



Activity of antibiotics and non-antibiotics substances on Staphylococcus aureus biofilms

Jingjing Liu

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THÈSE

En vue de l'obtention du
DOCTORAT DE L'UNIVERSITÉ DE TOULOUSE

Délivré par l'Université Toulouse 3 - Paul Sabatier

Présentée et soutenue par

Jingjing LIU

27 septembre 2021

**Activité d'antibiotiques et de substances non antibiotiques sur
des biofilms de *Staphylococcus aureus***

Ecole doctorale : **BSB - Biologie, Santé, Biotechnologies**

Spécialité : **PHARMACOLOGIE**

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INTHERES - Innovations Thérapeutiques et Résistances

AVB-Unité Antibiorésistance et Virulence Bactériennes

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Foreword

This PhD thesis was cooperatively established between the lab of Intheres, a mixed research unit INRAE/ENVT in Toulouse and the ANSES laboratory in Lyon. The first part of the project was done at Intheres on the design of antibiofilm strategies. The second part of the project should have been conducted at Anses-Lyon to explore the mechanisms which may cause the strain-specific of biofilm formation. However, the spread of COVID19 caused severe travel restriction and a shortage PCR material. I had to stay in Toulouse because moving to Lyon in the COVID time was impossible, and the delay of the second project is significant. Therefore, we only provided the preliminary results of the second project in this manuscript. Since the main experiments of the second project are finally in progress, we expect that the final results of the second project could be presented during the oral defense.

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Destruction of *Staphylococcus aureus* biofilms by combining an antibiotic with subtilisin A or calcium gluconate

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Glossary

Agr	Accessory gene regulator
AHLs	N-acyl homoserine lactones
AI	Autoinducer
AI-2	Autoinducer-2
AIP	Autoinducing peptide
ALT	Antimicrobial lock therapy
AMPs	Antimicrobial peptides
ATCC	American type cell culture
Bap	Biofilm-associated protein
Bbp	Bone sialoprotein-binding protein
BHI	Brain heart infusion
BJI	Bone and Joint Infections
BSI	Bloodstream infections
CaG	Calcium gluconate
CA-MRSA	Community associated MRSA
CC	Clonal complexes
CFU	Colony forming unit
Cna	Collagen adhesin
CRBSIs	Catheter-related bloodstream infections
CV	Crystal violet
CVC	Central venous catheters
CWA	Cell Wall-anchored proteins
DLVs	Double locus variants
ECM	Extracellular matrix
eDNA	Extracellular DNA
EDTA	Ethylenediaminetetraacetic acid
EPS	Extracellular polymeric substances

FnBPs	Fibronectin binding protein
LA-MRSA	Livestock associated MRSA
MIC	Minimum inhibitory concentration
MLST	Multilocus sequence typing
MRSA	Methicillin-resistant <i>S. aureus</i>
MSCRAMMs	Microbial surface component recognition adhesive matrix molecule
MSSA	Methicillin Susceptible <i>S. aureus</i>
NEAT	Near iron transporter
OD	Optical density
OTC	Oxytetracycline
PCR	Polymerase chain reaction
PIA	Polysaccharide intercellular adhesin
PII	Prosthetic implant infections
PNAG	Poly-N-acetyl- β -(1–6)-glucosamine
PRR	Proline-rich Repeats
PSMs	Phenol soluble modulins
QS	Quorum sensing
qPCR	Quantitative PCR
RT-PCR	Reverse transcription polymerase chain reaction
SD	Standard deviation
SdrP	Serine aspartate repeat protein
SLVs	Single locus variants
SPA	Staphylococcal protein A
ST	Sequence type
MBEC	Minimal biofilm eradication concentration
PK	Pharmacokinetic
PD	Pharmacodynamic
CLSM	Confocal laser scanning microscopy
PI	Propidium iodide

SCC <i>mec</i>	Staphylococcal chromosome cassette <i>mec</i>
PFGE	Pulsed-field gel electrophoresis
FnBPs	Fibronectin binding protein
SdrP	Serine aspartate repeat protein
Cna	Collagen adhesin
SPA	Staphylococcal protein A
Bap	Biofilm-associated protein
AtlA	Autolysin A
MMP	Modulate matrix metalloproteinases
AHC	Agglomerative hierarchical clustering

Bibliography

1. Biofilms: General considerations

1.1 Biofilm: definition

Bacteria can grow in the form of floating cells (planktonic cells) or biofilms. Biofilm is defined as a sessile community derived from microorganisms, represented by cells attached to a surface, or attached to each other, embedded in a matrix of extracellular polymeric substances (EPS). Biofilm is a microcolony with a three-dimensional structure. The formation of this structure follows a particular growth cycle and is affected by environmental factors. In the later stage of biofilm formation, biofilm bacteria transformed into a floating cell inside the biofilm matrix and will finally be released. Single-cell microorganisms can gather into biofilms because of the existing of extracellular polysaccharides, eDNA, proteins, flagellum, fimbriae, pili, and other multimeric cell appendages¹. This community life induces altered phenotype, modified gene expression and modified protein production². Unlike in a single cell floating state, the microbial populations that adhere to the carrier's surface exhibit a series of new biological characteristics and have a more vital ability to adapt to the external environment. These characteristics contribute to the persistence of microorganisms in presence of drugs and lead to major challenges for the treatment of infectious diseases. The bacteria in the biofilm use small molecular signals to control some group behaviors (aggregation or dispersion) of bacteria. From planktonic to sessile growth mode, the formation of biofilm is a complex and highly controlled process³. The development of surface-related biofilm observed *in vitro* can be mainly divided into three stages (Fig. 1): attachment, maturation, and dispersion⁴. *In vivo* biofilms can also often form small attached or free-floating aggregates^{5, 6}.

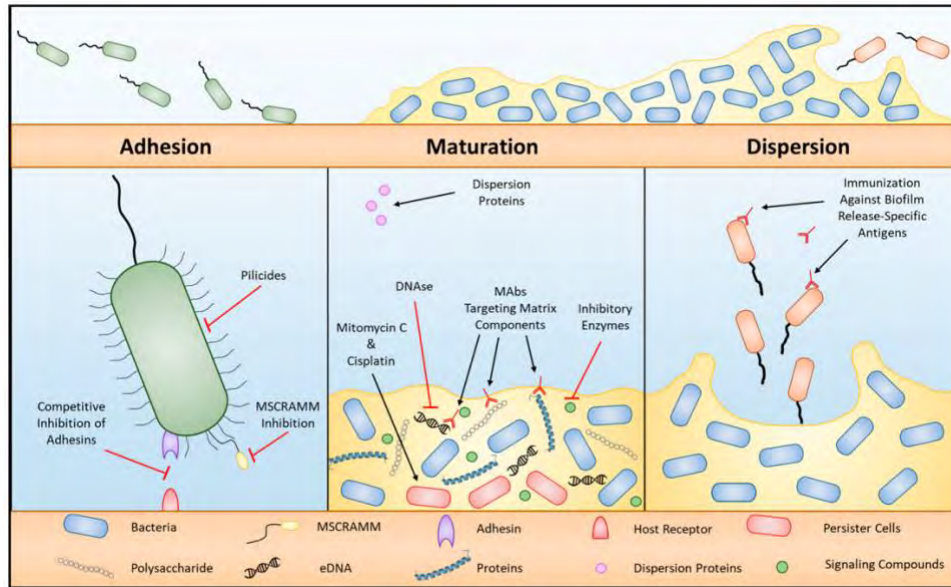


Figure 1 The formation of biofilm
(adapted from Beltelshees et al.⁷)

1.2 Biofilm: structure and composition

EPS matrix is one of the most crucial bacterial biofilm components in all bacterial species, which can protect against mechanical and chemical environmental pressures, including antimicrobial peptides, antibiotics, and phagocytic uptake. In biofilms, exopolysaccharides, proteins, and extracellular DNA (eDNA) are broadly viewed as the main components of the EPS matrix⁸. The morphology of the biofilm depends on the EPS components produced by itself. For example, in *Escherichia coli*, these EPS components are amyloid curli fibers and cellulose⁹. When the *E. coli* biofilm formed by curli fibers and cellulose, its morphology was long and tall like a ridge. On contrary, the biofilm composed of only curli fibers formed a pattern of broken concentric dome ring. EPS molecules fill in the space between biofilm cells to shape the spatial morphology of the entire biofilm¹⁰. This directly determines the living environment of the biofilm cells and provides biofilm elasticity and mechanical stability. For example, *Pseudomonas aeruginosa* maintains the integrity and stability of the biofilm structure by cross-linking the cationic extracellular polysaccharide Pel with eDNA¹¹.

1.3 Persistence of biofilm bacteria in presence of antibiotics

After the bacteria form a biofilm, their “resistance” to antibacterial substances, extreme temperature or pH, and other physical and chemical environmental factors is significantly enhanced. Bacteria embedded in biofilms are far more tolerant to antibiotics than planktonic bacteria, which is probably the main reason why antibiotics fail to treat chronic bacterial infections¹². It is worth noting that resistance to drugs or other agents means that bacteria can multiply and form colonies in the exposure of the drug or agents. Nevertheless, the "resistance" of bacteria to antibiotics caused by biofilms is a physiological state, is not caused by a modification of the genome through mutations or acquisition of new genes¹³. This unique "resistance" should now be more precisely described as persistence and corresponds to a bacterial culture that cannot be eradicated under the drug treatment while bacteria are classified as susceptible by standard susceptibility testing. The persistence of biofilm is conferred by various concomitant mechanisms, such as the biofilm matrix's composition and structure, the phenotypic changes of cells in the biofilm and the cooperation between regulatory systems. More details on the possible mechanisms of persistence to biofilm will be provided in section 3.3.

1.4 Horizontal gene transfer in biofilms

Griffith et al. first discovered horizontal gene transfer between bacteria in 1928^{14,15}. This transfer can occur between almost all microorganisms to accelerate the genetic variation and evolution of bacteria so that they can adapt to environmental changes in a short time¹⁶. There are three main ways of horizontal gene transfer between microorganisms: transformation, conjugation, and transduction¹⁷. For *S. aureus*, phage transduction is the main horizontal gene transfer mechanisms¹⁸. The efficiency of transformation consisting in ingesting naked DNA from the environment¹⁹ is usually low in but in the biofilm, the high concentration of cells can effectively improve the transformation efficiency. Studies have shown that the transformation frequency of

Streptococcus mutans in the biofilm state is 10 to 600 times higher than in the planktonic state²⁰. Savage et al.²¹ found similar results for *S. aureus* with bacteria embedded in a biofilm having a transfer frequency of drug-resistant plasmids 10,000 times higher than that of planktonic bacteria, which indicates that the biofilm promotes the horizontal spread of drug-resistant genes. Also, biofilms generally accumulate various microorganisms, which promotes the transfer of genes encoding resistance determinants between different bacterial species. For example, *S. aureus* can obtain the *vanA* gene from *Enterococcus* spp through horizontal gene transfer, thereby developing resistance to vancomycin^{22,23}.

As we mentioned earlier, biofilms contain a large amount of eDNA²⁴, which are released by bacteria through cell lysis or active secretion system and are retained in the extracellular matrix. High quantities of eDNA can be absorbed by transformation²⁵. In the process of biofilm formation, phages can also mediate bacterial lysis to form water channels in the biofilm²⁶. After lysing the host bacteria, new phages integrated within the host bacteria's DNA can be disseminated, infecting other bacteria²⁷. The close and stable cell-to-cell contact in the biofilm also efficiently enhances the horizontal transfer of plasmids in the biofilm²⁸.

1.5 Biofilm infections

Biofilms are able to cause acute and chronic infections (biofilm-related infection). Acute infection is a short-term infection, usually lasting less than six months, which occurs suddenly with microbial pathogens generally replicating rapidly. In contrast, chronic infection can last for years or even decades. The biofilm-related infections (chronic infection) can be separated in device-related infections and tissue-related infections²⁹. This indicates that the presence of a biotic or abiotic surface is no more considered as a hallmark for biofilms. Therefore, biofilm-related infection (chronic infection) is now defined as an infection that persists despite antibiotic treatment and the innate and adaptive immune response of the host and is characterized by persistent pathology³⁰.

Some biofilms adhere to artificial surfaces, causing chronic infections associated with medical devices. Other biofilm infections may be caused by aggregates related to the surface but do not adhere directly to the surface³¹. Lebeaux et al.²⁹ summarized chronic infections mainly caused by biofilms. It can be seen from Fig. 2 that the existence of biofilms is the key to chronic infections. And biofilm-related infections can be induced by one or multiple microorganisms. In animals, biofilm can also cause numerous infections and cause substantial economic losses in animal husbandry production, for instance, wound infection, mastitis bovine, enteritis in cattle, fleece rot in sheep, pneumonia, otitis, and urinary tract infection in companion animals³².

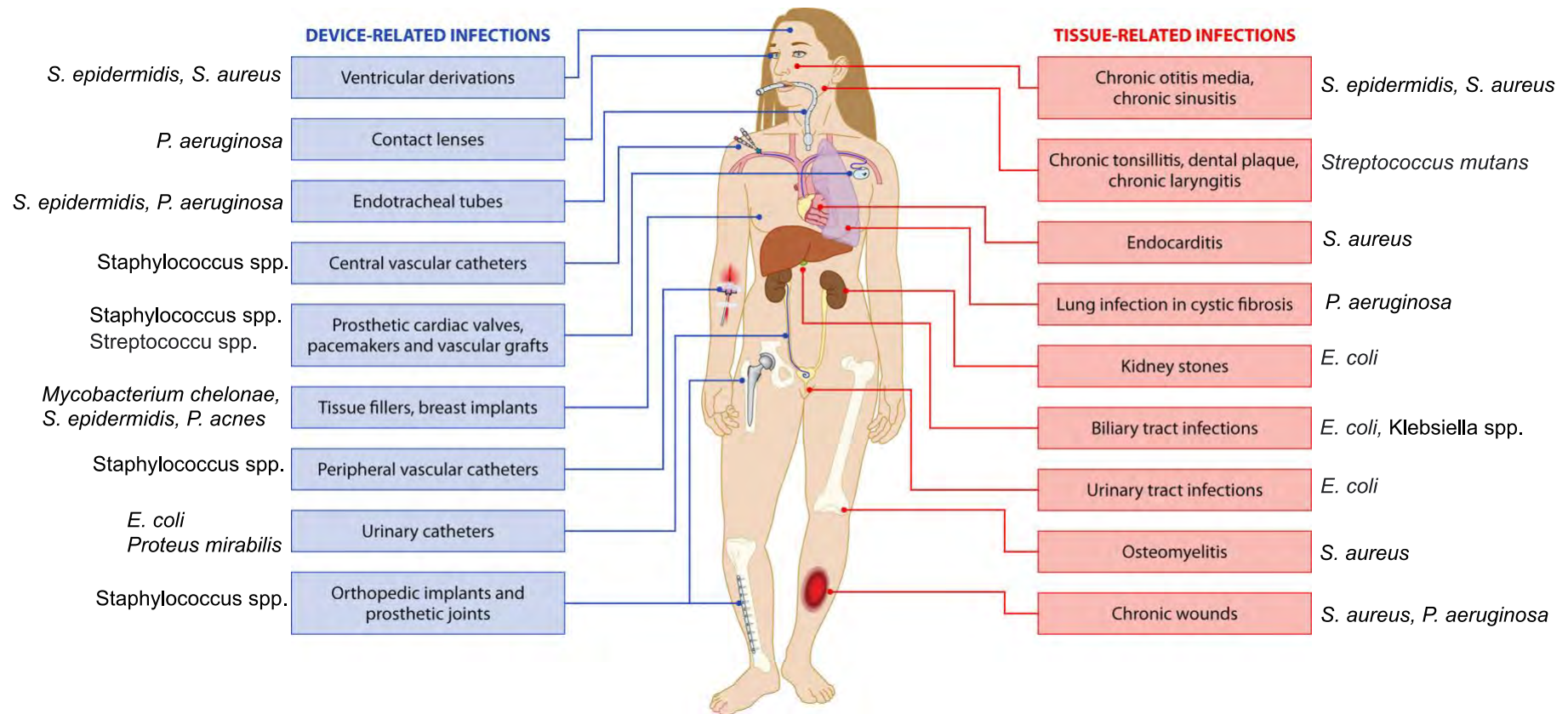


Figure 2 The biofilm related infection in human
(adapted from Lebeaux et al.²⁹)

1.6 *In vitro* model and assays for biofilm

Biofilm research relies on some characteristic experimental techniques. The *in vitro* model simulates the external conditions of the biofilm formed by microorganisms without directly using animal models or infected hosts as research carriers, including solid surfaces, specific nutrients and temperature, to study topics related to the formation of biofilms by microorganisms. The main techniques for *in vitro* model research include cell culture systems, cell labeling methods, and quantitative or qualitative detection methods. Below, we will focus on several of the most common *in vitro* biofilm models.

1.6.1 Static models

Several static biofilm culture systems use batch culture methods to observe the formation of bacterial biofilms within 1 to 2 days. These systems have inherent limitations, such as the fact that nutrients can only be added regularly and uncontrollable oxygen concentration. However, due to the low cost of replication and simple operation, static biofilm culture systems are particularly fitted for screening large sample collections. The most commonly used high-throughput biofilm model is microtiter plates, which can simultaneously detect biofilms formed by many different strains. Genevaux et al. first adopted 96-well microplates to cultivate bacterial biofilms and stained them with crystal violet for quantitative analysis³³. Since then, this combination of microtiter plates for biofilm culture and cell stains has been widely used in *in vitro* model of biofilms³⁴. Calgary biofilm device is a modified high-throughput biofilm model based on microtiter plates, also known as minimal biofilm eradication concentration (MBEC) biofilm inoculator (Fig. 3). In the MBEC device, the biofilms are established on the pegs, making the subsequent washing or transfer of the biofilm (only the cover is transferred) becomes fast, low risk of contamination and soft mechanical damage³⁵.



Figure 3 MBEC biofilm inoculator
(adapted from Innovotech.ca)

1.6.2 Dynamic models

The principle of the dynamic model is similar to continuous culture. In the reactor, the metabolic byproducts, planktonic bacteria, and dead cells are continuously replaced with the fresh culture medium. Environmental parameters are usually controllable in the model, such as the flow rate of the medium or drugs and the speed of stirring. Pereira et al.³⁶ found that a large amount of biofilm biomass formed by *Pseudomonas fluorescens* under the laminar flow, and the biofilm surface was flat. In contrast, a small amount of biofilm formed under turbulent flow with a rough surface. Dynamic models are widely used to study the physicochemical resistance of biofilms. However, due to the requirement of professional operation, high cost of experiments and long time-consuming, these systems are usually unsuitable for high-throughput analysis³⁷. At present, the most commonly used dynamic biofilm model is the flow cell system, which provides culture fluid to the biofilm at a fixed flow rate through a pump (Fig. 4). The biofilm grows at the gas-liquid interface to maintain a high gas flux. The Bioflux® device allows microscopic observation of the biofilm formed on the glass slide to realize non-destructive real-time monitoring of the biofilm formation.

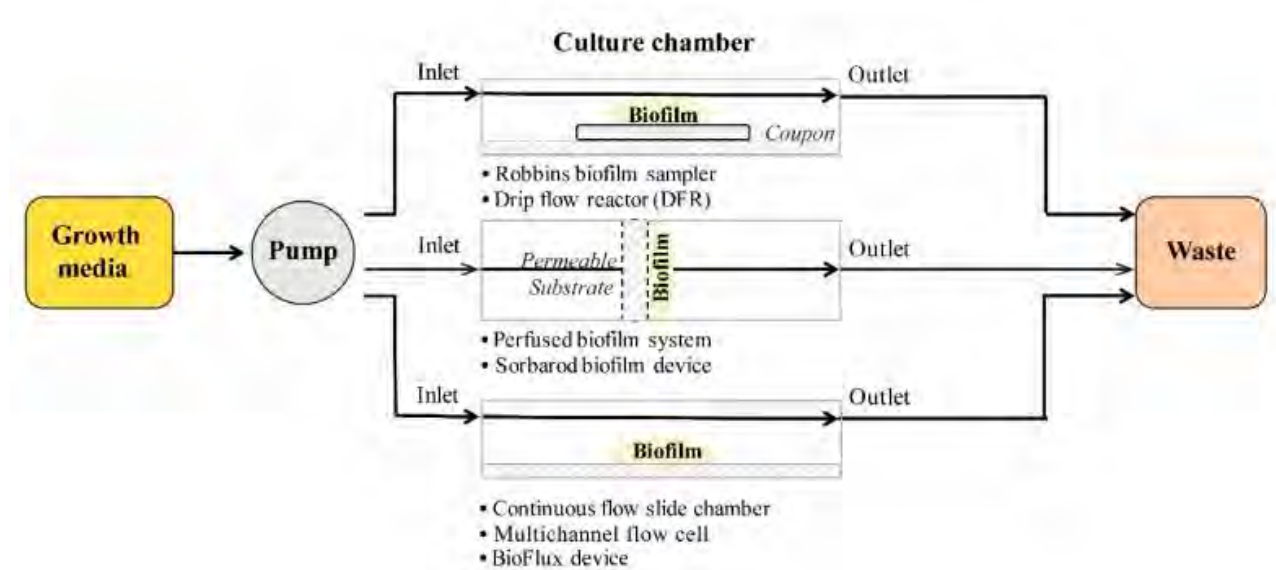


Figure 4 The general schematic of the flow cell system
(adapted from Tania F et al.³⁸)

Another dynamic biofilm model is called CDC biofilm reactor (Fig. 5), in which the biofilm formed on the coupons in the reactor³⁹. These coupons can be made of different materials and fixed to eight holder rods. The removal and subsequent processing of the biofilm only need to take out the whole rod and remove the fixed coupons. The CDC biofilm reactor is convenient for studying biofilms formed by a single strain with large biomass production.

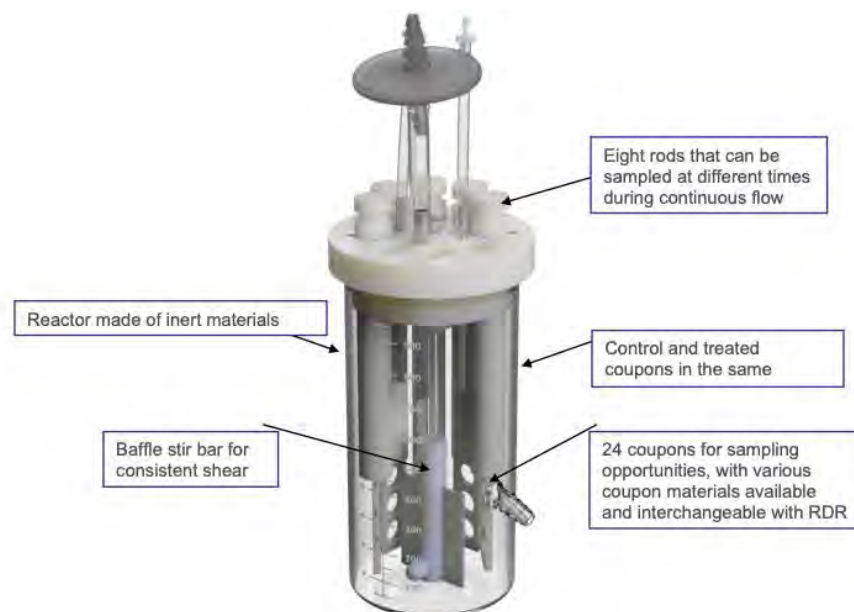


Figure 5 CDC biofilm reactor
(adapted from CDC Biofilm Reactor® Operator's Manual)

In the 1980s, Zinner et al.⁴⁰ first adapted hollow fiber cartridges for bacteria culture and testing. After that, the hollow fiber infection model was widely used to analyze the efficacy of antibiotics⁴¹. The biofilm-forming process in hollow fiber bioreactor (Fig. 5) is different from the above two dynamic models. Its core equipment is a cartridge containing thousands of hollow fibers, each of which has a diameter of only 200 microns⁴². Bacteria are inoculated in the extracapillary space of the cartridge where they can form biofilms. The culture medium from the reservoir continuously circulates through the cartridge to afford bacteria oxygen and nutrients (Fig. 6). Indeed, the pore size of the fiber allowed drugs and other small molecules to pass freely, whereas it stopped the bacteria.

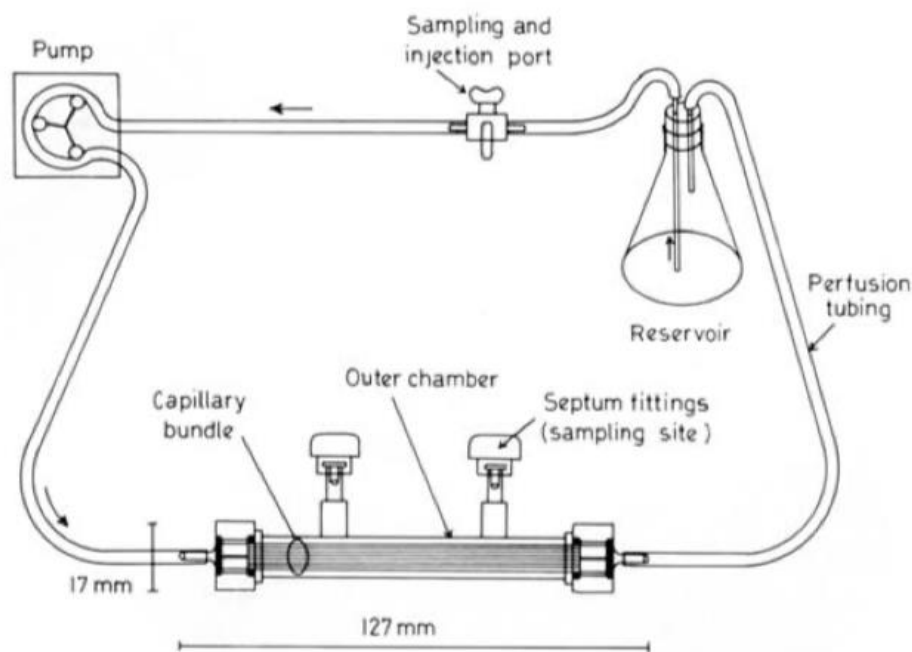


Figure 6 The original design of the hollow fiber infection model
(adapted from Zinner et al.⁴²)

The hollow fiber reactor has various advantages in establishing biofilm infection models and anti-biofilm drug efficacy testing. For example, it can accurately simulate the PK/PD of drugs in humans or animals, detect the therapeutic effects of two drugs simultaneously, mimic the dose curve and drug elimination curve, and check the growth of bacteria in different stages et al.. Nevertheless, the hollow fiber cartridge used for culturing bacteria is disposable and expensive, so the hollow fiber bioreactor normally cannot be employed for high-throughput testing and is only fitting for testing a single species of bacteria per time.

1.6.3 Confocal laser scanning microscopy (CLSM)

Since the early 1990s, confocal laser scanning microscopy (CLSM) has completely changed the investigation of the structure of hydrated microbial samples⁴³. Nowadays, CLSM is a standard technology in many laboratories, allowing advanced applications such as phase separation in binary polymer mixtures⁴⁴, visualization of water configuration⁴⁵, or skin cancer diagnosis⁴⁶. The core instrument of CLSMs is a conventional vertical or inverted fluorescence microscope for visual inspection of samples⁴⁷. CLSMs with multiple fluorescence channels can simultaneously detect and record fluorescence of different wavelengths, such as red, green, yellow, and blue with far apart wavelengths⁴⁸. For example, Live -Dead kit is very commonly used to label live and dead cells in bacteria. It contains green fluorescent dye SYTO9 and red fluorescent dye propidium iodide (PI). SYTO9 can penetrate all cell membranes, including live cell membranes and damaged cell membranes of dead cells. PI can only penetrate the damaged cell membrane and weaken the effect of SYTO9. When the two fluorescent dyes were applied in a specific ratio, live cells can appear green fluorescent, and dead cells are marked red. This fluorescent dye kit is often used to indicate the situation of survival cells in the bacterial biofilm after treatment⁴⁹.

2. *Staphylococcus aureus*

2.1 Brief introduction of *S. aureus*

S. aureus was first isolated from pure culture by German surgeon Alexander Ogston in 1886⁵⁰. It was named *S. aureus* because of the color of its colonies. With the development of science, people realized that the presence of coagulase (an enzyme that coagulates proteins in plasma) was related to this species. Then, a coagulase test was applied to the clinical diagnosis of *S. aureus* infection from the 1920s. Penicillin was introduced into clinical application and showed an excellent killing effect on *S. aureus* during the Second World War. However, with the extensive use of penicillin in hospitals, the rise and spread of drug-resistant strains began to exceed those of susceptible strains (Fig. 7), which advanced the invention and clinical use of semi-synthetic penicillin (e.g., methicillin).

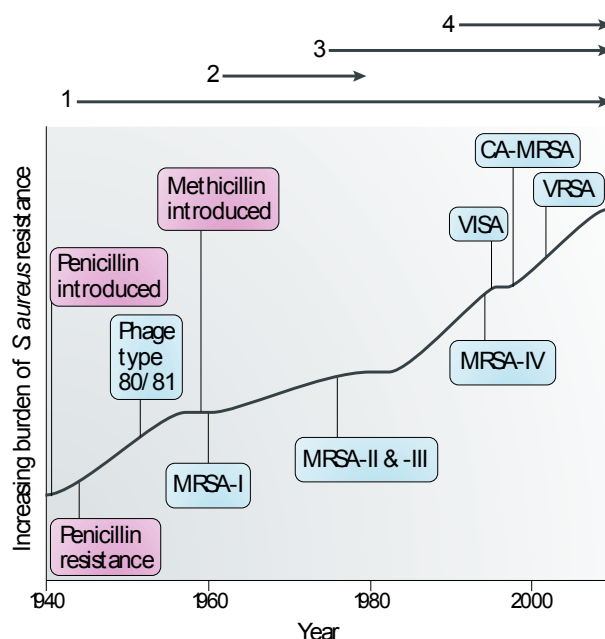


Figure 7 The evolution of antibiotic resistance in *S. aureus*
(adapted from Chambers et al. ⁵¹)

Wave 1: Shortly after penicillin was introduced into clinical practice in the 1940s, the first wave (indicated at the top of the chart) is still ongoing today. Wave 2: The second wave was from the presence of the first MRSA (ancient clone), which included the staphylococcal chromosome cassette *mec I* (SCC*mecI*) to the 1970s. Wave 3: The third wave followed the second one, when new MRSA strains appeared, including new SCC*mec* allotypes SCC*mecII* and SCC*mecIII*. Additionally, the emergence of vancomycin-intermediate *S. aureus* (VISA) was finally caused by the increasing use of vancomycin. Wave 4: From the mid of 1990s, the fourth wave started and marked the MRSA strains that appeared in the community (CA-MRSA).

Although this newly synthesized penicillin showed a good bactericidal effect on *S. aureus* initially, the hospital began to report the spread of methicillin-resistant *S. aureus* (MRSA) in the 1990s, and new cases of MRSA (CA-MRSA) related to the community appeared. After entering the 21st century, the strains of MRSA (LA-MRSA) related to livestock have been reported in various countries in the world. The emergence and spread of LA-MRSA may make it re-evolve to adapt to humans and lead to endemic and epidemic human infectious diseases (Fig. 8)⁵².

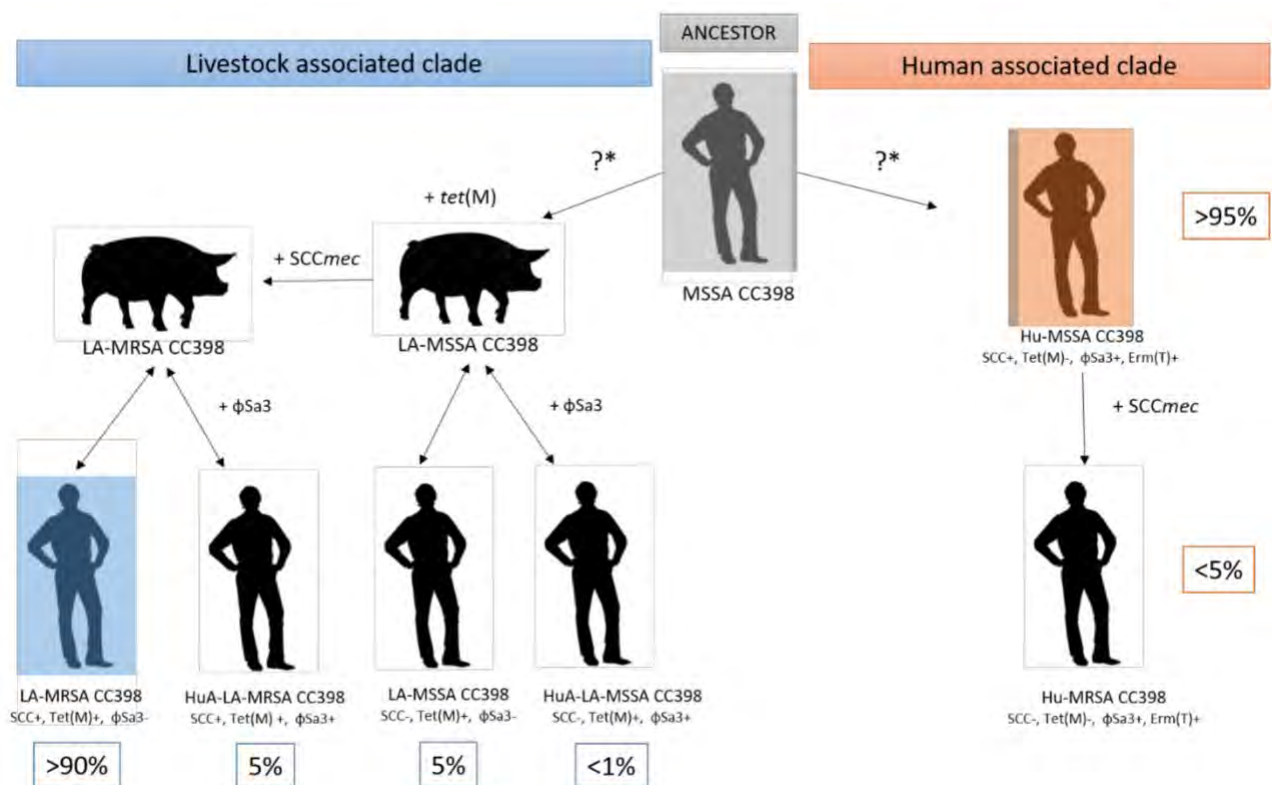


Figure 8 Schematic diagram of the evolution of different subgroups within the CC398 lineage (adapted from Bouiller et al.⁵³)

The last common ancestor of *S. aureus* CC398 may be the human adaptive immune escape cluster (IEC) MSSA CC398 strain. This strain acquired *SCCmec* in the later stage, resulting in the emergence of MRSA CC398 strain in humans (human-associated clade). On the other side, the ancestral MSSA CC398 strain transmitted to livestock with the loss of IEC and the acquisition of *tet(M)*, which awarded tetracycline resistance. The latter acquired *SCCmec*, which was conferred the resistance to methicillin, leading to MRSA CC398 related to livestock. However, the time of this emergence between humans and livestock remains unclear ("?"). Double arrows indicated the subgroups of clades linked to livestock. These transmissions may be caused by the acquisition of phage ϕ Sa3 in the livestock-associated clade, which distinguished the human *S. aureus* CC398 subpopulations from the adapted human livestock-associated *S. aureus* CC398 subpopulations.

2.2 Epidemiology of ST398 *S. aureus*

2.2.1 MRSA ST398

Molecular typing technology is widely used to analyze and monitor the epidemiological characteristics of *S. aureus* typing in different countries, different regions, and different locations (such as hospitals, farms, and slaughterhouses)⁵⁴. Multilocus sequence typing (MLST)⁵⁵ involves sequencing the 450-500bp DNA internal fragments of seven housekeeping genes. The different sequences that exist in each housekeeping gene of bacterial species are designated as different alleles. By comparing the sequence of the obtained gene to known alleles (<https://pubmlst.org/organisms/staphylococcus-aureus>) at each locus, belongs combination of different allele numbers can be obtained, which ultimately corresponds to a specific sequence type (ST). E-burst analysis classifies STs, SLVs (single locus variants), and DLVs (double locus variants) into Clonal complexes (CC). The CC's number depends on the ancestor ST, that is, the ST with the most different SLV and DLV⁵⁶. Enright et al.⁵⁷ first used MLST in 2000 to sequence 155 strains of *S. aureus* isolated from patients with community-acquired and hospital-acquired invasive diseases. The results indicated that MLST clearly distinguished these strains (MRSA and MSSA), 53 different alleles were identified, of which 17 were represented by at least two isolates. MLST can also effectively distinguish the type of *S. aureus* associated with livestock (LA-*S. aureus*) and has been widely used⁵⁴. LA-*S. aureus* associated with human infections is usually typed as ST1, ST8, CC5, ST121, and ST398⁵⁸.

MRSA ST398 was first discovered in the nasal isolates of healthy pig farmers⁵⁹ in a selected population from 7 departments in the central regions of French pig production. Fifty-seven percent (25/44) of the isolates from pig farmers had the same STs as the strains of pigs, of which 6 ST398 isolates came from swine farmers in four different departments. Sequence type was determined at that time because these swine isolates could not be typed using the formerly widely used Pulsed-Field Gel Electrophoresis (PFGE) method applying the *Sma*I restriction enzyme. Since then, more and more MRSA-ST398 isolates (swine host) with different *spa* types have been reported by research groups worldwide⁶⁰, including Denmark⁶¹, the Netherlands⁶², Belgium⁶³, and Germany⁶⁴,

Canada⁶⁵, the United States⁶⁶ and China⁶⁷. First described in swine, swine-related products, or people in contact with the swine production chain, MRSA ST398 has now been identified in numerous other animal hosts where it can be a commensal or cause infections. In a Brazilian farm, a research reported 11% of cows with mastitis carried MRSA-ST398, and one of the isolates contained *Isa*(E), *spw*, and *aadE* resistant genes⁶⁸. Vries et al.⁶⁹ identified that MRSA-ST398 isolated from cattle in Denmark started in the early 1990s. It may indicate that ST398 has spread among species in the early 1990s. Subsequently, related studies in the United States also proved the MRSA-ST398 isolates in turkeys⁷⁰. Meanwhile, MRSA-ST398 was also isolated from poultry, healthy broiler breeders, and slaughterhouse personnel among the six slaughterhouses in the Netherlands⁷¹, 5.6% (26/466) of the employed employees carried MRSA, and about 70% of 117 MRSA isolates were ST398. LA MRSA-ST398 was originally thought to be an animal clone.

Although ST398 isolates are considered less toxic (because of the rare occurrence of enterotoxin genes) and transmissible than other *S. aureus*, the MRSA-ST398 isolate shows resistance to many non- β -lactam antibiotics of both medical and veterinary interest, for instance macrolides, tetracycline, and gentamicin^{72,73}.

2.2.2 MSSA ST398

With the in-depth study of it, the colonization and infection of MSSA-ST398 in humans with no exposure to animals have been gradually discovered worldwide^{74, 75, 76}. Price et al.⁷⁷ completed the accurate phylogenetic reconstruction of the CC398/ST398 clone lineage using whole-genome sequence typing. The analysis results proved that this clonal lineage originated from human MSSA, and experienced rapid radiation accompanied by jumping from humans to livestock. In the process, resistance to tetracycline and methicillin was obtained randomly. In 2016, Uhlemann et al.⁷⁸ applied comparative whole-genome sequencing to analyze the epidemiological characteristics and geographic diversification of MSSA-ST398, which showed that this pandemic lineage was widely spread in both humans and livestock. Relevant reports about severe invasive infections caused by ST398 are not uncommon⁵³. For example, in a French four-year survey on the occurrence of 3-month bloodstream infections (BSI)⁷⁹, 18 cases were caused by MSSA-ST398, and this infection

trend had increased by seven times in the four years of investigation. These patients diagnosed with MSSA-ST398 BSI did not have livestock exposure. Bouiller et al.⁸⁰ identified 124 CC398/ST398 in 958 Bone and Joint Infections (BJI) associated to *S. aureus*. The proportion of CC398/ST398 increased from 4% to 26% over the seven years investigated, with only 4 isolates being MRSA-CC398/ST398.

2.2.3 *S. aureus* ST398 biofilm

Although the research on ST398 biofilm has only appeared in recent years^{81, 82, 83}, the early screening of genes encoding adhesion factors in ST398 indicated its inheritance of robust adhesion biofilms in livestock and human clones⁸⁴. In the genetic screening of 10 MRSA-ST398 (from poultry) isolates, Nemati et al.⁸⁵ found that all isolates contain *icaD*, *cna*, *ebpS*, *eno*, *fnbA*, *fnbB*, *clfA*, and *clfB*. These genes encode various components closely related to the formation of the biofilm matrix. Another study from New Zealand⁸⁶ demonstrated that 9 MRSA-CC398/ST398 isolates from humans did not contain genes encoding toxic shock syndrome toxin 1 or staphylococcal enterotoxin. In contrast, all isolates have multiple genes encoding biofilm formation, such as *clfA*, *clfB*, *cna*, *fnbA*, *fnbB*, and *bbp*. To sum up, even if ST398 lack of some virulence genes, it can form biofilm. And, to adapt to the selection pressure brought by different host environments, when LA MRSA-CC398/ST398 isolates are transmitted to humans, they can lose antibiotic resistance genes and gain the human-specific virulence genes of the IEC cluster in human-associated isolates⁸⁷. Therefore, biofilm generation may have enhanced the adaptability of LA MRSA CC398 lineage to different hosts, which has achieved rapid colonization and transmission.

3. *Staphylococcus aureus* biofilms

3.1 Factors affecting the formation of *S. aureus* biofilms

Many factors such as, pH, temperature, nutrition or antibacterial drugs can affect biofilm formation and influence every stage of formation.

The formation of biofilm requires an appropriate pH level. High acidity or strong alkalinity conditions are not conducive to the formation of biofilm of *S. aureus*. In contrast, weak acidity pH conditions are most suitable for *S. aureus* to form a stable biofilm⁸⁸. Concerning nutrients, Singh et al.⁸⁹ found that supplementing the culture medium with glucose, sucrose or NaCl can promote biofilm formation of MRSA and MSSA isolates. As an example, the addition of NaCl enhances the aggregation, stability and strength of biofilm in a dose-dependent manner, and strong biofilm of *S. aureus* harboring *icaA* can be formed by adding 7% NaCl⁹⁰.

Adhesion between bacteria and surface is the first step of biofilm formation and constitutes the first survival strategy in the evolution of bacteria⁹¹. In order to increase the rate of attachment, *S. aureus* produce different adhesion factors to attach to biotic (soft tissue or bone) or abiotic (artificial or ecological) surfaces, or even to self-aggregate. This characteristic enables bacteria to construct a biofilm community in every environment. Physical and chemical mechanisms such as cell surface charge, extracellular polymer and cell surface hydrophobicity play an important role in bacterial adhesion. Most bacterial cells are negatively charged, so bacteria are more likely to adhere to positively charged surfaces, while negatively charged surfaces are more resistant to bacterial adhesion⁹². Macromolecules on the cell wall of *S. aureus* can mediate the binding of bacteria to hydrophobic and hydrophilic surfaces through thermal fluctuations⁹³. On the hydrophobic surface, many hydrophobic macromolecules (teichoic acid, protein, or protein-associated molecules) at the surface of the bacteria as well as the capsule can contribute to the adhesion. On the contrary, only a few macromolecules (such as peptidoglycan) produced by *S. aureus* can adhere firmly to hydrophilic surface^{94,93}. Therefore, the hydrophobic structure on the surface of bacteria makes the adhesion easier on hydrophobic surfaces.

3.2 Development of *S. aureus* biofilm

3.2.1 Formation of *S. aureus* biofilm

In the initial stage, planktonic cells attach to the surface of abiotic or biotic substrates by a wide variety of mechanisms. The mechanism for promoting adhesion depends largely on the chemistry of the substrates composing the surface. Among them, electrostatic or hydrophobic interaction promotes the attachment of bacteria to abiotic surfaces; various noncovalent interactions mediate adhesion between adhesins and biotic surfaces⁸. The initial attachment of these cells may be irreversible. Cells can then undergo phenotypic changes that initiate biofilm formation. Bacteria can also aggregate to each other through various molecules, such as adhesion proteins or extracellular polymers such as polysaccharides^{95, 96, 97}. With the continuous increase of intercellular aggregation and the continuous production of biofilm matrix components, the biomass increases significantly and leads to a mature biofilm. In 1991, the architecture of biofilm formed by *Pseudomonas fluorescens* was published by Lawrence et al.⁹⁸. A mature biofilm can acquire the form of simple towers or mushrooms (Fig. 9), interspersed with water channels. These channels shown in Fig. 9 have no cells inside, and seem to constitute an original circulation system that can be responsible for transporting nutrients and removing wastes. These very complex and highly structured multicellular communities protect the embedded cells that show the greatest persistence to antibacterial agents^{99, 100}.

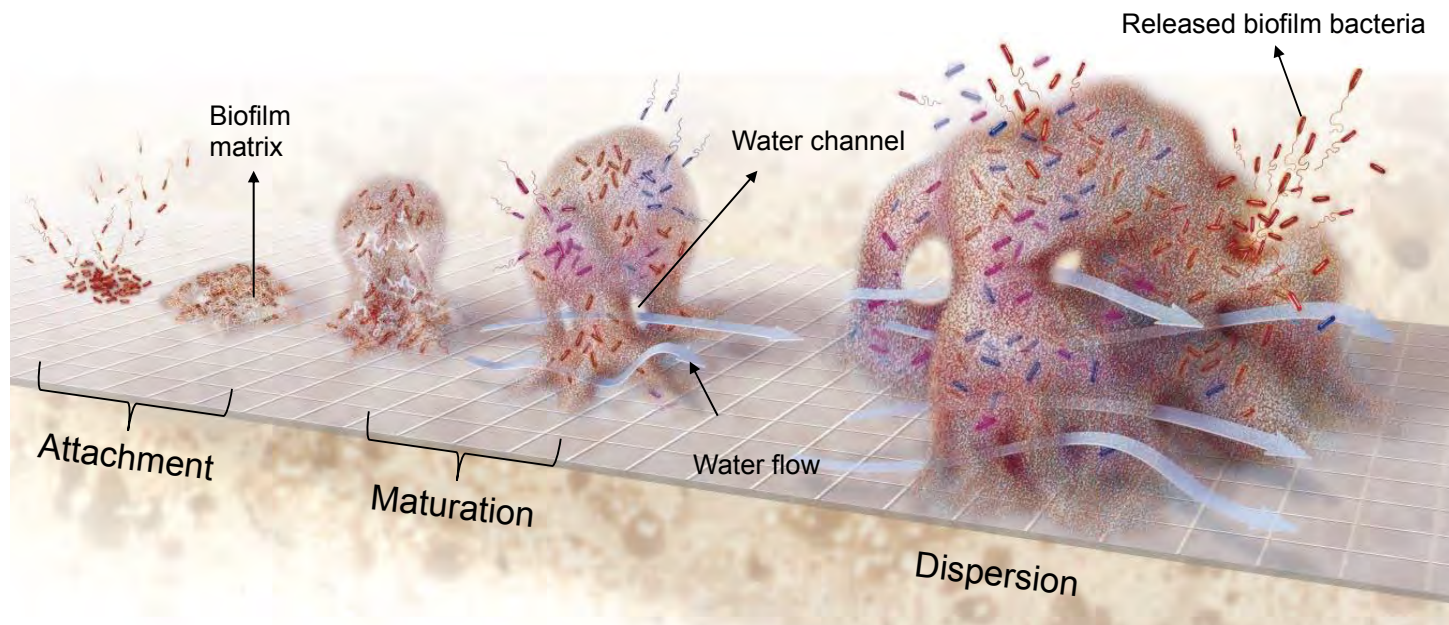


Figure 9 The formation and structure of biofilm
(adapted from Costerton et al.¹⁰¹).

For the dissemination of bacteria to other settlements, the detachment of biofilm is essential. It can occur through the detachment of aggregated cell clusters or single cells. When shear forces exceed the tensile strength of biofilm in the flow system, some cell clusters and their enveloping matrices will be transferred into the bulk liquid^{102, 103}. Such a process was suspected to cause the separation of biofilm fragments in endocarditis occurring on natural valve¹⁰⁴. Another more complicated process of biofilm detachment is the self-detachment of sessile cells (without shear forces). These embedded cells should return to the planktonic phenotype before releasing themselves from the biofilm matrix¹⁰⁵. This releasing process is controlled by a signal that triggers the synthesis or the release of enzymes that can degrade the complex polymers of biofilm matrix^{106, 107}. However, the factors that can initiate this release are still unclear.

3.2.2 Role of quorum sensing

Quorum sensing (QS) is a group behavior system by which bacteria communicate between or within species by transmitting pheromones to each other¹⁰⁸. Bacteria can spontaneously produce signal molecules (autoinducer, AI), which accumulate as the number of bacteria in the biofilm increases. When the concentration of AI reaches a certain threshold, they bind to signal receptors on the cell wall's surface to directly or indirectly activate the expression of related genes¹⁰⁹. The existence of quorum sensing makes bacteria have significantly different behaviors from planktonic bacteria¹¹⁰, such as the attachment of bacteria to the surface, the production of extracellular matrix, competition or metabolic—cooperativity between bacteria^{111,112} and the expression of virulence factors¹¹³. Quorum sensing regulates bacterial biofilms in two-way directions (aggregation or dispersion). When external environmental conditions are not conducive to bacterial growth, bacteria aggregate and form biofilms under the QS system's control. As the density of bacteria increases, the internal microenvironment of mature biofilms becomes poor. The quorum sensing will regulate the expression of genes related to the release of bacteria in the biofilm¹⁰⁸. This process converts bacteria from a biofilm state to a planktonic state, leading to the spread or recurrence of the infection.

Quorum sensing has a high degree of specificity in regulating bacterial group behavior, which

is essential for forming and maintaining multi-species biofilms. Autoinducers (AIs) produced in different bacterial species can communicate and influence each other in different signal pathways, forming a complex three-dimensional network system¹¹⁴. Currently known signal molecules can be divided into three categories: N-acyl homoserine lactones (AHLs, Gram-negative bacteria), autoinducing peptide (AIP, Gram-positive bacteria), and autoinducer-2 (AI-2, Gram-positive bacteria and Gram-negative bacteria)¹¹⁵. The extracellular concentration of the AIP is sensed and responded to by the accessory gene regulator (*agr*) system of staphylococci (Fig. 10).

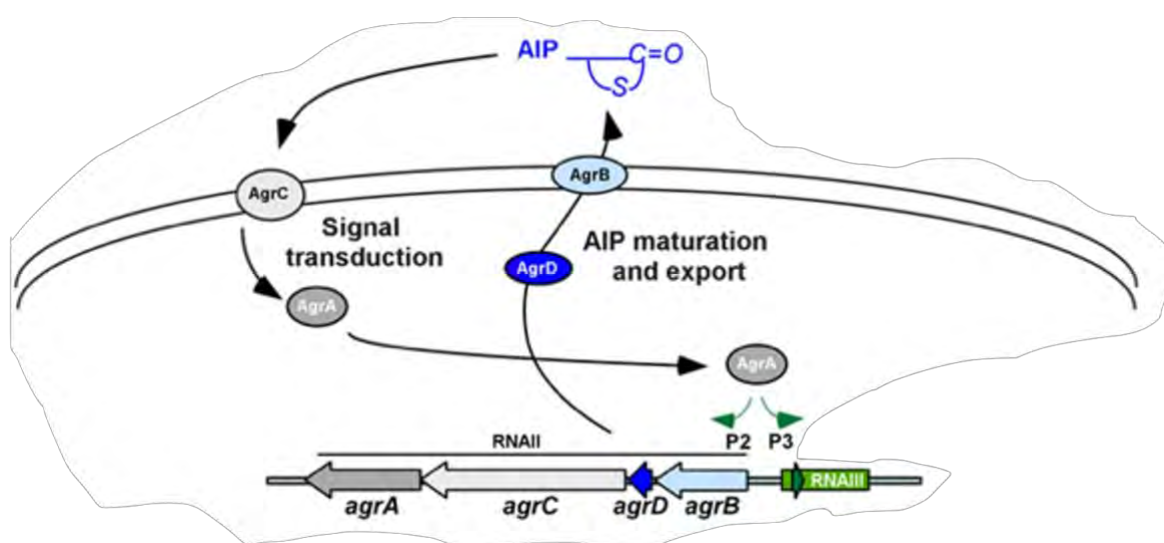


Figure 10 The quorum-sensing of *Staphylococcus* spp
(adapted from Le et al.¹¹⁶)

The final AIP signal is produced from the precursor (AgrD, propeptide) by the maturation and export of primary enzyme (AgrB) that is localized on the cell membrane. AgrC (membrane histidine kinase) and AgrA (response regulator) is a two-component system. After binding to AIP, by phosphorylates AgrA, AgrC activates the P2 and P3 promoters to drive the *agrBDCA* transcription and upregulate the transcription RNAII. The transcription from the P2 promotor activated the auto-feedback regulation.

The *agr* locus contains *agrBDCA*, which is considered as a part of the core genome but not on a pathogenicity island¹¹⁷. AIP is a stable self-induced peptide derived from the inner regions of AgrD through a series of processing dependent on AgrB¹¹⁶. The ATP-binding cassette transport system or other membrane proteins secrete AIPs outside the cell. The accumulated AIPs will initiate a two-component system composed of membrane-bound sensor kinase receptors and cytoplasmic transcription factors to regulate gene expression¹⁰⁸. Between the different

bacterial species, the type of two-component system is different. For instance, in *Streptococcus* spp, the two-component system comprises histidine protein kinase and response regulator protein¹¹⁸. The two-component system in *S. aureus* WalK/WalR, also called YycG/YycF is highly conserved and directly regulates the formation of biofilms¹¹⁹.

On the other hand, the quorum sensing system can regulate the expression of efflux pump genes. Researches demonstrated that the auto-inducing molecules could up-regulate the expression of the efflux pump gene *bmeB* and the multi-drug resistance pump MexAB-OprM (in *P. aeruginosa*) in quorum sensing^{120,121}. These bacterial efflux pumps can discharge both the auto-inducing molecules and the drugs and so, can lead to drug resistance. The discharged auto-inducing molecules accumulate outside the cell to promote the auto-feedback regulation of the quorum sensing system¹²².

3.2.3 Persister bacteria in biofilms

In 1944, Bigger et al.¹²³ found that less than 0.1% of a unique subpopulation of *S. aureus* showed significant survival in presence of antibacterial drugs without carrying genetic mutations or drug resistance genes. In another important early paper, Gunnison et al. 1964 also described a subpopulation of *S. aureus* that can survive to a concentration 1,000 times higher than the minimum inhibitory concentration (MIC) of penicillin. Even with the addition of streptomycin, the proportion of surviving bacteria remains the same. Both research groups proved that this tiny group of a highly tolerant subpopulation, named persister, are phenotypic variants and are not produced by stable genetic modifications. The formation of persisters was initially thought to be caused by changes of gene expression levels in individual cells with a genetically identical population¹²⁴, which is a random phenomenon. The follow-up studies proved that there are persisters in the biofilms formed by almost all pathogens. Even when exposed to 100 times the MIC of antibacterial drugs, persisters can still be cultivable¹²⁵. When antibiotic treatment is stopped, the surviving persisters begin to multiply again¹²⁶. The final recovery rate of persistent bacteria of the newly formed population is less than 1%¹²⁷, and is genetically the same as before exposure to the drug^{128,129}. Clinically, the presence of persisters

is the main factor that causes antibiotic failure and chronic infection intractability¹³⁰. Biofilm properties contribute to persister formation, including slow metabolism, oxidative stress, quorum sensing, and nutritional heterogeneity¹³¹. High concentrations of antibiotics can kill planktonic bacteria, while the persisters in the deep layer of the biofilm enter a dormant state. The biofilm matrix can help persisters escape the immune system, allowing them to survive after discontinuing therapy (Fig. 11). When the external environment becomes suitable again, the persister will end dormancy and resume growth¹²⁹. *In vitro* experiments have shown that the cell status of the persisters in the biofilm can be maintained for at least four weeks¹³².

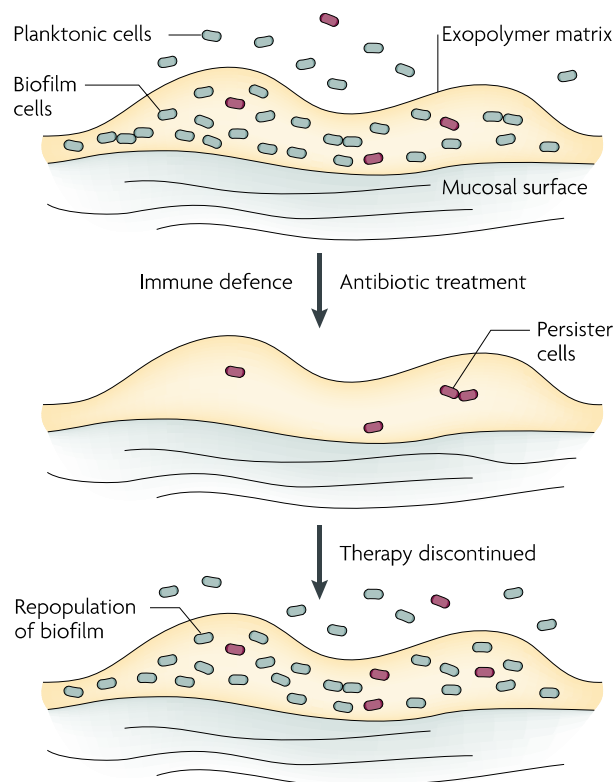


Figure 11 The formation of persisters when in vivo biofilm exposure to drugs
(adapted from Lewis et al.¹³³)

3.3 Persistence of *S. aureus* biofilm

3.3.1 Role of biofilm matrix

Bacteria secrete an extracellular matrix and wrap it to form a biofilm, which is the first line of defense against antibacterial drugs. As the biofilm matures, the polysaccharide complex layer increases and diversifies, which helps the bacteria to form a more complex biofilm structure¹³.

Among the biofilm matrix components, polysaccharides allow bacteria to adhere to and colonize both biotic and abiotic surfaces, which effectively improves the bacteria's resistance to physical stress forced by fluid movement. Several studies have shown that antibodies or inflammatory cells cannot effectively penetrate polysaccharides-dependent biofilms^{134,135,136}, resulting in low immune responses due to poor detection. In addition, polysaccharides-producing bacteria can also protect non-polysaccharides-producing bacteria in biofilms¹³⁷.

The mature biofilm has a highly organized three-dimensional structure, and the tortuous channels throughout its interior can prevent the penetration of macromolecular antibiotics¹³⁸. Due to this barrier to penetration, many antibiotics have difficulty reaching the deepest cells, resulting in a heterogeneous distribution of antibiotics in the biofilm. The cells in the deepest layer of the biofilm may be exposed to antibiotics below the inhibitory concentration for a long time. This gradual exposure to antibiotics may trigger the resistance mechanism of bacteria¹³⁹. In fact, the factors affecting the penetration of antibacterial agents into biofilms are very complex, including biofilm thickness, drug concentrations and exposure duration, effective diffusion rate of drugs, reactivity of the drug in the biofilm and the sorptive capacity of the biofilm for the drug. Research by Jefferson et al. found that the penetration time of antibiotics in biofilms ranges from a few minutes to almost 24 hours¹⁴⁰. There is a correlation between exposure duration and penetration time. Antibiotics with limited penetration in staphylococcal biofilms are oxacillin, cefotaxime, vancomycin, and delafloxacin, while other antibiotics such as amikacin and ciprofloxacin are unaffected¹⁴¹. For all antibiotics, the concentrations around deep-layer bacteria are gradually increasing and the early exposure to sub-inhibitory

concentrations can favor the entrance in persister state, thereby making them survive at the subsequent lethal concentrations¹⁴². So, the antibiotic concentration gradient may add extra selective pressure to the bacteria in biofilm. Of interest, the penetration of antibiotics (such as delafloxacin¹⁴³) can be improved when combined with some anti-biofilm compounds.

Penetration failure cannot be the main reason for the persistence of biofilms. Biofilm is made of 90% water and soluble molecules, so many antibiotics should diffuse easily through the matrix. Moreover, the water channels inside the biofilms are compared to the original circulatory system, further enhancing drug penetration¹⁴⁴. Therefore, the penetration of antibiotics in biofilms cannot be analyzed solely based on the physical barrier of biofilm matrix.

The biofilm is a negatively charged and can absorb the positively charged amino side chains of the polypeptide chains surrounding the biofilm to form a charge barrier and prevent hydrophilic antibacterial drugs from entering the bacteria and reduce their killing effect¹⁴⁵. Cationic antibacterial agents or biocides such as gentamicin¹⁴⁶ and chlorhexidine¹⁴⁷ can also bind to anionic sites in the biofilm matrix, and this process interferes with their penetration into the biofilm. Many enzymes able to degrade antibacterial drugs can also be excreted in the matrix by bacteria from the biofilm, such as β -lactamase¹⁴⁸ and aminoglycoside modifying enzymes^{149,150}. The effect of β -lactamase in biofilms is different from that in planktonic bacteria because the accumulation of enzymes in the biofilm matrix is sufficient to prevent any penicillin antibiotic penetration¹⁵¹. The eDNA in the biofilm matrix can also change the extracellular environment by chelating cations (such as magnesium ions). As an example, the reduction of Mg^{2+} leads to the synthesis of spermidine which can promote the protection of bacteria by reducing the permeability of aminoglycosides and other positively charged antibacterial agents¹⁵².

3.3.2 Role of the slow metabolism

The concentration of nutrients inside the biofilm presents a gradient. Bacteria on the surface have a quite easy access to nutrients and oxygen and so, their metabolism is relatively active.

In contrast, the deep-layer bacteria in the biofilm are primarily in a dormant or sub-dormant state with slow metabolism because they are in a microenvironment with nutrient deficiency, low oxygen, and large amount of metabolic wastes. As the growth rate is a critical factor in the susceptibility of bacteria to many antimicrobial agents, the inactive bacterial division of deep-layer bacteria may be the reason for the limited efficacy of some antibacterial drugs, called "drug indifference" by Jayaraman et al.¹⁵³. Studies have shown that the metabolism of *S. aureus* cells embedded in the deep layer of biofilm is slow, increasing the tolerance to bactericidal drugs¹⁵⁴. The oxygen gradient inside the biofilm makes the center of the deeper biofilm produce an anaerobic environment again favoring a lower metabolic activity of deep layer bacteria¹⁵⁵. Indeed, relevant studies have proved that the biofilms treated with multiple antimicrobial agents are more persistent under anaerobic conditions than the aerobic control treated with the same treatment¹⁵⁶.

3.3.3 Role of oxidative stress

In microbial metabolism, oxidative stress is produced under various environmental stresses, such as high and low temperature environments, ionizing and non-ionizing radiation environments. These factors lead to the formation of reactive oxygen species in cells (for example, free radicals, peroxide, and nitric oxide)¹⁵⁷. Oxidative stress is essentially due to the disturbance of the balance between reactive oxygen species and scavenger systems, leading to the accumulation of reactive oxygen species in cells¹⁵⁸. When reactive oxygen species concentration is too high, it causes damages to cell components including protein, DNA, and lipids and increases the mutagenic rate and cell death¹⁵⁵. Studies by Boles et al. showed that the endogenous oxidative stress of bacteria in biofilms could generate genetic diversification in biofilms by up regulating several genes related to oxidative stress such as the regulator of superoxide response SoxS¹⁵⁹. For exogenous oxidative stress, the biofilm matrix with complex composition and structure cannot prevent reactive oxygen species to enter the biofilm. However, biofilm matrix can slow down the metabolism of cells to limit endogenous oxidative stress¹⁶⁰. Walters et al.¹³ proved that the biofilm matrix could decrease the diffusion of exogenous active oxygen inside the biofilm so that the

bacteria in the center of biofilm have enough time to adapt and remove different forms of active oxygen.

4. *S. aureus* biofilm matrix

In *S. aureus* biofilm, poly-N-acetyl- β -(1–6)-glucosamine (PNAG; is also called polysaccharide intercellular adhesin PIA) helps biofilms form and enables bacteria to gain protection from the host immune system^{161,162}. Adhesion to the surface and initiation of biofilm formation¹⁶³ are related to the expression of numerous proteins, such as cell wall-anchored (CWA) proteins, phenol soluble modulins (PSMs) and recycled cytoplasmic proteins found in both methicillin-susceptible *S. aureus* (MSSA) and methicillin-resistant *S. aureus* (MRSA) isolates¹⁶⁴. The third main component, the extracellular DNA (eDNA) released from cells through controlled autolysis, is considered crucial for stabilising the structure of *S. aureus* biofilms¹⁶⁵.

4.1 Polysaccharides (PIA/PNAG)

4.1.1 Structure and function of PIA/PNAG

In 1996, Mack et al.⁹⁵ first isolated a specific polysaccharide antigen named PIA/PNAG from the biofilm of *S. epidermidis*. After that, PIA/PNAG also has been detected in various pathogens, including Gram-positive and Gram-negative bacteria, fungal pathogens, and protozoa¹⁶⁶. In *S. epidermidis*, PIA comprises at least 130 β -1,6-linked 2-deoxy-2-amino-D-glucopyranosyl residues, which include 80 to 85% N acetylated (polysaccharide I), and the rest part are non-N-acetylating and with positively charged. Polysaccharide II is an anionic portion with a lower content of non-N-acetylated D-glucosaminy residues and comprises phosphate and ester-linked succinate. *S. aureus* also produces polysaccharide-dependent biofilms, and the exopolysaccharide components, which have identical structures as PIA from *S. epidermidis*, were given the name of PNAG by Maira-Litran et al.¹⁶¹. The structure of PIA/PNAG polymer is shown in Fig. 12. About 10 to 20% of the amino groups are not acetylated, which could bring a net positive charge to some polymers in the polysaccharide complex that constitute an important feature for intercellular adhesive properties^{167, 162}.

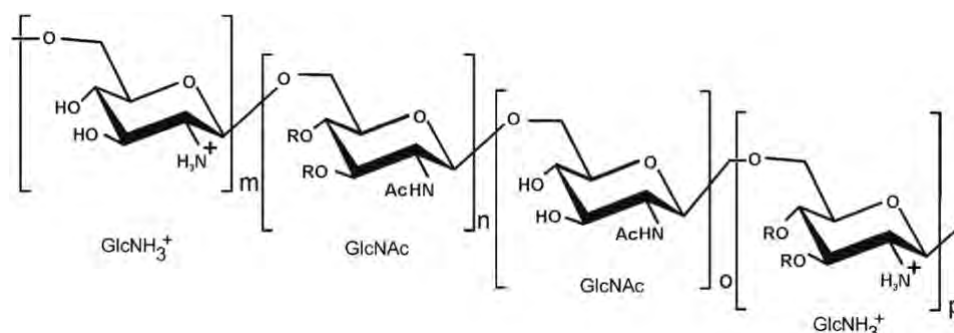


Figure 12 The structure of PIA/PNAG
(adapted from Rohde et al.¹⁶⁸)

About 10-20% of residual are deacetylated (GlcNH³⁺). R is indicated ester-linked succinate introducing negative charges into the polymer.

PIA/PNAG plays an important role in maintaining the stability of biofilm matrix structure by enhancing the intercellular adhesion between *S. aureus* cells in biofilm¹⁶⁹. Notably, the production of PIA/PNAG is crucial to the virulence in animal infection models^{170, 171}. The secreted form of PNAG can act as a trick by inducing the binding of potential opsonin antibodies away from the bacterial surface, thereby preventing the mediation of killing effect¹⁷². Deacetylation of N-acetylglucosamine residues in PIA/PNAG has essential biological significance. Releasing free amino groups charged at neutral or acidic pH introduces positive charges into neutral molecules, such as those found in human skin¹⁷³. Owing to the negative charge on the surface of bacterial cells, the cationic characteristics PIA/PNAG enable it to bond cells together through electrostatic interaction. For example, teichoic acid has a negative charge which can interact with the positively charged cell surface¹⁷⁴. So, PIA enables bacteria to effectively change their electrostatic characteristics of cell surfaces, leading to drug resistance to antibacterial peptides, which are usually cations¹⁷³.

4.1.2 Regulation of PIA/PNAG synthesis

PIA/PNAG is a product regulated and synthesized by the *ica* locus⁹⁵. Transforming the *ica* locus from wild-type *S. aureus* to *ica* knockout mutant strains can make them have a strong biofilm production again¹⁷⁵. The *ica* operon (Fig. 13) is located on the bacterial chromosome, including four functional genes of *icaADBC* connected in series^{176,177}. *In vitro* experiments have shown that

IcaA is the actual N-acetylglucosaminyl transferase, but its activity is weak. The *icaD* gene is located between *icaA* and *icaB*, and the encoded IcaD is an independent membrane-binding protein similar in size to the IcaA. The glycosyltransferase activity is fully activated and N-acetylglucosamine oligomer is formed only when *icaA* and *icaD* are co-transcribed¹⁷⁸. The product of *icaC* is a hydrophobic membrane protein with six predicted transmembrane helices¹⁷⁹. It is involved in the chain growth and externalization of the nascent polysaccharide¹⁸⁰. IcaB on the surface of the bacteria is a deacetylase that participates in the export of PIA/PNAG after the deacetylation process. The introduction of positive charge into PIA/PNAG by deacetylation changes the surface charge of bacterial cells, thereby increasing the persistence of biofilm in presence of antimicrobial peptides or phagocytic neutrophils¹⁷³. IcaR is a DNA binding protein encoded by *icaR*, which specifically binds to the *ica* promoter upstream of *icaA* to regulate the expression of *icaADBC* negatively¹⁷⁵. In *S. aureus*, IcaR had shown a weaker effect on its own expression, and its negative regulatory effect on the expression of the *icaADBC* operon is not significant. However, the regulatory effect of IcaR depends on the up-regulation of *icaR* expression by SarA and SigB¹⁸¹ with differential regulation between strains. The author tried to explain this strain specificity among *S. aureus* by biofilm productivity due to different *agr* types¹⁸².

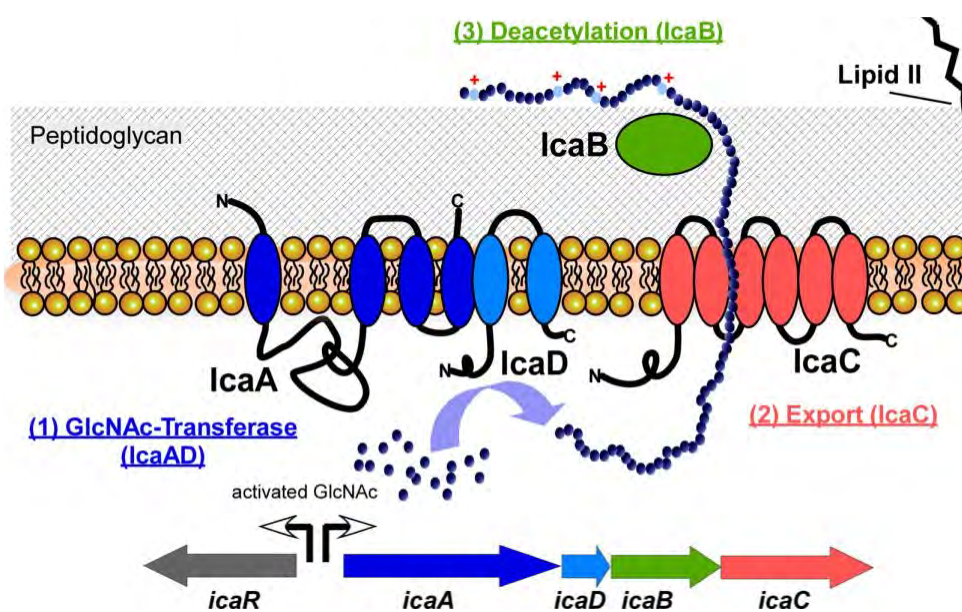


Figure 13 *ica* operon and regulation
(adapted from Otto Micheal⁸)

Some studies have demonstrated that the formation of biofilm *in vitro* and *in vivo* can be constructed in a form that is independent of *ica*. The *in vitro* formation of MRSA biofilm studied by Fitzpatrick et al.¹⁸³ found that the response of *icaADBC* to environmental changes in these clinical isolates did not consistently affect biofilm formation. For the laboratory strain BH1CC grown in BHI, BHI-NaCl, and BHI-glucose or the deletion of the *ica* locus did not result in a biofilm-negative phenotype. The three *S. aureus* Δ *ica::tet* mutants (BH1S, BH1P, and BH1Y) also presented a glucose-induced *ica*-independent biofilm phenotype. For the *S. aureus* isolated from the prosthetic joint infections after total hip arthroplasty and total knee arthroplasty, PNAG and proteins such as *S. aureus* surface protein G, SasG work synergistically in biofilm formation¹⁸⁴. The roles of these two factors in cell-cell adhesion of *S. aureus* were parallel¹³⁴.

The formation of PIA independent biofilm comprised other surface proteins, including SasC, protein A, fibronectin-binding proteins FnBPA and FnBPB, clumping factors A and B, the fibrinogen-binding protein Fib, and autolysins AtlA/E¹⁸⁵ will be introduced in detail later.

4.2 Cell Wall Anchored (CWA) proteins

Biofilm matrix of *S. aureus* is mainly composed of polysaccharides, proteins, teichoic acid, and eDNA. According to the composition of the EPS matrix, *S. aureus* biofilms can be divided into two classes: biofilms mainly constituted of polysaccharide matrix and biofilm mainly composed of a proteinaceous matrix^{186,187}. *S. aureus* can express various virulence factors and among them, there are the surface proteins covalently adherent to peptidoglycan, which are called cell wall anchoring (CWA) proteins¹⁸⁸. The expression of these surface proteins allows pathogens to attach to the surface and to initiate biofilm formation. CWA proteins have a Sec-dependent secretion signal sequence at the nitrogen terminal and an 'LPXTG' motif at the carbon terminal. The secreted proteins are cleaved by membrane-bound transpeptidase, Sortase A (SrtA), which catalyzes the attachment of the processed protein to cell wall peptidoglycan¹⁸⁹. Foster et al.¹⁶³ proposed that CWA proteins could be classified into four groups according to the existence of motifs determined by structure-function analysis: microbial surface component recognition

adhesive matrix molecule (MSCRAMMs), the near iron transporter motif family (NEAT), the tandemly repeated three-helical bundles (protein A), and the G5–E repeat family.

In bovine mastitis, CWA proteins of *S. aureus* can adhere to other cells such as mammary epithelial cells, endothelial cells, and fibroblasts, which make bacteria easily invade the host cells¹⁹⁰. This ability to invade host cells increases with the increase of the number of bacteria¹⁹¹. CWA proteins, which promote the biofilm formation of *S. aureus*, will be described below.

4.2.1 Microbial Surface Component Recognition Adhesive Matrix Molecules

In 1994, Patti et al.¹⁹² originally defined the cell surface adhesins that mediate microbial adhesion to the EPS matrix of host tissues as MSCRAMMs, which include the fibrinogen binding proteins (Fib, ClfA, and FbpA), the fibronectin binding proteins (FnbA and FnbB), the collagen binding adhesin (Cna), Serine–aspartate repeat protein (SdrC, SdrD, and SdrE) and elastin binding protein (EbpS)¹⁹³. The molecules classified as MSCRAMMs should have the following characteristics. First, the molecules must be located on the surface of microbial cells. Second, they must recognize a macromolecular ligand that can be found within the extracellular matrix (ECM) of the host's tissues. The ligand is a component of ECM and can be collagen, laminin, fibronectin or fibrinogen. Finally, the interaction between MSCRAMMs and extracellular matrix ligands should have high affinity and high specificity meaning that unrelated molecules cannot significantly interfere with the interaction between MSCRAMMs and ligands¹⁹².

In the process of the infection of the host, the adhesion of *S. aureus* to the tissues of the host is an essential factor to initiate the infection, which is mainly divided into two stages: reversible adhesion and then irreversible adhesion^{194,195}. Although various environmental factors influence this process, MSCRAMMs adhesion to matrix components play an important role^{196,192}. In the following, we will more deeply described the structure and function of some important proteins in MSCRAMMs.

4.2.1.1 Fibronectin binding protein (FnBPA/FnBPB)

Components in human plasma, such as fibrinogen and fibronectin, contribute to the adhesion of *S. aureus* to human cells, thereby enhancing the formation of biofilm and causing biofilm-related infections, such as the adhesion of *S. aureus* to medical devices mediated by human plasma^{197,198,199}. The interactions between *S. aureus* and human plasma components is thus, dependent for most of them, on the expression of fibronectin binding proteins FnBPA and FnBPB²⁰⁰. Their two closely-linked coding genes (*fnbA* and *fnbB*) show tremendous sequence homologies but are independently transcribed²⁰¹.

FnBPA and FnBPB have domains linked to specific proteins that promote bacterial accumulation^{199, 202}. The domain organizations of FnBPA and FnBPB (*S. aureus* 8325-4, Fig. 14) contain a secretion signal sequence at the N-terminus and a sortase pentapeptide recognition motif LPETG located at C-terminus. The sortase-mediated protein anchoring to the cell wall peptidoglycan requires a LPETG motif²⁰³. The N-terminal A domains of FnBPA and FnBPB fold into three sub-domains (N1, N2, and N3) similar to the A domain of clumping factor A (ClfA), which bind to fibrinogen²⁰⁴. Unlike ClfA, the A domains of both FnBPs also bind to immobilized elastin²⁰⁵. Among these N-terminal A domains, N2 and N3 combine with fibrinogen through the change of DLL (dock-lock-latch) mechanism to form a highly stable complex²⁰⁶. The D-domains of FnBPA and FnBPB are almost identical, are close to the cell wall spanning domain, and are usually regarded as the primary domain interacting with fibronectin²⁰⁷.

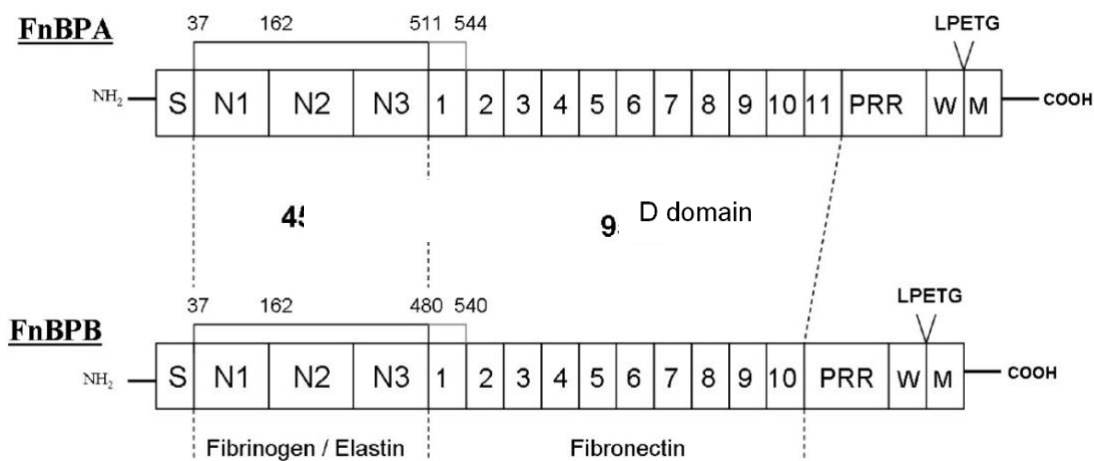


Figure 14 Schematic representation of FnBPA and FnBPB structural organization
(adapted from Bruke et al. ²⁰³)

The figure indicates the positions including a signal sequence (S), the A domain for fibrinogen and elastin binding (N1, N2, N3), and the fibronectin binding motifs (D domain). At the C-terminus are proline-rich repeats (PRR), cell wall (W), and transmembrane region (M). The LPETG motif is necessary for covalent attachment to the cell wall peptidoglycan.

In vitro experiments showed that FnBPA and FnBPB are essential factors in the initiation of *S. aureus* infection, which not only mediate bacterial attachment and binding to fibronectin but also help *S. aureus* to adhere to thrombus and to stay in contact with host cells for a long time^{208, 209}. In fact, *S. aureus* first binds to target proteins (including fibronectin) and reaches saturation of ligand binding sites before forming a biofilm on tissue or instrument surfaces^{210, 206}. In the presence of glucose, FnBPs have been proved to be part of bacterial membrane protein components. Under osmotic stress, bacteria can produce PIA/PNAG-dependent biological membranes²¹¹. This indicates that *S. aureus* can regulate metabolism, from producing protein to producing exopolysaccharides in the biofilm matrix, to adapt to external conditions. The research on MSCRAMMs of *S. aureus* has led to the emergence of more effective immunotherapy and preventive therapies²¹². For example, FnBPA and FnBPB can be used as target proteins for passive immunotherapy in the treatment of *S. aureus* infection²¹³.

4.2.1.2 Serine aspartate repeat protein (SdrP)

SdrP family is similar in structure to other surfaces covalent binding proteins, and its most prominent feature is that it contains a serine aspartate repeat region²¹⁴. ClfA and ClfB in this family

are present in the cell wall of *S. aureus* and both have the function of making bacteria adhere to matrix and lectin containing fibrinogen^{215, 216}. ClfA and ClfB have a cell wall spanned R domain located in A domain and include many serine-aspartic dipeptide repetitive sequences. The A domain binds to the ligand and depends on the support of the R domain²¹⁶. In the formation of *S. aureus* biofilm, when other matrix components such as poly- β (1,6)-acetyl-d-glucosamine (PNAG) or eDNA become ineffective, ClfB can promote the formation of biofilm *in vitro* in the absence of Ca^{2+} ²¹⁷. In the murine model, even though *S. aureus* expressing ClfB are less pathogenic than *S. aureus* expressing ClfA, both can cause endocarditis^{218, 219}. ClfA can also prevent phagocytosis of *S. aureus* by macrophages and can bind partial fibrinogen molecule. As other example, ClfB makes *S. aureus* adhere to the nasal cavity, which leads to the exfoliation of nasal epithelial cells. The above process is crucial for the proliferation of *S. aureus* in the nasal cavity and can mediate the binding process between ClfB and keratin on the surface of squamous cells⁹⁷.

SdrC, SdrD, and SdrE in *S. aureus* were identified for the first time by Josefsson et al.²¹⁴ in 1998 and also belong to Sdr proteins. Compared to ClfA and ClfB, the A domain of SdrC, SdrD, and SdrE are similar in length^{214, 220}, but the amino acid sequences of the A domain of ClfA and ClfB is very different²¹⁶. The only common feature in the amino acid sequence of the A domain between Clf and Sdr proteins (namely ClfA, ClfB, SdrC, SdrD, and SdrE) is that they all contain the TYTFTDYVD sequence. Therefore, the properties and functions of these three Sdr proteins are different. SdrC protein promotes *S. aureus* to adhere and colonize nasopharyngeal epidermal cells²²¹; SdrD protein is necessary for the formation of an ulcer in *S. aureus* infected host²²²; while SdrE protein helps *S. aureus* to escape the host immune response by capturing factors regulating complement²²³ and also independently induce platelet aggregation. Besides, SdrC, SdrD, and SdrE also have a B repeat region with 110-113 amino acid residues between the A and R domains²¹⁴. The repeat region of B contains EF-hand ring, and its affinity for calcium ion is higher than that of ClfA and ClfB in the A domain²¹⁴.

Bone sialoprotein-binding protein (Bbp) is also classified into the Sdr protein family because of its serine aspartate repeat sequence²¹⁴. Bone sialoprotein is a kind of non-collagen protein that is often found in bone tissue and dentin and abundant in newly formed bone tissue^{224, 225}. *S. aureus*

can selectively bind bone sialoprotein through Bbp, resulting in osteomyelitis or suppurative arthritis^{226,227}.

4.2.1.3 Collagen adhesin (Cna)

Collagen-binding adhesin Cna, a member of MSCRAMMs, has been proved to be necessary for *S. aureus* to bind collagen-rich matrix cartilage²²⁸. The collagen binding site of Cna is in the A domain, followed by the B domain with 187 amino acid repeats¹⁹². In different isolates of *S. aureus*, the repeat structure of the B domain varies from 1 to 4^{229, 230}. Cna can block part of the complement pathway activation²³¹ and has been proved to be a virulent factor in many different animal models of *S. aureus* infection, including osteomyelitis²³², septic arthritis²³³, endocarditis²³⁴, and mastitis²³⁵. Also, Nilsson et al.²³⁶ used recombinant Cna as an effective component of the vaccine and found that the antibodies secreted against Cna have protective effects in a murine model of *S. aureus* induced sepsis.

4.2.2 Staphylococcal protein A (SPA)

In the biofilm of *S. aureus*, a multifunctional CWA protein is called protein A. Protein A contains five homologous antibody binding domains at the N terminus²³⁷. Each domain is composed of a single independently folded triple helix that can bind multiple ligands¹⁸⁶. As a multifunctional virulence factor, protein A can bind to the Fc region of immunoglobulins and promote *S. aureus* immune evasion. Studies have also shown that protein A in renal epithelial cells can bind to actin, thereby indirectly assisting the invasion of pathogens^{238, 239}. Merino et al.'s²⁴⁰ experiments showed that whether *S. aureus* overexpresses protein A or adding an appropriate amount of soluble protein A to the culture medium can provoke bacterial aggregation and biofilm formation. Beside its role in biofilm formation, sequencing of a specific section of the *spa* gene is a widely used method of *S. aureus* typing.

4.2.3 Biofilm-associated protein (Bap)

Among the CWA proteins involved in the formation of biofilms, Bap is a surface protein with multi-domain characteristics which contains 13 repeats of 2276 amino acids and 86 residue sequences²⁴¹. The *bap* gene is mainly found in cattle from chronic mastitis associated to *S. aureus*²⁴². Bap promotes the initial attachment of bacteria to biological and non-biological surfaces through a mechanism independent of extracellular polysaccharides²⁴³. For *S. aureus*, the lack of Bap protein expression induces the formation of smaller and smoother colonies, the decrease of cell adhesion and the impairment of biofilm adhesion to abiotic surfaces^{244, 245}. The C-terminus of Bap contains a distinct cell wall attachment region containing the LPETG motif²⁴³. Taglialegna et al.²⁴⁶ researches have shown that the N-terminal region of Bap is delivered into the extracellular medium and assembled into amyloid-like fibers. In contrast, part of the C-terminal repeat region is anchored in the envelope. The number of repetitive sequences in the central region may differ in different species and isolated strains, leading to different extended structures and protein variants, which may help bacteria evade the host immune response^{247, 244}. During mastitis, Bap can enhance the adhesion of *S. aureus* to epithelial cells, thereby promoting its persistence in the udder. Bap can also bind to host receptors to prevent cell internalization, whereby interfering with the FnBPs mediated invasion pathway²⁴⁶. In general, Bap exhibits a dual function by mediating both interactions between bacteria themselves and between bacteria and host. Besides, Bap contains four EF-hand motifs (PS00018) that can bind to calcium ion, which suggests that calcium may inhibit *S. aureus* mediated biofilm formation by inducing conformational changes^{243,248}. Indeed, the mutation of two EF-hand motifs can eliminate the inhibitory effect of calcium ion on biofilm formation²⁴³. Calcium also can lessen the thickness of *S. aureus* biofilm²⁴⁸. Taglialegna et al.²⁴⁶ proved that under low pH and low calcium concentration, the fragments of Bap containing at least the N-terminus can self-assemble into amyloid-like structures. The self-assembly process of Bap fragments can be interrupted by binding of cations. These findings define that Bap could be a sensor and could, as a scaffold protein, promote biofilm production under particular environmental conditions.

4.3 Extracellular DNA in biofilm

Depending on the strain and the environment, the content of each biofilm matrix component varies and the content of eDNA can be as high as 15%²⁴⁹. eDNA is one of the crucial components in EPS, and its relevance to biofilm formation was first proposed by Whitchurch et al.²⁴ in 2002. The negative charge of eDNA polymer can be used as an electrostatically charged polymer to immobilize cells on the surface of materials or in a host²⁵⁰. Also, the presence of eDNA on the surface of bacteria could significantly enhance its adhesion force to a hydrophobic surface (more than to a hydrophilic surface), especially after bond maturation and at high ionic strength²⁵¹. It indicates that eDNA may be the main factor that triggers the initial biofilm formation under specific environmental conditions.

There are many ways to release eDNA, including cell lysis²⁵², active secretion²⁵³, regulation of release²⁵⁴ through a quorum sensing system, induction of bacteria to form a competent cell to release²⁵⁵, or secretion of vesicles through live cell secretion and transport²⁵⁵. In *S. aureus*, a murein hydrolase regulator (*cidA*) triggers bacterial cell death and lysis and thereby contributes to the eDNA releasing and biofilm development.²⁵⁶ eDNA not only participates in the initial adhesion of biofilms, but also participates in the structural maintenance of mature biofilms. In 2008, Izano et al.²⁵⁷ observed that the addition of DNase I to a *S. aureus* culture can inhibit the formation of biofilms and disperse the biofilms that have formed on polystyrene plates. The study of Puga et al.²⁵⁸ showed that after adding DNase I to mature biofilms alone, both the eDNA content and the protein content decreased significantly. After protease alone was added, similar results also appeared, indicating that in mature *S. aureus* biofilms, eDNA depends on the protein in the matrix to bind to cells²⁵⁴. This also proves that eDNA interacts with other molecules in the biofilm matrix to form a connecting scaffold between different components to further increase the structural stability of mature biofilms^{259, 260}. Because eDNA polymers have a negative charge, they have high ability to chelate cations. Therefore, eDNA can directly protect bacteria from the attack of antimicrobial peptides by chelating cationic antimicrobial peptides secreted by the host immune system^{260, 261}.

A large amount of eDNA found in bacterial biofilms also constitutes a dynamic gene pool, especially in the biofilms of strains with multiple characteristics^{262, 260}. This "dynamic gene pool" is beneficial to enhance horizontal gene transfer, thereby promoting the transfer and subsequent spread of antimicrobial resistance genes.

4.4 Other components in biofilm matrix

Phenol soluble modulins (PSM) are a group of secreted peptides that play an essential role in the formation and stabilization of *S. aureus* biofilm⁹⁶. In addition to proteases that can drive the degradation of the biofilm matrix in biofilm decomposition, PSMs are also an important factor in triggering the destruction and remodeling of the biofilm^{250, 263}. PSMs are amphiphilic and have properties similar to surfactants, so they can trigger the dispersion of biomolecules by disrupting the non-covalent interactions between matrix components^{264, 265}. The presence of nucleases and PSMs is a critical factor in forming the three-dimensional structure of *S. aureus* biofilm^{263, 264}. They are involved in forming mature biofilm channels and tower-like structures so that nutrients can be transported to the deep layers of the biofilm²⁶⁶. In a murine model of biofilm related infection, the preformed biofilm on the central venous catheter spreads to the secondary infection site due to the dispersion after addition of PSMs derived from *Staphylococcus* spp. on the biofilm²⁶⁷.

Teichoic acid is a sugar polymer that can be anchored to the cell wall through covalent bonds or to the cell membrane through lipid anchoring (lipoteichoic acid). It promotes the formation of staphylococcal biofilms^{268, 269}. Noticeably, teichoic acid helps the colonization of bacteria on abiotic surfaces, such as polystyrene and glass. Their net negative charge mediates surface adhesion of biofilm²⁶⁸.

Two peptidoglycan hydrolases, AtlA and Sle1, are involved in the cell separation of *S. aureus*. Autolysin AtlA is a bifunctional murein hydrolase with amidase and glucosaminidase activities²⁷⁰ that directly participate in the autolysis of *S. aureus* contrary to Sle1^{271, 272}. Although AtlA cannot act alone as an adhesin to promote the initial adhesion of bacteria²⁷³, it can still enhance the structure and formation of the biofilm matrix by degrading cell walls and releasing DNA²⁵⁶.

5. *S. aureus* biofilm infections and treatments

5.1 *S. aureus* biofilm in chronic infections

In 1982, Marrie et al.²⁷⁴ firstly used a scanning electron microscope to study the intracardiac part of pacemaker lead taken out from patients with recurrent *S. aureus* bacteremia. They found that the metal tips, inner surfaces, and inner wires were covered with a thick layer of bacterial biofilm. Despite intensive antibiotic treatment, these cells seemed to be intact. Many studies on device-associated infections further supported this observations^{275, 276, 277,278}.

Biofilm is also considered clinically significant in veterinary medicine³², by being involved in many animal disease²⁷⁹, such as equine wounds²⁸⁰, bovine mastitis²⁸¹, periodontal infection²⁸², otitis²⁸³ and gastrointestinal infections²⁸⁴. These biofilm infections have not only become a costly complication of health care²⁸⁵, but also caused a substantial economic loss in the production of livestock industries²⁸⁶. Mastitis is the most common disease in dairy cows, which has recognized harmful effects on animal well-being and profitability of dairy farms²⁸⁷. It leads to the decline of milk yield and quality, the increase of antibacterial drugs consumption, and the increase of veterinary costs²⁸⁸. *S. aureus* is one of the most common pathogens isolated from mastitis cases²⁸⁹. It adheres to the mammary gland epithelium in the primary step of the pathogenesis of mastitis. After about 24 h, the initial biofilm is formed and the bacterial clusters appear in the mammary alveoli, lactiferous ducts, and interstitial tissue²⁹⁰. The development of biofilm contributes to their persistence in the udder and leads to chronic infection²⁹¹. Although bovine mastitis has existed since 3100 BC²⁹², its overall cure rate is still disappointing. Epidemiological studies have reported the high uncertainty around the cure rates ranging from 0 to 80% that can be obtained by treating *S. aureus* mastitis by antibiotics^{293,294}. When mastitis develops into a chronic infection (with biofilm) after antibacterial treatment, udder health remains unsatisfactory in most cases, and the somatic cell count either keeps rising or a second increase is detected after the initial decline^{295, 288}. So, the culling of the cow is often the most favorable economic choice.

5.2 Current treatments of biofilm infections

The challenges for clinical treatment of biofilm infections rely on two main problems. First, it is tough to make timely clinical diagnoses of biofilm infections due to their high rate of false negative culture result, especially in the early stage of infection²⁹⁶. Second, biofilm bacteria can escape active host defenses and are highly resistant to conventional antibiotic therapies²⁹⁷, bringing significant challenges to the clinical cure of biofilm infections. In many cases involving infections of medical devices and damaged tissues^{298,299}, biofilm infections are diagnosed by experienced clinicians facing typical symptoms, while traditional diagnostic methods can sometimes not effectively detect microorganisms that cause infections due to negative culture results³⁰⁰. Rayner et al.³⁰¹ detected bacterial mRNA from otitis media effusion by RT-PCR in 1998, which proved that pathogens were present in the patient's ear exudate in a viable state even when the results of culture were negative. Later, Hall-Stodley et al.³⁰² also confirmed this observation through an electron scanning microscope. Agar plate culture technology has a history of nearly a hundred years, and it is the only laboratory technology approved by the US Food and Drug Administration to detect and identify bacteria that cause human infections. Nevertheless, this diagnostic methodology can only detect one or two out of dozens of bacteria (in an ideal state) that can be present, as an example, in an infected wound in plastic surgery³⁰³. In cases of infection around the prosthesis, the sensitivity of culture is only about 20%³⁰⁴ highlighting the challenge to accurately isolate and identify biofilm bacteria from deep tissues³⁰⁵. One explanation for this false-negative phenomenon is that prolonged use of antibiotics kills the planktonic bacteria and selects bacteria with slow growth³⁰⁴. Thus, a negative culture result does not indicate that there is no pathogen in the biofilm and conversely, the detection of bacteria by culture only means that they are mainly present in the infected tissue in a planktonic state. To further improve the accuracy of bacterial detection in biofilm infections, some new molecular biological detection methods have gradually begun to be used for clinical diagnosis of microbial infections such as PCR, 16S deoxyribonucleotide sequencing Technology (16S rRNA), denaturing gradient gel electrophoresis and peptide nucleic acid fluorescence in situ hybridization technology^{306,307,308}. However, due to their complicated operation and high price, their use in clinical practice remains rare.

Due to late diagnoses of biofilm infection and persistence of bacteria in biofilm, patients with biofilm infection usually have to receive high dose of long-term antibiotic treatment with a high risk of recurrence of infection. For most infections caused by bacteria, the antibiotic concentration must be few times greater than MIC to have an effect but to inhibit or kill bacteria in mature biofilms, the concentration of antibiotics must usually be 100-1,000 times the MIC^{309,99}. If the biofilm infection is not handled correctly, it may continue for months or even years²⁹⁷. As an example, Birolini et al.³¹⁰ reviewed patients who had experienced chronic mesh infection related to the formation of a biofilm in the past 16 years³¹¹. Intraoperative care to solve these infections were mandatory to remove the infected mesh, other foreign objects and fibrotic tissue because biofilm bacteria easily hide in them. These patients also usually required long-term follow-up (71% of patients undergoing hernia surgery are followed for more than three years) to monitor any recurrence of chronic infection after mesh replacement³¹⁰.

Catheter-related bloodstream infections (CRBSIs) have high morbidity and mortality rates, and is one of the most common causes of bloodstream infections³¹². In 1992, Passerini et al.³¹³ used scanning electron microscopy and transmission electron microscopy and concluded that biofilms were widely present in all infected central venous catheters (CVC). After insertion of CVC, the host-derived proteins quickly encapsulate the intravascular part of the device. These proteins then promote the adhesion of microorganisms on the tube wall and the formation of biofilms³¹⁴. The gold standard for the treatment of CRBSI is catheter removal and systemic antibiotic therapy³¹⁵. The replacement of CVC has a high mechanical risk and increases the risk of bloodstream infection³¹⁶. Patients with septic thrombosis or endocarditis need to receive antibiotics for 4-6 weeks after removing the catheter or device and in case of osteomyelitis, the antibiotic treatment should last 6-8 weeks after catheter removal³¹⁷. For some exceptional cases, such as long-term dialysis catheters, conservative treatment is recommended. For CVC that cannot be removed, antimicrobial lock therapy (ALT) can be considered. It consists in instillation of a highly concentrated antibiotic solution (up to 1,000 times the MIC) into the catheter for 12 to 24 hours. ALT combined with systemic antibiotic therapy had a success rate of 82.6% in 167 CRBSIs³¹⁸. However, this therapy is not ideal for some pathogens (including fungi and bacteria)^{319,320}.

Periprosthetic joint infection (PJI) is a potential complication of total joint arthroplasty. Its overall incidence is not high (approximately 1-2%), but the affected people will experience repeated pain and significant mobility loss³²¹. About 65% of PJIs are believed to be caused by bacterial biofilm formation in the joint space³²². The biofilm makes the sensitivity of synovial culture and even biopsy minimal (45-100%), especially if the patient has previously received antibacterial therapy, or if the infection is delayed after surgery. Trampuz et al.³²³ discovered that the treatment by ultrasonic of removed prosthesis (including screws and inlays) can improve detection sensitivity. When the PJI infection develops into a chronic infection (more than six weeks after the initial surgery or the blood-borne event), the failure rate, when implant is not removed, is unacceptable. It is because biofilm bacteria remaining on the implant cannot be entirely removed. So, the treatment of chronic PJI usually consists in one-stage exchange or two-stage exchange treatment strategies, i.e. a new prosthesis is re-implanted in the same or in a delayed procedure³²⁴. No antimicrobial therapy should be proposed for patients who need a two-stage exchange before tissue culture or ultrasonic liquid culture is obtained³²⁵. The objective is to reduce the incidence of false-negative culture results. For staphylococcal PJI, pathogen-specific intravenous antimicrobial therapy is recommended for six weeks after total joint replacement, followed by oral rifampicin and an adjuvant drug (ciprofloxacin or levofloxacin) twice a day for three months³²⁶. The two-stage exchange is the gold standard for PJI care; however, this method is critical to the patient's condition, and repeated operations will damage the bones and muscles to a certain extent, leading to poor postoperative function. If the patient's condition is not compatible with surgery, antibiotics can be used alone for a long time to, at least, control clinical symptoms. Once antibiotics are stopped, most patients (>80%) have a recurrence of infection³²⁶.

There is no specific strategy for clinical treatment of animal biofilm infections and the treatment is usually based on experience or learning from the human-related infections. Although many classes of antibiotics available in human medicine are also used to treat animals, there are not many antibiotics that can be selected to treat animal biofilm infections due to limited access to last-line antibiotics in veterinary medicine. For example, rifampicin can penetrate biofilms and has a high diffusion rate in host cells and bone tissues. It also has bactericidal activity against *S. aureus* in the stable growth stage. Thus, rifampicin combined with other antibiotics is often

recommended for the treatment of staphylococcal PJIs^{327,328}. It could potentially be use with success in similar biofilm-related infections in dogs but in France, the use of rifampicin is forbidden for dogs since 2016³²⁹.

Due to the particular structure of the mammary glands of bovine, treating bovine mastitis is utterly different from human mastitis and is often managed by intramammary administration of drugs³³⁰. This method consumes fewer quantities of antibiotics than the systemic administration and leads to very high local concentrations of antibiotics in the udder. The concentration of penicillin G in milk after intramammary administration can reach 100-1,000 times the concentration after systemic (parenteral) administration^{331,332}. To achieve a good efficacy on pathogens, the drugs should also be distributed throughout the udder after administration^{333,334}. However, due to udder inflammation or debris blocking the milk ducts, intramammary administration often does not evenly distribute the drug in the udder³³⁵ and the effects could be not as good as expected. Systemic treatments can also be implemented in case of mastitis. With these treatments, drugs need to pass through the blood-milk barrier³³⁶ by passive diffusion or active transport to effectively reach the infected lesions. The process depends on many factors, such as lipid solubility and protein binding of the drug³³⁷. None of the veterinary antibiotics has excellent pharmacokinetic and pharmacodynamic characteristics when administered systemically to treat bovine mastitis. Even the commonly used broad-spectrum antibiotics (such as oxytetracycline) are challenging to produce and maintain effective therapeutic concentrations in the udder³³⁸, and these antibiotics have little effect on the biofilm formed in the udder. Due to the limitations of available antibiotics, animal biofilm infections urgently need the implementation of new therapeutic strategies such as the development of non-antibiotic substances with anti-biofilm effects that could be used as auxiliary drugs.

5.3 New therapeutic strategies of biofilm infections

The existing clinical treatments have a limited effect on the inhibition and elimination of biofilms. This is not only due to the rapid increase of antibiotic resistance of pathogens³³⁹, but also due to the complex tissue structure of biofilm matrix and the high tolerance of biofilm bacteria to

antibacterial agents and disinfectants^{340,341}. In the previous section, we described the formation and composition of *S. aureus* biofilm and the current research on new strategies for the treatments of biofilm-related infections is also divided into prevention of biofilm formation and damage to the established biofilms. However, in clinics, the diagnosis of biofilm infection is extremely challenging and often the treatment has to deal with a well-established biofilm on the device or tissue surface. Thus, the development of novel therapeutic strategies probably needs to focus more on targeting established biofilms, including degradation of biofilm matrices, antibiofilm peptides, and dispersion inducers than on prevention of biofilms. In the following parts, we will present some selected strategies non-relying on antibiotics developed in recent years with relevant anti-biofilm effects.

5.3.1 Modified surfaces to prevent biofilm formation

To reduce bacterial adhesion and thus achieve the purpose of preventing biofilm formation, some new materials with adhesion resistance have been developed. Bacterial adhesion decreases when the hydrophobicity of cells and surface materials decreases. Thus, hydrophilic liners made by chemically modified surfaces, are beginning to be used in medical devices. Self-auto assembled monolayers with hydrophilic residues (hydroxyl and NH₂) could significantly reduce bacterial adhesion³⁴² and limit biofilm formation³⁴². The surfaces made of titanium nanotubes can also reduce bacterial adhesion because they are more hydrophilic than conventional titanium surfaces. Besides, the nanotubes can be filled by antibacterial agent that could further enhance the biofilm inhibition³⁴³.

The adhesion on the nanostructured surfaces also depends on the bacterial strains. *Pseudomonas aeruginosa* could not colonize on a super-hydrophobic surface with a nanostructure mimicking a lotus leaf, whereas *S. aureus* had a stronger adhesion on it than on a smooth surface³⁴⁴.

5.3.2 The strategies against mature biofilms

5.3.2.1 Enzymes

Biofilm matrix has complex structural components, including polysaccharides, proteins, eDNA, lipids, RNA, soluble nutrients, and host derivatives²⁶¹. Some biofilm matrix-degrading enzymes have been used to identify the structural components of biofilm matrices²⁵⁷ and they are now tested as new treatments of biofilm. These enzymes can promote the degradation of the established biofilm matrix, thereby dispersing the bacteria from the biofilm to the surrounding environment. In theory, the bacteria released biofilm bacteria should recover their susceptibility to the host immune system and to antibiotics. Therefore, biofilm matrix-degrading enzymes can be recommended to supplement antibiotic therapy to prevent and treat biofilm infection³⁴⁵.

5.3.2.1.1 Glycoside Hydrolases

The initial adhesion and formation of most *ica* dependent *S. aureus* biofilms rely on the secretion of extracellular polysaccharides³⁴⁶. These polysaccharides can maintain the stable structure of the biofilm and protect the bacteria in the biofilm from the host immune system and from the deterioration of the external environment (such as water shortage, lack of chemical reagents and nutrients)³⁴⁷. Therefore, many types of research target the extracellular polysaccharide of biofilm by using different glycoside hydrolases as the treatment for biofilm dispersal.

In vitro tests have shown that commercially available amylase compounds from different sources (human saliva, sweet potato, and *Aspergillus oryzae*) can effectively disrupt *S. aureus* (SH1000) biofilm (a reduction of 77%-89% of the biomass)³⁴⁸. These amylases also showed a significant dispersion effect on the biofilms produced by different *S. aureus* clinical isolates (a reduction of 65%-83%)³⁴⁸.

Dispersin B is a bacterial glycoside hydrolase (42 kDa) targeting PNAG³⁴⁹. Dispersin B is a soluble sugar citrus hydrolase produced by *Actinobacillus actinomycetemcomitans*, specifically cleaving β -1,6-glycosidic bonds of PNAG in the biofilm formed by *S. aureus*. Using 0.72mg/ml

dispersin B can reduce the biomass of *S. aureus* biofilm by 50%. When 0.75 mg/ml of tobramycin was combined with dispersin B, it can reduce biofilm bacteria by 7500-fold compare to the control group³⁵⁰. This enzyme also showed high activity against *S. epidermidis* biofilms but had a limited degradation effect on the biofilms formed by *Burkholderia cenocepacia* and *Achromobacter xylosoxidans*³⁵¹.

Cellulase is an enzyme that can hydrolyze β -1,4 bonds³⁵² and that can partially inhibit *P. aeruginosa* biofilms³⁵³. Fleming et al.³⁵⁴ discovered that α -amylase, cellulase, and a 1:1 solution of α -amylase and cellulase can induce a large dispersion of 48h-old *S. aureus* biofilm. These enzymes were also efficacious to disperse biofilms in a murine chronic wound infection model. Moreover, the addition a 1:1 mixture of α -amylase and cellulase to gentamicin improved the efficacy on biofilm bacteria compared with gentamicin alone³⁵⁴.

Exogenous addition of purified hyaluronidase significantly disperses biofilms containing hyaluronic acid which is a glycosaminoglycan with an average molecular weight of 7,000 kDa, composed of repeating disaccharide units of N-acetylglucosamine and glucuronic acid³⁵⁵. The hyaluronidase secreted by *S. aureus* first cleaves the hyaluronic acid in the host, and the cleaved hyaluronic acid can be integrated into the structure of the biofilm³⁵⁶.

5.3.2.1.2 Proteases

Proteases exist in all organisms and have many physiological regulatory activities. They degrade proteins by catalyzing peptide bond cleavage³⁵⁷. In nature, the protease can exist in intracellular or extracellular locations. Extracellular proteases have low selectivity for substrate recognition and can equally efficiently cleave self and non-self produced molecules. Thus, extracellular proteases are usually present as zymogens or inactive forms to prevent premature proteolytic activity, which is also harmful to the cell itself³⁵⁷. The activation of extracellular proteases is usually under a complex cascade, including automatic processing and proteolytic maturation³⁵⁸. In the gram-positive bacteria, and specifically in *S. aureus*, the extracellular proteases (self-secreted) were reported as a tool to regulate the detachment and dispersal of biofilm²⁶¹ by potentially degrading MSCRAMMs and by secreting proteins with matrix protein-binding domains²⁶⁵. These

proteases can also help bacteria survive by cleaving harmful antimicrobial peptides produced by other bacterial species. On the contrary, the absence of all these proteases produced by *S. aureus* could significantly increase the secretion of numerous virulence factors³⁵⁹.

There are ten extracellular proteases³⁶⁰ encoded in four separate transcripts of *S. aureus*, including seven serine proteases (Spl proteases and SspA), two cysteine proteases (Staphopain ScpA and SspB), and the metalloprotease aureolysin (Aur)³⁶¹. Although these self-secreted enzymes usually exist as inactive forms in biofilm, their purification or commercial product have shown a significant antibiofilm effect.

Studies have also shown that Spl proteases in diffuse lung injury caused by *S. aureus* are essential for the pathogenicity. Among them, SplA can promote the invasion and spread of *S. aureus* to multiple parts of the body by cleaving mucin 16 from epithelial cells of the eyes, airways, and female reproductive tract epithelial cells^{362, 363}.

Fibronectin binding protein (FnBP) is a vital adhesion factor in *S. aureus* biofilm. Adding exogenous SspA (V8 protease) can increase the loss of FnBP on the bacterial surface and reduce its adhesion³⁶⁴. SspA can also degrade the Bap protein in the biofilm of *S. aureus* and disrupt the established biofilm³⁶⁵.

In addition to the self-secreted enzymes by *S. aureus*, the proteases secreted by other species could also perform an ideal effect on biofilm prevention or destruction. Mootz et al. found that purified SspB or ScpA enzymes from *E. coli* inhibited the formation of biofilms, and that ScpA can destroy established biofilms of *S. aureus*³⁵⁹. However, the targets of SspB and ScpA in biofilms are currently unclear. The experiment of Marti et al. proved that in addition to SspA, aureolysin plays an essential role in the degradation of Bap-dependent *S. aureus* biofilm³⁶⁵.

Proteinase K produced by the fungus *Tritirachium album* Limber also belongs to serine protease with high activity. This enzyme can remain stable under various conditions¹⁰⁷, making it an ideal choice among proteases. Proteinase K can cleave peptide bonds of aliphatic, aromatic, or

hydrophobic amino acids³⁶⁶. By addition of proteinase K (2 µg/mL) to established *S. aureus* biofilm, a quick detachment of the biofilm is observed within 12 hours¹⁰⁷. Shukla et al.³⁶⁷ also demonstrated that proteinase K has a dispersing effect on the Bap-dependent biofilm formed by *S. aureus*. However, this dispersion effect did not enhance proportionally with the increase in the concentration of proteinase K. Besides, the combination of 150µg/ml gentamicin and proteinase K (2 µg/mL) reduced the biofilm cells of *S. aureus* by 3 logs.

5.3.2.1.3 DNase I

Extracellular DNA (eDNA) widely exists in various bacterial biofilm and is an essential component of biofilm formation and structure maintenance²⁴. In 1973, Liao et al.³⁶⁸ proved that DNase I of the bovine pancreas with a molecular weight of 33 kDa could cleave eDNA but not cause bacterial death. Tetz et al.³⁶⁹ cultured *S. aureus* with DNase I (5.0µg/mL) for 24h, and showed that DNase I could inhibit the formation of biofilm by 51%. DNase I also degraded the matrix of 24h-old *S. aureus* biofilm with dispersal of the biofilm and reduction of bacterial counts. After dispersal of biofilm, it cannot reform in the following 120h³⁶⁹. The combination of DNase I and levofloxacin or rifampicin increased the activity of antibiotics against some Gram-positive bacteria³⁶⁹.

5.3.2.2 Antibiofilm peptides

Antimicrobial peptides (AMPs) are short cationic amphiphilic peptides produced by various cells and tissues. It is an integral part of human innate immunity³⁷⁰. Unlike the enzymes described above, 83% of the antibacterial peptides have a bactericidal effect on bacteria³⁷¹. Treating established biofilms with these antimicrobial peptides can reduce the concurrent use of other antimicrobial agents. However, this bactericidal property may also lead to the development of bacterial resistance³⁷².

5.3.2.2.1 Cathelicidin

Defensins and cathelicidins are two groups of AMPs in mammals. In 1995, hCAP18/LL37

cathelicidin was first discovered in humans³⁷³. It is a human cationic AMP with a molecular weight of 18 kDa³⁷⁴. Studies have shown that the concentrations of LL37 needed to kill planktonic *S. aureus* or to reduce biofilm biomass were significantly different³⁷⁵ with lower concentrations needed to inhibit biofilm formation.

P60.4Ac and P10 are synthetic LL37-derived peptides, which retain the α -helical structure of LL37 and show better disruption effect on biofilms than LL37³⁷⁶. The maximum inhibitory effects of three antimicrobial peptides (LL37, P60.4Ac, and P10) on the biofilm formation of *S. aureus* were 83%, 86%, and 85%, respectively. However, at the concentration used to inhibit biofilm, none of the three antimicrobial peptides can kill bacteria.

5.3.2.2.2 Bacteriocins

Bacteriocins are a group of antibacterial peptides produced by bacteria and their primary function is to inhibit or kill bacteria from other species³⁷⁷. Bacteriocin lacticin 3147 is a two-component lantibiotic³⁷⁸ that can inhibit many gram-positive bacteria (such as *Streptococcus* and *Staphylococcus*)³⁷⁹. Klostermann et al.³⁸⁰ administered lacticin 3147 and antibiotics into bovine's udder to compare their therapeutic effects on clinical bovine mastitis. This experiment showed that lacticin 3147 had no significant difference in eliminating pathogenic bacteria of chronic subclinical infection compared with antibiotic treatment, and the somatic cell count remained unchanged before and after treatment.

5.3.2.3 Phage therapy

Phage was first discovered by Twort in 1915³⁸¹. Subsequently, d'Herelle³⁸² achieved encouraging results by using bacteriophages to treat people suffering from bacillary dysentery. Due to clinical failures, technological limitations, and the rapid development of antibiotics, the research of bacteriophages has stalled³⁸³. However, the year-on-year increase in the incidence of antibiotic resistance and the development of biotechnology have brought researchers back to the study of phage therapy. The ability of bacteriophages to replicate exponentially and kill pathogenic bacteria indicates that they should play an essential role in treating infectious diseases.

At present, bacteriophages are considered promising antibacterial agents for preventing and controlling biofilms³⁸⁴. Although single-species biofilms could be significantly reduced after phage treatment³⁸⁴, it is challenging to eradicate biofilms by single phage³⁸⁵. Hence, various possible methods have been proposed to elude phage specificity limitation and improve biofilm control effects, such as phage mixture, phage and antibiotic combination, and genetically engineered phage. Rahman et al.³⁸⁶ isolated the bacteriophage SAP-26 from clinical isolates of *S. aureus*, which has extensive lytic activity against MSSA and MRSA. In addition, the combined treatment of phage SAP-26 and rifampicin caused structural changes in the biofilm matrix and significantly reduced the number of biofilm bacteria.)³⁸⁷. the biofilm and persisters pretreated with 10⁵ PFU/mL of Sb-1, another phage specific of *S. aureus*, was also eradicated by the additional (or next) administration of antibiotics (fosfomycin and vancomycin)³⁸⁷.

Phages are specific and continue to evolve together with bacteria. The characteristics of phages make them resistant to bacteria that have developed resistance³⁸⁸. Still, it also means that bacterial pathogens must be identified at the strain level before selecting phage treatment. Since most biofilms are multi-species/strain communities, using a single phage to treat biofilms becomes tough. The phage mixture treatment was thus produced and named as phage cocktail. Phage cocktails includes multiple bacteriophages with activity against different targets, which can more effectively destroy the biofilm produced by various strains/species and reduce the production of bacteriophage-resistant bacteria^{389,390}. Phage K can kill a broad spectrum of pathogenic staphylococci³⁹¹. By multiple consecutive passages of the phage through strains entertaining restriction-modification systems, Kelly et al.³⁹² screened modified derivatives (phage K) with a broader spectrum than their parents. Kelly et al.³⁹³ further proved that phage K and its six modifications effectively prevented *S. aureus* from producing biofilms and reduced the density of established biofilms. Recently, Ferry et al.³⁹⁴ reported on the treatment status of three patients with recurrent *S. aureus* prosthetic knee joint infections who received phage therapy (followed a suppressive antimicrobial therapy) during the "Debridement Antibiotic and Implant Retention" (DAIR) surgery. These patients are elderly (over 80 years old) and cannot undergo transplantation. After the DAIR procedure and joint closure (PhagoDAIR procedure), the surgeon injected the phage cocktail (three phages, 1×10⁹ PFU/ml) directly into the patient's joint. After an extended follow-up, the results proved that none of the three patients

had persistent bacteria or relapsed infections. Thus, the final results of PhagoDAIR surgery combined with inhibitory antibiotics are encouraging.

With the rapid development of biotechnology and synthetic biology, the engineered bacteriophages have become possible. For instance, bacteriophages could be modified to express the most effective EPS degrading enzyme for the targeted biofilm. The engineered enzymatic phage realized the dual therapeutic strategy of bacterial lysis and biofilm matrix degradation through rapid replication. These genetically engineered phages have been utilized to remove biofilms in the industries³⁹⁵. This strategy effectively improves the eradication of biofilms by phages and intelligently circumvents some limitations of enzyme therapy, such as the difficulty of expression, purification, and high-dose enzyme delivery to specific infection sites. CRISPR-Cas9 genome editing is the technology that is applied to expand the host range of temperate bacteriophages and promote the bactericidal effect by modifying the tail fiber protein³⁹⁶. These genetically engineered bacteriophages showed a high level of *S. aureus* elimination *in vitro*. It also effectively reduced the bacteria on the skin of infected mice³⁹⁷. Cobb et al. used a similar method to obtain CRISPR-Cas9 modified bacteriophage which displayed an ideal effect in a rat model of soft tissue infection. Furthermore, the killing effect of CRISPR-Cas9 modified bacteriophage on *S. aureus* is similar to the results obtained with high-dose fosfomycin³⁹⁶.

5.3.3 Molecules triggering biofilm dispersal

In the non-antibiotic treatment to eliminate biofilms, there are also other molecules that can trigger the degradation of the biofilm matrix. In clinical medicine, ethylenediaminetetraacetic acid (EDTA) can modulate matrix metalloproteinases (MMP) and has been added to wound dressings to manage wound infections³⁹⁸. EDTA also has an additive effect with gentamicin³⁹⁹ and when EDTA is combined with tromethamine (Tris), it can increase the penetration of the drug into microorganisms by destroying the bacterial cell wall⁴⁰⁰. For treating chronic *Pseudomonas* otitis in canine, the combination of Tris and EDTA may be used as an effective adjuvant treatment⁴⁰¹. The combined use of EDTA and antiseptic substances also showed an additive effect against biofilms⁴⁰².

Finally, nitric oxide and D-amino acids can induce biofilms disruption and transform the phenotype of biofilm bacteria to planktonic bacteria⁴⁰³. The dispersion activity of D-amino acids on biofilms is achieved by the inhibition of the expression of genes related to biofilm matrix formation⁴⁰⁴. *In vitro* experiments have shown that 200 ppm NO can reduce the number of bacteria by 4 to 6 logs, and when the treatment time is about 5 hours, it can eradicate MSSA and MRSA⁴⁰⁵. For external wounds colonized by *S. aureus*, exposing it to 500 ppm NO for 60 seconds every 24-48 hours can speed up wound healing by 30%⁴⁰⁶. When treating *S. aureus* biofilm *in vitro*, the combination of D-amino acid and rifampin reduced the biofilm bacteria by more than 2 logs compared to using antibiotic alone⁴⁰⁷.

Despite the availability of antibiotics, biofilm-associated infections are associated to a low cure rate and frequent relapses. They give rise to major issues in human and veterinary medicine. This bibliography demonstrated that the high prevalence of persister cells in biofilms, as well as the complex chemical composition and structure of the extracellular matrix have been described as the key critical factors responsible for the very low activity of antimicrobial drugs on biofilms. However, the diversity of components needed to maintain the biofilm structure represents an advantage for the design of new therapeutic strategies.

Objectives of Thesis

The bibliography part highlighted that many factors (such as EPS matrix, low metabolism, and formation of persisters) contribute to the drug tolerance of biofilm and explain why biofilm-associated infections usually have a low cure rate and a high recurrence rate. In addition, animal biofilm-associated infections impact animal welfare and cause severe economic losses and food safety hazards. New strategies have been proposed against biofilms with a significant effect demonstrated for different substances on EPS matrix of *S. aureus* biofilm *in vitro*. However, almost all of the substances were only tested or validated using few reference strains or typical isolates, while the molecular composition of *S. aureus* biofilm can be strain specific. For example, *fnbpA/B* are expressed at higher levels in an FnBP-dependent biofilm-forming MRSA isolate while *fnbpA/B* are expressed at lower levels in laboratory strain SH1000. Consequently, the EPS-targeting strategies should be assessed on a high number of isolates with different biofilm production capacities to select them on their spectrum of efficacy. Therefore, the purpose of our first project was to design a broad-spectrum antibiofilm solution based on the characteristics of *S. aureus* biofilm. For that, eight non-antibiotics (proteinase K, Subtilisin A, DNase I, lactoferrin, EDTA, Phytosphingosine, Chlorhexidine, and Calcium gluconate) and eight antibiotics will be tested on 73 isolates originating from bovine mastitis and selected based on their biofilm masses. Furthermore, the optimal non-antibiotic substance will be combined with several antibiotics to investigate a potential synergy. The final aim is to discover whether one of the combinations will present a high biofilm destruction effect and simultaneously control the quantity of biofilm-embedded bacteria.

In the second part, we will focus on the specificity of low and high biofilm producers *S. aureus* strains. *S. aureus* ST398 is currently the most frequent livestock associated MRSA (LA-MRSA) found in animals⁴⁰⁸. Therefore, the research on *S. aureus* ST398 related virulence and survival mechanism is critical for eliminating or reducing their prevalence. It is well known that *S. aureus* causes numerous chronic infections in humans and animals by forming biofilms. These chronic infections are challenging to cure and have a high recurrence rate. So far, only a few studies^{81,408} have focused on the formation of *S. aureus* ST398 biofilm and its related mechanisms. Since ST398 is a frequent cause of animal infections, in particular cow mastitis, we chose this clone as the target strains for the second project. We plan first to prove that the specificity of biofilm

formation is not derived from differences in the ST-type of *S. aureus*. One hundred and five *S. aureus* ST398 (85 MRSA and 20 MSSA) isolated from various animal hosts (cat, dog, horse, and bovine) will be analyzed for their biofilm-forming capacity (crystal violet assay and bacteria counting). Then, PCR will be applied to detect ten biofilm adhesion-related genes (*icaA/D*, *fnbA/B*, *clfA/B*, *fib*, *ebps*, *eno*, *bbp*, *cna*, and *bap*), *spa* type, and *agr* type in all ST398 isolates. We will finally select the representative isolate and target genes to explore further the possible factors that cause the specificity of the *S. aureus* strains that leads to the differential formation of biofilms.

Our project may propose more details about the antibiofilm effect of a combination between antibiotics and non-antibiotic substances. Its destroying effect on the EPS matrix could promise and probably be readily applied for topical treatments, the systemic use being conditioned to the substances' stability and absence of toxicity. Moreover, the final results of the second project will be meaningful for establishing biofilm in vitro models and the detection of the efficacy of antibiofilm substances. In addition, it can provide new ideas for the clinical treatment of biofilm infections in the future, such as a more accurate classification of the pathogen to select more suitable treatment options.

Experimental part:

Chapter 1

1. The objective of project

We hypothesized that the destruction of the EPS matrix would enhance the detachment of biofilm bacteria. Meanwhile, due to this destructive effect, the released planktonic bacteria could be prevented from adhering and recolonizing. However, the drawback of such non-antibiotic substances could be the sudden massive release of bacteria after the disruption of the matrix, which can potentially lead to severe septicemia. Therefore, the substances specifically targeting matrix components should be combined with antibiotics, which are supposed to maintain or enhance the detachment's effect or kill biofilm bacteria.

To assess EPS-targeting combinations, we examined the destructive effects of eight non-antibiotic substances on the EPS matrix of biofilms formed by 24 representative isolates of *S. aureus*. By combining crystal violet staining, bacterial counts, and confocal laser-scanning microscopy (CLSM), we selected one protease and one non-enzymatic compound leading to significant degradation of the EPS matrix. The reduction of embedded biofilm bacteria was further tested by combining these substances with antibiotics.

2. Article

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Destruction of *Staphylococcus aureus* biofilms by combining an antibiotic with subtilisin A or calcium gluconate

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In *S. aureus* biofilms, bacteria are embedded in a matrix of extracellular polymeric substances (EPS) and are highly tolerant to antimicrobial drugs. We thus sought to identify non-antibiotic substances with broad-spectrum activity able to destroy the EPS matrix and enhance the effect of antibiotics on embedded biofilm bacteria. Among eight substances tested, subtilisin A (0.01 U/mL) and calcium gluconate (CaG, Ca^{2+} 1.25 mmol/L) significantly reduced the biomass of biofilms formed by at least 21/24 *S. aureus* isolates. Confocal laser scanning microscopy confirmed that they both eliminated nearly all the proteins and PNAG from the matrix. By contrast, antibiotics alone had nearly no effect on biofilm biomass and the selected one (oxytetracycline-OTC) could only slightly reduce biofilm bacteria. The combination of OTC with CaG or subtilisin A led to an additive reduction (average of 2 \log_{10} CFU/mL) of embedded biofilm bacteria on the isolates susceptible to OTC (MBC < 10 $\mu\text{g/mL}$, 11/24). Moreover, these two combinations led to a reduction of the embedded biofilm bacteria higher than 3 \log_{10} CFU/mL for 20–25% of the isolates. Further studies are now required to better understand the factors that cause the biofilm produced by specific isolates (20–25%) to be susceptible to the combinations.

Bacterial biofilms are surface-associated multicellular communities in which bacteria are embedded in a self-produced extracellular polymeric substance (EPS), a matrix mostly composed of exopolysaccharides and proteins¹. Owing to their recalcitrance to antibiotic treatments and to immune host defences, biofilm-associated infections are often chronic and a cause of frequent relapses². Such infections have been reported to be involved in 65% to 80% of all human bacterial infections³ and give rise to major issues in human and veterinary medicine^{4,5}. The high prevalence of persister cells in biofilms as well as the complex chemical composition and structure of the extracellular matrix have been described as the key critical factors responsible for the very low activity of antimicrobial drugs on biofilms^{5–8}.

The strategies to manage *S. aureus* biofilms include early physical removal of colonised materials or local delivery of high doses of antibacterial chemotherapy⁹ when possible. However, even after therapy with very high concentrations of antibiotics for several weeks, the clinical cure rate of *S. aureus* infections remains low¹⁰. Indeed, previous studies suggested that the EPS matrix that remains after the bacteria have been killed by antibiotic treatments could promote the re-colonisation of the surface by the same or other bacterial species, thereby causing infection recurrence or severe complications^{10–12}. Consequently, removing the residual EPS matrix could be at least as crucial as killing bacteria in the management of biofilm infections. Additionally, due to the variability in the composition of *S. aureus* EPS matrix and the interaction between their multiple components, the strategies to disrupt the matrix should ideally target several constituents of the EPS matrix simultaneously¹³.

In *S. aureus* biofilms, poly-N-acetyl- β -(1–6)-glucosamine (PNAG; also known as polysaccharide intercellular adhesin PIA), proteins and extracellular DNA (eDNA) are broadly viewed as the main components of the EPS matrix¹⁴. PNAG helps biofilms form and enables bacteria to gain protection from the host immune system^{15,16}. Adhesion to the surface and initiation of biofilm formation¹⁷ are also related to the expression of numerous proteins, such as cell wall-anchored (CWA) proteins, phenol soluble modulins (PSMs) and recycled cytoplasmic proteins found in both methicillin-susceptible *S. aureus* (MSSA) and methicillin-resistant *S. aureus* (MRSA)

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isolates¹⁸. The third main component, the extracellular DNA (eDNA) released from cells through controlled autolysis, is considered crucial for stabilising the structure of *S. aureus* biofilms¹⁹.

To avoid biofilm formation, previous studies have focused on the development of prophylactic therapies dedicated to limit the accumulation of EPS matrix components during bacterial growth, i.e. PNAG, biofilm-related proteins and eDNA^{20–23}. However, although most of the proposed compounds were successful in preventing biofilm formation, they were unable to destroy mature biofilms²⁴ and thus had only limited efficacy when tackling clinical infections. During the maturation of *S. aureus* biofilms, the amount of PNAG decreases while proteins gradually increase to finally play a critical role in the mature form²⁵. Up to 24 types of CWA proteins have been described as being implied in biofilm formation^{26,27} so proteases could be good candidates even if their efficacy can be lower in vivo than in vitro due to rapid elimination and instability²⁸. Several studies have already shown significant effects of proteases on the EPS matrix of *S. aureus* biofilm in vitro, but almost all of them were only tested or validated using a few reference strains or laboratory strains^{29,30} whereas the molecular composition of *S. aureus* biofilm can be strain-specific^{31,32}. EPS-targeting substances should consequently be assessed on many isolates with different biofilm production capacities to select those with the broadest spectrum of activity.

We hypothesized that the destabilization of proteins and/or PNAG would both enhance the destruction of the EPS matrix and limit the adhesion by the surviving biofilm bacteria released during this process. However, the destruction of EPS matrix may not be systematically correlated to a decrease in embedded bacteria and thus, in chronic infections associated to a biofilm, the addition of antibiotics is systematically required. The antibiotics should at least preserve or enhance the destruction effect of non-antibiotics on the EPS matrix and better, in optimal combinations, increase the detachment or kill embedded biofilm bacteria.

To assess EPS-targeting combinations, we examined the destructive effects of eight non-antibiotic substances on the EPS matrix of biofilms formed by 24 representative isolates of *S. aureus*. By combining crystal violet staining, bacterial counts, and confocal laser-scanning microscopy (CLSM), we selected one protease and one non-enzymatic compound leading to significant degradation of the EPS matrix. The reduction of embedded biofilm bacteria was further tested by combining these substances with antibiotics.

Results

Broad screening and classification of isolates based on their biofilm production. A total of 73 clinical isolates of *S. aureus* from bovine mastitis, including 54 MSSA and 19 MRSA, plus one laboratory strain (SH1000) were tested for their capacity to form a biofilm. The 24 h-old biofilms, which were maximised in BHI plus 1% glucose, were stained with crystal violet (CV) (four independent experiments in quadruplicate). Three distinct classes of *S. aureus* isolates related to their biofilm biomass were obtained by K-means and agglomerative hierarchical clustering (AHC, Fig. S1).

Based on the profile plot and variance decomposition, the isolates were categorised into low (42%, 31/74), medium (20%, 15/74), and high (38%, 28/74) biofilm producers (Table S1). Specifically, 45% of MSSA (25/55) and 32% of MRSA (6/19) isolates were clustered as low producers, 16% of MSSA (9/55) and 32% of MRSA (6/19) isolates as medium producers, 38% of MSSA (21/55) and 37% of MRSA (7/19) isolates as high producers. Biofilm biomass largely varied among strains, but there was no significant divergence between MSSA and MRSA (Fig. S2a). Among these 74 isolates, two isolates were chosen from the medium producers (one MSSA and one MRSA) and two from the high producers (one MSSA and one MRSA) to rapidly screen the substances. Another larger subset of 24 representative isolates (14 MSSA and 10 MRSA) from the three clusters was selected to further characterise the most effective substances. The biofilm biomasses and the counts of biofilm bacteria for these 24 representative isolates are represented in Fig. S2b. The quantity of biofilm bacteria in the 24 representative isolates ranged from 4 to 7 log₁₀ CFU/mL with weak linear correlation with the biofilm biomass assessed by CV staining ($R^2 = 0.486$) (Fig. S2b).

Selection of subtilisin A and calcium gluconate (CaG) out of eight non-antibiotic substances. The effect of eight different non-antibiotic substances (Fig. S3 and Table S2) on total biofilm biomass was quantified on the small subset of four isolates in order to determine which ones had the potential to destroy the EPS matrix. The results showed a dramatic reduction in the biofilm biomass with proteinase K and subtilisin A, both of which resulted in lower OD₅₉₅ values than with DNase I (Fig. S3a). Interestingly, calcium ions (1.25 mmol/L), which must be applied in combination with proteinase K to ensure its activity, also significantly reduced the total biofilm biomass (Fig. S4) and thus prevented the quantification of the effects of proteinase K alone. Consequently, subtilisin A was then selected and applied on the biofilms of the 24 representative isolates. This led to a reduction in total biofilm biomass for all 24 isolates (Fig. 1a). The lower relative efficacy observed for the low biofilm producers compared to the medium and high producers (Fig. S5) can be explained by the CV assay's detection limit, which prevented the observation of a further reduction in the biofilm biomass of these low producers. It should be noted that the effect of a medium concentration of subtilisin A (0.01 U/mL) was similar to that of the high concentration (0.1 U/mL) (Fig. S3a).

Control biofilm imaging. The 3D images captured with CLSM after Syto9/PI staining showed that in the control *S. aureus* biofilm of the MS3 isolate (a high producer with an average OD₅₉₅ value of 3.4 and average biofilm bacterial counts of 6.8 log₁₀ CFU/mL), the PI stained (red) and the Syto9 stained (green) components were mixed in a thick biofilm with a higher density of the PI stained components at the bottom of the biofilm. The two additional staining procedures with WGA (wheat germ agglutinin) and SYPRO Ruby matrix stain indicated that the EPS matrix contained large amounts of poly-*N*-acetyl- β -(1–6)-glucosamine (PNAG) exopolysaccharide and proteins (Fig. 2).

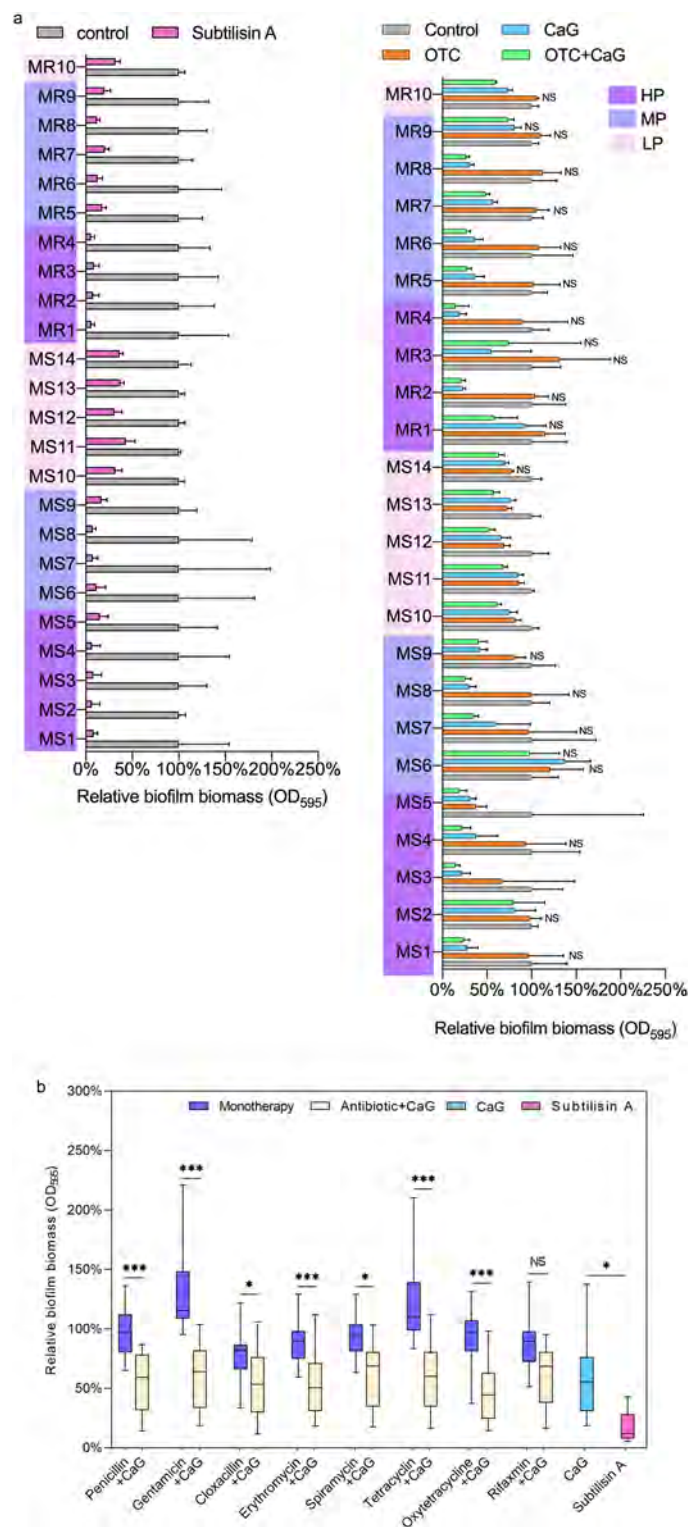


Figure 1. Reduction of *S. aureus* biofilm biomass by subtilisin A, Calcium gluconate (CaG), antibiotics alone and in combination. **(a)** Relative biomasses of 24 h-old biofilms of 24 isolates exposed to subtilisin A (0.01 U/mL), CaG (Ca²⁺ 1.25 mmol/L), OTC (10 µg/mL), and the combination of OTC and CaG for 24 h. The X-axes represent the percentage of biofilm biomass (OD₅₉₅) with the control (biofilm formed by the non-treated isolate) set at 100%. High (HP), medium (MP) and low (LP) biofilm producers are highlighted with different colors. Statistical significance was determined by the Mann-Whitney U test. Unmarked: $P < 0.05$. NS: not significant. Error bars indicate the SD. **(b)** Relative biomass of 24 h-old biofilms exposed to eight antibiotics, subtilisin A (0.01 U/mL), CaG (Ca²⁺ 1.25 mmol/L) and to the combination of each antibiotic with CaG for 24 h. Results from the 24 isolates are represented as boxplots. Statistical significance was determined by Friedman's test with a post hoc application of Nemany. * $P < 0.05$ ** $P < 0.01$. *** $P < 0.001$. NS: not significant. Other relevant P values are reported in Table S4. The Y-axis represents the percentage of OD₅₉₅ values relative to the control group set at 100%. Data are the means of values from two independent experiments in triplicate (n = 6).

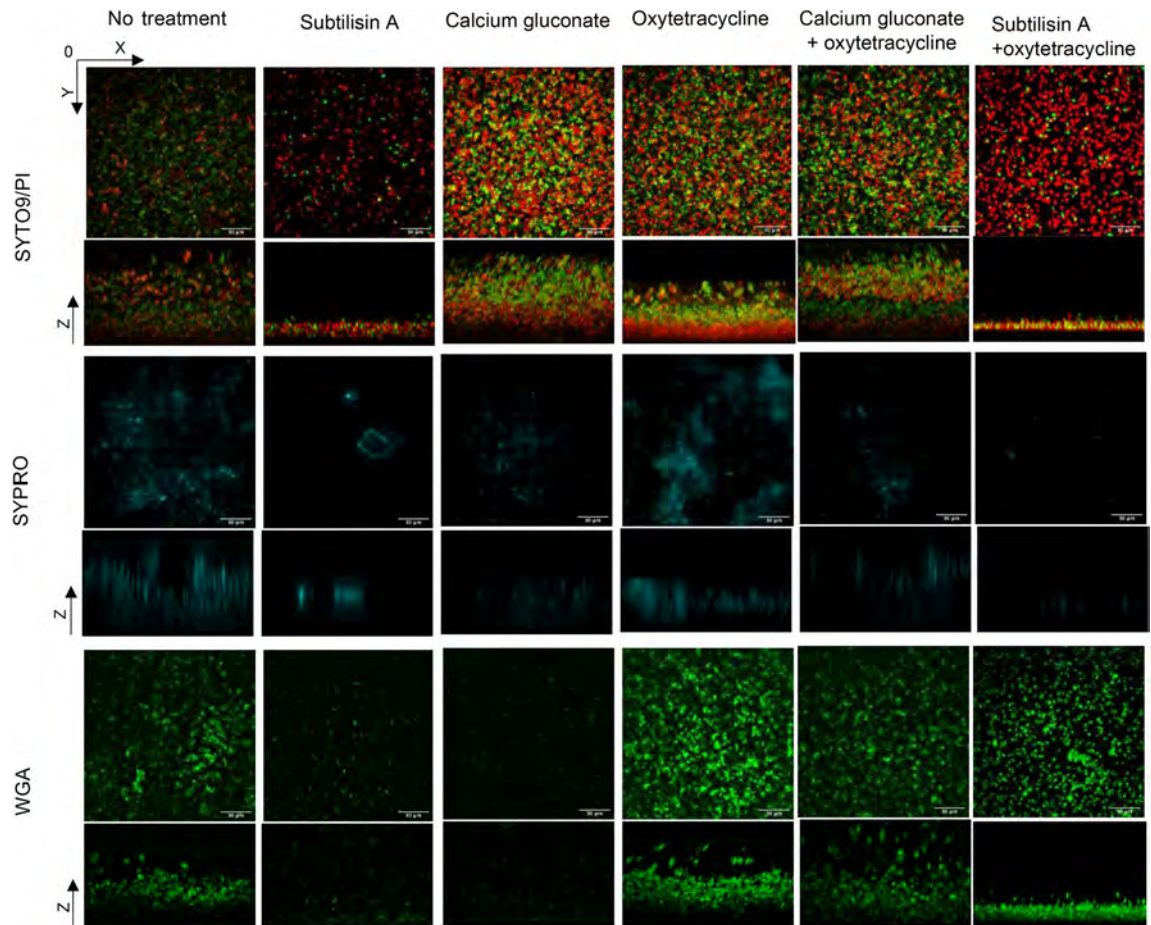


Figure 2. CLSM images of *S. aureus* biofilms exposed to the three substances tested. Representative 3D images of biofilms from MS3 isolate grown statically for 24 h and treated or untreated with subtilisin A (0.01 U/mL), CaG (Ca^{2+} 1.25 mmol/L), OTC (10 $\mu\text{g}/\text{mL}$) alone or in combination for 24 h. Biofilms were stained with the Syto9/PI, SYPRO Ruby biofilm matrix stain and wheat germ agglutinin (WGA). Scale bars, 30 μm .

Characterisation of subtilisin A activity on biofilm. The MIC and MBC of subtilisin A were both higher than 0.05 U/mL for all 24 *S. aureus* isolates (Table S3), meaning that the tested concentration of 0.01 U/mL should neither kill bacteria nor inhibit bacterial growth. Treating the biofilm with subtilisin A for 24 h resulted in an approximate average reduction of 0.6 \log_{10} CFU/mL in embedded biofilm bacteria that could be mainly explained by detachment (Fig. 3). The effect of subtilisin A was observed by CLSM on the MS3 isolate (Fig. 2), for which subtilisin A led to a relative biofilm biomass of 8% compared to the control set at 100% (Fig. 1a). Subtilisin A induced a dramatic decrease in biofilm thickness and the red and green stained components were limited to small individual pieces. The abundant proteins (SYPRO Ruby—blue stain) observed in the control were no longer visible. The PNAG (WGA-green stain) were rare and dispersed after subtilisin A monotherapy.

In parallel to the experiments on mature biofilm, subtilisin A (0.01 U/mL) added before incubation of bacteria for either 24 or 48 h significantly lowered the total biofilm biomass compared to the control for all the tested isolates, including high biofilm producers (Fig. S6).

Characterisation of CaG activity on biofilm. During the preliminary selection of substances to test on four isolates, the addition of calcium ions led to an unexpected and dramatic reduction in the biofilm biomass for three out of four isolates at a concentration similar to the plasma concentration (Ca^{2+} 1.25 mmol/L) (Fig. S3a). CaG was therefore tested on the 24 representative isolates and induced a significant decline in biofilm biomass for 21/24 isolates (Fig. 1a). Interestingly, CaG led to a significant increase in biofilm biomass for the laboratory strain SH1000 (named here MS6) and had no significant effect on a further two isolates (MR1 and MR9). The values for the MIC (Ca^{2+} > 112.5 mmol/L) and MBC (Ca^{2+} > 112.5 mmol/L) of CaG for all isolates were far higher than the tested Ca^{2+} concentration of 1.25 mmol/L (Table S3). The average reduction of embedded biofilm bacteria after treatment with CaG was around 0.4 \log_{10} CFU/mL (Fig. 3). CLSM performed on the MS3 isolate, for which CaG led to a relative biofilm biomass of 21% compared to the control set at 100% (Fig. 1a), showed that the live/dead staining in presence of CaG was quite similar to the control. The only difference was that PI-stained components appeared to be slightly more evenly distributed and aggregated at the bottom of the biofilm (Fig. 2). The marked difference between CaG and the control was observed for SYPRO Ruby and WGA

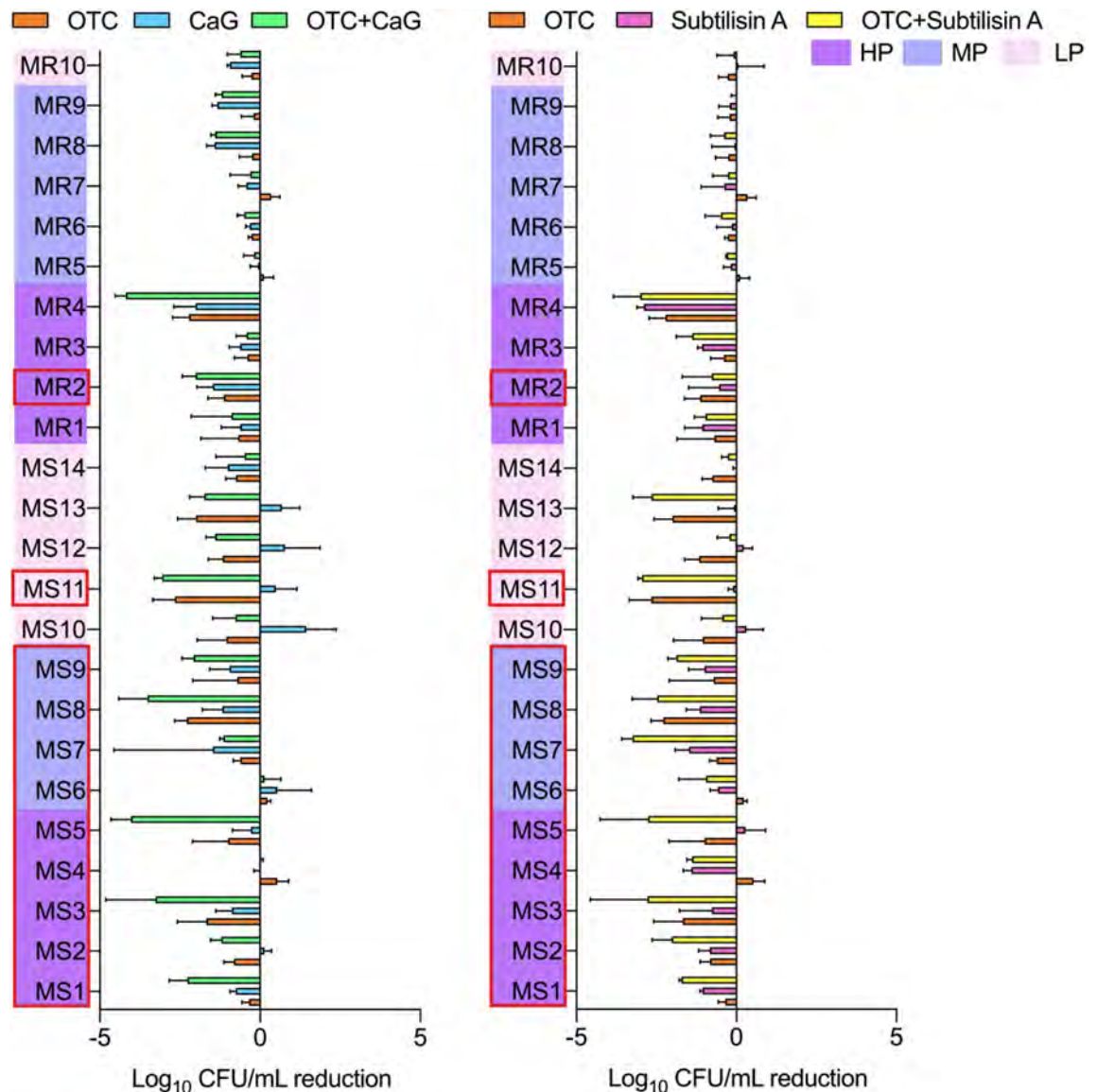


Figure 3. Reduction in the biofilm bacteria of 24 individual isolates by subtilisin A, CaG, and OTC alone or in combination compared to control. Data are the means of three independent experiments ($n=3$). Error bars indicate the SD. The isolates for which the MBC of OTC is less than 10 µg/mL are labelled in red frame. High (HP), medium (MP) and low (LP) biofilm producers are highlighted with different colors.

staining, with much fewer stained components compared to the control even if the thickness of the stained layer (z-axis) after CaG remained far higher than after subtilisin A.

Like subtilisin A, CaG inhibited biofilm formation (Fig. S6) as shown by the marked reduction in biofilm biomass after incubation for 24 h (22/24 isolates) or 48 h (21/24 isolates).

Selection of antibiotics to combine with subtilisin A and CaG. We initially assessed the ability of eight antibiotics alone at three different concentrations (Table S2) to reduce the biofilm biomass of the small subset of four isolates (Fig. S3b). Since the eight antibiotics failed to substantially reduce the biofilm biomass, only the highest concentrations were tested on the 24 representative isolates (Fig. 1b). Antibiotics alone led in the best cases to a very slight reduction in biofilm biomass (oxytetracycline (OTC), cloxacillin) and in the case of penicillin G, gentamicin and tetracycline, even led to a significant increase in biofilm biomass in respectively 5/24, 10/24 and 7/24 isolates compared to the control.

The very low OD values obtained after CV staining with subtilisin A alone were too close to the limit of detection to detect any enhanced effect by combining antibiotics with subtilisin A. Therefore, only the effects on biofilm biomass of a combination of antibiotics and CaG were explored by CV staining. The results, represented in Fig. 1b and Table S4, showed that the addition of antibiotics did not significantly reduce or enhance the efficacy of CaG on the biofilm biomass of the 24 isolates. Among all the combinations, OTC combined with CaG led to the lowest average for the relative biofilm biomass (46%) compared to the control set at 100% (Fig. 1b). Moreover,

this combination significantly decreased the individual biofilm biomass for 22 out of 24 isolates compared to the control (Fig. 1a). This effect was independent of the MSSA or MRSA status, and did not depend on biofilm production of the isolates (Fig. S5). As for CaG alone, the only isolate whose biofilm biomass actually increased after exposure to the combination was the laboratory SH1000 strain (MS6, Fig. 1a).

The MIC of OTC for the 24 isolates ranged from 0.0625 to 0.25 µg/mL for MSSA and from 0.0125 to 128 µg/mL for MRSA (Table S3). The MBC ranged from 0.25 to 32 µg/mL for MSSA and from 4 to > 128 µg/mL for MRSA with 11/24 isolates having an MBC lower than 10 µg/mL (Table S3). The average reduction of embedded biofilm bacteria exposed to 10 µg/mL of OTC alone was only of 0.8 log₁₀ CFU/mL (Fig. 4a) and was not correlated to the isolates' MBC (Fig. 4b).

Characterisation of the effects of the combination of subtilisin A or CaG with OTC on bio-film. Both combinations (OTC + CaG and OTC + subtilisin A) led to a significantly greater reduction in embedded biofilm bacteria by detachment or killing than OTC alone (Fig. 4a). OTC + CaG also showed a better effect than CaG alone, while OTC + subtilisin A showed no significant difference compared to subtilisin A alone (Fig. 4a). In order to identify factors explaining the differential effects of these two combinations on the representative isolates, we classified them depending on their susceptibility to methicillin (MSSA vs MRSA) and to OTC (MBC < 10 µg/mL vs > 10 µg/mL). The combination of OTC and subtilisin A had an additive effect (2 log₁₀ CFU/mL average reduction) on the embedded biofilm bacteria from the MSSA isolates and from the isolates with MBC of OTC lower than 10 µg/mL (Fig. 4b). The additive effect of OTC combined with CaG was only observed for the isolates with MBC of OTC < 10 µg/mL (Fig. 4b). The reduction of embedded biofilm bacteria exceeded 3 log₁₀ CFU/mL for 20%–25% of these isolates after the treatment by combinations (OTC + CaG and OTC + subtilisin A) (Figs. 3 and 4a).

After exposure to OTC alone, the PI staining of the MS3 isolate's biofilm (MBC of OTC < 10 µg/mL) corresponding to dead cells or extracellular DNA or RNA increased and was concentrated at the bottom of the biofilm compared with the control group (Fig. 2). The image of biofilm staining by Syto9/PI after the combination of OTC and CaG was quite similar to the image with CaG alone. When OTC was combined with subtilisin A, the images were different from those of the control group, subtilisin A alone or OTC alone. After combined exposure to OTC and subtilisin A, the PI staining became fragmented and isolated. The thickness of the biofilm also decreased and was even thinner than biofilm after subtilisin A alone. In the presence of SYPRO Ruby stain (blue), the CLSM images of OTC alone and the control were similar, suggesting that OTC had no impact on the proteins. In combination, OTC did not impact the ability of CaG and subtilisin A to extensively reduce the protein content. In parallel, WGA staining showed that OTC increased the PNAG content of the biofilm matrix compared to the control group. The combination of OTC with subtilisin A or CaG led to higher PNAG content than subtilisin A or CaG alone, even though the combination with subtilisin A seemed to limit WGA staining to the wall of the bacteria (almost only surrounding single cells).

Discussion

S. aureus infections associated with biofilms are difficult to eradicate because of the high tolerance of bacteria to antibacterial agents and to host immune defences. We selected and proved that subtilisin A (0.01 U/ml) and CaG (Ca²⁺ 1.25 mmol/L) inhibited the in vitro formation of the EPS matrix and, more importantly, destroyed the EPS matrix of several mature *S. aureus* biofilms, enabling for some isolates a significant reduction of embedded biofilm bacteria once combined with antibiotics.

In most studies related to *S. aureus* biofilms, assays were conducted on a few laboratory strains and especially on the NCTC 8325 lineage³³. Although this is relevant for comparisons between related studies, it can lead to a selection of drugs with a narrow spectrum of activity since the main biofilm structural components could depend on the strains³². To better identify broad-spectrum drugs, we decided to select 24 out of 73 clinical isolates from bovine mastitis (plus one laboratory strain, *S. aureus* SH1000) with different biofilm biomass productions to test both the non-antibiotic and antibiotic substances.

By comparing biomasses (OD values after CV staining) and bacterial counts for the biofilm formed by 24 isolates after 24 h, we observed that there was a weak linear correlation between biomass and bacterial counts, suggesting that both methods should be implemented in parallel to obtain information on the effects of substances on the matrix and the bacteria. A decrease in the biomass would indicate a decrease in the biofilm matrix content, in embedded dead cells or adherent living biofilm bacteria whereas a decrease in bacterial counts would indicate bacterial detachment or killing within the biofilm.

Among the eight non-antibiotic substances tested, subtilisin A (a serine endopeptidase produced by *Bacillus subtilis*) significantly reduced the biofilm biomass. The lower efficacy of DNase I could result from the supplementation with glucose in the broth, which was shown to reduce the release of eDNA from cells³². Since proteinase K needs calcium for its activity, a control experiment was conducted using calcium alone and revealed that the activity of proteinase K could be partly explained by calcium ions. Therefore, proteinase K was excluded while calcium gluconate (CaG) was kept for further experiments. Subtilisins have been described as having a broad-spectrum activity on proteins³⁴, and are already widely used to control biofilm in the food industry and to reduce water pollution^{35–37}. In our study, the reduction in biofilm biomasses by subtilisin A was not specific to either MSSA or MRSA. It did not depend on the different strains' production of biofilm either, thus supporting broad-spectrum activity. The ability of subtilisin A to prevent biofilm formation was also confirmed. Compared to the reduction in biofilm biomass, subtilisin A reduced cultivable bacteria inside the biofilm only slightly, with an average reduction of about 0.6 log₁₀ CFU/mL. This suggests that the decrease in biomass could be explained by degradation of the EPS matrix.

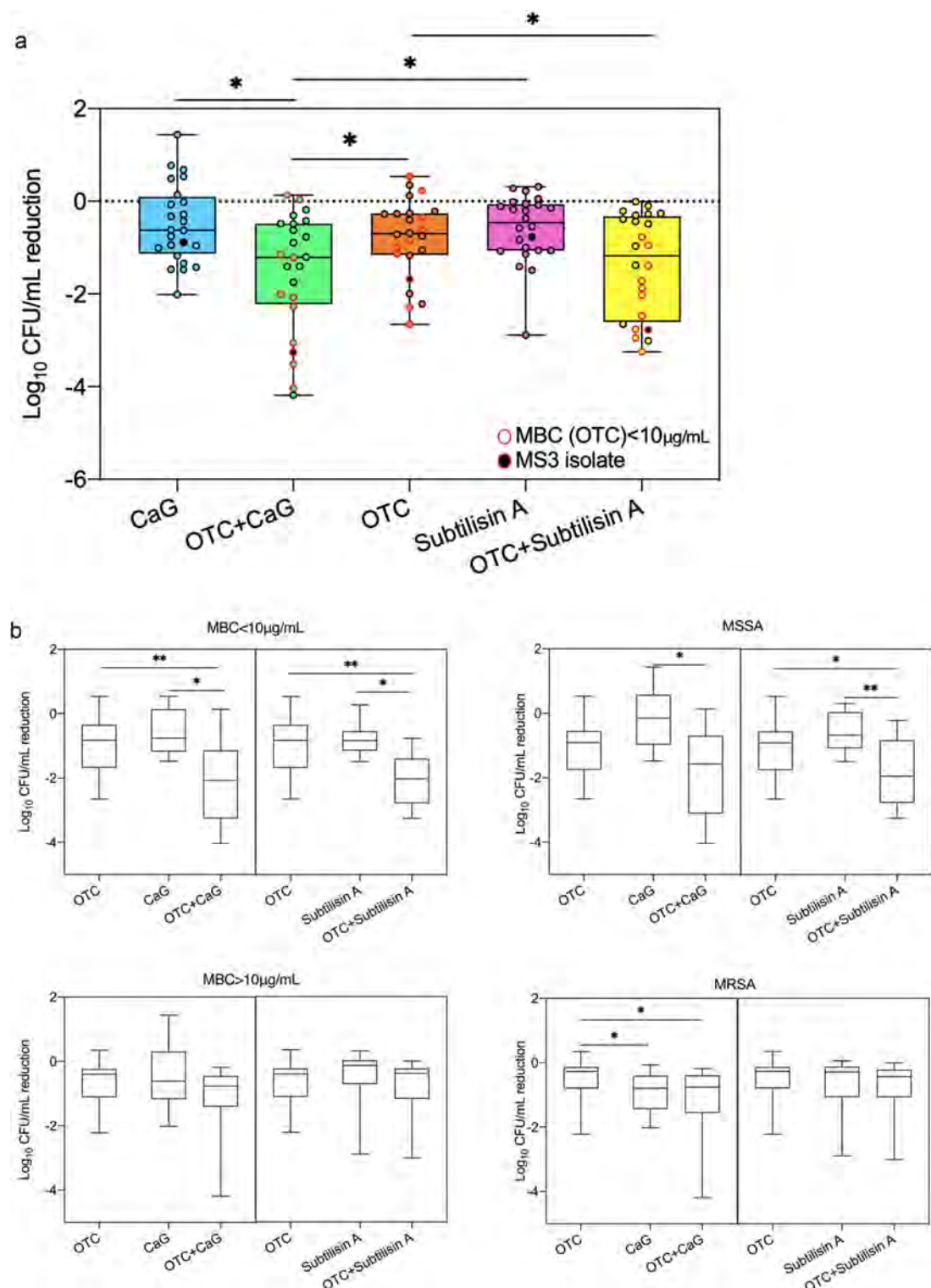


Figure 4. Reduction in *S. aureus* biofilm bacteria after exposure to subtilisin A, CaG, OTC alone and in combination. **(a)** Reduction in biofilm bacteria counts after exposure to OTC (10 µg/mL), subtilisin A (0.01 U/mL), and Ca²⁺ (1.25 mmol/L) alone and in combination represented as box plots for the 24 isolates. Each isolate is represented by a circle. Statistical significance was determined by Friedman's test with a post hoc application of Nemenyi. *P < 0.05. **(b)** Reduction in biofilm bacteria counts after exposure to OTC (10 µg/mL), subtilisin A (0.01 U/mL), and Ca²⁺ (1.25 mmol/L) alone and in combination represented as separate box plots according to the MBC of OTC (MBC < 10 µg/mL or MBC > 10 µg/mL) and MSSA/MRSA status for the individual isolates. Statistical significance was determined by the Friedman's test with a post hoc application of Nemenyi. *P < 0.05, **P < 0.01. No mark: not significant. Data are the means of three independent experiments (n = 3).

For the high biofilm producer isolate (MS3), CLSM demonstrated that, under control conditions, live biofilm cells were embedded in PI-stained components that can be dead cells, extracellular DNA or small parts of RNA^{19,38,39} gradually integrated in the biofilm matrix. The staining of PNAG and proteins also showed that MS3 control biofilm contained both components. The images suggested that subtilisin A destroyed the biofilm structure. A similar effect has previously been observed with high concentrations of ficin (1000 µg/ml), which is also a protease⁴⁰. Furthermore, a significant decrease in biofilm thickness was observed with subtilisin A while there was little bacterial reduction (0.76 log₁₀ CFU/ml) for the MS3 isolate's biofilm, thus confirming that the EPS matrix was massively destroyed. In accordance with the protease activity of subtilisin A, no protein was stained in the EPS matrix but more surprisingly, the PNAG content was also greatly reduced. One explanation could be that, by degrading proteins, subtilisin A triggered the collapse of the entire biofilm structure. However, the ability of subtilisin A to also degrade PNAG and other biofilm-related components needs to be further assessed.

CaG also significantly reduced the biomass of the mature biofilm for 21/24 isolates. The tested calcium concentration of 1.25 mmol/L was consistent with the concentration of free calcium ions in mammals' blood (strictly maintained between 1.1 and 1.3 mmol/L)^{41,42}, and slightly lower than the concentration of free calcium ions in human plasma (2.2 to 2.7 mmol/L). This concentration is far below the MIC of calcium, so it should have had no bactericidal or inhibitory effects⁴³ and suggested again that calcium can damage the formed EPS matrix. Like subtilisin A, CaG also prevented biofilm formation. Calcium ions mainly play a structural role in the maintenance of cell wall integrity (extracellular calcium-binding proteins)^{43,44}. In 2004, Arrizubieta et al.⁴⁵ demonstrated that calcium ions at 10 mmol/L inhibited Bap-mediated *S. aureus* biofilm formation by binding to the identified EF-hand-like domains of the Bap protein, thus rendering proteins incompetent for biofilm formation and intercellular adhesion. Similarly, in our experiments, CaG at 1.25 mmol/L (Ca²⁺) inhibited biofilm formation in 22/24 isolates. Interestingly, the only isolate that grew after exposure to CaG was laboratory strain SH1000. CaG did not reduce the biomass of this strain's mature biofilm but was still able to inhibit EPS matrix formation. It is unlikely that the inhibitory and destructive effects of CaG on the EPS matrix are due solely to the presence of the *bap* gene, as calcium can potentially bind to the EF-hand domain in other proteins. Abraham et al.⁴⁶ showed that calcium ions at 3.125 mmol/L disrupted established biofilms by binding to the Clf-B protein and a study by Lee et al.⁴⁷ also demonstrated the inhibitory effect of calcium ions on the biofilm formation of an isolate from a human lesion. The CLSM images showed that the decrease in protein and PNAG content within the EPS matrix with CaG was similar to the decrease observed with subtilisin A, even though the biofilm remained far thicker with calcium than with subtilisin A. One hypothesis is that the proteins in the EPS matrix could be degraded by subtilisin A, whereas calcium ions could cause a conformational change in Bap that affects its ability to form biofilms⁴⁵.

Since subtilisin A and CaG alone destroyed the matrix without extensively reducing embedded biofilm bacteria, we then investigated combining each of them with antibiotics. The first criterion for the antibiotic selection was that the addition of antibiotics should preserve or increase the destructive effect of subtilisin A and CaG on the EPS matrix. The eight antibiotics tested had very little effect on the biofilm biomass as previously demonstrated in many studies^{48–50}. Even if not statistically significant, the greatest reduction in biomass obtained by combining CaG with an antibiotic was obtained with OTC, which was thus selected for further experiments. We observed through CLSM that OTC alone killed very few biofilm bacteria. Interestingly, OTC led in parallel to a significant increase in PNAG compared to the control. Similar observations were also reported with penicillin G, which increased PNAG while reducing the bacterial population and biofilm biomass⁵¹. The slight killing effect associated with the increase in PNAG in presence of OTC could explain why the global biofilm biomass between OTC treatment and the control was similar, and supports the use of CLSM to clarify drugs' mechanism of action. Even if OTC can bind to calcium⁵², the combination of calcium and OTC led to a greater reduction in biofilm biomass than other combinations, and to a significant additive effect on the removal of embedded biofilm bacteria. The added calcium suppressed the enhanced effect of OTC on PNAG in the EPS matrix and the effect of the combination on proteins was similar to the effect of calcium alone. These effects on PNAG, proteins and bacteria were in accordance with the significant reduction in total biofilm biomass by a combination of OTC and calcium compared to the control. Similarly, the addition of subtilisin A inhibited or reduced the enhanced effect of OTC on PNAG as there were no more visible cell clumps after the combination of both. This might be explained by an increase in dead or detached bacteria or by extensive destruction of the matrix. The remarkable protein degradation of subtilisin A was preserved when used in combination with OTC and caused a significant decrease in total biofilm biomass and thickness. We further found that both combinations (OTC + subtilisin A and OTC + CaG) had a significant additive effect (killing or detachment) on the embedded biofilm bacteria for the 11/24 isolates having an MBC lower than the tested concentration of OTC (10 µg/mL). As MSSA are usually more susceptible to OTC^{53,54}, we had more MSSA included in the group of MBC < 10 µg/mL. However, there was no significant additive effect of OTC + CaG on the embedded biofilm bacteria when the isolates were classified as MSSA vs MRSA groups. Villa et al.⁵⁵ showed that the susceptibility of biofilm bacteria to ampicillin was enhanced when subtilisin A was used to prevent the biofilm formation of *Escherichia coli*. Another study showed that an engineered peptidoglycan hydrolase degrading the peptidoglycan structure of *S. aureus* could increase bacterial killing and biofilm eradication by gentamicin in animal models⁵⁶.

In conclusion, by using several approaches to explore biofilm in parallel, we characterised the mode of action of compounds with anti-biofilm activity and selected efficient combinations. This study specifically demonstrated that subtilisin A or calcium can extensively disrupt the matrix of many *S. aureus* isolates of animal origin. The combination of subtilisin A or CaG with OTC produced an additive reduction of embedded biofilm bacteria for isolates highly susceptible to OTC (MBC < 10 µg/mL). This suggests that subtilisin A and CaG may reveal the activity of OTC on biofilm bacteria and that they could also probably be combined to other antibiotics depending on the strain specificity.

Materials and methods

Bacterial strains. A total of 73 clinical isolates of *S. aureus* were included in this study. All the isolates were collected in France through the Resapath network for the surveillance of resistance in veterinary medicine (<https://resapath.anses.fr/>). Susceptibility testing using disc diffusion according to the guidelines of the French Society for Microbiology (CA-SFM) showed that 54 isolates were methicillin-susceptible *S. aureus* (MSSA), and 19 isolates were methicillin-resistant *S. aureus* (MRSA). The presence of the *mecA* gene was assessed by PCR. We used *S. aureus* SH1000 as the positive control to ensure that the low biofilm production observed for some isolates was not caused by the experimental conditions.

Biofilm formation. All the isolates were first cultured on Mueller Hinton Agar overnight at 37 °C. A few colonies were then diluted in sterile Brain Heart Infusion (BHI) broth plus 1% glucose to obtain a final suspension of $6 \log_{10}$ CFU/mL and 180 µL of this suspension was added to each well of a 96-well polystyrene microplate (ThermoFisher Nunc). Plates were incubated at 37 °C for 24 h to allow biofilm formation before adding substances, except for experiments carried out to assess the ability of subtilisin A and CaG to inhibit biofilm formation. In this case, the substances were added to the wells at the same time as bacteria.

Crystal Violet (CV) assay (biomass). After a further 24 h of incubation at 37 °C in the presence of substances or only BHI with 1% glucose (control), the microplates were turned upside down and tapped so that broth and most of the planktonic bacteria fell out onto the absorbent mat⁵⁷. The biofilms were then rinsed twice with 200 µL of sterile phosphate-buffered saline per well. Two hundred microlitres of CV solution (Sigma-Aldrich, 0.05% w/v) was added to each well and left for 3 min at 25 °C. The excess dye was then rinsed with 200 µL of sterile PBS, the wells were air-dried naturally, then 200 µL of 96% (w/v) ethanol was added to each well to resolubilise the residual dye. The biofilm's biomass was measured using a microplate reader (CLARIOstar Plus) with the value of absorbance set at 595 nm (OD₅₉₅).

Quantification of cultivable biofilm bacteria. After 24 h of incubation at 37 °C in the presence of substances or only BHI with 1% glucose (control), wells were rinsed twice with 200 µL of sterile PBS as above and the bacteria were then resuspended in 200 µL of sterile PBS. After 10 min of ultrasound at 40 Hz (Branson), the cultivable biofilm bacteria were counted by plating serial tenfold dilutions on tryptic soy agar plates⁵⁸. The reduction in biofilm bacteria was calculated as the difference between counts after exposure to substances and counts in control wells.

Classification of isolates depending on biofilm production and selection of subsets. The averages of four OD values obtained in quadruplicate for each of the 74 isolates without added substances were used to perform k-means clustering followed by agglomerative hierarchical clustering (AHC) in order to classify the isolates according to their biofilm biomass-forming capacity using XLSTAT (2019.4.2 version). The results of this classification followed by the counting of live bacteria in control biofilms led to the selection of two representative subsets of 4 and 24 isolates out of the 74 strains in total in order to conduct assays with the different substances.

Assessment of the efficacy of different substances. Proteinase K (Sigma-Aldrich, USA), subtilisin A (Sigma-Aldrich, USA), EDTA (Sigma-Aldrich, USA), phytosphingosine (Sigma-Aldrich, USA), lactoferrin (Sigma-Aldrich, USA), DNaseI (protease-free) (Roche Diagnostics, Germany), chlorhexidine digluconate (Intervet, France) and calcium gluconate (Lavoisier, France) were tested as non-antibiotic substances. The tested antibiotics were benzylpenicillin G, gentamicin, cloxacillin, spiramycin, erythromycin, tetracycline and oxytetracycline (OTC) purchased from Sigma-Aldrich (St. Louis, USA) and rifaximin obtained from TRC (Toronto, Canada). After 24 h of biofilm incubation, 20 µL of solutions containing the tested substances was added to each well. For the control wells, BHI with 1% glucose was added. After 24 h at 37 °C (except chlorhexidine digluconate, which had 2 min of treatment), the effect of treatment was measured by a CV assay and bacteria count. For the CV assay, three OD values in duplicate (n = 6) were obtained for each isolate and each condition. Triplicate bacteria counts (n = 3) were performed for each isolate under each tested condition. The preliminary experiments were conducted on four isolates and the effects of the substances with the highest efficacy were then assessed on 24 isolates.

The Minimum Inhibitory Concentration (MIC) of OTC, subtilisin A, and CaG was determined for the 24 isolates in triplicate by the microdilution method according to the CLSI⁵⁹. The Minimum Bactericidal Concentration (MBC) was tested in triplicate as described in CLSI document M26-A⁶⁰.

Confocal laser scanning microscopy. CLSM was used to examine 24 h-old biofilm exposed or not to substances. The selected isolate was the MS3 isolate which was representative of high producers of biofilm biomass. The biofilms were formed on a 6-well polystyrene microplate and washed with sterile PBS (except for the wheat germ agglutinin stain, for which sterile water was used) as described above. The biofilms were stained with Syto9/PI (Live/Dead kit, Molecular Probes), FilmTracer SYPRO Ruby biofilm Matrix stain (Molecular Probes), and wheat germ agglutinin AlexaFluor 488 conjugate (Invitrogen). Images were acquired on a spinning disc from Perkin Elmer with the CSU-X1 scan head. The biofilms were observed using a 40× water immersion objective (Fluor 40×/0.8W, Nikon). The biofilm image stacks were obtained at 499 × 500 pixels (two HAMAMATSU C9100-13 EMCCD cameras) in three different areas of each surface analysed. 3D reconstructions were created by ImageJ software.

Statistical analysis. Mann–Whitney U tests were performed to compare the biofilm biomasses (OD values) of each control and treated isolate, to compare MSSA and MRSA isolates, and to compare isolates with an MBC for OTC lower or higher than 10 µg/mL. A Kruskal–Wallis test with Dunn's multiple comparison post-hoc analysis was used to determine whether there was a statistically significant difference in the biofilm biomasses produced by the different groups according to their biofilm-forming capacity. For multiple comparisons of OD values and counts measured after different treatments, statistical significance was determined by a Friedman test with post hoc application of Nemanyi. A p value < 0.05 was considered statistically significant.

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Author contributions

J.J.L., J.Y.M., A.B., M.H. and A.A.F. conceived and designed the project. J.J.L. performed experiments. J.J.L., M.H. and A.A.F. analyzed the data. J.J.L., J.Y.M., A.B., M.H. and A.A.F. contributed to the final version of the manuscript. All authors approved the final manuscript.

Competing interests

The authors declare no competing interests.

Additional information

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Destruction of *S. aureus* biofilms by combining an antibiotic with subtilisin A or calcium gluconate

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FIGURES

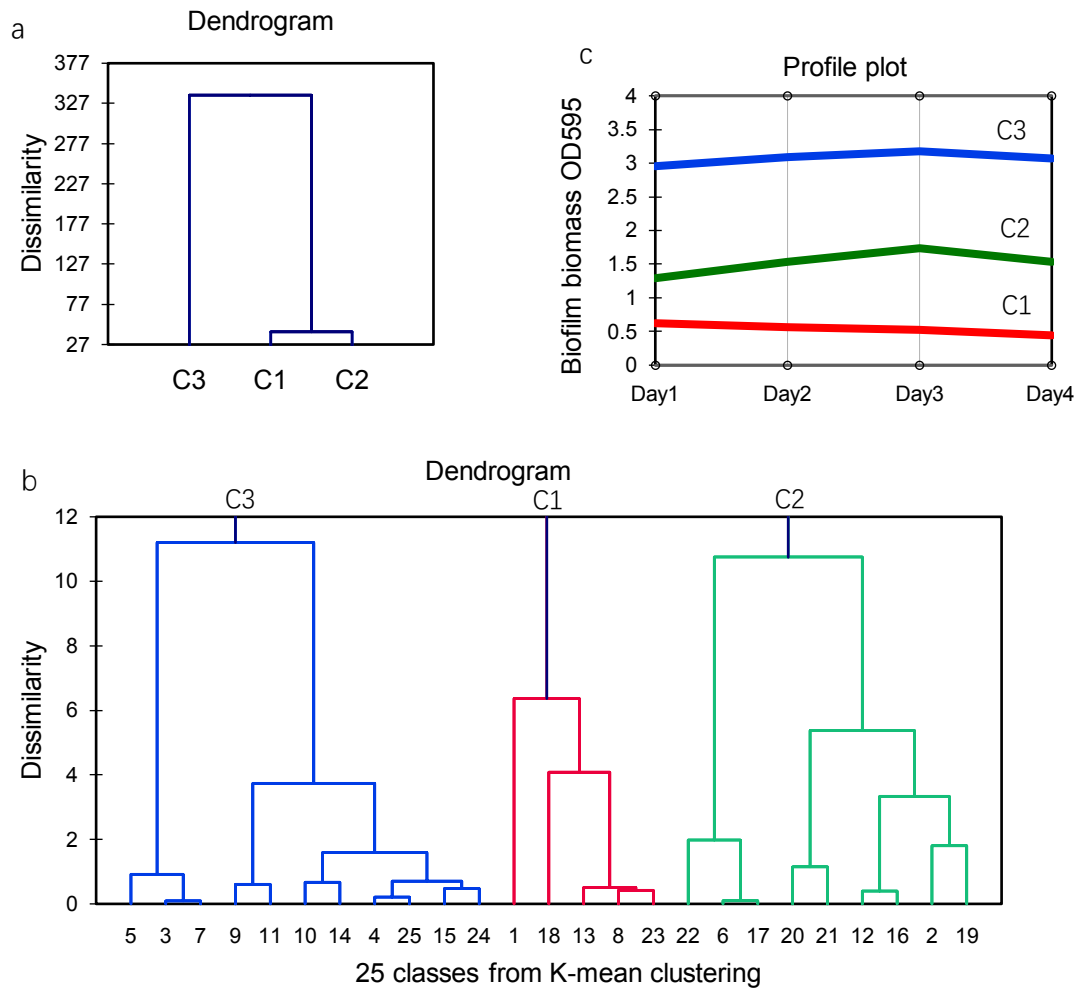


Figure. S1 Clustering of biofilm biomass producers. (a) The averages of the four OD values obtained each day for each isolate were first clustered into 25 classes by k-means. (b) The class centroids obtained were then analysed by agglomerative hierarchical clustering (AHC), which produced (c) three distinct clusters of *S. aureus* isolates related to their biofilm biomass. Four independent experiments in quadruplicate.

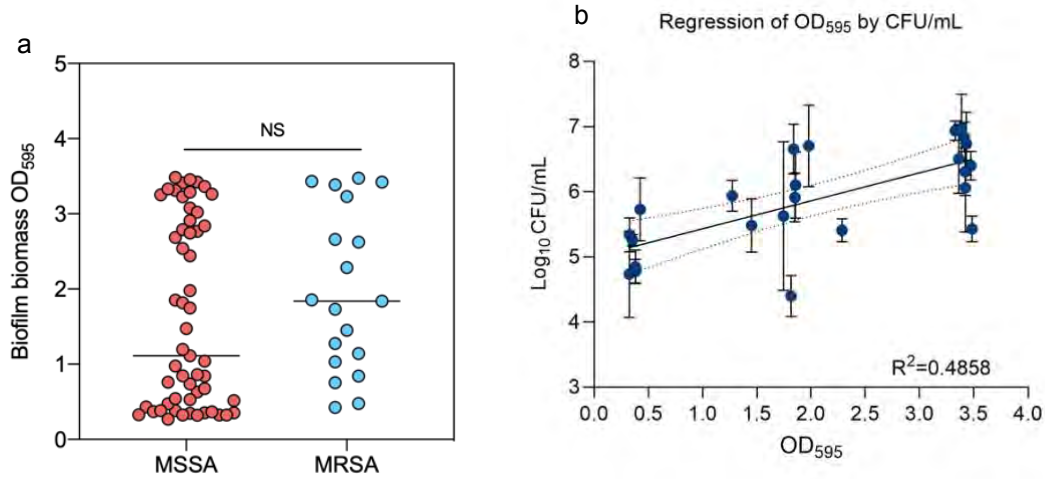
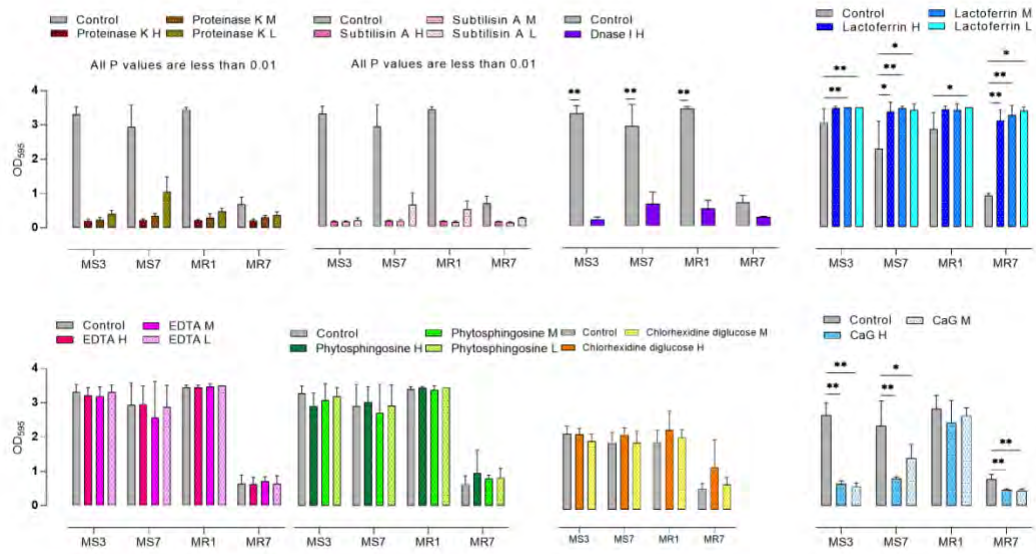


Figure S2. (a) The scattergrams of biofilm biomass production of MSSA and MRSA isolates. Data are the means of values from four independent experiments with four replicates. (b) Weak statistically significant fit for linear regression between biomass and biofilm bacteria counts. Biofilm biomass (OD₅₉₅ values) and counts of biofilm bacteria (log₁₀ CFU/mL) were obtained from 24 representative *S. aureus* isolates. OD values are the means of values obtained from four independent experiments with four replicates (n=16 for each isolate). The log₁₀ CFU/mL data are the means of values obtained in at least three independent experiments with replicates (n>=6 for each isolate). Error bars indicate SD.

a. Non-antibiotic substances



b. Antibiotic molecules

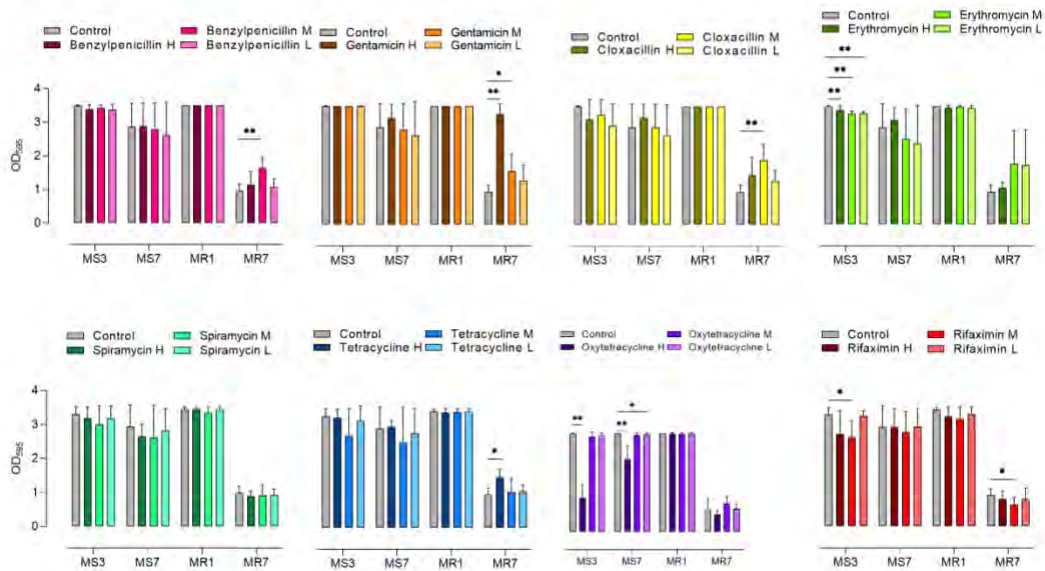


Figure S3. Preliminary selection of substances based on a reduction in biofilm biomass for two isolates (MS3 and MR1) from the high biomass producer group and two isolates (MS7 and MR7) from the medium biomass producer group out of 24 representative isolates. The destructive effect on biofilm biomass was measured (OD₅₉₅ value) after exposure of biofilms to (a) eight non-antibiotic substances (proteinase K combined with Ca²⁺ 1.25mmol/L, subtilisin A, Dnase I, lactoferrin, EDTA, phytosphingosine, chlorhexidine digluconate, and calcium gluconate (CaG)) and (b) eight antibiotic molecules (benzyl-penicillin, gentamicin, cloxacillin, erythromycin, spiramycin, tetracycline, oxytetracycline (OTC), and rifaximin). The concentrations (H: high; M: medium; L: low) tested for each substance are reported in Table S2. Statistical significance compared to control was determined by the Mann-Whitney U test. *P<0.05. **P<0.01. ***P<0.001. Without a mark: not significant. Data are the means of values from two independent experiments in triplicate (n=6). Error bars indicate the SD.

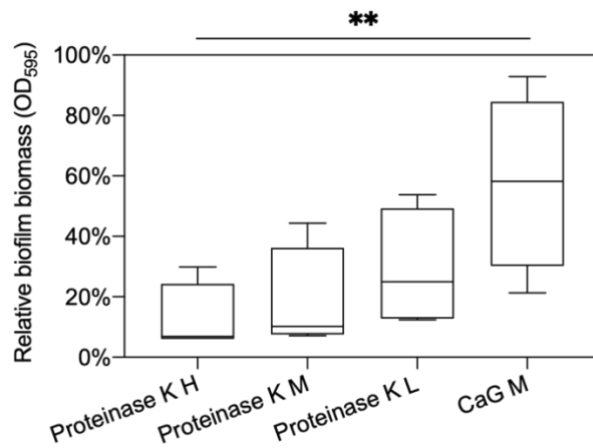


Figure S4. Reduction in biofilm biomass by CaG (1.25 mmol/L) alone or with proteinase K. Proteinase K was used with calcium chloride (Ca^{2+} 1.25mmol/L). H: high concentration; M: medium concentration; L: low concentration. Results from a preliminary selection (4 isolates) are represented as boxplots. Statistical significance between the different conditions was determined by Friedman's test with a post hoc application of Nemanyai. ** $P < 0.01$. Without a mark: not significant. The Y-axis represents the percentage of OD₅₉₅ value relative to the control group. Data are the means of values from two independent experiments in triplicate (n=6). Error bars indicate the SD.

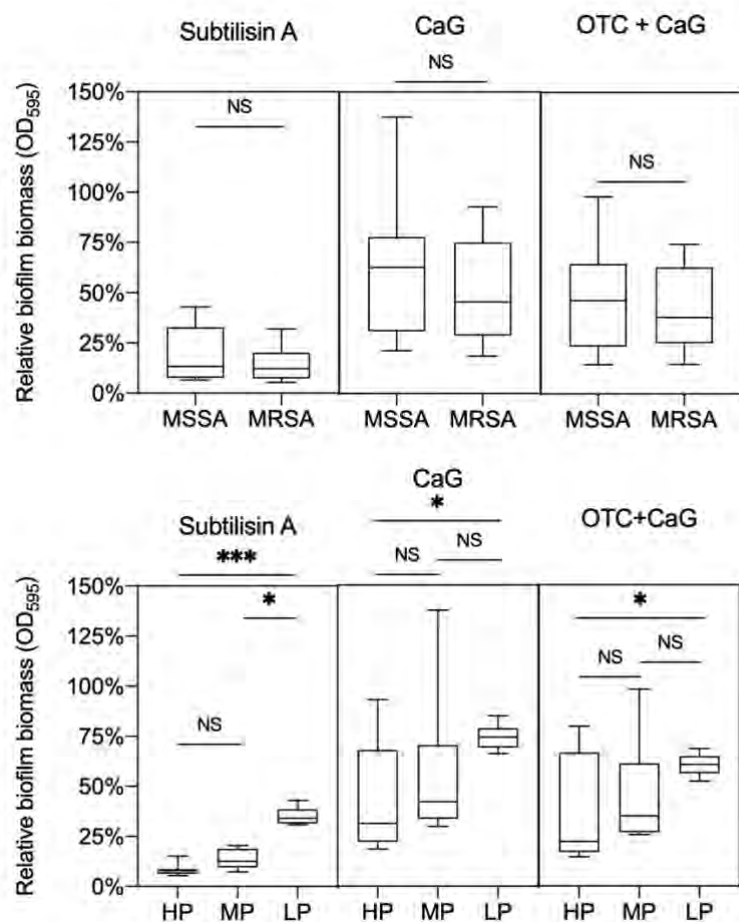


Figure S5. Reduction in biofilm biomass (24 isolates) after exposure to subtilisin A (0.01 U/mL), Ca^{2+} (1.25 mmol/L) or a combination of CaG and OTC (10 $\mu\text{g/mL}$) represented as separate box plots according to (a) MSSA and MRSA profiles or (b) to biofilm-forming capacity (high (HP), medium (MP), and low (LP) producer) for the individual isolates. Statistical significance between conditions was determined by the Mann-Whitney U test (for MSSA and MRSA) and Kruskal-Wallis test (for biofilm-forming capacity) respectively. * $P < 0.05$. ** $P < 0.01$. *** $P < 0.001$. NS: not significant. Data are presented as box plots with values from two independent experiments in triplicate ($n=6$).

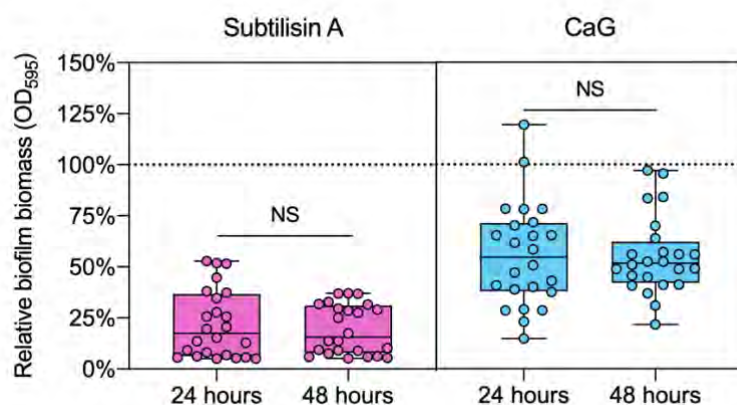


Figure S6. Inhibition of biofilm formation by subtilisin A and CaG. Relative biofilm biomasses from 24 representative isolates after addition of subtilisin A (0.01 U/mL) or CaG (Ca^{2+} 1.25 mmol/L) at the onset of the biofilm. The inhibitory effects on the biofilm biomass were measured after incubation for 24 hours or 48 hours. Statistical significance was determined by the Mann-Whitney U test. NS: not significant. The Y-axis represents the percentage of OD₅₉₅ value relative to the control group, itself set at 100%. Data are presented as box plots of two independent experiments in triplicate (n=6). Error bars indicate the SD.

TABLES

Table S1: Numbers and MSSA/MRSA profiles of the isolates according to their capacity to produce biofilm

Cluster of isolates	Total (74 isolates)		MSSA (55 isolates)		MRSA (19 isolates)	
	Percentage of isolates	OD ₅₉₅ range	Percentage of isolates	OD ₅₉₅ range	Percentage of isolates	OD ₅₉₅ range
Cluster 1: Low producer	42% (31/74)	1.1~0.3	45% (25/55)	0.9~0.3	32% (6/19)	1.1~0.4
Cluster 2: Medium producer	20% (15/74)	2.3~1.0	16% (9/55)	2.0~1.0	32% (6/19)	2.3~1.3
Cluster 3: High producer	38% (28/74)	3.5~2.4	38% (21/55)	3.5~2.4	37% (7/19)	3.5~2.6

Table S2: Tested concentrations for the eight non-antibiotic substances and eight antibiotics

Final concentration	Unit	High	Medium	Low
Proteinase K (with 1.25 mmol/L Ca ²⁺)	µg/mL	100	10	1
Subtilisin A	U/mL	0.1	0.01	0.001
DNase I (Buffer)	U/mL	100	N/A	N/A
Lactoferrin	µg/mL	500	50	5
EDTA	µg/mL	10	1	0.1
Phytosphingosine	µg/mL	1	0.1	0.01
Chlorhexidine digluconate	% (w/v)	0.005	0.0005	N/A
Calcium gluconate	Ca ²⁺ mmol/L	12.5	1.25	N/A
Benzyl-penicillin	µg/mL	100	10	1
Gentamicin	µg/mL	20	2	0.2
Cloxacillin	µg/mL	20	2	0.2
Erythromycin	µg/mL	200	20	2
Spiramycin	µg/mL	50	5	0.5
Tetracycline	µg/mL	10	1	0.1
Oxytetracycline	µg/mL	10	1	0.1
Rifaximin	µg/mL	5	0.5	0.05

Table S3: Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of OTC, subtilisin A, and CaG for the 24 isolates. The isolates for which the MBC of OTC is less than 10 µg/mL are labelled in red.

Isolate No.	Oxytetracycline (µg/mL)		Subtilisin A (U/mL)		Calcium gluconate (mmol/L)	
	MIC	MBC	MIC	MBC	MIC	MBC
MS1	0.125	0.25	>1.5	>1.5	>112.5	>112.5
MS2	0.125	8	>1.5	>1.5	>112.5	>112.5
MS3	0.125	4	>1.5	>1.5	>112.5	>112.5
MS4	0.125	8	>1.5	>1.5	>112.5	>112.5
MS5	0.125	8	>1.5	>1.5	>112.5	>112.5
MS6	0.125	0.5	>1.5	>1.5	>112.5	>112.5
MS7	0.25	4	>1.5	>1.5	>112.5	>112.5
MS8	0.125	2	>1.5	>1.5	>112.5	>112.5
MS9	0.125	2	>1.5	>1.5	>112.5	>112.5
MS10	0.0625	16	0.1875	0.1875	>112.5	>112.5
MS11	0.125	4	0.09375	0.09375	>112.5	>112.5
MS12	0.125	16	0.1875	0.1875	>112.5	>112.5
MS13	0.125	16	0.046875	0.046875	>112.5	>112.5
MS14	0.125	32	0.09375	0.09375	>112.5	>112.5
MR1	16	128	>1.5	>1.5	>112.5	>112.5
MR2	0.125	4	0.375	0.375	>112.5	>112.5
MR3	0.125	64	0.1875	0.1875	>112.5	>112.5
MR4	0.125	16	>1.5	>1.5	>112.5	>112.5
MR5	8	32	>1.5	>1.5	>112.5	>112.5
MR6	16	>128	>1.5	>1.5	>112.5	>112.5
MR7	128	>128	>1.5	>1.5	>112.5	>112.5
MR8	16	128	>1.5	>1.5	>112.5	>112.5
MR9	16	128	0.1875	0.1875	>112.5	>112.5
MR10	128	>128	>1.5	>1.5	>112.5	>112.5

Table S4: Statistical significance of Fig.2b. The table only shows statistically significant p-values and study-relevant p-values.

	PENG	GEN	CLOX	ERY	SPIR	TC	RIFX	OTC	CaG	Sub A
PENG +CaG	P <0.001								1.00	0.11
GEN +CaG		P <0.001							1.00	P <0.001
CLOX +CaG			P <0.05						1.00	0.28
ERY +CaG				P <0.001					1.00	0.40
SPIR +CaG					P <0.05				1.00	P<0.01
TC +CaG						P <0.001			1.00	P<0.05
RIFX +CaG							0.38		1.00	P <0.001
OTC +CaG								P <0.001	0.64	1.00
CaG	P <0.001	P <0.001	0.19	P<0.01	P <0.001	P <0.0001	P <0.05	P<0.01	1.00	<0.05
SubA	P <0.001	P <0.001	P <0.001	P <0.001	P <0.001	P <0.001	P <0.001	P <0.001	P<0.05	1.00

Antibiotics: PENG, benzyl-penicillin; CLOX, cloxacillin; GEN, gentamicin; ERY, erythromycin; SPIR, spiramycin; TC, tetracycline; RIFX, rifaximin; OTC, oxytetracycline.

3. Supplementary data

This part described supplementary data that were not published in the frame of the Scientific Report publication.

We examined, using the same methods as previously described, the combination of the 2 best substances (Ca and subtilisin A) and assessed the destructive effects of lactoferrin that was described as effective on *S. aureus* biofilms⁴⁰⁹.

3.1 Results

Our results demonstrated that there is no significant additive effect when subtilisin A and CaG were combined, while the addition of CaG did not reduce the destructive effect of subtilisin A on biofilm biomass (Fig. 15).

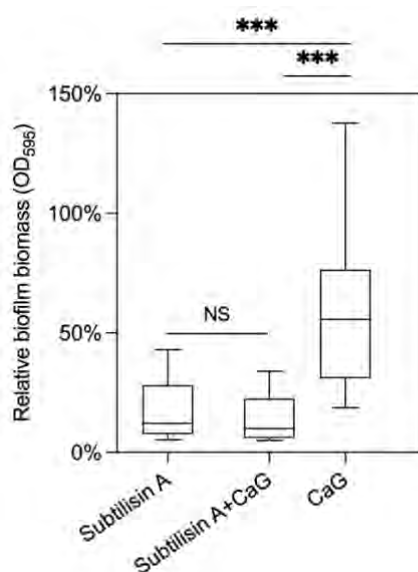


Figure 15 Reduction of *S. aureus* biofilm biomass after exposure to subtilisin A or CaG or subtilisin A plus CaG.

Reduction of biofilm biomass tested after exposure to subtilisin A (0.01U/mL), Ca²⁺ (1.25 mmol/L), and their combination represented as box plots for the 24 isolates. Statistical significance was determined by Friedman's test with a post hoc application of Nemanyi. ***P < 0.001. NS: not significant. The Y-axis represents the percentage of OD₅₉₅ values relative to the control group set at 100%. Data are the means of values from two independent experiments in triplicate (n = 6).

In the preliminary screening of non-enzymatic substances, we noticed that adding bovine

lactoferrin (500µg/mL, 50µg/mL, and 5µg/mL) on 24h-old biofilm significantly ($P<0.01$) increased the biomass of 18 out of 24 isolates (Fig. 16a). Culturing bacterial suspension with lactoferrin (50µg/mL) of 24 or 48 hours also significantly ($P<0.01$) promoted the formation of biofilm formation (15/24 isolates) (Fig. 16b). By contrast, the combination of calcium gluconate and lactoferrin on established biofilm resulted in the reduction of total biofilm biomass (Fig. 17). Nevertheless, this reduction effect is poorer than the monotherapy with calcium gluconate (Fig. 17).

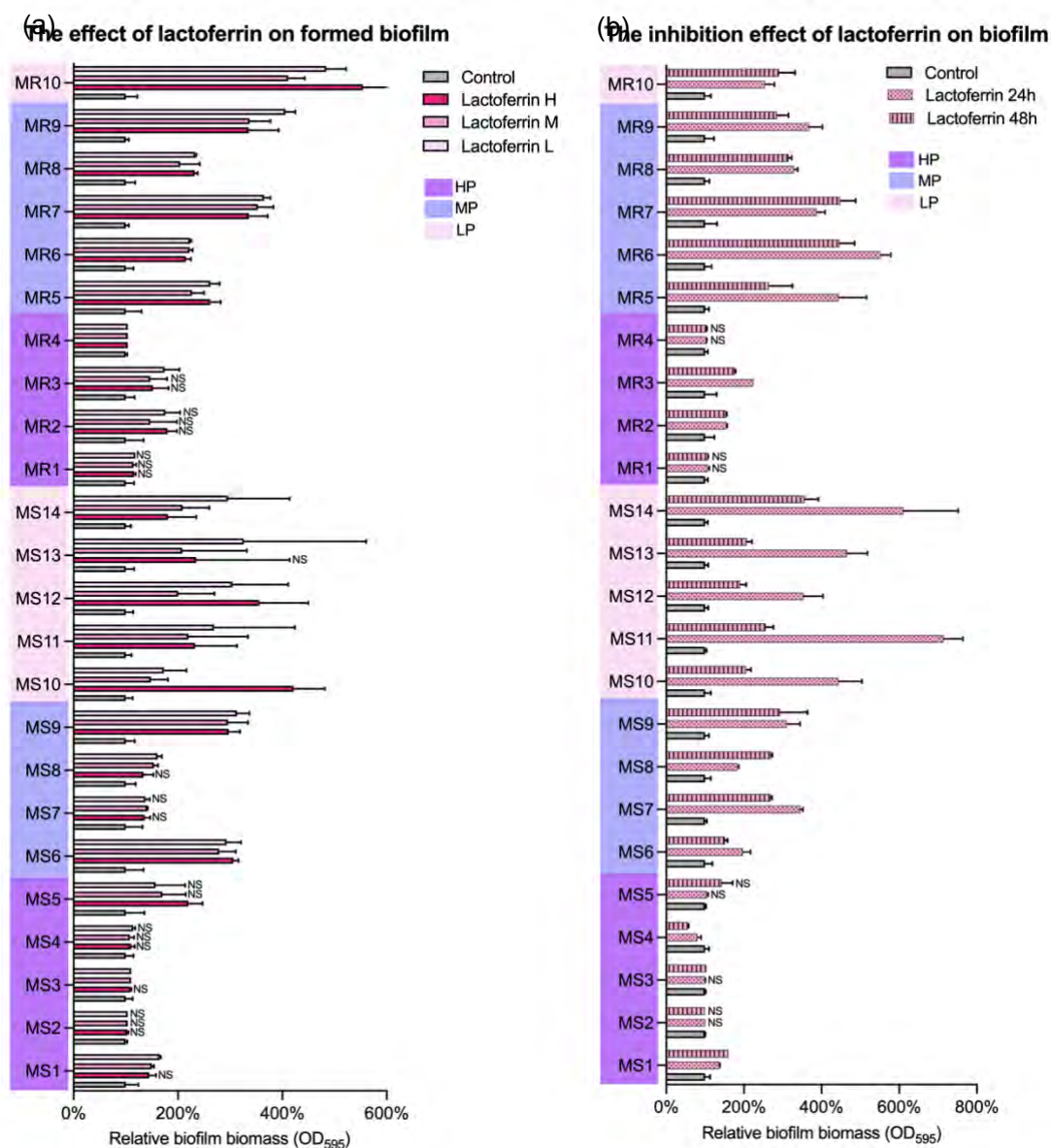


Figure 16 Enhance of *S. aureus* biofilm biomass by lactoferrin

(a) Relative biomasses of 24h-old biofilms of 24 isolates exposed to lactoferrin H (500µg/mL), lactoferrin

M (50µg/mL), and lactoferrin L (5µg/mL) for 24h. (b) Relative biomasses of biofilms of 24 isolates after culturing with lactoferrin M (50µg/mL) 24h or 48h. The X-axes represent the percentage of biofilm biomass (OD₅₉₅) with the control (biofilm formed by the non-treated isolate) set at 100%. High (HP), medium (MP) and low (LP) biofilm producers are highlighted with different colors. Statistical significance was determined by the Mann-Whitney U test. Unmarked: P<0.01. NS: not significant. Error bars indicate the SD. Data are the means of values from two independent experiments in triplicate (n=6).

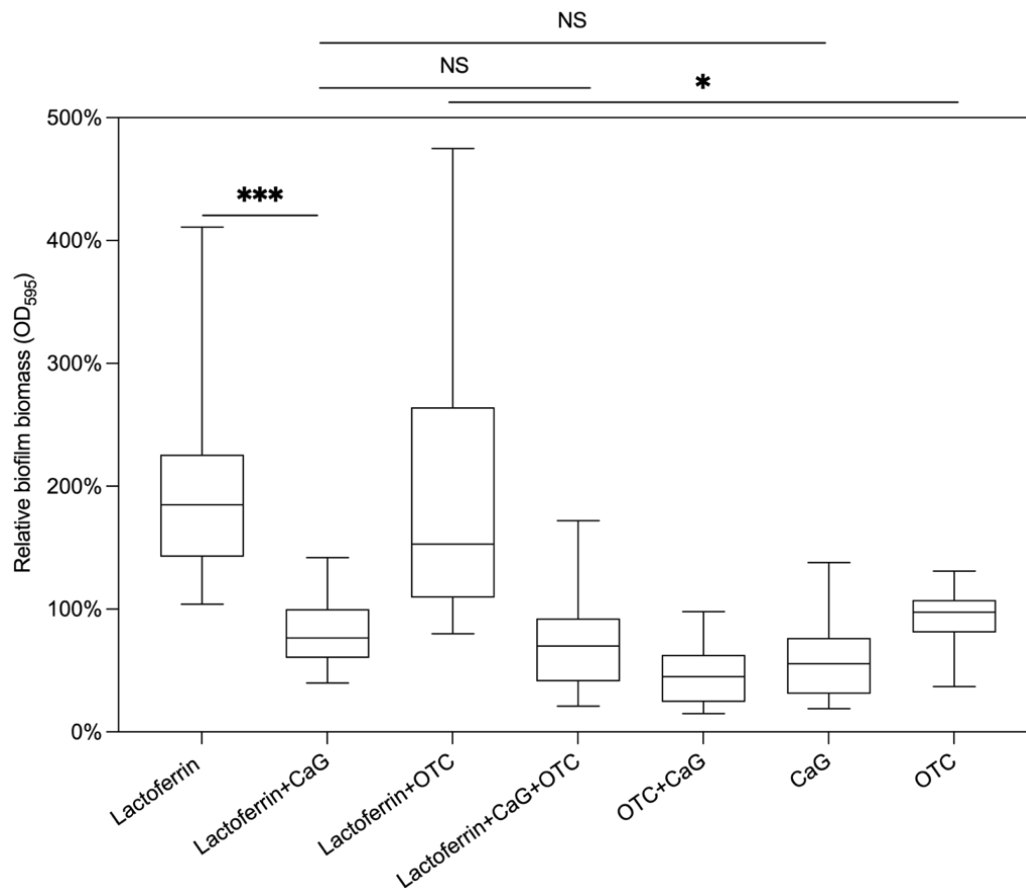


Figure 17 Effect on *S. aureus* biofilm after exposure to Lactoferrin, CaG, and their combination

Relative biomasses of 24h-old biofilms of 24 isolates tested after exposure to lactoferrin M (50µg/mL), CaG (1.25 mmol/L), and Lactoferrin M+CaG represented as box plots for the 24 isolates. Statistical significance was determined by Friedman's test with a post hoc application of Nemanyai. *P<0.05. **P<0.01. ***P<0.001. P values are presented in Table 1. Data are the means of values from two independent experiments in triplicate (n=6).

Table 1 Statistical significance of Fig.17

P value	Lacto+ CaG+OTC	Lacto+ CaG	Lacto+ OTC	OTC+CaG	CaG	OTC	Lacto
Lacto+ CaG+OTC			P<0.001	P<0.01			P<0.001
Lacto+ CaG			P<0.01	P<0.001			P<0.001
Lacto+ OTC	P<0.001	P<0.01		P<0.001	P<0.001	P<0.05	
OTC+CaG	P<0.01	P<0.001	P<0.001			P<0.001	P<0.001
CaG			P<0.001			P<0.05	P<0.001
OTC			P<0.05	P<0.001	P<0.05		P<0.01
Lacto	P<0.001	P<0.001		P<0.001	P<0.001	P<0.01	

Lacto: Lactoferrin

3.2 Discussion

Lactoferrin significantly increased the biofilm formation of almost all the *S. aureus* isolates. The possible explanation is that the production of *S. aureus* biofilm was induced under the iron-restricted conditions provided by the binding of free iron by lactoferrin⁴¹⁰. However, the factors, which could enhance the biofilm biomass, such as the enhancement of EPS matrix formation, an increase of dead cells embedded with matrix, or a bacterial growth were not explored separately. For example, the enhancement of the biofilm biomass (*S. aureus* ATCC6538) conducted to a significant rise in the OD value after polymyxin treatment while killing almost all bacteria⁴¹¹. Although we did not explore the factors of lactoferrin on biofilm biomass, we validated that calcium could still reduce the biofilm biomass in presence of lactoferrin.

3.3 Material and methods

The 24 representative isolates of *S. aureus* included in this part were identical to those used in the published article.

The substances that were used are same subtilisin A (Sigma-Aldrich, USA) and calcium gluconate (Lavoisier, France) as in the article and lactoferrin (Sigma-Aldrich, USA),

The process of CV assay and the related materials as well as statistical analysis were those described in the published article.

Experimental part:

Chapter 2

1. The objective of project

We hypothesize that the ability of *S. aureus* ST398 to produce surface-associated biofilms has significant strain specificity, which may be caused by the type of adhesion genes and the genes expression throughout biofilms forming. Therefore, we analyzed the correlation among various factors of 105 *S. aureus* ST398, including biofilm biomass forming capacity, the quantity of biofilm bacteria production, *spa* type, *agr* type, the types of related adhesion genes, the expression of genes related to adhesion during the biofilm formation. Specifically, the genes linked to surface adhesion which included in the study are *icaA* and *icaD* (intercellular adhesion A and B), *fnbA* and *fnbB* (fibronectin-binding proteins A and B), *fib* (fibrinogen binding protein), *clfA* and *clfB* (clumping factors A and B), *cna* (collagen-binding protein), *ebpS* (elastin binding protein), *bap* (biofilm-associated protein) and *eno* (laminin binding protein).

2. Results

2.1 Characterization of *S. aureus* ST398 isolates

Among the 105 *S. aureus* ST398 isolates, 85 were MRSA ST398 and 20 isolates were MSSA ST398. The donut chart (Fig. 18a) shows the proportion of various host resource of 105 *S. aureus*: 33 (31%) were from bovine, 35 (33%) were from horse, 16 (15%) were from cat, and 21(20%) were from dog. Within all the group of *S. aureus* ST398 by host source, the amount of MRSA ST398 are higher than the number of MSSA ST398, in particular, all the *S. aureus* ST398 isolated from dog were MRSA (33 isolates). Of the 105 *S. aureus* ST398 studied by PCR, the *icaA*, *icaD*, *fnbA*, *fnbB*, *eno* genes were all detected (100%), while none of the isolates contained the *bap* gene and only one isolate possessed *fib* gene as detected by PCR. The prevalence of other adhesion-related genes was as follows: 95% *clfA* (100 isolates), 98% *clfB* (103 isolates), 90% *bbp* (95 isolates), 98 % *cna* (103 isolates), 99% *ebpS* (104 isolates) and *bap* 0%.

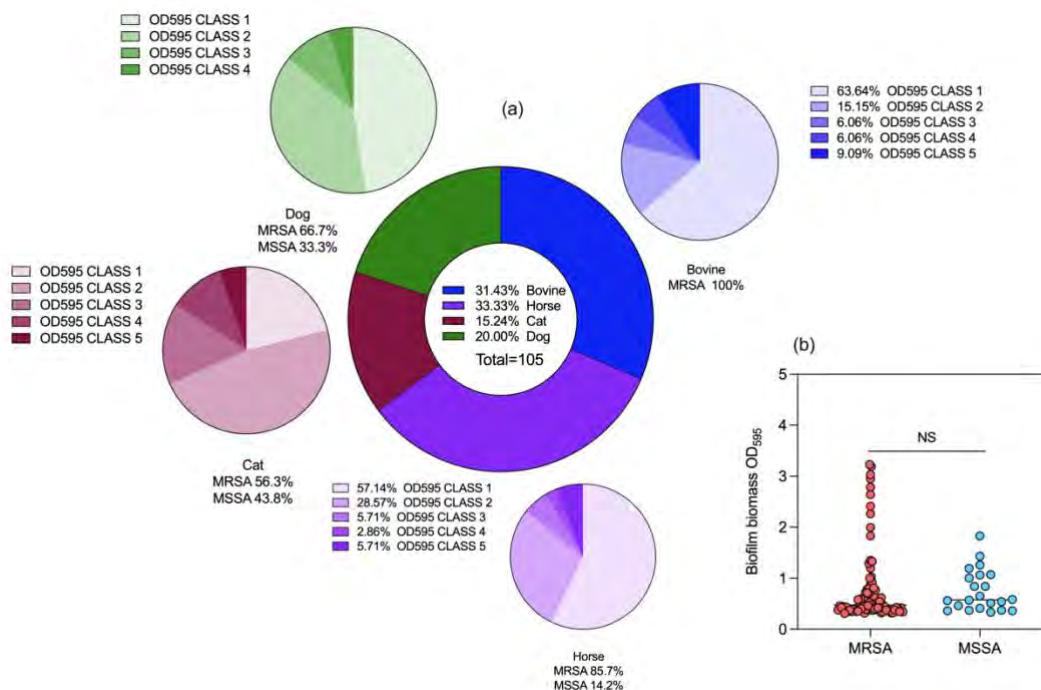


Figure 18 Differentiation between *S. aureus* ST398 isolates with different biofilm-forming capacities. (a) The percentage of 105 *S. aureus* ST398 isolated from the various hosts was presented in a donut chart, including bovine, horse, cat, and dog. The five class OD595 indicated the different biofilm-forming

capacities (24 h of culture) of 105 *S. aureus* isolates. The pie chart showed the percentage of each OD595 class within each host group. The distribution of MRSA and MSSA within each host group was also labeled in figure (b) The scattergrams of biofilm biomass (24 h) production of MSSA and MRSA ST398 isolates. Data are the means of values from four independent experiments with four replicates. Statistical significance was determined by the Mann–Whitney U test. NS: not significant.

2.2 Selection of isolates with the highest forming capacity of surface associated biofilm

By the same process of crystal violet (CV) assay, all *S. aureus* ST398 isolates produced biofilm but presented varying degrees of adherence to the surface of polystyrene microplate. The five classes of *S. aureus* ST398 were acquired from the results of OD595 analyzed by k means clustering with AHC optimization (Fig. 19a). The number of classifications from low to high (from class 1 to class 5) represented enhancing isolate's ability to produce surface-associated biofilms. Fig. 19b demonstrated the frequency of *S. aureus* ST398 in each classification in which more than half of the isolates belonged in class1. The isolates classified into class 5 were considered to own the strongest capacity to form surface- associated biofilms. Moreover, the frequency of *S. aureus* ST398 with different surface-associated biofilm forming abilities in different hosts is random (Fig. 18a) The frequency of *S. aureus* ST398 with different surface-associated biofilm forming abilities in various hosts is random (Fig. 18a) and no significant difference was observed between MRSA ST398 and MSSA ST398 (Fig. 18b).

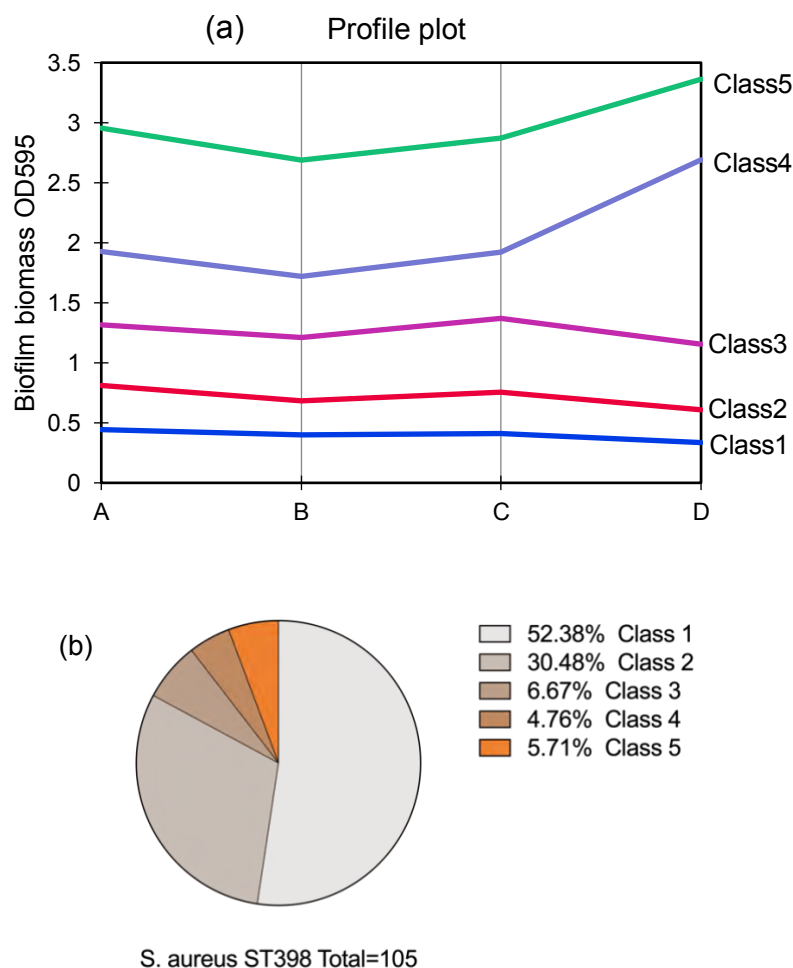


Figure 19 Classification of *S. aureus* ST398 biofilm biomass producers

(a) The averages of the four OD values obtained each day for each isolate were first clustered into 25 classes by k-means. Then, the class centroids obtained were analysed by agglomerative hierarchical clustering (AHC), which produced five distinct clusters of 105 *S. aureus* ST398 isolates related to their biofilm biomass. Four independent experiments (A, B, C, and D) in quadruplicate. (b) The proportion of each biofilm biomass category in 105 *S. aureus* isolates.

2.3 Quantitative analysis of biofilm bacteria

After the same pre-test treatment on formed biofilm as above, the biofilm bacteria produced by all *S. aureus* ST398 after 24 h incubation was counted. Most *S. aureus* ST398 (68 isolates, 64.76%) carried around $6 \log_{10}$ CFU/mL biofilm bacteria after the maturation of biofilm, while only 4 isolates (2 MRSA ST398 and 2 MSSA ST398) produced about $8 \log_{10}$ CFU/mL biofilm bacteria (Fig. 20a). The forming capacity of surface-associated biofilm between MRSA ST398

and MSSA ST398 has no significant difference (Fig. 20b). Also, by the same pre-test treatment, the number of biofilm bacteria in the 24 h biofilm formed by 105 *S. aureus* ST398 isolates ranged from 5 to 8 log₁₀ CFU/mL with no linear correlation with the biofilm biomass assessed by CV staining ($R^2 = 0.1636$) (Fig. 20c).

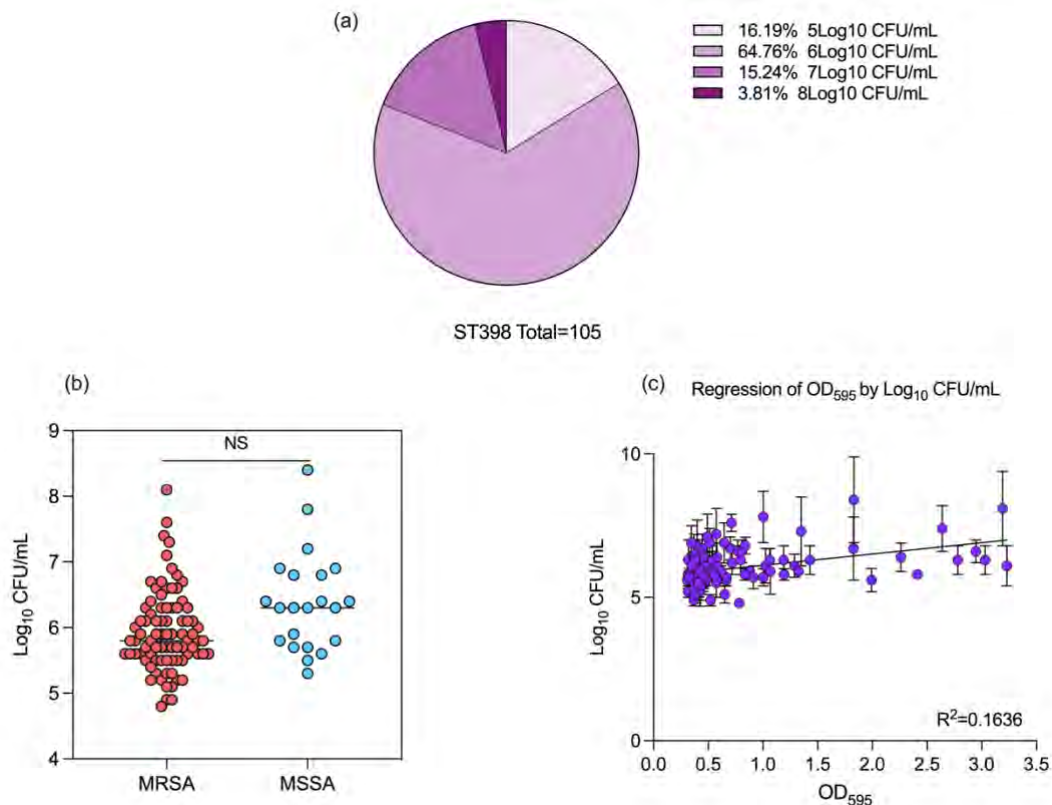


Figure 20 Quantitative *S. aureus* ST398 biofilm bacteria

(a) 105 *S. aureus* ST398 were classified depending on the number of biofilm bacteria after 24 hours of culture. The pie chart indicated the frequency of each log₁₀ CFU/mL class. The log₁₀ CFU/mL data are the means of values obtained in three independent experiments with three replicates. Statistical significance was determined by the Mann–Whitney U test. NS: not significant. (b) Non statistically significant fit for linear regression between biomass and biofilm bacteria counts. Biofilm biomass (OD₅₉₅ values) and counts of biofilm bacteria (log₁₀ CFU/mL) were obtained from 105 *S. aureus* ST398 isolates.

2.4 Selection of isolates with the original low forming capacity of surface associated biofilm

We next planned to select isolates with the original lowest ability to produce surface-associated biofilms. We canceled the biofilm washing step with PBS in the original CV assay protocol (the rest steps remain unchanged). After the new CV assay, k means clustering (the number of categories given is 25) was applied to statistically analyze the OD₅₉₅ values of all *S. aureus* ST398 isolates. In the profile plot of Fig. 21, most of the OD₅₉₅ values are higher than 2.5 due to the extremely low external stress of pre-test biofilm treatment. However, isolates that can still maintain a low OD₅₉₅ (around or lower than 2) were considered to have the lowest levels of their ability to produce surface-associated biofilms. Therefore, class7, class8, and class12 with the lowest and stable OD₅₉₅ were selected as the representative isolates with the potential lowest forming capacity surface-associated biofilm. Compared to the above selection (with PBS washing step), the isolates belonging to the class7/8/12 simultaneously belonged to the class1/2 from the original CV assay (PBS washing step).

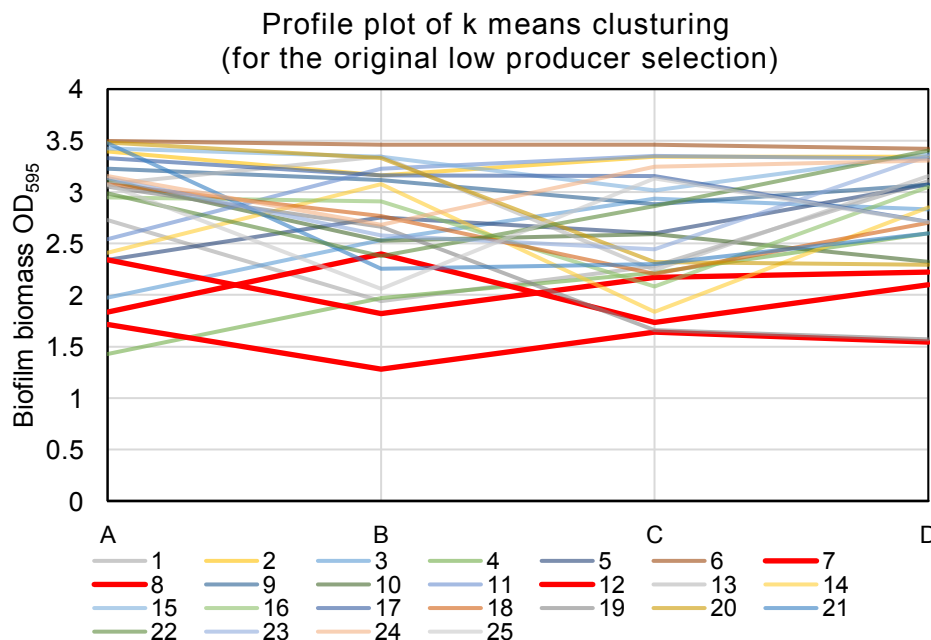


Figure 21 The selection of original low producer

The 24 h biofilm of 105 *S. aureus* isolates were obtained by the same process as previous screening but without the twice PBS washing step. The averages of the four OD values obtained each day for each isolate were first clustered into 25 classes by k-means. The classes in red were considered as the original low biofilm biomass producer. Data are the means of values from four independent experiments

with four replicates.

3. Brief discussion

The following discussion is only based on the current results. *S. aureus* ST398 can distribute in various hosts, including bovine, horse, cat, and dog. The types of biofilm adhesion-related genes contained in all the *S. aureus* ST398 isolates are highly similar and do not correlate with the ability to produce surface-associated biofilms. Therefore, the strain-specificity of surface-associated biofilms may be related to the expression of adhesion-related genes during the formation of biofilms. To prove this hypothesis, we need to compare the expression of target genes in two isolates with significantly different capabilities of surface-associated biofilm formation. Meanwhile, these two isolates should be the same MRSA or MSSA and have the same host, the same adhesion-related genes, the same *spa* and *agr* type, and the similar production level of biofilm bacteria.

In selecting high biofilm producers, the PBS washing step of the original CV assay brought intense external stress on mature biofilms. Under this external stress, the biofilms produced by class 5 isolates ($OD_{595} > 3$) are significant in amount and highly resistant to external stress, enabling most of the formed biofilm to remain on the surface of polystyrene. In contrast, the low OD_{595} (< 1) values obtained under intense external stress may come from two factors: first, the original ability of the isolate to produce biofilms is low; second, the biofilm produced by the strain was less resistant to external stress (like aggregated biofilm). To screen out isolates with the first factor, we removed the PBS washing step from the original CV assay to minimize the impact of external stress on mature biofilms. The absence of the washing step resulted in a significant increase in the OD_{595} value of most of the *S. aureus* ST398 (Fig. 4), which was caused by the residue of many planktonic bacteria and suspended biofilm fragments. Nevertheless, there are still some *S. aureus* ST398 (from class 7/8/12 and originally belong to the class 1/2) whose OD_{595} value presented a relatively low level ($OD_{595} < 2$), indicating their forming capacity of surface-associated biofilm is low, rather than the produced biofilm had poor adhesion.

4. Prospect

The *spa* type, *agr* type of all the *S. aureus* ST398 will be determined. Then, the exact representative isolates will be screened out, which will include three isolates: biofilm hog producer, biofilm low producer and isolate with no-susceptibility to CaG. The target adhesion related genes required for the RT-PCR test will be selected depending on the references and further results, probably including *icaA*, *icaD*, *fnbpA/B*, and *ebps*.

Each of the three selected isolates will be cultured under the same condition as previous experiments. Because the expression of biofilm-related genes is regulated up and down along with biofilm formation, we will detect the levels of gene expression in the biofilm bacteria formed under different culture times. Meanwhile, the expression level of the same gene in the planktonic bacteria will also be tested, which will serve as a comparison object. For the specific experimental arrangement of this part, please refer to the following flow chart (Fig. 22). The cultivation time of biofilm and the selection of genes will be adjusted based on preliminary experimental results. Related auxiliary experiments (such as the quantitative analysis of planktonic bacteria and biofilm bacteria) will also be added or deleted according to certain conditions. Depending on the gene expression results, other experiments may be jointed to prove the results further. Finally, we may introduce the hollow fiber biofilm infection model to compare the differences of gene expressions between instate system and dynamic system.

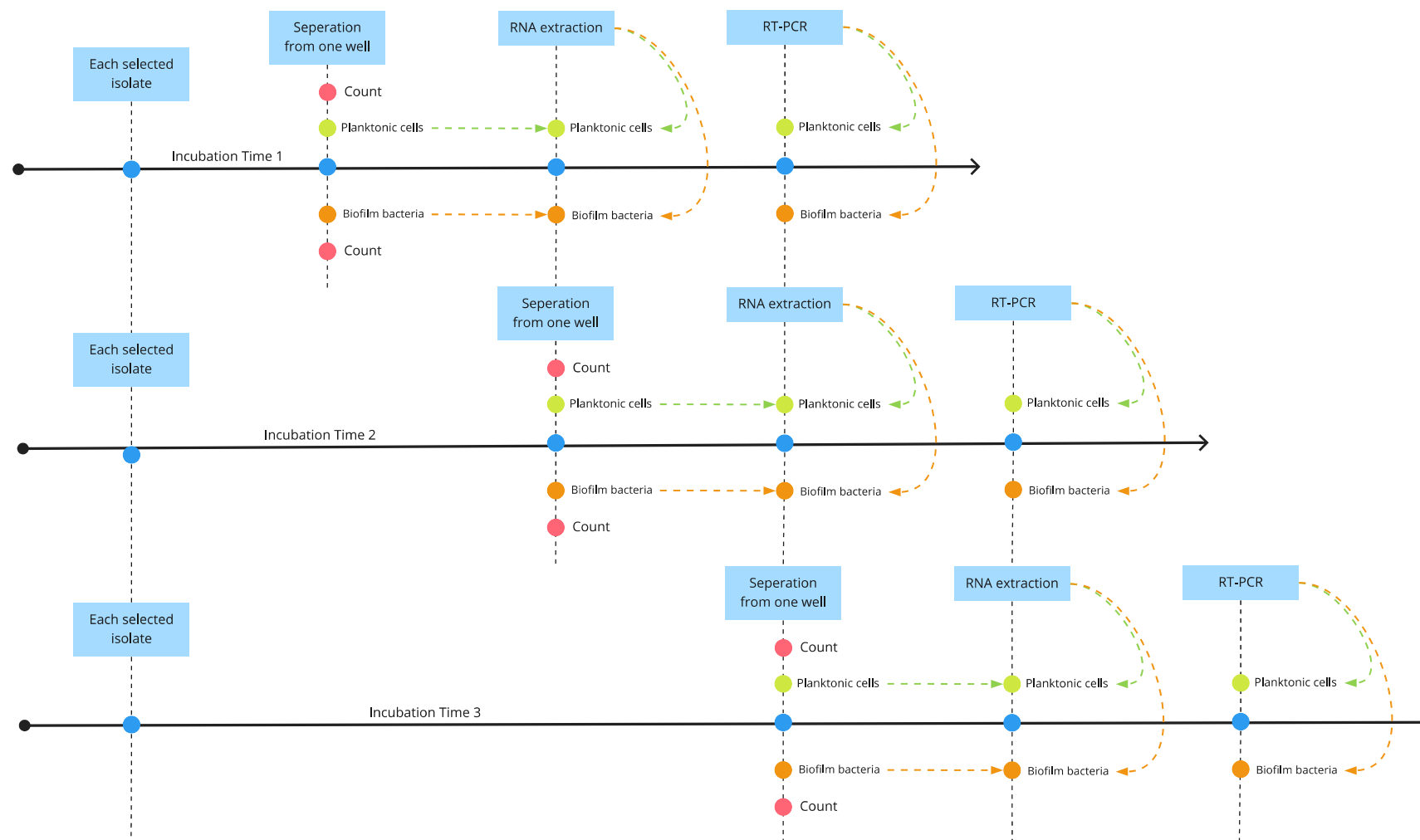


Figure 22 The flow chart for further experiments.

5. Materials and methods

5.1 Bacterial strains

A total of 105 clinical isolates of *S. aureus* were used in this study. All isolates were collected in France through the Resapath network for the surveillance of resistance in veterinary medicine (<https://resapath.anses.fr/>). All isolates were identified as ST398 using the ST398-specific PCR⁴¹². The presence of the *mecA* gene was assessed by PCR.

5.2 Biofilm formation

All the isolates were first cultured on Mueller Hinton Agar overnight at 37 °C. A few colonies were then diluted in sterile Brain Heart Infusion (BHI) broth plus 1% glucose to obtain a final suspension of 6 log₁₀ CFU/mL and 180 µL of this suspension was added to each well of a 96-well polystyrene microplate (Thermofisher Nunc). Plates were incubated at 37 °C for 24 h to allow biofilm formation.

5.3 Crystal Violet (CV) assay (biomass)

After 24 h of incubation at 37 °C, the microplates were turned upside down and tapped so that broth and most of the planktonic bacteria fell out onto the absorbent mat⁴¹³. The biofilms were then rinsed twice with 200 µL of sterile phosphate-buffered saline per well (Note: for the selection of original low biofilm producer, this PBS washing step was removed). Two hundred microlitres of CV solution (Sigma-Aldrich, 0.05% w/v) was added to each well and left for 3 min at 25 °C. The excess dye was then rinsed with 200 µL of sterile PBS, the wells were air-dried naturally, then 200 µL of 96% (w/v) ethanol was added to each well to resolubilise the residual dye. The biofilm's biomass was measured using a microplate reader (CLARIOstar Plus) with the value of absorbance set at 595 nm (OD₅₉₅).

5.4 Quantification of cultivable biofilm bacteria

After 24 h of incubation at 37 °C in the presence of substances or only BHI with 1% glucose (control), wells were rinsed twice with 200 µL of sterile PBS as above and the bacteria were then resuspended in 200 µL of sterile PBS. After 10 min of ultrasound at 40 Hz (Branson), the cultivable biofilm bacteria were counted by plating serial tenfold dilutions on tryptic soy agar plates. The reduction in biofilm bacteria was calculated as the difference between counts after exposure to substances and counts in control wells.

5.5 Classification of isolates depending on biofilm production

For the high biofilm producer selection, the averages of four OD values (with PBS washing step) obtained in quadruplicate for each of the 105 isolates were used to perform k-means clustering (giving 25 classes) followed by agglomerative hierarchical clustering (AHC) in order to classify the isolates according to their biofilm biomass-forming capacity using XLSTAT (2019.4.2version). For the original low producer selection, the averages of four OD values (without PBS washing step) obtained in quadruplicate for each of the 105 isolates were classified 25 classes by k-means clustering.

5.6 PCR assay

According to manufacturer's instructions DNA was extracted from each *S. aureus* ST398 isolate using the NucleoSpin Tissue kit (Macherey-Nagel, Hoerd, France) kit and stored at -80°C for further characterization.

The detection of *bap*, *icaA* and *icaD* was done by simplex PCR tests. The primers were included in Table 1 and the conditions of thermal cycling for simplex PCR tests were described by Vancraeynest et al⁴¹⁴. The detection of other adhesion-related genes was done by two multiplex PCR tests. The primer sets prepared for multiplex PCR were shown in Table 1: primer 1 to amplify *bbp*, *cna*, *ebpS*, and *eno* and primer 2 to amplify *fnbA*, *fnbB*, *fib*, *clfA*, and *clfB*. The conditions of thermal cycling for the two multiplex PCRs were the same as those described

by Tristan et al⁴¹⁵.

Table 2 Primers of simplex and multiplex PCR tests

Gene	Primer sequence	Amplicon size (bp)	Type of PCR	Reference
<i>icaA</i>	5'-CCTAACTAACGAAAGGTAG-3' 5'-AAGATATAGCGATAAGTGC-3'	1315	simplex	Vancraeynest et al ⁴¹⁴
<i>icaD</i>	5'-AAACGTAAGAGAGGTGG-3' 5'-GGCAATATGATCAAGATAC-3'	381	simplex	Vancraeynest et al ⁴¹⁴
<i>fmbA</i>	5'-GTGAAGTTTTAGAAGGTGGAAGATTAG-3' 5'-GCTCTTGTAAGACCATTTTTCTTCAC-3'	643	Multiplex2	Tristan et al ⁴¹⁵
<i>fmbB</i>	5'-GTAACAGCTAATGGTCGAATTGATACT-3' 5'-CAAGTTCGATAGGAGTACTATGTTTC-3'	524	Multiplex2	Tristan et al ⁴¹⁵
<i>fib</i>	5'-CTACAACTACAATTGCCGTCAACAG-3' 5'-GCTCTTGTAAGACCATTTTTCTTCAC-3'	404	Multiplex2	Tristan et al ⁴¹⁵
<i>clfA</i>	5'-ATTGGCGTGGCTTCAGTGCT-3' 5'-CGTTTCTTCCGTAGTTGCATTG-3'	292	Multiplex2	Tristan et al ⁴¹⁵
<i>clfB</i>	5'-ACATCAGTAATAGTAGGGGGCAAC-3' 5'-TTCGCACTGTTTGTGTTTGCAC-3'	205	Multiplex2	Tristan et al ⁴¹⁵
<i>cna</i>	5'-GTCAAGCAGTTATTAACACCAGAC-3' 5'-AATCAGTAATTGCACTTTGTCCACTG-3'	423	Multiplex1	Tristan et al ⁴¹⁵
<i>bbp</i>	5'-AACTACATCTAGTACTCAACAACAG-3' 5'-ATGTGCTTGAATAACACCATCATCT-3'	575	Multiplex1	Tristan et al ⁴¹⁵
<i>ebpS</i>	5'-CATCCAGAACCAATCGAAGAC-3' 5'-CTTAACAGTTACATCATCATGTTTATCTTTG-3'	168	Multiplex1	Tristan et al ⁴¹⁵
<i>eno</i>	5'-ACGTGCAGCAGCTGACT-3' 5'-CAACAGCATYCTTCAGTACCTTC-3'	302	Multiplex1	Tristan et al ⁴¹⁵
<i>bap</i>	5'-CCCTATATCGAAGGTGTAGAATTG-3' 5'-GCTGTTGAAGTTAATACTGTACCTGC-3'	971	simplex	Vancraeynest et al ⁴¹⁴

5.7 RNA extraction (prospect)

According to the manufacturer's instructions, RNA was extracted from biofilm bacterial cells using RNeasy Power Biofilm Kit (QIAGEN). RNA quantification will be quantified using the Qubit device and the corresponding Qubit kits (ThermoFisher). In addition, the ratio of A260/280 was calculated to assess sample integrity and protein contamination.

5.8 qRT-PCR (Prospect)

Real-time qRT-PCR technology will be used to quantify the expression of tested genes of *S. aureus* ST398 isolates. The StepOne Plus real-time PCR system and StepOne Plus software v2.3 (Applied Biosystems, Foster City, California, USA) will be used for DNA amplification. The transcript abundance of all target genes in *S. aureus* ST398 isolates will be obtained using a custom SYBR green gene expression analysis (purchased from Life Technologies). The *rpoB* gene encoding the RNA polymerase subunit was used as an internal reference. The primer and thermal cycling conditions will be set. To evaluate the relative expression of genes, the $2^{-\Delta\Delta C(T)}$ method will be used⁴¹⁶.

Discussion and perspectives

Chapter 3

3.1 Discussion

In our project, subtilisin A (0.01 U/ml) and CaG (Ca^{2+} 1.25 mmol/L) exhibited an inhibitory effect on biofilm formation *in vitro* and significantly destroyed the formed EPS matrix. The embedded biofilm bacteria produced by specific *S. aureus* isolates was significantly reduced by applying subtilisin A and CaG in combination with antibiotics.

When we studied the ability of 73 *S. aureus* originating from bovine mastitis to produce biofilm, we found that the biofilm-forming level of each isolate was different. Through the statistical analysis, we found that, according to the production of biofilms, the 73 *S. aureus* were mainly divided into three categories, namely high producers, medium producers and low producers. The use of such a large collection of isolates was a key feature of our work, allowing a generalization of our results. On the contrary, the majority of the researches related to *S. aureus* biofilms mostly used laboratory strains, such as NCTC8325, or a very small number of field isolates. Considering the diversity of biofilms formed by different clones of a single bacterial species; working on a restricted number of isolates implies that the observed effects of the antibiofilm substances might be limited to the small number of selected strains. It was thus challenging for us to infer from the relevant literature about the effect of these substances on the biofilms formed by large samples. Similarly, we also cannot speculate on their efficacy in treating biofilm infections caused by other *S. aureus* isolates as the capacity of *S. aureus* to form biofilm depends on the strains. Thus, we established an extensive collection (24 isolates) of isolates with significant differences in biofilm-forming abilities between isolates as a follow-up test of the efficacy of antibiofilm reagents.

In the quantitative analysis of the biofilm bacteria produced by 24 representative isolates, we did not notice significant linear relationship between OD value and CFU/mL. This result indicates that the decrease in OD value caused by the antibiofilm substance was only a consequence of the detachment effect of the substance on the biofilm but did not fully prove the substance's damaging effect on the biofilm matrix or the reduction of the bacteria embedded in the biofilm. In the same way, the decrease of CFU/mL only explained the reducing

effect of the substance on the biofilm mosaic bacteria, but it cannot explain its effect on the biofilm matrix. However, the reduction of biofilm bacteria does not imply that the antibiofilm effect of the substance in the *in vitro* test is ideal because large amount of residual biofilm matrix will make the biofilm infection more likely to re-occur, and the infection may become more complicated. In summary, we demanded to use CV staining, bacteria count, and CLSM imaging simultaneously to detect the antibiofilm effect of the substance and to comprehensively evaluate the influence of the substance on biofilm biomass, biofilm bacteria, and on the composition of the biofilm matrix.

The results of CLSM imaging of biofilms (high producer) showed that the mature biofilms tested contain many proteins, PNAG, and components that can be stained by PI (such as dead cells eDNA, and small parts of RNA). When mature biofilms were exposed to subtilisin A, a large amount of stained proteins disappeared, and the content of PNAG was also significantly reduced. In addition, exposure to subtilisin A could reduce the OD value of mature biofilms, and the concentration of subtilisin A used has no killing effect on *S. aureus*. The comprehensive analysis determined that subtilisin A was likely to cause the complete damage of the biofilm structure by degrading the protein in the mature biofilm instead of just the detachment of biofilm. CLSM images proved that, in the biofilm exposed to CaG, the proteins and PNAG of biofilm decreased, similarly to what has been observed with subtilisin A. However, the residual biofilm appeared much thicker than with subtilisin A. Moreover, the concentration of Ca^{2+} used in the assays is consistent with the concentration of free calcium ions in the blood of mammals. These indicate that calcium ions may affect the ability of Bap to help in the formation of biofilms by causing conformational changes, rather than directly reducing specific components in the biofilm matrix as enzymes.

Our results showed that CaG has a significant damaging effect on the biofilm formed by 21/24 isolates. For the other three *S. aureus* isolates, we noticed that they were not susceptible to Ca^{2+} treatment. Therefore, we are interested in which characteristics (such as particular genes or proteins) give them the ability to produce biofilms in the presence of Ca^{2+} . It also proved that using a large number and diversity of samples could detect the broad spectral antibiofilm

activity of the substances more accurately and screen out particular strains that are not susceptible to the substances.

In the first project, our results attested that even though the isolates were all *S. aureus* and isolated from bovine mastitis, the individual isolate's ability to produce biofilm was significantly different under the same culture conditions. Hence, we planned to explore further the possible principles that led to strain specificity. In addition, we also wanted to characterize the biofilm formation capacities of a major clone in animals, causing chronic animal infections. To achieve this goal, we first considered whether this difference was due to the different adhesion-related genes between the isolates. Therefore, we selected as the target species a collection of 105 *S. aureus* isolates that all belonged to the ST398 clone. Since more than half of *S. aureus* ST398 were classified as a category with a low level of biofilm production under the same pretreatment steps before biofilm testing, this was not conducive to screening out isolates with original low biofilm production capacity. The low OD value of these isolates may also indicate poor surface adhesion of the mature biofilm. To this end, we added an optimization step for the screening of the lowest-producing isolates, completely removing the biofilm washing step, which has minimized the destructive power of the external stress on the formed biofilm. Isolates that exhibited low biofilm productivity in both cases (high external stress and low external stress) were selected as low producers.

Due to the findings of the first project that the similar OD value did not systematically result in the same amount of biofilm bacteria produced by the isolates, we conducted the quantitative analysis (biofilm stain and bacterial counts) on 105 *S. aureus* ST398 biofilm bacteria. Among these strains, we observed that about half of the isolates produced $6 \log_{10}$ CFU/mL biofilm bacteria. This part of the isolates are our ideal selection objects due to their similar biofilm bacteria production, but significantly different biofilm biomass level. The PCR results of all *S. aureus* ST398 revealed that 90-95% of the isolates contained the same types of adhesion-related genes, which proved that our first hypothesis was invalid. The strain specificity of the biofilm-forming has no link to the kind of related adhesions genes contained and the number of biofilm bacteria. It then turned our hypothesis into whether the dynamic changes of

adhesion-related gene expression during the biofilm formation are related to the level of biofilm biomass produced by each isolate. Due to the biofilm heterogeneity, we will analyze the relevant gene expression of the planktonic bacteria in the upper layer of the biofilm to compare and prove that the difference in gene expression between the upper layer of bacteria and the lower layer of sediment is likely to come from biofilm bacteria. The actual conclusion depends on the final data analysis.

In the second project, we worked with two MRSA ST398 isolates having the same biofilm production level, containing the same adhesion-related genes, and isolated from bovines. Their biofilms presented an utterly different susceptibility to Ca^{2+} and it led us to further hypothesize that changes in the expression of related adhesion genes could also be responsible for the susceptibility of biofilms to Ca^{2+} . We believe that we will gradually unveil the mystery of *S. aureus* biofilm with the enrichment of data from the second project.

3.2 Perspectives

At the end of the PhD project, we still have some hypotheses to be explored and proved. Below we will discuss four perspectives related to this project in detail: (1) comparison of phenotypes between *S. aureus* aggregates and their surface-attached biofilm; (2) the possible design of antibiofilm materials with subtilisin A; (3) the further study on antibiofilm effect of calcium (4) biofilm study on the single cell level.

3.2.1 Comparison of phenotypes between *S. aureus* aggregates and their surface-attached biofilm

In a recent study⁴¹⁷, the tiny bacterial aggregates ($\sim 50 \mu\text{m}^2$) attached to abiotic surfaces were described to cause neutrophil lysis and persistence of bacteria. *In vivo* imaging revealed that the *S. aureus* aggregates on the mouse's skin were not detected by neutrophils within three hours and kept growing. This result indicated that the delayed recruitment of neutrophils for the detection of aggregates provides opportunities and time for bacteria to build new biofilms.

In our research, we only studied the mature biofilm in static culture and, more specifically, we focused on surface-attached biofilms because we always washed biofilms before assessing the antibiofilm effect of treatments. However, in addition to the surface attaching, bacteria may achieve mutual adhesion (aggregation/autoaggregation) through matrix components and mucus in the first step of biofilm formation⁴¹⁸. These aggregates have shown characteristics similar to biofilms, such as the difficulty of host's cells (such as phagocytes) to penetrate the barrier formed by them^{419,420}. Therefore, some chronic infections may be characterized by aggregates in host tissues or in organ cavities. So far, there are few studies on aggregates formed by *S. aureus*. When we used the CDC system to generate large-scale *S. aureus* biofilm (data not shown), we found that *S. aureus* also developed many

aggregates that gradually settled at the bottom of the reactor. Therefore, studying whether *S. aureus* aggregates demonstrate similar drug tolerance as biofilms would be of great value for establishing a *S. aureus* chronic infection treatment strategy.

In our subject, the general structure and composition of *S. aureus* biofilm were presented through CLSM with fluorescent staining. To compare the structural characteristics of *S. aureus* aggregates and its attached biofilm, we need to consider scanning electron microscopy (SEM) and atomic force microscopy (AFM). Alhede et al.⁵ employed SEM to image *Pseudomonas aeruginosa* aggregates (Fig. 23). The obtained images proved that cells within aggregates were connected by a fibrous structure, similar to the surface-attached biofilm in terms of visual structure. In contrast, in planktonic status, only single cells could be observed. It is worth noting that in the study of Alhede et al.⁵, the aggregates were obtained from static culture whereas the surface-adhesive biofilm were obtained from a flow-cell biofilm system. Therefore, to further distinguish whether the difference comes from the culture method (static culture vs. flow-cell biofilm system), we suggest that the detection of surface adhesion biofilm formed under static culture should be added.

AFM relies on measuring the change in force between the sample surface and the probe tip. Contrary to SEM, the application of AFM is less affected by sample pretreatment and sample damage⁴²¹. It can even operate on liquid surfaces to achieve *in situ* imaging⁴²². AFM also quantitatively evaluates biofilms' interaction with surfaces and the cohesion of biofilms⁴²³. Therefore, AFM is ideally applied as a supplementary method to quantitatively analyze the formation of biofilms (Fig. 24). However, the use of AFM to explore the features of *S. aureus* aggregates may be a challenge as these aggregates float.

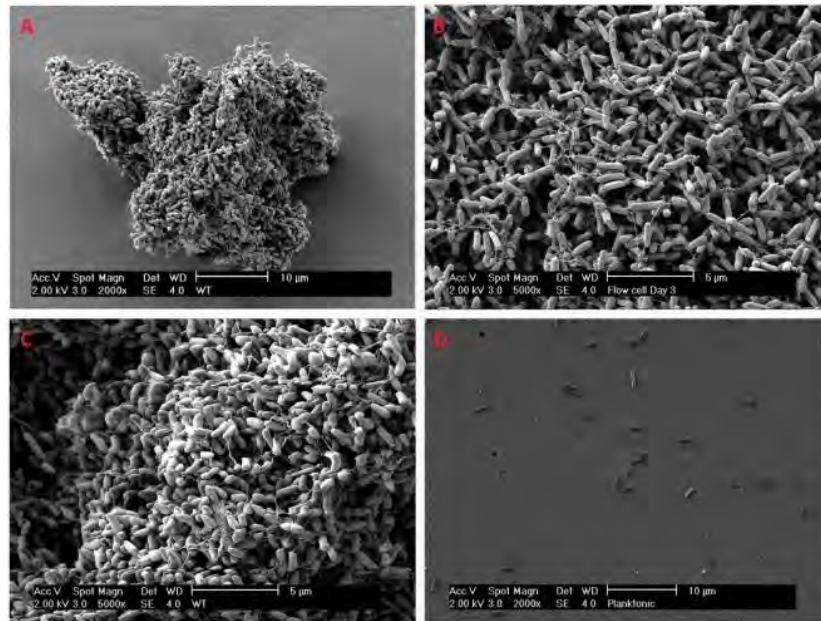


Figure 23 The *P. aeruginosa* under different cultures through SEM assay

A: Aggregate obtained by stationary culture after 48h. B: The biofilm was grown under the flow-cell system for 3 days. C: Details of stationary aggregate after 48h culture. D: Planktonic cells. (adapted from Alhede et al.⁵)

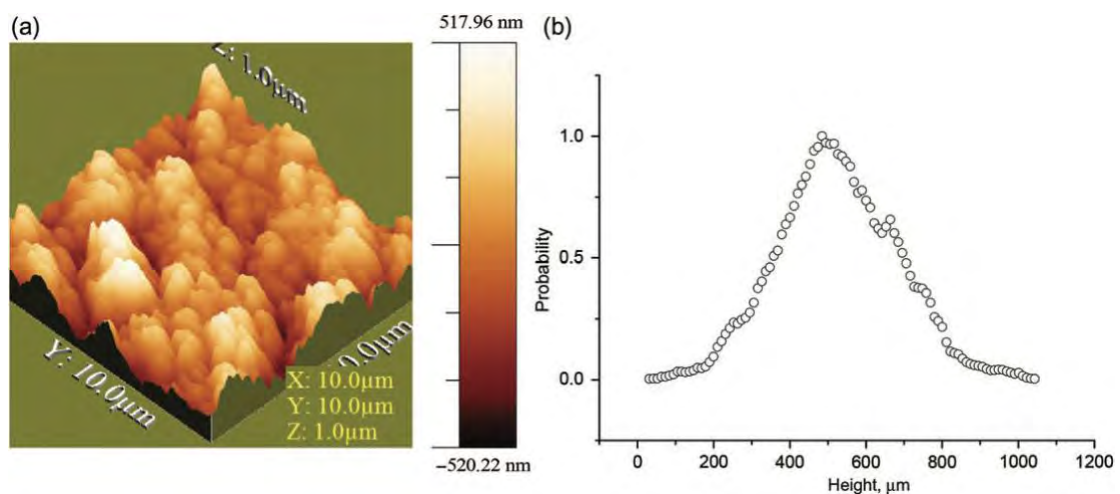


Figure 24 AFM image of *S. aureus* biofilm surface formed on the polycarbonate membrane
(a) Height topography in three dimensions (b) The height of biofilm distribution. (adapted from Chatterjee et al.⁴²⁴)

In our subject, *S. aureus* strains with different biofilm-forming capacities were already selected. We can further explore whether there are significant differences in *S. aureus* aggregates (size and structure) formed by high producers and low producers. *S. aureus* aggregates and their surface-attached biofilm could correspond to different

phenotypes of bacterial growth. Still, some aggregates (especially formed by autoaggregation) can colonized the surface and contribute as the initial step in forming a surface-attached biofilm. Therefore, the level of surface adhesion of biofilm produced by the isolate may have a particular relationship with its formation of aggregates, which needs to be further studied.

Bacterial aggregates are mostly suspended, or reversibly attached to the surface, while surface-adhesive biofilms irreversibly adhere to the surface since the beginning of formation. Therefore, it is reasonable to assume that the amount of PNAG and the abundance of related proteins in the two different phenotypes of bacterial clusters are dissimilar. The quantitative analysis of PNAG in *S. aureus* biofilm and *S. aureus* aggregates at different time intervals can be obtained by WGA staining combined with top fluorescence measurement. Moreover, by analyzing the content of biofilm by LC-MS/MS, we could get the relative abundance of proteins in each formed biofilm and *S. aureus* aggregates over time.

Finally, we should further prove whether *S. aureus* aggregates are already tolerant to PMNs before adhering to abiotic surfaces. We also need to examine whether the tolerance level of *S. aureus* aggregates to PMNs is similar to that of surface-attached biofilm. In addition, the susceptibility of *S. aureus* aggregates to the treatment of some antibiotics and antibiofilm agents is required to be assessed and compared to surface-attached biofilm.

3.2.2 The possible design of antibiofilm materials with subtilisin A

In our project, subtilisin A did not only destroy the established *S. aureus* biofilm formed by 24 representative isolates but also inhibit their biofilm formation. Thus, it makes subtilisin A owns value in the development of new antibiofilm strategies. Some studies have confirmed that using antimicrobial agents (such antibiotics, antimicrobial peptides,

or disinfectants) or enzymes to functionalize surfaces is an effective strategy to inhibit biofilm formation, disperse biofilms and increase susceptibility to antibiotics^{425,426}. Contrary to antimicrobials, enzyme coating does not have a killing effect on bacteria nor destroy the micro-ecological balance, and the risk for the selection of antimicrobial resistance is extremely low³⁴⁵. Ye et al.⁴²⁷ applied dopamine as an intermediate to immobilize the functional DNase I coating on the pretreated pure titanium coupon (Fig. 25). This coating presented a significant inhibitory effect on the formation of *S. aureus* biofilm. Compared with DNase I, subtilisin A has a higher tolerance to environmental factors and lower cost. Therefore, we believe that it is worthy to further explore the application of subtilisin A as an enzyme coating to gain the surface with antibiofilm properties. In the subsequent related research, Alves et al.⁴²⁸ used dopamine chemistry to immobilize both antimicrobial peptide Palm and DNase I on polydimethylsiloxane (PDMS) material, then obtained a coating with dual-functional properties (anti-adhesion and antibacterial). This research questioned us about a possible synergy/additive antibiofilm effect when subtilisin A combined with antimicrobial peptides by grafting on the surface. To realize the immobilization of subtilisin A on the surface, we cannot avoid multiple challenges, such as analyzing the molecular structure of subtilisin A, selecting intermediates, and analyzing high-resolution images of single-cell adhesion.

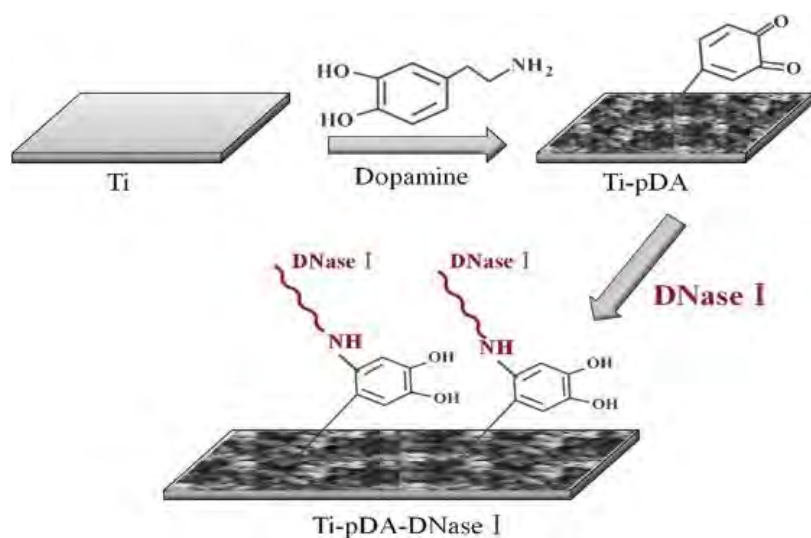


Figure 25 Using dopamine as an intermediate to immobilize DNase I to the Ti surface

(adapted from Ye et al.⁴²⁷)

Kingshott et al. discovered that branched poly(ethylenimine) has spontaneous physical adsorption property, producing a thin organic layer containing high-density primary amine functions⁴²⁹. Based on this, Caro et al.⁴³⁰ covalently grafted two hydrolases (lysozyme and trypsin) to the stainless-steel surface with a high-density primary amine layer through a glutaraldehyde cross-linker (Fig. 26), achieving both surface anti-adhesion and antibacterial properties. Notably, the covalent immobilization process led to solidly bound and maintained the active enzyme layer. Therefore, this method may be the ideal choice for subtilisin A immobilization, which could anchor subtilisin A away from the surface to maximize surface enzymatic activity.

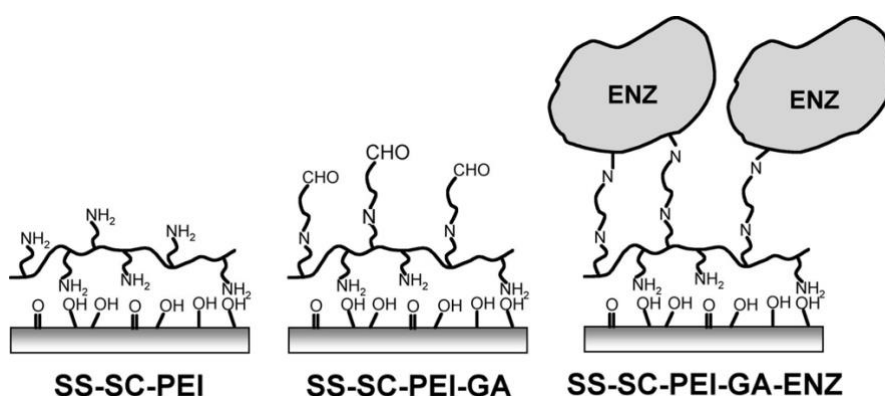


Figure 26 Stainless steel surface functionalization steps of hen egg white lysozyme or Trypsin
SS-SC: cleaned and pre-conditioned coupon. PEI: poly ethylene. GA: glutaraldehyde. ENZ: enzymes (adapted from Caro et al.⁴³⁰)

The newest method on surface protection against biofilms also provided us some fresh thinking and choices. Beaussart et al.⁴³¹ functionalized the surface with biotinylated lysozyme based on the high-affinity ligand-receptor interaction between biotin and streptavidin (Fig. 27). They utilized AFM (single-molecule force spectroscopy) to prove that grafting through ligand-receptor interaction retained the enzyme in the correct orientation on the substrate. Compared with the other grafting methods, this new type of surface grafted with oriented enzymes exhibited higher activity towards microbial targets. If subtilisin A can also be oriented, the ligand-receptor type of grafting may be applied to establish an antibiofilm surface with subtilisin A coating.

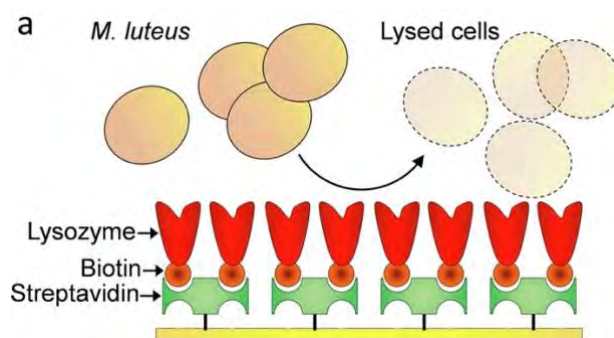


Figure 27 The surface protection principle of biotin-streptavidin grafting enzyme (adapted from Beaussart et al.⁴³¹)

3.2.3 The further study on antibiofilm effect of calcium

Calcium gluconate (0.233 mmol/L Ca^{2+}) is a calcium salt of gluconic acid used to treat hypocalcemia, cardiac arrest, and cardiotoxicity caused by hyperkalemia or hypermagnesemia⁴³². Calcium gluconate is usually better than calcium chloride because the risk of tissue necrosis is lower if the fluid is extravasated⁴³³. The systemic toxicity caused by calcium gluconate is the same as the systemic effects of hypercalcemia, usually manifested as fatigue, anorexia, muscle weakness, polydipsia, electrocardiogram changes, and other non-specific symptoms⁴³⁴. In general, calcium gluconate is a drug with minor side effects on humans and animals under strict administration and monitoring.

In recent years, several research results confirmed that calcium phosphate (coating or bone grafts) effectively inhibited the formation of clinical *S. aureus* biofilm and enhanced the susceptibility of *S. aureus* to antibiotics^{435,436,437}. Therefore, we believe that calcium has promising development prospects in the practical application of antibiofilm, especially in dentistry and orthopedics.

In our research, calcium ion has shown an excellent destructive effect on the formed *S. aureus* biofilm. However, before considering the practical use of calcium ion in

antibiofilm, we believe that calcium ion's antibiofilm spectrum and characteristics need to be further studied in at least the two following aspects.

In the first aspect, we mentioned (Chapter 2) that related studies inferred that calcium's damaging effect on *S. aureus* biofilm might be due to its potential to bind EF-hand-like domains of the Bap protein. Since the *bap* gene was more related to *S. aureus* isolated from mastitis bovine and quite rare, the antibiofilm effect of calcium ion could be limited to bacteria harboring a *bap* gene, which are apparently very rare⁴³⁸. However, in *S. aureus* biofilm, the proteins with EF-hands are not only Bap; another example is ClfB⁴³⁹. Therefore, in subsequent experiments, we could first obtain the sequences of the proteins involved in the formation of *S. aureus* biofilm by the UniProt *S. aureus* protein database⁴⁴⁰ (<https://www.uniprot.org/>). Then, the list of proteins contains EF-hands will be obtained by applying InterProScan⁴⁴¹. For the identified proteins that are not listed, sequenced proteins would be compared with different protein sequences containing EF-hands through BLAST, and the final identification will be made based on the similarity. We would next need to screen out two sets of *S. aureus* isolates, high and low susceptible to calcium ion, and analyze the proteins (type and abundance) involved in their formed biofilm. The final purpose would be to verify whether the antibiofilm effect of calcium ion on *S. aureus* biofilm could be explained by the type of proteins on which the matrix formation depends. For instance, in two closely related *S. aureus* strains, Newman and NCTC 10833, biofilm formation was inhibited in Newman in the presence of a chelator of calcium but enhanced in 10833. The 10833 strain produced ClfB-dependent biofilms that could be the cause of this discrepancy⁴³⁹.

In the second aspect, from previous related reports, the biofilm destruction effect of calcium was mainly demonstrated on *S. aureus* biofilm. In contrast, calcium ion is necessary and beneficial for some other microbe species to form biofilms. As early as 1986, Turakhia MH⁴⁴² discovered that calcium increases the carbon accumulation rate on the surface of *P. aeruginosa* cells and that free calcium is essential for maintaining the integrity of the biofilm. In 2014, Das et al.⁴⁴³ demonstrated that the addition of

calcium ion mediates bacterial aggregation and biofilm formation by binding to eDNA on the surface of *P. aeruginosa* and *Enterococcus faecalis*. Moreover, in the industrial environment, Guvensen et al.⁴⁴⁴ observed that the addition of calcium ions resulted in a significant increase in the formation of *Sphingomonas paucimobilis* biofilm. In contrast, the same concentration of calcium ions did not affect the growth of planktonic *S. paucimobilis* in the medium. In natural environment, the formation of biofilms of *Vibrio fischeri* that are polysaccharide-dependent or cellulose synthesis-dependent biofilms are induced through action of calcium at the transcription level of *syp* and *bcs* loci⁴⁴⁵. Significantly, the unique selected *S. aureus* strain in the study of Das et al.⁴⁴³ formed more biofilms after adding calcium ions. Since there is no detailed information on the strain, we cannot determine the source and the specificity of this *S. aureus* strain. In our project, the two isolates (MR1 and MR9) were also not susceptible to the calcium ions, which indicated that calcium ions did not act on the biofilm formed by all *S. aureus* isolates like subtilisin A. Therefore, the confirmation of the destructive mechanism of calcium ions on *S. aureus* biofilm is necessary.

For developing and utilizing calcium to treat biofilms, it is essential to identify the antibiofilm characteristics of calcium discreetly. Because, whether it is in biofilm infection, factory production, or in the natural environment, biofilms formed by multi-species are common. Before considering the application of calcium to treat biofilms, we suggest that the identification of bacterial species and even the specific related genes should be carried out. It is also an essential initial step before considering any antibiofilm strategies.

3.2.4 Biofilm study on the single-cell level

Although the second project is still in progress, we could still make a reasonable outlook on the further direction of the project. Traditional quantitative gene expression techniques (such as RNA sequencing, microarray, qPCR, and gene reporter fusions) are only proper for determining the average expression level of the clonal population.

Consequently, such analyzes may only give a partial picture of the expression pattern of a population, since gene expression in the clonal population (with the same genotype) is not a homogeneous process⁴⁴⁶. This substantial variation in gene expression between cells can be completely random or in response to fluctuations in the local microenvironment (phenotypic plasticity)⁴⁴⁷. In phenotypic plasticity, fluctuations in environmental signals directly lead to changes in gene expression and phenotype. This phenomenon is more evident in biofilms with complex and abundant heterogeneous conditions. In the bibliography, we mentioned gradients of multiple substances (oxygen, nutrients, and metabolites) in biofilms, which provide the individual cell with almost a single microecological habitat. These different microenvironments make the gene expression of individual cells exhibit phenotypic plasticity⁴⁴⁸. Our results recorded that the ability of *S. aureus* to produce biofilm is strain-specific (under the same culture conditions), but this specificity is a phenotypic difference between two independent strains. Even in a biofilm (similar to a clonal population) formed by the same isolate, cells produce different expression profiles and metabolic states depending on the location within the biofilm⁴⁴⁹. Robijns et al.⁴⁵⁰ developed a GFP promoter fusion library with 79 essential *Salmonella* biofilm genes. They managed the library combined with fluorescence microscopy and fluorescence-activated cell sorting (FACS) to analyze the local expression profile in *Salmonella* biofilms. The figure 28 shows that cells in different regions of the *Salmonella* biofilm presented different levels of *csgD* expressions. The traditional genes expression techniques cannot display variable expression profiles for complex populations (such as microbial biofilms), nor are they suitable for studying unique subpopulations (such as persisters) in biofilms. Biofilms urgently need to be analyzed at the single-cell level, especially for the study of multi-species biofilms. The use and innovation of single-cell gene expression technology is an indispensable development trend. The current commonly used single-cell isolation and analysis technique is flow cytometry. This technology uses continuous flow to isolate individual cells in tiny droplets to obtain different characteristics of the cells, including size, granularity, and general fluorescence information. This sorting technique based on fluorescence is called

fluorescence-activated cell sorting (FACS)⁴⁵¹. However, FACS is challenging to isolate sporadic cells (such as persisters) in the sample, and fluorescent reporter gene constructs are limited to recording the activity of individual genes in some genetically manageable model species, and it is still challenging to design for most bacteria.

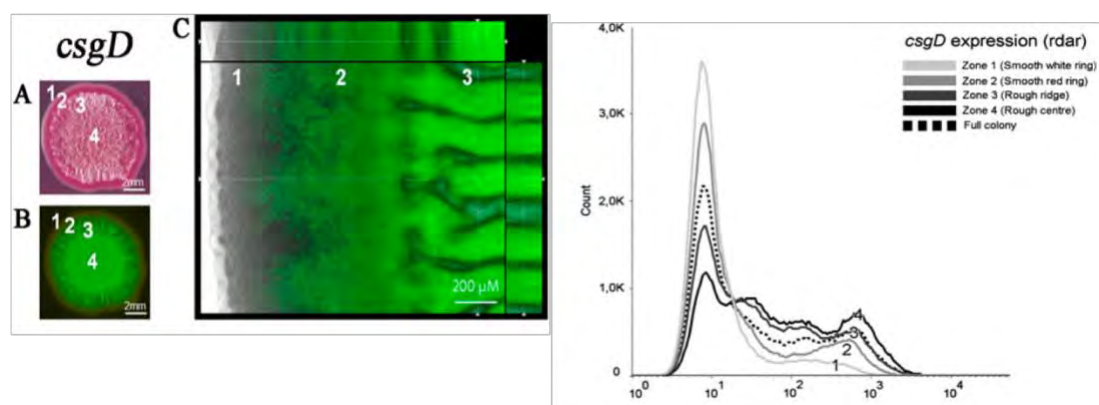


Figure 28 The variation of *csgD* expression inside a radar colony biofilm model. FACS profiles of different areas in the radar colony to determine local expression differences at the single-cell level (adapted from Robijns et al.⁴⁵⁰)

It is necessary to overcome several obstacles in bacterial single-cell transcriptomics to analyze single-cell gene expression in microbial biofilms. For instance, bacteria only contain RNA fragments, and the half-lives of bacterial mRNA are only a few minutes⁴⁵². Moreover, the bacterial single-cell RNA-sequencing (scRNA-seq) protocol needs to settle extremely low copy number mRNA, 0.4 copies/cell⁴⁵³ (14). Imdahl et al.⁴⁵² used a highly sensitive random hexamer, priming based on the initiated scRNA-seq protocol, and successfully obtained growth-dependent gene expression patterns from all RNA classes and genomic regions of a single *Salmonella* and *Pseudomonas*. According to the general workflow (Fig. 29), after a single cell was separated from the culture, it underwent a single tube of cell lysis, cDNA synthesis and amplification. The researchers used poly(A)-independent multiple annealing and dC-based tailing-based quantitative scRNA-seq (MATQ-seq) protocols to obtain enough cDNA from a single bacterium. Kuchina et al.⁴⁵⁴ developed a new high-throughput scRNA-seq with bacterial cells called microSPLIT (Microbial Split-Pool Ligation Transcriptomics). This new technology can analyze the gene expression status of more than 25,000 single

Bacillus subtilis cells and reveal the rare status present in less than 0.1% of the population. As shown in the figure 30, microSPLiT not only identified a rare cell subgroup (36 cells, expressing PBSX prophage genes) in a prophage-induced state but also analyzed the expression of major phage-related operons. This technological breakthrough may provide the possibility for the research of persister gene expression in biofilms.

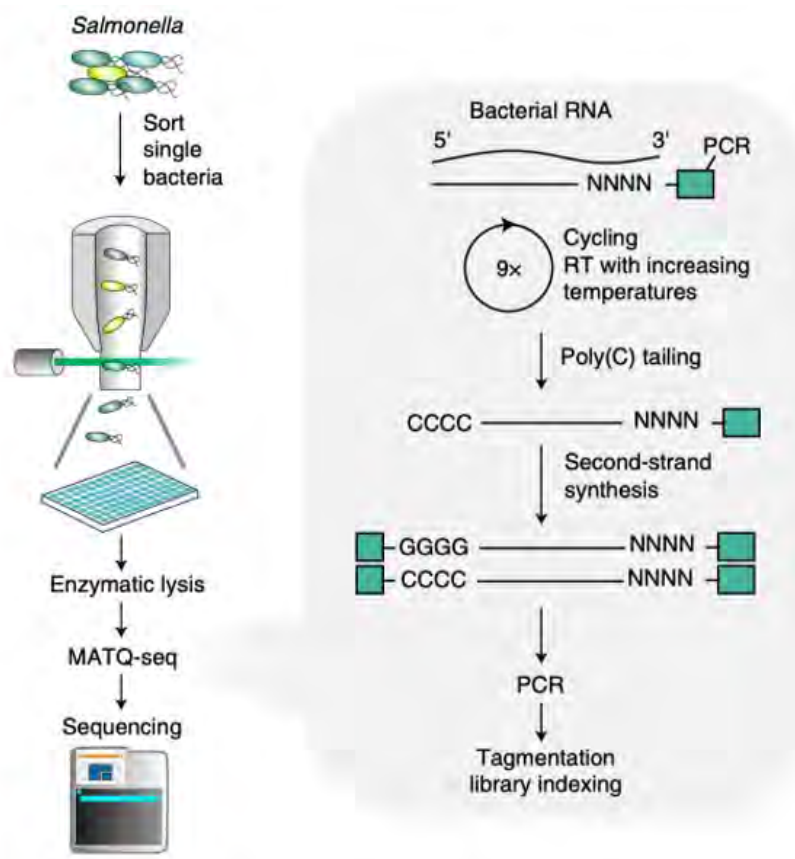


Figure 29 Workflow of bacterial scRNA-seq protocol adapted from Imdahl et al.⁴⁵²

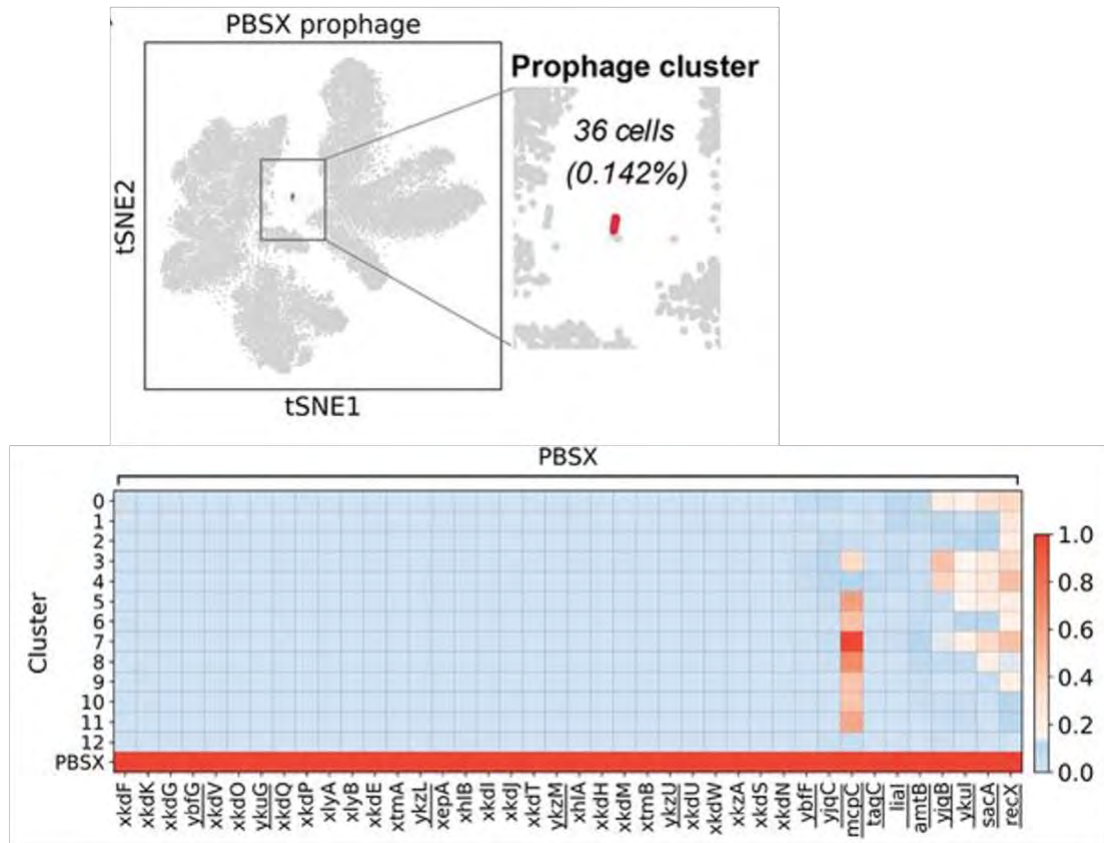


Figure 30 Rare cellular state quantified by microSPLIT

The PBSX prophage cluster (36 cells) shown on the t-SNE image (left). The normalized average expression of the enriched genes in the PBSX prophage cluster (right), adapted from Kuchina et al.⁴⁵⁴

With current gene expression quantitative technology, there are still some important difficulties in considering the heterogeneity of microbial biofilms. For example, isolating single cells may destroy the population environment in which the single cell is located and easily lead to cell gene expression changes. In addition, the tiny amount of DNA and RNA in prokaryotic cells makes subsequent amplification steps more error-prone, and random noise may be amplified. However, with the continuous improvement of the technological level, researchers can already predict that sequencing technology will overcome the above obstacles to achieve precise single-cell separation and sequencing in the next few years. We also believe that studying gene expression *in situ* at the single-cell level will make it possible to associate specific genotypes with specific biofilm niches accurately. The research on the formation of persisters in biofilms and their principle of drug tolerance that has annoyed people for many years

will be breakthrough progress.

In conclusion, based on our current results, we have given relatively feasible hypotheses and research directions to further explore and develop the application of subtilisin A and calcium in controlling *S. aureus* biofilm. As antibiotic resistance becomes worse and worse, researchers/medical doctors apprehended that effective treatment of chronic biofilm infections is essential for improving clinical cure and reducing antibiotic consumption. In recent years, strategies to control biofilm formed by *S. aureus* biofilm or other bacterial species have received extensive attention and development. However, due to the complex structure of biofilms and their high tolerance to external pressure, it is more challenging to control biofilms than to prevent their formation. In addition, simply killing most of the biofilm bacteria may leave biodegradable substrates that can then be reused by bacteria. Therefore, although the results of biofilm control in our subject are promising, the combination of antibiotics and non-antibiotics may not be sufficient to eradicate the biofilm infection. Currently, other antibiofilm methods have been proposed and include the application of modified antibacterial surfaces, natural compounds extracted from plants (phytochemicals), synthetic QS inhibitors, bioengineered phages and photodynamic therapy. Each of these methods has its advantages and disadvantages that include various physical, chemical, and biological technologies. Future research could consider a suitable combination of these methods to use the different effects they provide in biofilm control fully. Finally, we believe that the exploration of strain specificity of biofilm formation and the development of an effective assay for analyzing the characteristics of persisters are essential for developing new antibiofilm strategies.

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TITLE: Activity of antibiotics and non-antibiotics substances on *Staphylococcus aureus* biofilms

RESUME:

The purpose of our research was to design a new anti-biofilm strategy to effectively destruct mature biofilms formed by *S. aureus* and to explore the possible mechanisms which lead to the strain specificity of biofilm forming capacity at the molecular level. By confocal laser scanning microscopy, we found that subtilisin A and calcium ion significantly destroyed the biofilm matrix. Combining oxytetracycline with each of them enhanced the detachment of biofilm bacteria, while maintaining their destructive effect on the biofilm matrix. One hundred and five *S. aureus* ST398 isolates were then analyzed for their biofilm forming capacity and their biofilm-related genes. The results demonstrated that, two isolates with the same host source, the same type of biofilm-related genes, and the same *spa*-type could have significantly different biofilm-producing abilities. Further experiments will analyze the expression of biofilm-related genes and explore the possible mechanism of the difference in biofilm productivity between these two types of isolates. Our research proposed a new strategy to target biofilm matrix by combining antibiotics with non-antibiotics and may provide new targets for future research and development of antibiofilm strategies.

KEYWORDS : *Staphylococcus aureus*, biofilm, matrix, antibiofilm, antibiotics, non-antibiotic

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TITRE: **Activité d'antibiotiques et de substances non antibiotiques sur des biofilms de *Staphylococcus aureus***

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à l'Ecole Nationale Vétérinaire de Toulouse

RÉSUMÉ : Le but de notre recherche était de proposer une nouvelle stratégie antibiofilm pour détruire efficacement les biofilms matures formés par *S. aureus* et d'explorer les mécanismes moléculaires qui pourraient conduire à des spécificités de souche dans la capacité à former du biofilm. Nous avons constaté, par microscopie confocale à balayage laser, que la subtilisine A et l'ion calcium détruisaient de manière significative la matrice du biofilm. L'association de l'oxytétracycline à chacune de ces molécules a amélioré le détachement des bactéries du biofilm, tout en maintenant leur effet destructeur sur la matrice du biofilm. Cent cinq isolats de *S. aureus* ST398 ont ensuite été analysés pour leurs gènes et leur capacité à former du biofilm. Les résultats ont démontré que deux isolats provenant du même hôte avec les mêmes gènes impliqués dans le biofilm et le même *spa*-type pouvaient avoir des capacités de production de biofilm significativement différentes. D'autres expériences analyseront l'expression de gènes impliqués dans le biofilm et exploreront les mécanismes pouvant expliquer la différence de productivité du biofilm entre ces deux types d'isolats. Notre recherche a proposé une nouvelle stratégie pour cibler la matrice des biofilms en combinant des antibiotiques avec des substances non-antibiotiques et pourrait fournir de nouvelles cibles pour la recherche et le développement de stratégies contre les biofilms.

MOTS-CLES : *Staphylococcus aureus*, biofilm, matrice, antibiofilm, antibiotiques, non - antibiotique

DICIPLINE ADMINISTRATIVE : Pharmacologie- Microbiologie

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