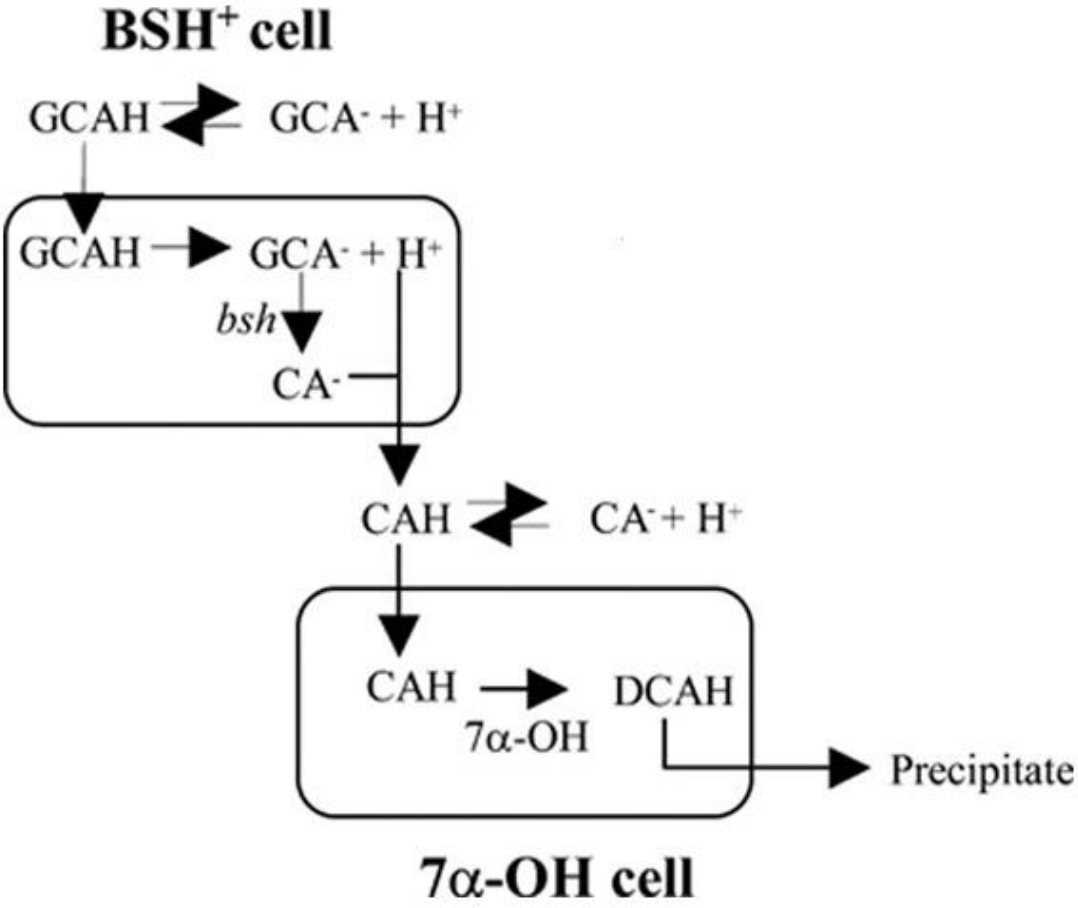


Figure 1.3 Schematic representation of detoxification mechanism of bile acid.
Bile salt hydrolase by BSH converts conjugated bile salts (GCA) that enter cells to their weaker unconjugated CA. 7α-dehydroxylating bacteria may dehydroxylate unconjugated bile acids and the resulting molecules precipitate at a moderately acidic pH. (Modified from Begley *et al*. (2005). Abbreviations: 7α-OH – 7α dehydroxylating, GCAH – glycocholic acid, CAH – cholic acid, DCAH – deoxycholic acid.

Bifidobacteria is another group of microbiota able to resist the detergent effect of bile. Ruiz *et al*. (2012) demonstrated that an up-regulation of genes encoding protein which is involved in cell envelope biosynthesis, membrane transport, protection against protein denaturation and DNA damage and oxidative stress response is a response to bile stress.

Ruiz *et al*. (2013) review and discuss bile the resistance mechanism in bifidobacteria and lactobacilli; the authors conclude that there are no bile sensing system or specific controlled transcriptional regulators in these bacteria.

1.9 Modification of bile acids by enteric bacteria

Modification of bile acid is one of the mechanisms by which enteric bacteria diminish the antibacterial activity of bile. The main ways of modification include hydroxylation (replacement of a hydrogen with a hydroxyl group) and dehydroxylation (replacement of a hydroxyl group with a hydrogen), oxidation (expulsion of H2), reduction (insertion ofH2), epimerisation (inversion of the stereochemistry of the hydroxyl groups at C-3, C-7 and C-12) and deconjugation (removal of the amino acid side chain) (Sistrunk *et al*., 2016; Begley *et al*., 2005).

Cholic acid and chenodeoxycholic acid are the primary bile acids in humans; these acids are modified by enteric bacteria to form secondary bile acids. Dehydroxylation converts CA and CDCA to DCA and LCA, respectively (Figure 1.4). A few species of intestinal bacteria can complete dehydroxylation; these include *Eubacterium* and *Clostridium* species. Dehydroxylation occurs in free bile salts for that it flow deconjugation (Urdaneta & Casadesús, 2017). Deconjugation is hydrolysis of the bond between an amino acid side chain and steroid nucleus. This reaction makes bile acid free (unconjugated) and is suitable for further modification by enteric bacteria **(Figure**

1.4). The main enzymes of gut microbes involved in the deconjugation and dehydroxylation reaction include bile salt hydrolase (BSH) and bile acid-inducible (BAI) (Long *et al*., 2017).

Another microbiological modification of bile acid is oxidation, which is strictly related to reduction. It is well documented that some types of bacteria (for instance Actinobacteria, Proteobacteria, Firmicutes and Bacteroidetes) have the ability to generate oxo- (or keto-) bile acids by oxidation of hydroxyl groups at ring position 3, 7, or 12 by using hydroxysteroid dehydrogenases (HSDHs) (Fukiya *et al*., 2009; Kisiela *et al*., 2012; Wahlström *et al*., 2016).

In reviewing bile acid biotransformation, Bortolini and colleagues (Bortolini *et al*.,1997), mention that the oxidoreductase activity of *Peptostreptococcus productus* began with the conversion of CA and CDCA. Recent studies highlight that mutation in a gene encoding a putative oxidoreductase results in significant changes in bile tolerance; this might be related to the ability of some kinds of enteric bacteria to modify bile salts through oxidation and reduction ((Bron, 2004; Le Breton *et al*., 2002; Pfeiler *et al*., 2007).

Epimerization is inversion of the stereochemistry of the hydroxyl groups. This reaction is associated with oxidation and characterised by the formation of a stable oxo-bile salt. *Clostridium, Peptostreptococcus, Bacteroides, Eubacterium,* and *coli* are enteric species with the ability to perform oxidation and epimerization of hydroxy groups at the positions C3, C7, and C12 of bile salts, generating isobile (β-hydroxy) salts.

The produced bile is recycled to the liver and re-joins the bile (Long et al., 2017; Ridlon, Kang, & Hylemon, 2006; Urdaneta & Casadesús, 2017).

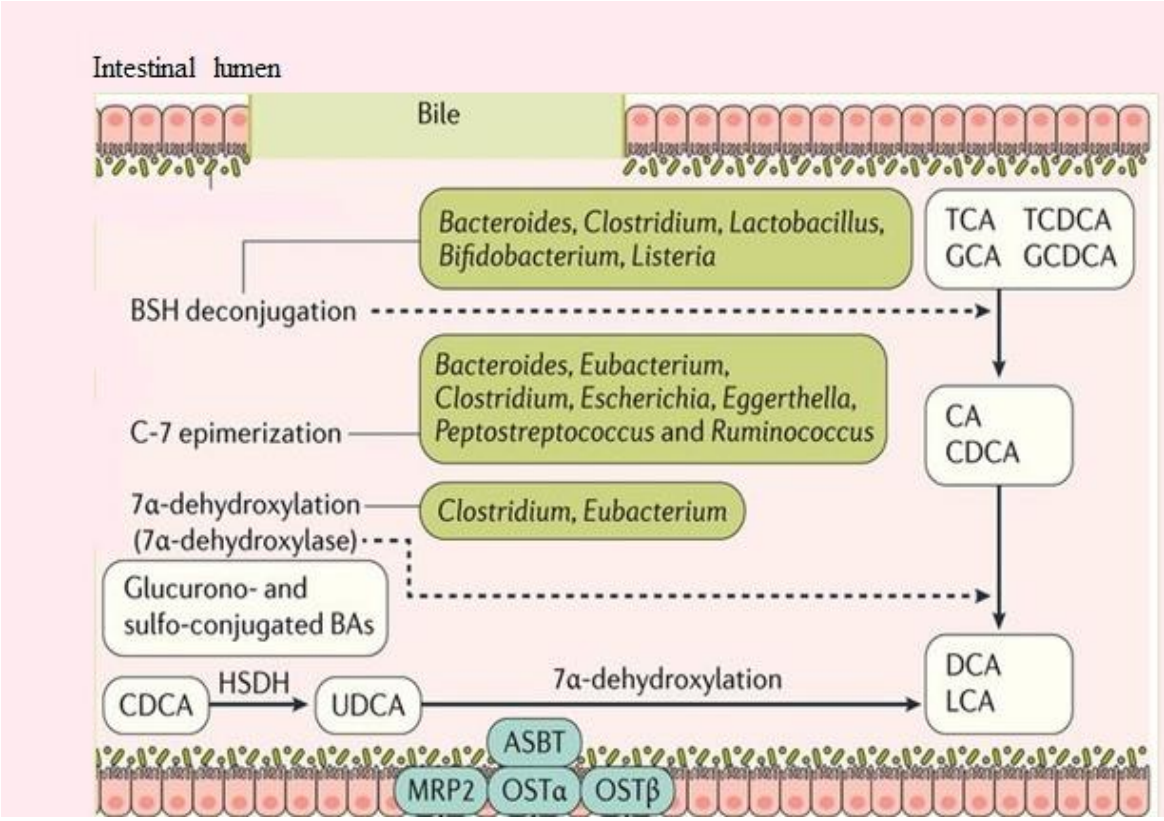


Figure 1. 4 Modification of bile salts by enteric bacteria.

The first stage is the deconjugation of bile salt to make it free (unconjugated) and suitable for further modification by enteric bacteria. Free primary bile acid (CA and CDCA) converts to secondary bile salt (DCA and LCA). (Modified from Jia *et al*., 2018)

Recent studies have highlighted that the modification of bile acid by enteric bacteria impacts the size and composition of the bile acid pool. Modification of bile acid by a certain type of enteric bacteria results in the inhibition of the nuclear receptor farnesoid X receptor (FXR). This receptor is responsible for the regulation of bile acid synthesis. Inhibition of FXR leads to a reduction in bile acid levels in the gut and then overgrowth of intestinal bacteria (Ridlon *et al*., 2014).

1.10 Metabolism of fatty acid in bacteria

In order to respond to changes in the environment, fatty acid metabolism is tightly regulated in many bacteria, especially *E. coli*. Fatty acid is one of the hydrophobic components of the membrane lipids (generally phospholipids) and one of the important sources of metabolic energy in almost all species of bacteria. For decades, *E. coli* was used as a model prokaryote for studying fatty acid metabolism because the genomic sequence of these bacteria was available and, furthermore, it is easy to grow and suitable for genetic manipulation. However, some genes and enzymes of lipid pathways in *E. coli* are not common in other bacteria. Recently, other bacteria were added to the list of model prokaryotes including *Bacillus subtilis* and *Streptococcus pneumoniae* (Parsons & Rock, 2013; Zhang, Marrakchi, & Rock, 2002; Lu & Rock, 2006).

The availability of fatty acid in bacterial cells is very important to maintain membrane lipid homeostasis. Fatty acid degradation and biosynthesis pathways switch on and off depending on the availability of these acids. There are two sources of fatty acid exogenous (in media) and endogenous (synthesised by the bacterial cell).

Bacterial cells incorporate synthesised fatty acid into phospholipid membranes while exogenously supplied fatty acid is used as a source of carbon. In *B.subtilis*, the biosynthesis and degradation are governed by two transcriptional global regulators

FadR (YsiA)and FabR (Matsuoka *et al*., 2007). There are two types of mechanism by which fatty acid degradation is regulated in *E. coli*, a nonspecific mechanism in which fatty acid is used as a substrate of low status by the bacterial cell and the *fad* genes are expressed as a response to strong regulation by the global cyclic AMP-dependent catabolite repression system; in the specific mechanism FadR represses the transcription of the *fadL, fadD, fadE, fadBA, fadH, fadI* and *fadJ* genes which plays an important role in fatty acid transportation, activation and β-oxidation (Campbell *et al*., 2003; Cherepanov & Wackernagel, 1995; Clark & Cronan, 2014; Iram & Cronan, 2006). Parsons *et al*. (2011) tested the ability of *S. aureus* to utilise fatty acid by adding a label to it and adding it to the *S. aureus* medium; the experiment results showed that 30% of fatty acids are incorporated into the cell membrane and the rest are recovered in the medium. The authors concluded that *S. aureus* does not degrade fatty acid and all exogenous sources are incorporated into the cellular component. In *E. coli*, exogenic fatty acid enters the bacterial outer membrane by diffusion and through a long chain fatty acid transport protein (FadL) and then passes to the inner membrane. In the inner membrane, fatty acids are converted to their CoA esters by a cytosolic acyl-CoA synthetase. The acyl-CoAs either degrade to acetyl-CoA by β-oxidation or are incorporated into membrane phospholipids (Campbell *et al*., 2003; Cronan, 2014; Garlid *et al*.,1996; Morgan-Kiss & Cronan, 2004). Parsons *et al*. (2011) concluded that there is no acyl CoA synthetase in *S. aureus* because the bacterium lacks an a β-oxidation pathway**(Figure1.5).** Furthermore, inactivation of the PlsX gene which is annotated as encoding an acyl co A synthetases does not affect the ability of *S. aureus* to take fatty acids (Parsons *et al*., 2014). However, Cronan (2014) mentions that fatty acids might directly convert to acyl-ACPs by an acyl-ACP synthetase and this possible mechanism was well defined in *Vibrio harveyi.*

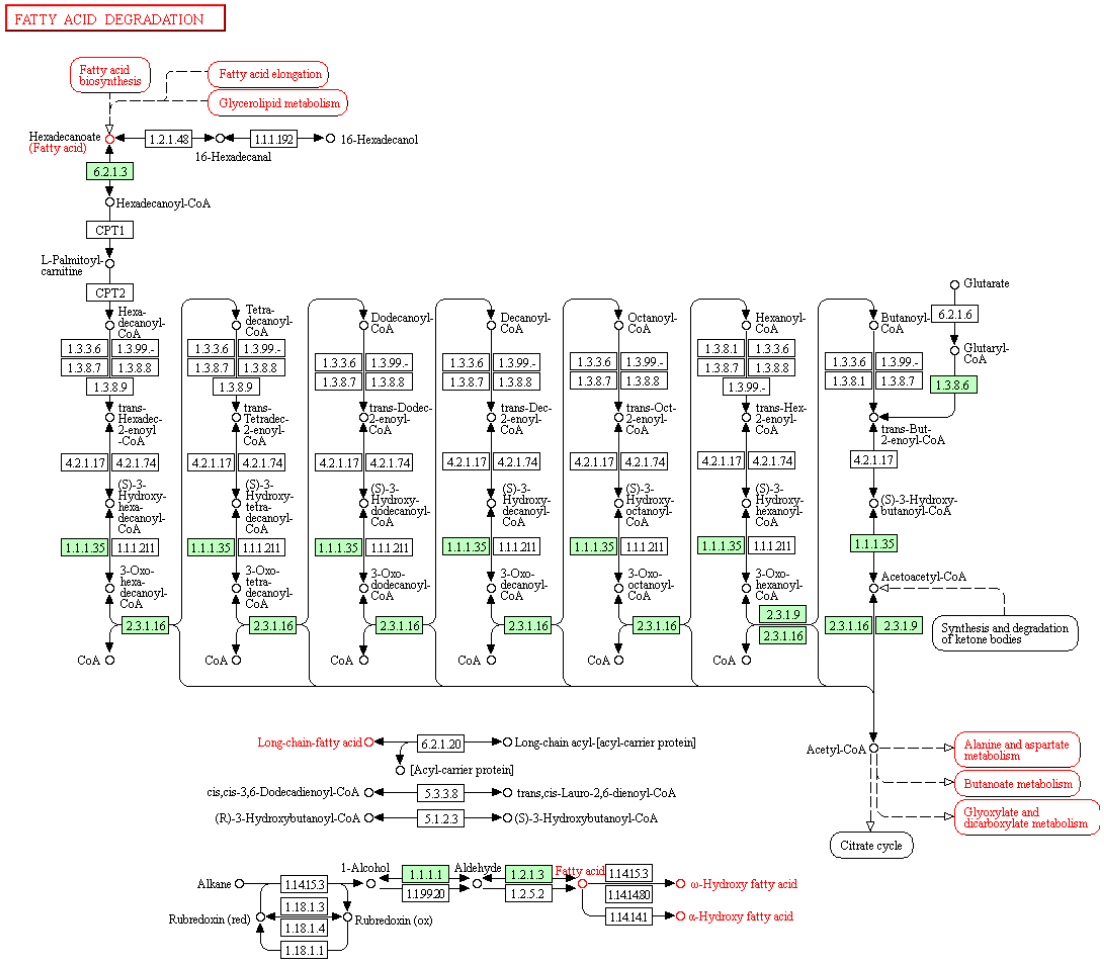


Figure (1.5) Fatty acid degradation – *S. aureus* subsp. aureus N315. KEEG (Parekh *et al.*,1970).

Moreover, Parsons *et al*. (2011) found that fatty acid in crude *S. aureus* extracts converted to acyl-ACP in a Mg^{2+} and ATP-dependent manner by an unknown protein. In *B. subtilis* there is a direct mechanism for degradation of exogenous fatty acid. However, genes involved in the β -oxidation of fatty acids, such as lcfA, lcfB (yhfL), f*adB* (*ysiB*), f*adN* (*yusL*), *fadA* (*yusK*) and *fadE* (*yusJ*) were found in this bacterium *(Kunst et al*., 1997; Matsuoka *et al*., 2007). The pathway of fatty acid metabolism in *E. coli* is summarised in **(Figure 1.6).**

A recent study at the transcriptional level in *S. aureus* shows upregulated expression of the *fadB* gene during fatty acid metabolism (Khairon *et al*., 2016). Similarly, Camargo *et al*. (2009) found that the *fadB* gene was upregulated as a part of the lipid metabolism response to Daptomycin , which is a lipopeptide antibiotic used in the treatment of systemic and life-threatening infections caused by Gram-positive organisms. This antibiotic disrupting multiple aspects of bacterial cell membrane function. It inserts into the cell membrane in a phosphatidylglycerol-dependent fashion, where it then aggregates. The aggregation of daptomycin alters the curvature of the membrane, which creates holes that leak ions.

1.11 Impact of membrane fatty acid composition on bile resistance in enteric bacteria

The cell membrane is the main target of the detergent action of bile. These actions include disturbing the lipid packaging and disrupting the proton motive force, causing cell death (Ruiz *et al*., 2013). In order to resist the stresses (including bile) in the human gastrointestinal tract, bacteria induce changes in the lipid and protein profile of their cell membrane. Changing the lipid profile mainly leads to alteration in the physical properties of a cell membrane (Couto *et al*., 1996).

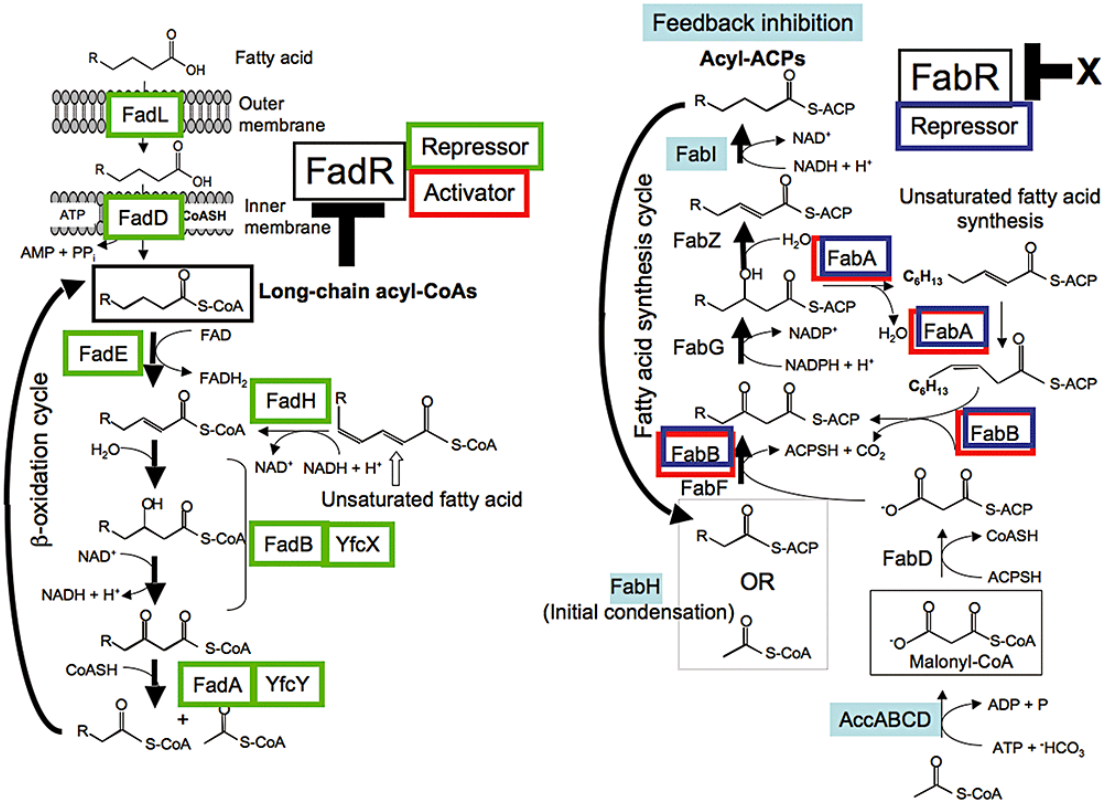


Figure 1. 6 Metabolism of fatty acid in *E. coli*.

Conversion of acyl-CoA to enoyl-CoA through acyl-CoA dehydrogenase (FadE) is the first step in the β-oxidation pathway. Then, enoyl-CoA hydratase/3-hydroxyacyl-CoA dehydrogenase (FadB) converts enoyl-CoA to 3-ketoacyl-CoA via 3-hydroxylacyl-CoA through hydration and oxidation. In the degradation pathway of unsaturated fatty acids, 2,4-dienoyl-CoA reductase (FadH) transforms 2,4-dienolyl-CoA derived from unsaturated fatty acids into enoyl-CoA. Meanwhile, the formation of malonyl-CoA from acetyl-CoA is catalysed by acetyl-CoA carboxylase (AccABCD) and this is the first step of the bacterial fatty acid synthesis pathway (FASII). FadR protein is responsible for the regulation of fatty acid metabolism in *E. coli* (Modified from Fujita, Matsuoka & Hirooka (2007)).

Kristofferson *et al*. (2001) found that the genes of *Bacillus cereus* involved in lipid metabolism are unregulated as a response to the destructive effect of bile. Similarly, Johnson *et al*. (2018) conclude that *fad* (fatty acid degradation) genes of *Salmonella enterica* are upregulated as a response to bile. Genes encoding proteins associated with lipid metabolism in multiple bacterial species (e.g. *Lactobacillus casei, Bacillus cereus* etc*)* are induced as a response to bile stress (**Figure 1.7**) (Bustos *et al*., 2018).

Bile promotes changes in the lipid metabolism through changes in the production of proteins involved in fatty acid metabolism (Ruiz *et al*., 2013; Sánchez *et al*., 2007a; Fernández *et al.*, 1999a). According to Taranto *et al*. (2003), bile causes severe changes in the lipid profile of *Lactobacillus reuteri*; these changes include decreases in phospholipids and a lower ratio of saturated: unsaturated fatty acids. The induced changes affect the physical properties of the cell membrane and might play a key role in the survival of these bacteria in the gastrointestinal tract.

Kimoto *et al*. (2009) showed that recovery of *E. coli* O157:H7 from low temperature treatment leads to increased sensitivity of these bacteria to bile salt, crystal violet, sodium chloride and ethanol.

According to the author, the reason for this is damage of the cell membrane which acts as a protective barrier in normal cells, during freezing treatment. It can thus be suggested that the cell membrane plays a critical role in resistance of bacteria to bile. It is well known that lipid contributes to the permeability and stability of bacterial membranes. Bacterial cells modify their fatty acid composition in order to maintain a constant membrane fluidity; this phenomenon called homeoviscous adaptation (Fernández *et al.*, 2001b).

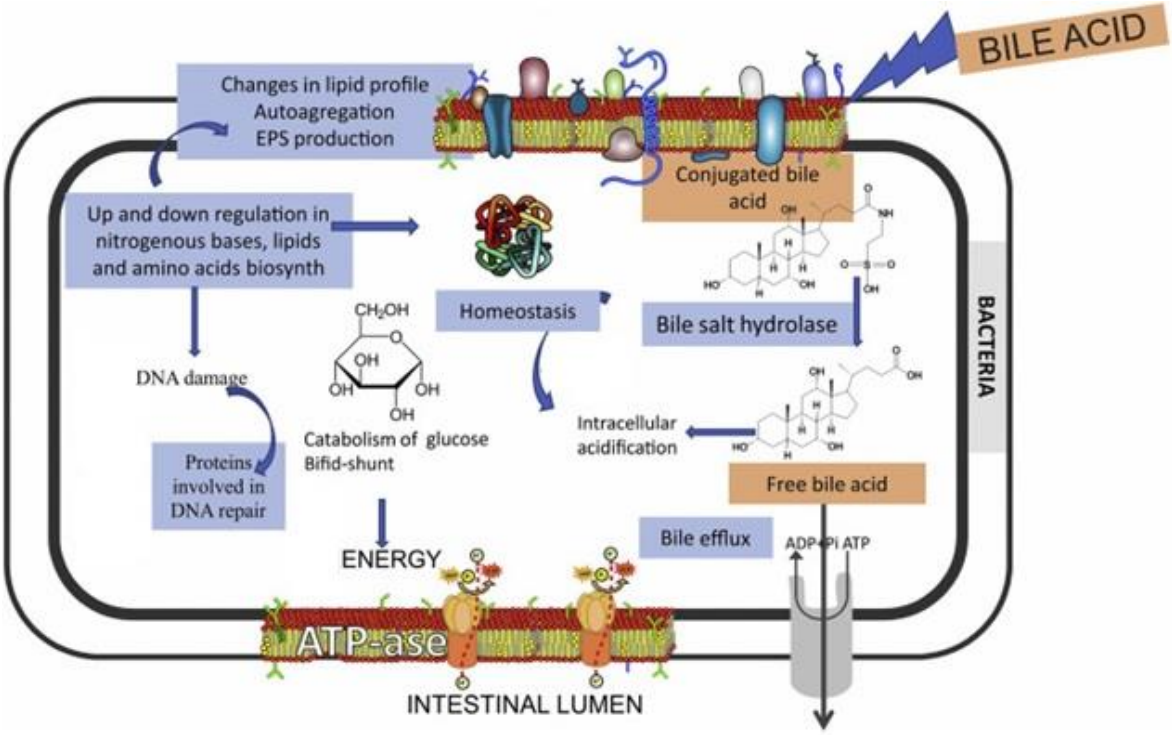


Figure 1. 7 Mechanisms of response of bacterial cells to bile stress.

Mechanisms of response of bacterial cells to the bile stress mediated by the presence of efflux pumps, the architecture and composition of the cell membrane, the action of the BSH enzyme and the intrinsic capacity of cells to maintain intracellular homeostasis. Also, bile acid regulated bacterial proteins including those involved in fatty acid, carbohydrate metabolism and amino acid and nitrogenous base biosynthesis, transporters and proteins related to general stress response are regulated by bile acids in bacteria (modified from Bustos *et al*. (2018)).

Tarantoet *et al*. (2003) suggest that changes in the lipid profile of *Lactobacillus reuteri* is considered mainly to be a physiological response of the cell for survival in the gastrointestinal tract. Several studies have highlighted that the level of bile resistance in multiple bacterial species (e.g., *E.coli*, *L. monocytogenes*, and *L. acidophilus*) is strongly related to the changes in fatty acid composition, lipid fluidity, lipopolysaccharide (LPS), hydrophobicity and membrane electric charge (Chou & Cheng, 2000; Fernández *et al*., 2001b; Urdaneta & Casadesús, 2017).

Kimoto *et al*. (2009) demonstrate that *Lactococcus lactisstrains cells* grown on a GM17 medium were more resistant to bile than cells grown on an LM17 medium. The authors conclude that bile-resistant lactococci had fatty acid compositions different from those of bile sensitive ones. In the same vein, the amount of C18:2 and C16:0 fatty acids of *Lactobacillus acidophilus* cells grown at 25°C was more than cells grown at 37°C. Because of the role of fatty acids in the stability of the lipid membrane, more fatty acids content cells are more resistant to bile than other cells (Kimoto *et al*., 1999).

Interestingly, the relationship between bile resistance and fatty acids might be useful for improving the bile resistance of some bile sensitive *Lactococcus* strains with antimicrobial activity and then these bacteria can reach the intestine and be involved in the imbalance of intestinal flora by permitting these sensitive bacteria to reach the intestine. An alternation of the culture condition for *Lactococcus* to increase the relative content of C18:1 and decrease that of Δ19:0 is one example of a mechanism for improved resistance of these bacteria to bile (Kimoto *et al*., 2009; Kimoto *et al*., 1999).

1.12 Aims of the study

The aim of the current study is to investigate the ability of *Staphylococcus aureus* to survive the innate defence to infection of the human gut and identify the main components of the bacterium which allow it to resist bile salts.

The aim of the study will be achieved by:

- Identifying genes involved in resistance by identifying natural protein diversity in response to bile stress
- Purification enzymatic analysis and phenotyping of the protein gene encoding by the gene identified
- Transcriptional analysis of the identified gene
- Testing the ability of any bile sensitive mutant to survive in *in vitro* models of the human gut.

Chapter Two : Material and Methods

2.1 Chemicals and reagents

The chemicals used were of analytical grade or higher and they were purchased from Bio-Rad, Sigma, Melford Laboratories, MP Biomedical or Fisher Scientific.

2.2 DNA modification and restriction endonuclease enzyme

All restriction endonucleases were purchased from Fermentas™. DNA polymerase and the quick ligase were both purchased from Bioline and Fermentas. Phusion® High Fidelity DNA polymerase was purchased from Thermo-Scientific and used according to the manufacturer's instructions. DNase I and RNA polymerase were purchased from Bioline.

2.3 DNA, RNA and protein markers

Fermentas GenerulerTM ladder (250-10,000 bp) was used for the accurate estimation of the size and quantity of DNA following gel electrophoresis. The gel was visualized using UV-induced fluorescence in the presence of 0.5ug/ml ethidium bromide (Fig 2.1). An RNA ladder (0.16-1.77kn) was purchased from Invitrogen for the in vitro transcription experiments. A PAGE ruler prestained protein ladder involving (10 to 180 kDa) from Fermentas was used to determine the size of the protein of interest and its quantity was determined using SDS page electrophoresis (Fig 2.2).

2.4 Microbiological growth media

The media was supplied by Sigma-Aldrich or Oxoid. The media was prepared according to the supplier's instructions. The media was sterilised by autoclave at 121ºC at 20 lbs/in2 for 20 min. The heat liable material was sterilised using 0.2 and 0.45 before being added to the media.

2.4 .1 Selective Agar

S.aureus selective agar medium was prepared by the addition of a sterile 0.01% potassium tellurite solution to a molten BHI agar at 55°C.

2.4.2 Phage buffer

The phage buffer was prepared by the addition of $MgSO_4$ (1 mM), $CaCl_2$ (4 mM), Tris-HCl (50 mM) and NaCl (100 mM) to a litre of distilled water. The pH was adjusted to 7.8 and the solution was sterilised by autoclaving.

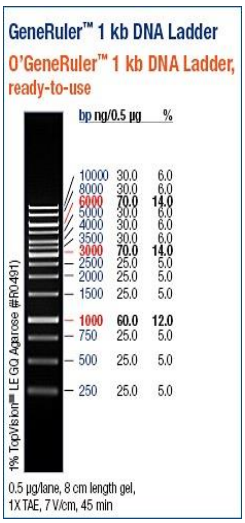


Figure 2. 1 Gene Ruler 1kb ladder from Fermentas.

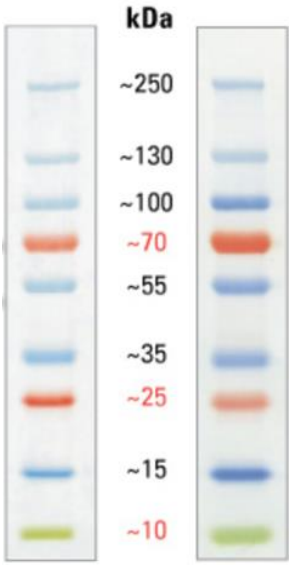


Figure 2. 2 Protein ladder. 10 to 180 kDa (Fermentas).

2.4.3 LB broth

LB was prepared by the addition of bacto-tryptone (5g), yeast extract (2.5g) and NaCl (5g) to a half litre of distilled water. The pH was adjusted to 7.5 and then autoclaved at 121ºC, 20 lbs/in^2 for 20 min.

2.4.4 LK broth

An LK broth consisting of tryptone (10 g), yeast extract (5 g) and potassium chloride (7 g) was added to a litre of distilled water and sterilised by autoclaving.

2.4.5 B2 medium

B2 was prepared by the addition of casein hydrolysate (1% w/v), yeast extract (2.5% w/v), K2HPO4 (0.1% w/v) and NaCl (2.5% w/v) in a litre of distilled water. The pH was adjusted to 7.5 and the medium was sterilised by autoclaving. Twenty-five ml of 20% (w/v) sterile glucose was added to medium before use.

2.4.6 SOC medium

The SOC medium was prepared by the addition of a yeast extract (0.5% w/v), tryptone (2% w/v), NaCl (10 mM), KCl (2.5 mM), $MgCl_2$ (10 mM), magnesium sulphate (10 mM), and glucose (20 mM). The medium was sterilised by autoclaving.

2.4.7 pH controlled faecal batch culture medium

The medium consisted of the following: (g/l) in distilled water: peptone water, 2.0 (Oxoid Ltd., UK); yeast extract, 2.0 (Oxoid Ltd., UK); sodium chloride, 0.1 (Thermo Fischer Scientific Inc., Loughborough, Leicestershire, UK); hydrogen di-potassium phosphate, 0.04 (Thermo Fischer Scientific Inc., UK); dihydrogen potassium phosphate, 0.04 (Thermo Fischer Scientific Inc., UK); magnesium sulphate heptahydrate, 0.01 (Thermo Fischer Scientific Inc., UK); calcium chloride hexahydrate, 0.04; sodium hydrogen carbonate, 2 (Thermo Fischer Scientific Inc., UK); Tween 80, 10ml; vitamin K, 10µl; L-cysteine hydrochloride, 0.5; bile salts, 0.5 (Thermo Fischer

Scientific Inc., UK) and a 4 ml/L concentration of a 0.025% (w/v) resazurin solution was added. The medium was autoclaved at 121°C for 15 min.

2.5 Antibiotics

The stocks of antibiotics were prepared using a suitable solvent (water or ethanol) and then sterilised by 0.22μm filtration. The antibiotics used in this study have been listed in Table 2.1.

Table 2. 1: Antibiotic solutions used in this study

Antibiotic	Abbreviation	Solvent	Stock concentration (mg/ml)
Ampicillin	AMP	Water	1mg/mL
Chloramphenicol	CHL	Ethanol	10mg/mL
Tetracycline	TET	Ethanol	0.035 μg/mL
Kanamycin	KAN	Water	50 mg/ml
Erythromycin	ERY	Ethanol	5 mg/ml

2.6 Bacterial strains and plasmids

The strains used in this study have been listed in Table 2-2. Stocks of these strains were stored at -80˚C in 20% glycerol. All plasmid stocks were maintained at -20°C in ultra-pure water. The plasmid details have been listed in Table 2.3.

2.7 Primers

The primer used for the DNA procedure and the oligonucleotide probes for the fluorescent in situ hybridisation procedures were ordered from Eurofin (formerly MWG, Germany). All DNA primers used in this study have been listed in Table 2.3 and all oligonucleotide primers used in the fluorescent in situ hybridisation procedures in this study have been listed in Table 2.4.

Table 2. 2: Bacterial Strains and Plasmid used in this study

Strain	Genotype	Ref. /Source
S. aureus SH1000	Functional *rsbU+* derivative of 8325-4	Horsburgh *et al.*, 2002
S. aureus SH1000 Δ*srt*	Sortase mutant derivative of SH1000	Heilbronner *et al*., 2013
S. aureus SH1000 Δ*fadB*	*fadB* mutant derivative	This study
S. aureus RN4220	*Restriction deficient derivative of 8325-4, rK− mK+*	Novick et al., 1993
S. aureus Newman	*Staphylococcus aureus subsp. aureus Newman*	Baba *et al*., 2008
S.aureus SA113	*Staphylococcus aureus Rosenbach 1884*	Weiss *et al*.,1980
E. coli BL21(λDE3)	F^-, *ompT*, *hsdS*$_B$, (r_B^-, m_B^-), *dcm*, *gal*, λ DE3.	Promega
E. coli TOP10	F^-, *mcrA*, Δ(*mrr-hsdRMS-mcrBC*), Φ80*lacZ*ΔM15, *recA1*, Δ*lacX74*, *endA1* , *nupG*, *galU*, *galK*, Δ(*araleu*)7697, *rpsL* (Str^R), *araD139*.	Invitrogen
E. coli JW3822	BW25113 Δ*fadB*::*kan*	Baba *et al*., 2006
E. coli BW25113	F^- DE(araD-araB)567lacZ4787(del):rrnB-3 LAM^- rph-1 DE(rhaD-rhaB)568 hsdR514	Baba *et al*., 2006

Table 2. 3: Plasmids used in this study

Plasmid name	Genotype	Selection	Reference/source
pJET1.2/blunt	Cloning vector (Appendix 1)	Amp^R	Fermentas
pMAD	Temperature sensitive (30°C) shuttle vector. pE194ts:: pBR322, *ermC*, *bgaB*	ErmR	Arnaud *et al.*, 2004
pBAD His A	An expression vector containing an *araBAD* promoter	AmpR	Gunzman *et al.*, 1995
pJET-*fadB*	pJET1.2 plus *fadB* gene from *S. aureus* (Appendix)	Amp^R	This study
pMAD-fadB	pJET1.2 plus *fadB* gene from *S. aureus*	Amp^R	This study
pfadB	*araBAD*, *fadB*, Amp	Amp	This study
pET21a	Over-expression vector Amp^R, *lacI*, pBR322 ori	Amp	Novagene (lab stock)
pET21a-fadB	Over-expression vector Amp^R, *lacI*, pBR322 ori	Amp	This study

Table 2. 4: Oligonucleotide primers used in the DNA procedures

Primer name	Sequence and restriction site	Restriction enzyme
FadB upstream For	5'-CTAAATGGATCC ACAGTCACATGAACTGCG	*Bam*HI
FadB upstream Rev.	5'-TTACCCGGGTTGTCATAGTGATTCCTCCAATTTAGTTG	*Sma*I
FadB downstream For	CATTACCCGGGCGTAATTAAAAGATAGTCATTAAGAGAGG	*Sma*I
FadB downstream Rev.	CGTTTGGGATCCAGAAGCAAATGCTTCGTTCAATTCG	*Bam*HI
FADB clone For.	CTAAGAGCTCATTGGAGGAATCACTATGACAATTAATAAAG	*Sca*I
FADB clone Rev.	GACTAGGTACCTCTTTTAATTACGTAATGGCTTACCAG	*Kpn*I
FADB overexpression For.	GGAGATATACATATGATTGGAGGAATCACATATGAC	*Nde*I
FADB overexpression Rev.	GTGGTGGTGCTCGAGATTACGTAATGGCTTA	*Xho*I

2.8 DNA and RNA methods

2.8.1 Extraction of the genomic DNA

The gnomic DNA was isolated from *S. aureus* using the DNeasy® Blood & Tissue kit (Qiagen). One colony was inoculated in 10mL of pre warmed TSB and then incubated for 16h at 37°C. The culture was harvested by centrifugation at 7500rpm for 10 min and then resuspended in a 200 ml buffer (PBS) containing 5µl of lysostaphin (5 mg/ml). The suspension was transferred into the DNeasy® Mini Column and centrifuged at 8000 RPM for 1 min. Then, 500 µl of AW1 buffer were added and the column was centrifuged again at 8000 RPM for 1min. The AW2 buffer was added and the column was centrifuged at 14 RPM for 3 min. Following this, 200 µl of AE buffer was added and incubated at room temperature for 1 min. Then the column was centrifuged at 8000 rpm for 1 min and the elutant was transferred to the freezer at -20 ºC for later use.

Table 2. 5: Oligonucleotide primers used in FISH procedures

Short Name	Target genus	Sequences
Sau	Staphylococcus aureus	5'-GAAGCAAGCTTCTCGTCCG-3'
EUB338	Most Bacteria	5'-GCTGCCTCCCGTAGGAGT-3'
EUB338II	Most Bacteria	5'-GCAGCCACCCGTAGGTGT-3'
EUB338III	Most Bacteria	5'-GCTGCCACCCGTAGGTGT-3'
Non Eub	-	5'-ACT CCT ACG GGA GGC AGC-3'

2.8.2 Isolation of plasmid DNA

A single colony carrying the desired plasmid was used to inoculate 5 ml LB containing the appropriate antibiotic and it was incubated overnight at 37 ºC with shaking at 250 RPM. The culture was harvested by centrifugation and then resuspended in a 200 buffer (PBS) containing 5µl of lysostaphin (5 mg/ml) . plasmid DNA was isolated using the GeneJET^{TM} plasmid Miniprep kit (Fermentas) according to the manufacturer's instructions.

2.8.3 DNA digestion with restriction endonucleases

All restriction endonucleases were purchased from the Fisher Thermo Scientific. The DNA digestion of the PCR products or plasmids was used to confirm the presence of the desired insert, namely DNase from GE and AMV reverse transcription from Promega.

2.8.4 Gel extraction

The extraction of the DNA fragments from the agarose gels was achieved by the excising of the right size DNA fragment using a scalpel blade. The fragment was then purified using the QIA quick gel extraction kit.

2.8.5 DNA clean up

The GeneJET PCR Purification Kit (Fermentas) used to purify the PCR products and restrict digestion. One volume of binding buffer for the PCR product was combined and up to 800 µl of the re-suspension solution was transferred to the Gene JET purification column which was then centrifuged for 45 s. Following this, 700 µL of wash buffer was added and the column was then centrifuged again for 45 s. The column was centrifuged for 1 min, then 50 µl of ultra-pure water was added and the column was incubated at room temperature for 2min and then centrifuged for 2 min.

2.8.6 DNA ligation

The PCR amplified DNA and vector were ligated at room temperature for 1 hour. The total reaction was 20µL and it consisted of 50-100 ng of the digested PCR product and 50 ng of the digested plasmid, 4 µL of 10x reaction buffer (Fermentas) and 1 µL of T4 DNA ligase (Fermentas).

2.8.7 RNA extraction

RNA was isolated from *S. aureus* using the RNeasy Mini Kit® (Qiagen). One colony was inoculated in 10mL of pre-warmed TSB and then incubated for 16h at 37°C. The culture was diluted 1- 100 in 100ml of fresh media and incubated at 37°C until 0.5 OD; 12 ml of culture was added to an equal amount to the Invitrogen™ RNAlater™ Stabilisation Solution (Fisher) and incubated at R.T for 5min. The mixture was then harvested by centrifugation at 7500 RPM for 10 minutes. The bacterial pellet was resuspended in 200 µl of PBS containing 5 µl of lysostaphin (5 mg/ml) and incubated for at least 30 minutes at 37°C. The RNA was then isolated according to the manufacturer's instructions.

2.8.8 RNA quality analysis

High-quality RNA is required for many downstream applications. The RNA 600 LAB Chip kit was used for quality and quantity analysis of the RNA. According to the manufacturer's instruction, 1µL RNA 600 Nano Dye concentrate was added to the RNA 600 Gel Matrix (65 µL) and 9 ul of the mix were loaded onto the RNA chip on the chip priming station. After this, 1 µL of the RNA 6000 ladder and RNA sample (1 µL) was loaded into the well of the chip followed by 5 µL. The chip was vortexed for 1 min (2400 RPM) and analysed in the Bio-analyser for 5 min.

2.8.9 Design and optimise primers

The oligonucleotide primers for qPCR were designed using an online qPCR assay designing tool from the Eurofin website (formerly MWG, Germany).

2.8.10 Polymerase chain reaction (PCR)

DNA fragments of interest were amplified from the genomic DNA. A standard volume of 50 µL reaction containing 20 µL Pwo water, 2 µL forward primer, 2 µL reverse primer, 1 µL template DNA and a 25 µL Pwo master mix containing Pwo DNA polymerase (Roche) were prepared. The amplification of the DNA was performed in the initial denaturing step at 95°C for 1 min, followed by 30 cycles at 95°C for 1 min (denaturation), at 52°C for 1 min (annealing), at 72°C for 1 min (extended) and in the polymerization step at 72°C for 5 min.

2.8.11 Colony PCR

Colony PCR was used to screen multiple colonies for successful plasmid construct following ligation and transformation. Typical reaction volumes used for the colony PCR were 20 µl reactions consisting of 2 µl of 10 x Taq buffer, 2 µl dNTP mix (2 mM each), 0.6 µl of 50 mM MgCl2, 0.4 µl forward primer, 0.4 µl reverse primer, 1 µl colony water (prepared from single colony), 14.5 µl nuclease free water and 0.1 µl Dream Taq DNA polymerase was prepared. The enzyme was always added in the final step. Prior to the addition of the enzyme, one colony was selected from its plate using a sterile tip. The tip was touched to a separate agar plate (so then a stock of the colony was preserved) before being dipped into an aliquot (50 µl) of sterile water and stirred gently. PCR amplification was carried out as described above.

2.8.12 Transformation reaction

The transformation was achieved by combining 5 µl of the ligation reaction to 100 µl of chemically competent cells incubated on ice for 30 min. After incubation, the

mixture was subjected to heat shock at 42°C for 2 min and it was then incubated on ice for 5 min. 1ml of LB was added and gently mixed. The mixture was incubated at 37°C for 1 h. Following incubation, the cells were centrifuged at 13,000rpm for 5 min, then 900 ul of supernatant was removed and the pellet resuspended with the remaining supernatant. Following this, 100 µl of the reaction mixture was spread on the surface of an agar plate and incubated at 37°C overnight.

2.9 Preparation of competent cells

2.9.1 Chemically competent *E. coli* cells

Briefly, chemically competent *E. coli* TOP10 was created by taking1 ml of fresh overnight culture and inoculating 100 ml of prewarmed fresh LB grown up to OD_{600} of 04 at 37 ºC in a Gallen Kamp orbital shaker (250 RPM). The cultures were chilled on ice for 15 min. The cells were harvested at 4 ºC and 5000 RPM for 5 min. The cells were re-suspended in 1.6 ml of ice-cooled 0.1 M $MgCl_2$ and incubated on ice for 30 min. The cells were centrifuged again, the pellet re-suspended in 1.6 ml ice cold 0.1 M of $CaCl_2$ and it was then incubated on ice for 20 min. The pellets were re-suspended in 0.5 ml ice cold 80% glycerol. Then the cells were divided into 100µl aliquots (Lederberg & Cohen, 1974).

2.9.2 Electrocompetent *S. aureus* cells

Overnight cultures of *S.aureus* RN4220 were grown in 10 ml of TSB. Four ml of overnight culture were inoculated into 200 ml of pre-warmed B2 medium, and then the culture incubated at 37°C until reaching optical density 0.35-0.40 (at 600 nm). Then, 30 ml of culture were then transferred aseptically into 50 ml Falcon tubes centrifuged at 4000 RPM for 10 minutes at room temperature. The pellet was resuspended in 30 ml of sterile water and centrifuged again. After three washing steps and the careful removal of the supernatant, the bacterial pellet was resuspended in 10 ml of 10% glycerol,

centrifuged at 4000 rpm for 10 min and the supernatant was discarded. The pellet was then resuspended in 5 ml of 10%(v/v) glycerol, incubated for 15 minutes at room temperature. The pellet was centrifuged again at 10000 RPM for 3 minutes in Eppendorf tubes and resuspended in 100 µl of 10%(v/v) glycerol before being flash frozen in liquid nitrogen and stored at -80°C until use.

2.10 Construction of S. *aureus* Δ*fadB*

S. aureus SH1000 *ΔfadB* was constructed through the allelic replacement of chromosomal genes using an *E.coli – S. aureus* shuttle vector pMAD as previously described (Arnaud et al., 2004).

2.10.1 Primer design and the PCR amplification of the *fadB* gene

Primers containing restriction sites were designed to amplify ~1000 bp regions upstream and downstream of the *fadB* gene. The regions of the *fadB* gene were amplified using a PCR reaction.

2.10.2 Sub-cloning into a blunt cloning vector

The PCR amplified regions of the upstream and downstream *fadB* gene products were sub cloned into the pJET1.2/blunt cloning vector. The positive clones in the transformed *E.coli* TOP10 cells were identified through colony PCR and confirmed with restriction mapping with *Bgl*II restriction digestion.

2.10.3 Construction of the pMADΔ*fadB* vector

The pMAD*ΔfadB* vector was constructed in two steps. In the first step, the pJET1.2/blunt vector containing the upstream region of the fadB gene was restricted with *Bam*HI and *Sma*I. It was ligated into the pMAD vector restricted with *Bgl*II and *Sma*I. The ligated pMAD vector with an upstream region related to the fadB gene was transformed into the *E. coli* TOP10 cells. The presence of the *fadB* upstream region in

the transformed cells containing the pMAD upstream *fadB* fusion construct was confirmed using PCR amplification using the *fadB* upstream For and *fadB* upstream Rev oligonucleotide primers.

In the second step, the pJET1.2/blunt vector containing the downstream region of the *fadB* gene was restricted with *Bamh*I and *SmaI*. It was ligated into the pMAD upstream *fadB* fusion construct and restricted with *Bam*HI and *Sma*I to generate the pMADΔfadB vector. The ligation product was transformed into the *E. coli* TOP10 cells. The presence of upstream and downstream *fadB* fusion was confirmed by PCR amplification using *fadB* upstream For and fadB downstream stream Rev primers.

2.10.4 Transfer of the pMADΔ*fadB* vector into *S. aureus*

2.10.4.1 Electrotroporation

The pMADΔ*fadB* vector was introduced into *S. aureus* RN4220 cells by electroporation at 100 Ω, 2.3 KV and 25 μF using a Bio-Rad electroporator. The cells were recovered in a B2 medium for 2 h at 30°C and plated on TSA plates containing erythromycin.

2.10.4.2 Transduction

The erythromycin resistance colonies that appeared on the TSA were used to generate phage lysate (Φ 85) propagated on *S. aureus* RN4220 cells. They were then transduced into *S. aureus* SH1000 cells using erythromycin (5 μg/ml) in LK agar. The plates, were incubated at 30°C for 24 to 48 h.

2.10.5 Generation and confirmation of Δ*fadB S. aureus*

The successful transduced colonies were plated onto TSA-Xgal-erythromycin plates (50 μg/ml and 5 μg/ml, respectively) and incubated at 30°C for 24 h. One blue colony

was streaked onto the same medium, incubated at 42°C for 24 h. Growth at a non-permissive temperature (42°C) leads to the integration of the pMADΔfadB into the chromosome by a single crossover event. Light blue colonies were picked and plated into TSB and incubated at 30°C overnight. Growth at the permissive temperature (30°C) leads to a second recombination event, resulting in generation of mutant. Serial dilutions after growth at 30°C were plated onto TSA-X- gal plates and incubated at 42°C for 24 h which resulted in the excision of the plasmid. The white colonies that appeared on the TSA-X gal plates were checked for erythromycin sensitivity.

The white colonies representing gene deletion (Δ*fadB*) were confirmed by PCR amplification using the *fadB* upstream For and *fadB* downstream stream Rev oligonucleotide primers.

2.11 Expression of upstream, downstream and the *fadB* encoding gene.

S. aureus was grown in the presence of 8% bile (a sub inhibitory concentration of bile) until it reached an OD_{600} of 0.5. Culture medium without bile was used as a control. Following this, 12 ml of culture were removed and mixed with 24ml of the RNA protect reagent, before being incubated for 5 min at R.T. The mixture was centrifuged and the pellet resuspended with a TE buffer containing 25mg/ml lysozyme and 12.5mg/ml lysostaphine. 20 µL of proteinase k was added and then incubated at 30°C for 1h. RNA was isolated from *S. aureus* using RNeasy Mini Kit® (Qiagen) according to the manufacturer's instructions. RNase-free DNase was used to remove any residual DNA and then 3µg of RNA was reverse transcribed into single-stranded cDNA using a high-capacity cDNA archive kit (Applied Biosystem).

Real time PCR was performed in an ABI Prism 7500 fast real- time PCR system (Applied Biosystem). A standard volume of 25 µL reaction containing 12.5ul of SYBR

green master mix (Applied biosystem) and 1 µL of cDNA, 200nM of each primer was used.

The amplification of the DNA was performed in the initial denaturing step at 95°C for 10 min, followed by 35 cycles at 95°C for 15s (denaturation) and 60°c for 1 min (annealing), followed by melting-curve analysis. The expression level was determined through relative quantification using the threshold cycle method in which the expression level in the control culture (without bile) was arbitrarily set to 1. The expression level in the sample was calculated relative to the control. Housekeeping genes (HKG) *gmk, gyrA*, *proC* and *tpiA* were used as an endogenous control using the previously described primers. All of the experiments were carried out in triplicate.

2.12 Polyacrylamide gel electrophoresis (SDS PAGE)

2.12.1 Preparation of SDS page

The SDS PAGE is a technique commonly used for the separation of proteins according to their molecular weight. The SDS PAGE gel in a single electrophoresis was divided into a stacking gel and separating gel. 12% of acrylamide was used for this. Briefly, the separating gel solution (2 ml H_2O, 1.67 ml 30% acrylamide, 1.25 ml 1.5 M Tris (pH 8.8), 100 µl 10% SDS, 15 µl 10% ammonium persulfate and 10 µl TEMED) was poured between two glass plates of casting frames. After waiting for 30 minutes, the stacking gel (2.088 ml H_2O, 30% acrylamide/Bis, 0.375 ml of 1 M Tris (pH 6.8), 100 µl of 10% (w/v) SDS, 30µl of 10% ammonium persulfate and 15µl TEMED) were added. The well-forming comb was inserted without trapping air under the teeth. After waiting for 10 - 15 min, the gel was transferred to a vertical electrophoresis cell (Bio-Rad).

2.12.2 Protein electrophoresis

The sample was put through electrophoresis using the method of Laemmli (1979). The whole cell samples were boiled in an SDS sample buffer (62.5mMTris-HCl pH

6.8,2.5%SDS,0.00125% Bromophenol Blue,1% β-mercaptoethanol and10%glycerol) by heating it to 95 °C for 7 min and it was then centrifuged for 2 min prior to loading. An aqueous sample was boiled in an equal amount of 2x SDS; 15 ul of prepared sample was loaded into each well of the gel. The electrophoresis tank was filled with 1x running buffer (25mM Tris, 192mM glycine, 0.1 $_{W/V}$ SDS) and the cell was then connected to the power supply ~230 V for 45min. The protein of interest was detected using Coomassie blue staining (0.2% w/v Coomassie blue R, 30% methanol, 10% acetic acid) for 1 h. This step was followed by the overnight destaining of the gel in destain (10% v/v acetic acid , 30% v/v methanol) with gentle agitation.

2.13 Protein overexpression and purification

2.13.1 Small scale overexpression

An overnight culture was prepared by inoculating a single colony of E. coli BL21 carrying a pET21a-*fadB* plasmid for protein expression from a freshly streaked plate into a 5 ml L-broth containing the appropriate antibiotic. A 0.5 ml of this culture was added to 50 ml of LB -AMP. The culture was grown at 37°C until the OD600 reached 0.5. At this point, the culture was induced by adding 50 µl of 1M of IPTG. A sample was taken every hour for 4h and one sample was taken after the overnight growth. The samples were taken at a standardised volume for the optical density units (OD_{600} 0.5) and they were pelleted by centrifugation at 13,000 rpm for 5 min. The supernatant was removed and the pellet was re-suspended in 100 µl of a 1x SDS sample loading buffer denaturated by being heated at 100 °C for 10 min; 15 µl of sample was loaded in SDS gel to get the determination of the expression.

2.13.2 Large scale overexpression

Once an appropriate time point for the maximum protein expression post-IPTG induction had been determined from the small scale over-expression trial, large scale

overexpression was performed. One colony were inoculated in 50 ml of LB-AMP into 250 sterile flasks, grown overnight with shacking. Overexpression was performed in 4L in 8 2L flasks. Each flack contained 500 ml of LB-AMP. Each flask was inoculated with 5 ml of overnight culture grown at 37°C with shaking for 3 - 4 h. Once the optical density had been reached, 0.5 of OD600, the cultures were induced with IPTG and grown for a further 4 h. Then the cells were harvested by centrifuging them at 5,000 rpm at 4 °C for 20 min. The supernatant was quickly removed and the pellets were resuspending with 35ml of buffer A2 (50 mM Tris, 15 mM mannitol and 15 mM imidazole,5% glycerol, pH 8.0) for Nickel chromatography.

2.13.3 Nickel affinity chromatography

FadB-His6 (His-tagged protein) was purified using a column prepacked with Ni-Sepharose via the HR workstation (Bio-Rad). The column was recharged with 0.1 M $NiSO_4$ and washed with 3 column volumes of ultrapure degassed water to remove any unbounded Nickel. The column was equilibrated with 1 column volume of elution buffer (50 mM Tris, 15 mM mannitol and 1M imidazole, 5% glycerol, pH 8.0) and 3 volumes of binding buffer (50 mM Tris, 15 mM mannitol and 15 mM imidazole, 5% glycerol, pH 8.0). The protein sample obtained from the cell lysis was loaded onto the column. The column was then washed with a binding buffer until the A280 of the flow-through was <0.01. The baseline was zeroed at this time. FadB- His6 was then eluted with a linear gradient of 0-1.0M imidazole using a 3 - 4 column volume of elution buffer. The elutant was collected in 7 ml fractions using a plastic tube. A peak present on the chromatogram corresponded to the potential protein elution from the column. The suspected fractions were analysed by SDS-PAGE to confirm if they contained FadB - His6 and they were stored at -80°C.

2.13.4 Dialysis and concentration of protein

The protein samples were dialysed thoroughly against exchange the buffers to remove any unwanted salt molecules or low mass contaminants. Dialysis membrane tubing of an appropriate molecular weight cut off for the respective protein of interest was employed by Fisher Scientific. The dialysis tubing was boiled in qH_2O containing one small spatula of EDTA to ensure that the tubing was sterile and the excess dialysis tubing was stored in 30% ethanol at 4 °C. Appropriate lengths of dialysis tubing were cut and sealed at one end. The tubing was tested for leaks by filling them with qH_2O. The water was subsequently removed and the tubing gently squeezed to remove any excess. The protein sample was then placed carefully into the tubing and the other end was tightly sealed to ensure that the protein was contained. The sealing tube was then placed in 500 ml of the appropriate buffer which was gently stirred on a magnetic stirrer using a small-size flea. Dialysis was performed at 4 °C in ~5L of buffer. The buffer was changed every 6h until 5 L of buffer had been used. After dialysis, the protein was concentrated using a Vivaspin 20 (50,000 MWCO) (Satorius Stedim Biotech) centrifugal concentrator according the manufacturer's instructions.

2.13.5 Determination of protein concentration

The protein concentration was estimated using a Bradford assay with bovine serum albumin (BSA) as the protein standard. BSA was diluted in a series ranging from 0.025 to 5 µg/ml. The dye reagent was prepared by adding 1 ml of reagent to 4 ml of water. Then, 10µl of each BSA dilution and 10µl of each unknown sample solution were added to a 96-wells microtiter plate. Subsequently, 190 µl of diluted dye reagent was then added to each well. The microtiter plate was then incubated at R.T for 5min. The absorbance was measured at 595 nm using a microtiter-plate reader. Using the measurement obtained from the standards, a standard curve was created from which the

protein concentration was calculated. The protein concentration was also assessed using Nandrop ND-1000 (Nanodrop Technologies), which measured the absorbance of 2 µl of protein sample at 280 nm. The data from the Nanodrop and Bradford assay were compared to ensure consistency and accuracy.

2.13.6 Cell lysis and protein extraction

The cell pellet and the French component were maintained at 40°C to minimise protein degradation and to slow the enzymatic processes during cell lysis. The lysis procedure was repeated twice. Following that, the cells were centrifuged using a rotor SS34 (8 x 50 ml) at 51,428 x g for 40 min. The supernatant was quickly retained for further purification.

2.13.7 Mass spectrometry analysis of FadB

The protein sample was sent for mass spectrometry analysis at an advanced mass spectrometry facility at the University of Birmingham. The protein sample was digested with trypsin and a mass of the recovered peptide was determined using LC-MS (liquid chromatography mass spectrometry).

2.13.8 Western blotting

2.13.8.1 Electrotransfer

Following electrophoresis, the protein was transferred from the gel to the nitrocellulose membrane by electroblotting. Blotting was performed using Trans-Blot® SD Semi-Dry Electrophoretic Transfer Cell and according to manufacturer's instruction (BioRad). The blot was conducted at 18V for 1h.

2.13.8.2 Immunodetection

Following the electroblot, the nitrocellulose membrane was washed in 1x TBS (20 mM Tris, 500 mM NaCl, pH 7) for 10 min on a shaking platform. The nitrocellulose

membrane was then blocked in a blocking solution (1% BSA-TBS) for 1 h at room temperature or overnight at 4 ºC while shaking gently before being washed twice in TTBs (20 mM Tris, 500 mM NaCl, 0.05% Tween 20, pH 7.0) for 10 min for each wash. Then, 15 ml of antibody buffer (1%BSA-TTBS) containing an appropriate antibody diluent was incubated with the nitrocellulose membrane for 1 h at room temperature. After washing them twice with TBST, the membranes were incubated for 1 h with the secondary antibody conjugated to horseradish peroxide (HRP) or alkaline phosphatase diluted in an antibody buffer (1%BSA-TTBS). This was then twice washed with TBST for 10 min each wash. Then the protein of interest was detected by a ChemiFast Chemiluminescence Substrate (Syngene) according to the manufacturer's instructions. The images were visualised and recorded using a G:BOX Chemi (Syngene).

2.13.9 Enzymatic assay

A continuous spectrophotometric assay was used to measure the activity of the enzyme (FadB) based on Lynen and Wieland's (1995) method. The enzyme converts acetoacetyl-Co to ß-hydroxy butyryl-CoA in the presence of ß-NADH. This activity was determined by measuring the decrease of NADPH absorption at A340nm. The reaction contains 0.2mM acetoacetyl-CoA (substrate) and 0.1mm NADH and 0.7 unit of the enzyme. The mixture was incubated at 37 ºC and it decreased in A340nm; this was recorded for up to 5 mins. One unit of enzyme activity was defined as the unit that converted 1.0 µmole of acetoacetyl-CoA to ß-hydroxybutyryl-CoA per minute at pH 7.3 at 37°C in the presence of NADH. The reaction was performed in triplicate and the data has been presented as a mean with standard deviation.

2.13.10 Surface plasmon resonance (SPR)

The protein sample was sent for SPR analysis at Creative Peptides company –USA. They use this protocol : Various concentrations of protein dissolved in water were manually printed onto the bare gold-coated (thickness 47 nm) PlexArray Nanocapture Sensor Chip (Plexera Bioscience, Seattle, WA, US) at 40% humidity. Each concentration was printed in replicate, and each spot contained 0.2 mL of sample solution. The chip was incubated in 80% humidity at 4°C for overnight, and rinsed with 10× PBST for 10 min, 1× PBST for 10 min, and deionized water twice for 10 min. The chip was then blocked with 5% (w/v) non-fat milk in water overnight, and washed with 10× PBST for 10 min, 1× PBST for 10 min, and deionized water twice for 10 min before being dried under a stream of nitrogen prior to use. SPRi measurements were performed with PlexAray HT (Plexera Bioscience, Seattle, WA, US). Collimated light (660 nm) passes through the coupling prism, reflects off the SPR-active gold surface, and is received by the CCD camera. Buffers and samples were injected by a non-pulsatile piston pump into the 30 mL flowcell that was mounted on the coupling prim. Each measurement cycle contained four steps: washing with PBST running buffer at a constant rate of 2 mL/s to obtain a stable baseline, sample injection at 5 mL/s for binding, surface washing with PBST at 2 mL/s for 300 s, and regeneration with 0.5% (v/v) H3PO4 at 2 mL/s for 300 s. All the measurements were performed at 25°C. The signal changes after binding and washing (in AU) are recorded as the assay value. Selected protein-grafted regions in the SPR images were analyzed, and the average reflectivity variations of the chosen areas were plotted as a function of time. Real-time binding signals were recorded and analyzed by Data Analysis Module (DAM, Plexera Bioscience, Seattle, WA, US). Kinetic analysis was performed using BIAevaluation 4.1 software (Biacore, Inc.).

2.13.11 Cholic acid coupled -sepharose affinity chromatography

Cholic acid was coupled to ethyl-3-Qxlimetbylaminopropyl carbodimide HCI and the pH of the coupled mixture was adjusted to 7. It was then slowly added to amino hexylamino (AH)-Sepharose 4B and incubated for 24h with shaking. The mixture was then packed into the 50ml chromatography column. The purified protein was added to the column and washed with phosphate saline buffer to remove any non-bound proteins. After this, 5 mM of cholic acid was used to elute the binding proteins.

2.14 pH controlled faecal batch cultures

The batch culture was anaerobically carried out in fermentation vessels containing 135 mL of sterile basal medium. Nitrogen gas was used to provide an anaerobic condition in the vessels. pH was maintained at 6.8 via the pH electrodes between pH 6.7 – 6.9. The distal large intestine has a neutral pH and this was automatically adjusted by adding 0.5 mM-NaOH and HCl to the vessels when required. The temperature was maintained at 37°C via a circulating water bath. Each vessel was inoculated with 15 mL faecal slurry (1% final concentration). The batch culture fermentations were run for 48 h and the samples (5 ml from each vessel) were collected at 0, 4, 8 and 24, 30 and 48 h for the analysis of the bacterial populations and metabolite production. Fermentation experiments were performed in triplicate.

2.15 Enumeration of major colonic bacterial population fluorescence in situ hybridisation (FISH)

All of the probes were fluorescently labelled (Alexa488, Alexa488) and synthesised by Eurofin (formerly MWG, Germany). Briefly, 375 µL of the batch culture samples were fixed in three volumes of ice-cold 4% (w/v) paraformaldehyde for 4 h at 4 °C. The cells were then pelleted by centrifugation at 13000 g for 5 min and washed twice

in 1 ml of sterile PBS. Then the washed cells were resuspended in 150 µL of sterile PBS to which 150 µL of ethanol was added. The sample was then mixed by vortexing and stored at -20°C. For permeabilization, 50 µl aliquot of the 4% PFA fixed samples was added to 500µL of PBS and we then vortexed the sample. The cells were pelleted by centrifugation at 13000 g for 3min. The supernatant was removed and the pellets were resuspended in 100µL of TE-FISH containing lysozyme (1mg/ml), incubated in the dark for 10 min at R.T. The Sau probes required hybridisation pre-treatment with 20 µl lysozyme followed by 20 µl lysostaphin (0.5 mg/ml) respectively to ensure the sufficient permeabilization of the cell envelope. After treatment with the enzyme, the mixture was centrifuged and washed with BPS(Phosphate Buffered Saline). For the situ hybridization, the pellet was resuspended in a 150µL hybridisation buffer and then centrifuged. The pellets were resuspended in 1 ml of hybridisation buffer. In a 1.5 ml Eppendorf tube, 50µL of sample was added to 4µL of the appropriate probe and incubated overnight at 37°C. Following incubation, 150ml of hybridisation buffer was added to each tube and vortexed gently. The sample was then plated through centrifugation and then washed with 200ul of washing buffer solution and incubated for 20min at 37°C in the heating block/hybridisation oven. After incubation, the mixture was centrifuged and the supernatant was dicard. 200µL of PBS were added to the control tube.

The sample was analysis with a flow cytometer to identify the appropriate amount of PBS to then distribute to the sample. Sample were kept in - 40°C and covered with aluminium foil until it was measured with a cytometer.

2.16 Determination of minimum inhibitory concentration (MIC)

The MICs of the selected bile salts, sodium cholate (CA), sodium chenodeoxycholate (CDCA) and sodium deoxycholate (DCA), were determined by the broth dilution

method. The MICs were determined by doubling up the dilution. The two-fold dilution of bile salt was incubated with *S. aureus* at a density of 106 CFU/mL after incubation for 16h in 37°C , tubes with no visible growth that was read as the MIC value. The MICS were reproduced in three independent experiments.

2.17 Time-course viability upon exposure to bile salts

Bacterial grown cells were cultured until mid-exponential phase in fresh BHI broth at 37°C under aerobic conditions. Then cell harvested and washed three times in sterile 5 mM HEPES buffer (pH 7.2) with 10 mM glucose and resuspended in the same buffer to an OD600 of 0.5. This bacterial suspension was used to inoculate various concentrations of CA, DCA, CDCA, GCA, and TCA in peptone saline diluent then the mixture was incubated 30 minutes at 37°C. At 10-minute intervals until 30 minutes. Cells from appropriate dilutions were plated onto tryptic soy agar (TSA) plates, and incubated overnight at 37°C. Colonies were counted and the percentage viabilities were calculated based on the counts from initial untreated cell suspension.

2.18 Adhesion of bile treated staphylococcus

HT-29 is a human colorectal adenocarcinoma cell line (Cohenet al., 1999). HT-29 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) and supplemented with 10% fetal bovine serum (FBS) and 10000 IU/ml penicillin in tissue culture flasks at 37 °C in a 5% CO_2 humidified atmosphere for two days. The cells were deattached using trypcin before they (5×10^5cells/well) were seeded in a 24-well plate in a DMEM medium with a 10% fetal bovine serum (FBS) and antibiotic mix. This was incubated at 37 °C in 5% CO_2 for 48 h before the addition of the bacteria.

For the determination of total adherent bacteria, the cell monolayers were washed thrice with DMEM without an antibiotic to flush the unbound extracellular bacteria. The cells

were lysed with ice cold 0.2% Triton X-100 in sterile PBS (pH 7.2) to release the intracellular bacteria. The number of adherents was then counted and summarised after plating the lysates on TSA.

2.19 Haemolysis assay

4 mL overnight was centrifuged at 12 500×*g* for 5min, then 0.1 of supernatant were taken and treated with 1 mL of haemolysin buffer (0.145M, NaCl, 20mM $CaCl_2$). 25 μL of defibrinated rabbit blood were added to the mixture and then incubated for 15min at 37 °C. The mixture was centrifuged at 5500g at room temperature for 1min. The optical density of the supernatant was measured at 543nm. The percentage of haemolysis was calculated thus: percentage of haemolysis = 100× (absorbance of sample – absorbance of negative control) / (absorbance of positive control – absorbance of negative control).

2.20 Bacteria Cell Envelope Extraction

Proteins were extraction by using the fastprep method (Larsen et al.,2006). Briefly, pre-warmed BHI was inoculated and grow it until mid-exponential phase at 37 °C with shaking. bacterial suspension was centrifuge at 8000xg for 5 min. The pellets resuspended in buffer including 50mM Tris(pH 7.5), 0.1 M NaCl, 0.5 mM PMSF and 1mg/mL iodacetamide.cells were lysed fastprep using fastprep-24 device (set speed to 60 and time to 40s and repeate 10 times) . Supernatants taken and treated with 5 mL of cold 50mM Tris and 0.1M NaCl, then centrifuged at full speed for 10 min at 4°C. The pellets re-suspended in 1mL of cold 50mM Tris and centrifuged to give native cell wall.

2.20 Statistical analysis

GraphPad Prism 5 software was used in statistical analysis. T test and one-way ANOVA were used for analysis of experimental data. P value <0.05 was considered as statistically significant difference.

Chapter Three: Interaction between bile and *S.aureus*

3.1 Introduction

Bile is a complex combination of organic and inorganic molecules that is stored in the gallbladder and released into the proximal small intestine (Hofmann & Eckmann, 2006). Bile consists of bile acids, bilirubin, amino acids, phospholipids, cholesterol, steroids, enzymes, porphyrins, vitamins and heavy metals (Begley *et al*., 2005).

In the liver, primary bile salts (cholic and chenodeoxycholic acids) are conjugated to the amino acids, glycine or sulphate (Ameri, 2015). Based on the conjugation with amino acids and a degree of hydroxylation, bile salts are categorised into three groups; trihydroxy conjugates (such as taurocholate and glycocholate), dihydroxy conjugates (such as glycodeoxycholate, glycochenodeoxycholate and taurodeoxycholate) and unconjugated forms (such as colette, deoxycholate and chenodeoxycholate) (Moghimipour & Handali., 2015).

After bile is secreted to the intestine, the normal commensal bacterial flora of the human gut produces secondary bile salts by deconjugation, namely 7α-dehydrogenation and 7α-dehydroxylation, of the primary bile salts (Ridlon *et al* ., 2016).

The concentration of bile salts in the gastrointestinal tract is variable and differs according to the different bodily components. However,the physiological concentration of bile in the human intestine ranges from 2 – 30mM depending on nutrition and metabolic condition (Martínez-Augustin & de Medina, 2008).

Intestinal enterocytes reabsorb about 95% of bile salts from the intestinal lumen, with the remaining excreted via faeces. The absorbed bile salts enter the blood stream and they are then taken by the hepatocytes, before being reconjugated and resecreted into the bile. Ninety percent of bile salts are cleared from hepatic circulation for reuse (Mertens et al., (2017; Begley et al., 2005).

3.1.1 Antibacterial activity of bile

Bile salts (BS) are bio-surfactants molecules that play a vital role in the emulsification and solubilisation of dietary lipids (Maldonado-Valderrama., 2011). They possess potent antimicrobial activity due to their peculiar amphipathic structure and detergent function. The mode of bile action on microbial cells may be related to oxidative DNA damage, disrupting the cell membranes and cellular homeostasis (Jakob *et al.,* 2014).

Watanabe *et al*. (2017) evaluated the antibacterial activity of bile by monitoring intracellular pH, membrane integrity and the viability of a human intestinal bacterium. The authors investigated the toxicity of 14 human and rodent samples of free bile acid. They found that deoxycholic and chenodeoxycholic acid both have a high toxicity effect on the tested bile salts and that one of the possible bacteriostatic mechanisms of these acids is related to the damaging of the bacterial cell membrane. The hydrophobicity nature of bile acid molecule gives it a high affinity to the phospholipid bilayer of the bacterial cell membrane which results in damage to the cell membrane (Watanabe, Fukiya, & Yokota, 2017). Several studies have investigated the role of bile in regulating the composition of the gut microbiota. Islam *et al*., (2011) found that feeding rats with CA lead to alternation in the microbiota population in the rat gut in the same vein. Yokota et al. (2012) demonstrated that a high fat diet in a mouse model, which dramatically causes an increase in the excretion of bile in the intestine, can result in an alteration of the microbiota composition. The authors suggest that the anti-bacterial effect of bile might be responsible for the alternations in the microbiota as a response to the increase in HFD (Atsushi Yokota *et al*., 2012).

Furthermore, it has been documented that a decrease in the bile pool, which is associated with liver disease and cirrhosis, results in an increase and overgrowth of pathogenic bacteria (Ridlon *et al*., 2013).

3.1.2 Impact of bile on staphylococcus virulence

Certain types of enteric bacteria not only have the ability to resist bile, but also uses bile as a signal for the regulation of virulent gene expression for efficient infection (Sistrunk *et al*., 2016). Hamner *et al*. (2013) suggested that E. coli O157:H7 uses bile as a signal to adapt to changing conditions in the small intestine. The authors found that the transcriptional profile of the bile-treated bacteria using qPCR and microarray analysis showed that bile induces significant changes in 41 genes which were related to the encoding proteins associated with virulence. The screening of the genome sequencing of pathogenic *E. coli* strains shows that the majority of genes associated with gastrointestinal infection, particularly fimbriae and enterotoxins, are carried on mobile genes that are lacking in commensal strains (Croxen *et al*., 2013; Fegan *et al*., 2014; Sahl & Rasko, 2012; Torres *et al*., 2007).

Adherence to the gut epithelium is one of the most important virulence factors related to establishing infection and promoting survival in the gut. Faherty *et al.* (2012) demonstrated that bile induces the adherence factor of *Shigella flexneri,* which facilitates attachment to colonic epithelium cells. The authors found that the adherence of *Shigella flexneri* to T84 cells increased after exposure to the bile salts.

On the other hand, bacteria attached to the surface produce an extracellular polysaccharide which results in a biofilm which provides a barrier against the host's immune defences and antimicrobial therapy (Branda *et al* 2005). Ulluwishewa et al., (2016) investigated the effect of bile on the *S.aureus* biofilm formation. The authors

found that the subinhibitory concentration of bovine bile stimulates biofilm formation in most of the clinical and laboratory strains of *S.aureus*.

In this study, some aspects of the relationship between bile and *S.aureus* were studied. Theses aspects include the antibacterial effect of bile against *S.aureus*, the factors that impact on bile resistance (strains & media), the effect of bile on some of the virulence aspect of *S.aureus* and the bile efflux pump of *S.aureus* .

3.2 Results

3.2.1 Antibacterial activity of bile

Bile has potent antimicrobial activity against a wide range of enteric bacteria. The effect of bile on the viability of *S. aureus* can be evaluated by detecting the minimum inhibitory concentration of bile and the time-course viability upon exposure to bile salts.

3.2.1.1 MICs of bile salts

The MICs of bile were within a minimum concentration of bile salts that completely inhibit the growth of *S. aureus SH1000*. The MICs of the crude bile, cholic acid, deoxy cholic acid, chenodeoxycholic acid, glycocholic acid and taurocholic acid were determined through serial twofold dilutions in a TSB broth.

The MICs values were read after 16h of incubation at a cell density of 10^6 CFU/ml. The MICs of bile salts have been summarised in the **Table 3.1.** The MICs were determined after three independent experiments.

As shown in **Table 3. 1**, *S. aureus* was less susceptible to conjugated bile acids (GCA and TCA) as a comparison with unconjugated bile acids (CA, CDCA and DCA). The MIC of CA was higher than the MIC of CDCA and DCA.

On the basis of these results, it can be concluded that unconjugated bile acid has a more potent antimicrobial effect than conjugated bile acid. The results agree with the findings of other studies which show that unconjugated bile acids have greater inhibitory effects on bacteria than conjugated bile acids (Begley *et al*., 2006; Sannasiddappa *et al*., 2017).

Table 3. 1- MICs of bile salts for *S. aureus SH1000*

Bile salt	Wild type (mM)
Cholic acid (CA)	22
Deoxycholic acid (DCA)	1.2
Chenodeoxycholic acid (CDCA)	1.2
Glycocholic acid (GCA)	>200
Taurocholic acid (TCA)	>200

Data represents triplicate independent experiments.

3.2.1.2 Time-course viability upon exposure to bile salts

In this study, the viability of *S. aureus* under the MICs and sub-MICs of bile were determined using viable plate count methods. *S.aureus* was cultured up until the mid-exponential phase in the BHI medium at 37°C .The harvested bacteria were then washed twice with a 5 mM HEPES buffer (pH 7.2) with 10 mM glucose. They were then resuspended in the same buffer; A_{600} of 0.5. Portions of this cell suspension were incubated in various concentrations of CA, CDCA, DCA, GCA and TCA for 30 minutes at 37°C. Every ten minutes, the cell suspension was taken and diluted with a sterile peptone saline diluent. Appropriate dilution was plated onto the TSA plate and incubated for 24h at 37°C. The colonies were counted and the viabilities (%) were calculated based on the count taken from the initial untreated cell suspension.

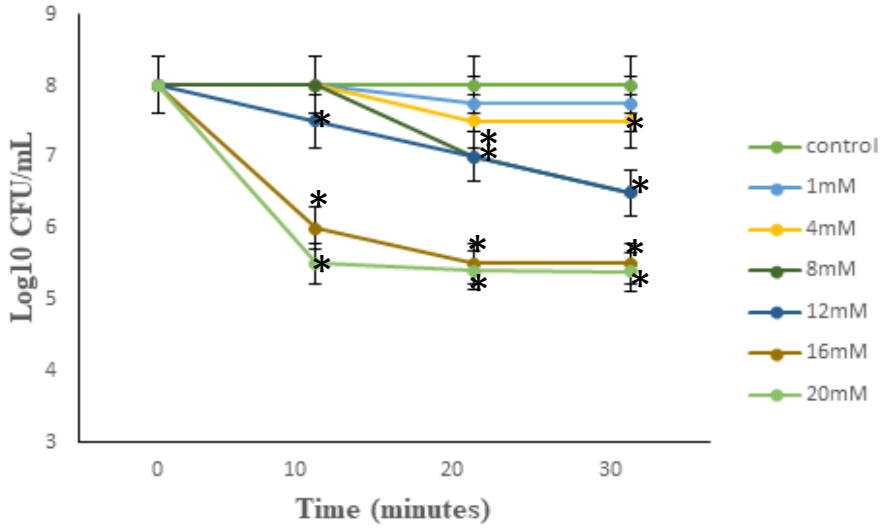
The results as shown in (**Figure 3.1 A)** indicate that the CA at a concentration of 1Mm causes a 20 - 41.9% reduction in the viability of *S. aureus* while nearly killing it at 20mM ($p<0.05$). The viability was decreased 72.9% ($P<0.05$) in the case of 0.2mM of DCA and 71.2 % ($P<0.05$) in the case of 0.2 CDCA over 30 minutes of exposure. Both bile salts showed complete death at their MICs ($P<0.05$) (**Figure 3.1 B, C)**.The viability of *S.aureus* was decreased by only 40% when exposed to the highest concentration of conjugated bile acid(20mM) (GCA and TCA) (**Figure 3.1 D, E)**.

In summary, these results show that unconjugated bile acids were more potent when compared to conjugated bile acid in terms of kill kinetics. The findings of the current study are consistent with those of Sannasiddappa *et al*. (2017), who stated that the subinhibitory concentration of bile acids was able to inhibit *S.aureus* growth. However, unconjugated bile acids show a higher level of inhibitory action than conjugated ones.

The reason for this is not clear but might be related to differences in pKa value between them. The decrease of intracellular pH was more marked with unconjugated bile salts (CA and DCA) compared to conjugated bile salts (GCA and TCA). This difference could be attributed to the higher pKa values of unconjugated bile salts (6.4 and 6.58 for CA and DCA, respectively) compared to conjugated bile salts (4 and 2 for GCA and TCA, respectively), meaning they are less dissociated at pH 7 and hence more able to cross the hydrophobic cell membrane . Conjugated bile salts are stronger acids having lower pKa values, and at physiological pH 7 are effectively fully ionized, unable to cross cell membrane unless a specific transporter is available(Thippeswamy H. Sannasiddappa et al., 2017).

It can thus be conceivably hypothesised that during the passage through the gastrointestinal tract, bile acids are chemically modified by the host or microbiota. Based on the type of modification, bile acids are different in their antimicrobial efficacy (Sannasiddappa et al., 2017).

A


Log10 CFU/mL
9
8
7
6
5
4
3
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10
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30
Time (minutes)
control
1mM
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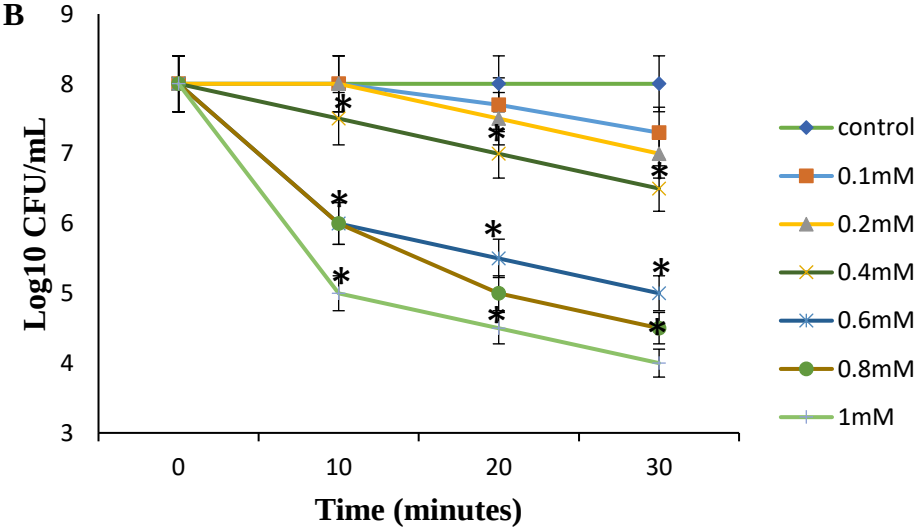
12mM
16mM
20mM

B


Log10 CFU/mL
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Time (minutes)
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0.8mM
1mM

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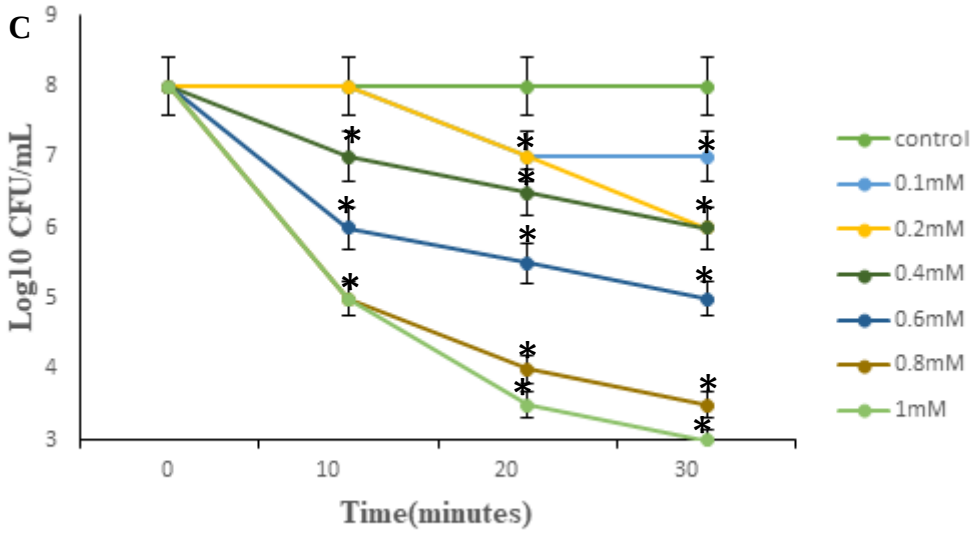
Log10 CFU/mL
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Time(minutes)
control
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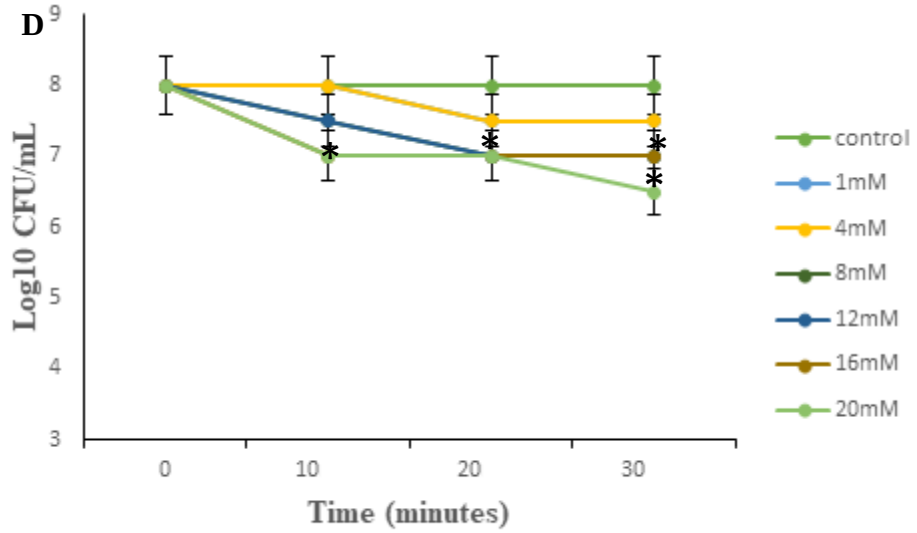
0.4mM
0.6mM
0.8mM
1mM

D


Log10 CFU/mL
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Time (minutes)
control
1mM
4mM
8mM
12mM
16mM
20mM

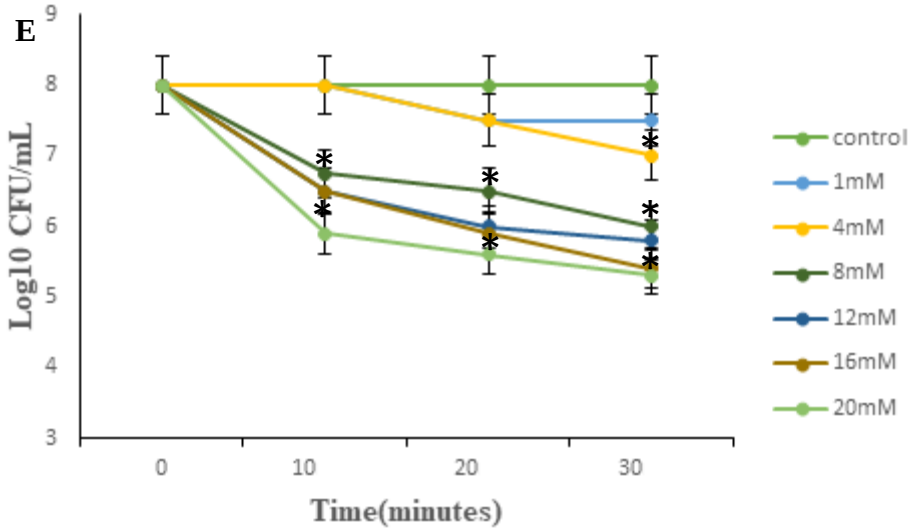


Figure 3. 1 *In vitro* antibacterial and time-kill assay of bile salts treated with S. aureus SH1000

Experiments were performed at a cell density of 10^8 CFU/ml in peptone buffered saline supplemented with 2 mM CA (A), 0.25 mM DCA (B) and 20 mM GCA (C) determined by the viable plate count method. The data represents the mean ± standard error of the mean from three independent experiments. ∗ ($p<0.05$)

3.2.2 Factors affecting bile resistance

In order to colonise in the gastrointestinal tract, enteric bacteria must resist considerable stresses including the detergent effect of bile salts. Studying the mechanism by which enteric bacteria resist bile might inspire a novel strategy for gastrointestinal diseases. It is also one of the criteria for choosing probiotic species.

Several of the limitations involved in studying, documenting and understanding bile resistance in enteric bacteria is related to the strain specificity of bile resistance and the differences in the experimental conditions (type of bile and growth media act). This allows for a comparison of the results between enteric bacteria and even between the strains in one species, which in many cases is unworthy (Margolles *et al*., 2003; Lee & Salminen, 1995; Gilliland *et al*., 2010).

3.2.2.1 Strain specificity of bile resistance

In this study, the strain specificity of bile resistance in *S.aureus* was assessed by determining the minimum inhibitory concentration of bile for a number of bacteria strains.

Five strains of *Staphylococcus* were studied for their bile tolerance. The strains were grown anaerobically at 37°C in 10 ml of TSB. The strains cultures then were used to inoculate the fresh media supplanted with different concentrations of bile. The MIC values were read after 16h of incubation at a cell density of 10^6 CFU/ml.

The results, as shown in **Table 3.2**, indicate that there was no significant difference in the MIC among the studied strains.

A limitation of this study is that the number of strains was relatively small and that these findings cannot be extrapolated to all *Staphylococcus* strains. However, it might suggest that none of these strains has a strain-specific mechanism to resist bile.

These results are in contrast to several studies which investigated bile resistance in enteric bacteria and that suggest that bile resistance is a strain-specific trait and, thus, that the resistance of species cannot be generalised (Buck & Gilliland, 2010; Chateau et al., 1994; Crittenden et al., 2001; Ibrahim & Bezkorovainy, 1993; Jacobsen et al., 1999; Walker & Gilliland, 2010; Zárate et al.,2000)**.** Similarly, Guariglia-Oropeza *et al*. (2018) found phenotypic, genotypic, and ecologic differences among the *Listeria monocytogenes* strains in response to bile stress. In the same vein, Chateau et al. (1994) observed heterogeneity of bile sensitivity in *Lactobacillus rhamnosus* strains which was isolated from colostrum.

3.2.2.2 Nutrient composition affects bile resistance in S. aureus

In this experiment, the effect of the type of media on the level of bile tolerance in *S. aureus* was assessed using an experimental design developed by Kimoto-Nir *et al* (2009) with little modification. BHI, TSB, LB and M9 were prepared according to the manufacturer's instruction and then a sterile bile solution (Oxgall) was added to each medium to make up the final concentration 6% (v/v). Each medium was inoculated with *S.aureus* at a cell density of 10^6 CFU/ml before being incubated at 37°C for 16 h.

The *S.aureus* growth characteristics in BHI , TSB, LB and M9 with or without bile are shown in **Table 3.3.** Bile resistance varies depending on the media. *S. aureus* was unable to grow in M9 containing bile while it grew by more than 60% of the control in BHI or TSB with bile. The growth rate of the bacterium in the LB containing bile was 38% of the control. From the data in **Table 3.3**, it was apparent that the growth media might be related to the bile resistance of *S.aureus*. These results match those observed in earlier studies which suggest that bacteria need energy to exclude bile and that it might use the acquired energy from the media (Yokota *et al.*, 2000).

Table 3. 2- MICs of the bile salts for some *S. aureus stains*

(mM)	*S.aureus SH100*	*S.aureus Newman*	*S.aureus Rn4420*	*S.aureus RN6390*	*S.aureus SA113*
CA	22	22	22	22	22
DCA	1.2	1.2	1.2	1.2	1.2
CDCA	1.2	1.2	1.2	1.2	1.2
GCA	>200	>200	>200	>200	>200
TCA	>200	>200	>200	>200	>200

Data represents triplicate independent experiments.

Table 3.3- Effect of the growth medium on the bile resistance of *S. aureus SH1000.*

Growth media	Control OD_{600}	Bile OD_{600}	Bile resistance (%)	Bile tolerance
BHI	13	8	67	R
TSB	8	5	67	R
LB	8	3	38	S
M9	2	0.0	-	S

BHI, TSB, LB and M9 media containing 6% crude bile or that were bile-free (control). Bile resistance was measured as a percentage of the growth of the control. The growth of *S.aureus* was determined by measuring the absorbance at a wavelength of 600 nm after 16-h incubation at 37 °C. All values are the mean of three samples.

3.2.3 Effect of bile on *S. aureus* virulence

3.2.3.1Adhesion of bile treated staphylococcus to the human intestine epithelium

In this study, we investigated whether the adherence of *S. aureus* to the human intestine epithelial HT-29 cell line was affected by the presence of bile. HT-29 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) 10000 IU/ml penicillin. The cells were grown in tissue culture flasks at 37 °C in a 5% CO_2 atmosphere. The cells were detached by using trypsin and then (5×10^5cells/well) were seeded in a 24-well plate in a DMEM medium with a 10% fetal bovine serum (FBS) and antibiotic mix incubated at 37 0C in 5%CO_2 for 48 h before the addition of the bacteria **(Method 2.23).**

5.0×10^6 CFU/ml of *S. aureus* was inoculated into plate wells containing a monolayer of bile treated and bile free cells (control) that were then incubated by 37 °C in 5% CO2 atmosphere for up 3 h. The cells were washed three times with DMEM (without antibiotic) to flush the unbound extracellular bacteria.

The total amount of adhering cfu was determined as described in **Methods 2.23**. As shown in Figure 3.1, no significant differences were observed between the treated bile and the control related to adherence capacity, indicating that the bile had no effect on the adhesion of S. *aureus* to the intestinal epithelial cells.

In contrast, several studies have demonstrated that bile induces the adherence of bacteria to intestinal epithelial cells. Paceet al. (1997) found that bile treated *Vibrio parahaemolyticus* showed a higher adherence capacity than untreated bacteria. The author concluded that bile enhances the adherence of *Vibrio parahaemolyticus* to the epithelial cells. Similarly, Faherty *et al*. (2012) demonstrated that Shigella utilises bile as an environmental signal to induce the adhesion factors involved in the adhesion of bacteria to the colonic epithelium.

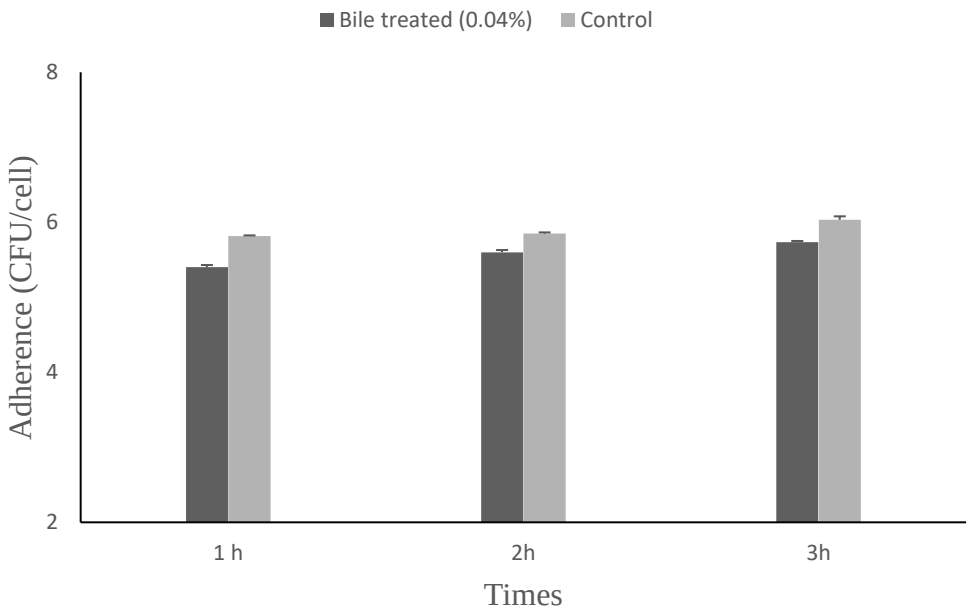


Figure 3. 2 Effect of bile on the adherence of S.aureus in the HT -29 cell line.
The adherence assay with *S. aureus* for up 3 hours in the presence of the physiological concentration of bile (0.04%).

3.2.3 .1 Influence of bile salts on virulence factor

The present study was designed to determine the ability of bile to effect expression of virulence factors in *S. aureus* of this bacteria to bile. It is well established that *S. aureus* has the ability to produce several toxins and virulence factors which causes lyses of RBCs (Otto, 2014). A haemolysis assay was used to determine if the bacteria excreted these factors into the environment (growth media) as a response to sensing the bile.

As mentioned previously in Method 2.19, the overnight growth of *S.aureus* was cultured for 16 h in TSB broth both with and without bile. The culture was span and 0.1 of the supernatants was taken and treated with 1 mL of haemolysis buffer. Two controls were used to determine if the RBC haemolysis was as a result of the effect of toxins and the virulence factor excreted from *S. aureus* or from the effect of the bile molecules themselves. In C1 (control 1), bile was added to the bile free supernatant while C2 contained a bile solution only. Defibrinated rabbit blood was added to the treated and control tubes and then incubated for 15 min at 37°C. The tubes were centrifuged at 5500g, at room temperature for 1 min and the optical density of the supernatant was measured at 543 nm. The percentage of haemolysis was calculated thus: percentage of haemolysis = 100× (absorbance of sample – absorbance of negative control) / (absorbance of positive control – absorbance of negative control).

As shown in **Figure 3.3,** the haemolysis percentage of the bile-treated supernatant was 82% while the bile free supernatant recorded 38.9%. The percentage in C1 and C2 was 80% and 89% respectively. In summary, these results show that significant differences ($P<0.05$) of the haemolysis between the bile-treated bacteria and the non-treated bacteria occur. The effect is due to the bile molecule residue in the media which might be as a haemolytic reaction to the RBCs. Hemolysin is one of the important virulence factors for *S. aureus.* Several studies suggested that rabbit red blood cells are highly

sensitive to α-hemolysin. . γ-hemolysin can damage the red blood cells of human and animals . It was not possible to conclude that bile does not induce haemolysin in *S.aureus* because a not all *S.aureus* strains have this virulence factor and usually restricted to clinical isolated for that further work is required to establish this.

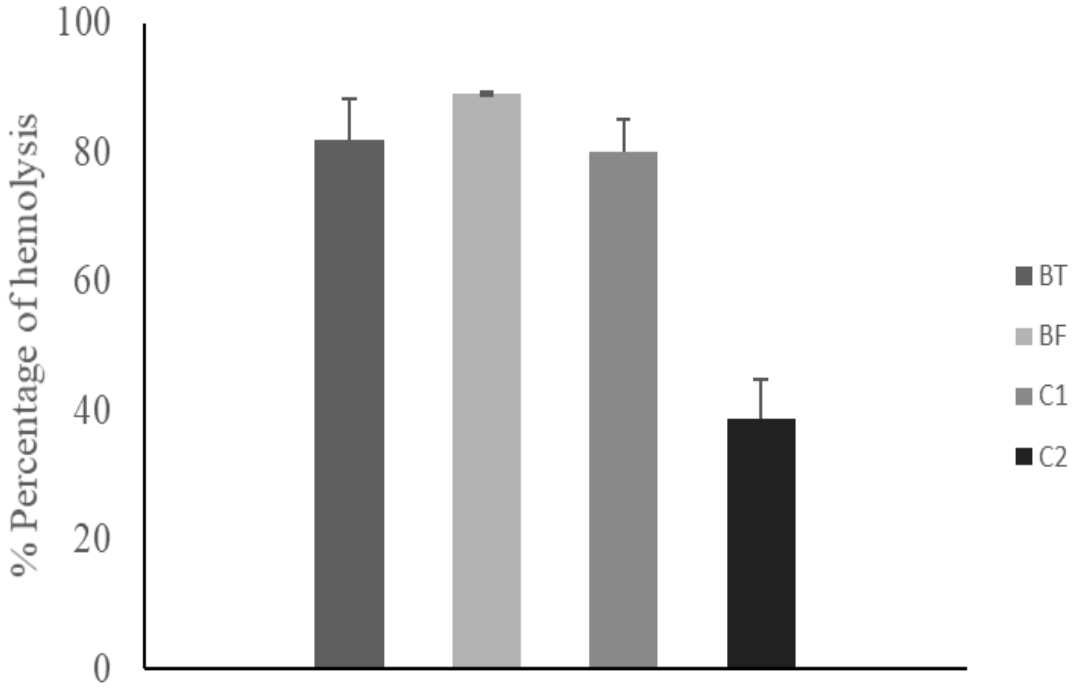


Figure 3. 3 Haemolysis of rabbit blood by a supernatant of S. aureus SH100 in the presence or absence of crude bile.

Supernatant from the overnight culture of *S.aureus* with bile (BT) or without (Bf), and a bile solution (bile dissolves in water) (C1). A bile solution added to the portion of supernatant from non-treated bacteria (C2).The samples added to the defibrinated rabbit blood and incubated for incubated for 15 min at 37°C. Haemolysis was calculated using the following equation. The percentage of haemolysis = 100× (absorbance of sample – absorbance of negative control) / (absorbance of positive control – absorbance of negative control) which was modified from Sowemimo-Coker (2002) & (Evans et al., 2013). The experiment was normalized using the following controls:

-Control positive represented 100% of the haemolysis (added 20% Triton X-10 to the whole blood).

-Negative control represents 0% of the haemolysis (we added a haemolysis buffer to the whole blood).

3.3 Conclusion and discussion

In this investigation, the aim was to assess some of the aspects of interaction between bile salts and *S.aureus*. These aspects include the antibacterial activity of bile on *S.aureus*, the strain specificity of the bile salts, the effect of the type of media on the bile resistance and the effect of bile on the virulence factors of *S.aureus*.

The antibacterial activity of bile and *S.aureus* tolerance to bile was assessed by detecting the MICs of bile and the time course violability of *S.aureus* upon exposure to bile salts. One of the more significant findings to emerge from the study of the MICs of bile found that unconjugated bile acid (CA, CDCA and DCA) has a more potent antimicrobial effect than conjugated bile acid.

It is encouraging to compare this result with that found by Sannasiddappa *et al*., (2017). They found that *S.aureus* was more sensitive to the antimicrobial effect of unconjugated bile acids than conjugated acids. In the same vein, Begley *et al*. (2005) reviewed the bile tolerance of enteric bacteria and found that conjugated bile acids are weaker in their antibacterial effect than unconjugated bile acid. The results of the viability of *S.aureus* upon exposure to bile acids showed that all of the tested bile acids cause a reduction in the viability in *S.aureus* at their subinhibitory concentration and complete death at MICs. Similar to the MICs experiment, the viability test of *S. aureus* showed that the inhibition effect of unconjugated bile acid against *S. aureus* growth is more potent than that of conjugated bile acids. The earlier study shows that the subinhibitory concentration of both types of bile acid (conjugated and unconjugated) can reduce *S.aureus* growth. However, unconjugated bile acids show a higher inhibitory action than the conjugated type (Thippeswamy H. Sannasiddappa et al., 2017).

The major limitation of studying bile resistance in enteric bacteria is related to difficulties in comparing the results between bacterial species and even between the

strains of one species. These difficulties are related to the strain-specificity of bile resistance in a wide range of bacterial species and different experimental designs (type of bile and growth media act) (Margolles *et al*.,2003; Lee & Salminen, 1995; Gilliland *et al*., 2010).

Extensive research has been carried out on bile resistance mechanism in *Listeria monocytogenes* and *Lactobacillus rhamnosus*. These results conclude that bile tolerance in these bacteria differ according to their strains and thus the resistance of the species cannot be generalised (Chateau *et al*., 1994; Guariglia-Oropeza *et al*., 2018). In contrast to the previous research studies, the current study shows no differences in the bile tolerance among the studied strains of *S. aureus.* However, these findings cannot be extrapolated to all *Staphylococcus* strains.

On the other hand, the effect of the type of growth media on the level of bile tolerance in *S.aureus* has been studied and the results show that the bile tolerance in *S.aureus* varies with the type of media. As shown in **Table 3.3**, the growth density of *S.aureus* in the presence of bile in the BHI and TSB media was greater than in the LB medium. The bacteria failed to grow in M9. It might be that the type and concentration of the nutrients in the media governs the rate of bile tolerance in *S. aureus*.

Kimoto-Nira *et al*. (2009) demonstrated that the bile resistance of lactococci can be altered by differences in the growth media. The author suggested that the acquired energy from the media enhances the bacteria's ability to resist bile. The nutrient consistency of the growth media, especially glucose and lactose, has an impact on fatty acid composition. Fatty acids are important for maintaining membrane structure and stability in Gram positive bacteria when it interacts with the detergent effect of bile which acts on the stability of the cell membrane.

Chapter Four: FadB confers bile salt resistance in *S.aureus*

4.1 introduction

4.1.1 Membrane architecture and composition plays a role in bile resistance to enteric bacteria

In order to survive and colonise the human gut, enteric flora and pathogens must resist various stresses and challenges throughout the human gastric tract including variations in pH, low oxygen level, high osmolarity, nutrient limitation, local gut immunity and the potent antimicrobial activity of bile (Sannasiddappa, 2014; Gunn, 2000; Begley *et al*., 2005). The main function of bile is to act as a biological detergent (Begley *et al*.,2008). Because bile is surface active and made up amphipathic molecules, it acts as a detergent gives bile a potent antibacterial activity. For this reason, resistance to bile salts is an essential requirement for flora and enteric pathogens to survive in the human intestine (Hofmann, 1999; Gunn, 2000). Thus, resistance to bile salts is an essential requirement for commensal and enteric bacteria to survive in the human intestine. It has been reported elsewhere that commensal and enteric bacterial pathogens have developed multiple bile resistance mechanisms such as detoxification of bile salts by producing bile salt hydrolases (Begley *et al*., 2005a; Coleman & Hudson, 1995; De Boever *et al*., 2000; Grill et al., 2000; Gunn, 2000), modulating synthesis of cell wall lipopolysaccharide and porins, and efflux pumps (Begley *et al*., 2005a; Gunn, 2000).

Changes in the architecture/composition of the cell membrane and cell wall are one of the most important mechanisms in bile resistance. It has been demonstrated that bile has a destructive effect on cell membranes and it also has the ability to disorganise the structure of the cell membrane (Ruiz *et al*., 2012a, b; Kristoffersen *et al* , 2007;Sannasiddappa *et al*., 2015). Fernández (1999) suggested that the bile tolerance of *L. monocytogenes* alters as a result of changes in the lipopolysaccharide (LPS),

membrane electric charge, hydrophobicity, lipid fluidity and fatty acid composition. Taranto *et al*, (2013) found that bile induces changes in the lipid profile in the cell membrane of *L. reuteri* CRL 1098 and that this change is considered to be one of the main physiological responses of *L. reuteri* related to survival in the gastrointestinal tract.

4.1.2 Bile resistance in gram positive bacteria

Bile represents a serious challenge to enteric pathogens**.** The specific mechanisms by which Gram-positive bacteria resist bile are not fully understood. Bacterial membrane architecture and composition plays an important role in the bacterial tolerance to bile (Begley *et al*., 2005a; Kristoffersen *et al*, 2007). Fatty acid, lipid species and its molar ratios have an effect on the permeability of the cell membrane (Anibal & Simon, 2017). It has been shown conclusively that the genes and proteins involved in lipid metabolism are induced in response to bile salt exposure (Alcantara & Zuniga, 2012; Begley *et al*., 2005a; Kristoffersen *et al*., 2007; Wu *et al*., 2010). Previous research has indicated that the genes in *Staphylococcus*, in which the genes encode major lipid metabolising enzymes such as 3-hydroxyacylCoA dehydrogenase, have a positive impact on the resistance of these bacteria to antibiotics and biocides. No role in bile resistance has been illustrated (Jang *et al*, 2008).

In this study, a bile regulated protein in *S. aureus* was identified and its gene was expressed in a heterologous host in order to study its functional effect. In *S. aureus* the gene was deleted and the *in vitro* sensitivity of the mutant to various bile salts and antibiotics was tested. Furthermore, the survivability of the mutant in the human was tested.

4.2 Results

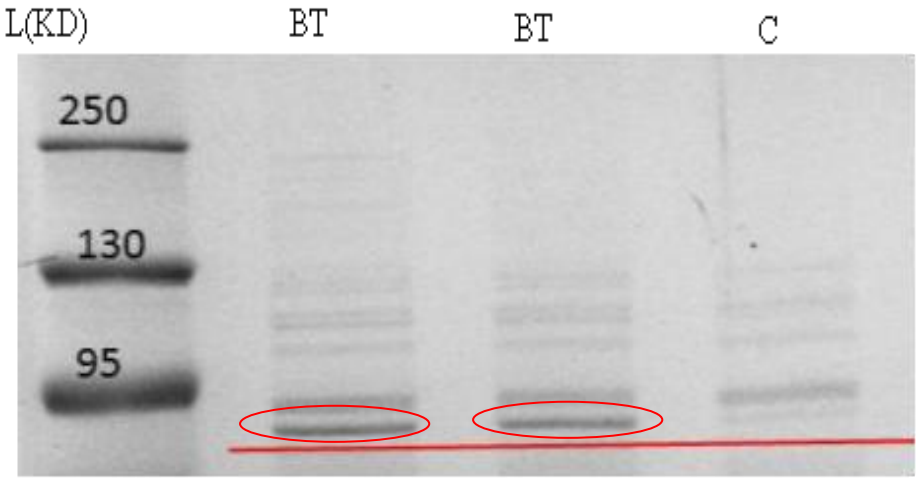
4.2.1 Identification of the up regulated protein in response to bile

A comparative study was performed between bile-treated and untreated. *S. aureus* SH1000 cultured under bovine bile stress (8% w/v) at 37^0c until the stationary phase in the same condition of non-bile treated bacteria. The proteins were extracted from the cell wall using fast prep methods**(Material 2.20)**. To determine differences in protein expression in the presence or absence of bile, the extracted proteins were separated using Coomassie Stain one-dimensional gel (SDS-Page) **(Figure 4.1-A).** Band differences were identified between the bile treated and non-treated bacteria. The unique protein band of ~ 85 kDa was found in the treated bacteria but not in the non-bile treated bacteria. The unique protein band was extracted and analysed using mass spectrometry (MS). The mass and bioinformatics results (**Figure 4.1-B**) show that a putative 3-hydroxyacyl-CoA dehydrogenase had the highest score and coverage compared with the other analysed protein.

3 hydroxyacyl-CoA dehydrogenase encoded by the *fadB* gene has a potential role in lipid transfer and metabolism. The molecular function of 3-hydroxyacyl-CoA dehydrogenase is oxidoreductase (Jang *et al*., 2008). The metabolism of lipids in bacterial cells is complex and it quickly adapts to changes in the bacterial cell environment. In E.coli , 3 hydroxyacyl-CoA dehydrogenase is one of stress-response proteins and it might have a role in the survival of bacteria under various stress factors (DiRusso *et al*., 1992).

Jane and his colleagues (2008) found that hydroxyacyl-CoA dehydrogenise is involved in the regulation of *S. aureus* and its survival under exposure to triclosan as a stress factor. Multiple researchers (Alcantara & Zuniga, 2012; Begley *et al*, 2005; Kristoffersen *et al*, 2007; Wu *et al*, 2010) found that the genes involved in lipid metabolism, cell membrane biogenesis and efflux transporter were induced as a response to bile exposure. Furthermore, in many bacterial species, bile resistance levels alter as a result of changes in their lipopolysaccharide (LPS), lipid fluidity and fatty acid composition (Urdaneta & Casadesús, 2017). Therefore, the homologous *fadB* (ID: 5331895) in *S. aureus* SH1000 was deleted and its phenotype was analysed accordingly.

A



B

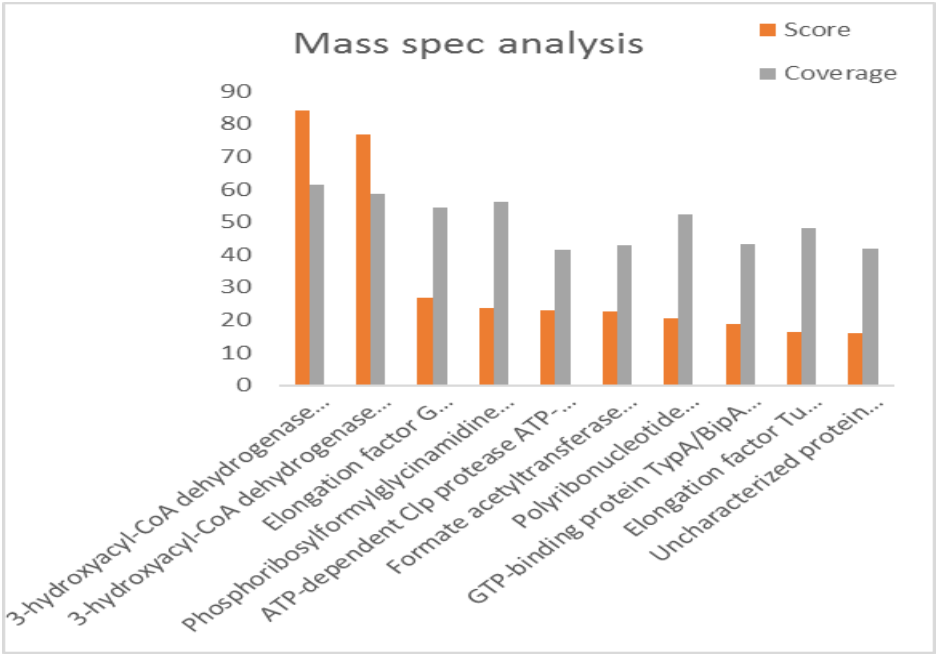


Figure 4. 1 A Coomassie-stained 1D electrophoresis gels of the cell membrane fraction proteins extracted from *S. aureus SH1000* cells both untreated (C) and treated with 8% bile (BT).

The figure shows that the protein band of the soluble fraction was significantly more abundant in the bile-treated cells (Red circle). **B *Mass Spectrometry analysis*** for the protein band showed the highest score and coverage for the 3-hydroxyacyl-CoA dehydrogenase protein.

4.2.2 Construction of *fadB* mutant *S. aureus*

PMAD is a temperature sensitive shuttle vector **(Figure 4.2)**. This vector was used to construct *fadB* mutant *S. aureus* via allelic replacement. As described by Arnaud *et al*., (2004), pMAD was constructed for the gene mutants in low-GC-content, gram-positive bacteria. To generate the chromosomal *fadB* mutant in *S.aureus*, we constructed the pMAD-carrying part of 1000bp both up and downstream of *fadB* **(Figure 4.3)**.

4.2.2.1 PCR amplification of fadB upstream and downstream region

The primers were designed to amplify by at least 1000bp both up and downstream of fadB**.** The amplified fragments were analysed through agarose gel electrophoresis to estimate their size before being cloned in a pjet blunt vector **(Figure 4.4).** The presence of purified ~10000 bp up or downstream of *fadB* in pjet was confirmed by restriction mapping with *Bgl*II and subsequent nucleotide sequencing **(Figure 4.5).**

4.2.2.2 Construction of the pMADΔfadB vector

After the subcloning up and downstream of the *fadB* fragment in the pjet blunt vector, the upstream fadB in pjet was digested with *Bgl*II and *Sma*I and ligated with the pMAD vector. This was digested with the same restricted enzyme. The resulting pMAD/upstream *fadB* was digested with *Sma*I and *BamH*I and ligated with downstream fadB fragment in pjet, which was digested with *Sma*I and *BamH*I. The presence of *fadB* up and downstream fusion construct in the recombinant pMAD vector was confirmed by PCR amplification using P1 and P4 primers(Appendix 1) which result in the amplification of ~2000 bp *fadB* up and downstream and subsequent nucleotide sequencing (Figure 4.3).

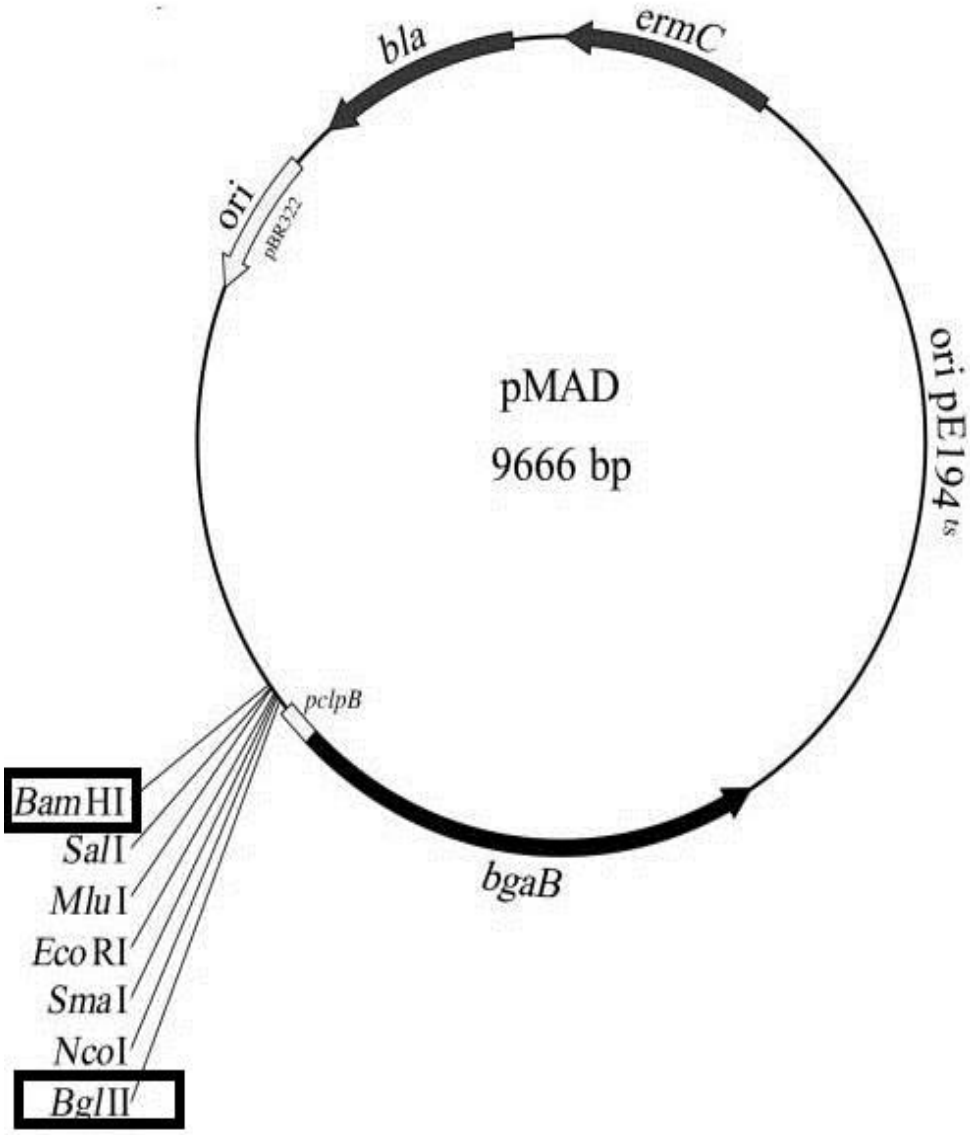


Figure 4. 2 Physical map of the pMAD vector.

The *bga*B – DNA fragment containing the promoter-less *bga*B gene encoded a thermostable βgalactosidase; *erm*C – erythromycin selective marker; the *pclp*B – DNA fragment from *S. aureus* contained the *clp*B promoter. The unique restriction sites have been indicated. The *fadB* up and downstream fusion construct was cloned between the *Bam*HI and *Bgl*II site (Arnaud et al., 2004).

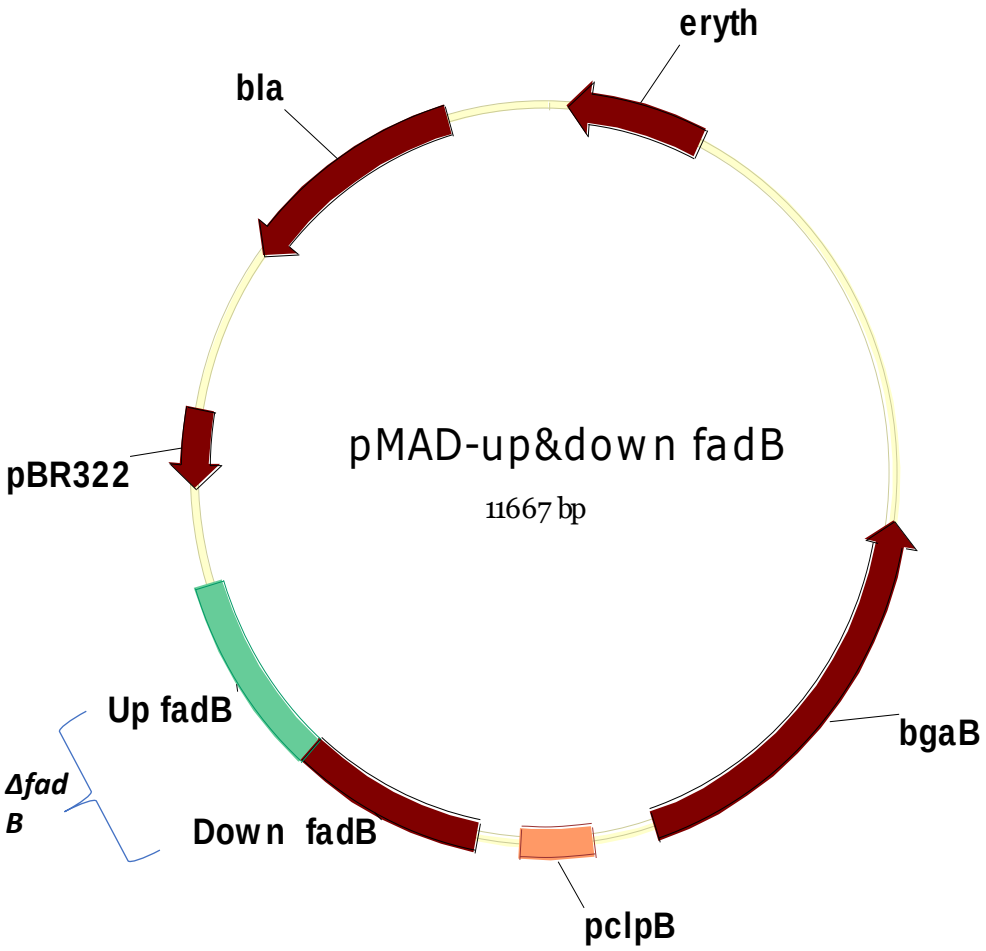


Figure 4. 3 Physical map of the pMAD up & down *fadB* plasmid.

The Δ *fadB* gene represents a fusion of the up and downstream regions of *fadB*.

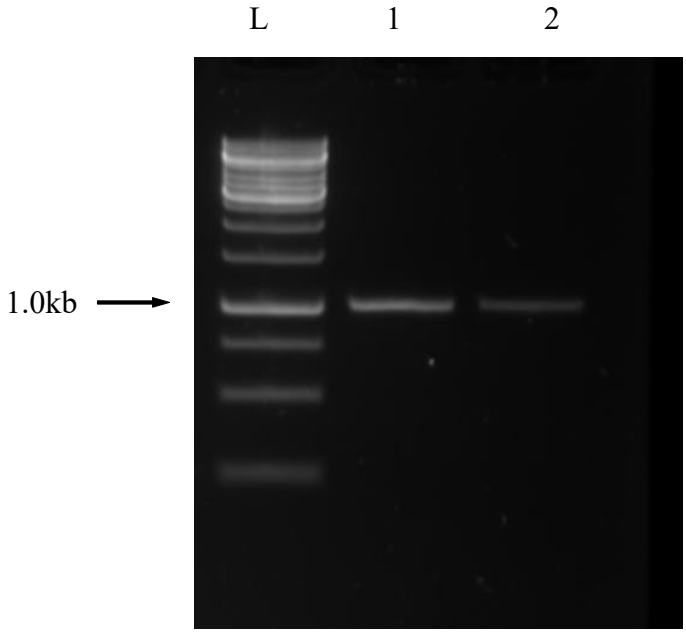


Figure 4. 4 Analysis of the PCR products of *fadB* up and downstream regions.

Lanes 1 and 2 represent the ~1000 bp band of the *fadB* upstream and downstream fragments respectively in 1% agarose gel. Lane L represents the standard 1 Kb DNA ladder (Thermo Scientific).

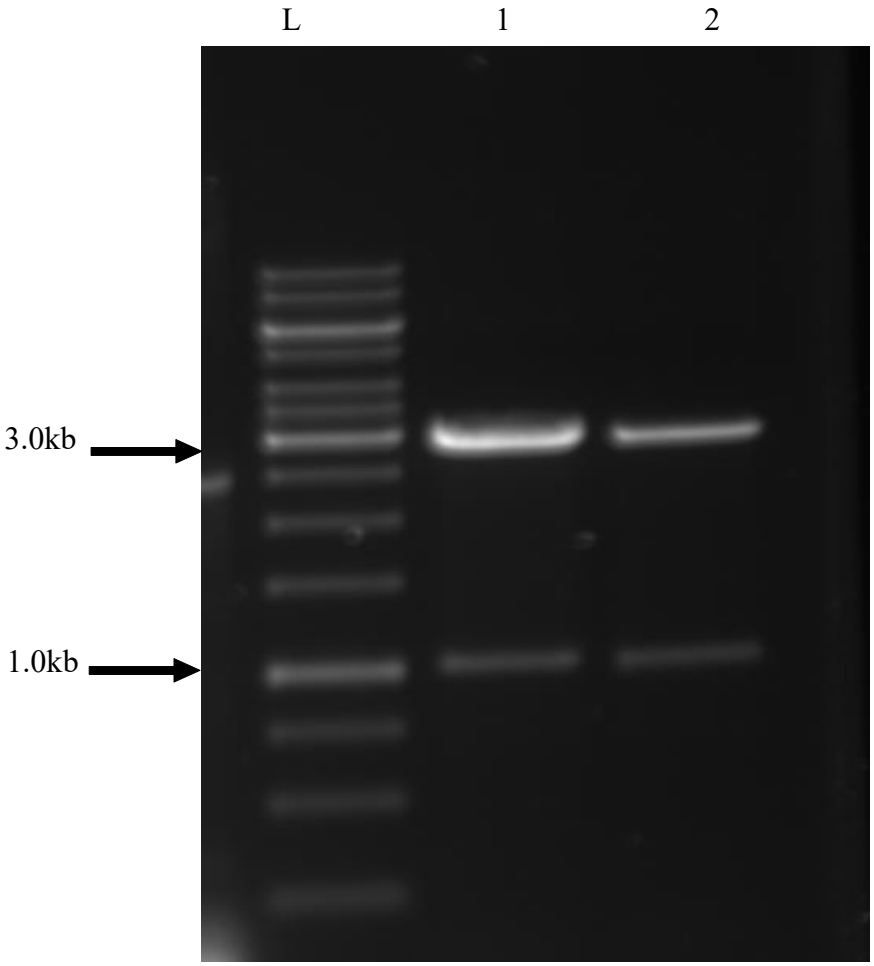


Figure 4.5 Restriction mapping of *fadB* up and downstream regions in pJET1.2/blunt cloning vector.

Lane 1 represents the pJET1.2/blunt cloning vector containing the fadB upstream region restricted with *Bgl*II. Lane 2 represents the pJET1.2/blunt cloning vector containing fadB downstream region restricted with *Bgl*II. The presence of *fadB* upstream and downstream regions in the vector was indicated by the ~1000 bp band with a 3000 bp vector backbone.

4.2.2.3 Mutagenesis and confirmation fadB deletion in *S. aureus*

Ampicillin resistant colonies *E.coli* top 10 appeared on the nutrient agar plates which contain pMAD/up-down was selected. The extracted pMAD/up- down fusion *fadB* was electroporated into *S. aureus* RN4220 (Methods 2.9.2). Erythromycin resistant colonies were selected and the plasmids were transduced from *S. aureus* RN4220 through to *S. aureus* SH1000 (Methods 2.10.4.2 Transduction). The transduced *S. aureus* SH1000 pMADΔ*fadB* was grown at a non-permissive temperature of 42°C for 24h. A few light blue colonies represented the co-integrates of the plasmid into the *S. aureus* chromosome. The selected colonies were then grown at 30°C, the permissive temperature, and then grown again at 42°C. In this step, the second homologous recombination event resulted in the generation of both ΔfadB and wild type strains and the plasmid was eliminated **(Figure 4.6)**. White colonies were candidates for erythromycin sensitivity. Erythromycin labial colonies were selected to confirm the deletion of fadB by PCR using P1and P4 primers and comparing the PCR product with the wild type. The wild type shoed the PCR product band of ~4260 bp, which represents ~2000 bp up and downstream of fadB and ~2260 *fadB* gene. Δ*fadB* showed a DNA band of ~2000 bp which represents the up and downstream of *fadB* and frame deletion of fadB (Δ*fadB*) **(Figure 4.7)**.

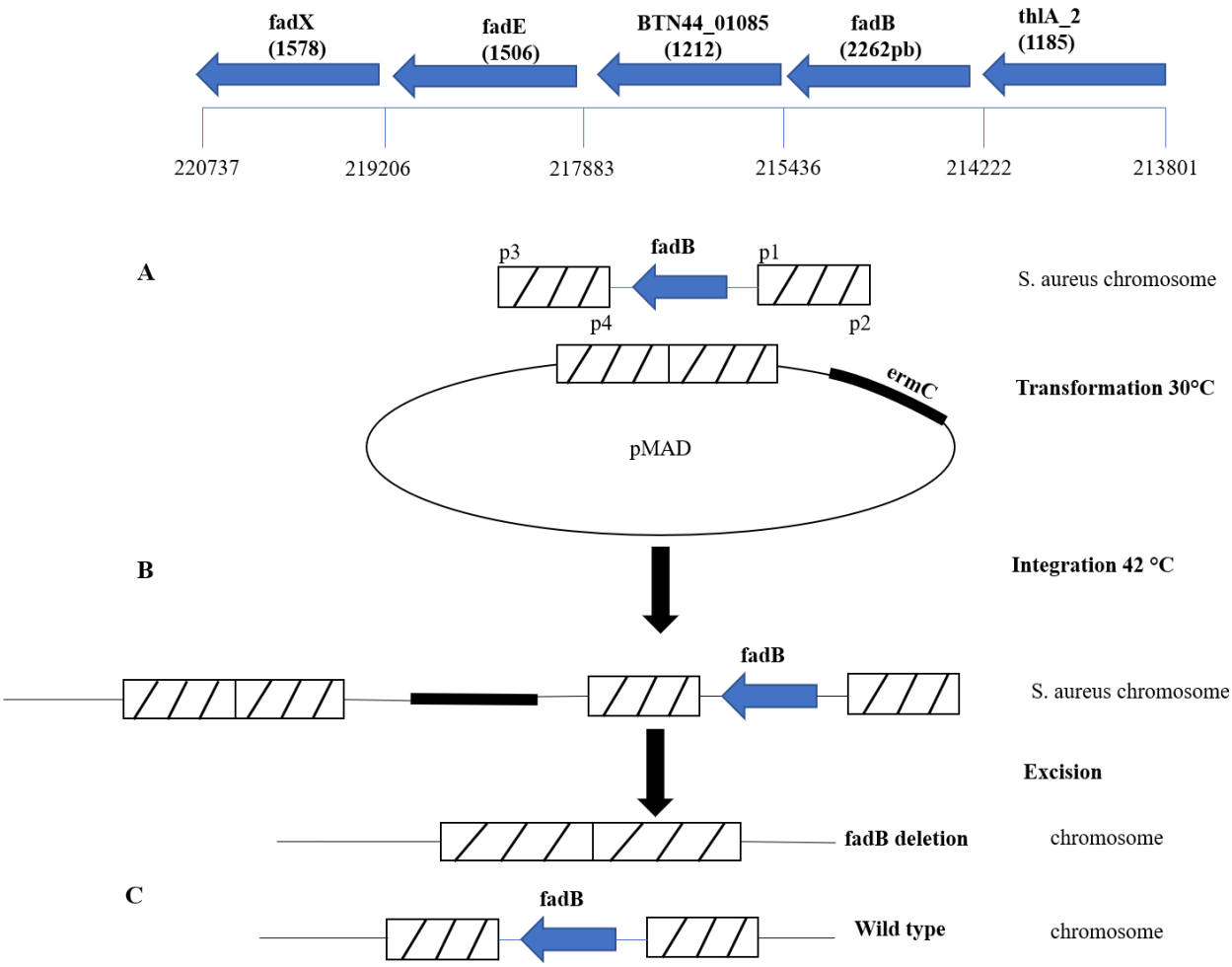


Figure 4. 6 Schematic representation of allelic replacement.

(A) A DNA fragment flanked by two homologous regions encoding upstream and downstream of fadB gene cloned into the pMAD vector and introduced into *S. aureus* at 30 °C onto a TSB plate containing erythromycin. (B) The integration of the plasmid into the chromosome through homologous recombination achieved by shifting them to 42 °C, a non-permissive temperature for plasmid replication, while maintaining selection for erthromycine resistance. The first recombination event occurred in either the upstream or downstream region. (C) The excision of the plasmid region in the chromosomes by a double-crossover event in the opposite side of the region. This is as the first integration event leads to markerless gene deletion. Homologous recombination on the same side of the region leads to the regeneration of the wild type sequence.

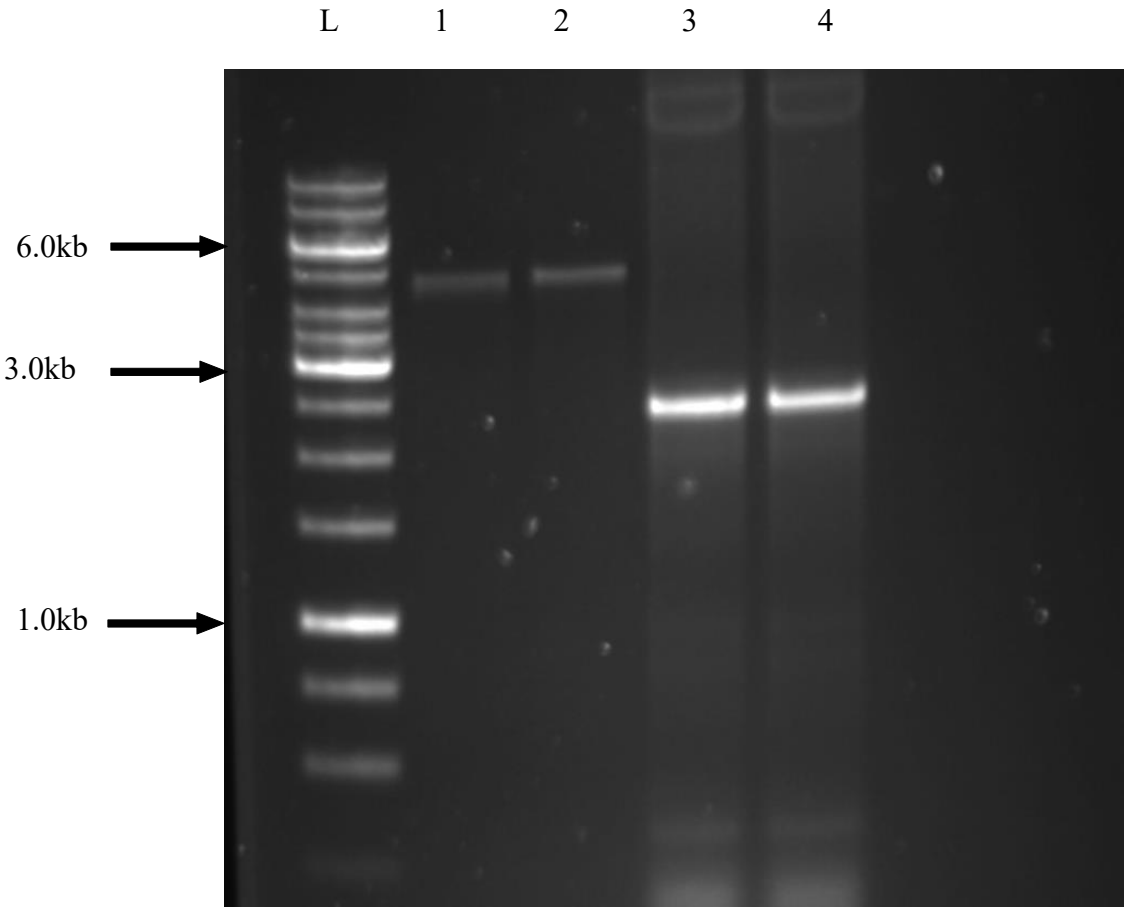


Figure 4. 7- PCR confirmation of S. aureus Δ*fadB*

The PCR products amplified by primer pairs P1 and P4 were analysed through agarose gel electrophoresis (1% agarose). Lanes 1 and 2 represent the ~4600 bp band of wild type *S. aureus* with an intact *fadB* gene up and downstream of *fadB*. Lanes 4 and 5 represent the ~2000 bp band of Δ*fadB* in *S. aureus*. Lane L represents a standard 1 Kb DNA ladder (Thermo Scientific).

4.2.3 Controlled expression of *S. aureus fadB* in *E. coli JW* Δ*3822*

To enable us to study the functional effect of *fadB*, the gene was cloned under the control of the arabinose inducible PBAD promoter. This allows for the controlled expression of genes in E. coli in response to the arabinose levels (**Figure 4.8**). The *fadB* gene encoding region (~2262 bp) was amplified from *S.aureus* SH1000's genomic DNA using *fadB* For and *fadB* Rev primers (**Figure 4.9**). The purified and confirmed PCR product was then subcloned into the pJET1.2/blunt cloning vector and then transformed into *E.coli* TOP10. The pJET1.2/blunt cloning vector with the *fadB* insert released by *Sac*I and *kpn*I digestion and ligated with pBAD/HisA restricted with *Sac*I and *kpn*I. After the transformation into competent *E. coli* TOP10, the AmpR transformants were selected on agar plates. Positive clones were confirmed by restriction mapping with *Sac*I and *kpn*I. The *Sac*I and *kpn*I digestion of pBAD/His A resulted in the generation of 2 bands of ~4100and ~2260bp respectively **(Figure 4.10).** The complete sequence of the insert was subsequently obtained and found to match that expected.

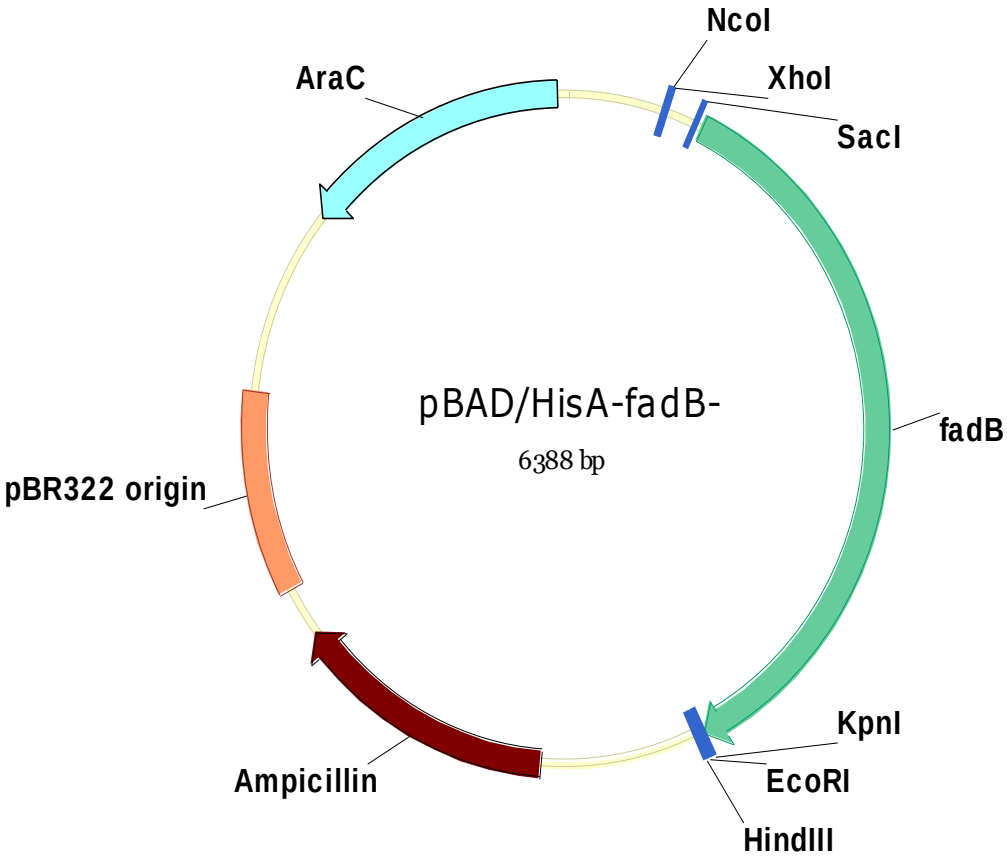


Figure 4. 8 Physical map of pBAD/ His A expression vector.

S. aureus *fadB* was cloned into multiple cloning sites (MCS) between the SacI and KpnI restriction site (Invitrogen).

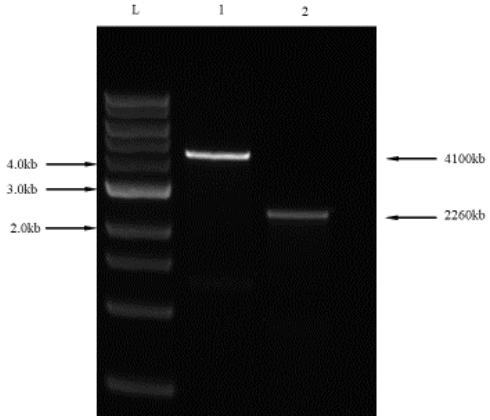


Figure 4. 9 Gel electrophoretic analysis of the pBaD *and fadB* PCR products.

Lanes 1 represents the ~4100 bp band of pBaD **/HisA**. Lane 2 represents the ~2260 bp band of the *fadB* PCR products. Lane L represents the standard 1 Kb DNA ladder (Biolab).

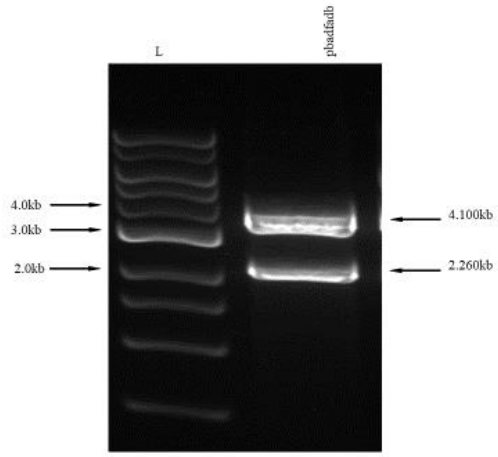


Figure 4.10 Gel electrophoretic analysis of pBaD-*fadB*//HisA restriction digestion by *Sac*I and *kpn*I.

L represents the marker (GeneRuler 1 kb DNA ladder, Biolab). Lane 1 possesses two DNA bands of size ~4.1 and ~2.2 Kb from the SacI restriction of pBaD-*fadB* /HisA. Electrophoresis was performed using a 0.8% agarose TAE gel.

4.2.4 Growth and MICs of the bile salts for *S. aureus* wild type and Δ*fadB*

The MIC of bile salts was determined through a serial twofold dilution in BHI. *S. aureus* SH1000 and *S. aureus* SH1000 Δ*fadB* were grown in normal laboratory media (TSB) and both strains showed a normal growth rate without any growth defects **(Figure 4.11).** The MICs of bile salts for both the wild type and *S. aureus* Δ*fadB* were read after an overnight incubation at 37C, with each tube inoculated with 10^6 CFU/ml in BHI medium. The MICs of the bile salts have been summarised in **Table 4.1** Result are from three independent experiments. The MICs of the bile salts for *S. aureus* Δ*fadB* decreased threefold for cholic acid and twofold for deoxycholic acid and chenodeoxycholic acid respectively when compared to wild type cells. Notably, the MICs of glycocholic and taurocholic acid were unchanged.

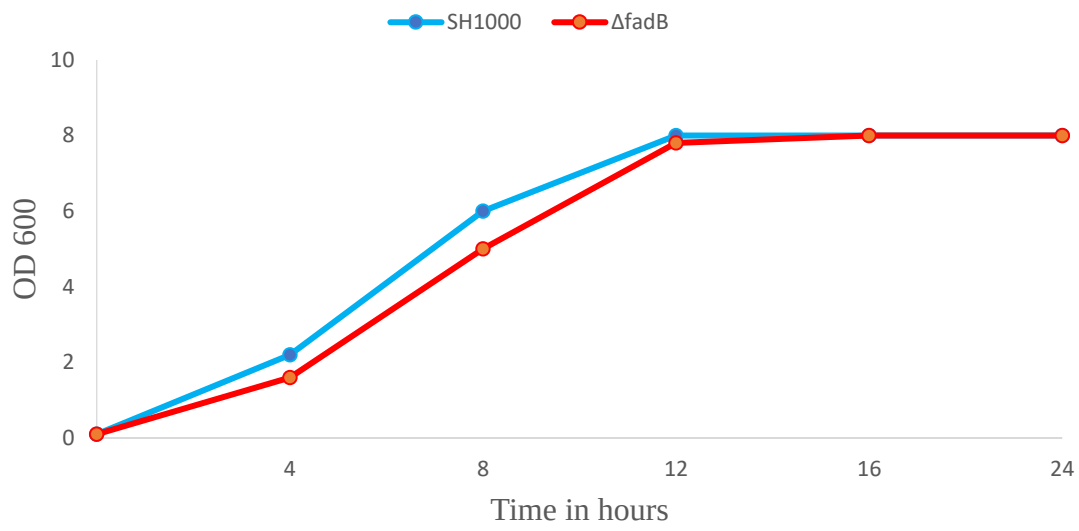


Figure 4.11 Growth of wild type and *S. aureus* SH1000 and Δ*fadB* in TSB media.

S. aureus SH1000 and ΔfadB were inoculated into the TSB media at 1% inoculum before being grown at 37°C for 24 h in an orbital shaker.

Table 4. 1- MICs of bile salts for *S. aureus* SH1000 and Δ*fadB*

Bile salt	Wild type (mM)	ΔfadB (mM)
CA	22	7*
DCA	1.2	0.6*
CDCA	1.2	1.2
GCA	>200	>200
TCA	>200	>200

The data represents three independent experiments. *P<0.005

4.2.5 MICs of bile salts for wild type and recombinant *E. coli JW* Δ*fadB* expressing *S.aureus fadB.*

To test the involvement of the *fadB* gene product to bile resistance, the growth of wild-type *E. coli* BW25113 and *E. coli* JW ΔfadB, expressing *S. aureus fadB* under the control of the arabinose-inducible PBAD promoter under bile salt stress, was investigated by measuring the minimum inhibitory concentration (MIC) of each bile salt (**Table 4.2**). As shown in **Table 2**, *S. aureus fadB* expressed fadB in *E. coli* JW Δ*fadB*, which resulted in a phenotype that was more than one-fold resistant to cholic acid than E. coli JW Δ*fadB*. It was found to be close to the parent strain BW25113. In the case of deoxycholic acid and chenodeoxycholic acid, there was no significant difference among the strains. This result clearly shows the expressed *fadB* enhanced resistance of *E .coli* to bile salt and especially to cholic acid.

Table 4. 2The minimum inhibitory concentration (MIC) end points of the bile related to the wild type and recombinant *E. coli* strains expressing *fadB* at different levels of arabinose induction.

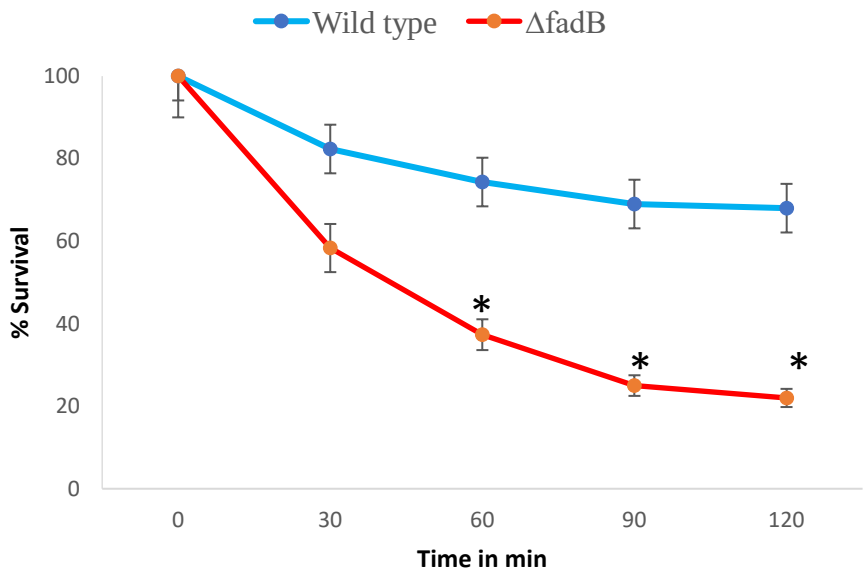
Bile salt	Wild type BW25	*Vector* control* (*JW* pBAD)	*JW* Δ*fadB*	Recombinant		
				JW Δ*fadB* pBAD *S.a fadB*		
				0% Arabinose	0.02% Arabinose	2% Arabinose
CA	60	30	30	30	50	50
DCA	4	4	4	4	4	4
CDCA	4	4	4	4	4	4
GCA	120	80	80	80	100	100
TCA	120	80	80	80	100	100

* Arabinose was not added to vector control . It has been reported elsewhere that empty pBAD has no effect on Bile resistance in *E.coli* (Sannasiddappa *et al*., 2015).

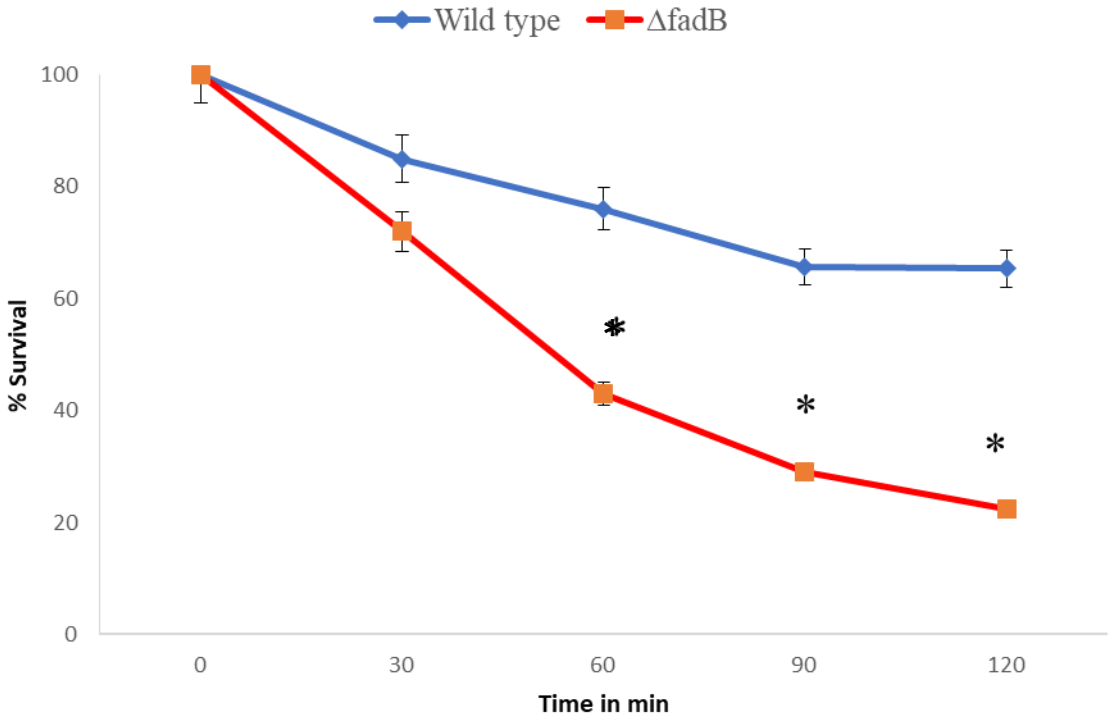
4.2.6 Time-course in vitro kill kinetics of *S. aureus* Δ*fadB* in bile salts

The in vitro antibacterial and time-kill assay of bile salts on wild and ΔfadB *S. aureus* were tested at the sub-MIC level of CA, DCA and GCA for 120 min. Killing assays were performed as described previously (Method 2.19). Peptone buffer saline containing 2,.25 and 20mM of CA, DCA and GCA were respectively inoculated at a cell density 108 CFU/ml. As shown in Figure 4.12, the viability of *S. aureus* Δ*fadB* significantly decreases ($P<0.05$) in the presence of CA and DCA over 120 min of exposure in comparison with the wild type. Δ*fadB S. aureus* showed an 80% reduction in their viability when exposed to CA and DCA for 120 min. The decrement of viability was 60 - 65 in the case of wild type *S. aureus*. No significant differences were found between wild and *fadB* mutant *S. aureus* in the case of GCA **(Figure 4.12)**. Overall, the reduction of the MIC of the bile acid and the killing time of *fadB* mutant *S. aureus* as a comparison to the wild type indicated that the *fadB* has a role in the resistance of *S.aureus* to bile.

A


Wild type
ΔfadB
100
80
60
40
20
0
% Survival
0
30
60
90
120
Time in min
*
*
*

B


Wild type
ΔfadB
100
80
60
40
20
0
% Survival
0
30
60
90
120
Time in min
*
*
*

C

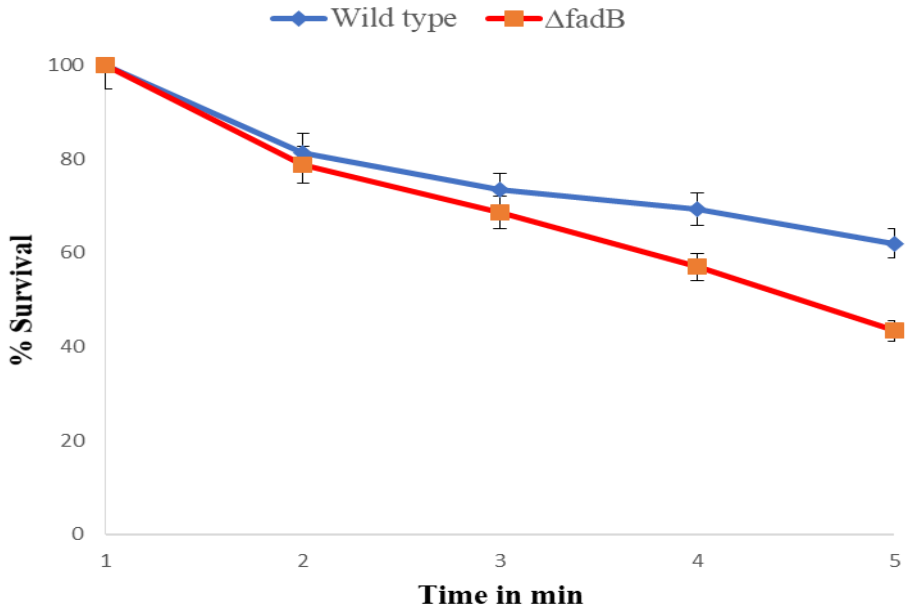


Figure 4. 12 In vitro killing of wild and ΔfadB S. *aureus* to bile salts. Viability of wild and Δ*fadB S. aureus* cells inoculated at cell density of 10^8 CFU/ml in peptone buffered saline supplemented with 2 mM CA (A), 0.25 mM DCA (B) and 20 mM GCA (C). Cell counts were then determined by viable plate counting. Data represents mean ± standard error of mean from three independent experiments.

4.2.7 *In vitro* killing of *E. coli* JW *3822* by bile salts.

The viability of *E. coli Jw3822* expressing *fadB* under the control of the arabinose-inducible PBAD promoter was tested to further support the *fadB* dependent increase in the MICs of bile salts. *E.coli* Jw3822 was grown at 37 °C for 16h in the LB medium and then the culture was used to inoculate the LB medium containing the sub-MICs of CA, GCA or DCA. TCA and 0.02 and 2% arabinose were used as an inducer. The results are as shown in **Figure 4.13** indicating the viability of the recombinant strain (as a control) with and without arabinose. They are similar, with no noticeable effect of arabinose in relation to the survival of recombinant *E. coli* JW3822 cells. A significant increase ($P<0.05$) in the viability of E. coli JW3822 fadB cells was induced by 2% arabinose when exposed to 10Mm of CA compared with E. coli JW3822 cells (**Figure 4.13)**. There were no significant differences between *E. coli* JW3822 f*adB*+0.02% arabinose and E. coli JW3822 when exposed to 10Mm of CA(**Figure 4.13)**. In the case of DCA, no significant differences ($P>0.05$) in viability were found between E. coli JW3822 and arabinose-inducted *E. coli* JW3822 *fadB*. In the same vein, *E. coli* JW3822 fadB+0.02% arabinose showed no significant increment of viability when exposed to 50mM of GCA or 50mM of TCA compared with *E. coli* JW3822 (**Figure 4.13)**. The viability of *E. coli* JW3822 fadB+2% arabinose significantly ($P<0.05$) increased when exposed to 50mM of GCA or 50mM of TCA compared with *E. coli* JW3822 (**Figure 4.13)**.

Overall, these results indicate that the heterologous expression of *FadB* from *S. aureus* in *E. coli Jw3822* increase resistance of the recombinant strains to bile salts, specifically CA, TCA and GCA.

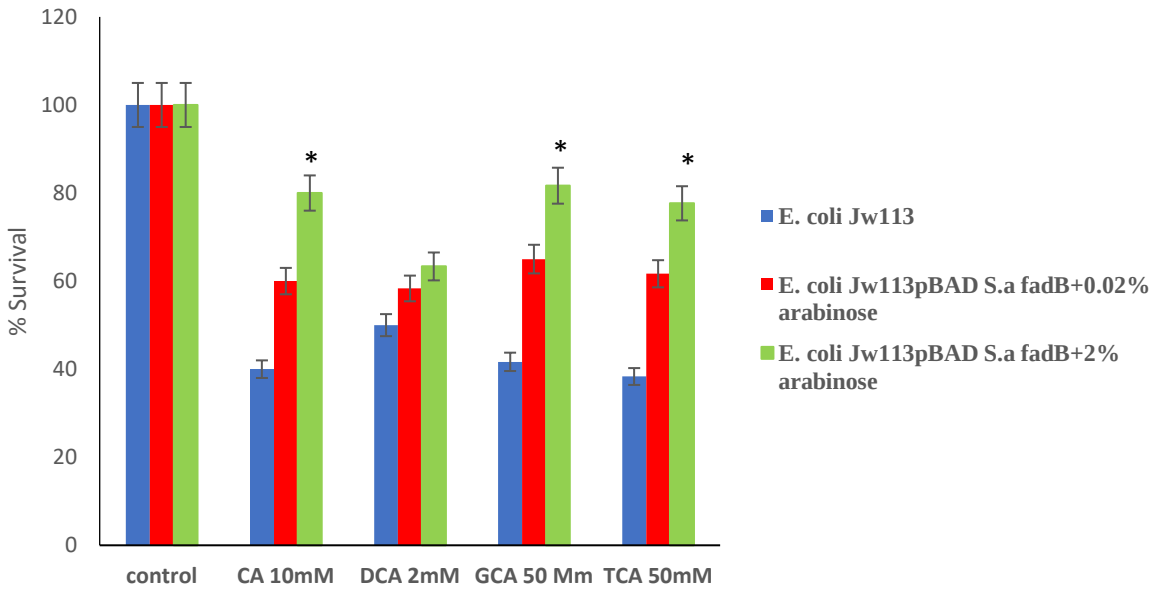


Figure 4. 13Comparison of the survival rates after 16h of exposure to bile salts for E. coli Jw3822 and JW3822 fadB.

Viability of wild type *E. coli Jw3822* pFadB cells inoculated at a cell density of 106 CFU/ml in LB medium containing either 0.02 or 2% arabinose supplemented with CA (10 and 20 mM), DCA (2 and 4 mM) and GCA (25 and 50 mM) and then grown for overnight at 37°C. Cell counts were then determined by viable plate counting. Data represents mean ± standard error of mean from three independent experiments. *P<0.005

* This experiment lack to vector control . It has been reported elsewhere that empty pBAD has no effect on Bile resistance in *E.coli* (Sannasiddappa *et al*., 2015).However the role of *fadB* in bile resistance needs to be confirmed by vector control.

4.2.8 Bioinformatic of the FadB protein

4.2.8.1 Characteristics of primary and secondary structure of FadB

ProtParam tool provided by the bioinformatics resource portal ExPASy were used to analyse of the amino acid sequence of FadB. The analysis shown that FadB is predicted 753 amino acid with a predict molecular weight of 84608.42 Da and a predicted isoelectric point of 5.63. Instability index predict that FadB is stable protein. Leu consist 16% of Amino acid composition which considered as a most dominant amino acid. The total number of negative charge residue (Asp + Glu) is 102 with 85 positive residues (Arg + Lys) is 85 resulting in a net negative charge for FadB at pH 7.

The primary and secondary structure of the FadB protein was predicted using the ExPAsy Protein Knowledge database (UniprotKB) (http://www.uniprot.org/uniprot/) and TMHMM server (http://www.cbs.dtu.dk/services/TMHMM) available on the World Wide Web.

A predicted secondary structure of FadB predict that no transmembrane helices. The majority of the amino acid residues found consisted of hydrophobic residues and FadB is predicted to be a cytoplasmic protein .

Impact of *fadB* for *S. aureus* survival in the *in vitro* human colonic model.

In this study, the single-batch fermentation system was used to simulate the human colon. The role of the fadB gene in the survivability of staphylococcus was tested in a molecular quantitative study of an *S. aureus* Δ*fadB* population as a comparison with the wild type and in the presence of Microbiota. Wild type and *S. aureus* Δ*fadB* were grown in TSB media adjusted to a pH between 5-8.5(**Figure 4.1 5**). The resulting growth curve showed no effect from pH on the growth of wild type and ΔfadB *S. aureus*. There were no significant differences in the growth profile between the two strains **(Figure 4.1 6).** Subsequently, it was discovered that neither the acidic pH nor basic pH had any impact on the development of *S. aureus* ΔfadB. Notwithstanding, the decreased development of *S. aureus* ΔfadB in the human colonic media that was enhanced with and without bile salts contrasted with the wild sort ($P<0.05$). **Figure 4.16** proposed that *fadB* might be required for *S. aureus* survival in the human colon.

To test this plausibility, a single-batch fermentation system (as in vitro human colonic) was used to evaluate the role of fadB in relation to the survival of *S. aureus* in the human gut. The anaerobic batch culture systems were supplied with basal media (material 2.4.7) and then inoculated with 5ml of 10% faecal slurry from healthy adult volunteers prepared with anaerobic phosphate-buffered saline (PBS, 0.1 M, pH 7.2).

A colony count was performed to check the presence of any detectable *S. aureus* in the system. A single dose of *S. aureus* wild type and Δ*fadB* (10^{10} CFU/mL) were used to inoculate the vessel and then 5ml of sample was taken over a period of 4 - 48h. The numeration of the wild type and ΔfadB were detected using a flow cytometer fluorescence in situ hybridisation (Flow-FISH). The results show that the total counts of the wild type were 5.9 and 6.1 Log10 CFU/ml after 4 and 8 h of incubation respectively. The population then stabilised at 7 Log10 CFU/ml

over a period of 24 to 30 h and 6 at 48h of incubation. The Δ*fadB* counts decreased significantly ($P<0.05$) over a period of 24 to 48h incubation compared to the wild type **(Figure 4.16)**. At 4 to 8h of incubation, there was no significant change in the population of Δ*fadB* compared with the wild type **(Figure 4.16)**.

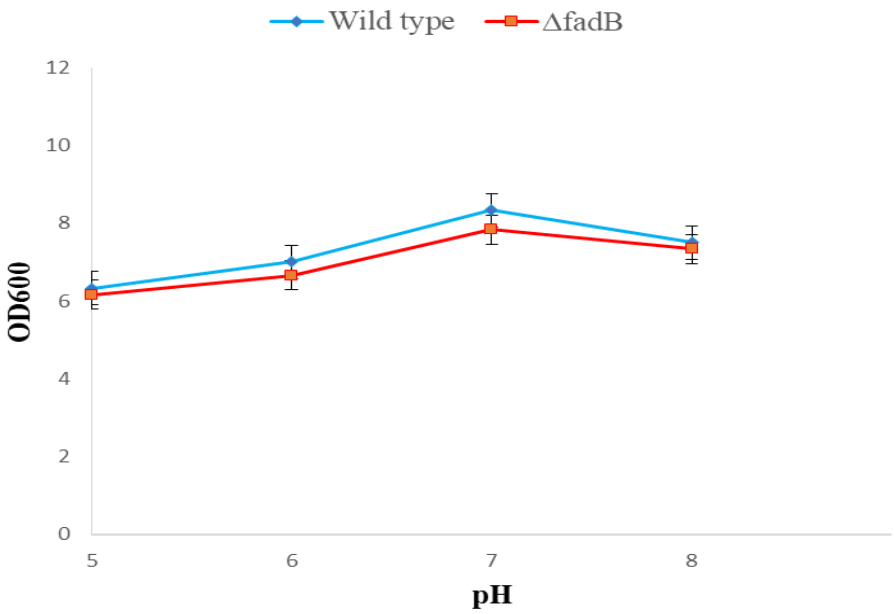


Figure 4. 14 Growth dynamics of S. aureus SH1000 and ΔfadB in a media with various pHs.

Wild type and Δ*fadB* cells were grown in the TSB media adjusted with either HCl or NaOH to the values of 5, 6, 7 and 8 for 24 h at 37°C. The data represents the mean ± standard error of the OD600 values from three independent experiments.

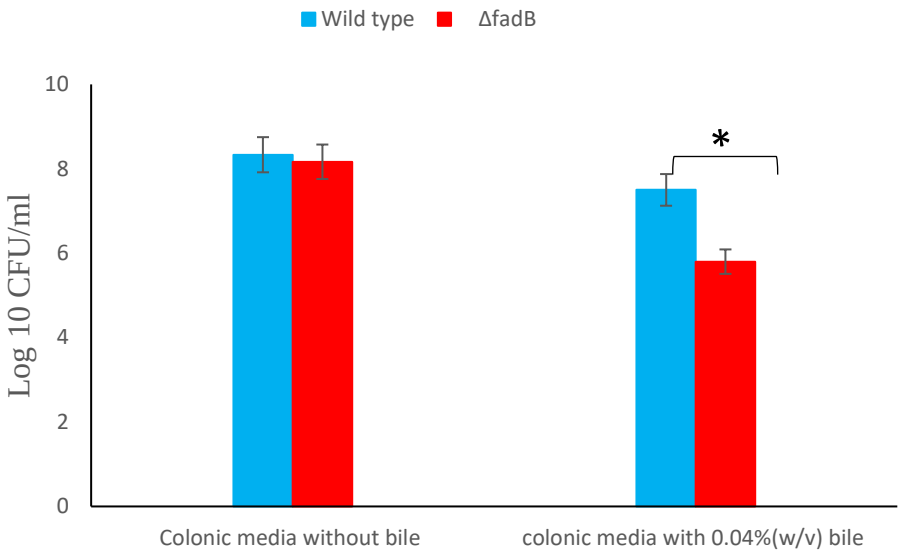


Figure 4. 15 Survival of S. aureus wild type and ΔfadB in the colonic media.

S. aureus wild type and Δ*fadB* populations were detected in culture broths recovered from the culture flasks containing colonic media with and without bile salts after 24 h anaerobic incubation with no shaking to determine a viable plate count. The results were reported as the means (Log10 CFU/mL) of the data of three colonic models ± standard error of mean (*P<0.05).

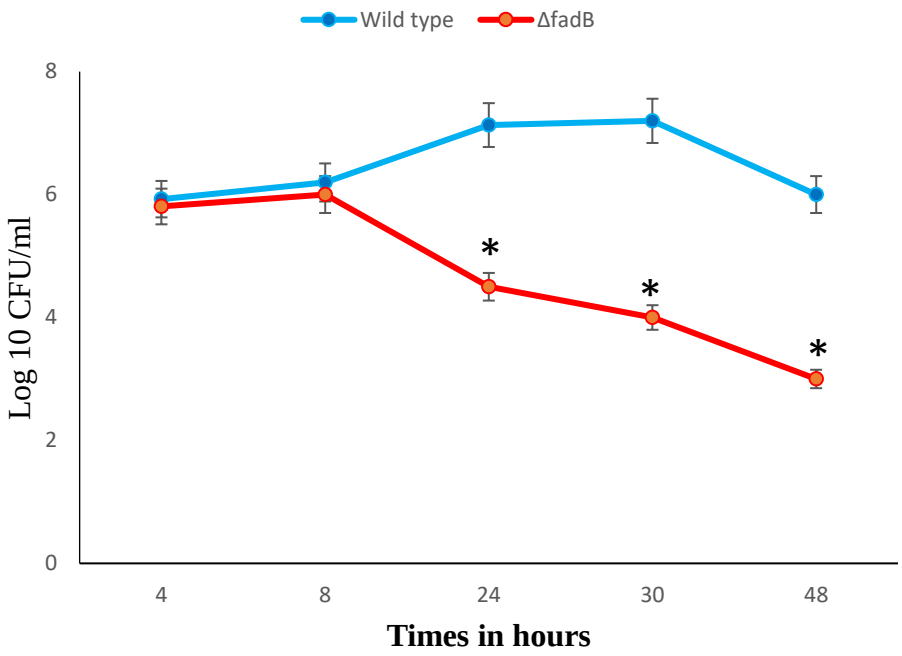


Figure 4. 16 Survival of *S. aureus* wild type and Δ*fadB* in a gut microbiota model (batch cultures).

Mean populations of wild type and ΔfadB *S. aureus* in a culture recovered from a batch culture vessel containing adult faecal inoculum and basal media. The results are the means (models ± standard error of mean. P<0. 05.log10 per ml) of *S. aureus* wild type and ΔfadB by flow FISH enumerated after 4, 8, 24, 30 and 48 h post-infection.

4.3 Conclusions and discussion

In the present study, a bile resistance gene in *S. aureus* was distinguished and then the gene was expressed in a heterologous host in order to study its functional effect. The gene was deleted and the in vitro sensitivity of the mutant to various bile salts and antibiotics was tested. Furthermore, the survivability of a bile sensitive mutant in the human colonic model compared with a wild type was determined.

The deletion of *fadB* in the strain of *S. aureus* produced a mutant strain more sensitive to bile than the mother strain. The sensitivity of the fadB mutant to bile was assessed using MIC and a killing time assay. The result showed that MIC viability significantly decreased ($P<0.05$) in the mutant compared with the wild type specific to the conjugated bile acid.

In *S. aureus*, Khairon et al. (2016) found upregulated expression of the *fadB* during fatty acid metabolism. Only two studies have attempted to investigate a role of FadB in *S. aureus* Camargo *et al*. (2009) found that fadB gene was upregulated as a part of the lipid metabolism response to Daptomycin stress, while Jang et al. (2008) documented that *fadB*, among genes encoding lipid metabolism enzymes, was down-regulated in response to triclosan, which is a broad-spectrum antimicrobial agent. The authors suggest that triclosan inhibits production of fatty acid metabolism-related genes of *S.aureus*. The inhibition of fatty acid synthesis killing this pathogen by interfering with its ability to form cell membranes. However, no previous study has investigated the role of *fadB* gene in bile resistance in *Staphylococcus*

Furthermore, the heterologous expression of *fadB* gene in this work suggested that *fadB* has a role in the survivability of *S. aureus* under bile salt stress. FadB was expressed in *E. coli* strains under the controlled and regulated pBAD expression vector upon the induction with arabinose. MIC and a viability course test were used to assess the role of *fadB* in the resistance of bile. The E. coli strains expressing *fadB* showed enhanced MICs related to CA, TCA and GCA. The

bacterial viability upon exposure to CA, TCA and GCA significantly increased ($P<0.05$) compared to wild type *E. coli* strains lacking *fadB.*

Thirdly, the colonisation of *S. aureus* wild type and *fadB* in the batch culture as a human colonic model demonstrated a significant reduction in the populations of *S. aureus* Δ*fadB* during the post-infection period (**Figure 4.16**). Overall, these results suggest that *fadB* plays a role in *S. aureus* colonisation in the human gut.

Chapter Five : Expression of *fad* genes in *S. aureus*

5.1 Introduction

Bioinformatic analysis revealed that the *fadB* from *S.aureus* contains homology to the lipid metabolism genes in *E. coli.* A number of researchers have reported induction of genes and proteins involved in lipid metabolism as a response to bile acid exposure in some bacterial species (Alcantara & Zuniga, 2012; Begley *et al*., 2005; Kristoffersen *et al*., 2007; Wu *et al*., 2010).

In Escherichia coli, the main player in transcription regulation of fatty acid metabolism is the FadR protein. FadR protein involved in negative regulation of fatty acid degradation and in positive and negative regulation of the cellular processes related to it. The FadR protein is highly conserved in many Gram-positive bacteria including *Bacillus, Clostridium, Streptomyces* and other related genera. It is notable that FadR is not conserved in the *Listeria* and *Staphylococcus* genera, which possess FapR homologues. *Bacillus subtilis* possesses two global transcriptional regulators, FadR (YsiA) and FapR. B. subtilis FadR represses fatty acid degradation, whereas FapR represses almost all the processes in the biosynthesis of saturated fatty acids and phospholipids.(Yasutaro *et al*.,2007).

Johnson (2018) concluded that fatty acid degradation (*fad*) genes were upregulated as a response to bile in all studied isolates of *Salmonella*. The major constituents of bile are bile salts, phospholipids, cholesterol, proteins and biliverdin pigment. Since twenty-two percent of bile is a phospholipid, upregulation of the *fad* genes in bile-treated *Salmonella* isolates might be related to the ability of these bacteria to use phospholipid in bile as a source of carbon (Johnson, 2018; Antunes et al., 2011).

Furthermore, FadB contains homology to oxidoreductase of *E.coli*. Some studies have suggested that proteins with oxidoreductase function are induced as a result of bile stress in

some bacterial species. Some species of enteric bacteria have the ability to modify bile salts by oxidation and reduction (Begley *et al*., 2005).

Pfeiler *et al*. (2007) studied the effect of bile on the transcriptional profile of *Lactobacillus acidophilus*. Their study showed that a gene that encoded a protein with oxidoreductase function was significantly upregulated in the presence of bile. Le Breton *et al*. (2002) and Bron (2004) found that inactivating a gene with oxidoreductase homology in *Enterococcus faecalis* or *Lactobacillus plantarum*, respectively, led to increased sensitivity of these bacteria to bile. Several studies have revealed significant changes in lipid composition of several bacterial species as a response to bile. Among the genes and proteins that are involved in the response to stress, a fatty acid biosynthesis enzyme was significantly downregulated as a response to bile stress in *Lactobacillus* and *Bacillus* species (Alcantara & Zuniga, 2012; Kristoffersen et al., 2007). For this chapter, the role of the *fadB* gene of *S. aureus* in bile resistance was investigated by studying the gene expression of *fadB* and genes upstream and downstream of *fadB***(Figure 5.1)** upon exposure to a sublethal dose of bile.

5.2 Real-time quantitative reverse transcription polymerase chain reaction (qRT-PCR) analysis of lipid metabolism genes in *S. aureus*

Real-time quantitative PCR, or qPCR is a powerful technique that provides accurate and fast measurements of gene expression. It provides high specificity and sensitivity of transcript detection as a compared with other methods, such as traditional PCR, Northern blotting and slot blotting (Derveaux *et al*., 2010).

qPCR was performed to quantify the transcript level of genes involved in fat metabolism (*fadB, fadX, fadE, BTN44 01085* and *thlA_2*) in *S. aureus* under bile stress. The expression profiles of these genes were determined in the presence of 6% crude bile (w/v) and compared to that of a control lacking bile. Housekeeping genes (HKG) *gmk*, *gyrA*, *proC* and *tpiA* were used as endogenous references for the relative quantification of the target genes. Amplification

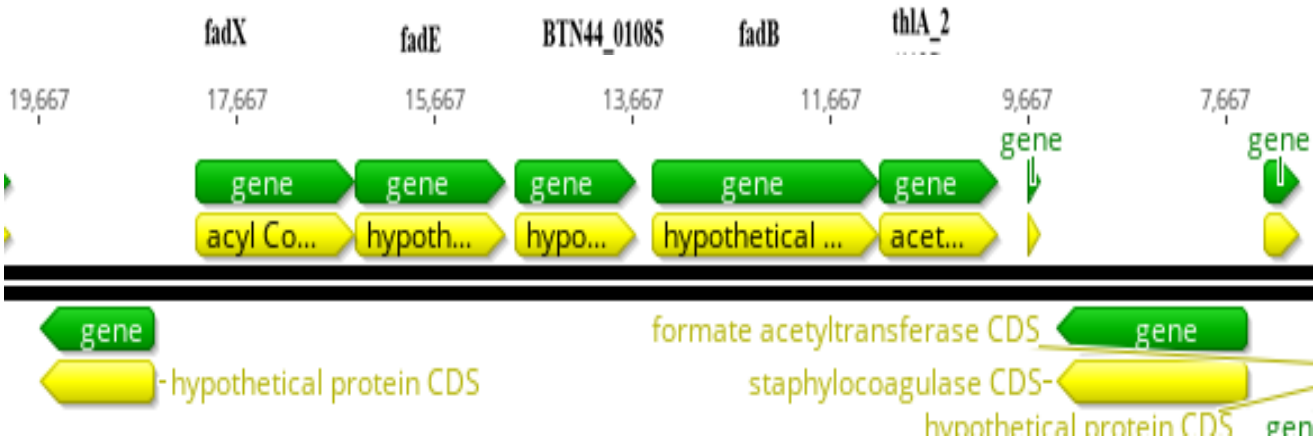


Figure 5.1: Schematic representation of the *fadB* region (gene PA5248) in *the S.aureus.*

efficiencies were determined for reference and target genes. To determine any changes in expression of *fad* genes in response to bile stress, relative expression of *fadB* was measured using qPCR. RNA levels of the target genes were measured and normalized to those of the house keeping gene HKG(*gmk*, *gyrA*, *proC* and *tpiA*).

5.2.1 Experimental design and growth conditions

The *S. aureus* strain SH1000 was inoculated from a fresh culture plate into 10 ml tryptic soy broth (TSB) and grown for 16 h at 37°C. One ml of overnight culture was subcultured into 100 ml (1% v/v) of TSB with or without 6% bile and grown at 37°C until mid-log phase (OD600 = 0.5). At this point 12 ml of culture were taken for RNA isolation, to which were directly added 24 ml of RNAlater™ (Thermofisher Scientific), a reagent that inactivates RNases and stabilizes RNA within tissues or cells. The mixture was incubated at room temperature for 5 min and then centrifuged (5000 g, 10 min, 4°C) and washed with PBS, pH 7.4. The cell pellet was resuspend with TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0) containing lysostaphin (10 mg/ml) and then incubated for 1h at 37°C. RNA was isolated from cells of *S. aureus* using the RNeasy Mini Kit (Qiagen) by following the protocol suggested by the manufacturer. Total

RNA was purified using the Invitrogen™ TURBO DNA-free Kit (Invitrogen) to eliminate ribosomal RNA and then stored at -80°C for future use.

5.2.2 Selection of primers

The primers were designed by using the Primetime® Predesigned qPCR Assays online tool from IDT® (Integrated DNA Technologies). Oligonucleotide primers details are listed in Table 2.4. Primer lengths were 18–23 base pairs and resulting DNA fragment lengths were 80–132 base pairs. The GC content of the primers was 43–50% and the T_m was 53.7–60°C. Primers were tested by using standard PCR and genomic DNA from *S. aureus.* The PCR product generated was the expected size.

5.2.3 RNA quality analysis

The purified RNA integrity was first assessed using agarose gel electrophoresis (**Figure 5.2**), followed by assessment using an RNA 600 Nano kit (Agilent), which is designed for use with an 2100 Bioanalyzer (Agilent). The agarose gel and images from the Agilent 2100 Bioanalyzer showed no mRNA smearing, indicating that there was no degradation of the RNA **(Figure 5.3).** Purified RNA and primers were tested using standard PCR to confirm there was no contamination with genomic DNA. Intact RNA was selected for cDNA synthesis using the Tetro cDNA synthesis kit (Bioline).

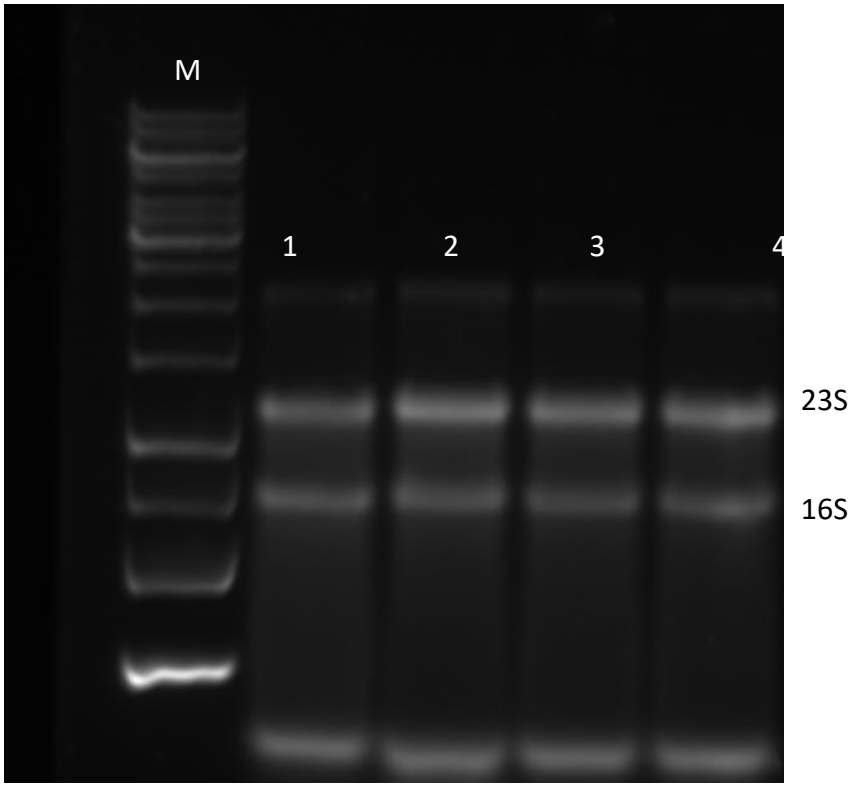


Figure 5. 2 Gel electrophoresis of total RNA which showing integrity of RNA isolate recovery from *S. aureus* SH1000.

M represents the standard marker (GeneRuler 1 kb DNA ladder, Biolab). Lanes 1 and 2 represent total RNA from bile-treated *S. aureus* SH1000, and lanes 3 and 4 represent RNA from the control (untreated *S. aureus* SH1000).

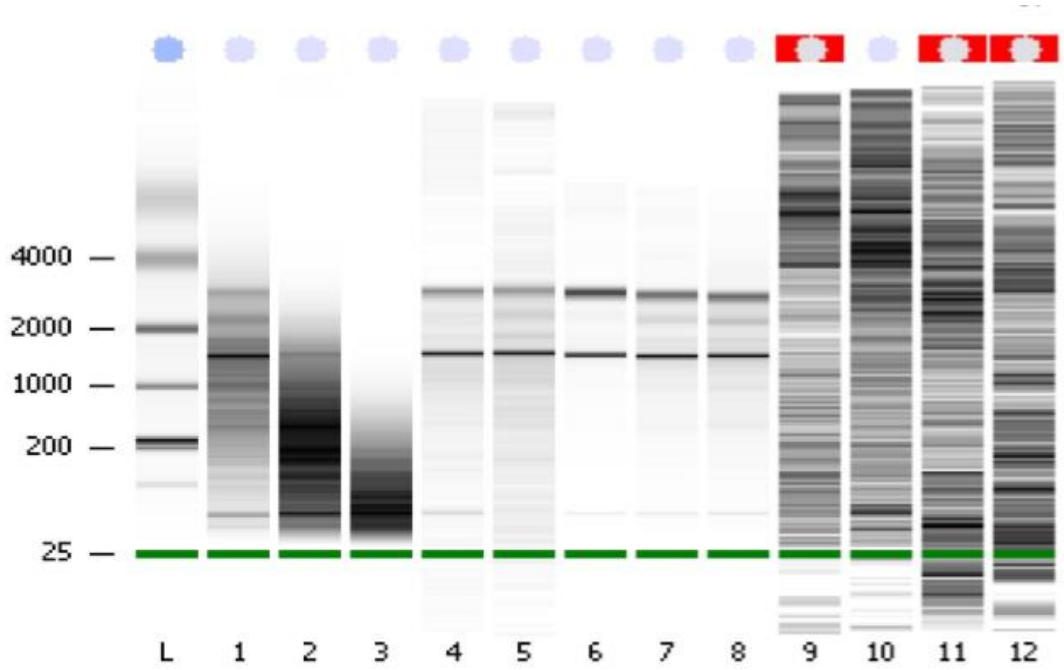


Figure 5.3 Gel image from the Agilent 2100 Bioanalyzer of enriched mRNA preparation. Lane 1, standard ladder; lanes 2–5, intact RNA extracted from *S. aureus* culture; lanes 2–5, intact RNA extracted from bile-treated *S. aureus* culture. Lanes (1,2,3,9,10,11 and 12) which represented RNA sample were discarded due degradation of RNA

5.2.3 Amplification specificity and primer efficiency

Testing of primer efficacy is essential for the relative quantification of expressing genes. Target and housing keeping gene primer were tested using standard curve. Four dilution of cDNA were used (25,2.5,0.25 and 0.02ng). qPCR reaction was set according to master mix supplier company. genes were analysed separately; the standard curve of the genes were giving an amplification efficacy between 90-102% which indicate that tested primer in accepted range and can be used it for gene amplification **(Figure 5.4)**.

5.2.4 Data analysis

Quantitative RT-PCR reaction were performed in a BioRad My iQTM Real time detection system. The measurement of expression density of each gene is expressed in Cycles to Threshold (Ct) of PCR, which refer to the cycle number where the level of fluorescence passes the threshold. In this study, relative quantification method was used to measured expression level of target gene (*fadB, fadX, fadE, BTN44 01085* and *thlA_2*) using the ΔΔCt method (Pfaffl,2001). RNA levels of the target genes were normalized to four HKG (*gmk, gyrA, proC* and *tpiA*) , this gene was previously tested in previous study (Theis et al .,2007). Results show that expression level of these genes does not affected by presence of bile. Data analysis were based on Three independent experiment (biological replicate) .

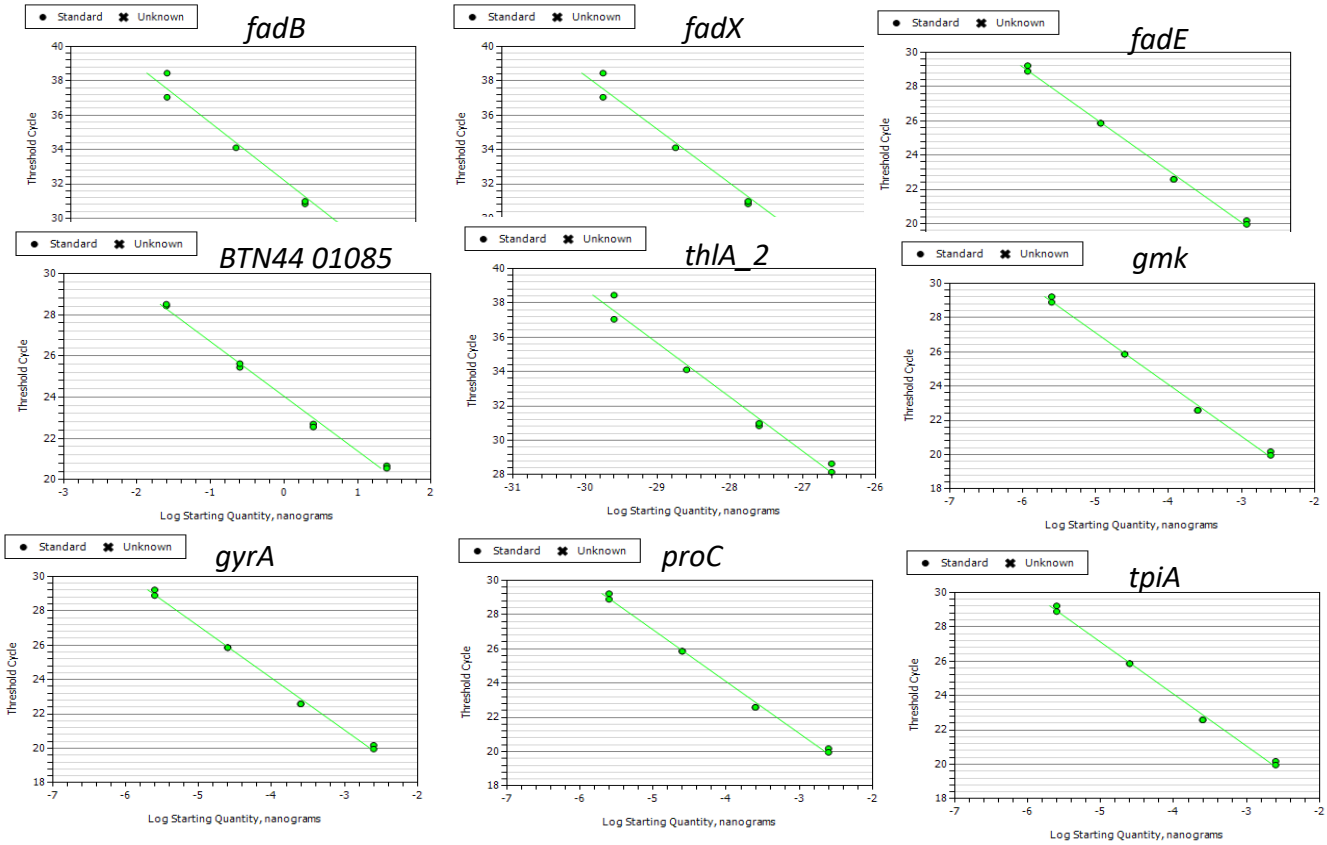


Figure 5. 4 Standard curve of primer efficiency of the qPCR for each gene.

The primer efficiencies were tested in triplicate for each cDNA sample, by using a dilution series (10^1-10^5) from the template cDNA of the wildtype strain for each target gene. the standard curve of the genes was giving an amplification efficacy between 90-102% which indicate that tested primer in accepted range and can be used it for gene amplification.

6.2.5 Transcriptional analysis of lipid metabolism genes in *S. aureus*

In order to determine if *fad* genes (*fadB, fadX, fadE, BTN44 01085* and *thlA_2*) in *S. aureus* are induced as response to bile stress, transcription analysis of theses gene were performed in presence sublethal dose of crude bile using qPCR. mRNA level of the genes was determined RT-qPCR in mid log phase culture of *S. aureus* SH1000 grown with 6% (v/w) of crude bile. Results indicated in (**Figure 5.5**) show *fadB* gene induce under bile stress. This gene code for 3-hydroxyacyl-CoA dehydrogenase which involved in fatty acid beta-oxidation, which is part of *S. aureus* lipid metabolism. The expression level of *fadB* was increase by more than 3.5-fold relative to control in the same time point. Overall, genes up and downstream of *fadB* shows down-regulated expression as a response to bile stress. *fadX* gene code 3-ketoacid CoA transferase shows 0.5-fold decrease in the expression level in presence of bile. The expression level of *fadE, BTN44 01085* and *thlA_2*down-regulated by 0.25, 0.15 and 0.4-fold respectively under bile stress conditions.

The proteomic study (chapter 4) of bile which was performed by comparing protein from cell samples after bile exposure, shows that the putative protein (3-hydroxyacyl-CoA dehydrogenase) was strongly upregulated in the presence of bile. Combined with these findings the conceptual premise that *fadB* might have a role in staphylococcal bile resistance is strengthened.

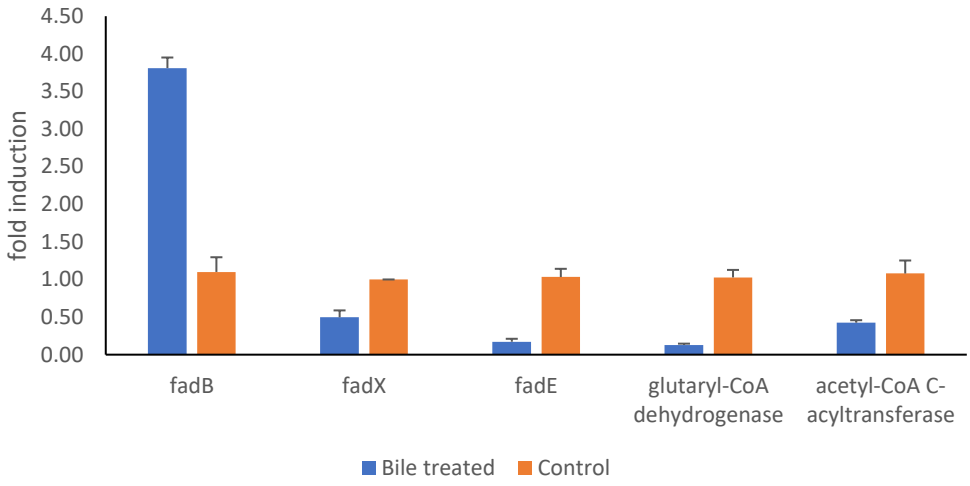


Figure 5. 5Transcriptional analysis of the *fadB, fadX, fadE, BTN44 01085* and *thlA_2.* genes in the wt *s.aureus* SH1000 strain in TSB culture with or without crude bile in mid log phase .RNA level obtained from the strain measured by qPCR . data correspond to the results of at least three independent experiments.

Table 5. 1: Fold differences and *P* values for expression levels of *fad* genes between the bile treated wildtype *S.aureus* SH1000 bile and non-treated control .

Gene	**Fold difference**		***P* value**
	Up regulated	***Down regulated***	
fadB	3.5	-	*P*<0.001*
fadX	-	0.5	*P*<0.05
fadE	-	0.4	*P*<0.06
BTN44 01085	-	*0.25*	*P*<0.08
thlA_2	-	*0.15*	*P*<0.09

* Significant difference

6.3 Conclusions

Transcriptional analysis of *fadB* and its up and downstream genes in *S.aureus* SH100 grown in TSB with or without 6%(w/v) bile was used to detect if these genes were induced in response to bile stress. Strongly upregulated were observed in expression level of *fadB* gene response to bile. *fadB* code for a putative 3-hydroxyacyl-CoA dehydrogenase which may be involved in the pathway fatty acid beta-oxidation and in lipid metabolism *S. aureus* SH100 and molecular function is oxidoreductase. The recent study documented that the transcription of the putative 3-hydroxyacyl-CoA dehydrogenase among of genes encoding lipid metabolism enzymes down-regulated in response to triclosan, which is a broad-spectrum antimicrobial agent (Jang *et al*.,2008). No previous study has investigated the role of fadB gene in bile resistance in *staphylococcus*. As was mentioned in the chapter four(4.2.8.2), bioinformatics analysis of FadB sequence predict that function of this protein is oxireductase. Some studies have suggested that proteins with oxidoreductase function, are induced as a result of bile stress in some bacterial species. It is documented that some species of enteric bacteria have the ability to modify bile salts by oxidation and reduction (Pfeiler, *et al.,* 2007; Begley *et al.*, 2005, Bortolini *et al.,* 1997). Furthermore FadB involved in the pathway fatty acid beta-oxidation, thus increased transcription *fadB* in presence of bile might be related to ability of bacteria to use bile as a source of carbon and energy (Johnson, 2018; Antunes et al., 2011). Genes up and downstream of *fadB* shows down-regulated expression as a response to bile stress. *fadX* gene code 3-ketoacid CoA transferase shows 0.5-fold decrement in the expression level in presence of bile. The expression level of *fadE, BTN44 01085* and *thlA_2* down-regulated by 0.25,0.15 and 0.4-fold respectively under bile stress conditions. Taken together, these results suggest that *fadB* encoding protein involved in bile resistance in *S.aureus.*

Chapter Six: Overexpression and analysis of FadB

6.1 Introduction

The putative function of *S.aureus* FadB is inferred from homology, it contains homology to FadB in *E.coli.* FadB function in *S.aureus* is unknown but according to computerized analysis(Chapter four -section 4.2.8.2) of its sequencing, FadB is involved in the pathway fatty acid beta-oxidation which is a part of lipid metabolism (www.uniprot.org).

A number of researchers have reported a relation between FadB and fatty acid metabolism in *S.aureus*. Khairon *et al*.,(2016) demonstrated upregulated expression of *fadB* during fatty acid metabolism in *S.aureus*. Also, Jang *et al*. (2008) suggest that *fadB* was downregulated as a response to triclosan, which is a broad-spectrum antimicrobial agent. Resulting in inhibition of fatty acid synthesis in *S.aureus*.

No research has been conducted to the role of FadB in bile resistance in enteric bacteria. However, several studies documented that a viability of fatty acids in bacterial membrane is crucial to maintain membrane lipid homeostasis and resist of bacteria to the environmental stress including bile salts (Williams *et al*., 2014).

In this study, FadB was overexpressed and purified to provide evidence for its role in bile resistance in *S. aureus*. The encoding gene, *fadB* was expressed with tight control to allow for the purification of enough quantity protein. To achieve this, the target encoding gene was cloned into a vector containing T7 RNA polymerase promoter.

6.2 Amplification of the encoding gene

The gene fragment encoding *fadB* was amplified by PCR (primers: *fadB*F, *fadB*R; Table 2.3). Forward primers were designed so then the natural N-terminus of the overproduced protein was preserved. The reverse primers were designed such that the His6 tag, encoded on the vector, would be fused to the C-terminus of the overproduced protein. *fadB* was amplified using High Fidelity Hot start Phusion® DNA polymerase (Thermo Scientific). The amplified fragments were analyzed by agarose gel electrophoresis to contain the size of the amplified fragment **(Figure 6.1).**

6.3 Constructing pET21-fadB

The pET21a vector and fragment were both double digested with *Nde*I and *Xho*I and ligated with *Nde*I and *Xho*I digested by *fadB (***Figure 6.2**). The amplifying fragment was extracted from the gel. 3 µl of around 195 ng of the fragment was mixed with 3 µl of around 150 ng of the linearized vector (pET21a) in molar at a ratio of 3:1 (insert: vector). Four µl of the rapid ligation buffer and 1 ul of T4 ligase were added and the total reaction volume was adjusted to 20 µl with water. The mixture was incubated at 25 for 10 mins before being transformed into chemically competent *E. coli* TOP10 cells as described in chapter 2 section 2.9.1.

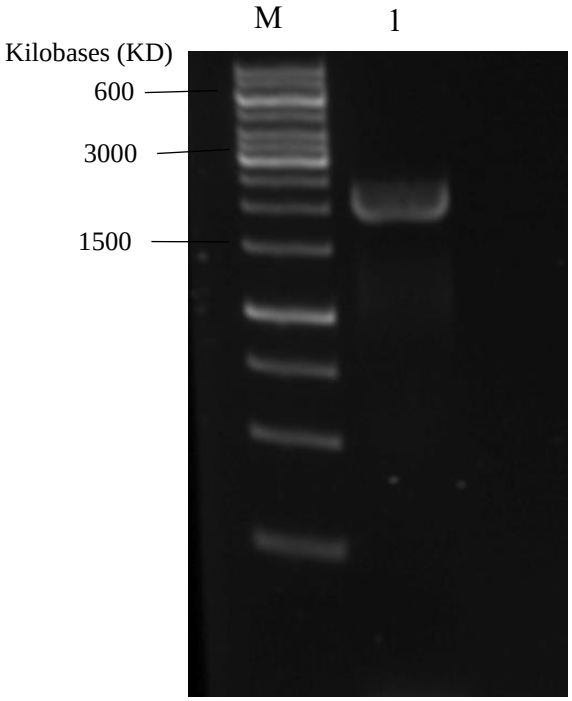


Figure 6. 1 Analysis of the *fad*B PCR products by agarose gel electrophoresis.
Lanes 1 represents the ~2.26 kb band of *fadB* in 1% agarose gel. Lane M represents a standard 1 KB DNA ladder (Thermo Scientific).

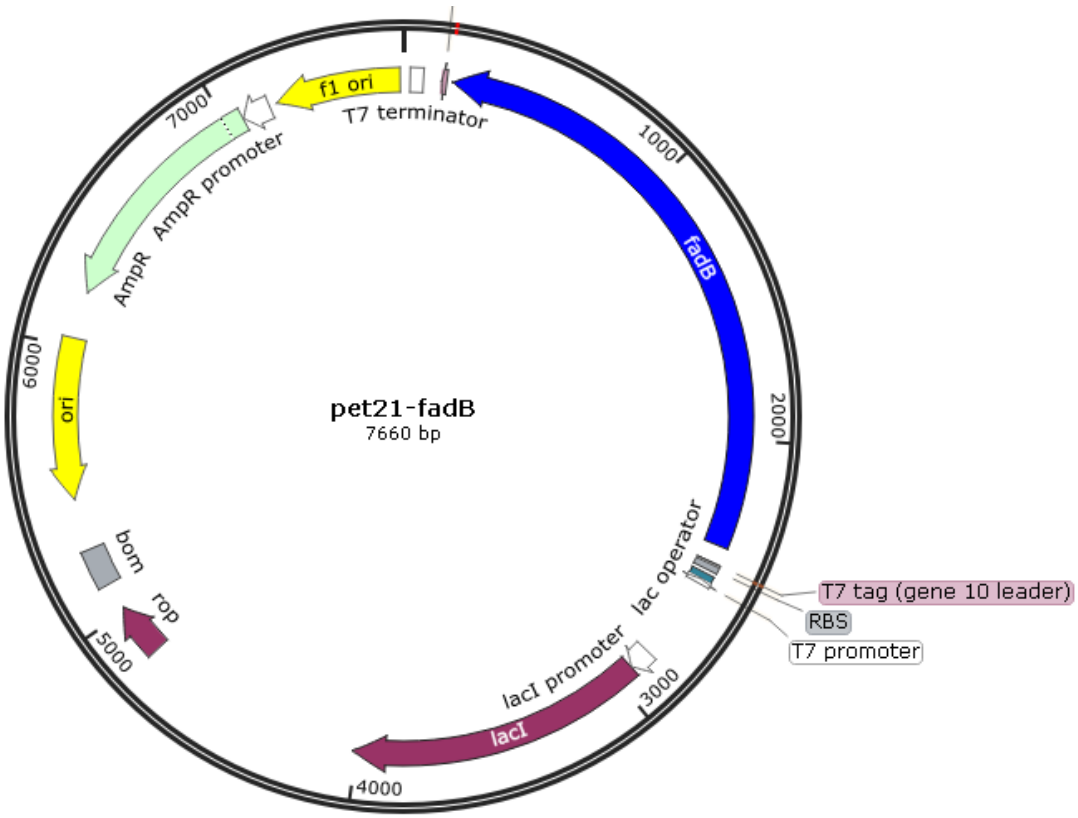


Figure 6. 2 Cloning of *fadB* variants into pET21a.

S. aureus fadB was cloned into multiple cloning sites (MCS) between the *Nde*I and *Xho*I restriction sites (Invitrogen).

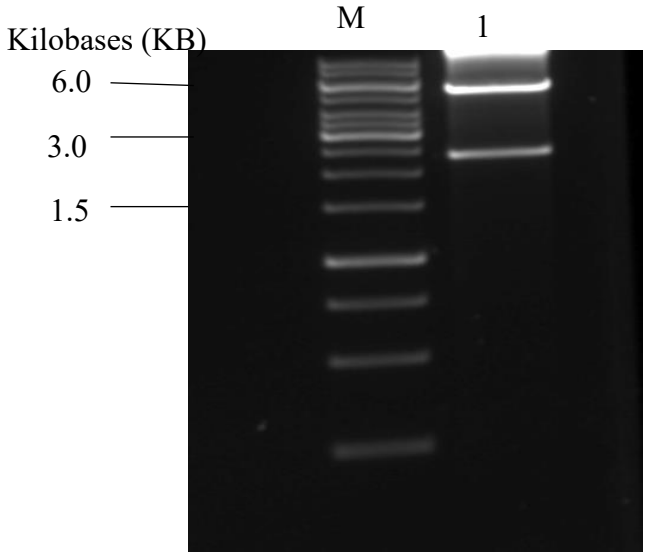


Figure 6. 3 *Nde*I restriction mapping of pET-21 a (+) and *fadB*.

Lane 1 possesses two DNA bands of size ~5.4 and ~2.26 KB from the NdeI restriction of pET-21 a (+) from *E. coli* TOP10. Lane M represents the standard 1 KB DNA ladder (Thermo Scientific). The complete sequence of the insert was subsequently obtained and found to match that expected

6.4 Overexpression trial of FadB

The pet21-*fadB* plasmid was transformed into *E. coli* BL21/λDE3. The plasmid was then extracted and digested with a restriction enzyme (*Xho*I and *Nde*I*)* to ensure that they contained the correct band size, representative *fadB*. One milliliter of the transformant was used to inoculate 100ml of fresh media containing 100 μg/ml of the amp. The culture grown at 37 °C for around 3 hours or until the OD adjusted to 0.5. thenIPTG to induce expression was added to each milliliter of culture. Samples were taken at 1, 2, 3, 4, 5 and 6. SDS-page analysis showed an over-expressed band around 85 kDa **(figure6. 3)** And maximal over-expression was achieved at 2h post induction. This time point was selected for large scale over-expression for further protein purification.

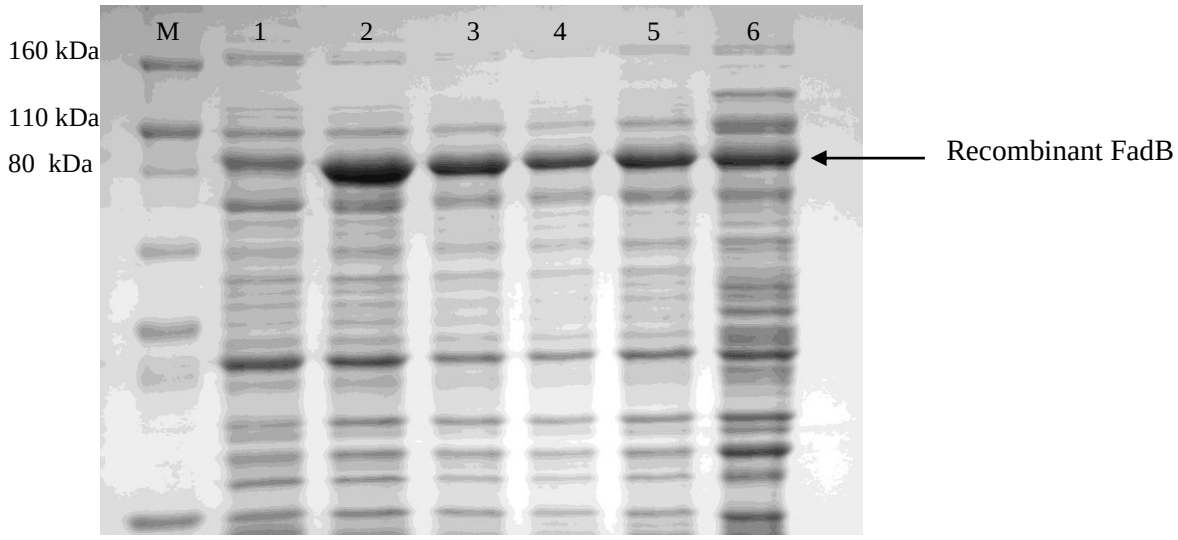


Figure 6. 4: SDS-PAGE analysis of FadB in a small-scale over-expression trial.

Over-expression produced major bands of recombinant protein (~ 85 kDa). The strain used was *E. coli* BL21/λDE3. The numbers 1-6 represent the time (hours) after induction by IPTG. Maximum over-expression production was achieved at 2 h post induction. M indicates the Unstained Protein Molecular Weight Marker (Thermo Scientific).

6.5 Solubility of recombinant FadB

The solubility of recombinant FadB was determined as described in section 2.14.2. A cell lysis reagent (Bug buster -Novagene) was used for the extraction of the cytoplasmic protein. Bug buster is a mild cell lysis reagent used for the disruption of bacterial cell membranes and the release of cytoplasmic proteins. IPTG inducing cells, which carry pET21a-*fadB*, were taken and treated with Bugbuster. The supernatant and pellets were collected and fractionated by SDS to compare the relative percentage of soluble and insoluble protein.

The results show (**Figure 6.5**) a bold band at 85 KD in the supernatant, particularly 2h post induction. About ~ 50% of the protein was observed in the soluble fractions. The percentage of soluble protein was considered to be acceptable for purification.

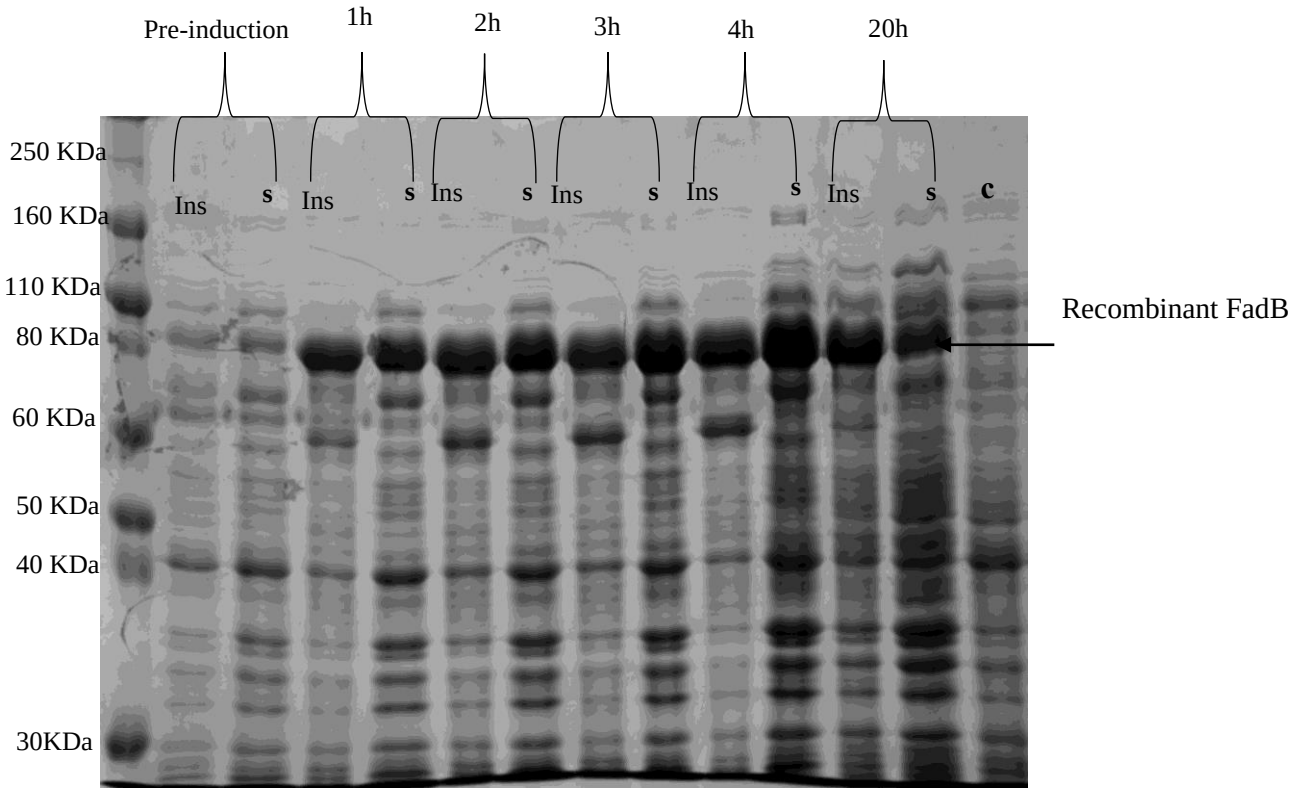


Figure 6. 5: Analysis of recombinant FadB solubility.

E. coli BL21 (DE3) cell were harvested at various time points post-IPTG induction. The harvested cells were treated with 1X Bugbuster® to generate insoluble pellet (Ins) and a soluble supernatant (S). A band of the expected size (~85 kDa) was observed in the supernatant and pellet fractions, indicating that the recombinant protein is soluble and thus acceptable for purification.

6.6 Large-scale overexpression of the recombinant protein

The large-scale overexpression of *E. coli* BL21/λDE3 (pET21a-*fadB*) was performed after confirmation of the expression and the solubility of the recombinant protein (method 2.14.2). Five liters of culture were used for the purification of the protein. After inoculating e LB media with the bacteria, the culture was incubated at 37c for ~ 3 h (OD_{600} reached around 0.5) and then inducted with IPTG. At 2h post-induction, the cells were harvested as determined by small scale overexpression (method 2.14.2). The pellets were resuspended in buffer A (50 mM Tris, 15mM mannitol, 15Mm imidazole and 5%(v/v) glycerol, PH 8) and then the cells were lysed mechanically using a French cell press at 20000 psi three times. The cell was centrifuged and the supernatant was taken and filtered before being loaded into the nickel affinity chromatography column.

6.7 Nickel affinity chromatography

Nickel affinity chromatography was used to separate N- terminally hexa-histidine tagged *fadB* from other soluble proteins. The tagged protein was eluted using imidazole. This is because its affinity to nickel is greater than histidine, therefore it displaces any histidine-bound protein. The cell lysate was filtered through a 0.45 μm syringe filter being loaded into the column. Nickel chromatography was performed as in methods 2.14.3. The column was equilibrated with buffer A2 (50mM Tris, 15mM mannitol, 5% glycerol(v/v), PH 8.0) and then the protein eluted with buffer B2 (50mM Tris, 1M mannitol, 5%(v/v) glycerol, PH 8.0). The results showed a single peak in the chromatograph chart. Fractions 8-16 were analyzed by SDS-Page (**Figure 6.6**). The SDS-Page of the selected fraction showed high concentrations of the recombinant protein (86 KD). The positive fractions were recombinant together for centrifugation.

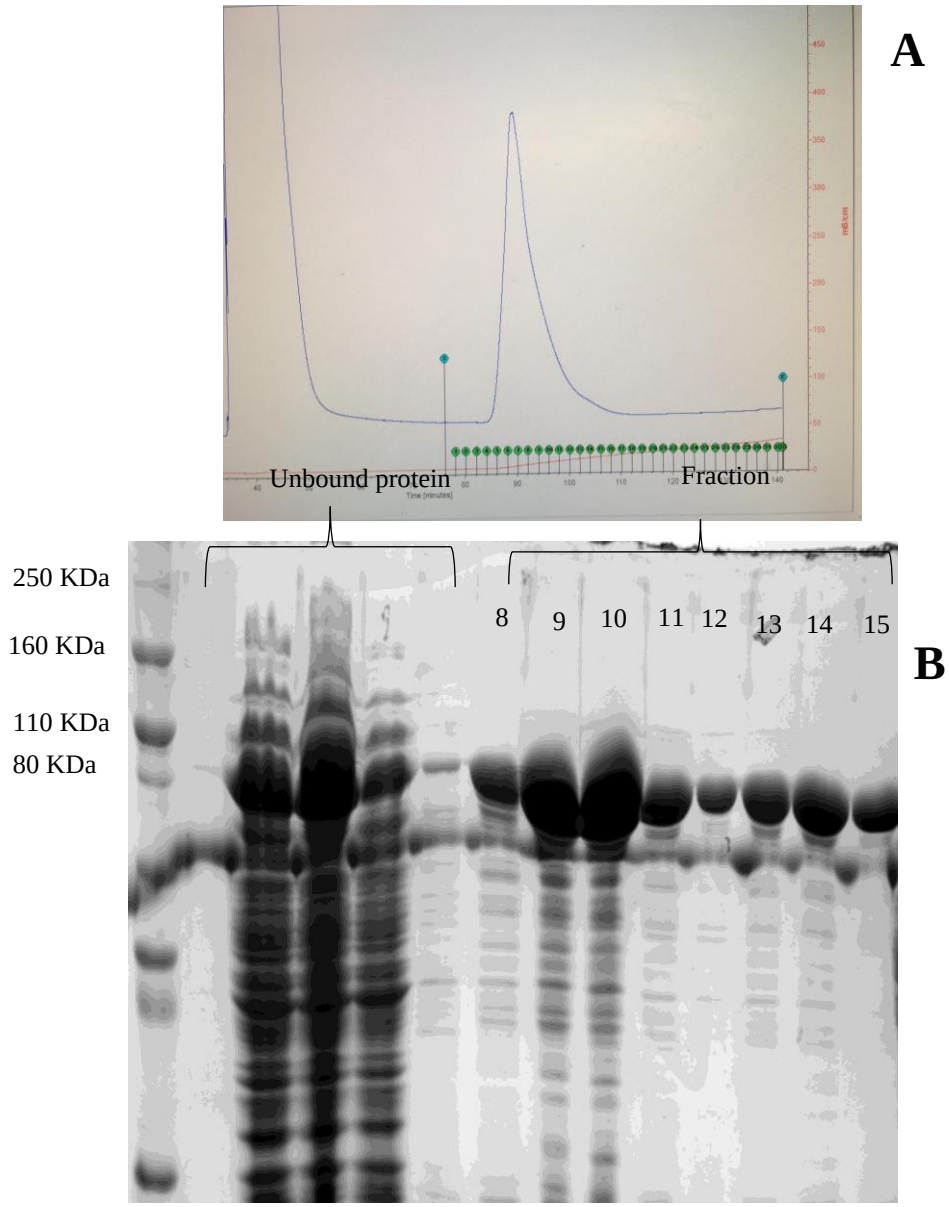


Figure 6.6: Elution profile and SDS-PAGE analysis of selected nickel affinity chromatography.

(A); Elution profile obtained from recombinant-(His-tagged) protein from the nickel Sepharose™ column. Conductivity (mS/cm) (red line) and absorbance at 280 nm (blue line). (B); the fractions obtained during the recombinant protein purification; 15ml of both the fraction and control were analyzed by 12% SDS page. The selected fractions showed a major band corresponding to a mass of ~85 kDa, similar to what was expected 85 kDa (FadB). The corresponding protein band sizes of the unstained protein molecular weight marker (thermos scientific) have been labelled on the left-hand side of the image.

6.8 Protein centrifugal concentration

To increasing protein purity, A second purification of the recombinant protein was conducted using a centrifugal concentrator (**Figure 5.7**). Fractions of the recombinant protein were combined and concentrated using a Vivaspin 50 (50,000 MWCO) (Satorius Stedim Biotech). The recombinant protein solution was loaded into a concentrate and centrifugation was 4000rpm for 20 minutes. Flow thought was kept for SDS-page analysis to be sure of the absence of the recombinant protein. The protein concentration was measured using a Bradford assay. Eventually, ~2.50 mg/ ml of the recombinant protein was obtained. The purified protein was sent for mass spectrometry analysis and Western blotting.

6.9 Mass spectrometry analysis of FadB

In order to confirm the identity of the purified protein, mass spectrometry of the protein was performed. After the further purification and concentration of the recombinant protein, the protein eluting buffer was exchanged with 100mM ammonium bicarbonate. The protein sample was sent for mass spectrometry analysis at an advanced mass spectrometry facility at the University of Birmingham. The protein sample was digested with trypsin and a mass of the recovered peptide was determined using LC-MS (liquid chromatography mass spectrometry). Mascot search engine was performed against the uniport database using FadB sequence.

MALDI-TOF analysis confirmed the identity of the purified protein, it showed that the sample contained FadB from *S. aureus*. Protein score was 1430 with a random probability lower than 0.05. According to Mascot Score Histogram, protein scores higher than 1430 is highly significant and is considered to have been successfully identified if it had hit with a random match probability lower than 0.05.

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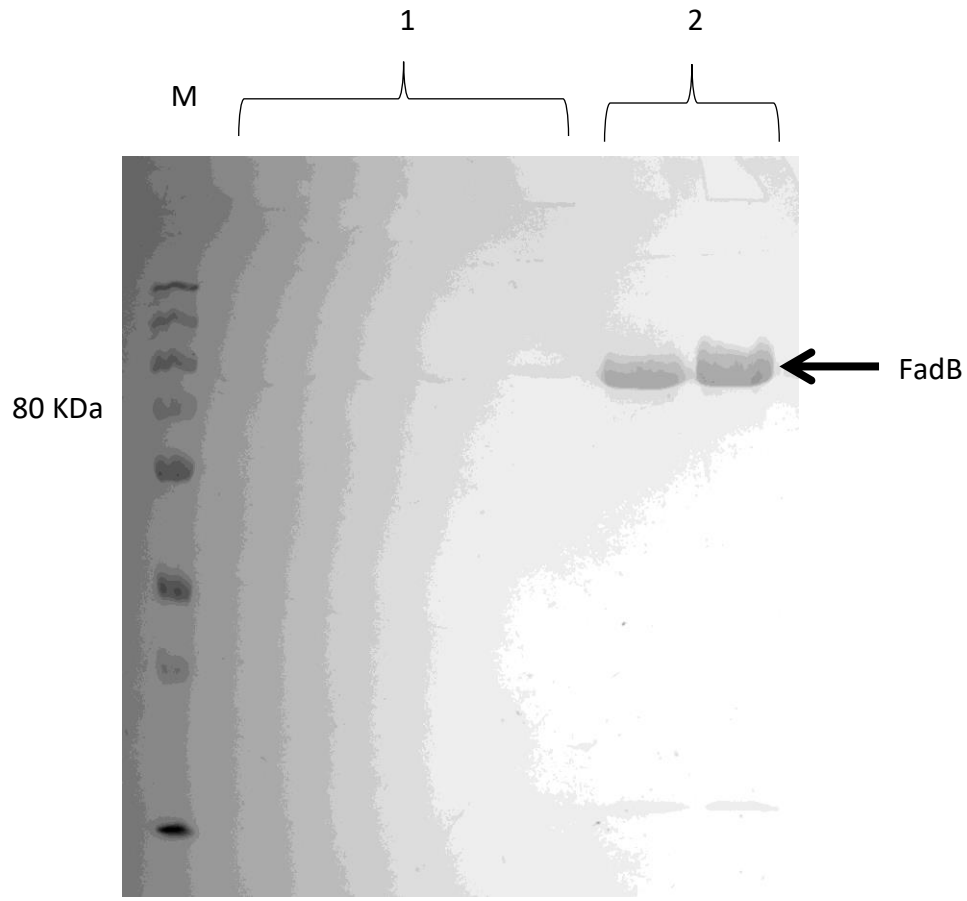

Figure 6. 7: SDS-PAGE analysis of concentrated protein.

A second purification of the recombinant protein was conducted using a centrifugal concentrator. Fractions of the recombinant protein were combined and concentrated using Vivaspin 50 (50,000 MWCO) (Satorius Stedim Biotech); M indicates the Unstained Protein Molecular Weight Marker (Thermo Scientific), 1 shows the concentrator flow and 2 shows the concentrating protein.

6.10 Enzymatic activity of recombinant FadB

FadB of 753 amino acids (AA) with a theoretical molecular weight of 84.6 and PI 5.68. The putative enzyme activity has the ability to convert acetoacetyl-Co to ß-hydroxy butyryl-CoA in the presence of ß-NADH **(Figure 6.8).** This activity was determined by measuring the decrease of NADPH absorption at A340nm and the calculation of activity was performed as unit/ml according to Lynen and Wieland's (1995) method. The enzyme shows optimal activity starting at 0.2mM acetoacetyl-CoA (substrate) in the presence of 0.1mm of NADH (Figure **6.9**). As shown in Figure 5.9, the optimum pH for enzyme is 6.5 and the enzyme activity decreased in an acidic pH. The lowest activity of the enzyme was recorded at pHs 8 and 5.

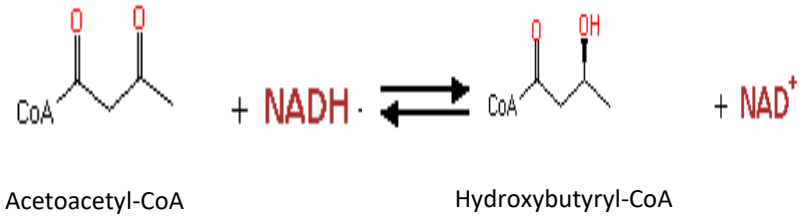

Figure 6. 8 Acetoacetyl-CoA and hydroxybutyryl-CoA dehydrogenase reaction of glutaryl-CoA degradation by *fadB*.

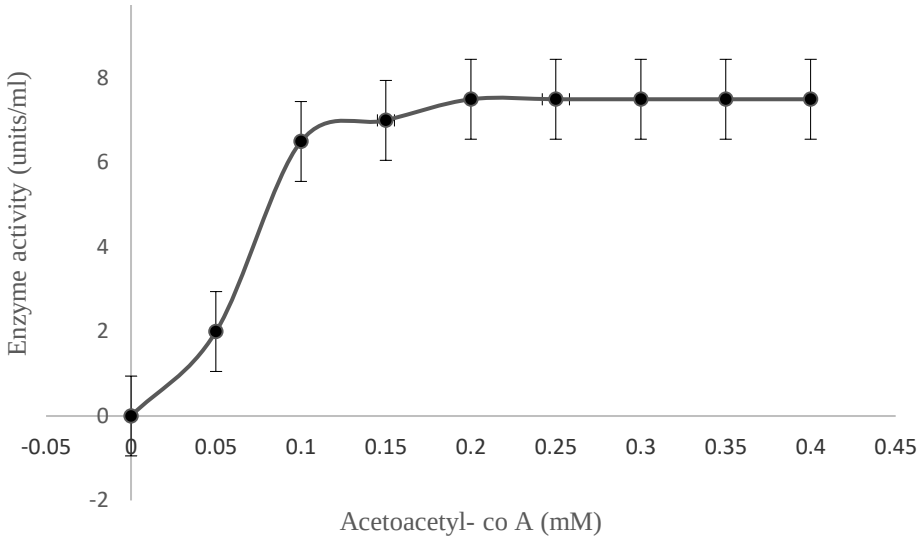

Figure 6. 9 Hydroxyacyl-CoA dehydrogenase enzyme assay of purified FadB

The catalytic activity of the enzyme by converting acetoacetyl-CoA to hydroxybutyryl-CoA in the presence of NADH as a cofactor was spectrometrically determined. The serial dilution of the substrate (acetoacetyl-CoA) was used to measure the activity rate of the enzyme. The points are the mean ±SE of the three replicates. Progress curve Appendix 4

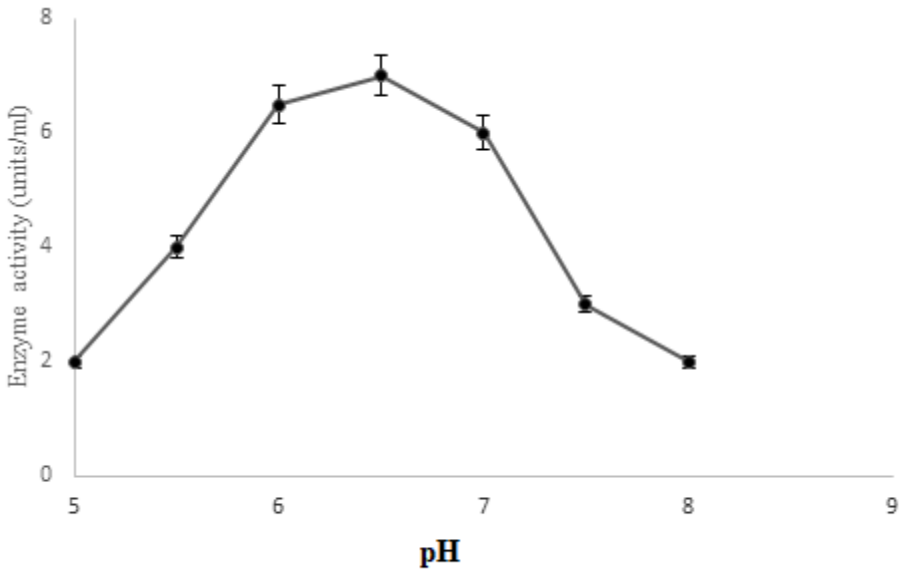

Figure 6. 10 Effect of pH on the activity of hydroxyacyl-CoA dehydrogenase.

Acetoacetyl-CoA were used as a substrate in the presence of NADPH and the effect of pH on the catalase reaction rate was assessed using continuous optical spectrometer. The points indicate the mean ±SE of the three replicates.

6.11 FadB binding to cholic acid

6.11.1 Surface plasmon resonance (SPR)

The binding of FadB to cholic acid was monitored using Surface Plasmon Resonance imaging (SPRi). SPR is a Realtime, label-free, and high-throughput technique which is used to study biomolecular interactions based on detecting the refractive index changes resulting from molecular binding.

The SPR technique allows the monitoring of the reflectivity variation at a single incident angle. This variation of reflectivity is correlated to the amount of analyte that reacts on each spot per surface unit. In this study SPR was used to provide experimental evidence for estimating the binding affinity between FadB and cholic acid. The protein sample was sent for SPR analysis at Creative Peptides company –USA. . Real-time binding signals were recorded and analyzed by Data Analysis Module(DAM, Plexera Bioscience, Seattle, WA, US). Kinetic analysis was performed using BIAevaluation 4.1 software (Biacore, Inc.).

Figure 6.11 shows SPR results at different concentration of FadB. The *K*d value of FadB bound by Cholic acid, calculated from the equilibrium binding isotherms using a simple binding model (a1:1 Langmuir binding model), was 2.25×10-3 S-1. Basing on the results from surface plasmon resonance (SPR) experiments, the current study found that FadB can bind to cholic acid.

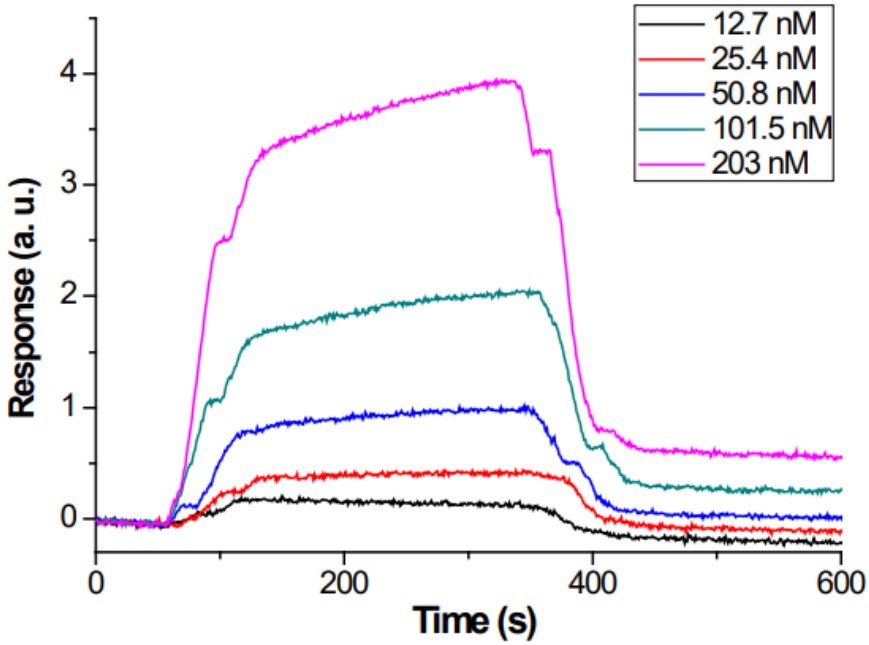

Figure 6. 11 Evaluation of the binding affinity of FadB to cholic acid .

The equilibrium dissociation constant (K_D Value) 1.28×10^{-6} µM (Ka = 1.76×10^{3} M^{-1}. S^{-1} , K_d =2.25×10^{-3} S^{-1})

6.11.2 Cholic acid coupled -sepharose affinity chromatography

To determine the interaction between cholic acid and the purified protein, ligand-protein binding was assessed using cholic acid coupled with sepharose affinity chromatography. This type of chromatography was first used by Pattinson and his colleague (1980) to purify albumin from the serum depending on the high affinity of this protein to binding bile acids. The affinity of FadB to cholic acid was tested using the same principle as Pattinson's method. As mentioned in the methods chapter, in the first step, the cholic acid was coupled to ethyl-3-Qxlimetbylaminopropyl carbodimide HCI and the pH of the coupled mixture was adjusted to 7. It was then slowly added to amino hexylamino (AH)-Sepharose 4B and incubated for 24h with shaking. The mixture was then packed into the 50ml chromatography column. The purified protein was added to the column and washed with phosphate saline buffer to remove any non-bound proteins. After this, 5 mM of cholic acid was used to elute the binding proteins. The protein fractions that bound to the cholic acid that conjugated in the EAH Sepharose 4B column were analysed by using SDS-PAGE (Fig. 6.12). As shown in Fig. 6.12, the bile acid-binding proteins of a molecular weight of 84 kDa were eluted and disappeared in the unbound protein fraction. The retention of the FadB to cholic acid might refer to the interaction between this protein and bile acid (cholic acid). Pattinson and his colleague (1980) demonstrated that cholic acid coupled Sepharose may prove a useful tool in studying binding sites on albumin. The gel has already proved useful in the isolation of receptor/carrier proteins involved in the enterohepatic transport of bile acids. It seems possible to use crude extract from bile treated S.aureus to primary detection of cholic acid binding protein in the bacteria.

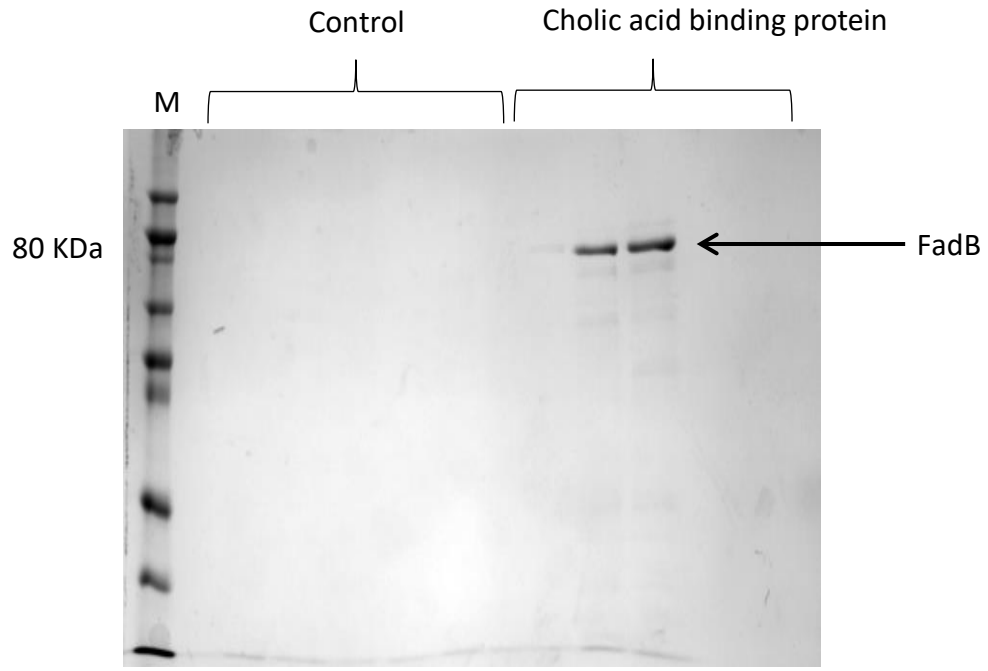

Figure 6.12 binding of FadB to cholic acid using Cholic acid-conjugated column chromatography.

Elution profile obtained for FadB from cholic acid coupled - sepharose affinity chromatography. The selected fractions showed a major band corresponding to a mass of ~85 kDa, similar to what was expected - 85 kDa (FadB). The corresponding protein band sizes of the unstained protein molecular weight marker (thermos scientific) have been labelled on the left-hand side of the image.

6.12 Protective activity of purified recombinant protein

In this study, the effects of the purified protein on the viability of wild and Δ*fadB S. aureus* were examined by adding a purified protein to inoculated media. As was mentioned in the methods chapter (2.19), the overnight culture of the bacteria was washed three times with a HEEPS buffer supplanted with glucose. A part of this mixture was used in an inoculated peptone saline solution containing a sub-inhibitory concentration of cholic acid (0.8%) with or without the protein. This was then incubated for up to 30 minutes. As shown in Figure 6.13, the recombinant purified protein slight enhances both strains when it comes to surviving in the presence of bile acid (cholic acid) as a comparison with control. Unfortunately, none of these effects were statistically significant. Recombinant FadB contains a homology to oxidoreductases. Some researchers have reported that enteric bacteria have the ability to modify bile acid through oxidation and reduction (Pfeiler *et al.*, 2007; Begley *et al.*, 2005, Bortolini *et al.*, 1997). It is possible, therefore, that the purified protein has a modification effect on colic acid but that the effect cannot be observed *in vitro*.

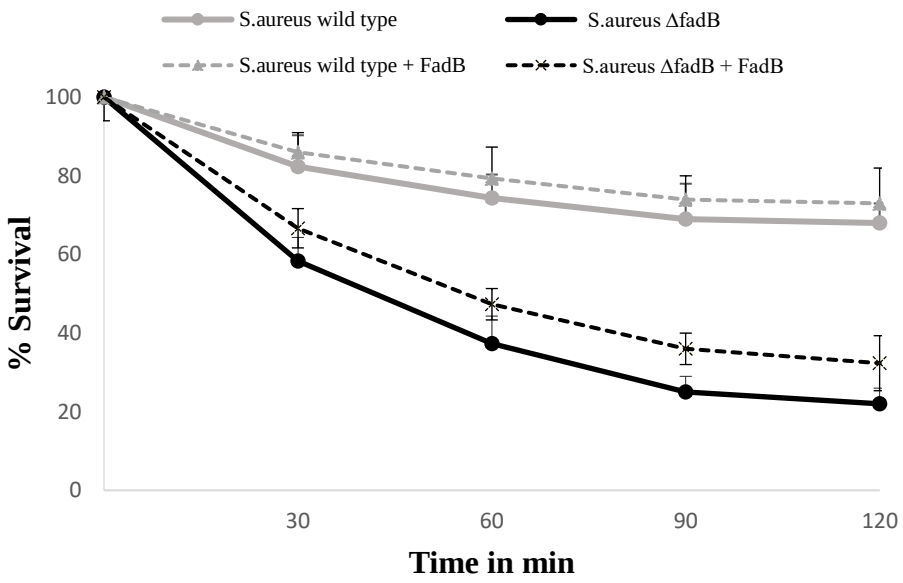

Figure 6. 13*In vitro* antibacterial and time-kill assay of bile salts on wild and ΔfadB *S. aureus* at a cell density of 10^8 CFU/ml in peptone buffered saline supplemented with 0.8 %CA (A) with or without the purified protein(2.5mg/ml). Viability was determined by viable the plate count method. The data represents the mean ± standard error of the mean from three independent experiments.

6-2 Conclusions and discussion

To determine whether FadB has a role to play in bile resistance in *S. aureus*, the 3hydroxy co a dehydrogenase protein was tagged with histidine and then purified. To achieve this, the target encoding gene was cloned into pET21a vector and expressed in *E. coli* BL21/λDE3.

Upon the expression of the recombinant protein, the maximum production of the protein was obtained at two hours post-IPTG induction. The solubility of the protein was assessed and the results of the solubility test showed that about ~ 50% of the protein was observed in the soluble fractions and that this percentage of soluble protein was considered to be acceptable for purification. Therefore, the recombinant protein was transferred to large-scale overexpression and purification via nickel affinity chromatography. Eventually, ~2.50 mg/ ml of the recombinant protein was obtained. The purified protein was sent for mass spectrometry analysis. MALDI-TOF analysis confirmed the identity of the purified protein; it showed that the protein sample contained FadB (theoretical mass 84.6 KDa) from *Staphylococcus aureus.*

Several studies demonstrate that bacterial *fad* (fatty acid degradation) which include *fadL*, *fadD, fadE, fadBA, fadH, fadI* and *fadJ* play an important role in fatty acid transportation, activation and β-oxidation (Campbell *et al*., 2003; Cherepanov & Wackernagel, 1995; Clark & Cronan, 2014; Iram & Cronan, 2006).Campbell *et al.* (2003) demonstrated that *fadB* plays a role in the anaerobic fatty acid degradation in *E.coli* and *fadBA fadR* mutants of *S. enterica* are unable to grow on media containing fatty acids (hexanoate or octanoate). In *S. aureus*, Khairon *et al*. (2016) found upregulated expression of the *fadB* during fatty acid metabolism.

The enzymatic activity of the purified protein was performed by measuring its ability to convert acetoacetyl-Co to ß-hydroxy butyryl-CoA in the presence of ß-NADH. The enzyme showed optimal activity starting at 0.2mM acetoacetyl-CoA (substrate) in the presence of 0.1mm of

NADH. The dependence of enzyme activity on temperature shows there to be no relation between the changing temperature and the increase of enzyme activity. The optimum pH for the enzyme was 6.5 and the enzyme activity decreased in an acidic pH. The lowest activity of the enzyme was recorded at pH 8 and 5.

The biding affinity between FadB and cholic acid were investigated using SPR assay and Cholic acid coupled -sepharose affinity chromatography. SPR results, as shown in **Figure 6.10**, indicate that FadB can bind to cholic acid at 25°C. Furthermore, FadB- cholic acid binding were tested using cholic acid coupled with sepharose affinity chromatography. The results suggested that the retention of FadB to the cholic acid might refer to an interaction between the protein with bile acid (cholic acid).

Furthermore, the effects of the purified protein on the viability of wild and Δ*fadB S. aureus* was examined in the presence of a sublethal dose of colic acid. The results of the viability test show no significant difference in terms of survivability between both the strain and the control. FadB has homology of oxireductase, they could play a role in bile salt modification, as it is known that some species of intestinal bacteria possess the ability to modify bile salts through oxidation and reduction. Le Breton's and his colleague (2002) and Bron (2004) found that an inactivated gene with oxidoreductase homology in *Enterococcus faecalis* and *Lactobacillus plantarum* respectively led to increase sensitivity of these bacteria to bile. However, the molecular mechanism is still poorly understood.

Chapter Seven : General discussion and future work

7.1 Introduction

This study aimed to investigate the ability of *Staphylococcus aureus* to survive the innate defences to infection of the human gut and identify components of the bacterium which confer bile salt resistance. The findings of this study have a number of important implications, which are described in the previous Chapters. Conclusions which can be drawn from the present study as described below.

7.2 *S. aureus* is able to survive under bile salts conditions

Bile resistance is a complex phenomenon and its mechanisms are not fully understood at the molecular level. Furthermore, such resistance is affected by several factors including bacterial strain, type of bile, growth media and experimental design (Chateau *et al*., 1994; Gunn, 2000). The mechanism by which *S. aureus* resists bile have not been fully established.

The current study concludes that bile salts have an antibacterial effect on *S. aureus* and can reduce the viability of the bacteria at subinhibitory concentration . However, *in vitro* MICs study of bile salts shows that *S. aureus* can resist bile salts at concentrations greater than 33 times their physiological concentration in the human gut (0.04% w/v).

These results agree with the findings of other studies, which shows that enteric bacteria such as *Salmonella, Escherichia, Vibrio* and *Campylobacter* are able to survive in bile at concentrations exceeding the *in vivo* physiological level (Nikaido *et al*., 2008; Picken & Beacham,2009 ; Prouty *et al*.,2002; Ramos-Morales, 2013; Cheng & Nikaido, 1997; Van & Gunn, 1999).

This finding, while preliminary, suggests that *S. aureus* can survive in the human. However, further *in vivo* studies required to establish this .

7.3 Bile acids are different in their antibacterial activity on *S. aureus*

Primary bile salts are immediate products resulting from cholesterol degradation .A certain type of microbiota can modify primary bile acid to secondary and conjugated bile salts to unconjugated (Urdaneta & Casadesús, 2017) . The modification of bile acids results in changing to their antibacterial activity and inhibition of the nuclear receptor farnesoid x receptor (FXR) in the human gut. This receptor is responsible for the regulation of bile acid synthesis. Inhibition of FXR lead to a reduction in bile acid level in the gut and then overgrowth of intestinal bacteria (Ridlon *et al*., 2014).

The most interesting finding of the current study was that unconjugated bile acid has a more potent antimicrobial effect against *S. aureus* than conjugated bile acids. It is encouraging to compare this result with that found by Sannasiddappa *et al*. (2017) who found that *S. aureus* are more sensitive to antimicrobial effect of unconjugated bile acids than conjugated one. In the same vein, Begley *et al*. (2005) review bile tolerance enteric bacteria and found that conjugated bile acids are weaker in its antibacterial effect than unconjugated bile acid. However, further research should be done to investigate the ability of *S. aureus* to modifying bile acids or regulate bile synthesis in the host. On the other hand, BSHs are involved in deconjugation of conjugated bile salts.BSHs have been implicated in negating intracellular acidification caused by dissociation of conjugated bile salts inside bacterial cells. The action of BSHs results in formation of weaker unconjugated bile salts (De Boever & Verstraete, 1999).

7.4 strain specificity of bile resistance in *S. aureus*

The current study shows no differences in the bile tolerance among the studied strains of *S. aureus*. However, these findings cannot be extrapolated to all *Staphylococcus* species. No previous study has investigated strain specificity of bile resistance in this bacterium. In contrast ,extensive research has been carried out on bile resistance mechanism in *Listeria*

monocytogenes and *Lactobacillus rhamnosus* concludes that bile tolerance in these bacteria differs by amongst strains and resistance of species cannot be generalized (Chateau *et al.*, 1994; Guariglia-Oropeza *et al* ., 2018).

7.5 Impact of growth media on the level of bile resistance in *S. aureus*

Several studies suggest that level of bile tolerance in the enteric bacteria is related to the growth media used (Chateau *et al*., 1994; Gunn, 2000). Kimoto-Nira *et al*, (2009) demonstrated that bile resistance of *Lactococci* altered, dependent on growth media. The authors suggest that acquired energy from medium enhanced the ability to resist bile and that nutrients within growth media, especially glucose and lactose, have an impact on membrane fatty acids composition. Fatty acids are important for maintaining membrane structure and stability in Gram positive bacteria. Since bile acids is a detergent , bile resistance is thought to be associated with membrane fatty acid availability and stability of the cell membrane (Kimoto-Nira *et al.*, 2009). The current study found that growth density of *S. aureus* in presence of bile in the BHI and TSB media are greater than LB medium, while the bacteria fail to grow in M9. It might that type and concentration of nutrient in growth media govern the rate of bile tolerance in *S. aureus*. Hence, it could conceivably be hypothesised that nutrient concentration affects bile resistance in *S. aureus*. It is difficult to explain this result, but it might be related to changing in cellular fatty composition which play a key role in the stability of cell membrane and resist to detergent effect of bile.

7.6 Impact of bile on the virulence of S. aureus

Certain type of the enteric bacteria not only have the ability to resist bile, but theses bacteria also use bile as a signal for regulation of virulence gene expression for efficient infection (Sistrunk *et al.*, 2016).

In *E. coli* O157:H7 about 41 genes encoding proteins associated with virulence are induced as a response to bile stress. Further screening of genome sof pathogenic *E. coli* strains show that the majority of genes which associated with gastrointestinal infection particularly fimbriae and enterotoxins are carried on mobile genes are lacking in commensal strains(Croxen *et al*., 2013; Fegan *et al*.,2014; Sahl & Rasko, 2012; Torres *et al*., 2007).

A recent study by Ulluwishewa *et al.,* (2016) showed that sub-inhibitory concentrations of bovine bile stimulated biofilm formation in clinical and laboratory strains of *S. aureus*. In this study, the effect of bile on adherence of *S. aureus* to human intestine epithelial HT-29 cells was investigated and showed that bile had no effect on adhesion of *S. aureus* to the intestinal epithelial cells. Furthermore, it was shown that bile has no effect on the ability of *S. aureus* to lyse erythrocytes.

7.8 FadB is involved in bile resistance in *S. aureus*

Bacterial *fad* (fatty acid degradation) genes are a group (*fadL*, *fadD*, *fadE*, *fadBA*, *fadH*, *fadI* and *fadJ*) which play an important role in fatty acid transportation, activation and β-oxidation (Campbell *et al*., 2003; Cherepanov & Wackernagel, 1995; Clark & Cronan, 2014; Iram & Cronan, 2006).

Campbell *et al.* (2003) demonstrated that *fadB* plays a role in the anaerobic fatty acid degradation in *E.coli* and *fadBA fadR* mutants of *S. enterica* are unable to grow on media containing fatty acids (hexanoate or octanoate).

In *S. aureus*, Khairon *et al*. (2016) found upregulated expression of the *fadB* during fatty acid metabolism. Only two studies have attempted to investigate a role of FadB in *S. aureus* where Camargo *et al*. (2009) found that the *fadB* gene was upregulated as a part of the lipid metabolism response to Daptomycin stress, while Jang *et al*. (2008) documented that *fadB*, among genes encoding lipid metabolism enzymes, was down-regulated in response to triclosan,

which is a broad-spectrum antimicrobial agent. The authors suggest that triclosan inhibits production of fatty acid metabolism-related genes of *S. aureus*. The inhibition of fatty acid synthesis killing this pathogen by interfering with its ability to form cell membranes. However, no previous study has investigated the role of *fadB* gene in bile resistance in *Staphylococcus*.

Several studies documented that fatty acid metabolism in many types of bacteria is tightly regulated as a response to changes in the environment (Parsons & Rock, 2013; Zhang, Marrakchi, & Rock, 2002; Lu & Rock, 2006). Furthermore, the availability of fatty acids in bacterial cells is important to maintain membrane lipid homeostasis and fatty acid degradation and biosynthesis pathways switch on and off depending on the availability of these acids (Matsuoka *et al*., 2007). Bile promotes changes in lipid metabolism through changes in the production of protein involved in fatty acid metabolism (Ruiz *et al*., 2013; Sánchez *et al*., 2007a; Fernández *et al.*, 1999a). According to Taranto *et al*. (2003), bile causes severe changes in the lipid profile of *Lactobacillus reuteri*; these changes include decreases in phospholipids and a lower ratio of saturated: unsaturated fatty acids. The induced changes affect the physical properties of the membrane.

Several studies have highlighted that the level of bile resistance in multiple bacterial species (e.g., *E. coli*, *L. monocytogenes*, and *L. acidophilus*) is strongly related to the changes in fatty acid composition, lipid fluidity, lipopolysaccharide (LPS), hydrophobicity and membrane electric charge (Chou & Cheng, 2000; Fernández *et al*., 2001b; Urdaneta & Casadesús, 2017). Interestingly, changes in fatty acid composition are closely related to bile sensitivity of *Lactobacillus* and *E. coli* (Kimoto-Nira *et al*., 2009b)

In this study, a bile resistance gene *fadB* in *S. aureus* SH1000 was initially identified by investigating the natural protein diversity within the *Staphylococcus* in response to bile challenge. The gene was deleted and sensitivity to bile salts was assessed by MIC and viability

test. *S. aureus fadB* mutant cells record a several fold decreases in their MICs and a significant decrease in the viability when challenged with bile salts, compared to wild type cells.

Furthermore, heterologous expression of *S. aureus fadB* in *E. coli*, suggest that *fadB* has a role in the survivability of *S. aureus* under bile salts stress. FadB was expressed in *E. coli* under the control of the pBAD expression vector which is induced with arabinose. MIC and viability tests were used to assess the role of *fadB* in resistance of bile. *E. coli* strains expressing *fadB* showed enhanced MICs to CA, TCA and GCA and bacterial a viability upon exposure to CA, TCA and GCA significantly increased ($P<0.05$) as a compared to wild type *E. coli* strains lacking *fadB*.

Moreover, this study found upregulated expression of *fadB* in *S. aureus* upon exposure to sub-lethal dose of bile. Johnson *et al*. (2018) concluded that *fad* (fatty acid degradation) genes were upregulated as a response to bile in all studied isolates of *Salmonella*. The major constituents of bile are bile salts, phospholipids, cholesterol, proteins and biliverdin pigment. Twenty two percent of bile is phospholipid, thus up-regulation of *fad* genes in bile treated *Salmonella* isolates might be related to the ability of these bacteria to use of phospholipids as a source of carbon (Johnson, 2018; Antunes *et al*., 2011).

Interestingly, this study found that FadB has affinity to bile acid (cholic acid) . furthermore, it has has homology of oxireductase, so could play a role in bile salt modification, as it is known that some species of intestinal bacteria possess the ability to modify bile salts through oxidation and reduction. Le Breton's and his colleague (2002) and Bron (2004) found that inactivation of genes with oxidoreductase homology in *Enterococcus faecalis* and *Lactobacillus plantarum* respectively led to increase sensitivity of these bacteria to bile. However, the molecular mechanism is still poorly understood.

7.9 Utility of *In vitro* human intestinal models

in vitro gut models have been extensively used for screening a large number of substances such as dietary ingredients, pathogens, drugs and toxic or radioactive compounds, to assess how they alter and are altered by gut environments and microbiota populations. *In vitro* gut models range from a simple batch fermentation system to sophisticated pH controlled three stage continuous culture systems, such as the three-stage continuous culture system, SHIME®, Entero Mix, Lacroix model and TIM-2. Choosing a model system depends completely on the goals of the study.

The batch fermentation model is simplest and most commonly used for studying the gut microbiome. This model is inexpensive, easy to operate, reproducible and fast. furthermore, in this model, fermentability of various substrates can easily be determined within a short time (Allison *et al*., 1989; Macfarlane *et al*., 1994). However, short-term fermentation and weakness in microbiological control are main disadvantage of this model.

The current study use batch culture as a human colonic model. *S.aureus* show ability to survive in this model in presence of the human gut microbiota.

7. 10 FadB is important for *S. aureus* survival in the human colon.

Several recent studies prove that *S. aureus* is often one of the members of gut flora (Acton *et al.*, 2009; Misawa *et al.*, 2015; Schaffer *et al*., 2006; Vesterlund & Ouwehand, 2006). In a study, Lindberg and colleagues (2000) identified *S. aureus* and superantigenic toxins (SEA, SED and TSST) in stool samples from 49 Swedish infants. They found 47 of 49 samples contained *S. aureus* producing TSST-1 and enterotoxin during their first year, without any gastrointestinal problems. *S. aureus* is a versatile pathogen and emerges as a member of the normal flora in infants may be of clinical importance (Lindberg *et al*., 2000; Reddy *et al*., 2017).

Infection of *S. aureus* wild type and *fadB* mutants in the batch culture as a human colonic model, demonstrated a significant reduction in the populations of *S. aureus* Δ*fadB* during post infection period. Overall, these results suggest that FadB plays a role in *S. aureus* colonization in the human gut.

Taranto *et al*., (2003) demonstrated that changes induce in the lipid metabolism or lipid profile in the cell membrane of *L. reuteri* might probably constitute one of the main physiological responses of the cells for survival in the gastrointestinal tract.

7.11 Future work

7.11.1 Global transcriptional response of *S. aureus* to bile

To better understand the mechanisms of bile resistance in enteric bacteria, the global transcriptional response of these bacteria to bile has been extensively studied. Several such studies suggest that genes which encode proteins involved in cell envelope and cell membrane biogenesis, carbohydrate and lipid metabolism, efflux transporters, protein modification and degradation of bile salts were induced in response to bile salts (Alcántara & Zúñiga, 2012; Begley *et al*., 2005; Kristoffersen *et al*., 2007; Wu, *et al* .,2010).

Analysis of the global transcriptional response of *S. aureus* to sub-lethal doses of bile salts could be useful to identify which genes respond to bile and provide a better understanding the mechanism of bile resistance in this bacterium.

Fujita *et al*. (2007) demonstrate that the availability of fatty acid in bacterial cells is very important to maintain membrane lipid homeostasis. Fatty acid degradation and biosynthesis pathways can switch on or off depending on the availability of these acids. Suzuki *et al*. (2014) suggests that cellular fatty acid plays a key role in bile resistance of *Lactobacillus brevis*.

Studying the relationship between bile resistance and cellular fatty acids composition might be useful for identify the mechanism by which *S. aureus* resist bile.

7.11.2 Profiling of bile acid interacting proteins in *S. aureus*

In vivo characterization of bile interacting proteins in *S. aureus* would be a powerful methods to better understand the mechanisms by which *S. aureus* resists bile. Zhuang *et al*. (2017) demonstrated novel method to study bile acids - interacting proteins in mammalian cells using clickable and photoreactive bile acid-based chemical probes in combination with quantitative mass spectrometry. The author successfully identified a large number of novel bile acid-interacting proteins including chaperone binding proteins involved in ER stress response, key metabolic enzymes in diverse pathways of lipid metabolism, and functional targets strongly associated with metabolic syndrome, neurodegenerative diseases, and diarrhoea. The identified BA-interacting protein involved in understanding of BA-mediated pathways in human metabolism and disease(Zhuang et al., 2017).

Using this strategy to study bile resistance in *S. aureus* might be useful to identifying proteins inside the cell that interact with bile acids and then understanding the possible mechanisms of bile resistance in the bacteria.

7.11.3 Effect of bile on the lipid cell membrane of *S. aureus*

The relationship between architecture and composition of the envelope (membrane and cell wall) and bile resistance have been documented in a wide range of bacteria (Begley *et al*., 2005; Ruiz *et al*.,2013; Sánchez *et al*., 2007). Taranto e*t al*., (2003) demonstrate that bile induces changes in the lipid profile of *Lactobacillus reuteri* which play a crucial role in the response of this bacteria to environmental stress. Furthermore Kimoto-Nira *et al.*, (2009) suggest that changes to the cellular fatty acid composition in *Lactococcus lactis* result in decrease in resistance of theses bacteria to bile.

Evaluating the effect of bile in the lipid profile of *S. aureus* and determined the relationship between these effect and bile tolerance in theses bacteria might be important to understand the mechanism of bile resistance.

Furthermore, studying lipid profile of *S. aureus* wt and Δ*fadB* will help to understand the mechanism by which this gene involvoled in bile resistance and survivae of the bacteria in the human gut.Complementation of *S. aureus* Δ*fadB* with *fadB* gene needed to confirm the parental bile salt resistance phenotype.

7.11.4 In vivo gastrointestinal survival studies

The effect of gastrointestinal colonisation as a risk factor for *S. aureus* infection was neglected for many years then it was refocused on after the appearance and spread of methicillin resistance *S. aureus* and antibiotic (MRSA) (Acton *et al*., 2009). Several studies suggested that *S. aureus* could colonize and survive in the human gastrointestinal tract. *In vivo* models are used to study carriage and survival of *S. aureus* in the human intestine. However, to date no suitable model can perfectly reproduce the complex microbial ecosystem or the physicochemical environment of the human gut (Sannasiddappa *et al*., 2011).

Pang *et al*., (2007) demonstrate that the human flora associated (HFA) piglet model, colonized by human flora can reproduce complex microbial ecosystem and mimics the *in vivo* digestive physiology of the human intestine. This model could be suitable for studying survival of *S. aureus* wild type and Δ*fadB* in the human gut. Further evalution of the effect of defect on the cellular fatty acid or lipid metabolism composition on the level of bile tolrence in *S. aureus* and ability to survive in the human gut.

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Appendix

Appendix 1 : Mutagenesis strategy

tep1 : amplified 1k upstream , fadb and 1kb downstream of fadb

P1

CTAAATTACAGTCACATGAACTGCGTACATGCTTTGCTTTAACTGAACCAGAACACGGTTCGGACGTTGC
GGGAGGTCTTGAAACAGTCGCTGAACGCCAAGGCGATACTTGGGTTATCAATGGTGAAAAGAAATGGATT
GGTGGTGCACATGTATCTGATGTCATTCCAGTATTCGCAGTAAATAAAGAAACTGGCAAACCCCATTGCT
TTGTAGTCAGACCAGAACAAGATGGCGTCGATATTGAAGTCATTGATAATAAAATCGCACTTCGCATTGT
TCCTAACGCCCTAATTAAATTAACTAATGTCAAAGTAGATGAAGCGGATCGCTTACAAAACATAACAAGC
TTTAAAGATATTGCCAAAATTCTTTATTCAACGAGAGCAGGCGTTGCTTATATGGCTACAGGTGGTATGG
CTGGCGCTTTACGTGCCACATTAGATTATGTCACTGAGCGTAAGCAATTCGGCAAACCAATTAGTAAATA
TCAGTTAATACAAGAAAAGCTAGCAATGATGCAAGGTAATTTAGCTCAAGCAATGGCAACATGTGCTCAA
TTAGCTAATATGCAAGCACATGGTGAATATGACGAGGTTGCAACTTCAACGGCGAAGATGATGAATGCCT
TACGTTTGCGTGAGACAGTAGCTATGGGCCGCGGTATTACAGGTGGTAATGGCATACTAGCTGACGATTA
TGATATTGCACGTTTCTTCTCTGATGCAGAAGCGATTTACACGTACGAAGGTACACATGAAATTAATGCC
TTAGTAATTGGACGCGCTTTGACTGGAGATTCTGCTTTCGTATAAATAGCAAATAATTATATGAGATGCA
TTAATTTCACTAAAAAAGACTTATTTTAAGCATAAAGCTTTTTCCTTAAATAAGAGGCTAAGATGACTGT
CAAAGATACTTAATTAATTTTATAAAATAGCAACGTTATTCCAATTATCTTAATGGTTATCTTATCCTCA

P2

ACTAAATTGGAGGAATCACTATGACAATTAATAAAGTAACCGTTCTTGGCGCAGGCACAATGGGCGCTCA
ACTGGCAGCACTTTTTGTGAATGCTGGACTTAAAGTAAAACTATTAGATATTGTAGTGGACAAAAACGAT
CCAAATCTCATTGCGAAAAAATCTTACGATAAAATTACAGATAAGAAACGGCCGCTACTATTCGACTTAA
ATCTAGCGAGTCATTTAACATATGGTAATTTTGATGATGACTTGGTAAATGATGATGCTGATTTATATAT
CGAAGCAGTCAAAGAAGATATTGAAATTAAGCATGCTGTTTGGCAACAAGTTCTACAACATGCTAAAGAA
GATGCTTTATTCGCTACAAATACATCAGGTATTCCAATTAATGCGATTGCTCAAGCATTTAACGAGAAGG
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GTCATTGTCGTCAATGATGTGCCTGGCTTTGTCGCAAATAGAGTCGGCACGCAAACAATGAATGATATTA
TGTATCGCGCCGAGCAACACAAGATAAGCATTGTAGATGTGGATGCTTTAACTGGGCAAGCGATTGGTCG
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ATGCAACAAGTACCTGAAGAAACACCTTATTTTCATGATGTCAAAATTGTAAATACGTTGTTTGACAATG
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AGACGGTCTTGTAGATGATGTCGTTGCACAATCAATTGATAAATTACATTATAGCTTTAATCGTTTGAAG
TATAGTTTGAAACCAGTAGTCACAGCTGTTCAAGGTCGTGCCTTAGGCGGTGGCTGTGAGCTTGTACTTT
ACTCACCTATTGTTGTCGCTGCAAGTGAAACATATATCGGTCTTGTTGAAGCAGGTGTTGGCTTATTACC
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GTTATGGTTATTTACGTGATACAGATACGATTATTTTCAATACAGCACAACGTGTCGAAGTTGCGCTCAA
ACGTGCGAAATATGAAGCAGAAACAAACTATATTCCGAATCCTAGACATCAATATATCGCTTTAGGTGAA
GACTTCAAAGCATTGATCCAAGGACAATTAGATGCGCAAAGACGGGGTCATTTTATTAGCGACCATGATT

fadB

P3

ATCATATTGCCTTAAATATCGCCACAATTTTAGCGGGTGGTGATTTACCAAGAAATACATTTATCAATCA
ACGTTACATTCAATCGTTGGAGAAAATTGGCTTTATTGACTTACTAAAATCTAAAAAATCATATGAAAGA
ATTGCACATATGTTAAAAACTGGTAAGCCATTACGTAATTAAAAGATAGTCATTAAGAGAGGATGATAAC
CATGCAAGAAGCATACATTGTAGCTTATGGGCGTTCAGCCGCAGCGAAAGCAAAGCAAGGCGCATTATTC
CACGAAAGACCTGATGATGTCGCAGCCAAAGTATTACAAGGCGTATTGAAACGTATTGACGGAAAATTCA

Appendix

ATAAGAATATGATTGAAGATGTCATTGTTGGTACGGCTTTTCCAGAAGGATTACAAGGCCAAAACATTGC
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TCAGGATTACAAACCATCGCGATTGCAGCCAATCAAATTATGGCTGGTCAAGGAGATATACTTGTAGCTG
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TCAAAGATGAAATTATTCCAATACAAGTAAACTCAGTTGAATATACAAACGCAGGACCAAAAGTACACAC
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TTTTAATGTCTGGAGATAAAGTGAAAGAACTCGGCGTGACACCTATTGCACGATTCGTTGGTTTTAAGGC
AGTAGGCGTTGACCCGAAAATTATGGGTATTGGGCCTGCATATGCGATTCCTGAAGTATTGTCACTCAGC
AATCTATCTGTTGAAGACATTGATTTGATCGAATTGAACGAAGCATTTGCTTCTCAAACGAT

P4

Figure 8.1 Primer designing strategy to construct S. aureus ΔfadB the fadB gene sequence (in red box) with extended 1kb sequence on either side of left and right gene coordinates.

P1

CTAAAT GGATCCTACAGTCACATGAACTGCG

L:31;GC:45;Tm:61.7

P2

ATTAACCCGGGTTGTCATAGTGATTCCTCCAATTTAGTTG

L:40;GC:40;Tm:64.5

P3

CATTA CCCGGGCGTAATTAAAAGATAGTCATTAAGAGAGG

L:40;GC:40,Tm:64.5

P4

CGTTTGGGATCCAGAAGCAAATGCTTCGTTCAATTCG

L:37;GC:46;Tm:65.6

BAMH1 GGATCC

Sma1CCCGGG

Appendix 2 : Vectors and constructed plasmids

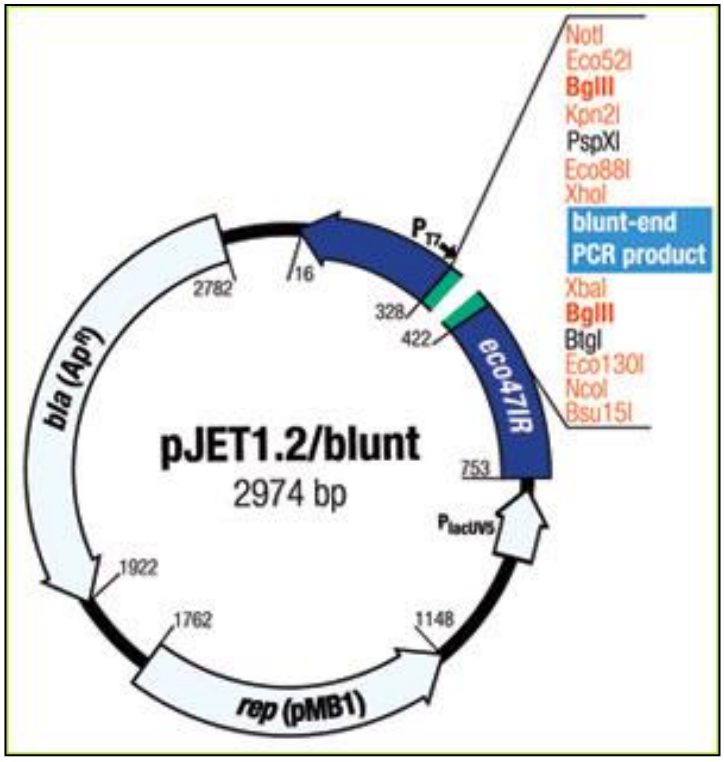


Figure 2.1 Restriction map of pJET1.2/blunt, plasmid used for cloning PCR fragments of *fadB*

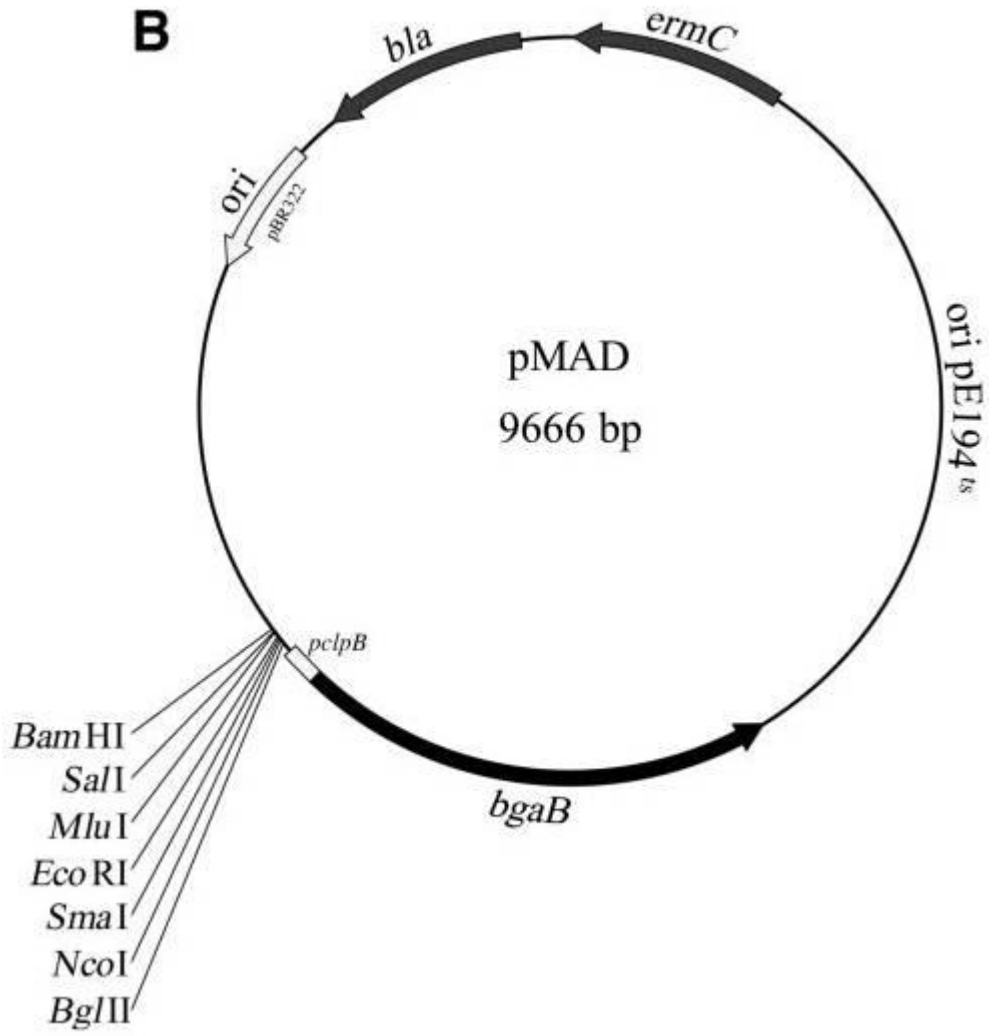


Figure 2.2 Restriction map of pmad plasmid used for cloning PCR fragments of *fadB*

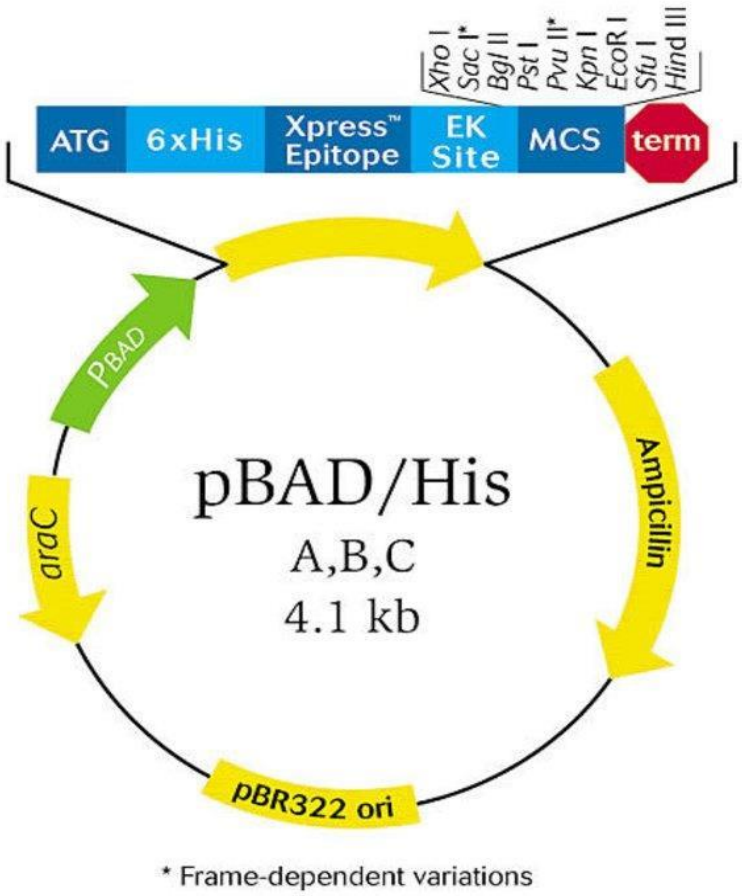


Figure 2.3 Restriction map of pBAD$_{ara}$. The inducible araBAD promoter is shown ('ARA_promoter'). Source: Thermo Fisher Scientific.

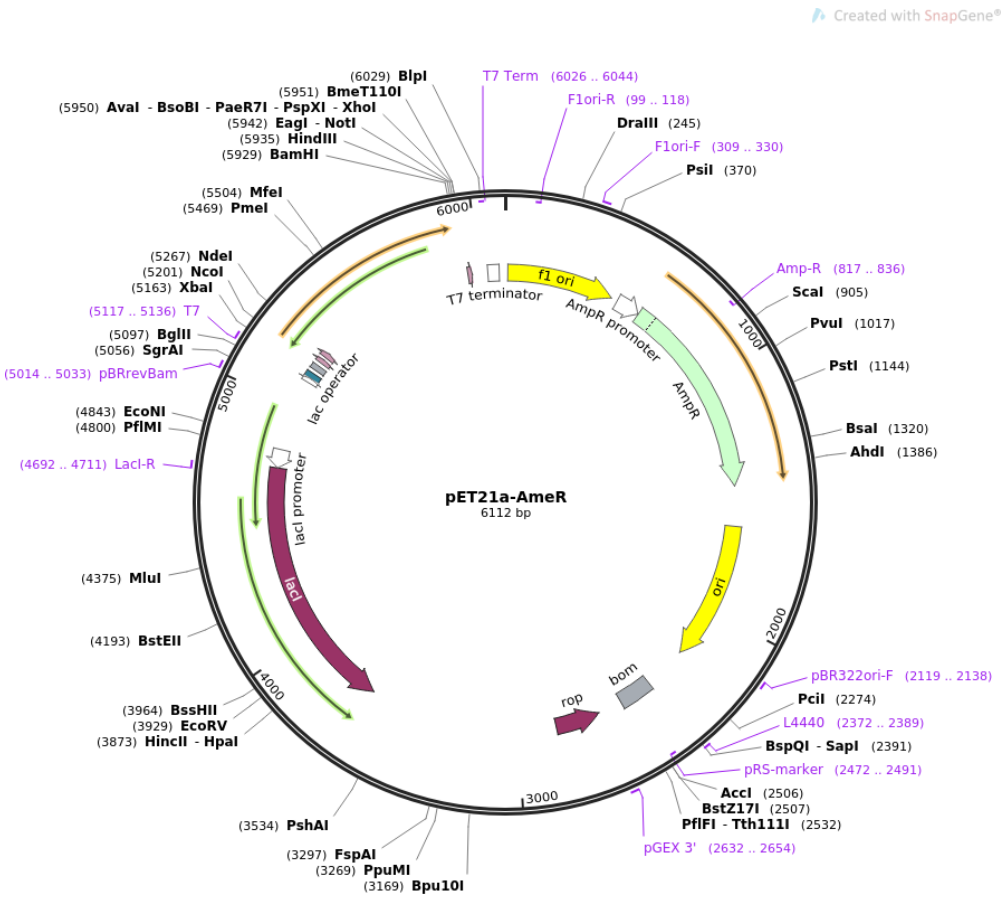


Figure 2-4: Restriction map of pET21-cyto c used for overexpression of FadB

Appendix 3 :Bioinformatic analysis of FadB

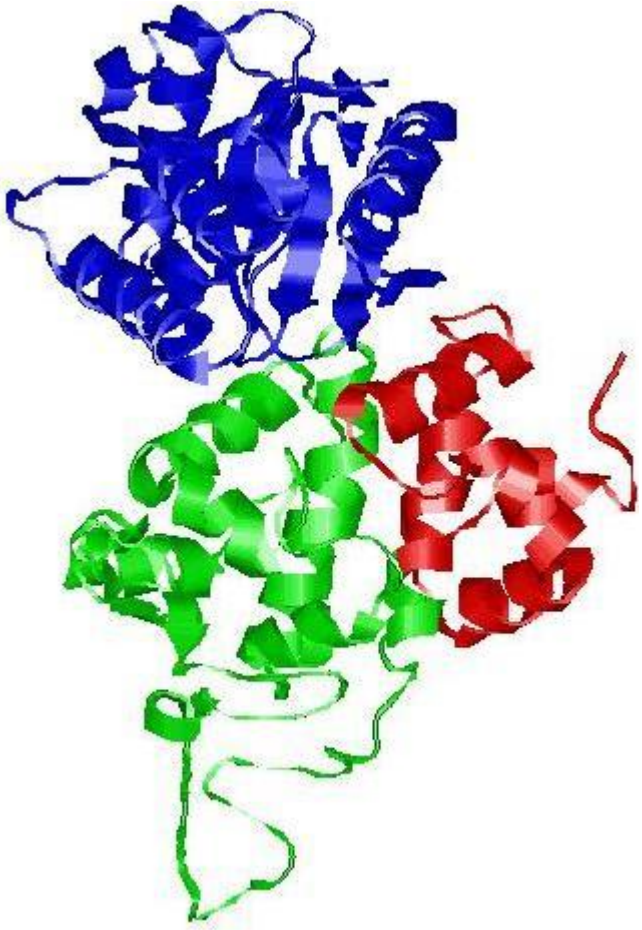


Figure 3.1 Domain predication of FadB *S.aureus*

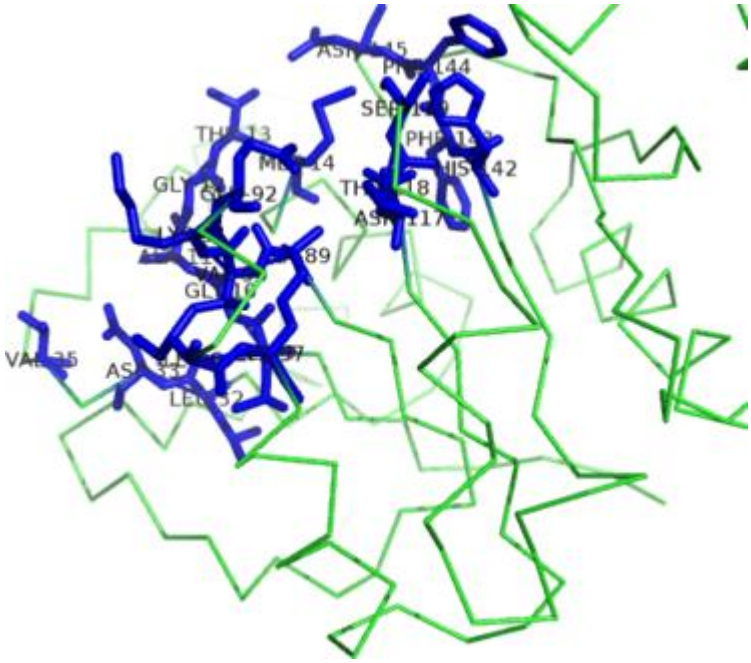


Figure 3.2 PyMOL generated image of ligand binding residues prediction for FadB_protein_domains

Predicted ligand binding residues are shown as blue sticks in the image above.
Binding site: 9, 10, 11, 12, 13, 14, 32, 33, 34, 89, 90, 91, 92, 97, 117, 118, 119, 142, 143, 144, 145, 229
Most likely ligands at each site (Type): NAD
Centroid ligands at each site (TypeID): NAD420
All ligands in clusters (Type-Frequency): NAD-5, CAA-1
Likely+centroid ligands at each site: NAD420
EC numbers: 4.2.1.17;5.3.3.8;1.1.1.35;5.1.2.3
GO terms:
0000166;0003824;0003857;0004165;0004300;0005515;0006629;0006631;0006635;0008152;0008692;0009062;0016042;0016491;0016507;0016616;0016829;0016853;0050662;0055114;0005634;0005737;0005739;0005743;0005759;0009725;0014823;0032868;0042493;0044255;0044281;0046676;0070403;0005730;0046872;

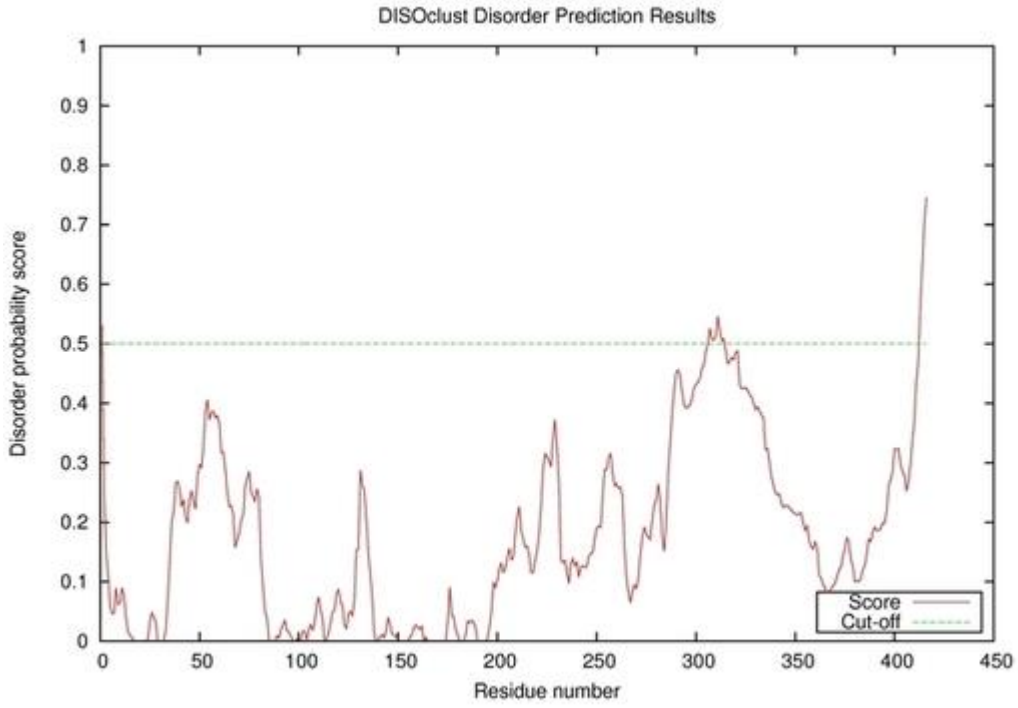


Figure 3.3 Disorder prediction for FadB_protein_domains

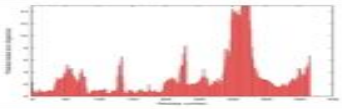
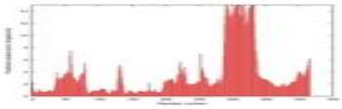
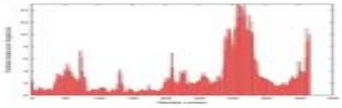
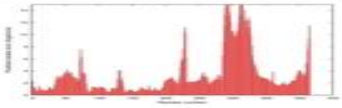
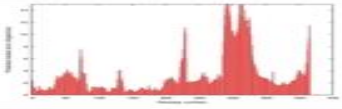
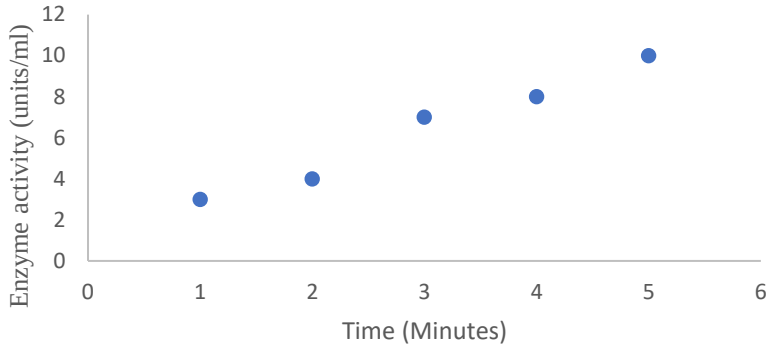
Model ID and PDBsum links for all templates used	Confidence and P-value	Global model quality score	Local model quality plot (click images to download plots)	Model coloured by local quality (click images to view models, local errors and target coverage interactively)
IntFOLD3 dPPAS2 multi8 TS1 3mogA 4j0eA 1zcjA	CERT: 7.195E-4	0.6862		
IntFOLD3 wPPAS multi8 TS1 3mogA 1zcjA	CERT: 7.306E-4	0.6848		
IntFOLD3 IntFOLDTS130 multi4 TS1 1wdkA 3mogA	CERT: 7.543E-4	0.6817		
IntFOLD3 MUSTER multi6 TS1 3mogA	CERT: 7.567E-4	0.6814		
IntFOLD3 MUSTER multi2 TS1 3mogA	CERT: 7.567E-4	0.6814		

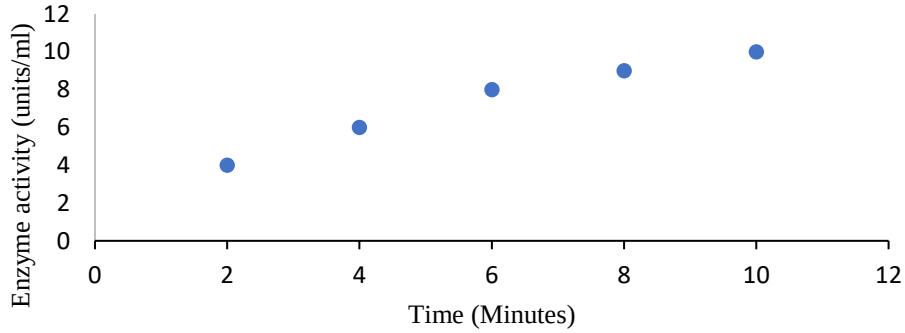
Figure 3.4 **Top 5 multi-template 3D models for FadB_protein_domains**

Appendix 4

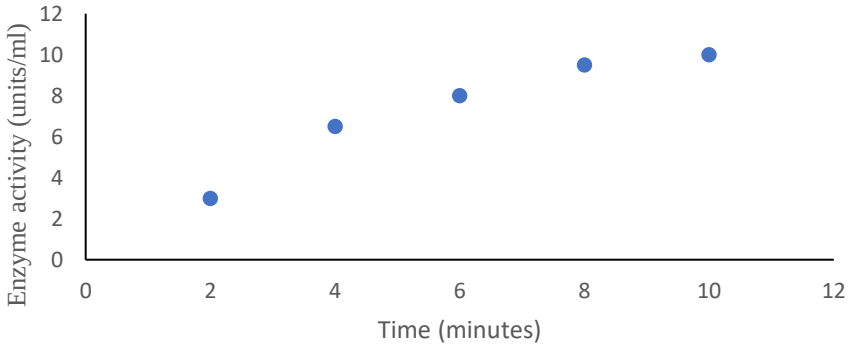
A



B



C



Progressive curve of FadB activity. The catalytic activity of the enzyme by converting acetoacetyl-CoA to hydroxybutyryl-CoA in the presence of NADH as a cofactor was spectrometrically determined. The substrate (acetoacetyl-CoA) was used to measure the activity rate of the enzyme at concentration 0.2 mM(A) , 0.2 Mm (B) and 0.3 mM (C).