

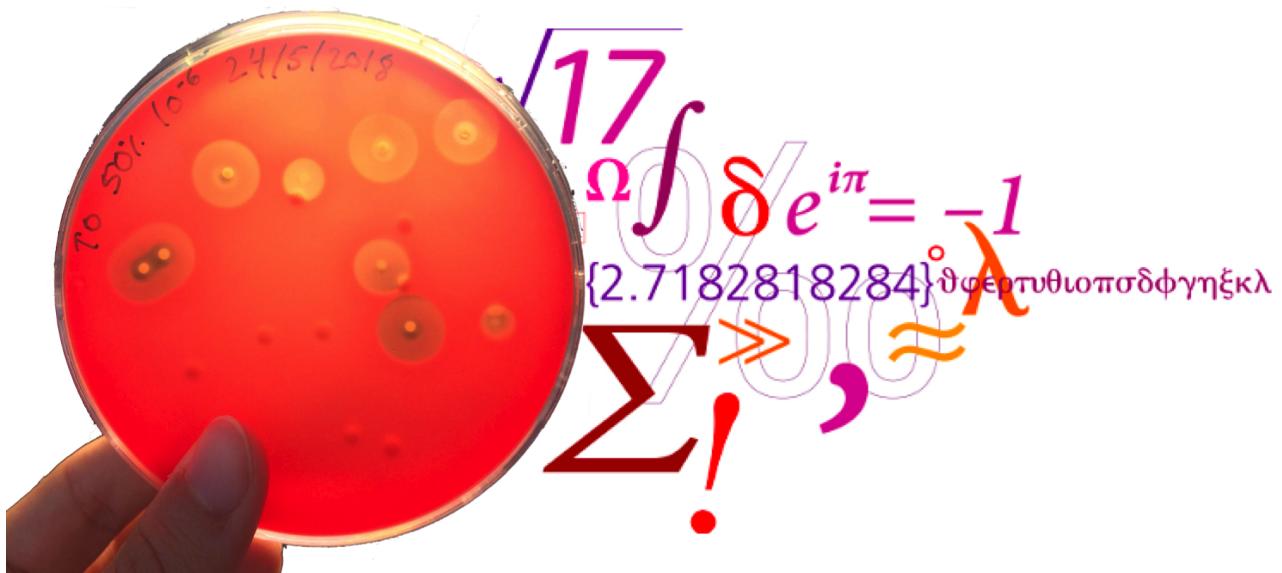
Technological University of Denmark

BACHELOR PROJECT

Investigating the MRSA antagonistic properties
of bacteria isolated from MRSA-positive pigs

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1 Abstract

In recent years, Livestock-Associated Methicillin-Resistant *Staphylococcus aureus* (LA-MRSA) has emerged as a prevalent problem across Europe. Efforts to find a way to combat the progressively more resistant bacteria have increased, but so far none have posed a permanent solution. In the present study, bacteria were collected from nasal, skin and faecal samples from Danish pigs. These bacterial isolates were then identified using MALDI-TOF, and subsequently screened for antagonistic properties against a strain of LA-MRSA CC398. From these screenings, six MRSA-antagonistic candidates were selected: *S. arlettae*, *S. chromogenes*, *S. hyicus* and three strains of *S. saprophyticus*. Of these six isolates, *S. arlettae* was the most potent antagonist. It was able to antagonise LA-MRSA in liquid culture and when cultured on blood agar plates. The strains of *S. saprophyticus* only displayed antagonistic properties when cultured on blood agar plates. Finally, *S. chromogenes* and *S. hyicus* were capable of inhibiting MRSA haemolysis on blood agar plates.

2 Dansk Sammenfatning

I de seneste år er husdrysassocieret Methicillinresistent *Staphylococcus aureus* (kendt som LA-MRSA) blevet et større problem i Europa. Indsatsen for at bekæmpe problemet med de stadig mere resistente bakterier er blevet forhøjet, men ingen har fundet en permanent løsning endnu. I dette studie er bakterieprøver blevet taget fra næsen, huden og fæces fra danske grise. Bakterierne blev identificeret ved MALDI-TOF og derefter screenet for antagonisme mod en stamme af LA-MRSA CC398. Fra disse screeninger blev seks potentielle MRSA-antagonistiske kandidater udvalgt: *S. arlettae*, *S. chromogenes*, *S. hyicus* og tre stammer af *S. saprophyticus*. Af disse seks isolater var *S. arlettae* den mest potente. Stammen havde antagonistiske evner i flydende kultur samt når den var dyrket på blodagarplader. *S. saprophyticus* stammerne viste kun antagonistiske evner på blodagarplader. Endeligt var *S. chromogenes* og *S. hyicus* i stand til at inhibere hæmolyse hos MRSA på blodagarplader.

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Contents

1 Abstract	2	8.1	The correlation between MRSA colonisation and antagonism	27
2 Dansk Sammenfatning	2	8.2	Evaluation of antagonistic potential	27
3 Acknowledgements	2	8.3	The supernatant experiment	28
4 Hypothesis and Objectives	4	8.4	S. arlettae: Liquid culture antagonism	29
5 Introduction	5	8.5	Possible antagonistic mechanisms	29
5.1 The genomics of MRSA	5	8.6	Applications of antagonistic bacteria	30
5.1.1 SCCmec typing of MRSA	5	9 Conclusion	30	
5.1.2 Other molecular typings of MRSA	6	10 Future perspectives	30	
5.2 Livestock-associated MRSA	6	11 Manufacturers	32	
5.2.1 The molecular typing of LA-MRSA	6	12 Abbreviations	32	
5.2.2 Transmission and colonisation	7	13 Bibliography	33	
5.2.3 The clinical relevance of LA-MRSA	7			
5.3 General mechanisms of antagonism	8			
5.3.1 Quorum sensing and biofilm	8			
5.3.2 Forms of competition between bacteria	9			
5.3.3 Bacteriocins	10			
5.4 Antagonistic species of bacteria	11			
5.5 Methods of testing	11			
5.5.1 Media for culture and selection of bacteria	11			
5.5.2 Identification using MALDI-TOF	12			
5.5.3 Screening bacteria for MRSA-antagonism	12			
5.6 Gaps in literature	13			
6 Materials and methods	14			
6.1 Sample collection and processing	14			
6.2 Isolation and selection of bacteria	14			
6.3 Transfer tests on MRSA-coated plates	14			
6.4 Agar plug diffusion experiment	16			
6.5 Supernatant experiment	16			
6.6 Liquid culture ratio experiment	17			
7 Results	17			
7.1 Identified species of bacteria	17			
7.2 Prevalence of MRSA in samples	17			
7.3 MRSA-antagonism screening results	21			
7.3.1 Drip test results	21			
7.3.2 Agar plug diffusion results	22			
7.3.3 Supernatant experiment	24			
7.4 Liquid culture ratio results	24			
8 Discussion	24			

4 Hypothesis and Objectives

Through the last decade the prevalence of methicillin-resistant *S. aureus* in European swine production has increased. Since LA-MRSA is able to transmit to humans and cause disease, it constitutes a potential hazard. As such, the search for new methods for treatment and prevention have been brought into focus. Due to the knowledge that neighbouring bacteria are in constant competition with each other, it has been suggested that bacteria co-living with *S. aureus* might possess competitive strategies that could be utilised in the search for new ways to combat multi-resistant strains of *S. aureus*.

The hypothesis of the present study is that:

Bacteria co-living with MRSA on pigs will utilise competitive strategies, such as production of antimicrobial compounds, which are effective in the prevention and treatment of LA-MRSA.

Following questions will be investigated:

- Are there species of bacteria co-living with LA-MRSA which show LA-MRSA antagonistic abilities?
- What genus most frequently displays LA-MRSA antagonistic traits?
- Is there any correlation between the prevalence of LA-MRSA and the prevalence of antagonistic bacteria in the samples?
- What are the possible antagonistic properties of the isolated bacteria?

5 Introduction

Staphylococcus aureus is a Gram-positive bacteria that can act as an opportunistic pathogen for both humans and animals. *S. aureus* is often found colonising the skin and mucous membranes of the nose and throat (1,2,3). It commonly causes a variety of infections in skin and soft tissues, including impetigo, folliculitis, and wound infections. However, it might also cause more serious infections such as bacteremia, pneumonia, and toxic shock syndrome (4).

Historically, human infections of *S. aureus* were regularly encountered in hospitals, where it was well known for causing invasive infections - particularly at surgical sites (2,3,5). With the emergence of penicillin in the 1940s, it became possible to treat *S. aureus* infections with a much higher efficiency than before. However, after only a few years, resistant strains were discovered. In response to the spread of penicillin resistance, the antibiotic *methicillin* was developed in the early 60s and was heavily used in hospital wards. But, just like with penicillin, it was not long before a new challenge emerged in the form of *methicillin-resistant S. aureus* (MRSA) (3,4). As the prevalence of MRSA infections in hospitals increased dramatically in the following years, it became apparent that *S. aureus* has an unfortunate tendency to rapidly develop resistances towards antimicrobials (3,6). Today official hospital guidelines have been established in many countries to stifle the number of nosocomial MRSA infections. Nevertheless, MRSA has prevailed and continues to rapidly adapt to new treatments. Many resources are therefore spent combating these multi-resistant *S. aureus* strains (5,6,7).

In the last decade, new epidemiological groups of MRSA have been identified, which appear to be genetically distinct from the well known *hospital-associated MRSA* (HA-MRSA) variant (8). One of these groups is commonly referred to as *livestock-associated MRSA* (LA-MRSA). As the name suggests, LA-MRSA was first isolated from species of livestock. This raises the issue of MRSA from animals potentially transferring to humans. As such, interest has been taken into testing alternative methods for prevention or removal of LA-MRSA colonisation (1,7,8).

5.1 The genomics of MRSA

Most β -Lactam antimicrobials, such as penicillin, affect bacteria by binding to penicillin-binding pro-

teins that are essential for cell wall biosynthesis. This generally results in bacterial cell lysis. However, the *mecA* gene encodes the production of an alternative penicillin-binding protein known as either PBP2a or PBP2'. Since this protein has low binding affinity towards most β -Lactam antimicrobials, bacteria carrying *mecA* is rendered resistant to almost all members of this widely used class of antibiotics (9,10).

When the prevalence of the *mecA* gene among staphylococci species was discovered, it was hypothesised that the gene was carried by some kind of mobile genetic element that could be transferred between species. Eventually the genetic element was identified as the *staphylococcal cassette chromosome mec* (SCCmec). This cassette carries the *mecA* gene, and is now considered to be one of the defining features of MRSA. The emergence of MRSA lineages is thus due to the insertion of SCCmec elements into the chromosomes of *S. aureus* strains (4,11).

Since its discovery, the detection of either *mecA* or PBP2A has been considered one of the most reliable ways to confirm MRSA positivity. It should therefore be noted that *S. aureus* strains which exhibit MRSA-like resistance without the *mecA* gene exist, most notably those that carry the divergent *mecA* gene known as *mecC*. Although strains of *mecC*-reliant MRSA are still relatively rare, they represent a potential problem if *mecA* is too heavily relied on for diagnostics (10).

5.1.1 SCCmec typing of MRSA

Although SCCmec elements can vary in both size and gene content, they often share some defining structural characteristics. Firstly, SCCmec elements usually contain a *mec* gene complex comprised of a *mecA* gene and its regulatory genes *mecR1* and *mecI*. The regulatory genes are however not always intact for all recognised SCCmec classes. Secondly, SCCmec elements contain a *cassette chromosome recombinase* (*ccr*) gene complex which holds site-specific recombinase genes that catalyses the movement of the SCCmec. Finally, the cassette typically contains two to three joining (J) regions. Although these J regions are said to be non-essential regions in the cassette, they remain significant, as they can encode additional resistances to antibiotics and heavy metals (4,11).

Since the structural composition of the SCCmec elements is highly diverse, SCCmec elements have been classified into various types based on the combination of *ccr* and *mec* complexes present. As of

now, at least 11 different *SCCmec* types have been discovered (8). The greatest variations between *SCCmec* types is commonly seen in the J regions, and subtypes are therefore established based on J region differences (4,9,11).

5.1.2 Other molecular typings of MRSA

Besides *SCCmec* typing, three other common typing methods for identifying and classifying MRSA strains exist.

The first one is *pulsed-field gel electrophoresis* (PFGE). This technique can provide a DNA fingerprint of a genome by separating DNA fragments in a gel matrix exposed to alternating electric polarities. For PFGE typing of *S. aureus* strains, the restriction enzyme *SmaI* is used as a standard (9,12).

The second method is *multilocus sequence typing* (MLST), which is based around the sequencing of DNA fragments from seven housekeeping genes. The sequences are assigned a sequence type (ST) through comparisons to other alleles in an *S. aureus* MLST database. Sequence types and strains that differ with one or two loci are grouped together in clonal complexes (CC). The clonal complexes are then named after the sequence type with most single and double loci variants (9,12).

The final method is *spa*-typing, which analyses variations in the DNA sequence of a particular region in the staphylococcal protein A (*spa*) gene. This region contains a number of repeating sequences. The *spa* type is then determined based on the total number of repeats as well as the sequence of each repeat (9).

5.2 Livestock-associated MRSA

During the 20th century, there were only a few instances of MRSA isolated from animals. These sporadic cases were generally assumed to involve MRSA strains of human origin. The situation changed in the mid 2000s, when an increasing number of reports began suggesting that MRSA was much more common in livestock than initially presumed (1,7).

One of the most notable cases of LA-MRSA in humans was documented in 2004, when a young Dutch girl and her parents were discovered to be colonised with a strain of MRSA. Several consecutive attempts at decolonisation were made, but the girl remained MRSA positive. In the Netherlands, the prevalence of MRSA in clinical isolates is generally below 1% (13), and the family had not recently interacted with foreign health care settings. It was

therefore deemed unlikely that they had contracted the bacteria from any hospital. As the family was known to raise pigs on their home farm, it was proposed that the pigs could have been the source of this unusual MRSA variant. This led to a study, which eventually concluded that the frequency of MRSA among a group of Dutch pig farmers was over 760 times higher than in the general Dutch population (13). Later, another study confirmed that the prevalence of MRSA in Dutch slaughter pigs were significantly higher than expected (14).

Following the studies in the Netherlands, LA-MRSA was soon reported to be found in the pig communities of many other nations across the globe. Consequently, studies were conducted in order to assess the identity and clinical relevance of this new MRSA variant (7,15).

5.2.1 The molecular typing of LA-MRSA

MLST typing has now identified that the most common LA-MRSA clonal complex in Europe is CC398. Other notable livestock-associated *S. aureus* clonal complexes include CC97, CC1 and CC9 (16). The CC398 complex is considered to have low host specificity, and is not a clonal complex typically associated with MRSA isolates from humans (8). LA-MRSA ST398 has subsequently been identified as the most prevalent sequence type among pigs in Europe (17,18,19). ST398 was also the first documented occurrence of LA-MRSA in Denmark, where it was discovered in Danish slaughter pigs in 2006 (20).

A distinct trait of LA-MRSA ST398, is its inability to be typed by the standard method of PFGE. This is due to ST398 containing a DNA methyltransferase which modifies the recognition sequence of the *SmaI* enzyme. However, it is now possible to type ST398 variants with PFGE using a different restriction enzyme (3,13,21).

A wide variety of different *spa*-types have been documented for LA-MRSA ST398. In 2008 a survey across 26 European countries was conducted by the European Food Safety Authority. This survey estimated the t011 *spa*-type to be the most dominant, while t108 and t034 was also found in relatively high numbers (17). However, the number of *spa*-types are continuously increasing as new types are discovered and reported (7,16).

Studies have indicated that there is a large amount of variety in the *SCCmec* elements of CC398 in general. The *SCCmec* types IV and V have been determined to be the most dominant *SCCmec* types

among LA-MRSA isolates (7,16). In comparison, HA-MRSA is generally associated with SCC_{mec} type I to III which are larger and presumably less mobile. In the case of LA-MRSA ST398, it is mostly encountered as SCC_{mec} type V, while the type IV variants are rarer. LA-MRSA ST398 type V was one of the first strains to be discovered in the Netherlands, and is nowadays often isolated in Western Europe, but also in countries such as Canada, The United States, Australia and New Zealand (22).

Most ST398 type V isolates carry the *mecA* gene as well as the *tet(M)* gene, which confers resistance to tetracyclines. However, LA-MRSA ST398 type V is also known to carry a wide variety of additional resistance genes. CC398 strains in general are often identified as carriers of genes that are associated with resistance to heavy metals. An example would be the zinc and cadmium resistance gene *czcC*, which has been identified within the type V SCC_{mec} cassette. The tendency to acquire heavy metal resistances is relatively common among LA-MRSA, and has in recent years been observed as increasing (22,23).

5.2.2 Transmission and colonisation

Direct exposure to MRSA positive animals is widely considered to be the primary source of LA-MRSA human colonisation (24). Studies in Europe have thus observed that people with occupations that involve high levels of animal contact, such as farmers or veterinarians, generally have higher rates of LA-MRSA colonisation (3,7). Exposure to LA-MRSA contaminated areas, such as farms and pig stables, is likely also a source of human colonisation. Dust in the stables of MRSA positive pigs has been found to be heavily contaminated. People working in the swine production environment could thus also contract LA-MRSA through the inhalation of the contaminated dust (8,25). Additionally, there has been documentations of LA-MRSA in raw meat. Human infections may therefore also occur through the handling of contaminated meat products (8).

Compared to HA-MRSA infections, it can be difficult to assess the epidemiology of LA-MRSA. Incidences of human LA-MRSA colonisation are not uniformly spread, but are instead often confined to regions with high densities of swine production (26). When studying the occurrence of human-to-human LA-MRSA transmissions in the households of MRSA-positive farms, it can therefore be difficult to distinguish the human LA-MRSA transmissions from the environmental transmissions (27). Nevertheless, a considerable amount of cases involving

human transmissions and human LA-MRSA carriage without animal contact have been documented (8,26,28). On the other hand, it has been estimated that LA-MRSA is much less likely to be transmitted in hospital settings than other MRSA genotypes (28,29,30). Studies have also suggested that LA-MRSA carriage seems to be strongly related to continuous exposure to infected animals. LA-MRSA colonisation rates in MRSA-positive farmers have thus been observed to rapidly decrease in periods of absence from MRSA-positive animal contact (24,31). Although LA-MRSA is generally not thought to cause much disease in pigs, it appears to be very efficient at colonising them (32,33). Based on these observations it appears likely that LA-MRSA CC398 is naturally adapted to pigs and other animals. Thus LA-MRSA is generally less adept at human transmission and colonisation compared to other MRSA variants (34,35,36).

The specific origin of LA-MRSA CC398 is still widely debated. Data from whole-genome analyses have suggested that LA-MRSA ST398 originated from human-associated strains of *S. aureus* (8,35,36). One hypothesis proposes that CC398 initially occurred in humans as methicillin-susceptible *S. aureus* strains carrying an *immune evasion gene cluster*. Most animal isolates of LA-MRSA CC398 lack the immune evasion associated genes, while human isolates of *S. aureus* more frequently contain it. Presumably, as the CC398 strains adapted to livestock hosts, they lost the immune evasion cluster and gained the SCC_{mec} cassette (8,36,37). Other studies have demonstrated that loss and gain of certain CC398 genomic elements, such as resistance genes and immune evasive genes, appear to follow host transfers between humans and pigs. However, more research is needed in order to fully understand the mechanisms behind LA-MRSA host adaption (37).

5.2.3 The clinical relevance of LA-MRSA

Although LA-MRSA CC398 often appears to lack some of the virulence-associated genes commonly found in other MRSA variants, LA-MRSA has retained the ability to act as an opportunistic pathogen in humans (26,38). Like many other strains of *S. aureus*, LA-MRSA infections often manifest as skin and soft tissue infections. There are also documented cases of CC398 causing more severe infections in humans, such as pneumonia, bloodstream infections, and necrotising fasciitis (8,23,26). Currently, most human LA-MRSA infections occur in healthy working adults due to occupational expo-

sure. However, as there are examples of LA-MRSA spreading in communities without direct animal contact, there is a risk of infections developing in more vulnerable population groups, such as sick or elderly people (26,38).

Despite generally being less dominant in humans than other MRSA variants, LA-MRSA has proven to be capable of establishing itself in hospital settings (1,8). LA-MRSA strains being introduced into hospitals by human carriers has therefore become a potential hazard to the patients. Particularly healthcare workers with direct or indirect animal contact could prove to be a significant source of LA-MRSA transmission. As such, a healthcare worker was presumed to be the cause of one of the first outbreaks of LA-MRSA in a Dutch hospital (28). The Netherlands has chosen to manage the issue of human carriers by classifying livestock farmers as a risk group. Like other high-risk patients, they must therefore be screened for MRSA carriage when admitted to hospitals. However, such an increase in screening procedures will inevitably put a burden on the health care system (7,39).

The rate of LA-MRSA infections could perhaps be reduced by preventing the development of MRSA in livestock populations. In the swine production industry, antibiotics are often used in pigs as a way to promote growth and prevent disease (40). However, a strong positive correlation between the intensive use of antibiotics and increased frequency of MRSA in pigs has been demonstrated. It seems likely that extensive antibiotic treatments promote the spread of LA-MRSA through selection (8,40,41). Heavy metal compounds such as zinc oxide have been used as an alternative to antibiotics. Yet studies have shown that this might actually add to the selective pressure. This is most likely due to the common presence of the *czzC* zinc and cadmium resistance gene within the SCCmec type V cassette. Increased zinc levels will subsequently co-select for SCCmec, thus further spreading the *mecA* gene as well as other resistance genes that might be present within the cassette (22,23,42). Pigs can therefore provide a reservoir of resistance genes, which could potentially be transferred to humans and other animals (40). As such, there is need for alternative ways to treat diseases in livestock without promoting co-selection.

Managing the emergence of LA-MRSA has proven difficult. Increasing security measures at hospitals may prevent nosocomial outbreaks, but it expends many resources and will not remove the source of the problem (7). Meanwhile, strategies for preventing the emergence of LA-MRSA in livestock populations

should avoid increasing selective pressure (1). For these reasons, it has become progressively more important to develop new alternative strategies for controlling LA-MRSA development. This project will investigate such a strategy by screening for bacteria with MRSA-antagonistic potential.

5.3 General mechanisms of antagonism

Bacteria have several forms of antagonistic mechanisms that are all necessary for one reason: competition. Across genera of bacteria, competition is mediated in numerous ways. It can both be chemical and non-chemical, exploitative and interfering (43). Antimicrobial production, space competition, predation and rapid growth rate are all factors that might put a bacterium ahead of others when fighting for nutrients (44). Depending on what kind of competition is at play, different factors matter. This section will explore these different factors.

5.3.1 Quorum sensing and biofilm

Some species of bacteria engage in so-called *quorum sensing* when the population cell density is high enough. Quorum sensing is a cell-cell communication mechanism used for the regulation of gene expression. Quorum sensing bacteria produce signal molecules called autoinducers. As the population size increases, so will the concentration of the autoinducer. When the threshold is reached, the signal molecule will interact with a receptor protein causing a coordinated change in gene expression across the population (45,46,47,48).

Communication and synchronised behaviour, like the one displayed in quorum sensing bacteria, is largely beneficial and a factor in the competition against other bacteria. These benefits are both in host colonisation, defence against competitors, and adaption to changing environments. Many social activities between bacteria are regulated by quorum sensing as well: symbiosis, formation of spores and fruiting bodies, bacteriocin production, genetic competence, apoptosis, formation of biofilms and their matrix, virulence factors, and DNA conjugation (45,47).

Three different types of quorum sensing bacteria exist. They are divided into classes based on what system they use for their regulation. The first group consists of Gram-negative bacteria that use acyl-homoserine lactones as signal peptides. The second group are Gram-positive bacteria with peptide based regulation (45,47). The third type of regulation is

found in both Gram-positive and Gram-negative bacteria, where the signal peptide is the *luxS*-encoded autoinducer 2 - called AI2. AI2 is generally regarded as the "universal language" of quorum sensing bacteria, and is used for cross-species communication. It has been found to be produced and detected by a large number of bacteria, implying that bacteria are able to assess cell density of other species in a community (47,48).

S. aureus is a quorum sensing bacteria, which uses the signal peptide based systems classic to Gram-positive bacteria. The specific system of *S. aureus* is called the *accessory gene regulator* (*agr*) system (47). The quorum sensing signal is produced by AgrB and AgrD. The prosignal is encoded by *agrD* and then exported and modified by AgrB. When the concentration of the signal is sufficient, the signal peptide binds to the surface receptor AgrC, a histidine kinase, which activates a response regulator AgrA. This affects gene expression through a small RNA molecule called RNAIII. Furthermore, *S. aureus* has been found to possess a functional *luxS* gene, enabling it to produce AI2 (45,46,47).

One of the possible outcomes of quorum sensing is the formation of biofilm. Biofilm is a cooperative group behaviour in which bacteria are embedded in a self-produced extracellular matrix. Forming biofilm can be highly beneficial to bacteria, as they are protected from environmental stresses such as desiccation, attack by immune systems, ingestion by single celled eukaryotes, and antimicrobials. The progress of a biofilm can be divided into stages: initial surface attachment of cells, physiological changes in the organism, multiplication of cells to micro-colonies, maturation of the biofilm, and in the end disassembly of the matrix and dispersion of bacteria (46,49).

Considering the benefits of intercommunication, it may sound like bacteria prefer to cooperate, but usually bacteria would choose competition over co-operation (43). This is evident in the fact that the signal peptides *S. aureus* produce during quorum sensing not only activates the *agr* system in their own strain, they also inhibit the same activation in strains different from the producer (47).

5.3.2 Forms of competition between bacteria

Competition between bacteria is usually divided into two forms: Exploitative and Interference competition. *Exploitative competition*, also called scramble competition, is the passive form of competition that depletes the surrounding nutrients

so competitors cannot utilise them. *Interference competition*, also called contest competition, is the active form of competition in which antagonistic factors play a role to impede competitors (43,44).

Antimicrobial compounds

The antagonistic factors can occur in the form of secondary metabolites as well as other antimicrobial compounds that are bioactive against competitors. Depending on the compound, antimicrobials can have a widely different target specificity. Some may target across species, others only strains within the same species and others yet may only target genetically identical individuals in a population. Reaching the sufficient quantity of the compound is important to inhibit competitors. This may require a population of bacteria to come together to produce the compound, and therefore production of antimicrobial compounds is often regulated by quorum sensing (43,44).

A subset of antimicrobial compounds are secondary metabolites. Secondary metabolites are synthesised outside the primary metabolism and are involved in other biological processes. In exploitative competition, they may alter the external environment through metabolic functions and thereby prohibit the growth of bacteria other than their producer. Meanwhile, the use of secondary metabolites in interference competition may seem obvious: many secondary metabolites have growth inhibitory or antibiotic properties, but the properties of secondary metabolites are plentiful. Just to name two sides of the same coin, some metabolites induce biofilm formation while others derail the quorum sensing and thereby inhibit it (43,44).

The specificity of microbial compounds might depend on the habitat and lifestyle of the bacteria. Bacteria found in a broad spectrum of environments will meet many potential competitors, and therefore a broad-spectrum production of antimicrobial compounds would be beneficial. Bacteria specialised for a specific environment would want to do the opposite and produce antimicrobials with specific targets (44).

Another group of compounds similar to those mentioned in this section are the *bacteriocins* which are discussed further down.

Steadfastness and transportation:

Motility and steadfastness also play important roles when competing other bacteria for nutrients and space.

A stable positioning at favourable sites prove a

good way of doing well as a bacterium. Again, we have two different ways to attain a good positioning: exploitative, in which bacteria colonise when new areas are available, and interfering, in which an active displacement of earlier competitors has taken place. When the bacteria are in position it is crucial to hold it as well. Numerous species do this by producing adhesins or receptors that bind to the host surface (44). An example are the species of *Lactobacillus* that bind to human epithelial cells and produce specific exterior glycoproteins that prevent potential pathogens (50,51).

Some bacteria use their motility to compete, while others might use it to avoid having to compete. For example, *Pseudomonas aeruginosa* moves around to find the most ideal places for biofilm formation before its competitors do. Motile organisms are at higher risk of finding new potential competitors, but as these are often alone and not parts of an entire population the options for coordinated competitive strategies are limited (44).

5.3.3 Bacteriocins

Bacteriocins are small, heatstable ribosomally synthesised antimicrobial peptides. They are produced by bacteria and are active antimicrobially against other bacteria. The producer of the bacteriocin will itself have a specific immunity mechanism, so the bacteriocin will not hurt it (52,53,54,55).

Bacteriocins are produced by all major lineages of bacteria and archaea and have a large diversity in size, structure, mechanisms of action, inhibitory spectrum, immunity mechanisms, and target cell receptors (53,54,55) but can be divided into two classes. Class I contains the lantibiotics which contain unusual amino acids such as lanthionine and dehydroalanine because of the post translational modifications they go through. These unusual amino acids contribute to the high stability. Lantibiotics are often characterised by cyclic structures. Class II are small unmodified peptides. Class II bacteriocins are further divided into four subgroups, while lantibiotics are divided into two subgroups (54,55,56).

Regarding the classification of bacteriocins, there is some disagreement. Some classify them as secondary metabolites (57) while others classify them as primary, arguing that they are ribosomally synthesised, sensitive to proteases and gene encoded (58,59).

An example of a class II bacteriocin is one produced by *Lactobacillus salivarius* which is regulated by a quorum sensing mechanism and is active against

Listeria monocytogenes. The production is highest in the beginning of the stationary phase and is catalysed by the accumulation of an induction peptide (52). The induction peptide is just one mechanism of regulation of bacteriocins, but there are various others. Regulation is in general complex and depends on environmental conditions like pH, temperature, and growth medium (54).

The target range of a bacteriocin varies greatly between species and compounds. Bacteriocins from Gram-positive bacteria have been shown to be both bacteriocidal and bacteriostatic across species or genera, but the effects are usually limited to other Gram-positive bacteria (52). While the targeting of bacteriocins varies greatly between the species of the producers, some bacteriocins only target strains that are relatively close to themselves and yet others might target across genera (53,55,60).

Bacteriocins have several different possible mechanisms of actions. Some are *colonising peptides* which contribute to the interference competition for space and facilitate the introduction of their producer or dominance of their producer (53). An example of this is a bacteriocin produced by *E. coli* which was shown by Gillor et al. (61) to be a significant factor to *E. coli* in colonising the gastrointestinal tract.

Others are *antimicrobial* or *killing peptides* which directly inhibits competing strains or present pathogens (53). An example of this would be the bacteriocin produced by *Lactococcus lactis* which has been shown to be effective against *L. monocytogenes* and *Clostridium perfringens* *in vitro* although it was shown to be ineffective *in vivo* (62).

Lastly there are *signalling peptides*. These are used for signalling other bacteria through quorum sensing and cross talk within bacterial communities. The benefits of quorum sensing is explained above. In this case, some bacteriocins might act as both inhibitors at high concentrations and as inducers at low concentrations (53).

In addition to the aforementioned species producing bacteriocins that have proven useful against pathogenic species, many species of the *Bacillus* genera are reported to produce bacteriocins or bacteriocin-like substances. Furthermore, bacteriocins from lactic acid bacteria are already used in food industry as natural preservatives (55).

More important to this report: two bacteriocins produced by *Staphylococcus epidermidis* called epidermin and Pep5 have been proven to have potential applications against MRSA (60). Furthermore, mersacidin which is produced by a *Bacillus* strain has also been proven to be active against MRSA (53).

5.4 Antagonistic species of bacteria

Due to the target specificity of some bacteriocins being limited to other bacteria within the same genus of the producer, it would make sense for other staphylococci to show antagonistic properties against *S. aureus*. However, other bacteria from completely different genera have been shown to exhibit inhibitory properties towards or even lyse *S. aureus* (44). In this section, further examples of bacteria which have proven to be antagonistic against *S. aureus* are listed and explained.

Staphylococcus

The bacterial genus *Staphylococcus* are Gram-positive cocci. They are nonmotile, non-spore forming and facultatively anaerobic (63).

In addition to the aforementioned epidermin and Pep5 (60) *S. epidermidis* has also been proven to secrete a serine protease which degrades *S. aureus* biofilms by inactivating autolysins, thereby preventing release of DNA which is an important component to the biofilm extracellular matrix (43).

The bacteriocin nukacin ISK-1 is produced by *S. warneri* and binds lipid II, a membrane-bound precursor of the cell-wall, thereby inhibiting synthesis of the cell-wall but it only has a bacteriostatic effect on *S. aureus* (56).

Lactobacillus

Species found in the genus *Lactobacillus* are Gram-positive, immobile, non-spore forming, mostly facultative anaerobic, acid tolerant, and negative catalase bacteria. They are carbohydrate fermenting bacteria and their main final product is lactic acid, making them a lactic acid bacterium. The genus is usually valued for its properties as a probiotic and are generally found in the same habitats of *S. aureus* (64).

Many bacteria within the *Lactobacillus* genus has been found to act as antagonists to *S. aureus*. Strains of *L. casei*, *L. acidophilus*, *L. plantarum* and *L. reuteri* have all been found to inhibit growth of *S. aureus* (65).

Other examples

Aside from the species of more the widely researched genera mentioned above, several stand-alone examples from different genera have also been found.

Nisin A and lacticin Q of *Lactococcus lactis* have been shown to be bacteriocidal to both planktonic *S. aureus* and its biofilm. Like nukacin ISK-1 of

S. warneri, nisin A targets the membrane-bound precursor of the cell-wall lipid II. This bacteriocin both inhibits the synthesis of the cell-wall and forms pores that rapidly kills cells (56).

Bifidobacterium longum, *Bifidobacterium bifido*, and a species of *Brevibacillus* have all been found to inhibit the growth of *S. aureus* (55,65) and *Pediococcus* strains produce pediocin which has a wide inhibitory spectrum against Gram-positive bacteria, *S. aureus* included (66).

Furthermore, other species have been found to take a more direct approach. *Streptococcus pneumoniae* has been found to efficiently eradicate biofilm and biofilm-released planktonic cells of *S. aureus* through physical contact (67) while *P. aeruginosa* is known to lyse *S. aureus* cells to gain free iron (44).

5.5 Methods of testing

During the experimental phase of this study, a variety of materials and methods were used in order to select, identify and screen bacterial isolates. In this section the most important ones will be introduced and explained.

5.5.1 Media for culture and selection of bacteria

While culturing the bacteria isolates, an assortment of media with different properties were used. The following will be a brief explanation of the features of each medium.

Blood Agar (BA):

Most of the solid culturing in this project was performed on BA plates. Blood agar is a versatile enriched medium containing between 5% and 10% blood - in this case calf blood. It is useful for growing a multitude of different organisms, and is often used for fastidious species. It can also be used to differentiate species based on their haemolytic properties.

Slanetz and Bartley Medium (SB):

The main feature of the SB medium is its high selectivity towards species of enterococci. However, it is also often used for detection of streptococci. Colonies of these two genera typically appear red or maroon while growing on this medium. However, pale colonies can occur (68,69).

SASelect Medium (SAs):

The SAs medium uses a combination of salts, antibiotics and antifungal growth inhibitors to

select for staphylococci. When cultured on the medium, species of staphylococci appear in various colours, which makes it easier to differentiate between them. Colonies of *S. aureus* thus usually develop a pink to orange colour, while other species tend to appear blue, purple, white or yellow (70).

MRSA 2 Agar (MRSA2):

The *Brilliance* MRSA2 medium is designed to specifically select for MRSA. All MRSA colonies grown on this medium will yield a dark blue colour due to targeting of a specific enzyme that is present in all MRSA variants. The medium also contains a combination of antimicrobials that inhibit a variety of competitive microorganisms. Non-MRSA colonies that manage to grow regardless can be distinguished by their pinkish colour (71,72).

Luria-Bertani Medium (LB):

LB medium is a common nutritionally rich type of medium that is often used for culturing and growing a variety of bacteria. In this study, bacterial isolates were mostly cultured in this media due to its versatility.

Müller-Hinton Medium (MH):

MH medium is well known for being a widely used standard medium when testing for antibiotic susceptibilities. It is also commonly used as a non-selective growth medium. In this study, the LA-MRSA test strain was primarily cultured in MH containing 6.5% NaCl. This was optimal, as *S. aureus* grows well in saline conditions, while most potential contaminants do not (73,74).

Brain Heart Infusion Medium (BHI):

BHI medium is a nutritionally rich growth medium, which can be used to culture fastidious and non-fastidious organisms. It contains infusions of brain and heart - usually from either cows or pigs.

5.5.2 Identification using MALDI-TOF

For identifying isolated bacteria *matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry* (MALDI-TOF MS) was used. The technology is based on comparison between the peptide fingerprint of the sampled microbial cells and a database of peptide fingerprints from a variety of species. MALDI-TOF is regarded as a soft ionisation technique because of its minimal damage to samples. This allows analysis of larger biomolecules like ribosomal proteins (75,76).

Samples are prepared for identification by mixing them with a saturated, energy-absorbant solution of low-mass organic compounds called the *matrix*. The sample-matrix mix is then spotted onto a metal target plate and dried. During the drying process the sample and matrix will co-crystallise, so a solid deposit of the sample will be embedded into the matrix (76,77).

The dried sample and matrix are then irradiated with a UV laser beam causing it to ionise and sublimate directly from the solid phase to gas phase. Desorption and ionisation with the laser beam creates singly protonated ions in a plume (76,77).

The protonated ions in the plume are then accelerated through a time of flight tube at a fixed speed and detected at the end of the tube. The time it takes for each ion to travel to the end of the tube is called the *drift time*. The drift time is determined by the size of the ion: larger ions will have longer drift times and vice versa for smaller ions. The drift time is directly proportional to the mass-to-charge (m/z) ratio which is another measurement for how quickly a protonated ion travels through the time of flight tube (76) and is used to create the spectra for each sample. These spectra are called peptide mass fingerprint (PMF) and to identify the sample in hand, the sample spectrum is compared to a full database of spectra. Usually, a mass range m/z of 2-20 kDa is used. This mainly represents ribosomal proteins enabling identification all the way down to species and strain level (77).

5.5.3 Screening bacteria for MRSA-antagonism

When a number of bacterial isolates had been selected and identified, screenings were performed in order to assess their antagonistic potential towards LA-MRSA. Several different screening methods were used, so that the isolates were tested under various conditions. The following will account for the four major experimental setups utilised in this project.

Drip experiment:

The experimental setup of the drip experiments is quite simple. Bacterial isolates are cultured in liquid medium, and then dripped onto agar plates newly coated with LA-MRSA culture. During the incubation of these plates, the isolate bacteria within the drops will compete with the surrounding LA-MRSA. Bacteria with antagonistic features would then ideally be identified due to a lack of MRSA growth in the areas surrounding the isolate

droplets. These clearing zones would presumably occur due to the isolate bacteria secreting competitive molecules, such as bacteriocins, which in turn would inhibit or kill the adjacent MRSA.

Agar plug diffusion experiment:

This method is commonly used to demonstrate the antagonistic properties between different microorganisms (78). The experimental setup was designed to give the bacterial isolates more of a chance to produce competitive metabolites before encountering LA-MRSA. As such, the isolates are all cultured individually on separate plates. While the isolates grow, the molecules they secrete will diffuse down into the agar medium. After incubation, small cylinders of agar - so-called agar plugs - are cut from the isolate plates and then deposited on top of agar plates freshly inoculated with LA-MRSA. The secreted molecules should then diffuse from the plug to the LA-MRSA plate below. If any of these molecules are competitive towards MRSA, it would be detected as MRSA clearing zones surrounding the plug (78).

Supernatant experiment:

The idea behind this experimental setup is to investigate the properties of metabolic compounds separately from their bacteria of origin. This is accomplished by removing all cells from overnight cultures using centrifugation and filters. Ideally, this should produce a sterile supernatant containing the metabolic products of the culture. The supernatant is then applied to an LA-MRSA coated agar plate. If the supernatant contains any competitive molecules, this should result in visible inhibition of MRSA growth in the affected areas.

This experiment should be performed twice for each isolate: once where the supernatant has been heat processed, and once where it remains unprocessed. This can be used to determine the general structure of the compound. If MRSA inhibition occurs with the unprocessed supernatant, but not with the heat processed one, it indicates that the antagonistic molecule is a larger peptide, which has been denatured by the heat. If inhibition occurs with both supernatants, it instead suggests that the antagonistic molecule is much smaller and heat stable - like bacteriocins.

Liquid culture ratio experiment:

This experiment pits a bacteria culture against LA-MRSA in a liquid culture setup. This is performed by diluting an overnight culture of each

bacteria down to the same OD, and then incubating them together in one tube. After the two cultures have been mixed, samples are then continuously collected and plated on agar plates for 24 hours. The development of the mixed culture can thus be tracked based on how many colonies of each species are present on the plates. If a species is competitively viable against LA-MRSA, it will grow to become the dominant species within the growth tube. As time progresses, the percentage of LA-MRSA colonies on the agar plates should therefore be observed as decreasing.

This experiment should be performed with several different ratio mixes of the two cultures. As a minimum, there should be three ratios: one where LA-MRSA is dominant, one where the tested bacteria is dominant, and one where they are equal. This makes it possible to document the differences in antagonistic efficiency, based on how long it takes for the isolate in each ratio to overpower LA-MRSA. It also accounts for bacterial isolates that may have competitive properties, but only successfully outcompete LA-MRSA when they have a starting advantage.

5.6 Gaps in literature

Although research has come a long way since the discoveries of LA-MRSA in 2004, much still remains to be understood regarding the development and spread of this persistent MRSA variant.

As previously mentioned in section 5.2.2, the origin of LA-MRSA CC398 has not been fully determined. Uncovering the starting point of LA-MRSA may shed some light on its earliest developments. Additionally, it might also bring some insight to the host adaption process that potentially occurred. However, as of now, further studies are required in order to determine the phylogeny behind LA-MRSA isolates from both humans and animals (37). It should also be noted that while LA-MRSA CC398 is the most dominant LA-MRSA worldwide, it is not the only variant. Other clonal clusters, such as CC9, has not received nearly as much attention due to the prevalence of CC398. As such, the development and significance of these alternative MRSA variants are much less documented (38).

Since the amount of LA-MRSA infections outside of risk groups appear to have been increasing, it has become more important to investigate other potential LA-MRSA transmission sources (26,39). Understanding the host adaption process from animal to human may grant significant insight when

trying to locate the source of an outbreak. Additionally, knowledge of the adaption and transmission procedures of LA-MRSA may prove useful when establishing preventative measures for development and spread of LA-MRSA. Although the exact molecular mechanisms behind the host adaption process are not yet completely understood, the discovery of host specific genetic elements suggest that certain genetic host factors are important for human colonisation and pathogenicity. However, little is currently known in regards to which host factors are significant and what roles they play. Consequently, more research must be performed in order to characterise the genomic changes that accompany host jumps (37).

Besides the exact mechanism of LA-MRSA transmission, it is also necessary to understand the frequency of these transmissions. Many studies have investigated the transmission rates in hospitals and pig farms. However, LA-MRSA transmission in general populations is much less studied. Larger long-term studies in hospitals, communities and livestock are thus needed in order to better document the changes in LA-MRSA carriage rates over time (28,34,41).

6 Materials and methods

During the present study, bacteria were isolated from the skin, nostrils, and faeces of pigs. Certain bacteria were then selected for further experiments with the aim to assess their MRSA-antagonistic capabilities. The isolation and selection processes along with additional experiments are detailed in the following sections. A representation of the experimental progress can be seen in figure 1. Details regarding the suppliers, materials and the LA-MRSA CC398 test strain used in the experiments can be found in section 11.

6.1 Sample collection and processing

The samples studied in this project originated from five weeks old weaned pigs. Samples were collected from 5 pigpens containing 10-20 pigs each. Two pigs per pigpen were randomly chosen for sampling, meaning that samples were taken from 10 pigs in total. Each pig was assigned a number so that samples could be differentiated later.

From each pig two types of samples were collected: skin and nasal. Both of these were sampled using ESwabs. The skin samples were taken by wiping the swab on the skin behind one ear, while the nasal

samples were taken by rubbing the swab within both nostrils of the pig. Two faecal samples were subsequently collected from the ground of each pigpen and stored in plastic containers.

Each sample was labelled with a letter that denoted the sample type - N (nasal), S (skin) or F (faeces) - as well as the number of the pig the sample was collected from. It was however not possible to assess which pig the faecal samples originated from. These samples were therefore labelled with the pigpen number instead.

Afterwards the samples were treated with glycerol to inhibit further growth during storage. 35 mg of the faecal samples were extracted and diluted in Phosphate Buffered Saline (PBS) before being supplied to a 25% glycerol solution. The skin and nasal samples were simply supplied to a 25% glycerol solution without any further processing. All samples were then stored at 5°C.

6.2 Isolation and selection of bacteria

The samples were vortexed carefully and centrifuged at 53 rpm for 5 minutes. Serial dilutions up to dilution 10^{-6} was then done in PBS for each sample. These dilutions were plated on different media. Which dilutions were plated on which types of media depended on the sample type (see table 1). The plating was done by transferring 100 μ l of sample dilution to the corresponding plate and then spreading it thoroughly with a Drigalski spatula until the plate was dry. The plates were then left to incubate upside-down overnight at 37°C.

Following incubation, four colonies from each sample were selected from the SB, SAs and BA plates. The colonies were mainly chosen based on accessibility and morphology, with the intention of collecting as many different species as possible. These colonies were then streaked onto new BA plates and left to incubate upside-down at 37°C overnight. The isolated species were then identified using MALDI-TOF with the HCCA-matrix (79).

Furthermore, identified species of *S. aureus* were plated onto MRSA2 plates to test if they were MRSA-positive. These were incubated overnight at 37°C upside-down.

6.3 Transfer tests on MRSA-coated plates

Bacteria from each isolate were transferred to a growth tube with 5 ml LB medium. Additionally, an LA-MRSA strain from storage was transferred to 5 ml liquid MH medium. All liquid cultures were

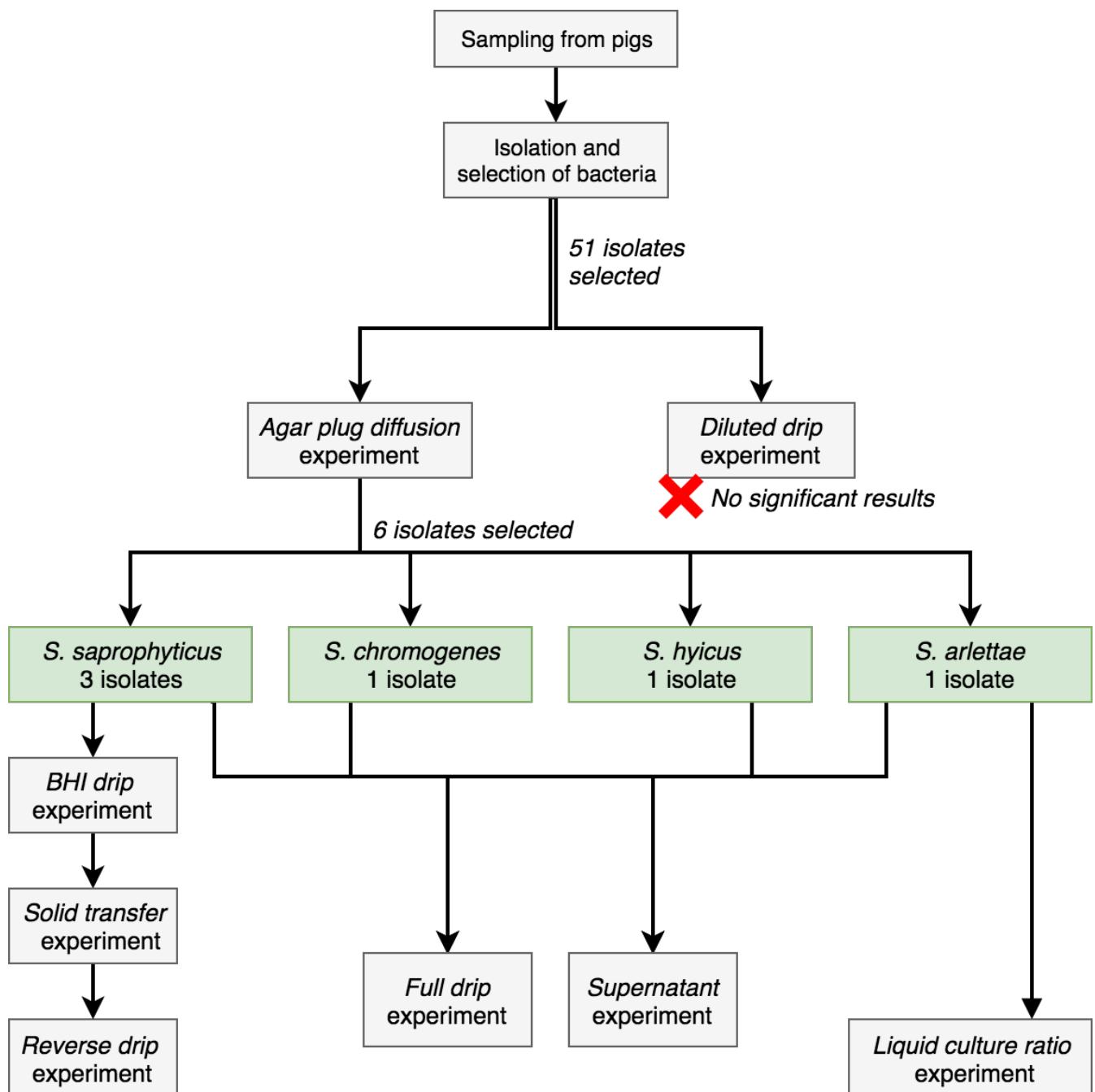


Figure 1: A chart depicting the workflow of the experimental phase.

Table 1: The dilutions that were plated for each sample type along with the media types they were plated on. The faeces samples was initially only plated from 10^{-4} to 10^{-6} . The remaining dilutions were plated later.
BA: Blood Agar, **SB:** Slanetz-Bartley Medium, **SAs:** SSelect Medium, **MRSA2:** MRSA 2 Agar.
*MRSA2 was only plated with 10^0 , 10^{-1} , and 10^{-2} .

Sample type	Dilutions plated	Types of medium
Skin	10^0 , 10^{-1} , 10^{-2} , 10^{-3} , 10^{-4}	BA, SB, SAs, MRSA2*
Nasal	10^0 , 10^{-1} , 10^{-2} , 10^{-3} , 10^{-4}	BA, SB, SAs, MRSA2*
Faeces	10^0 , 10^{-1} , 10^{-2} , 10^{-3} , 10^{-4} , 10^{-5} , 10^{-6}	BA, SB

then left overnight to incubate at 37°C while shaking at 135 rpm.

After incubation, $100 \mu\text{l}$ of MRSA were transferred to BA plates - one for each isolate to be tested - and spread with a Drigalski spatula. $10 \mu\text{l}$ of each isolate were then dripped onto their corresponding blood agar plate twice. The plates were left to incubate at 37°C .

This experiment was conducted several times with variations:

- **Diluted drip:** all of the overnight liquid cultures were diluted to an OD_{600} of 0.6 before the screening.
- **Full drip:** none of the overnight liquid cultures were diluted before the screening.
- **Solid transfer:** Isolate cultures from a clean streak on a BA plate were transferred to the MRSA-coated plate twice using an inoculation loop.
- **BHI drip:** The isolates were cultured in liquid BHI medium instead of LB, and no dilutions of the liquid cultures were made.
- **Reverse drip:** The BA plates were coated with undiluted overnight cultures of the isolates. $10 \mu\text{l}$ of undiluted MRSA was then dripped onto the plates.

6.4 Agar plug diffusion experiment

Isolates were transferred to growth tubes containing 5 ml LB medium. The growth tubes were all incubated at 37°C overnight while shaking at 135 rpm.

$100 \mu\text{l}$ of each overnight isolate culture was plated onto BA plates using a Drigalski spatula. The plates were then left to incubate for two days at 37°C upside-down.

The day before the screening, LA-MRSA was inoculated in five growth tubes containing 5 ml MH

medium each and incubated at 37°C overnight while shaking at 135 rpm.

To initiate the screening, $100 \mu\text{l}$ LA-MRSA culture was plated onto BA plates. Two agar plugs were then extracted from each prepared isolate plate and transferred onto the MRSA-coated plates to lie on top of the MRSA lawn. The plug puncher was sterilised between each plug using ethanol and fire. The plates were incubated right-side up overnight at 37°C .

6.5 Supernatant experiment

Isolates were each inoculated in four growth tubes, all containing 5 ml LB medium. The growth tubes were then left to incubate at 37°C while shaking at 135 rpm. Two tubes per isolate were retrieved after incubating for one night, while the remaining two tubes were incubated through two nights. Additionally, for each night, LA-MRSA was inoculated in a growth tube containing MH and left to incubate under the same circumstances. The following experiment was then performed twice: once for the one-night cultured isolates and again for the two-nights cultured isolates.

For each isolate the two growth tubes were poured together into one new tube and mixed until homogeneous. The isolate tubes were then centrifuged at 4000 rpm for 15 minutes. The pH of each isolate supernatant were measured. Afterwards, the supernatants were filtered through a $0.22 \mu\text{m}$ sterile filter. Each filtered supernatant was then split into two new tubes using a pipette - one tube was left as is, while the other was heat processed in an 80°C water bath for 5 minutes.

Blood agar plates were plated with $100 \mu\text{l}$ of an LA-MRSA overnight culture using a Drigalski spatula - two plates per isolate. Two evenly spaced holes were then punched in the agar. The hole puncher was sterilised with ethanol and fire between each plate. $40 \mu\text{l}$ of the unprocessed supernatant were poured into the holes of a plate, and $10 \mu\text{l}$ of the un-

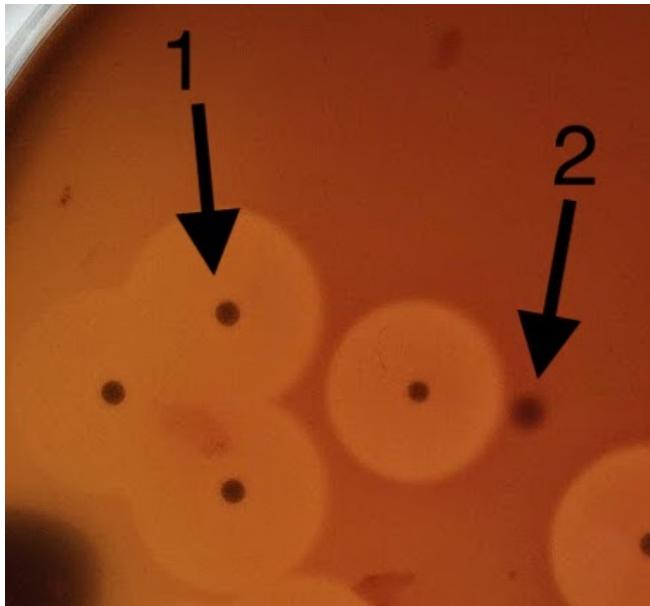


Figure 2: A segment of a BA plate coated with $100 \mu\text{l}$ 10^{-6} dilution from a freshly mixed culture containing 25% *S. arlettae* and 75% LA-MRSA. MRSA colonies (1) have a distinct clearing zone, while *S. arlettae* colonies (2) do not.

processed supernatant were dripped onto the plate twice. The process were then repeated with the heat processed supernatant on another plate. This was done for all isolates. The plates were incubated overnight right-side up at 37°C .

6.6 Liquid culture ratio experiment

A strain of *S. arlettae* and a strain of LA-MRSA were inoculated in two growth tubes each, all containing 5 ml LB medium. The four tubes were then incubated overnight at 37°C shaking at 135 rpm.

Before initialisation, both cultures were diluted to an OD₆₀₀ of 0.2. The two cultures were then mixed in five separate tubes with five different ratios of *S. arlettae* and MRSA. Each mix were consequently referred to by the percentage of *S. arlettae* present in the culture:

0% 25% 50% 75% 100%

A sample was immediately taken from each tube and the tubes were then left to incubate at 37°C while shaking at 135 rpm. New samples were continuously extracted from the tubes for the next 24 hours. At each sample extraction, the cultures were diluted in a serial dilution up to 10^{-6} . Dilutions 10^{-3} and 10^{-6} were then plated onto BA plates using a Drigalski spatula.

All plates were incubated for roughly 12 hours at

37°C . The colonies were then counted for each 10^{-6} dilution plate. The colonies of the two species could be distinguished due to the presence of a characteristic haemolytic clearing zone around each MRSA colony when cultured on BA. In comparison, *S. arlettae* made no clearing zones (see figure 2).

This experiment was conducted three times. The first experiment only tested with 25%, 50% and 75% *S. arlettae* ratios, and samples were extracted at the hours 0, 1, 2, 5, 12, and 24. The second and third experiments tested with all five ratios, and samples were extracted at the hours 0, 1, 2, 5, 8, 10, 12, and 24.

7 Results

The following sections will report the results of the identification of bacteria isolated from 10 sampled pigs, as well as the initial screenings of the isolates for MRSA-antagonistic abilities and the further experiments conducted on selected strains.

7.1 Identified species of bacteria

A total number of 139 strains of bacteria from the skin, nasal and faecal samples were successfully identified using MALDI-TOF. These can be seen in table 2. A graphical representation of the distribution of genera in each sample can be seen in figure 3.

Staphylococcus was by far the most represented genus in both skin and nasal samples, with *Enterococcus* as the second contender. Meanwhile, the faecal samples were mainly dominated by *Enterococcus* and *Escherichia*. *Streptococcus* was mainly isolated from the nasal samples, as this genus had very little presence in the other sample types.

Furthermore, all of the identified *S. aureus* strains were confirmed as MRSA-positive based on the MRSA2 platings.

Out of the 139 identified strains, 51 strains were chosen to be screened for MRSA-antagonistic capabilities. These specific strains, as well as their sample origin, can be seen in table 4.

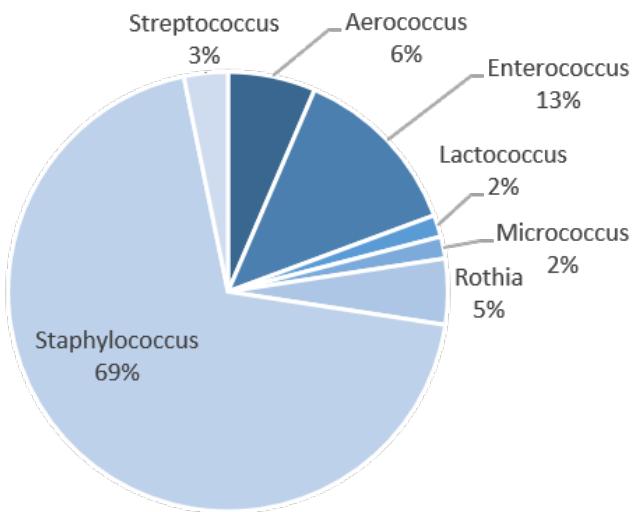
7.2 Prevalence of MRSA in samples

The 10^0 , 10^{-1} and 10^{-2} dilutions were also plated onto MRSA2 plates for a sense of how much MRSA could be found in the samples. The 10^0 samples can be seen on figure 4. Sample S5 was damaged by frost and S9 was unintelligible on the camera, and was thus flipped upside down with the counted number of colonies written.

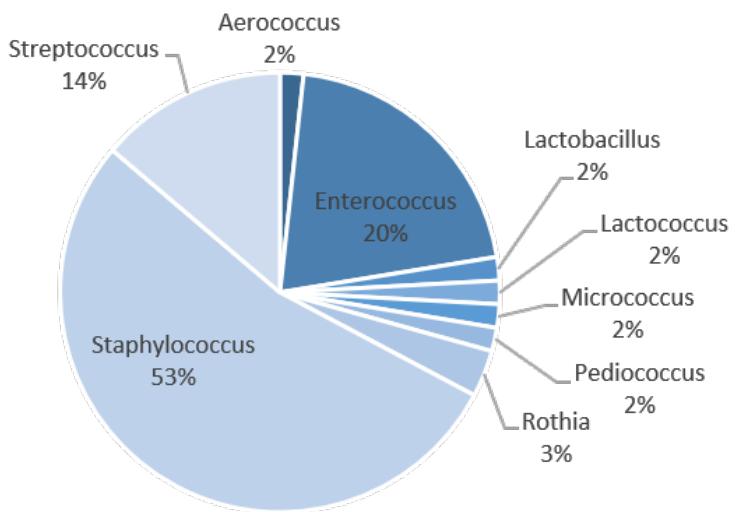
Table 2: The total number of bacteria strains identified from each sample type.

Species	Skin	Nasal	Faeces
<i>Aerococcus viridans</i>	4	1	0
<i>Bacillus subtilis</i>	0	0	1
<i>Enterococcus avium</i>	0	1	0
<i>Enterococcus faecalis</i>	5	2	4
<i>Enterococcus faecium</i>	0	5	4
<i>Enterococcus gilvus</i>	2	0	0
<i>Enterococcus hirae</i>	0	2	1
<i>Enterococcus malodoratus</i>	0	1	0
<i>Enterococcus mundtii</i>	1	1	0
<i>Escherichia coli</i>	0	0	8
<i>Lactobacillus johnsonii</i>	0	1	1
<i>Lactococcus lactis</i>	0	1	0
<i>Lactococcus raffinolactis</i>	1	0	0
<i>Micrococcus luteus</i>	1	1	0
<i>Pediococcus pentosaceus</i>	0	1	0
<i>Rothia nasimurium</i>	3	2	0
<i>Staphylococcus arlettae</i>	1	0	0
<i>Staphylococcus aureus</i>	11	7	0
<i>Staphylococcus chromogenes</i>	1	0	0
<i>Staphylococcus cohnii</i>	1	1	0
<i>Staphylococcus epidermidis</i>	5	4	0
<i>Staphylococcus equorum</i>	1	1	0
<i>Staphylococcus haemolyticus</i>	6	4	0
<i>Staphylococcus hyicus</i>	5	1	0
<i>Staphylococcus nepalensis</i>	1	0	0
<i>Staphylococcus pasteurii</i>	3	0	0
<i>Staphylococcus pettenkoferi</i>	1	0	0
<i>Staphylococcus saprophyticus</i>	1	3	0
<i>Staphylococcus simulans</i>	1	0	0
<i>Staphylococcus xylosus</i>	5	10	0
<i>Streptococcus agalactiae</i>	2	0	0
<i>Streptococcus canis</i>	0	2	0
<i>Streptococcus hyovaginalis</i>	0	2	0
<i>Streptococcus parauberis</i>	0	1	0
<i>Streptococcus suis</i>	0	3	0

Identified genera from skin samples



Identified genera from nasal samples



Identified genera from faecal samples

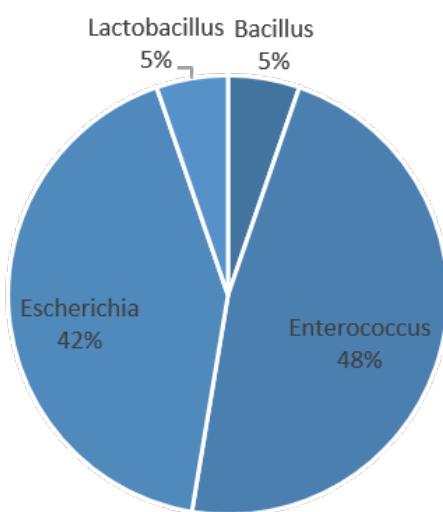


Figure 3: Distribution of genera identified from skin, nasal and faecal samples from pigs.

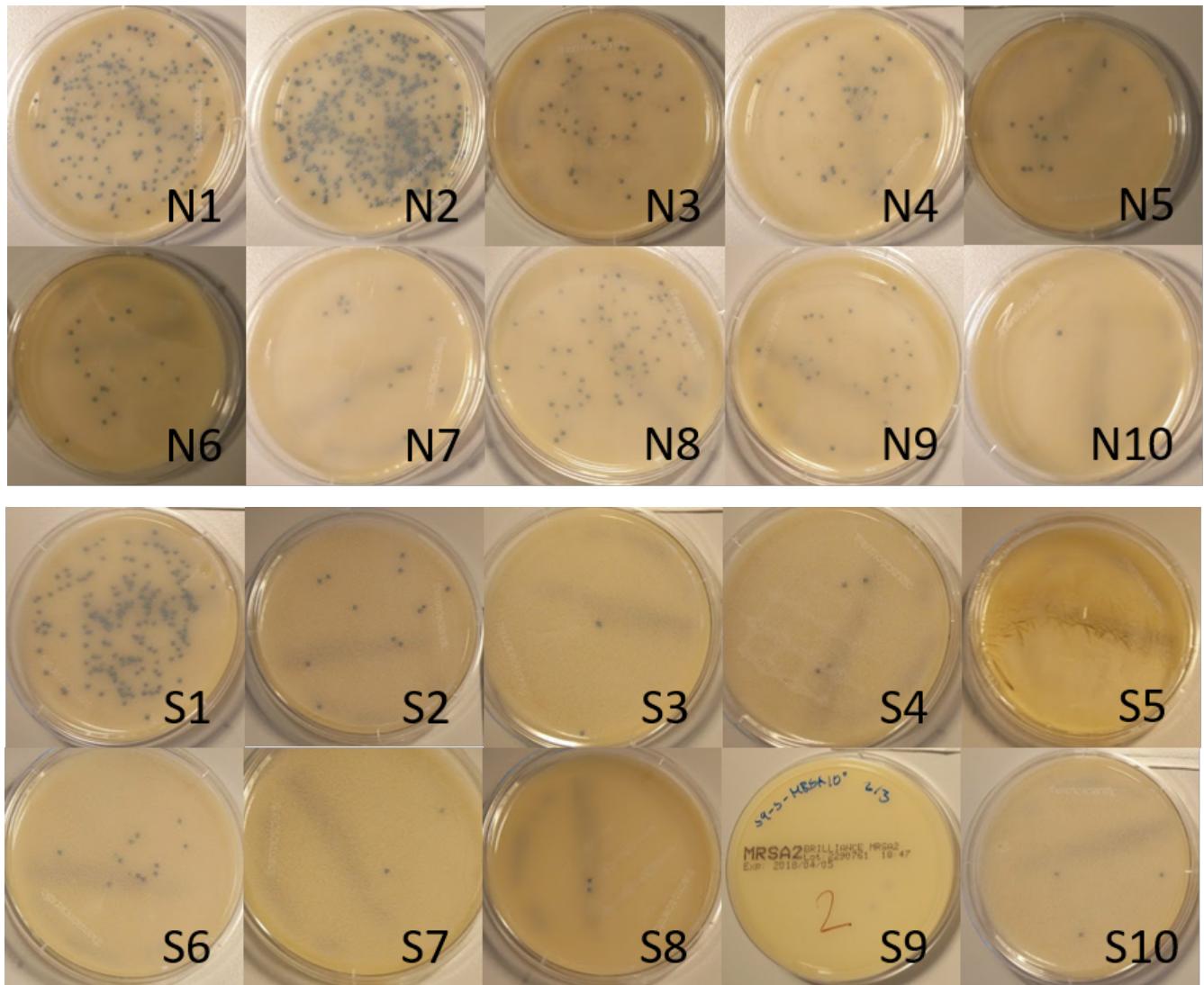


Figure 4: *MRSA found in nasal and skin samples from a 10^0 dilution. N1-10 and S1-10 indicates the pig number and sample type (N: Nasal, S: Skin). Frost damage on S5 is visible, and S9 was not intelligible on the camera and was flipped upside down for that reason.*

Table 3: Results of different screening tests on the four species of interest.

Yes: signs of antagonism, **No:** no signs of antagonism, **Haemolysis:** inhibition of MRSA haemolysis, **"-":** not tested.

Species / Test	<i>S. saprophyticus</i>	<i>S. chromogenes</i>	<i>S. hyicus</i>	<i>S. arlettae</i>
Diluted drip	No	Haemolysis	Haemolysis	No
Plug diffusion test	Yes	Haemolysis	Haemolysis	Yes
Full drip	No	Haemolysis	Haemolysis	Yes
Solid transfer	Yes	-	-	-
BHI drip	No	-	-	-
Reverse drip	No	-	-	-
Supernatant (day 1)	No	No	No	No
Supernatant (day 2)	No	No	No	No

The correlation between the samples and the pens they belong to are in general not very prevalent. N1 and N2 are from the same pen, as are N3 and N4, and N5 and N6 belong together as well. On these samples the correlation is very visible while the rest are more unclear.

This is most obvious when comparing S1 and S2, which are from the same pen as N1 and N2. First, there is close to no similarity between N1 and N2, and second, there is little similarity between N2 and S2. N2 and S2 are nasal and skin samples from the same pig, making this lack of correlation notable.

In general the prevalence of MRSA positive *S. aureus* strains is moderate to low with the exception of N1, N2, N8 and S1.

7.3 MRSA-antagonism screening results

This section will detail the results of the MRSA-antagonism screenings. During the initial screenings six isolates with MRSA-antagonistic tendencies were identified and tested further. These isolates of interest consisted of four different species:

- *Staphylococcus saprophyticus*
3 isolates, nasal samples (pig no. 6, 7, and 8).
- *Staphylococcus chromogenes*
1 isolate, skin sample (pig no. 8).
- *Staphylococcus hyicus*
1 isolate, skin sample (pig no. 7).
- *Staphylococcus arlettae*
1 isolate, skin sample (pig no. 10).

A brief overview on how these specific isolates performed in each screening can be seen in table 3.

7.3.1 Drip test results

Following is a description of the results from each variation of the drip test experiment.

The *diluted drip* experiment was conducted on all 51 isolates seen in table 4.

The *full drip* experiment was performed on all six isolates of interest.

The *solid transfer*, *BHI drip* experiment, and *reverse drip* experiment were performed on the three *S. saprophyticus* isolates only.

Diluted drip:

When all cultures were diluted down to an OD₆₀₀ of 0.6 before screening, none of the 51 isolates showed any notable signs of MRSA growth inhibition. Many isolates were capable of growing on top of the MRSA-lawn, however no clearing zones were visible. A few species - most notably the isolates of interest *S. chromogenes* and *S. hyicus* - inhibited the formation of MRSA haemolytic clearing zones.

Full drip:

Since the *diluted drip* experiment showed very few results, it was investigated whether or not an undiluted overnight culture of each of the six isolates of interest would create a response.

S. chromogenes and *S. hyicus* strongly inhibited MRSA clearing zones, but neither prevented MRSA from growing.

All three isolates of *S. saprophyticus* seemed to struggle with growing and had no visible influence on the surrounding MRSA growth.

However, the *S. arlettae* drop was surrounded by a faint clearing zone, indicating that growth of MRSA was successfully inhibited by the isolate.

Table 4: 51 isolates first screened for MRSA-antagonism

Pig	Species	Type
1	<i>Enterococcus faecium</i>	Nasal
1	<i>Lactobacillus johnsonii</i>	Nasal
2	<i>Staphylococcus equorum</i>	Skin
2	<i>Staphylococcus cohnii</i>	Nasal
2	<i>Enterococcus faecium</i>	Nasal
2	<i>Streptococcus hyovaginalis</i>	Nasal
2	<i>Streptococcus hyovaginalis</i>	Nasal
3	<i>Staphylococcus saprophyticus</i>	Skin
3	<i>Staphylococcus pettenkoferi</i>	Skin
3	<i>Staphylococcus pasteuri</i>	Skin
3	<i>Rothia nasimurium</i>	Skin
3	<i>Lactococcus lactis</i>	Nasal
4	<i>Enterococcus hirae</i>	Nasal
5	<i>Lactococcus raffinolactis</i>	Skin
5	<i>Pediococcus pentosaceus</i>	Nasal
5	<i>Streptococcus suis</i>	Nasal
5	<i>Staphylococcus xylosus</i>	Nasal
6	<i>Enterococcus faecalis</i>	Skin
6	<i>Staphylococcus epidermidis</i>	Skin
6	<i>Staphylococcus saprophyticus</i>	Nasal
6	<i>Rothia nasimurium</i>	Nasal
7	<i>Staphylococcus cohnii</i>	Skin
7	<i>Staphylococcus pasteuri</i>	Skin
7	<i>Staphylococcus hyicus</i>	Skin
7	<i>Enterococcus gilvus</i>	Skin
7	<i>Enterococcus gilvus</i>	Skin
7	<i>Enterococcus mundtii</i>	Skin
7	<i>Staphylococcus saprophyticus</i>	Nasal
7	<i>Staphylococcus xylosus</i>	Nasal
7	<i>Enterococcus malodoratus</i>	Nasal
7	<i>Staphylococcus xylosus</i>	Nasal
8	<i>Staphylococcus simulans</i>	Skin
8	<i>Staphylococcus chromogenes</i>	Skin
8	<i>Enterococcus faecalis</i>	Skin
8	<i>Staphylococcus haemolyticus</i>	Skin
8	<i>Staphylococcus haemolyticus</i>	Nasal
8	<i>Staphylococcus saprophyticus</i>	Nasal
8	<i>Streptococcus parauberis</i>	Nasal
9	<i>Staphylococcus xylosus</i>	Skin
9	<i>Staphylococcus haemolyticus</i>	Skin
9	<i>Aerococcus viridians</i>	Skin
9	<i>Enterococcus hirae</i>	Nasal
9	<i>Staphylococcus epidermidis</i>	Nasal
10	<i>Staphylococcus arlettae</i>	Skin
10	<i>Staphylococcus nepalensis</i>	Skin
10	<i>Aerococcus viridians</i>	Skin
10	<i>Staphylococcus epidermidis</i>	Skin
10	<i>Staphylococcus pasteuri</i>	Skin
10	<i>Staphylococcus equorum</i>	Nasal
10	<i>Enterococcus mundtii</i>	Nasal
10	<i>Enterococcus avium</i>	Nasal

Solid transfer:

Due to the lack of activity from the *S. saprophyticus* isolates further experiments were conducted on these isolates. It was tested, if *S. saprophyticus* would produce clearing zones if transferred from a blood agar plate to an MRSA-coated plate. These transferred *S. saprophyticus* colonies managed to produce small clearing zones surrounding the areas where they grew.

BHI drip:

The isolates were also tested in an alternative drip-experiment. It was tested if culturing *S. saprophyticus* in BHI liquid medium would produce alternative results than to the ones in LB medium. However, the medium change did not make any difference. The *S. saprophyticus* isolates did still not grow well and they had no impact on MRSA growth.

Reverse drip:

This experiment was a simple test to see if MRSA had any notable impact on the growth of *S. saprophyticus*. Although MRSA was capable of growing on top of the *S. saprophyticus*-lawn, there were no notable clearing zones or similar signs towards MRSA antagonising the *S. saprophyticus* isolates.

7.3.2 Agar plug diffusion results

This experiment was performed on all 51 isolates seen in table 4. Most species showed no signs of antagonism towards MRSA. The agar plugs would be colonised with the isolate, but the surrounding area would be colonised with MRSA.

However, two isolates - *S. chromogenes* and *S. hyicus* - seemed to strongly inhibit MRSA haemolysis. This ability was also observed previously in section 7.3.1.

Three isolates of *S. saprophyticus* had a small, but noticeable, clearing zone around their plugs with a radius of roughly 0.4 cm (from the center of the plug).

Finally, a strain identified as *S. arlettae* showed a significant clearing zone with a radius of around 0.8 cm for each plug. Although MRSA still seemed to be growing within the zone, the MRSA density was notably smaller than in the surrounding areas. Photos of the results for the six isolates of interest can be seen in figure 5.

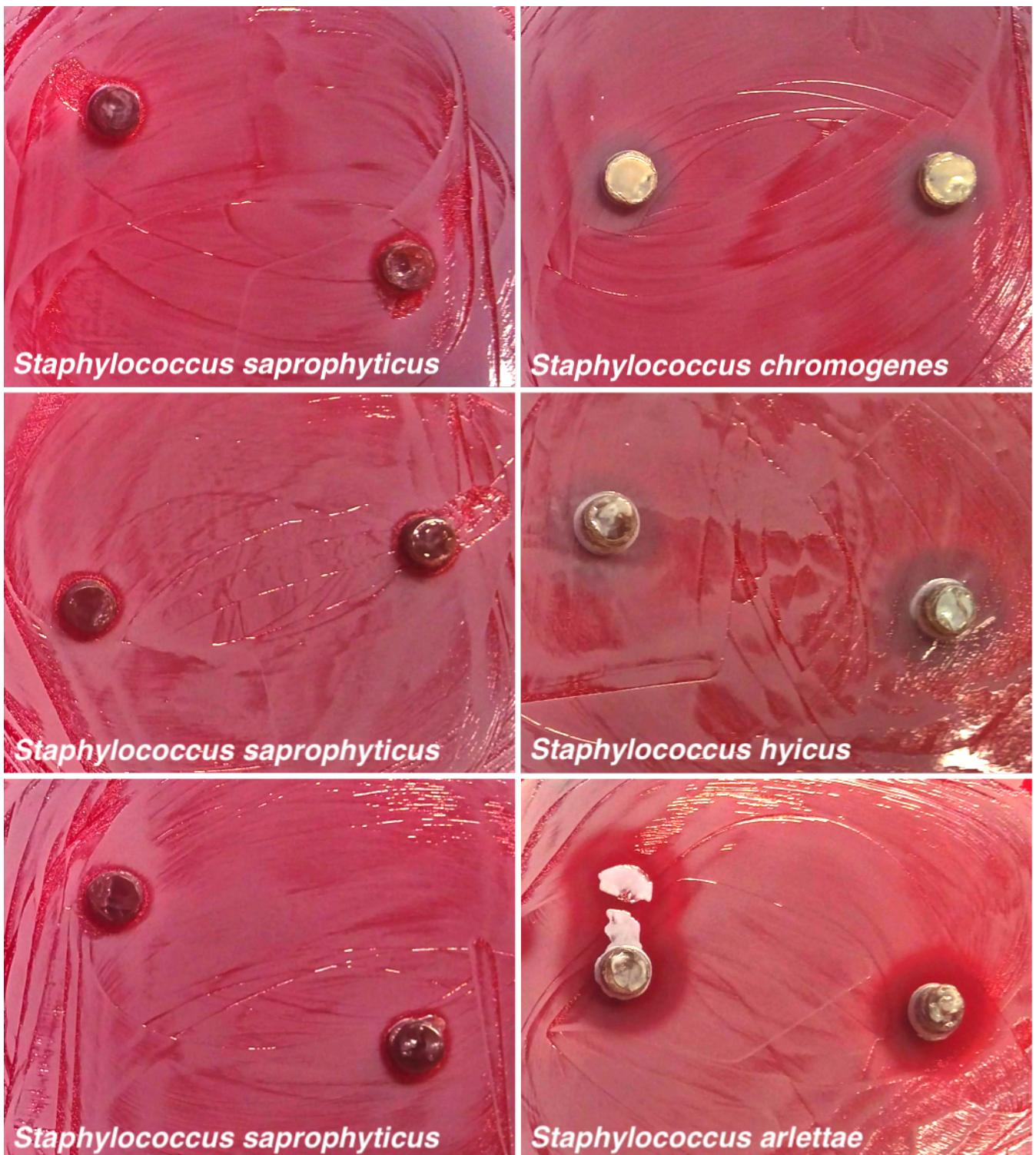


Figure 5: Agar plug diffusion results. Note the darker areas surrounding the *S. chromogenes* and *S. hyicus* plugs where MRSA clearing zones were inhibited. The left *S. arlettae* plug was accidentally tipped while being placed, resulting in the "spilled" bacteria growth seen above.

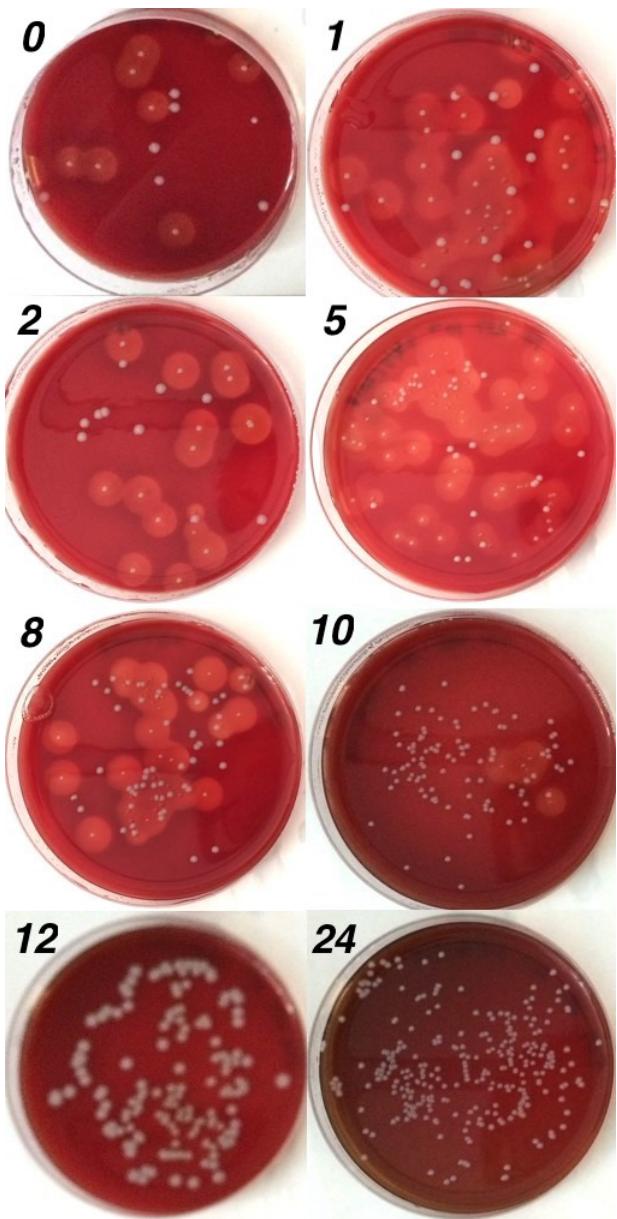


Figure 6: The 10^{-6} dilution plates for a growth tube containing 50%/50% *S. arlettae* and MRSA at the hours 0, 1, 2, 5, 8, 10, 12, and 24.

7.3.3 Supernatant experiment

This experiment was performed on all six isolates of interest. None of the MRSA-coated plates showed any visible signs of inhibition for any isolate - neither from the supernatant-filled holes nor the supernatant-drops placed onto the MRSA-lawn. It made no difference whether the supernatant was heat processed or not, or if the cultures had been incubated for one or two days.

7.4 Liquid culture ratio results

This experiment was performed three times using the *S. arlettae* isolate of interest against MRSA.

Figure 6 shows the 10^{-6} dilution plates for one of the 50% tubes. It can be seen how the amount of MRSA colonies producing haemolytic clearing zones decreases with time. In the case of this particular growth tube, the signs of LA-MRSA presence were gone at the 12 hour mark. The tendency for all other mixed growth tubes was ultimately the same. All ratios in which MRSA and *S. arlettae* were mixed would eventually end with *S. arlettae* outcompeting the other.

In the tube containing 25% *S. arlettae*, it would generally take at least ten hours for *S. arlettae* to dominate.

In the 50% tube, *S. arlettae* and MRSA would usually struggle for around five hours before *S. arlettae* finally took control.

In the 75% tube, MRSA numbers would rapidly drop and never recover.

In the two experiments conducted with a 0% tube containing only LA-MRSA, it was shown that LA-MRSA would generally grow well when left alone. The same could be said for the 100% tube containing only *S. arlettae*.

A graphical representation of the progress within the growth tubes for all the experiments can be seen in figure 7 and 8.

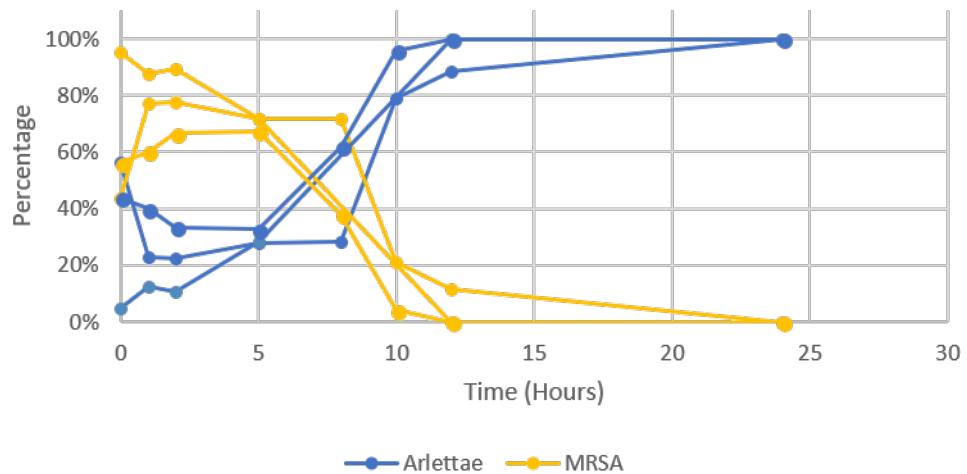
In the case of both of these experiments, the 100% tube was contaminated with MRSA at some point during the later extractions. This is only visible in the final data of one experiment, due to none of the MRSA contamination being present on the 10^{-6} dilution plate in the other experiment. This contamination was thus only discovered due to the presence of clearing zones on the 10^{-3} dilution plate for the 24 hour sample (see figure 9).

8 Discussion

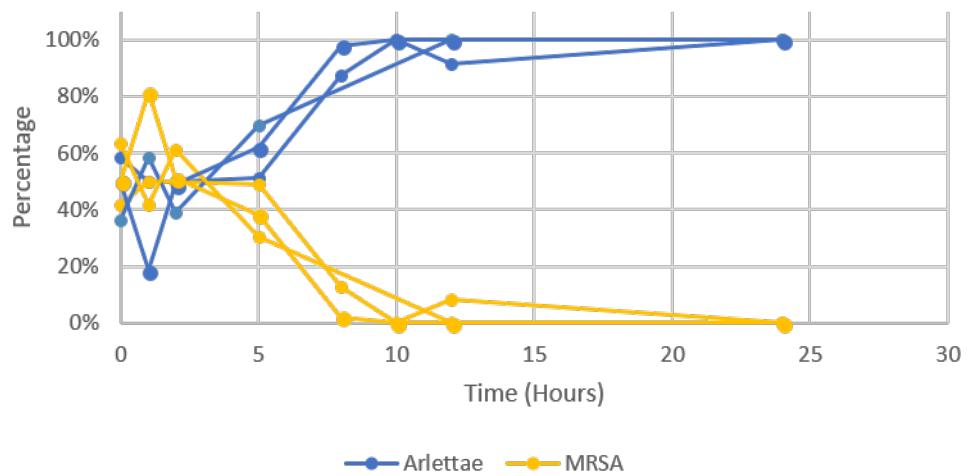
The present study successfully isolated and identified 51 strains of bacteria from nasal and skin samples collected from weaned pigs. These strains represented a variety of different genera, of which the majority belonged to *Staphylococcus*, *Enterococcus* or, in the case of the faecal samples, *Escherichia*.

Interestingly, not many streptococci were isolated despite the fact that the selection process deliberately tried to cultivate species of *Streptococcus*. This could be due to a variety of reasons, such as issues with the SB medium selection, randomness in the selection of colonies to identify, or perhaps simply that the samples did not contain many streptococci. Furthermore the bacteria chosen for antagonistic

25% arlettae



50% arlettae



75% arlettae

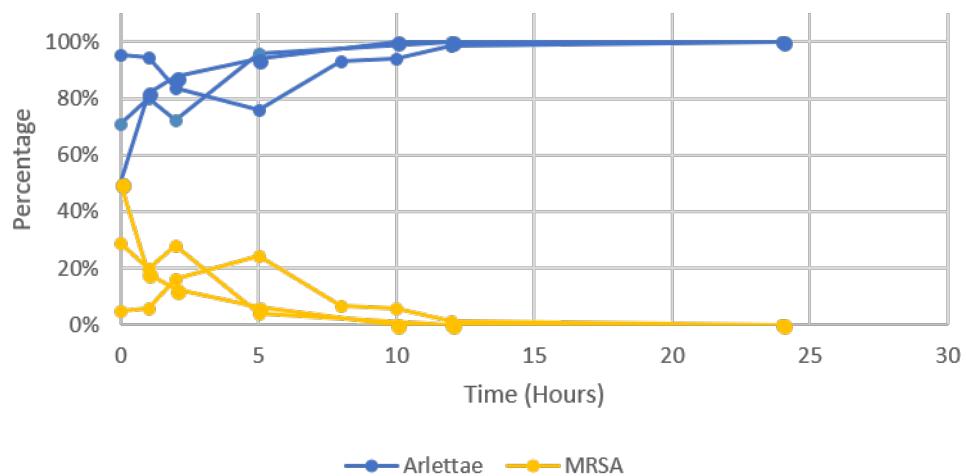


Figure 7: Percentage of LA-MRSA and *S. arlettae* colonies present on the 10^{-6} dilution BA plates at each extraction. The graphs show the accumulated data of three separate experiments, all performed with growth tubes that initially contained 25%, 50%, and 75% *S. arlettae*

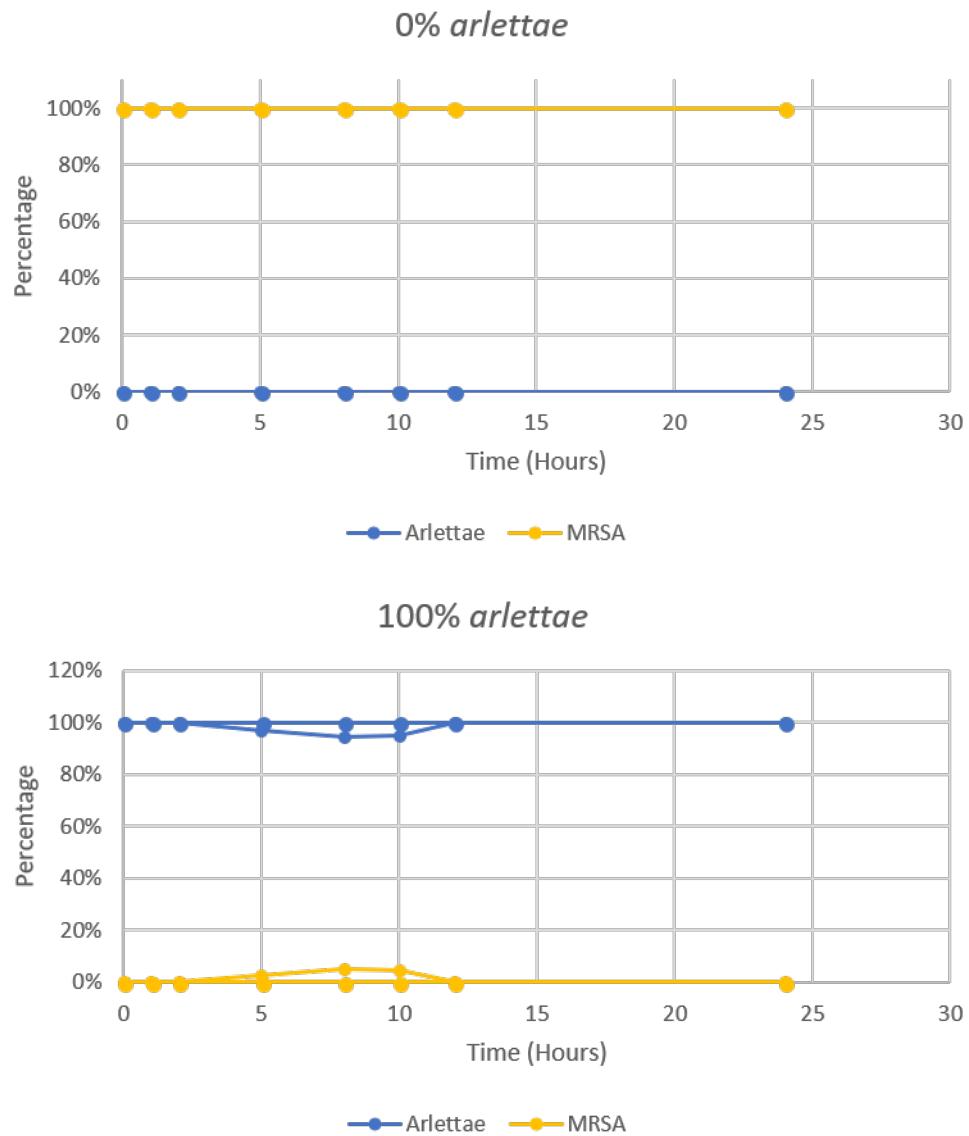


Figure 8: Percentage of LA-MRSA and *S. arlettae* colonies present on the 10^{-6} dilution BA plates at each extraction of the 0% and 100% growth tubes during two separate experiments. Note the fluctuation one of the two curves in the 100% diagram. This is attributed a technical error in the form of contamination by MRSA.



Figure 9: Signs of contamination on the 24 hour 10^{-6} dilution plate for a 100% *S. arlettae* growth tube. The clear spots indicate the presence of LA-MRSA colonies.

screening were the ones successfully identified using MALDI-TOF. Strains not identified were not used and therefore some strains may have been lost. Another strange occurrence was the general lack of bacteria in the faecal samples. It was expected that there would be an abundance of bacteria in the faeces, which is why the faecal samples initially were only plated from the dilutions 10^{-4} to 10^{-6} . When these plates only produced few colonies, the samples were retrieved in order to plate the 10^0 to 10^{-3} dilutions. However, even these dilutions yielded few species. This is presumably due to errors during the pre-processing of the faecal samples. The faecal bacteria may not have been properly released into the sample buffer before the centrifugation process, resulting in the majority of the bacteria being removed with the pellet. Since the few species that were uniquely isolated from the faecal samples held little interest, it was decided that only skin and nasal isolates would proceed to the screening phase. Consequently, 51 identified skin and nasal isolates were chosen. From all of these isolates, six showed notable results when screened for antagonistic potential towards a strain of LA-MRSA CC398. These isolates consisted of four species of staphylococci: *S. chromogenes*, *S. hyicus*, *S. saprophyticus*, and *S. arlettae*.

Staphylococcus being the only genus to exhibit antagonistic properties among the isolates seems curious, as many isolated species belonged to genera

that have been found to be antagonistic towards MRSA (see section 5.4). Numerous species of the mentioned genera were actually represented in the isolated bacteria but showed no antagonistic properties. In relation to bacteriocins it does make sense that the six isolates were all *Staphylococcus*. Some bacteriocins are known to be extremely specific and only affect bacteria within the same genus as the producer. So it seems that even though other species are known to produce bacteriocins across genera, this study found no such species.

8.1 The correlation between MRSA colonisation and antagonism

This study originally hypothesised that bacteria co-living with MRSA would show a greater competitive potential. It would therefore be interesting to investigate if there is any correlation between the properties of the six MRSA-antagonistic isolates, and the amount of MRSA located on the spots where they were originally isolated.

The three *S. saprophyticus* isolates were collected from the nasal samples of pig no. 6, 7, and 8. As illustrated in figure 4, the amount of MRSA present in these samples is fairly moderate, especially compared to the nasal samples of pig no. 1 and 2.

The *S. hyicus*, *S. chromogenes*, and *S. arlettae* isolates originated from the skin of pig no. 7, 8, and 10 respectively. All of these samples contained significantly low amounts of MRSA.

As such, the results of this study does not provide any evidence that high levels of MRSA make the neighbouring bacteria more competitively viable. On the contrary, none of the most MRSA-abundant samples, such as those from pig no. 1, provided any MRSA-antagonistic isolates. Perhaps the antagonistic bacteria in this sample were missed during the selection of the isolates to be screened. However, these results could also indicate that MRSA-antagonistic bacteria are more likely to be found in areas with a lower than expected amount of MRSA contamination. This is plausible, as these competitive bacteria would presumably prevent growth of MRSA in the environment. Thus it could be hypothesised that MRSA-antagonistic bacteria are more likely to be found in areas with lower MRSA-levels than expected.

8.2 Evaluation of antagonistic potential

S. chromogenes and *S. hyicus* displayed similar properties. Neither were actually capable of killing or

inhibiting LA-MRSA. However, they were both efficient at inhibiting the formation of the haemolytic clearing zones that usually accompanies MRSA on BA plates. This was particularly apparent in the plug diffusion experiment and full drip test. Therefore, it was hypothesised that these two species perhaps affected the MRSA metabolism. On the other hand, it is also possible that they simply produced something that affected the haemolysins that MRSA produce. Based on the results of this study, the latter seems like the most likely option. No screenings ever managed to demonstrate any correlation between the lack of haemolysis and a failure of MRSA to thrive. Since both *S. chromogenes* and *S. hyicus* were quite capable of colonising in very close proximity to LA-MRSA, it seems that they are capable of co-living with MRSA without the need for antagonising strategies.

S. saprophyticus was the most difficult species to work with. Unlike other isolates, *S. saprophyticus* seemed to thrive less in liquid culture, and tended to form dense masses of biofilm. This made it difficult to both culture and dilute these isolates in liquid medium. Nevertheless, three out of four isolated *S. saprophyticus* managed to produce notable clearing zones in the agar plug diffusion experiment. Based on this, it was decided that further tests on these three specific isolates were needed. However, it proved to be difficult to recreate the results of the plug experiment. It was only in the solid transfer experiment that *S. saprophyticus* managed to produce clearing zones. Whenever liquid cultures of *S. saprophyticus* were applied to MRSA-coated BA plates, the cultures would seemingly fail to grow. Changing the culturing media from LB to BHI did not make any notable difference. Furthermore, the reverse drip experiment demonstrated that MRSA was not actively antagonising *S. saprophyticus*. It thus appears that MRSA is simply more efficient at growing. Based on all of these observations, it seems plausible that the mechanism of antagonism observed from *S. saprophyticus* in the agar plug diffusion experiment is linked to stable biofilm. Since *S. saprophyticus* generally did not thrive in liquid medium, the cell density may not have been sufficiently high. Additionally, before transferring liquid *S. saprophyticus* culture to MRSA-coated plates, the biofilm matrix of the culture was usually destroyed. This could possibly have given *S. saprophyticus* a disadvantage from which it could not recover. As such, only the *S. saprophyticus* cultures collected from agar plates had the adequate levels of cell density and biofilm to be able to produce antagonistic

molecules. In the end, *S. saprophyticus'* issues with liquid media was its greatest hindrance.

S. arlettae was the most efficient MRSA-antagonist of all species screened in this study. This isolate consistently produced very notable clearing zones, no matter if it was cultured on BA plates or in liquid medium. These clearing zones were all much wider than the ones observed from *S. saprophyticus*. However, the antagonistic mechanism of *S. arlettae* appeared to be less potent. In the plug agar diffusion experiment, MRSA can still be seen growing within the clearing zone, although it is much less prominent than the surrounding MRSA growth (see figure 5). This may suggest that the antagonistic molecule produced by *S. arlettae* is inhibitory. It is also possible that *S. arlettae* is producing a bactericidal compound, but in too low concentrations to kill all MRSA. Interestingly, *S. arlettae* did not display any notable antagonistic features in the diluted drip experiment. This could perhaps suggest that a higher cell density is required for the production of the antagonistic metabolite, hinting at a mechanism regulated by quorum sensing.

8.3 The supernatant experiment

Of the experiments performed on all six isolates of interest, this was the only one to give completely negative results. Unfortunately, this is probably due to faults in the experimental design.

While running the experiment, it was assumed that the isolates would produce competitive molecules when cultured separately in liquid medium. However, the way the culture supernatants were applied to the MRSA-coated BA plates was probably too inefficient. A single drop or a hole filled with supernatant did most likely not contain nearly enough compound to have any visible impact on the growth of a full dose of LA-MRSA overnight culture. The two-day cultures were supposed to counter this issue, in case the one-day cultures had not produced enough compound, but there was no guarantee that an adequate concentration would have been reached within two days either. It is also possible that no antagonistic molecules were present in the isolate cultures at all. It is not unreasonable to argue that the isolates would only produce such compounds when exposed to a stress factor, such as a competitive microorganism.

In the end, this experiment failed at taking too many factors into account, and the results are therefore found lacking. A better approach could perhaps be to identify the compounds present in the

supernatant by utilising techniques such as high-performance liquid chromatography.

8.4 *S. arlettae*: Liquid culture antagonism

Based on previous results, it became apparent that the *S. arlettae* strain was the one which showed the greatest antagonistic potential towards LA-MRSA. Three liquid culture ratio experiments were therefore conducted in order to estimate the durability of *S. arlettae* and its antagonistic properties. All three experiments demonstrated that *S. arlettae* is highly efficient at outcompeting LA-MRSA in liquid culture, even when starting out substantially outnumbered. This, combined with the previously observed clearing zones, strongly suggests that *S. arlettae* indeed produces some sort of potent compound which targets MRSA.

It should be noted that the graphs seen in figure 7 and 8 are not an accurate representation of the development within the growth tubes. Due to the sampling essentially relying on randomness, there is a risk that the resulting agar plates may not reflect the true distribution of LA-MRSA and *S. arlettae*. These data can thus only be used as a broad estimate of how the general concentrations develop. Nevertheless, the 10^{-6} dilution plates of the 24 hour samples always ended up only producing colonies of *S. arlettae*. As such, it is reasonable to conclude that *S. arlettae* is the more dominant species.

The MRSA-contaminations that occurred within the 100% growth tubes were unexpected, but they also managed to provide some interesting insight regarding the potential mechanism of antagonism in *S. arlettae*. On 10^{-3} dilution plates heavily dominated by *S. arlettae* it was not unusual to see small haemolytic clearing zones produced by single colonies of MRSA (see figure 9). This was interesting, as the agar plug experiment and full drip experiment suggested that MRSA colonies growing near *S. arlettae* would be strongly inhibited. As such, it would be fair to assume that small MRSA colonies would not be able to colonise among such dense *S. arlettae* growth. However, it seemed that these isolated MRSA colonies were managing quite well. Additionally, when observing the MRSA contamination that occurred within one of the 100% growth tubes, it is notable that the presence of MRSA briefly appeared to be increasing after its initial detection at the 5-hour mark (see figure 8). This brief rise suggests that MRSA might have been thriving in the growth tube for a while, indicating

that *S. arlettae* was initially not producing any competitive molecules. However, five hours later the contamination levels were observed as falling, and after seven hours no trace of the contamination remained. The rise and fall of the MRSA level could potentially indicate that a certain level of MRSA was required in order to trigger a sufficiently competitive response from *S. arlettae*. Thus, the single MRSA colonies observed on the 10^{-3} plates were perhaps not generating enough of a response for *S. arlettae* to react antagonistically. However, as previously mentioned, the agar plating method does not produce a reliable representation of the contents within the growth tube. The observed rise and fall of MRSA growth is, at best, an estimation. As such, more research into the competitive mechanism of *S. arlettae* is required before any definite conclusion can be drawn.

8.5 Possible antagonistic mechanisms

Although little is known about the exact mechanism(s) *S. arlettae* utilises against MRSA, some suggestions can be made.

As previously discussed, a trigger may be necessary to start production of the assumed antimicrobial compound. Unpacking this hypothesis, the identification of the trigger would be the ideal place to start. The trigger of the production seems to stem from *S. arlettae* discovering that high levels of MRSA are present. As mentioned in section 5.3.3, *S. aureus* does have a mechanism for inter-species communication as it encodes the gene *luxS* enabling it to produce AI2. Should *S. arlettae* be able to recognise AI2 it would be able to assess the cell density of *S. aureus* and other bacteria. Thus, when the concentration of *S. aureus* reaches a critical point *S. arlettae* could react and start producing the antimicrobial compound. This may sound plausible when put up like that but it would require *S. arlettae* to be quorum sensing and able to recognise AI2. As of now, little is known regarding the quorum sensing properties of *S. arlettae* and in the laboratory it was not obvious whether or not quorum sensing was a property of *S. arlettae* as it did not seem to produce a biofilm matrix.

Comparing the results of the agar plug diffusion experiment and the liquid culture ratio experiment it would be relevant to consider the possible differences in the antagonistic mechanism. It is definitely not clear whether or not the mechanisms used in each experiments are the same. In that connection, studying the mechanism of *S. saprophyticus* would

also be relevant. As mentioned in section 5.3.2 the competition between bacteria does not have to be chemical. It could also be physical. This might be an explanation of the smaller clearing zone around the agar plug with the noticeably abrupt and defined stop.

8.6 Applications of antagonistic bacteria

When competitively viable bacteria have been successfully identified and characterised, this inevitably leads to the question of how they can be properly utilised against MRSA. The exact possibilities can vary a lot depending on the species and the exact antagonistic mechanism.

Since the *S. arlettae* investigated in this study is a natural inhabitant on pig skin, it could potentially be used as a preventative coloniser of pigs. Ideally, *S. arlettae* colonised pigs would be less MRSA contaminated due to *S. arlettae*'s competitive behaviour. This would off course only be applicable, if the bacteria respond in a natural setting as it would in a laboratory setting. Even then, it would also require that *S. arlettae* does not display any pathogenic traits, which could potentially harm the pigs. Furthermore, there is also a risk that the *S. arlettae* strains may develop antibiotic resistances, for example through the acquisition of the SCC_{mec} cassette. As such, thorough research should be performed before attempting counter-colonisation as a preventative measure.

Another approach could be to identify the genes encoding potential MRSA-antagonistic compounds. This would be particularly relevant in cases, where the antagonistic bacteria is pathogenic or simply difficult to work with, such as the case for *S. saprophyticus*. The genetic information can provide an idea of which kind of antagonistic molecule is at play. Furthermore, it opens up the option for transferring the genes to more convenient host organism using genetic engineering. This would make it easier to perform antagonistic screenings, and could be potentially useful for optimising production of the compound.

Finally, if a competitive bacteria is known to produce an antagonistic molecule, it could prove useful to isolate and characterise this compound. Compounds that are particularly efficient at inhibiting or killing MRSA could potentially be utilised as medicines for treating MRSA infections. This would however require that it is relatively easy and cost efficient to produce large amounts of the compound. Alternatively, it could be relevant to study the exact

mechanism with which the antagonistic compound targets MRSA. The antagonistic effect could then possibly be replicated by designing synthetic compounds that mimic the structure and mechanism of the original compound.

9 Conclusion

The present study successfully isolated six strains of bacteria which displayed antagonistic potential towards LA-MRSA. These were all isolated from MRSA-positive pigs, confirming the hypothesis that bacteria co-living with MRSA may exhibit competitive behaviour. All competitive strains isolated in this study belonged to the genera *Staphylococcus*, and consisted of four species: *S. saprophyticus*, *S. chromogenes*, *S. hyicus* and *S. arlettae*.

All six isolates were collected from samples which contained moderate to low prevalence of LA-MRSA. This may be due to the fact that MRSA-antagonistic species were present in the samples. However, other samples containing low prevalence of MRSA did not produce any antagonistic species. Based on these results, it is therefore difficult to assess whether or not there is any correlation between LA-MRSA prevalence levels and the occurrence of antagonistic bacteria.

The six MRSA-antagonistic strains displayed a variety of different antagonistic traits during the antagonistic screenings. The most likely antagonistic property for *S. arlettae* would be an antimicrobial compound, while *S. saprophyticus*, *S. chromogenes* and *S. hyicus* are all more uncertain.

The *S. arlettae* isolate displayed antagonistic behaviour towards LA-MRSA while cultured in both liquid LB medium and on BA. The three *S. saprophyticus* isolates were only capable of antagonising LA-MRSA when cultured on BA. Finally, the *S. chromogenes* and *S. hyicus* strains were potent inhibitors of MRSA haemolysis on BA, but did not seem to inhibit growth of MRSA itself.

10 Future perspectives

Although this study managed to determine a number of potential MRSA-antagonistic candidates, much remains to be investigated regarding these bacterial isolates.

The MALDI-TOF method was chosen for the identification of each isolate due to its remarkable speed and efficiency. However, some reads - particularly that of the *S. arlettae* strain - came out with a

notable degree of uncertainty. Additional identifications of these isolates should therefore be performed before proceeding with any further studies.

In the case of the *S. saprophyticus* isolates, these did show some antagonistic potential. Unfortunately, the methods used in this study was not suitable for these strains. Additional studies on these isolates should therefore take their reliance on biofilm formation into consideration. Furthermore, optimising the growth conditions of these isolates may provide more satisfactory results. Perhaps there are other media types more suited for *S. saprophyticus*. However, if they continue to be troublesome, it may be better to sequence them and investigate them genetically instead.

The *S. arlettae* isolate was the most promising candidate of all the isolates. Based on this study, it seems probable that this *S. arlettae* strain is producing a competitive molecule. As such, further studies should focus on isolating and characterising this compound. A good starting point would be to perform a whole-genome sequencing in order to assess the various properties of *S. arlettae*. It could also be interesting to perform additional experiments in order to determine the presence of a potential trigger-mechanism for the production of the molecule. These should study the possibility of inter-species communication being part of the trigger. Furthermore, the antagonistic properties of *S. arlettae* should be tested under a variety of different circumstances in order to understand the versatility of the bacteria. One of such experiments could test *S. arlettae* against LA-MRSA that has formed a biofilm matrix. This would be interesting, as some antimicrobial compounds are effective on planktonic MRSA, but not on its biofilm.

Future studies searching for MRSA-antagonistic bacteria might benefit from specifying the hypothesis used in this study. The hypothesis that MRSA-antagonistic bacteria are to be found in places with MRSA was confirmed, but it might be interesting to study if there is an optimal correlation between MRSA levels and the prevalence of antagonistic bacteria. It may be that areas with lower prevalence of MRSA than statistically expected is the most optimal place to isolate MRSA antagonists, as low MRSA levels could indicate that antagonistic bacteria was keeping MRSA in check. Another possibility is that places with a high prevalence of MRSA also has a high prevalence of antagonistic bacteria, because they would need to defend themselves against the plentiful MRSA. The results of this study points towards competitively viable bacteria being more

commonly found in areas where lower amounts of MRSA is present, but more data is required to confirm any correlation.

11 Manufacturers

Following is a list of manufacturers, as well as the materials they supplied for this study:

SSI Diagnostica, Herredsvejen 2, 3400 Hillerød, Denmark:

- ESWAB regulær flocked (Art. no. 82332)

Statens Serum Institut, 5 Artillerivej, 2300 Copenhagen S, Denmark:

- Calf blood (for blood agar plates)

The National Veterinary Institute/Center For Diagnostics, Kemitorvet 202, 2800 Lyngby:

- LA-MRSA strain (ID: 61597 11-86H posCC398kon 21/3/15)
- Calf blood agar, 9 cm plates (SOP V01-05-003)
- Slanetz and Bartley agar, 9 cm plates (SOP V01-05-112)
- Luria Bertani boillion (liquid medium)
- Müller Hinton with 6.5% NaCl (liquid medium)
- Brain Heart Infusion (liquid medium)

Bio-Rad laboratories, Symbion Science Park, Fruebjergvej 3, 2100 Copenhagen E, Denmark:

- SASelect plates (Art. no. 63748)

OXOID A/S, c/o Thermo Fisher Scientific, Kamstrupvej 90, 4000 Roskilde, Denmark:

- Brilliance MRSA 2 agar plates (Art. no. PO5310A)

12 Abbreviations

agr	Accessory Gene Regulator
BA	Blood Agar
BHI	Brain-Heart Infusion Medium
CC	Clonal Complex
<i>ccr</i>	Cassette Chromosome Recombinase
HA-MRSA	Hospital-Associated Methicillin-Resistant <i>Staphylococcus aureus</i>
LA-MRSA	Livestock-Associated Methicillin-Resistant <i>Staphylococcus aureus</i>
LB	Lysogeny Broth Medium
MALDI-TOF MS	Matrix-Assisted Laser Desorption/Ionisation Time-Of-Flight Mass Spectrometry
MH	Müller-Hinton Medium
MLST	Multilocus sequence typing
MRSA	Methicillin-Resistant <i>Staphylococcus aureus</i>
MRSA2	MRSA 2 Agar
PBS	Phosphate Buffered Saline
PFGE	Pulsed-Field Gel Electrophoresis
PMF	Peptide Mass Fingerprint
SAs	SAselect Medium
SB	Slanetz-Bartley Medium
SCCmec	Staphylococcal Cassette Chromosome <i>mec</i>
ST	Sequence Type

13 Bibliography

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