# *EFFECT OF HAMAMELITANNIN ON STAPHYLOCOCCUS AUREUS BIOFILM SUSCEPTIBILITY*

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# Summary

*S. aureus* infections can cause life threatening infections or colonize medical devices, requiring the removal of the device. These infections are hard to treat, due to biofilm formation and resistance. Since only few new antibiotics are being developed, there is a need for novel therapeutic approaches. One of these is the inhibition of quorum sensing (QS), since QS is involved in biofilm formation and virulence regulation.

Hamamelitannin (HAM) is a compound that possibly inhibits the QS system of *S. aureus*. The effect of HAM was not observed in planktonic bacteria, but only observed on biofilm bacteria. HAM increases biofilm susceptibility towards vancomycin for *S. aureus* strains with different *agr* types, suggesting that the effect of HAM is *agr* type-independent.

Similar effects were observed when HAM was combined with linezolid, tigecycline and chloramphenicol for treatment of *S. aureus* Mu50 biofilms. HAM does not enhance biofilm susceptibility towards daptomycin, fusidic acid, ciprofloxacin and doxycycline. However, these experiments should be repeated with different antibiotic concentrations before we can fully exclude the presence of an added value for combination therapy of HAM and one of these antibiotics.

Previous research showed that VAN treated biofilms resulted in an upregulation of hemolytic genes. In contrast, HAM treated biofilms or biofilms treated with the combination resulted in a downregulation of hemolysin gene expression (Brackman *et al.,* unpublished data). VAN treatment resulted in an increase of hemolytic activity, while the combination treatment of *S. aureus* Mu50 biofilms resulted in the inhibition of the VAN induced hemolytic activity. So, HAM does not only enhance biofilm susceptibility towards different antibiotics, but it also suppresses virulence caused by VAN treatment.

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# List of abbreviations

*agr* : accessory gene regulator

AI-2 : auto-inducer 2

AIP : autoinducing peptide

*C. elegans* : *Caenorhabditis elegans*

CA-MRSA : community acquired - MRSA

CFU/mL : colony forming units/milliliter

CHL : chloramphenicol

CIP : ciprofloxacin

CLSI: Clinical and Laboratory Standards Institute

Comb : combination treatment of HAM and VAN or other antibiotics

CTB : Cell Titer Blue

CTRL : control group

DAP : daptomycin

DMSO : dimethyl sulfoxide

DNA : deoxyribonucleic acid

DOX : doxycycline

eDNA : extracellular DNA

Em : emission wavelength

Ex : extinction wavelength

FIC : fractional inhibition concentration

FUS : fusidic acid

*G. mellonella* : *Galleria mellonella*

GC : growth control

GISA : glycopeptides intermediate *Staphylococcus aureus*

HAM : hamamelitannin

HA-MRSA : hospital acquired - MRSA

LIN : linezolid

MH : Mueller Hinton broth

MIC : minimum inhibitory concentration

MQ : Milli-Q water

MRSA : methicillin resistant *Staphylococcus aureus*

MSCRAMM : microbial surface components recognizing adhesive matrix molecule

ND: not determined

nm : nanometer

PBP: penicillin binding protein

PBS : phosphate buffered saline

PI : propidium Iodide

PIA : polysaccharide intercellular adhesin

PSM : phenol soluble modulin

PW : physiological water

QS : quorum sensing

RAP : RNAIII activating protein

RIP : RNAIII inhibiting peptide

RNA : ribonucleic acid

Rpm : rounds per minute

*S. aureus* : *Staphylococcus aureus*

SasG : *S. aureus* surface protein G

SCCmec : *Staphylococcal* cassette chromosome *mec*

SD: standard deviation

SDS : sodium dodecyl sulfate

SEM : standard error of means

TIG : tigecycline

TRAP : target RNAIII activating protein

TSA : tryptone soy agar

TSS: toxic shock syndrome

TSST-1 : toxic shock syndrome toxin-1

VAN : vancomycin

VISA : vancomycin intermediate resistant *S. aureus*

VRSA : vancomycin resistant *S. aureus*

# 1. Introduction

## 1.1 *Staphylococcus aureus*

### 1.1.1 Characteristics

*Staphylococcus aureus (S. aureus)* are a facultative anaerobic Gram-positive cocci. Staphylococci are highly resistant to heat, high concentrations of salt and high osmotic pressure. They are catalase-positive and oxidase-negative. *S. aureus* can be differentiated from other Staphylococcus species (e.g. *Staphylococcus epidermidis*) by their coagulase activity (Harris *et al*., 2002).

### 1.1.2 Staphylococcal infections

*S. aureus* is present on the skin and in the nares of humans as a commensal and opportunistic pathogen (Kim *et al.*, 2014). *S. aureus* is associated with many infections such as skin, soft tissue, respiratory, bone, joint and endovascular infections (Lowy, 1998). *S. aureus* can also cause food poisoning by producing enterotoxins A and B. Toxic shock syndrome (TSS) can also be caused by *S. aureus* through toxic shock syndrome toxin-1 (TSST-1) production (Krishnamurthy *et al.,* 2014).

### 1.1.3 Resistance

Methicillin resistance is frequently found in *S. aureus* (methicillin-resistant *S. aureus* or MRSA). Resistance is obtained by the integration of resistance gene *mecA* in the *S. aureus* genome. The gene is found on Staphylococcal cassette chromosome *mec* (SCC*mec*), which is a mobile genetic element. The m*ecA* gene encodes for a different penicillin binding protein 2a (PBP2a) that has a lower affinity for β-lactam antibiotics. PBP2a takes over the production of peptidoglycan while the original PBP is inhibited by the antibiotics (Berger-Bächi and Rohrer*,* 2002). Historically, MRSA infections were only hospital acquired (HA-MRSA). In 1999, several community-acquired MRSA infections (CA-MRSA) were reported among young adults and children. CA-MRSA strains are more virulent, but are more susceptible to non-β-lactam antibiotics (Pantosti *et al.,* 2007).

Vancomycin (VAN) is the standard treatment for a MRSA infection, but the first case of VAN resistance was already reported in 1996 in Japan. In this case the minimal inhibitory concentration (MIC) of VAN was slightly raised (Hiramatsu *et al.,* 1997). This *S. aureus* isolate with decreased VAN susceptibility was called vancomycin intermediate resistant *S. aureus*

(VISA). The National Committee for Clinical Laboratory Standards state that *S. aureus* strains that require 8 – 16 µg/mL of VAN to achieve growth inhibition are defined as VISA. When the MIC is elevated to 32 µg/mL these *S. aureus* strains are considered vancomycin resistant *S. aureus* (VRSA). When a VISA isolate is also less susceptible to other glycopeptides such as teicoplanin, it is defined as glycopeptides intermediate *S. aureus* (GISA) (Linares, 2001). Next to its resistance profile, treatment of *S. aureus* infections is even more complicated due to biofilm formation at the infection site.

## 1.2 Biofilms

A biofilm is an organized bacterial community. Bacteria are embedded in a biopolymer matrix consisting of self-produced exopolysaccharides, proteins and extracellular DNA (eDNA) (Høiby *et al.,* 2010). Within a biofilm, bacteria are protected against the host's defense mechanisms, against hostile environments and against antibiotics. The first phase of biofilm formation is the reversible attachment of planktonic cells to a surface (figure 1.1 step 1). The attachment to an abiotic surface depends on the bacterial surface, as well as on the characteristics of the material itself. Abiotic attachment occurs through van der Waal's forces and hydrophobic and ionic interactions (Heilmann *et al.,* 1997). A study by Gross *et al.* (2001) showed that teichoic acids also play a major role in attachment. As a medical device is inserted, it quickly becomes covered by host matrix proteins. *S. aureus* can also attach to host tissue by expressing microbial surface components recognizing adhesive matrix molecules (MSCRAMM's) (Patti *et al.,* 1994).

In the second stage of biofilm formation bacteria bind irreversibly to the surface and they proliferate to form microcolonies (figure 1.1 step 2). Microcolonies are obtained by intercellular adhesion. This is accomplished through the production of polysaccharide intercellular adhesin (PIA) by *S. aureus*. Because of its positive charge it can adhere to a bacterium's negatively charged surface (Otto, 2013). Next to PIA other intercellular adhesion proteins are produced, such as protein A (Merino *et al.,* 2009), *S. aureus* surface protein G (SasG) (Corrigan *et al.,* 2007) and fibrinogen-binding proteins in some MRSA isolates (O'Neill *et al.,* 2008). The bacteria in a microcolony produce the previously described biopolymeric matrix that increases biofilm stability (Høiby *et al.,* 2010).

The third phase of biofilm formation is called maturation. In this stage bacteria continue to multiply to form a mature biofilm. Water channels are formed within the biofilm for the delivery of nutrients to each layer of the biofilm (figure 1.1 step 3). The final phase of biofilm formation is dissemination. In this stage, a cluster of planktonic cells is released, spread out and is capable of establishing an infection at other body sites (figure 1.1 step 4) (Otto, 2013). Biofilm formation is a complex and regulated process. It is controlled by quorum sensing.

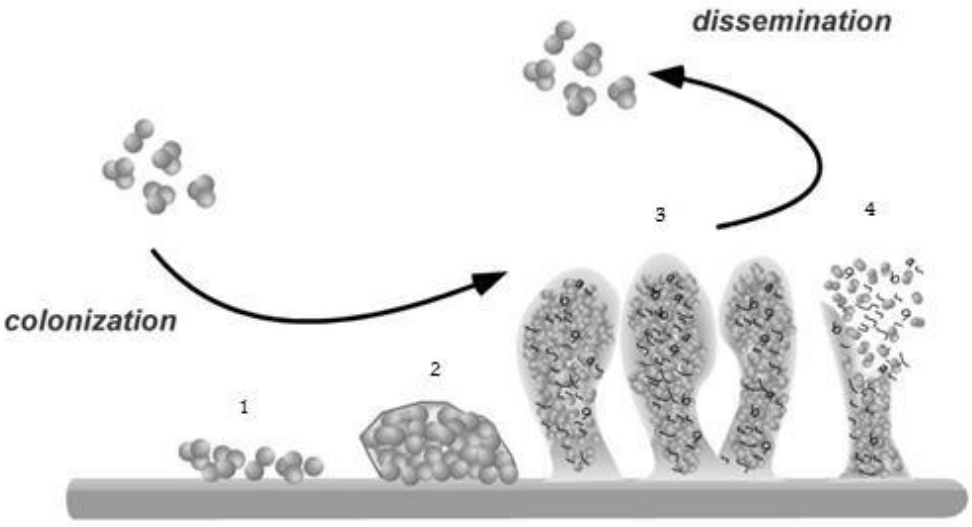


**Figure 1.1. The 4 stages of biofilm development (Otto, 2008).**

## 1.3 Quorum sensing

Bacteria are able to communicate with each other through the production of signaling molecules. When the number of bacteria is high enough, the signal concentration threshold is reached. This leads to a change in gene expression in the bacteria. This cell density dependent bacterial communication is called quorum sensing (QS). QS is mandatory for biofilm formation.

## 1.4 Role of QS in biofilms

QS regulates the production of adhesion molecules and virulence factors according to cell density in a biofilm. In *S. aureus* there are two major QS systems, the accessory gene regulator (*agr*) system and the RAP/TRAP system. There is also a LuxS system, but its mechanism is not yet fully understood in *S. aureus*. *Agr* and RAP/TRAP are both two-component systems. By using these QS systems, bacteria are able to respond to an external signal through a change in gene expression (Junecko *et al*., 2012).

### 1.4.1 *Agr* system

The *agr* QS system (figure 1.3, at the top) is a global regulatory system which consists of two primary transcripts, RNAII and RNAIII. Transcription of RNAII depends on the activation of its promoter P2. The RNAII locus includes four genes that are co-transcribed: *agrA*, *agrB*, *agrC* and *agrD*. The transcription of *agrD* results in the production of an autoinducing peptide precursor. AgrB cleaves the precursor peptide and forms a thiolacton ring of 8 amino acids. This substrate is called the autoinducing peptide (AIP). AgrB's second function is to transport AIP to the outside of the cell. AIP binds to AgrC histidine protein kinase receptor when the concentration threshold is reached (Laverty *et al.,* 2013). There are four different AIP types (type I–IV). Each AIP type is transcribed from its own *agr* operon. The four types of AIP exist as a result of sequence variation and have the following amino acid sequences: YSTCDFIM for AIP type I, GVNACSSLF for type II, INCDFLL for type III and YSTCYFIM for type IV (figure 1.2). Each AIP is able to bind to its corresponding sensor kinase AgrC (Novick and Geisinger, 2008).

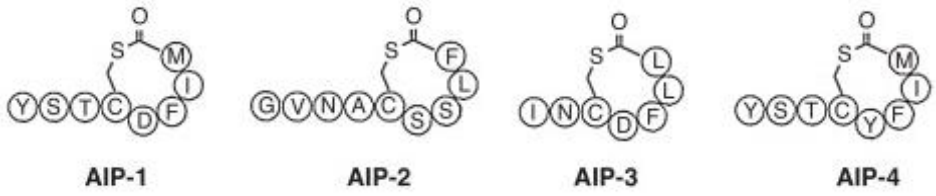


**Figure 1.2. Chemical structure of the auto-inducing peptides in *S. aureus* quorum sensing (Zhu and Kaufmann, 2013).**

AgrC is a transmembrane receptor that undergoes a conformational change upon binding of AIP. Due to this binding, the receptor is auto-phosphorylated and can consequently phosphorylate AgrA. Binding of another AIP type to AgrC leads to a conformational twist of its helix in the opposite direction. This twist disables auto-phosphorylation and the consequent signaling cascade. In this way different AIP types of *S. aureus* compete with and are able to inhibit each other (Painter *et al*., 2014). This event is called bacterial interference (Boles and Horswill, 2008). This observation led to the insight of using this mechanism as a therapeutic opportunity.

AgrA is a cytoplasmic DNA-binding response regulator. When activated AgrC phosphorylates AgrA, AgrA dimerizes and binds to an intergenic region between P2 and P3. This results in

the transcription of the *agr*ABCD operon and creates a positive feedback loop. It also leads to the activation of promoter P3. This results in the transcription of RNAIII, which is the second major unit of the *agr* QS system (Painter *et al.,* 2014).

RNAIII is the major effector of the *agr* system. It is a regulatory RNA molecule that regulates the production of various virulence factors, such as enterotoxin B (Laverty *et al.,* 2013), α-hemolysin, δ-hemolysin (Painter *et al.,* 2014) and various proteases. The production of cell surface proteins and adhesins decreases in presence of RNAIII. That is the reason why these proteins are only produced during the attachment phase of biofilm development (Laverty *et al.,* 2013). *Agr* activation seems to have no effect on PIA production (Vuong *et al.,* 2000).

The RNAIII gene also encodes for δ-toxin, which is a phenol-soluble modulin (PSM) (Laverty *et al.,* 2013). Research showed that AgrA directly controls PSM transcription. PSM's are α-helical amphipatic peptides acting as surfactants. They have proinflammatory characteristics and have the ability to lyse the host's neutrophils and erythrocytes (Wang *et al.,* 2007). Furthermore PSM's proved to be mandatory for biofilm dispersal in an *in vivo* mouse catheter infection model (Periasamy *et al.,* 2012). These properties designate PSM's as important virulence factors. The *agr* system is primarily activated at the end of the exponential growth phase and the start of the stationary phase. In these phases, AIP reaches the critical threshold concentration and is able to activate AgrC and set the whole signaling cascade in motion (Laverty *et al.,* 2013). According to Boles and Horswill (2008) *agr* activation is necessary for biofilm dispersal in several *S. aureus* strains.

### 1.4.2 RAP/TRAP system

The second QS system also consists of 2 units, RNAIII activating protein (RAP), a QS activator, and the target RNAIII activating protein (TRAP). This system is shown in figure 1.3 at the bottom. RAP is a protein of 33 kDa and is secreted by bacteria as they grow (Kiran *et al.,* 2008). When the critical threshold concentration of RAP is achieved in the mid-exponential growth phase, RAP induces TRAP phosphorylation. TRAP is a 21 kDa, highly conserved, constitutive, membrane-associated protein (Gov *et al.,* 2004; Han *et al.,* 2005). Research by Balaban *et al.* (2001) suggested that RAP induces RNAIII production via TRAP phophorylation, since RNAIII production does not occur in TRAP mutants. Their research also indicated that RNAIII synthesis induced by RAP or AIP are the result of different signaling pathways.

Research by Kiran and Balaban (2009) further specified the role of TRAP phosphorylation as a protective measure against oxidative stress and against mutations of DNA in the *agr* locus. TRAP phosphorylation decreases in the mid-exponential phase of growth as a result of *agr* induction and AIP production (Balaban *et al.,* 2001).

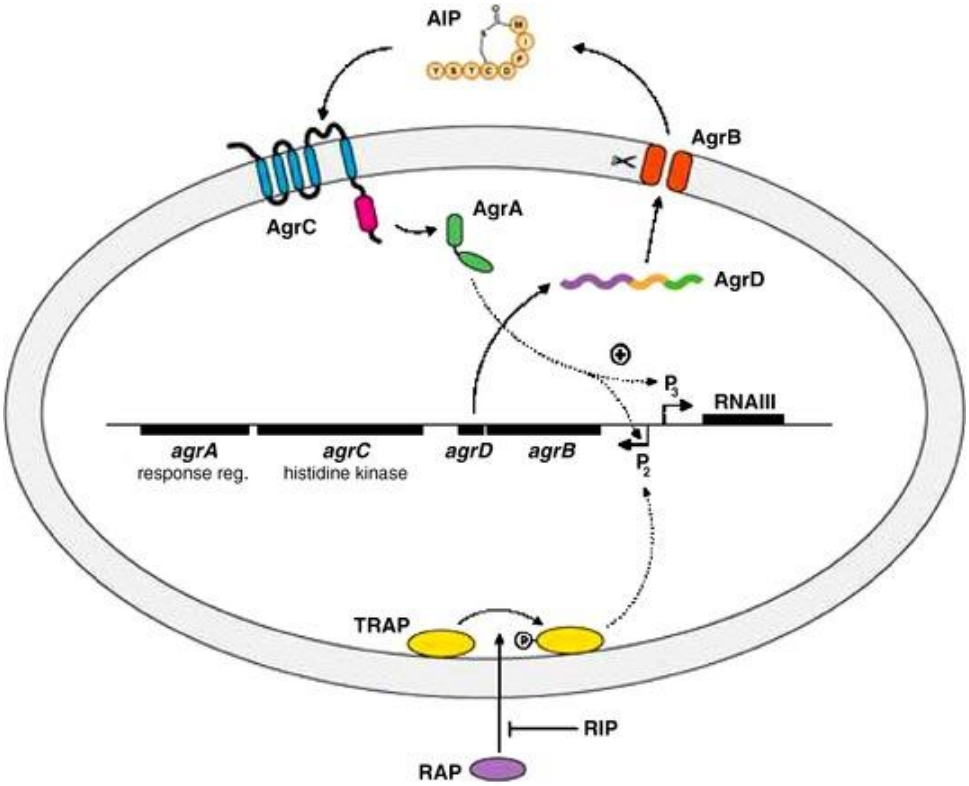


**Figure 1.3. The *agr* and RAP/TRAP QS system and their suggested interactions (Horswill *et al.,* 2007).**

### 1.4.3 *luxS*/AI-2 system

*S. aureus* carries a *luxS* gene. Its transcript is involved in the production of autoinducer-2 (AI-2). Although this gene is widely conserved, the signal transduction mechanisms of AI-2 have only been described in *Vibrio spp*., *Escherichia coli* and *Salmonella spp.* The QS function of *luxS*/AI-2 remains controversial in other species. In addition, genomic analysis revealed that no such receptors are present in *Staphylococcus spp.* Several studies indicate that the LuxS/AI-2 system is involved in interspecies signaling, as it is present in Gram-positive and negative bacteria (Vendeville *et al.,* 2005).

## 1.5 Quorum sensing inhibition

While the excessive use of antibiotics resulted in a large resistance problem and inefficiency in *S. aureus* infection treatments, biofilms themselves are already very resistant to antibiotic therapy. To increase biofilm susceptibility towards antibiotics and to reduce virulence factors, QS inhibition is presented as an alternative to combat bacterial infections. The *agr* operon of *S. aureus* bacteria has been widely researched in relation to QS inhibition. By inhibiting the activation of the *agr* QS system, virulence and biofilm formation can be decreased significantly. QS can be inhibited at different levels of the QS *agr* and RAP/TRAP systems. It can be blocked at the level of the signal, at the signal receptors or at the level of signal transduction.

Park *et al.* (2007) administered a monoclonal antibody against the signaling molecule AIP (type IV) to *S. aureus* strains with *agr* type IV. This resulted in decreased virulence factor expression. Co-administration of a *S. aureus* inoculum and the anti-AIP antibody to mice protected the host against a fatal infection. At the AgrC receptor level, different AIP types cause an AgrC receptor twist in the opposite direction. This prevents phosphorylation, as described above (Painter *et al.,* 2004) and hinders QS. The signal transduction pathway can be blocked by the small molecule inhibitor savirin. Its target is the DNA-binding response regulator AgrA. A functional AgrA is crucial for the activation of P2 and P3. Savirin binding to AgrA leads to a blockage of the transduction pathway and consequently leads to QS inhibition. This results in a decreased virulence gene expression (Sully et al., 2014).

The whole QS transduction can also be suppressed by RNAIII-inhibiting peptide (RIP). RIP is a heptapeptide with amino acid sequence: YSPXTNF (Gov *et al.,* 2001). The amino acid sequence of RIP is close to the amino acid sequence of RAP, with RAP serving as an agonist and RIP operating as an antagonist for TRAP. Binding of RIP to TRAP results in a decreased phosphorylation and activation of TRAP and subsequently causes QS inhibition (Kiran *et al.,* 2008). Balaban *et al.* (2000) show that RIP is able to inhibit RNAIII production and virulence very efficiently *in vitro*. A similar effect was seen in *in vivo* models, where administration of RIP was able to suppress *S. aureus* infections in cows, mice and rabbits. They declare that the effect of RIP is not *S. aureus* strain specific, possibly because of the highly conserved TRAP in *Staphylococci*.

### 1.5.1 Hamamelitannin

Hamamelitannin (HAM) or 2',5'-di-O-galloyl-D-hamamelose is a non-peptide analog of RIP (figure 1.4). It is a natural polyphenol, belonging the tannin family. HAM is derived from the bark of the witch hazel shrub (*Hamamelis virginiana*). HAM is able to inhibit and compete with RAP for RNAIII production, like RIP. It is also able to inhibit bacterial attachment and virulence. It acts as an antagonist for by inhibiting its phosphorylation and serves as a QS inhibitor. HAM seems to have no direct effect on bacterial growth even in very high concentrations (as high as 2.5 mM) therefore HAM cannot be considered as a traditional antibiotic (Kiran *et al.,* 2008).

As bacterial growth is not influenced by increasing HAM concentrations, HAM is unlikely to have membrane disrupting properties. The inhibition of RNAIII expression caused by treatment with HAM results in a decrease of virulence, for example in a reduction of δ-hemolysin production (Kiran *et al.*, 2008). Blocking QS not only decreases virulence and biofilm cell attachment, it also leads to an increase in susceptibility to antibiotics. By combining a QS inhibitor and an antibiotic, an interaction can take place. This interaction can probably reduce the resistance development further (Defoirdt *et al.,* 2010).

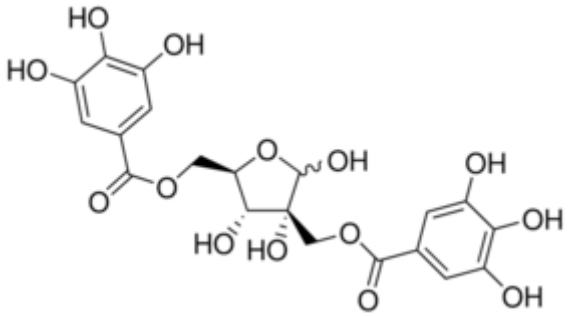


**Figure 1.4. Structure of HAM (Kiran *et al.,* 2008).**

Research by Brackman *et al.* (2011) showed that the combination of HAM and VAN treatment resulted in a significantly higher killing rate of *S. aureus* Mu50 biofilm cells *in vitro,* when compared to the antibiotic treatment alone. The same effect was seen in *in vivo* experiments, where an increased survival of *Caenorhabditis elegans* (*C. elegans*) and *Galleria mellonella* (*G. mellonella*) was observed for the combination treatment, when compared to VAN treatment alone.

HAM may also be useful to prevent biofilm formation on indwelling medical devices. In a study by Kiran *et al.* (2008), grafts were submerged in increasing concentrations of HAM before implantation. When the graft was removed, there were significantly less biofilm bacteria present on the graft with rising concentrations of HAM. This may suggest a new purpose for HAM, as a coating for indwelling medical devices to prevent infections.

# 2. Objectives

Infections with MRSA are very hard to treat and are life threatening. These high risks are caused by the high resistance of *S. aureus* and the ability to form biofilms at the infection site. *S. aureus* use a bacterial communication system called quorum sensing (QS) to regulate biofilm formation and virulence. Previous research showed that biofilm formation and virulence can be decreased by blocking the QS system (Kiran *et al.,* 2008; Brackman *et al.,* 2011). HAM was successfully used to increase *S. aureus* Mu50 biofilm susceptibility towards VAN and clindamycin (Brackman *et al.,* 2011).

In this thesis we want to investigate if the increased susceptibility to VAN is strain dependent and/or dependent on the *agr* type. We are also curious whether HAM could increase *S. aureus* Mu50 biofilm susceptibility towards other antibiotics.

Finally, we want to determine phenotypically whether HAM could affect the hemolytic activity of *S. aureus* Mu50. When *S. aureus* Mu50 biofilm bacteria are treated with VAN, an up - and downregulation of genes takes place (Brackman *et al.,* unpublished data). This change in gene expression mostly results in an increase of *S. aureus* virulence and one of these upregulated genes codes for hemolysin. Meanwhile, treatment with HAM or the combination of HAM and VAN results in a downregulation of various resistance and virulence genes. The combination treatment could therefore result in an inhibition of augmented hemolytic activity.

# 3. Materials and Methods

## 3.1 *S. aureus* strains

Different strains of *S. aureus* are used to investigate the role of *agr* types on the effect of HAM (table 3.1).

**Table 3.1. Overview of *S. aureus* strains and their *agr* type**

| | Strain | Number | *agr* type |
|---|---|---|---|
| 1 | *S. aureus* | NRS 384 | type I |
| 2 | *S. aureus* | Mu50 | type II |
| 3 | *S. aureus* | NRS 149 | type II |
| 4 | *S. aureus* | NRS 123 | type III |
| 5 | *S. aureus* | NRS 112 | type III (mutation) |
| 6 | *S. aureus* | NRS 153 | type IV |

## 3.2 Media

All media are prepared as described by the producer and sterilized by autoclavation for 20 minutes at 15 psi above atmospheric pressure.

- *S. aureus* isolates are plated on Tryptone Soy Agar (TSA) (LabM, Heywood, UK). TSA is a general purpose agar. It contains 1.5 % tryptone, 0.5 % soy peptone, 0.5 % sodium chloride and 1.2 % agar no. 2. The suspension is boiled and after sterilization the agar is cooled down to 48 °C.

- Mueller Hinton broth (MH) (LabM, Heywood, UK) is used as a culture medium for overnight cultivation and biofilm formation. It contains 0.2 % beef extract, 1.75 % acid hydrolyzed casein and 0.15 % starch.

- Double concentrated Mueller Hinton broth (2x MH) is used in MIC experiments.

## 3.3 Solutions

All the solutions are autoclaved for 20 minutes at 15 psi above atmospheric pressure unless stated otherwise.

- Physiological water (PW) is prepared by dissolving 9 g of sodium chloride (Novolab, Geraardsbergen, Belgium) in 1 L of MQ.

- MilliQ water (MQ) is obtained by the MilliQ Advantage A10 Ultrapure Water Purification System (Millipore, Brussels, Belgium).

- Phosphate Buffered Saline (PBS) is a physiological buffer that consists of the acid component $NaH_2PO_4–H_2O$ (Sigma-Aldrich, St-Louis, USA) and the base component $Na_2HPO_4$ (Sigma-Aldrich, St-Louis, USA). 0.69 g of $NaH_2PO_4–H_2O$ is dissolved in 100 mL of MQ and 1.06 g $Na_2HPO_4$ is dissolved in 150 mL of MQ. The acid is added to the base component until a pH of 7 is reached.

- A 25 mM stock of HAM is prepared by adding 364 µL of dimethyl sulfoxide (DMSO) to 5 mg of HAM powder (Fluka Gmbh, Buchs, Switzerland). The stock solution is diluted in PW for biofilm treatment or in MQ for MIC evaluation. The diluted stock solution is filtered through a 0.22 µm pore size cellulose acetate filter (Merck Millipore, Tullagreen, Ireland) and stored in the fridge.

- The VAN stock solution of 800 µg/mL is prepared by dissolving 8 mg of VAN (Sigma-Aldrich, St-Louis, USA) in 10 mL of PW. This stock solution is filter sterilized and stored in the fridge. The stock solution is diluted in PW for biofilm treatment or in MQ for MIC evaluation.

- A 10 % sodium dodecyl sulphate (SDS) in PBS solution is made by dissolving 1 g SDS (Sigma-Aldrich, St-Louis, USA) in 10 mL of PBS. This solution is not filter sterilized or autoclaved. The solution is diluted in PBS for the membrane integrity assay and the hemolytic activity assay.

- The linezolid (LIN) stock solution of 0.512 mg/mL is prepared by dissolving 2.048 mg of LIN powder (Sigma-Aldrich, St-Louis, USA) in 4 mL MQ for MIC determinations. For

biofilm treatment, LIN powder is dissolved in PW. These solutions are filter sterilized and stored in the fridge.

- The tigecycline (TIG) stock solution of 1.024 mg/ml is prepared by dissolving 2.048 mg of TIG (Sigma-Aldrich, St-Louis, USA) 2 mL MQ for MIC evaluations. For biofilm treatment, TIG powder is dissolved in PW. These solutions are filter sterilized and stored in the fridge.

- The daptomycin (DAP) stock solution of 20 mg/mL is prepared by dissolving 20 mg of DAP powder (Tokyo Chemical Industry, Tokyo, Japan) in 1 mL of MQ for MIC experiments. DAP is dissolved in PW for biofilm treatment. This solution is filter sterilized and stored in the freezer.

- The fusidic acid (FUS) stock solution of 5 mg/mL is prepared by dissolving 5 mg of FUS powder (Sigma-Aldrich, St-Louis, USA) in 1 mL of MQ for MIC determinations. FUS is dissolved in PW for biofilm treatment assays. This solution is filter sterilized and stored in the fridge.

- The ciprofloxacin (CIP) stock solution of 2.5 mg/mL is prepared by adding 12.5 mg of CIP powder (Fluka Gmbh, Buchs, Switzerland) to 5 mL of MQ. To dissolve CIP in MQ, 38 µL of 0.1 M HCL is added. The stock solution is diluted in PW for biofilm treatments. The solution is filter sterilized and stored in the fridge.

- The doxycycline (DOX) stock solution of 2 mg/mL is prepared by dissolving 2 mg of DOX powder (Sigma-Aldrich, St-Louis, USA) in 1 mL MQ. For biofilm treatment assays, DOX is dissolved in PW. The solution is filter sterilized and stored in the fridge.

- The chloramphenicol (CHL) stock solution of 100 mg/mL is prepared by dissolving 100 mg of CHL powder (Sigma-Aldrich, St-Louis, USA) in 1 mL ethanol. This solution is diluted in MQ for MIC experiments. CHL is diluted in PW for biofilm treatment.

## 3.4 Membrane integrity assay

Membrane integrity is evaluated using propidium iodide (PI) staining. *S. aureus* Mu50 bacteria are cultured overnight in MH and set to an optical density of 0.4 at 590 nm in PBS. Aliquots of 1 mL are treated with 100 µM HAM and positive controls are heat or 0.1 % SDS treatment. The heat treatment samples only receive 10 min of treatment, while SDS treatment samples receive 10 min or 24h of treatment at 37 °C. Samples are spotted on a microtiter plate and 10 µL of a diluted PI (Invivogen, Toulouse, France) solution (3/1000 in PBS) is added to the wells to evaluate bacterial membrane permeability. The plate is incubated for 10 min at 37 °C. After incubation, fluorescence (ex 485 nm, em 635 nm) is measured using the EnVision microtiter plate reader.

## 3.5 MIC determination

The MIC is defined as the lowest concentration of an antibiotic where bacterial growth is completely inhibited. The MIC's of planktonic *S. aureus* bacteria for HAM and 8 antibiotics are determined using 50 µL of serial twofold dilutions in MQ in a round bottom microtiter plate. The used concentration range of HAM and antibiotics are shown in table 3.2. The MIC assay also includes a growth control (GC). Bacteria are cultured overnight in MH and set to an optical density of 0.2 at 590 nm. The cell suspension is diluted 1/100 in 2x MH and 50 µL is added to the microtiter plate. After 24h of incubation at 37 °C, the absorbance (ex 590 nm) is measured using the EnVision microtiter plate reader.

**Table 3.2. The concentration range used in MIC determination assays.**

| Antibiotic | Concentration range |
|---|---|
| Hamamelitannin | 0.5 – 512 µM |
| Vancomycin | 0.25 – 512 µg/ml |
| Linezolid | 0.25 – 256 µg/ml |
| Tigecycline | 0.5 – 512 µg/ml |
| Daptomycin | 0.5 – 512 µg/ml |
| Fusidic acid | 0.5 – 512 µg/ml |
| Ciprofloxacin | 1 – 1024 µg/ml |
| Doxycycline | 1 – 1024 µg/ml |
| Chloramphenicol | 0.5 – 512 µg/ml |

## 3.6 Synergy testing against planktonic bacteria

The checkerboard titration method is used to determine whether there is a synergistic effect against planktonic bacteria. A synergistic interaction between HAM and VAN is determined by calculating the fractional inhibition concentration (FIC). The following formula is used: FIC = ($MIC_{HAM,Comb}/MIC_{HAM}$) + ($MIC_{VAN,Comb}/MIC_{VAN}$). Synergy is defined as FIC<0.5. An additive effect is defined as FIC>0.5. There is no interaction between HAM and VAN on planktonic cells when FIC>1.

Twofold serial dilutions of HAM and VAN are prepared in falcon tubes, with concentration ranges of 32 – 1024 µM and 1 – 512 µg/mL respectively. Twentyfive µL of each concentration of HAM is spotted along the y-axis, while 25 µL of each concentration of VAN is spotted along the x-axis. GC, HAM controls and VAN controls are included in the assay (table 3.3: location of HAM and VAN concentrations). Bacteria are cultured overnight in MH and set to an optical density of 0.2 at 590 nm. The cell suspension is diluted 1/100 in 2x MH and 50 µL is added to the microtiter plate. The ultimate concentration range is 8 – 256 µM HAM and 0.25 – 128 µg/mL VAN. The plate is incubated for 24h at 37 °C. After incubation, the absorbance (ex 590 nm) is measured using the EnVision microtiter plate reader and the content of a selection of wells is plated on TSA to obtain the number of Colony Forming Units/mL (CFU/mL).

**Table 3.3. Checkerboard titration method: location of VAN (µg/mL) and HAM (µM) concentrations.**

| | VAN | 0 | 0.25 | 0.5 | 1 | 2 | 4 | 8 | 16 | 32 | 64 | 128 | GC |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| HAM | | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
| 256 | A | | | | | | | | | | | | |
| 128 | B | | | | | | | | | | | | |
| 64 | C | | | | | | | | | | | | |
| 32 | D | | | | | | | | | | | | |
| 16 | E | | | | | | | | | | | | |
| 8 | F | | | | | | | | | | | | |
| 0 | G | | | | | | | | | | | | |
| | H | Blank | | | | | | | | | | | |

## 3.7 Biofilm treatment and quantification

### 3.7.1 Biofilm formation

Biofilms are grown in a round bottom 96-well microtiter plate (SPL Life Sciences, Pocheon-Si, South-Korea) in MH. The outside wells of the microtiter plate are filled with 200 µL MQ as an evaporation buffer. The bottom row is filled with 100 µL of MH, serving as blanks. The other wells are filled with 100 µL of cell suspension. This cell suspension is set to an optical density of 0.2 at 590 nm. The plate is incubated for 4 hours at 37 °C after which the medium is removed. The biofilms are rinsed with 100 µL PW to remove non-adherent cells and 100 µL of fresh MH is added. The plate is incubated for another 20 hours at 37 °C.

### 3.7.2 Treatment

After 24h of incubation the medium is removed and the plate is rinsed with 100 µL of PW. The rinsed biofilms are treated with either an antibiotic VAN solution, a HAM solution, the combination (Comb) or a control solution (CTRL). The treatment is added to the microtiter plate as mentioned in table 3.4. The final treatment concentration is 20 µg/mL VAN and 250 µM HAM. The plate is incubated for 24h at 37 °C.

**Table 3.4. Treatment of biofilm with VAN: location of CTRL, HAM, VAN and combination solutions a microtiter plate. The same locations are used with other antibiotic treatments. B: 200 µL MQ buffer.**

<table>
<tr><td></td><td>1</td><td>2</td><td>3</td><td>4</td><td>5</td><td>6</td><td>7</td><td>8</td><td>9</td><td>10</td><td>11</td><td>12</td></tr>
<tr><td>A</td><td>B</td><td>B</td><td>B</td><td>B</td><td>B</td><td>B</td><td>B</td><td>B</td><td>B</td><td>B</td><td>B</td><td>B</td></tr>
<tr><td>B</td><td>B</td><td colspan="2" rowspan="5">100 µl CTRL</td><td colspan="2" rowspan="5">50 µL CTRL +<br>50 µL HAM</td><td colspan="3" rowspan="5">50 µL CTRL<br>+ 50 µL VAN</td><td colspan="3" rowspan="5">50 µL HAM<br>+ 50 µL VAN</td><td>B</td></tr>
<tr><td>C</td><td>B</td><td>B</td></tr>
<tr><td>D</td><td>B</td><td>B</td></tr>
<tr><td>E</td><td>B</td><td>B</td></tr>
<tr><td>F</td><td>B</td><td>B</td></tr>
<tr><td>G</td><td>B</td><td colspan="10">Blank 100 µL CTRL</td><td>B</td></tr>
<tr><td>H</td><td>B</td><td>B</td><td>B</td><td>B</td><td>B</td><td>B</td><td>B</td><td>B</td><td>B</td><td>B</td><td>B</td><td>B</td></tr>
</table>

For treatment with other antibiotics (LIN, TIG, DAP, CIP, FUS , DOX and CHL), biofilms are treated with 4x, 10x, 50x and 100x the MIC of the specific antibiotic. The antibiotic concentration that results in a minimal 0.5 log reduction in CFU/mL is considered as responsive to the antibiotic treatment. Biofilms are treated with these responsive antibiotic solutions and 250 µM HAM to assess the effect of the combination treatment.

### 3.7.3 Quantification

After 24h the treatment is removed and biofilms are rinsed with 100 µL PW. In order to quantify the number of metabolically active cells, 100 µl of a diluted cell titer blue (CTB) (Promega, Fitchberg, USA) solution (1/6 in PW) is added to the rinsed biofilms in the wells. The plate is wrapped in aluminum foil and is incubated for 30 min at 37 °C. The fluorescence (ex 535 nm, em 590 nm) is measured using the EnVision microtiter plate reader (PerkinElmer, Waltham, USA). This method is used for the quantification of *S. aureus* biofilm bacteria after treatment with VAN. Another quantification method is the pour plate method that is described below. This method is used for the quantification of *S. aureus* Mu50 biofilm bacteria treated with antibiotics LIN, TIG, DAP, CIP, FUS , DOX and CHL.

## 3.8 CFU/mL determination

The pour plate method is used to quantify biofilm bacteria and to determine the number of CFU/mL of planktonic cells after checkerboard titration. For biofilms, the medium is removed and 100 µL PW is added. The biofilm is detached by 5 min of shaking and 5 min of sonication. The detached biofilm is collected in 9.9 mL PW. Of this suspension, 1 mL is added to a petridish and 1 mL is diluted 1/10 in PW (figure 3.1). This same process is repeated until a dilution of $10^{-7}$ is reached. TSA (48 °C) is poured in the petridishes and the petridishes are swirled. They are incubated overnight at 37 °C and colonies are counted to determine the CFU/mL present in the original well. The same protocol is used after checkerboard titration, without the biofilm detachment step.

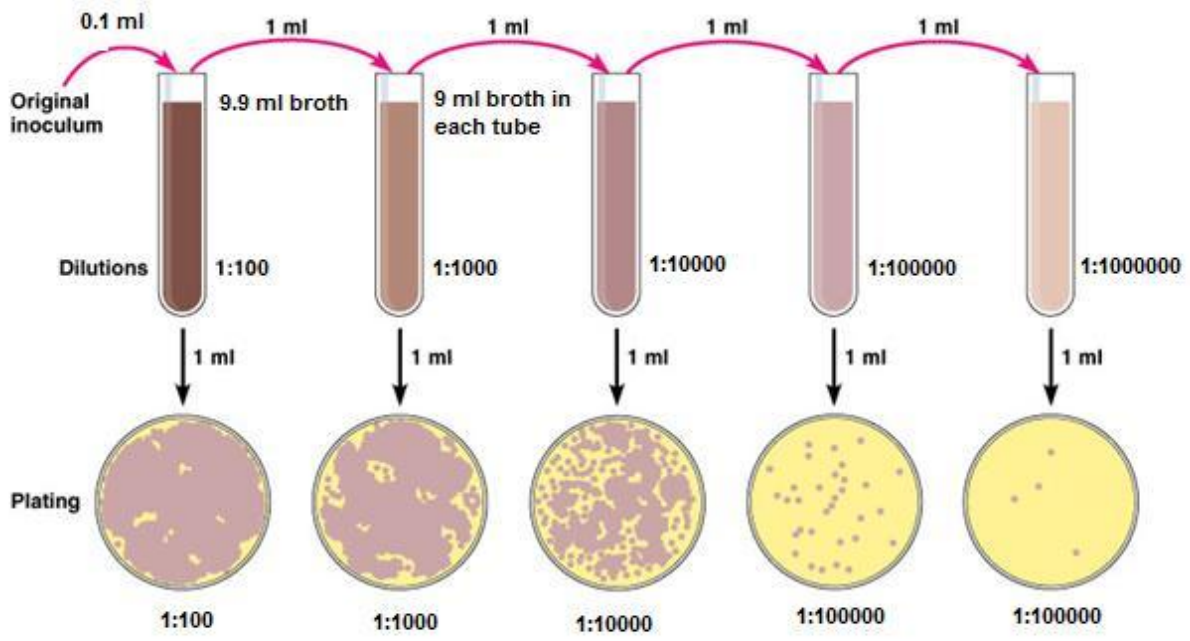


**Figure 3.1. Serial dilution of a bacterial suspension using the pour plate method to determine CFU/mL.**

## 3.9 Hemolytic activity assay

Hemolytic activity is evaluated by incubation of biofilm bacteria that were treated with HAM, VAN or the combination, together with rabbit blood erythrocytes. *S. aureus* Mu50 biofilms are prepared as in 3.7.1 and treated as in 3.7.2. After 24h of incubation, the treatment is removed and 100 µL of PW is added to the wells. Biofilms are detached from the wells by shaking (5 min) and sonication (5 min) and collected in falcon tubes. The detached cells are spotted on a microtiter plate and absorption (ex 590 nm) is measured using the EnVision microtiter plate reader. This absorption value is used to correct for the number of bacteria. Samples of 200 µL of each treatment condition are incubated with 800 µL of 4% rabbit blood (Biotrading, Mijdrecht, The Netherlands). A 10 % SDS in PBS solution serves as a positive control, while PW is used as a negative control. Aliquots are incubated for 4h or 24h at 37 °C.

After incubation, 100 µL of the supernatant is spotted on a microtiter plate. The absorbance (ex 590 nm, 420 nm and 450 nm) of the supernatant is measured using the EnVision microtiter plate reader. Samples are centrifugated for 2 min at 1000 rpm using the Eppendorf centrifuge 5427 R (Eppendorf, Hamburg, Germany) and absorbance is measured as mentioned above.

## 3.10 Statistical analysis

SPSS is used for the statistical analysis of obtained results. Normal distribution is evaluated by the Shapiro-Wilk test. Groups are compared using a non-parametric independent T-test, followed by a Mann-Whitney U test or a parametric ANOVA test, followed by a Dunnet's test.

# 4. Results

## 4.1 Effect of HAM on membrane integrity as determined by PI staining

PI is a nucleic dye that intercalates with DNA in lysed cells and thereby yields a fluorescent signal. No significant difference in fluorescence signal was observed after 10 min or 24h incubation of the CTRL. A significantly higher fluorescence value ($p<0.05$) was observed for bacteria receiving a treatment with 0.1 % SDS for 10 min or 24h (13816 ± 8727 or 13463 ± 2772 respectively) or 10 min of heat treatment (13443 ± 2270). In contrast, no significant increase in fluorescence was observed in bacteria receiving HAM treatment (100 µM) for 10 min or 24h 100 µM HAM treatment (figure 4.1).

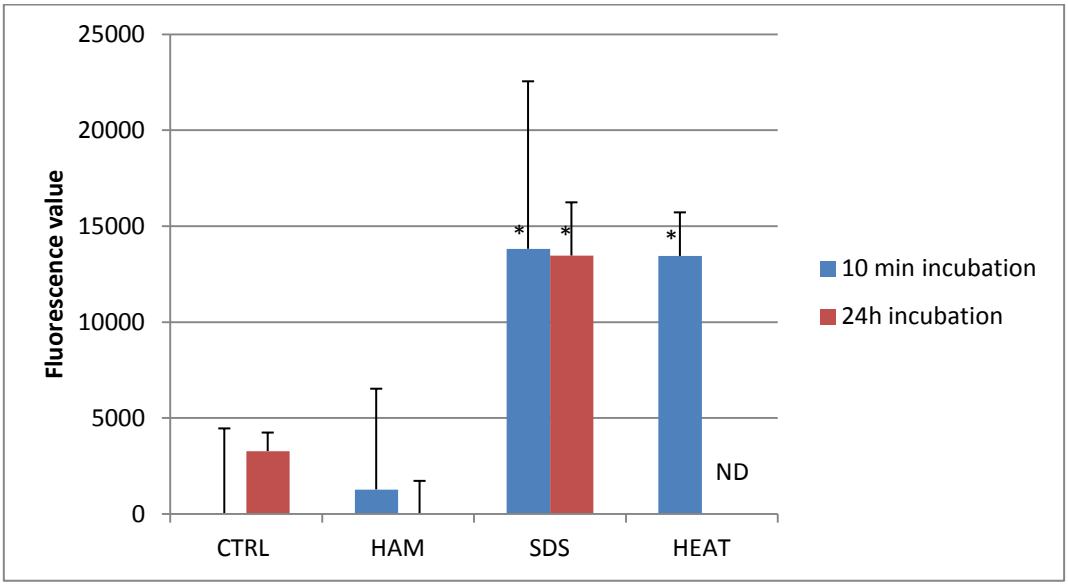


**Figure 4.1. Fluorescence values after 10 min or 24h of 100 µM HAM, 0.1 % SDS or heat treatment with standard deviation (SD). *: significantly different ($p<0.05$) compared to CTRL; ND: not determined.**

## 4.2 Inhibition of bacterial growth: MIC determination

### 4.2.1 MIC determination of HAM for *S. aureus* strains

HAM does not cause growth inhibition in the range of 0.5 – 512 µM HAM (figure 4.2) in the 6 tested *S. aureus* strains. The MIC of HAM is higher than 512 µM for all strains.

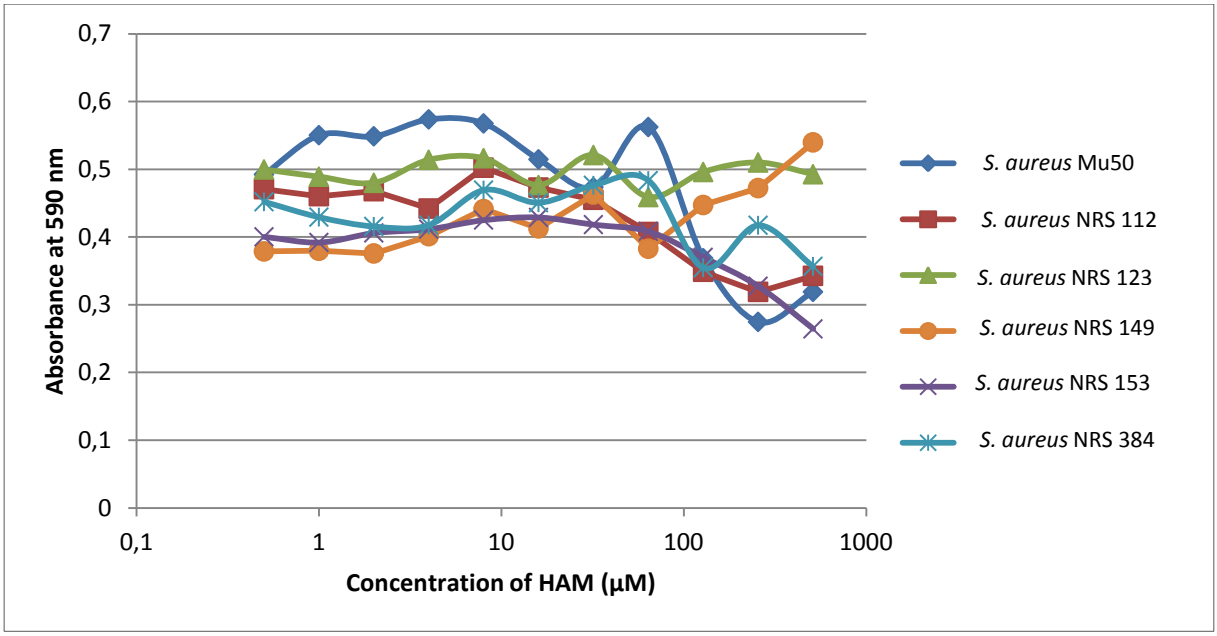


**Figure 4.2. The MIC determination of HAM in for *S. aureus* Mu50, NRS 112, NRS 123, NRS 149, NRS 153 and NRS 384.**

### 4.2.2 MIC determination of VAN for *S. aureus* strains

The MIC of VAN is investigated in *S. aureus* Mu50, NRS 112, NRS 123, NRS 149, NRS 153 and NRS 384 (table 4.1). The MIC of VAN for *S. aureus* Mu50 is 4 µg/mL. This MIC is 4x higher than the MIC's of the other investigated *S. aureus* strains (1 µg/mL). The *S. aureus* Mu50 strain is intermediate resistant towards VAN, while the other strains are VAN sensitive according to the Clinical and Laboratory Standards Institute (CLSI, 2012).

**Table 4.1. The MIC of VAN for 6 investigated *S. aureus* strains and their sensitivity interpretation.**

| *S. aureus* strain | MIC value of VAN | MIC standards interpretation |
|---|---|---|
| Mu50 | 4 µg/mL | Intermediate |
| NRS 112 | 1 µg/mL | Sensitive |
| NRS 123 | 1 µg/mL | Sensitive |
| NRS 149 | 1 µg/mL | Sensitive |
| NRS 153 | 1 µg/mL | Sensitive |
| NRS 384 | 1 µg/mL | Sensitive |

### 4.2.3 MIC determination of other antibiotics for *S. aureus* Mu50

The MIC of other antibiotics is only examined in *S. aureus* Mu50 (table 4.2). The *S. aureus* Mu50 strain is sensitive to LIN, FUS and CHL. This strain is intermediate resistant towards DOX and resistant towards TIG, DAP and CIP according to CLSI (2012).

**Table 4.2. The MIC values of 7 antibiotics for *S. aureus* Mu50 and their sensitivity interpretation.**

| Antibiotic | MIC value for *S. aureus* Mu50 | MIC standards interpretation |
|---|---|---|
| Linezolid | 1 µg/mL | Sensitive |
| Tigecycline | 1 µg/mL | Resistant |
| Daptomycin | 4 µg/mL | Resistant |
| Fusidic acid | 0.03 µg/mL | Sensitive |
| Ciprofloxacin | 32 µg/mL | Resistant |
| Doxycycline | 8 µg/mL | Intermediate |
| Chloramphenicol | 8 µg/mL | Sensitive |

## 4.3 Change in MIC of VAN in combination with HAM

The MIC of VAN, when combined with HAM treatment, is determined for planktonic *S. aureus* Mu50, NRS 112 and NRS 123 bacteria. The checkerboard titration is used. The used treatment range of VAN and HAM is 0.25 – 128 µg/mL and 8 – 256 µM respectively. For *S. aureus* Mu50, the MIC of VAN is 4 µg/mL. When HAM is added to the treatment, the MIC remains the same (figure 4.3). However, the absorbance values decrease when HAM concentrations increase, suggesting an increased effect on growth for VAN when combined with HAM. As such, we determined CFU/mL to confirm if this correlates with a lower CFU/mL (figure 4.4). This is repeated for the other *S. aureus* strains to investigate the change in absorbance.

The CFU/mL is 1.36 x $10^9$/mL for the growth control (figure 4.4). There is a significant decrease ($p<0.05$) in CFU/mL for 256 µM HAM (2.31 x $10^8$/mL) and 256 µM HAM + 1 µg/mL VAN (1.31 x $10^8$/mL) treatment, when compared to the growth control. There is no significant difference in other conditions.

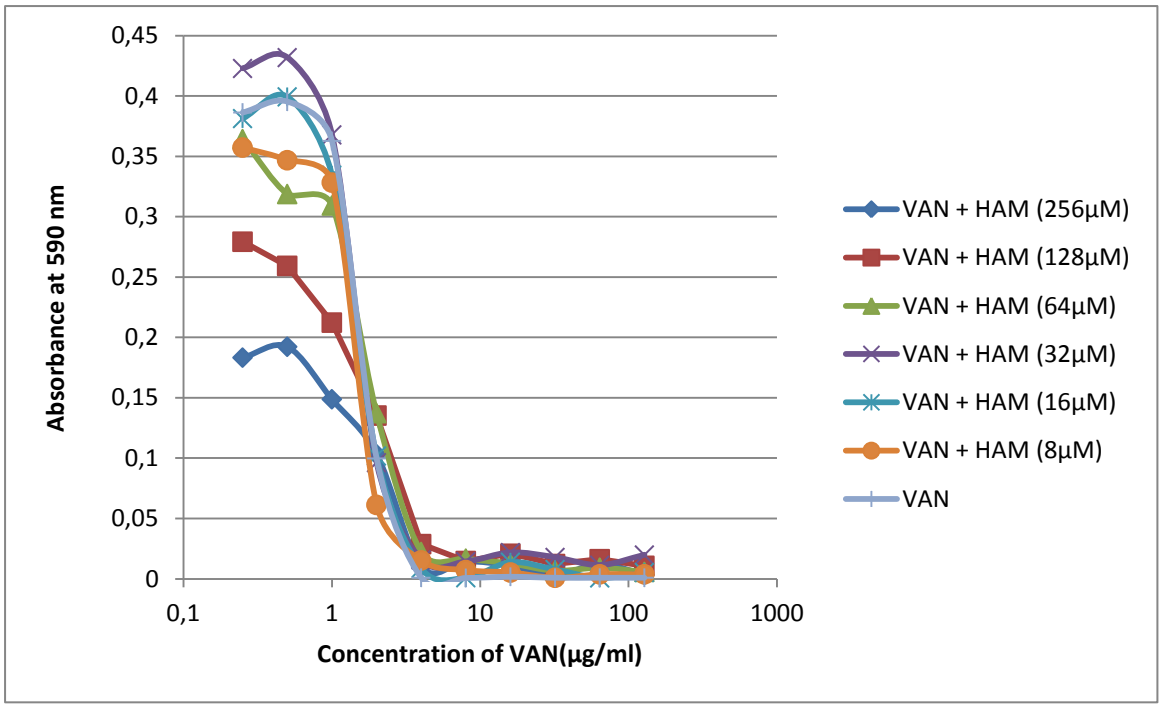


**Figure 4.3. MIC determination of VAN for *S. aureus* Mu50, in the absence or presence of HAM.**

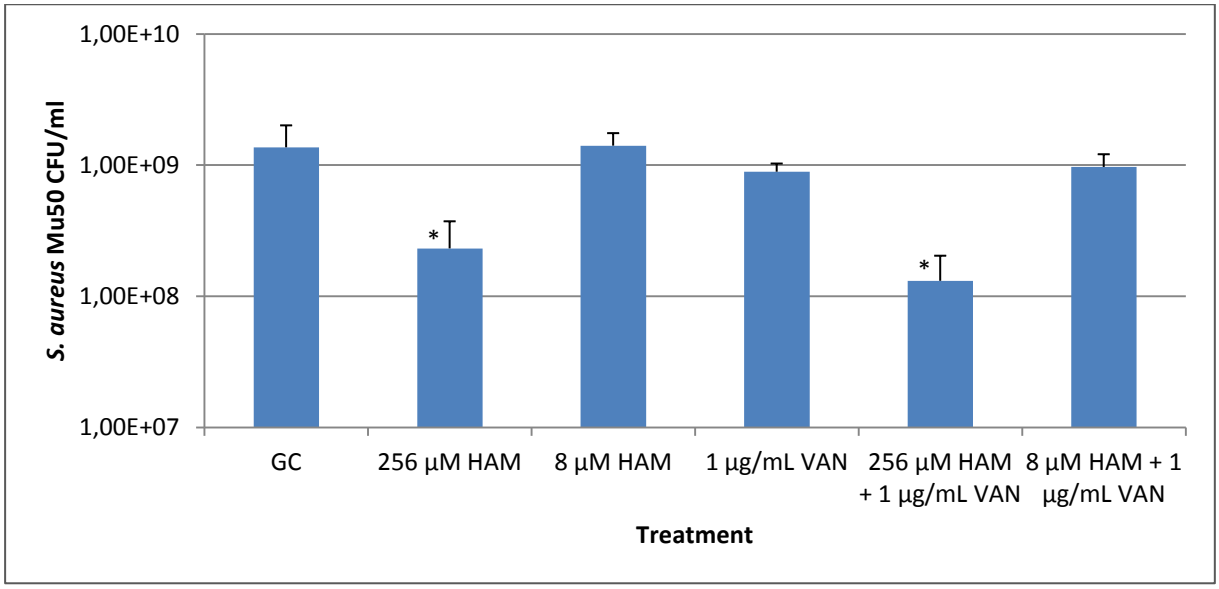


**Figure 4.4. *S. aureus* Mu50 CFU/mL for selected combinations of HAM and VAN with SD. *: significantly different ($p<0.05$) compared to GC.**

For *S. aureus* NRS 112, the MIC of VAN is 1 µg/mL. The MIC remains the same when combining VAN and HAM (figure 4.5). As in *S. aureus* Mu50, there is a decrease in absorbance with increasing concentrations of HAM. CFU/mL of *S. aureus* NRS 112 growth control is 2.47 x $10^9$/mL (figure 4.6). There is a significant difference between the conditions

containing HAM and the growth control ($p<0.05$). There is no significant difference between the 0.5 µg/mL VAN treatment and the growth control.

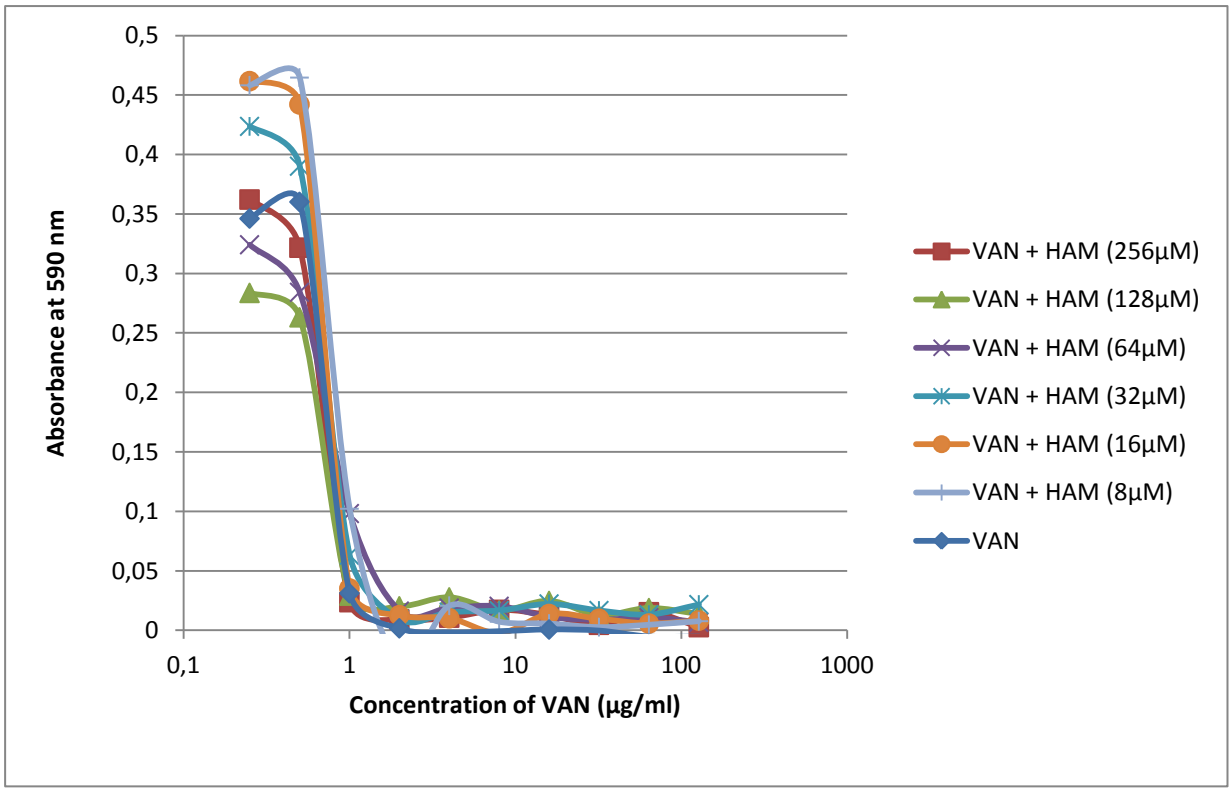


**Figure 4.5. MIC determination of VAN for *S. aureus* NRS 112, in the absence or presence of HAM.**

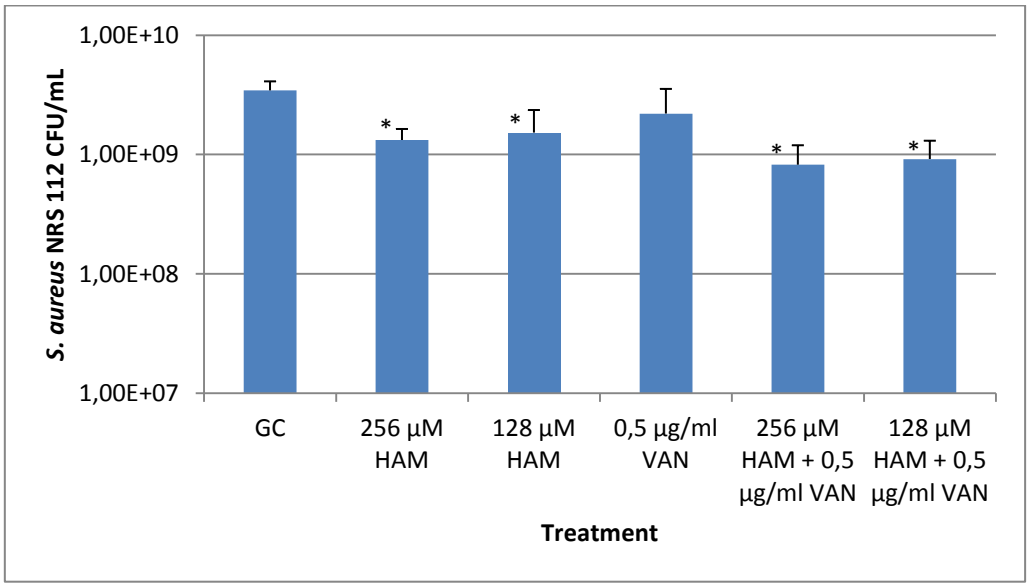


**Figure 4.6. *S. aureus* NRS 112 CFU/mL for selected combinations of HAM and VAN with SD. *: significantly different ($p<0.05$) compared to GC.**

The MIC of VAN for *S. aureus* NRS 123 is 1 µg/mL. When combining with HAM, the MIC does not change (figure 4.7). The CFU/mL of *S. aureus* NRS 123 growth control is 1.10 x $10^9$ /mL (figure 4.8). There is a significant difference in CFU/mL between the growth control and 256 µM HAM + 1 µg/mL VAN treatment (3.54 x $10^8$/mL) ($p<0.05$).

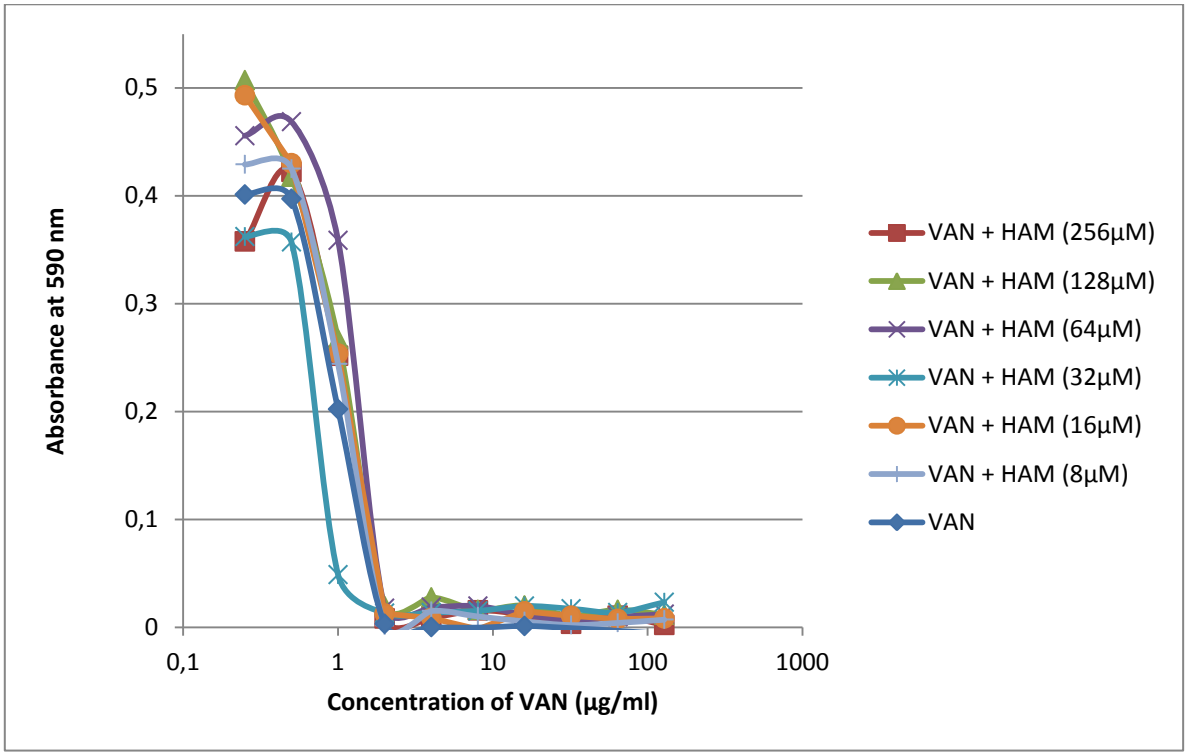


**Figure 4.7. MIC determination of VAN for *S. aureus* NRS 123 in the absence or presence of HAM.**

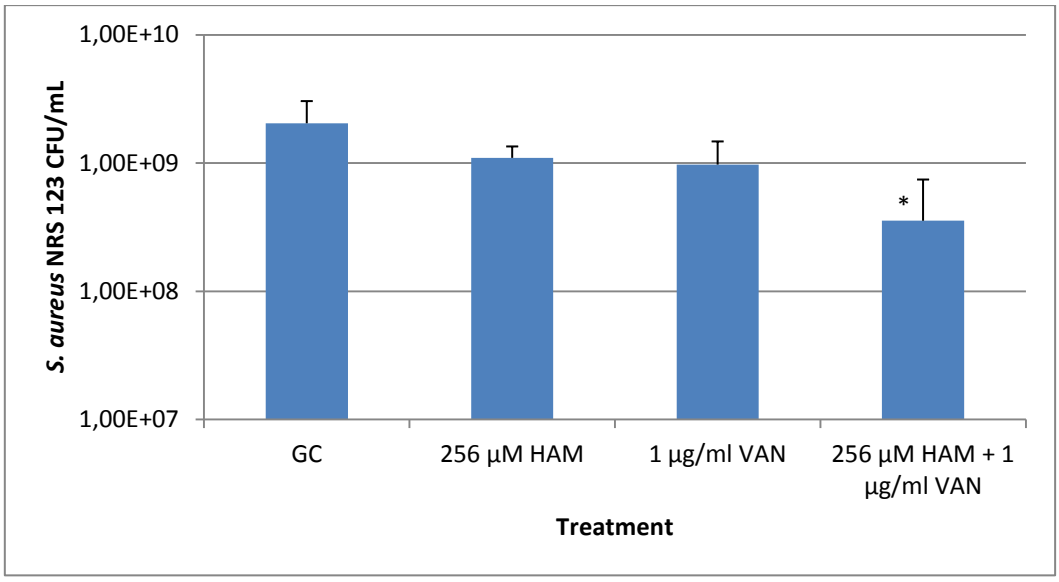


**Figure 4.8. *S. aureus* NRS 123 CFU/mL for selected combinations of HAM and VAN with SD. *: significantly different (p<0.05) compared to GC.**

## 4.4 Effect of HAM on biofilm susceptibility towards VAN

*S. aureus* biofilms are treated with 20 µg/mL VAN, 250 µM HAM or the combination. Cell viability is assessed with CTB and fluorescence is measured. The absolute fluorescence value obtained for CTRL is set at 100 %. The values obtained for the treated samples are compared to this CTRL.

### 4.4.1 Susceptibility change of *S. aureus agr* type II bacteria in biofilms

In *S. aureus* Mu50, there is no significant difference between CTRL and HAM treatment ($p>0.05$) (figure 4.9). While VAN treatment results in a significant reduction ($p<0.05$) of 30 % of fluorescence, combining it with 250 µM HAM results in a significant ($p<0.05$) additional reduction of 48 %.

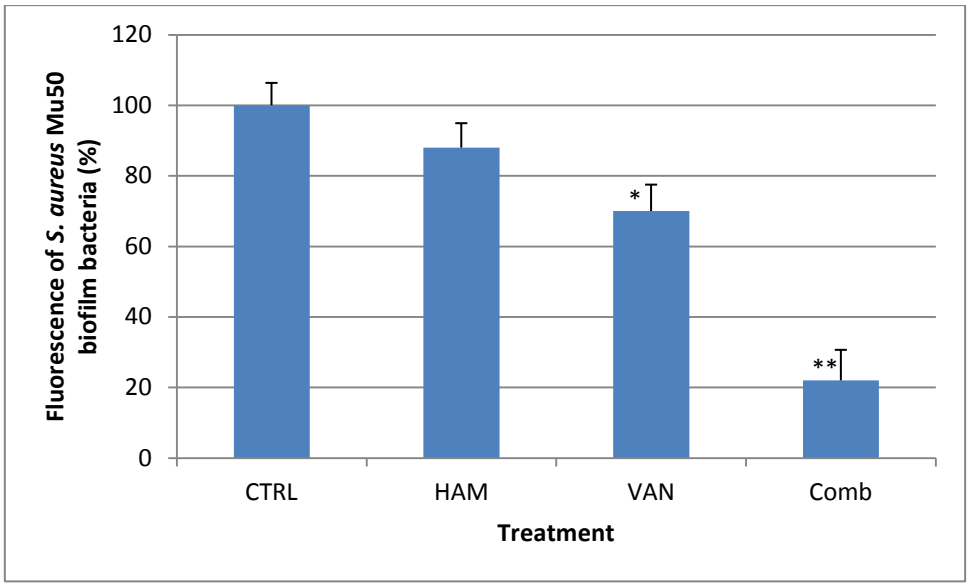


**Figure 4.9. Relative fluorescence of *S. aureus* Mu50 biofilm bacteria after 24h of treatment with 250 µM HAM, 20 µg/mL VAN or the combination (Comb) with standard error of means (SEM). *: significantly different ($p<0.05$) compared to CTRL; **: significantly different ($p<0.05$) compared to VAN treatment alone.**

In *S. aureus* NRS 149, there is a significant difference of 24 % between CTRL and HAM fluorescence ($p<0.05$) (figure 4.10). VAN treatment results a significant ($p<0.05$) fluorescence reduction of 70 %. However, the combination of VAN and HAM does not reduce fluorescence any further ($p>0.05$).

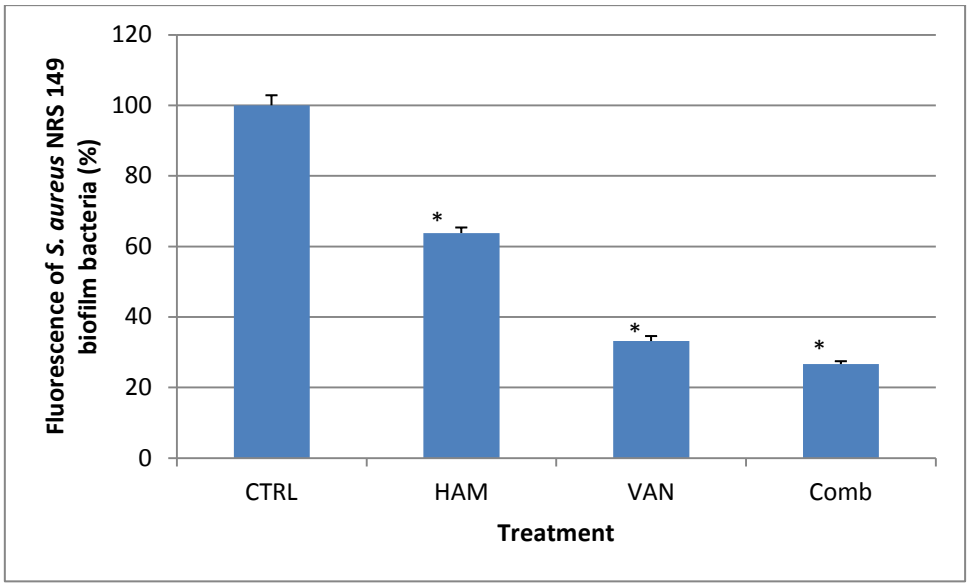


**Figure 4.10. Relative fluorescence of *S. aureus* NRS 149 biofilm bacteria after 24h of treatment with 250 µM HAM, 20 µg/mL VAN or the combination (Comb) with SEM. *: significantly different ($p<0.05$) compared to CTRL.**

4.4.2 Susceptibility change of *S. aureus agr* type I bacteria in biofilms

HAM treatment results in a significant fluorescence reduction of 26 % of *S. aureus* NRS 384 biofilm bacteria ($p<0.05$). After 24h of 20 µg/mL VAN treatment, the fluorescence is reduced by 58 % when compared to CTRL ($p<0.05$). The combination treatment results in an additional significant ($p<0.05$) reduction of 18 % (figure 4.11).

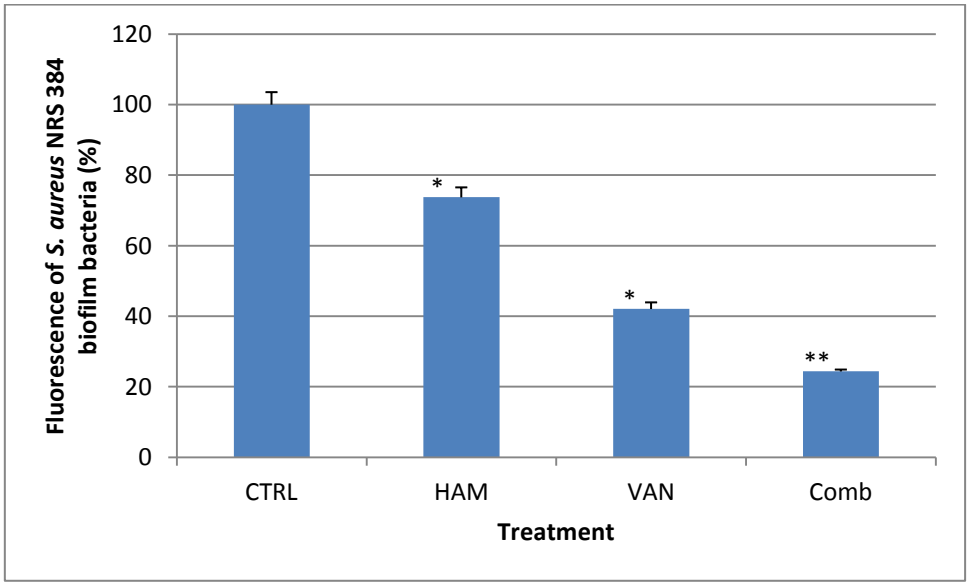


**Figure 4.11. Relative fluorescence of *S. aureus* NRS 384 biofilm bacteria after 24h of treatment with 250 µM HAM, 20 µg/mL VAN or the combination (Comb) with SEM.**

***: significantly different (p<0.05) compared to CTRL; **: significantly different (p<0.05) compared to VAN treatment alone.**

### 4.4.3 Susceptibility change of *S. aureus agr* type III bacteria in biofilms

In *S. aureus* NRS 123, there is no significant difference between CTRL and HAM (p>0.05) (figure 4.12). While VAN treatment results in a significant reduction (p<0.05) of 46 % in fluorescence, the combination with 250 µM HAM results in a significant (p<0.05) additional reduction of 19 %.

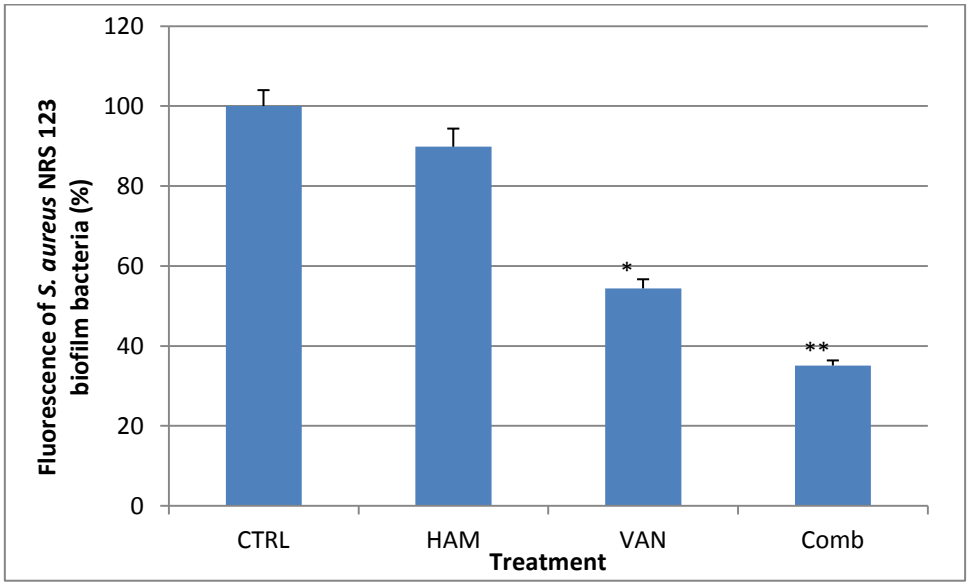


**Figure 4.12. Relative fluorescence of *S. aureus* NRS 123 biofilm bacteria after 24h of treatment with 250 µM HAM, 20 µg/mL VAN or the combination (Comb) with SEM. *: significantly different (p<0.05) compared to CTRL; **: significantly different (p<0.05) compared to VAN treatment alone.**

### 4.4.4 Susceptibility change of *S. aureus agr* type IV bacteria in biofilms

In *S. aureus* NRS 153, treatment with 250 µM HAM results in a significant reduction of 26 % in fluorescence (figure 4.13). Twenty-four hours of VAN treatment result in a significant fluorescence reduction of 72 % when compared to CTRL (p<0.05). The combination treatment results in an additional reduction of 11 % (p<0.05).

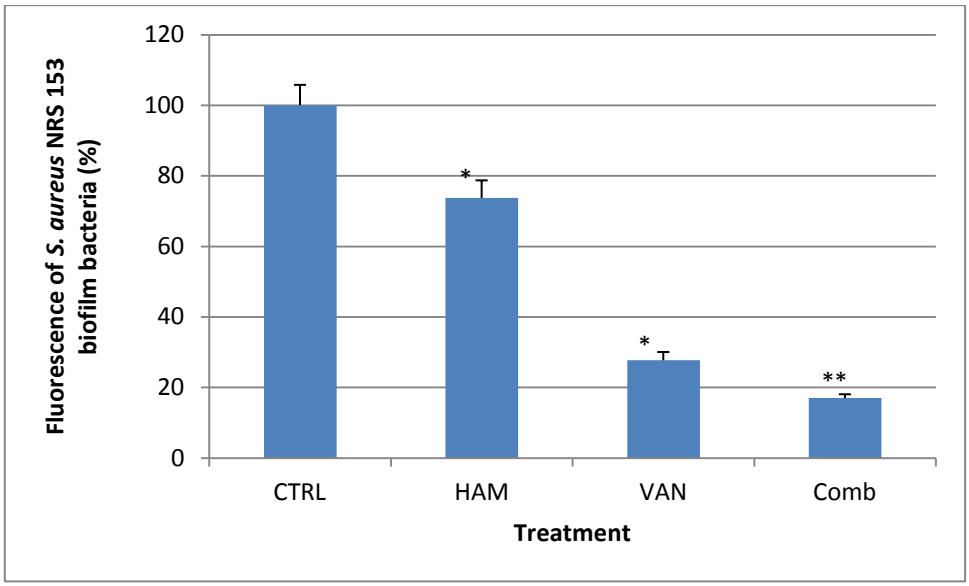


**Figure 4.13. Relative fluorescence of *S. aureus* NRS 153 biofilm bacteria after 24h of treatment with 250 µM HAM, 20 µg/mL VAN or the combination (Comb) with SEM. *: significantly different ($p<0.05$) compared to CTRL; **: significantly different ($p<0.05$) compared to VAN treatment alone.**

### 4.4.5 Susceptibility change of *S. aureus agr* type III mutant bacteria in biofilms

No significant difference in *S. aureus* NRS 112 biofilm bacteria is observed between CTRL and HAM ($p>0.05$) (figure 4.14). The fluorescence of *S. aureus* NRS 112 biofilm bacteria is significantly reduced by 59 % after VAN treatment. The combination treatment also results in a significant reduction of 68 %. However, this difference is not significant compared to VAN treatment alone ($p>0.05$).

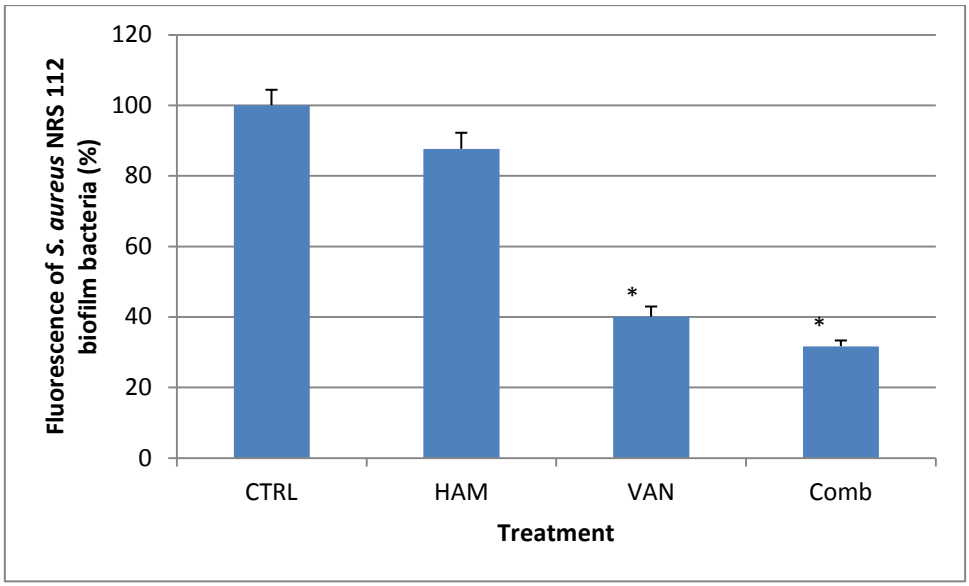


**Figure 4.14. Relative fluorescence of *S. aureus* NRS 112 biofilm bacteria after 24h of treatment with 250 µM HAM, 20 µg/mL VAN or the combination (Comb) with SEM. *: significantly different ($p<0.05$) compared to CTRL.**

## 4.5 Effect of HAM on biofilm susceptibility towards other antibiotics

*S. aureus* biofilms were treated with 4x, 10x, 50x or 100x MIC of the specific antibiotic (figure 4.15). Bacteria are quantified using the pour plate method. The antibiotic concentration that results in a 0.5 log reduction in CFU/mL is considered as responsive and is used in further experiments to determine the effect of the combination treatment with HAM.

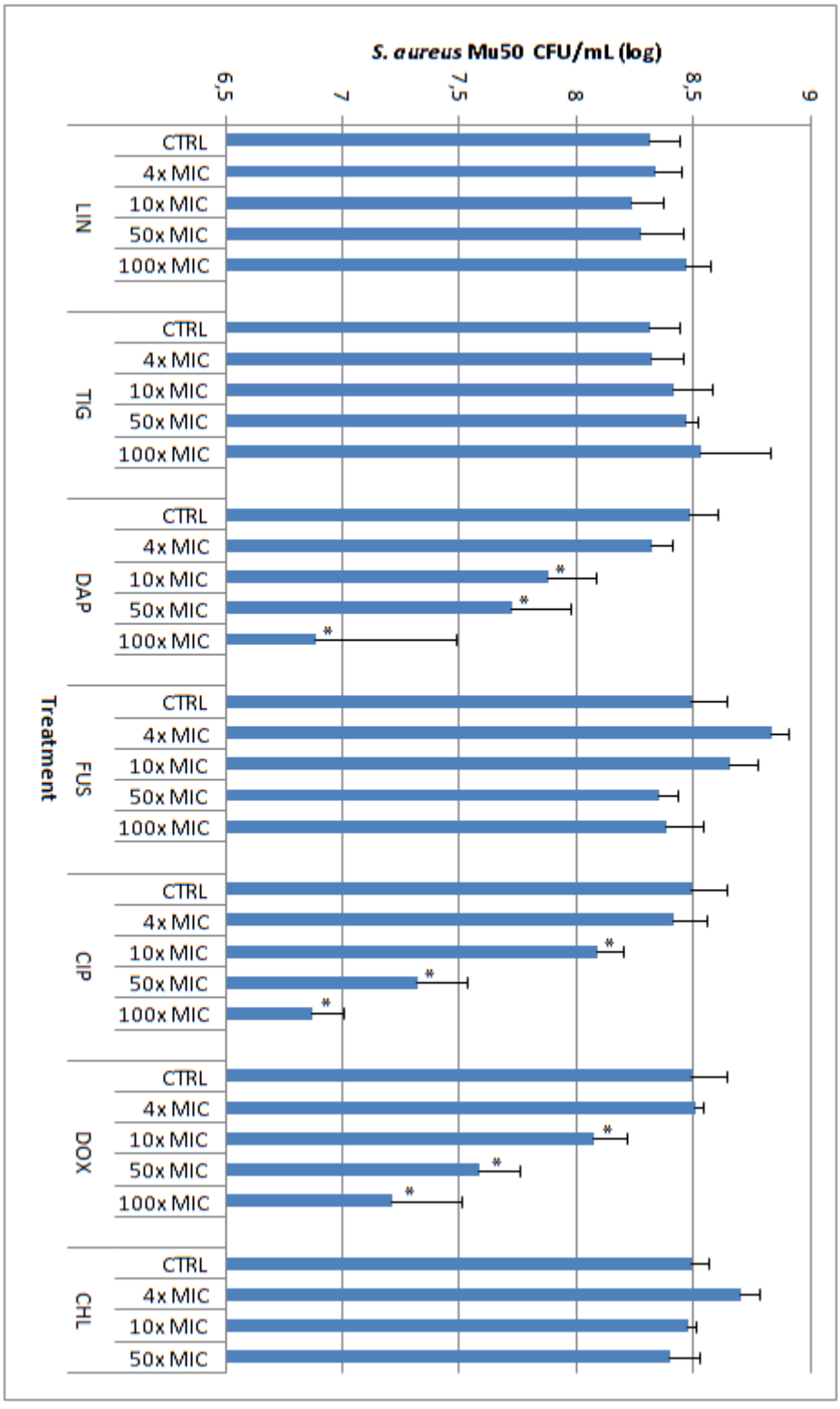


**Figure 4.15. *S. aureus* Mu50 CFU/mL (log) after 24h of treatment with LIN, TIG, DAP, FUS, CIP, DOX or CHL with SD. *: significantly different ($p<0.01$) compared to CTRL.**

*S. aureus* Mu50 biofilms treated with 100x the MIC of LIN, TIG or FUS (100 µg/mL, 100 µg/mL or 3.1 µg/mL respectively) or 50 x the MIC of CHL (400 µg/mL) do not result in a reduction in CFU/mL (figure 4.15). To discover the effect of HAM on biofilm susceptibility, 250 µM HAM is added to these concentrations. Biofilm bacteria receiving 10x the MIC of DAP (40 µg/mL) or 50x the MIC of CIP or DOX (1600 µg/mL or 400 µg/mL respectively) do result in a reduction of 0.5 log CFU/mL. These concentrations are combined with 250 µM HAM to test the change in susceptibility. The CFU/mL value obtained for the GC is set at 100 %, being the CTRL group. The CFU/mL obtained for treated biofilms are compared to this CTRL.

### 4.5.1 Susceptibility change of *S. aureus* Mu50 biofilm bacteria towards LIN

Treatment with HAM or LIN (100 µg/mL) does not result in a significant change of CFU/mL compared to CTRL ($p>0.05$). In contrast, combination treatment (100 µg/mL LIN + 250 µM HAM) causes a significant reduction ($p<0.05$) of 37 % of *S. aureus* Mu50 biofilm bacteria, compared to CTRL (figure 4.16).

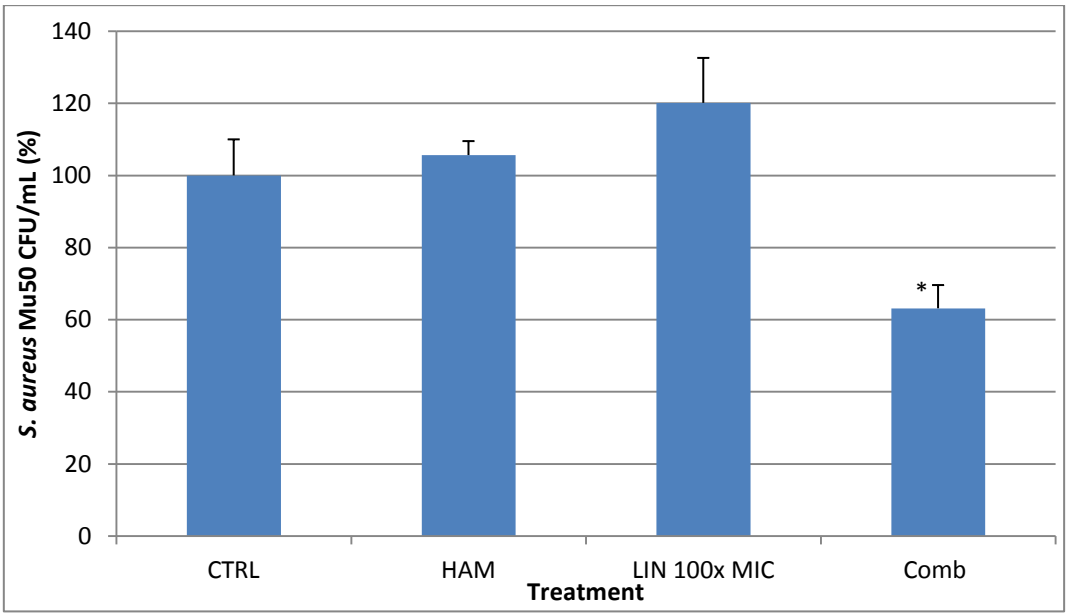


**Figure 4.16. Percentage of *S. aureus* Mu50 CFU/mL compared to CTRL, after 24h of treatment with 250 µM HAM, 100 µg/mL LIN or the combination (Comb) with SEM. *: significantly different ($p<0.05$) compared to CTRL and LIN treatment alone.**

### 4.5.2 Susceptibility change of *S. aureus* Mu50 biofilm bacteria towards TIG

There is no significant difference between untreated CTRL and groups treated with HAM or 100 µg/mL TIG ($p>0.05$). In contrast, combination treatment (100 µg/mL TIG + 250 µM HAM)

results in a reduction of 30 % biofilm cells compared to CTRL (figure 4.17). This reduction is significantly different ($p<0.05$) from CTRL and treatment with TIG alone.

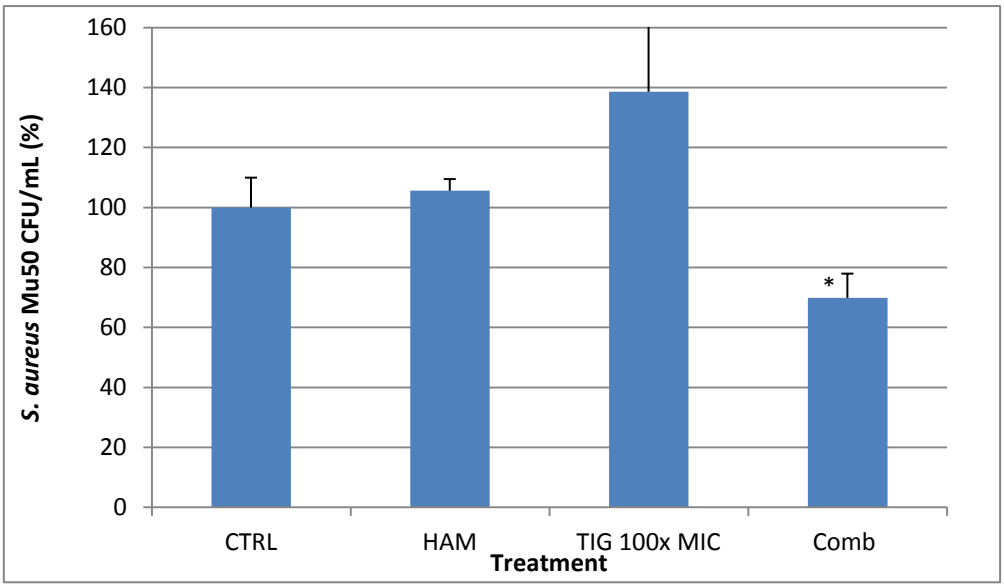


**Figure 4.17. Percentage of *S. aureus* Mu50 CFU/mL compared to CTRL, after 24h of treatment with 250 µM HAM, 100 µg/mL TIG or the combination (Comb) with SEM. *: significantly different ($p<0.05$) compared to CTRL and TIG treatment alone.**

### 4.5.3 Susceptibility change of *S. aureus* Mu50 biofilm bacteria towards DAP

Treatment with HAM does not result in a significant change of CFU/mL ($p>0.05$). Biofilm treatment with DAP 10x MIC (40 µg/mL) causes a significant CFU/mL reduction ($p<0.05$) of 69 % (figure 4.18). In contrast, there is no significant change in CFU/mL between DAP 10x MIC and the combination treatment (40 µg/mL DAP + 250 µM HAM) ($p>0.05$).

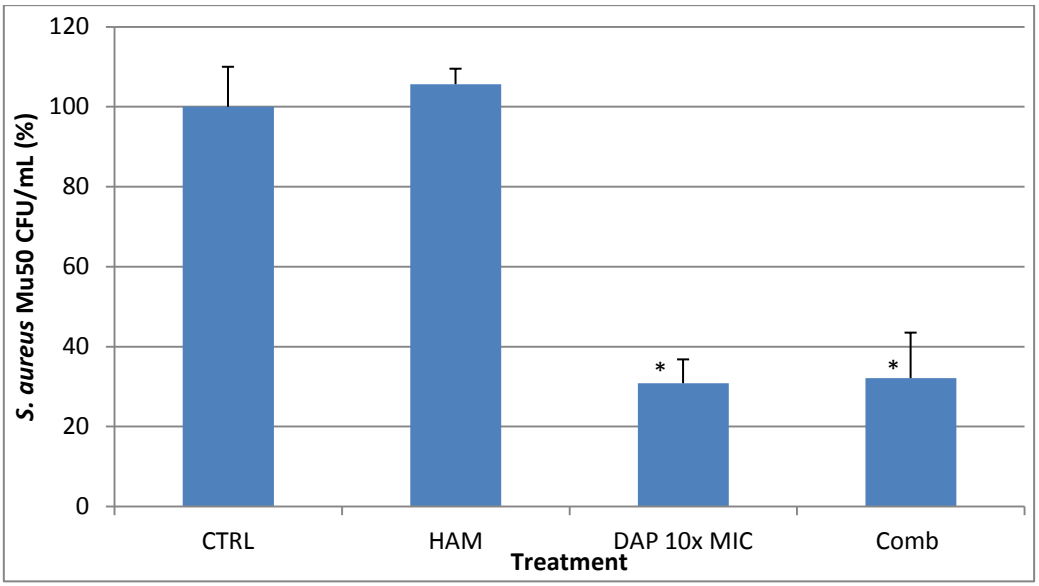


**Figure 4.18. Percentage of *S. aureus* Mu50 CFU/mL compared to CTRL, after 24h of treatment with 250 µM HAM, 40 µg/mL DAP or the combination (Comb) with SEM. *: significantly different (p<0.05) compared to CTRL.**

### 4.5.4 Susceptibility change of *S. aureus* Mu50 biofilm bacteria towards FUS

There is no significant difference between the untreated CTRL and groups treated with HAM, FUS or the combination of both (p>0.05) (figure 4.19).

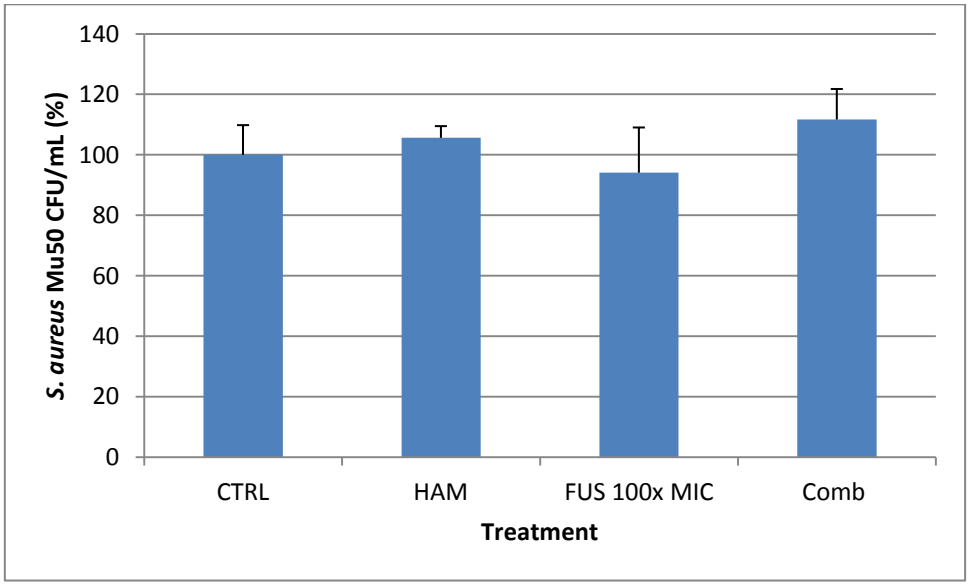


**Figure 4.19. Percentage of *S. aureus* Mu50 CFU/mL compared to CTRL, after 24h of treatment with 250 µM HAM, 3.1 µg/mL FUS or the combination (Comb) with SEM.**

### 4.5.5 Susceptibility change of *S. aureus* Mu50 biofilm bacteria towards CIP

No significant difference is observed between CTRL and HAM treatment ($p>0.05$). CIP 50x MIC (1600 µg/mL) treatment results in a significant reduction of 91 % of CFU/mL compared to CTRL (figure 4.20). However, the combination treatment (1600 µg/mL + 250 µM HAM) does not give rise to a significant extra reduction in CFU/mL ($p>0.05$).

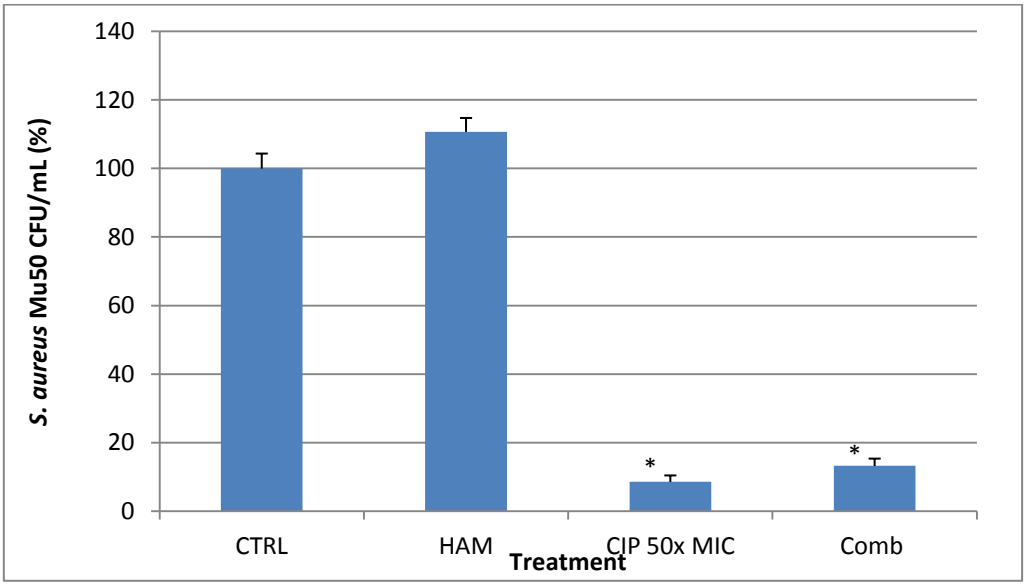


**Figure 4.20. Percentage of *S. aureus* Mu50 CFU/mL compared to CTRL, after 24h of treatment with 250 µM HAM, 1600 µg/mL CIP or the combination (Comb) with SEM. *: significantly different ($p<0.05$) compared to CTRL.**

### 4.5.6 Susceptibility change of *S. aureus* Mu50 biofilm bacteria towards DOX

There is no significant difference in CFU/mL between untreated *S. aureus* Mu50 biofilms and those receiving HAM treatment. Biofilm treated with DOX 50x MIC result in a significant decrease of 85 % of biofilm cells when compared to CTRL (figure 4.21). There is no significant difference in biofilm bacteria between DOX 50x MIC and combination treatment (400 µg/mL + 250 µM HAM) ($p>0.05$).

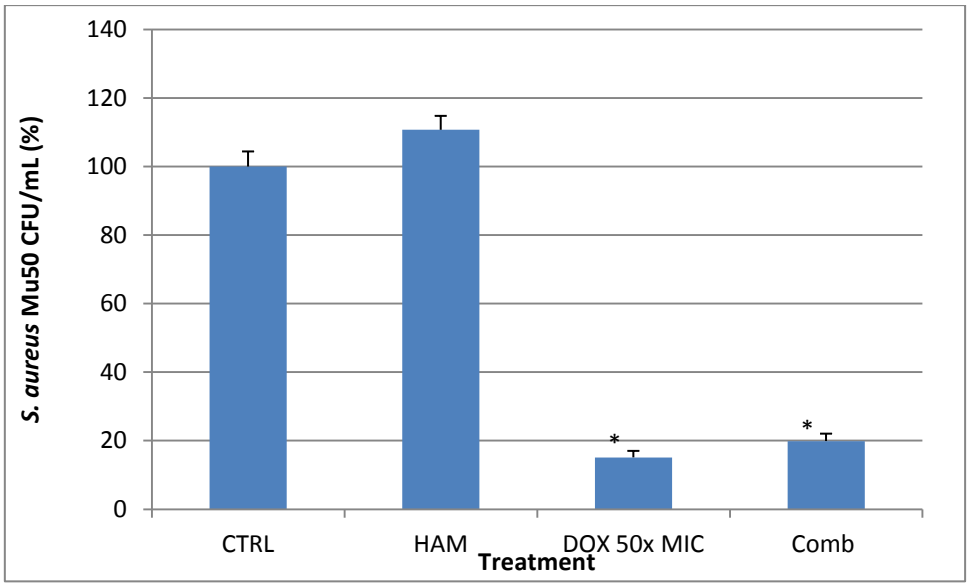


**Figure 4.21. Percentage of *S. aureus* Mu50 CFU/mL compared to CTRL, after 24h of treatment with 250 µM HAM, 400 µg/mL DOX or the combination (Comb) with SEM. *: significantly different (p<0.05) compared to CTRL.**

### 4.5.7 Susceptibility change of *S. aureus* Mu50 biofilm bacteria towards CHL

There is no significant difference in biofilm bacteria between CTRL, HAM and CHL 50x MIC (400 µg/mL) (p>0.05) (figure 4.22). In contrast, the combination treatment (400 µg/mL + 250 µM HAM) leads to a significant reduction of 35 % of *S. aureus* Mu50 biofilm cells (p<0.05).

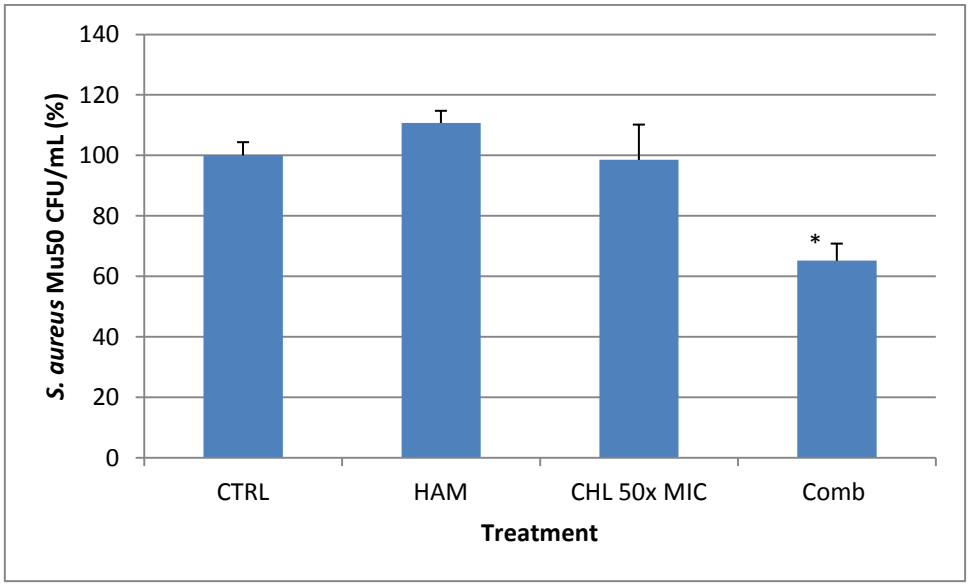


**Figure 4.22. Percentage of *S. aureus* Mu50 CFU/mL compared to CTRL, after 24h of treatment with 250 µM HAM, 400 µg/mL CHL or the combination with SEM. *: significantly different (p<0.05) compared to CTRL and CHL treatment alone.**

## 4.6 Optimization of hemolytic activity assay

Twenty-four hours old biofilm bacteria are incubated with rabbit erythrocytes at 37 °C. Aliquots of the supernatant are taken at different time points (4h and 24h) and hemolytic activity is determined by measuring the absorbance of these aliquots at different wavelengths (420 nm, 450 nm and 590 nm). No significant signal is observed when the supernatant is measured after 4h of incubation (figure 4.23), which indicates that hemolytic activity is only limited. In contrast, significant absorbance values are observed after 24h. After 24h of incubation, the absorbance value measured at 420 nm (0.506 ± 0.204) and 450 nm (0.328 ± 0.113) are significantly higher than the absorbance values measured at 590 nm (0.078 ± 0.012). Next we determine whether centrifugation of the supernatant at 1000 rpm would impact the absorbance value. The absorbance is higher in aliquots that are centrifugated.

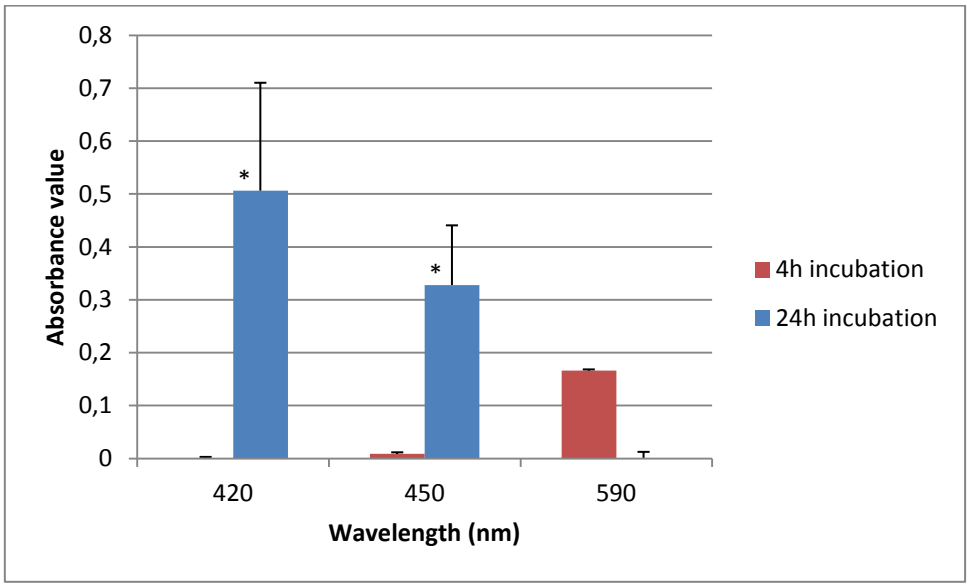


**Figure 4.23. Absorbance values of the CTRL supernatant after 4h and 24h of incubation, measured at 420 nm, 450 nm and 590 nm, without prior centrifugation with SEM. *: significantly different ($p<0.05$) compared to 4h incubation.**

## 4.7 Hemolytic activity of VAN treated biofilms

The addition of a 20 µg/mL VAN solution, a 250 µM HAM solution or the combination of both to rabbit erythrocytes does not increase hemolytic activity. In contrast, the addition of SDS does induce hemolysis (2.327 ± 0.817) (figure 4.24).

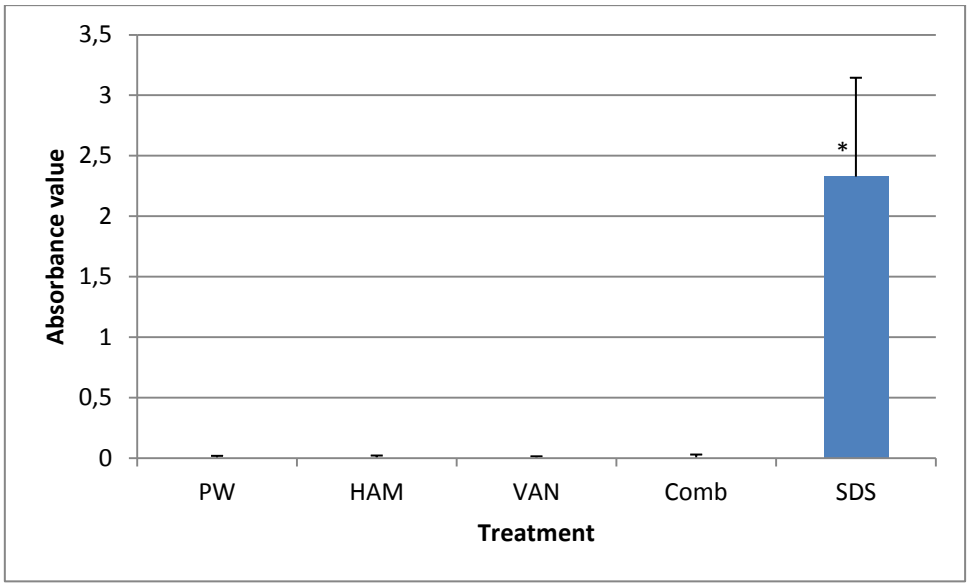


**Figure 4.24. Absorbance values of rabbit blood erythrocytes after 24h of treatment with 250 µM HAM, 20 µg/mL VAN, the combination (Comb) or a 10 % SDS solution , measured at 420 nm without prior centrifugation with SEM. *: significantly different ($p<0.05$) compared to CTRL.**

*S. aureus* Mu50 biofilms are treated with 20 µg/mL VAN, 250 µM HAM or the combination. Detached biofilm bacteria are incubated with rabbit blood for 24h and the absorbance of the supernatant is measured at 420 nm with centrifugation of the samples. Biofilm bacteria that received VAN treatment display a significantly increased hemolytic activity (absorbance value of 2.773 ± 0.477) when compared to the non-treated CTRL biofilms (0.506 ± 0.204) ($p<0.05$) (figure 4.25). In contrast, a decreased hemolytic activity is observed for biofilm bacteria receiving HAM treatment (0.154 ± 0.060) or combination treatment (0.226 ± 0.056).

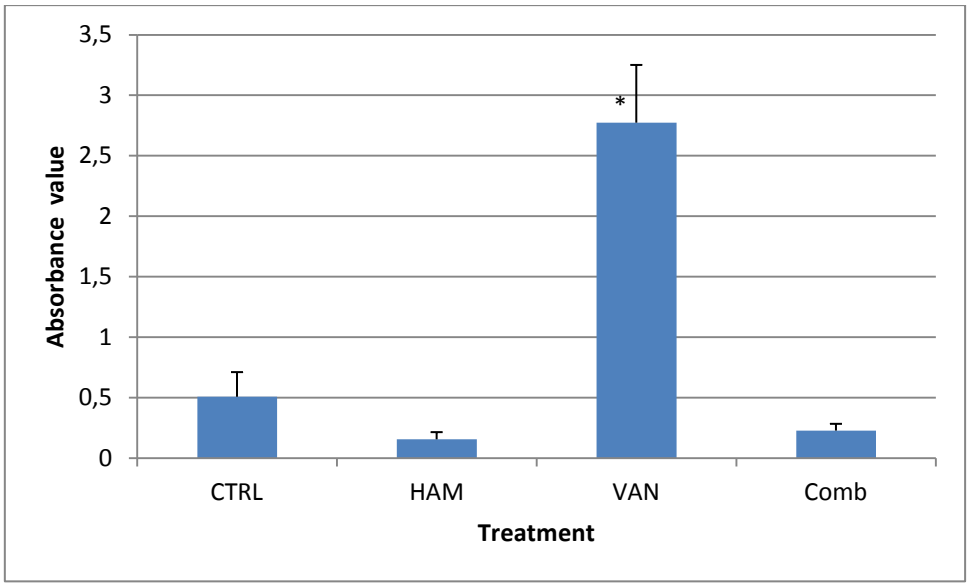


**Figure 4.25. Absorbance values of the supernatant after 24h of treatment with 250 µM HAM, 20 µg/mL VAN or the combination (Comb), measured at 420 nm with centrifugation with SEM. *: significantly different ($p<0.01$) compared to CTRL.**

# 5. Discussion

Infections with MRSA are difficult to treat due the high resistance profile of *S. aureus* and their ability to form biofilms. Therefore there is a search for novel methods to combat these types of infections. Since QS is known to regulate biofilm formation and virulence, targeting QS is considered as a possible new antibacterial strategy. In the research by Brackman *et al.* (2011), a QS inhibitor HAM was able to boost *S. aureus* Mu50 biofilm susceptibility towards VAN. However, it was unclear how HAM affected the susceptibility of biofilm cells and if HAM was able to increase biofilm susceptibility for other *S. aureus* strains against different types of antibiotics.

We first wanted to exclude the possibility that HAM indirectly affected bacterial growth by increasing membrane permeability. Membrane integrity was assessed with PI staining. PI is a nucleic staining dye that can only bind to nucleic acids in cells with compromised cell walls. As such, an increase of fluorescence in treated bacteria would indicate an increased uptake of PI by these cells, which indicates an increased cell permeability due to the treatment. We observed no significant change in membrane integrity after treatment with HAM compared to CTRL, which indicates that HAM has no effect on membrane integrity. However, higher concentrations of HAM should be evaluated to really exclude any effect on membrane permeability.

The MIC of HAM was higher than 512 µM. This confirms the observation of Kiran et al (2008) that HAM does not affect bacterial growth. Since HAM has an effect on the susceptibility of *S. aureus* Mu50 biofilm bacteria, we wanted to investigate whether HAM also has this effect on planktonic bacteria. HAM did not increase the susceptibility of planktonic cells towards VAN. There seems to be a minor, although significant, difference in CFU/mL (figure 4.4, 4.6 and 4.8) after treatment with HAM or after the combination treatment compared to GC. However, these reductions are biologically not relevant. This leads to the conclusion that HAM has no synergistic effect on planktonic *S. aureus* bacteria treated with VAN.

In previous research HAM was shown to increase biofilm susceptibility of both a methicillin resistant and sensitive *S. aureus* strain (Brackman *et al.,* 2011). In addition, recent research suggest that QS systems in *S. aureus* might play a role in this effect (Brackman *et al.,* unpublished data). At this point the effect of HAM was only investigated against *S. aureus*

strains with *agr* type II, however *S. aureus* use 4 types of *agr*. So it was unclear if HAM would have the same effect on *S. aureus* strains with other *agr* types.

An additional effect of HAM on biofilm susceptibility towards VAN was observed for *S. aureus* NRS 384 (*agr* type I), NRS 123 (*agr* type III) and NRS 153 (*agr* type IV) strains. This suggests that the effect of HAM is probably independent from *agr* type. However, HAM did not increase biofilm susceptibility towards VAN in *S. aureus* NRS 112 (*agr* type III mutant) and NRS 149 (*agr* type II). For *S. aureus* NRS 112 the absence of an added value can be explained by the fact that this strain has a mutation in *agr* that results in the attenuation of RNAIII. In addition, although *S. aureus* NRS 149 contains a functional *agr* system, it cannot be excluded that other genes, which might play a role in the effect of HAM, are mutated or differentially regulated in *S. aureus* NRS 149 compared to the other strains. In conclusion it can be said that an intact *agr* QS system is important for the effect of HAM, but that the *agr* type probably is not and that other genes might play a role in this effect.

Next we wanted to evaluate whether HAM could also affect biofilm susceptibility towards other antibiotics. Until now the increased susceptibility was only observed for the combination of HAM and VAN or HAM and clindamycin (Brackman *et al.,* 2011). However, recent research indicated that HAM also affects the expression of resistance genes of other antibiotics. Therefore it was interesting to evaluate the effect of HAM on biofilm susceptibility towards other antibiotics such as LIN, TIG, DAP, FUS, CIP, DOX and CHL. The observed MIC's for *S. aureus* Mu50 towards these antibiotics were in accordance to literature (Kwon and Lu, 2007; Mercier *et al.*, 2002; Pillai *et al.,* 2002; Cafiso *et al.,* 2012).

From our research it was clear that *S. aureus* Mu50 biofilms are highly resistant to antibiotic treatment. As expected there was no killing effect observed for biofilms treated with LIN, TIG, FUS (even at 100x MIC) and CHL (50x MIC), since these antibiotics are bacteriostatic. Only a moderate killing effect was observed for treatment with DAP (10x MIC) , CIP and DOX (50x MIC). This observation highlights the need for potentiators capable of increasing biofilm susceptibility. HAM was able to increase the susceptibility of *S. aureus* Mu50 biofilm bacteria towards LIN, TIG and CHL. However, the effect of the combination of these antibiotics with HAM should be evaluated on other *S. aureus* strains to exclude a strain specific effect. In contrast, the combination treatment of HAM with FUS, DAP, CIP and DOX does not result in

an added value. These combinations should be re-evaluated with a higher concentration of FUS and lower concentrations of DAP, CIP and DOX before we can conclude that there is no improved killing for these combinations against biofilm bacteria.

The decrease in CFU/mL could be explained by previous research (Brackman *et al.,* 2009), where the QS inhibitor cinnamaldehyde is able to loosen the *Burkholderia spp.* biofilm structure, thereby increasing the fraction of planktonic and thus more susceptible bacteria. This could lead to the loss of planktonic bacteria during rinsing steps. This has not been proven for HAM on *Staphylococcus spp.* biofilms, but it can be done by plating the rinsing solution and the medium of treated and untreated biofilm bacteria in further research. Another explanation could be that HAM alters the characteristics of the antibiotics from bacteriostatic to bacteriocidal. At this moment we do not know what explanation is most suitable for the decrease in CFU/mL.

Finally, HAM treatment of *S. aureus* Mu50 affects gene expression (Brackman *et al.,* unpublished data). In addition, treatment of *S. aureus* biofilm bacteria with VAN results in the upregulation of genes encoding hemolysins. However, it was unclear whether these observed differences in gene expression would also result in a phenotypically measurable difference. In order to measure this, we had to implement and optimize a bioassay to measure hemolytic activity of biofilm bacteria.

We first investigated the incubation time needed for measurable hemolytic activity values, the optimal wavelength for measurement and whether it was necessary to centrifuge the samples after incubation. We defined the best properties as those in which the highest absorbance value is obtained after incubation and where the biggest difference between a positive and negative control is measured. Our results indicate that hemolytic activity of biofilm bacteria should be measured after 24h of incubation at 420 nm. Our results further indicate that centrifugation gives rise to higher absorbance values but this could be explained by the fact that centrifugation itself can cause cell lysis. Centrifugation causes smaller differences between positive and negative controls. Therefore centrifugation is excluded from this protocol.

Next we wanted to evaluate whether biofilms receiving no treatment or a treatment with 250 µM HAM, 20 µg/mL VAN or the combination would display different hemolytic activities

in this assay. In order to exclude any effect of the treatment itself, we first investigated the hemolytic activity of HAM, VAN and the combination solutions incubated with rabbit erythrocytes and there was no difference compared to CTRL samples (figure 4.24). VAN treated biofilm bacteria clearly have a higher hemolytic activity compared to untreated CTRL biofilms (figure 4.25), so VAN can induce virulence in *S. aureus* Mu50 biofilms. In contrast, the addition of HAM in the treatment prevents the increase in hemolytic activity and the augmented virulence.

# 6. Conclusion

HAM is able to increase biofilm susceptibility towards VAN for *S. aureus* strains with different *agr* types. This suggests that the observed effect is independent of the *agr* type, although an intact *agr* QS system should be present. Other genes might also play a role in this susceptibility increase.

HAM increases *S. aureus* Mu50 biofilm susceptibility towards other antibiotics, such as LIN, TIG and CHL as well. In contrast, no effect of HAM on biofilm susceptibility towards DAP, FUS, DOX or CIP has been observed. However, different concentrations of the antibiotic should be combined with HAM before a change in biofilm susceptibility due to HAM can be excluded.

*S. aureus* Mu50 biofilm treatment with VAN induces the upregulation of hemolysin genes. This upregulation result in a measurable phenotypical change and increased hemolytic activity. In contrast, HAM is able to suppress this increase in virulence when combining HAM with VAN treatment.

# 7. Sources


Agwuh, K., N., MacGowan, A., 2006. “Pharmacokinetics and pharmacodynamics of the tetracyclines including glycylcyclines”, J Antimicrob Chemother. 58(2):256-65.

Balaban, N., Collins, L., V., Cullor, J., S., Hume, E., B., Medina-Acosta, E., Vieira da Motta, O., O'Callaghan, R., Rossitto, P., V., Shirtliff, M., E., Serafim da Silveira, L., Tarkowski, A., Torres, J., V., 2000. “Prevention of diseases caused by *Staphylococcus aureus* using the peptide RIP”, Peptides 21(9):1301-1311.

Balaban, N., Goldkorn, T., Gov, Y., Hirshberg, M., Koyfman, N., Matthews, H., R., Nhan, R., T., Singh, B., Uziel, O., 2001. “Regulation of *Staphylococcus aureus* pathogenesis via target of RNAIII-activating Protein (TRAP)”, J. Biol. Chem. 276:2658–2667.

Berger-Bächi, B., Rohrer, S., 2002. “Factors influencing methicillin resistance in *staphylococci*”, Arch. Microbiol. 178:165–171.

Boles, B., R., Horswill, A., R., 2008. “agr-Mediated Dispersal of *Staphylococcus aureus* Biofilms”, PLoS Pathog. 4(4): e1000052.

Brackman, G., Hillaert, U., Van Calenbergh, S., Nelis, H., J., Coenye, T., 2009. “Use of quorum sensing inhibitors to interfere with biofilm formation and development in *Burkholderia multivorans* and *Burkholderia cenocepacia*”, Res Microbiol. 160(2):144-151.

Brackman, G., Cos, P., Maes, L., Nelis, H., J., Coenye, T., 2011. “Quorum sensing inhibitors increase the susceptibility of bacterial biofilms to antibiotics in vitro and in vivo”, Antimicrob Agents Chemother. 55(6):2655-2661.

Cafiso, V., Bertuccio, T., Spina, D., Purrello, S., Campanile, F., Di Pietro, C., Purrello, M., Stefani, S., 2012. “Modulating Activity of Vancomycin and Daptomycin on the Expression of Autolysis Cell-Wall Turnover and Membrane Charge Genes in hVISA and VISA Strains”, PLoS One. 7(1):e29573.

Clinical and Laboratory Standards institute, 2012, http://antimicrobianos.com.ar/ATB/wp-content/uploads/2012/11/M100S22E.pdf, visited on 22/12/2014.

Corrigan, R.,M., Rigby, D., Handley, P., Foster, T.,J., 2007. “The role of *Staphylococcus aureus* surface protein SasG in adherence and biofilm formation”, Microbiology. 153:2435–2446.

Defoirdt, T., Boon, N., Bossier, P., 2010. “Can bacteria evolve resistance to quorum sensing disruption?”, PLoS Pathog. 6(7):e1000989.

Gonzalez, M., A., Uribe, F., Moisen, S., D., Fuster, A., P., Selen, A., Welling, P., G., Painter, B., 1984. “Multiple-dose pharmacokinetics and safety of ciprofloxacin in normal volunteers”, Antimicrob. Agents Chemother. 26(5):741-744.

Gov, Y., Bitler, A., Dell'Acqua, G., Torres, J., V., Balaban, N., 2001. "RNAIII inhibiting peptide (RIP), a global inhibitor of *Staphylococcus aureus* pathogenesis: structure and function analysis", Peptides. 22(10):1609-1620.

Gov, Y., Borovok, I., Korem, M., Singh, V., K., Jayaswal, R., K., Wilkinson, B., J., Rich, S., M. & Balaban, N., 2004. Quorum sensing in *Staphylococci* is regulated via phosphorylation of three conserved histidine residues", J. Biol. Chem. 279:14665–14672.

Gross, M., Cramton, S., E., Gotz, F., Peschel, A., 2001. "Key role of Teichoic Acid Net Charge in *Staphylococcus aureus* Colonization of Artificial Surfaces, Infect. Immun. 69:3423-3426.

Han, Y., H., Kim, Y., G., Kim, D., Y., Ha, S., C., Lokanath, N., K., Kim, K., K., 2005. "The target of RNAIII-activating protein (TRAP) from *Staphylococcus aureus*: purification, crystallization and preliminary X-ray analysis", Biochim Biophys Acta. 1748(1):134-136.

Harris, L.G., Foster, S.J., Richards, R.G., 2002. "An introduction to *Staphylococcus aureus*, and techniques for identifying and quantifying *S. aureus* adhesions in relation to adhesion to biomaterials: review", European Cells and Materials 4:39-60.

Heilmann, C., Hussain, M., Peters, G., Götz, F., 1997. "Evidence for autolysin-mediated primary attachment of *Staphylococcus epidermidis* to a polystyrene surface", Mol. Microbiol. 24:1013-1024.

Hiramatsu, K., Hanaki, H., Ino, T., Yabuta, K., Oguri, T., Tenover, F., C., 1997. "Methicillin-resistant *Staphylococcus aureus* clinical strain with reduced vancomycin susceptibility", J Antimicrob Chemother. 40(1):135-136.

Høiby, N., Bjarnsholt, T., Givskov, M., Molin, S., Ciofu, O., 2010. "Antibiotic resistance of bacterial biofilms", Int J Antimicrob Agents. 35(4):322-332.

Horswill, A., R., Stoodley, P., Stewart, P., S., Parsek, M., R., 2007. "The effect of the chemical, biological, and physical environment on quorum sensing in structured microbial communities", Anal Bioanal Chem. 387(2):371-380.

Junecko, J., M., Zielinska, A., K., Mrak, L., N., Ryan, D., C., Graham, J., W., Smeltzer, M., S., Lee, C. Y., 2012. "Transcribing virulence in *Staphylococcus aureus*", World J Clin Infect Dis. 2(4): 63-76.

Kim, H.K., Missiakas, D., Schneewind, O., 2014. "Mouse models for infectious diseases caused by *Staphylococcus aureus*", J Immunol Methods 410:88-99.

Kiran, M., D., Balaban, N., 2009. "TRAP plays a role in stress response in *Staphylococcus aureus*", Int J Artif Organs. 32(9):592-9.

Kiran, M., D., Giacometti, A., Cirioni, O., Balaban, N., 2008. "Suppression of biofilm related, device-associated infections by staphylococcal quorum sensing inhibitors", Int J Artif Organs. 31(9):761-770.

Kiran, M., D., Adikesavan, N., V., Cirioni, O., Giacometti, A., Silvestri, C., Scalise, G., Ghiselli, R., Saba, V., Orlando F., Shoham, M., Balaban, N., 2008. "Discovery of a quorum-sensing inhibitor of drug-resistant staphylococcal infections by structure-based virtual screening", Mol Pharmacol. 73(5):1578-86.

Krishnamurthy, V., Saha, A., Renushri, B.V., Nagaraj, E.R., 2014. "Methicillin Resistant *Staphylococcus aureus* Carriage, Antibiotic Resistance and Molecular Pathogenicity among Healthy Individuals Exposed and Not Exposed to Hospital Environment", J Clin Diagn Res. 8(7):DC04-8.

Kwon, D., H., Lu, C., D., 2007. "Polyamine Effects on Antibiotic Susceptibility in Bacteria", Antimicrob. Agents Chemother. 51(6):2070-2077.

Laverty, G., Gorman, S., P., Gilmore, B., F., 2013. "Biomolecular mechanisms of staphylococcal biofilm formation", Future Microbiol. 8(4):590-524.

Linares, J., 2001. "The VISA/GISA problem: therapeutic implications", Clin Microbiol Infect. 7(4):8-15.

Lowy, F., D., 1998. "*Staphylococcus aureus* infections", N Engl J Med. 339(8):520-32.

Mercier, R., C., Kennedy, C., Meadows, C., 2002. "Antimicrobial activity of tigecycline (GAR-936) against Enterococcus faecium and Staphylococcus aureus used alone and in combination", Pharmacotherapy 22(12):1517-1523.

Merino, N., Toledo-Arana, A., Vergara-Irigaray, M., Valle, J., Solano, C., Calvo, E., Lopez, J., A., Foster, T., J., Penadés, J., R., Lasa, I., 2009. "Protein A-mediated multicellular behavior in *Staphylococcus aureus*", J Bacteriol. 191(3):832-843.

Nahata, M., C., Powell, D., A., 1983. "Chloramphenicol serum concentration falls during chloramphenicol succinate dosing", Clin Pharmacol Ther. 33(3):308-313.

National Committee for Clinical Laboratory Standards, 2000. "Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically", 5th edn. Approved standard M7-A5.

Novick, R., P., Geisinger, E., 2008. "Quorum sensing in *staphylococci*", Annu Rev Genet. 42:541-564.

O'Neill, E., Pozzi, C., Houston, P., Humphreys, H., D., Robinson, A., Loughman, A., Foster, T., J., O'Gara, J., P., 2008. "A Novel *Staphylococcus aureus* Biofilm Phenotype Mediated by the Fibronectin-Binding Proteins, FnBPA and FnBPB", J Bacteriol. 190(11):3835–3850.

Otto, M., 2008. "Staphylococcal biofilms", Curr Top Microbiol Immunol. 322:207-228.

Otto, M., 2013. "Staphylococcal infections: mechanisms of biofilm maturation and detachment as critical determinants of pathogenicity", Annu. Rev. Med. 64:175-188.

Pantosti, A., Sanchini, A., Monaco, M., 2007. “Mechanisms of antibiotic resistance in *Staphylococcus aureus*”, Future Microbiology 2(3):323-334.

Painter, K., L., Krishna, A., Wigneshweraraj, S., Edwards, A., M., 2014. “What role does quorum sensing accessory gene regulator system play during *Staphylococcus aureus* bacteremia”, Trends Microbiol. [Epub ahead of print].

Park, J., Jagasia, R., Kaufmann, G., F., Mathison, J., C., Ruiz, D., I., Moss, J., A., Meijler, M., M., Ulevitch, R., J., Janda, K., D., 2007. “Infection control by antibody disruption of bacterial quorum sensing signaling”, Chem Biol. 2007 Oct;14(10):1119-1127.

Patti, J., M., Allen, B., L., McGavin, M., J., Höök, M., 1994. “MSCRAMM-mediated adherence of microorganisms to host tissues”, Annu Rev Microbiol. 48:585-617.

Periasamy, S., Joo, H., S., Duong, A., C., Bach, T., H., Tan, V., Y., Chatterjee, S., S., Cheung, G., Y., Otto M., 2012. “How *Staphylococcus aureus* biofilms develop their characteristic structure”, Proc Natl Acad Sci U S A. 24;109(4):1281-1286.

Pillai, S., K., Sakoulas, G., Wennersten, C., Eliopoulos, G., M., Moellering, R., C., Jr, Ferraro, M., J., Gold, H., S., 2002. “Linezolid resistance in Staphylococcus aureus: characterization and stability of resistant phenotype”, J Infect Dis. 186(11):1603-1607.

Singlas, E., Kitzis, M., D., Guibert, J., Taburet, A., M., Acar, J., F., 1988. “Pharmacokinetics of sodium fusidate in man after single and repeated infusions”, J Pharmacie Clinique 2:3341.

Sully, E., K., Malachowa, N., Elmore, B., O., Alexander S., M., Femling, J., K., Gray, B., M., DeLeo, F., R., Otto, M., Cheung, A., L., Edwards, B., S., Sklar, L., A., Horswill, A., R., Hall, P., R., Gresham, H., D., 2014. “Selective chemical inhibition of agr quorum sensing in *Staphylococcus aureus* promotes host defense with minimal impact on resistance”, PLoS Pathog. 10(6):e1004174.

Sun, H., K., Ong, C., T., Umer, A., Harper, D., Troy, S., Nightingale, C., H., Nicolau, D., P., 2005. “Pharmacokinetic profile of tigecycline in serum and skin blister fluid of healthy subjects after multiple intravenous administrations”, Antimicrob Agents Chemother. 49(4):1629-1632.

Traunmüller, F., Schintler, M., V., Spendel, S., Popovic, M., Mauric, O., Scharnagl, E., Joukhadar, C., 2010. “Linezolid concentrations in infected soft tissue and bone following repetitive doses in diabetic patients with bacterial foot infections”, Int J Antimicrob Agents.36(1):84-86.

Vendeville, A., Winzer, K., Heurlier, K., Tang, C., M., Hardie, K., R., 2005. “Making ‘sense’ of metabolism: autoinducer-2, LuxS and pathogenic bacteria”, Nat. Rev. Microbiol. 3:383-396.

Vuong, C., Saenz, H., L., Götz, F., Otto M., 2000. “Impact of the *agr* quorum-sensing system on adherence to polystyrene in *Staphylococcus aureus*”, J Infect Dis. 182(6):1688-1693.

Wang, R., Braughton, K., R., Kretschmer, D., Bach, T., H., Queck, S., Y., Li, M., Kennedy, A., D., Dorward, D., W., Klebanoff, S., J., Peschel, A., DeLeo, F., R., Otto, M., 2007. “Identification of novel cytolytic peptides as key virulence determinants for community-associated MRSA”, Nat Med. 13(12):1510-1514.

Zhu, J., Kaufmann, G., F., 2013. “Quo vadis quorum quenching?”, Curr Opin Pharmacol. 13(5):688-698.