UNIVERSITÀ DI PARMA

UNIVERSITÀ DEGLI STUDI DI PARMA

DOTTORATO DI RICERCA IN SCIENZE DEGLI ALIMENTI

CICLO XXXI

Food-Related *Staphylococcus aureus* Biofilms: Characterization and Control

Coordinatore:
Chiar.mo Prof. Furio Brighenti

Tutore:
Chiar.ma Prof.ssa Adriana Ianieri

Dottorando: Angelo Colagiorgi

Anni 2015/2018

Angelo Colagiorgi

Food-related *Staphylococcus aureus* **biofilms** – characterization and control

Thesis supervisor
Prof. Adriana Ianieri
Full Professor of Hygiene
and Inspection of Foods of Animal Origin
University of Parma

To my beloved family

"The most beautiful thing we can experience is the mysterious.
It is the source of all true art and science."
Albert Einstein

"A scientist in his laboratory is not a mere technician:
he is also a child confronting natural phenomena
that impress him as though they were fairy tales"
Marie Skłodowska-Curie

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ABSTRACT

Staphylococcus aureus is an ubiquitous and opportunistic pathogen. *S. aureus* is frequently involved in clinical infection, but it is also an important foodborne pathogen. In fact, some strains of *S. aureus* have enterotoxigenic capacity and can be potentially responsible for food intoxication (staphylococcal food poisoning). Furthermore, another challenge is represented by methicillin-resistant *S. aureus* (MRSA). Initially associated with nosocomial infections, it has been demonstrated that such β-lactams resistant bacteria can be present in retail foods, including pork, beef, and dairy products.

The capability of microorganisms to produce biofilms – complex three-dimensional structures of cells embedded in self-produced exopolymeric substances, strictly adhered to a surface – is an important persistence and dissemination mechanism of some foodborne bacteria, including *S. aureus*.

Different researchers studied the biofilm formation by members of *Staphylococcus* genus, but many of these investigations were focused on clinical aspects of this process. Furthermore, many studies that investigated the ability of dairy-related MRSA to form biofilm were focused on isolates collected from the herd, and not from the final products, which are more relevant in a food safety perspective.

This thesis dealt with the molecular characterization of methicillin -sensitive and -resistant *S. aureus* (MSSA and MRSA) isolated from food, food environments, and food handlers, in relationship with their ability to produce biofilms on common food-contact surfaces.

For the first time, the relationship between *S. aureus* genotypes (obtained by Ribosome-Spacer PCR) and biofilm formation was investigated. MSSA and MRSA isolates were tested for the capacity to produce biofilms on food contact surfaces, increasing the

knowledge about the molecular mechanisms potentially involved in this process.

The second part of the thesis (chapters 6 and 7) dealt with the problem of antimicrobial resistance in *S. aureus*, and with the role of biofilms in enhancing this process. Consequently, a novel strategy for the control of *S. aureus* biofilms was studied and applied. Results obtained represent a starting point for the development of a variety of application dealing with the increase of the shelf-life and the safety of food products.

SOMMARIO

Staphylococcus aureus è un patogeno ubiquitario e opportunista. Frequentemente coinvolto in infezioni cliniche legate all'ambiente ospedaliero, è anche un importante patogeno alimentare. Infatti, alcuni ceppi di *S. aureus* sono in grado di produrre enterotossine potenzialmente in grado di causare una forma di intossicazione alimentare nota come "intossicazione stafilococcica". Inoltre, una ulteriore problematica è rappresentata dallo *S. aureus* resistente alla meticillina (Methicillin-Resistant *S. aureus* – MRSA). In un primo momento associati alle infezioni nosocomiali, è stato dimostrato che questi ceppi resistenti agli antibiotici β-lattamici possono essere presenti in alimenti venduti al dettaglio, inclusa la carne suina e bovina, e i prodotti lattiero-caseari.

La capacità dei microrganismi di formare biofilm – complesse strutture tridimensionali di cellule inglobate in una matrice di sostanze esopolimeriche autoprodotte, strettamente adese a una superficie – rappresenta un importante meccanismo di persistenza e disseminazione di alcuni patogeni alimentari, tra cui *S. aureus*.

Diversi ricercatori hanno studiato la formazione di biofilm da parte di membri del genere *Staphylococcus*, ma molti di questi studi sono incentrati sugli aspetti clinici di questo processo. Inoltre, molti degli studi che hanno analizzato la capacità di MRSA da settore lattiero-caseario di formare biofilm hanno a che fare con ceppi isolati dagli animali e non dai prodotti finiti, che sono più rilevanti dal punto di vista della sicurezza alimentare.

Questa tesi ha come oggetto la caratterizzazione molecolare di ceppi di *S. aureus* meticillino –sensibili e –resistenti (MSSA e MRSA) isolati da alimenti, ambienti di lavorazione e maestranze, in relazione alla capacità degli stessi di formare biofilm su alcune delle superfici comunemente utilizzate nell'industria alimentare.

Per la prima volta è stata studiata la relazione tra i diversi genotipi (ottenuti attraverso Ribosome-Spacer PCR) di *S. aureus* e la

formazione di biofilm. Ceppi MSSA e MRSA sono stati testati per la capacità di produrre biofilm su superfici a contatto con gli alimenti, incrementando le conoscenze riguardanti i meccanismi molecolari potenzialmente coinvolti in questo processo.

La seconda parte di questa tesi (capitoli 6 e 7) affronta la problematica della resistenza agli antimicrobici in *S. aureus*, e il ruolo del biofilm nell'aumentare tale fenomeno. Di seguito, è stata quindi studiata ed applicata con successo una nuova strategia per il controllo dei biofilm di *S. aureus*. I risultati ottenuti rappresentano un punto di partenza per lo sviluppo di una serie di applicazioni finalizzate all'aumento della durata di conservazione e della sicurezza dei prodotti alimentari.

CHAPTER 1

General Introduction

1.1 *Staphylococcus aureus*

1.1.1 *Staphylococcus aureus*: an overview

Staphylococcus aureus (from the Greek staphyle - bunch of grapes - and kokkos - berry) was firstly described by the Scottish surgeon Alexander Ogston in 1880, who observed grape-like clusters of bacteria in pus from a surgical abscess in a knee joint (Ogston, 1882). In 1884, the microorganism was named "*aureus*" by the German physician and microbiologist Friedrich Julius Rosenbach because of the golden appearance of its colonies on solid media (Rosenbach, 1884).

S. aureus belongs to genus *Staphylococcus*, family Staphylococcaceae, order Bacillales, class Bacilli, phylum Firmicutes (Schleifer & Bell, 2009). Genus *Staphylococcus* comprises more than 50 species and subspecies that are divided into two groups, based on the ability to clot blood plasma by the action of the enzyme coagulase: coagulase-positive staphylococci (CoPS), and coagulase-negative staphylococci (CoNS). *S. aureus* belongs to CoPS and is the major pathogen within the genus (Harris *et al*., 2002; Foster, 2009). It is a round-shaped, facultative anaerobe, Gram-positive bacterium, characterized by a grape-like cluster arrangement, able to survive to dry conditions and high salt concentrations, although it is not a spore-forming microorganism. It can grow at a wide range of temperatures – 6-48 °C, optimal growth at 35-41 °C –, pHs – 4-10, optimum at 6-7 –, water activities – a_w = 0.83 ≥ 0.99, optimum at 0.99 – (Jay *et al*., 2005; Hennekinne *et al*., 2012). The secretion of diverse enzymes (e.g. thermonucleases, proteases, lipases, hyaluronidases, catalases, collagenases) and cytolytic exotoxins such as hemolysis allow the microorganism to achieve nutrients required for its growth (Dinges *et al*., 2000; DeLeo *et al*., 2009).

S. aureus is an ubiquitous microorganism, and can be found in the air, soil, water, sand, dust, sewage, vegetal. The main habitat for such microorganisms are the skin and upper respiratory tract of

many warm-blooded animals (Grace & Fetsch, 2018). In particular, human skin, throat, nose, hair, nails, axillae and perineum are usually asymptomatically colonized by *S. aureus* (Wertheim *et al.*, 2005). When it has the opportunity (e.g. in presence of damaged skin or mucosal membranes), *S. aureus* can cause a wide variety of infection, since a large number of virulence factors – both cell-associated and extracellular – enables it to overcome the host-immunity defense and to invade and colonize tissues (Foster, 2009). Staphylococcal infections include infection of superficial soft tissue and skin, such as pimples, boils and abscesses, but also severe systemic infections, i.e. endocarditic, bacteremia, pneumonia and toxic shock syndrome (Otto, 2012).

1.1.2 *S. aureus* virulence factors

S. aureus virulence factors are encoded in phages, plasmids, pathogenicity islands, and in the staphylococcal cassette chromosome.

The colonization and invasion of host tissues are mediated by the production of a variety of molecules, known as MSCRAMMs – microbial surface component recognizing adhesive matrix molecules – (Foster & Höök, 1998; Garzoni & Kelley, 2009; Bien *et al.*, 2011). These surface associated adhesins include (**Figure 1**):

Fibronectin binding proteins (FnBPs). FnBA and FnBB are involved in the attachment of *S. aureus* to fibronectin and plasma clot. Furthermore, since they are able to bind immobilized fibronectin, bacteria could adhere to implanted medical devices, such as proteases and catheters (Ton-That et al., 2001).

Collagen binding proteins (Cna). Cna is responsible for adherence of *S. aureus* to collagenous tissues and cartilage (Ton-That et al., 2001).

Clumping factor proteins (Clf). In presence of the fibronectin, ClfA and ClfB mediate clumping and adherence to fibrinogen (Ton-That et al., 2001).

Staphylococcal protein A (Spa). Spa is able to bind immunoglobulins (especially IgGs), inhibiting opsonization and phagocytosis (Ton-That et al., 2001).

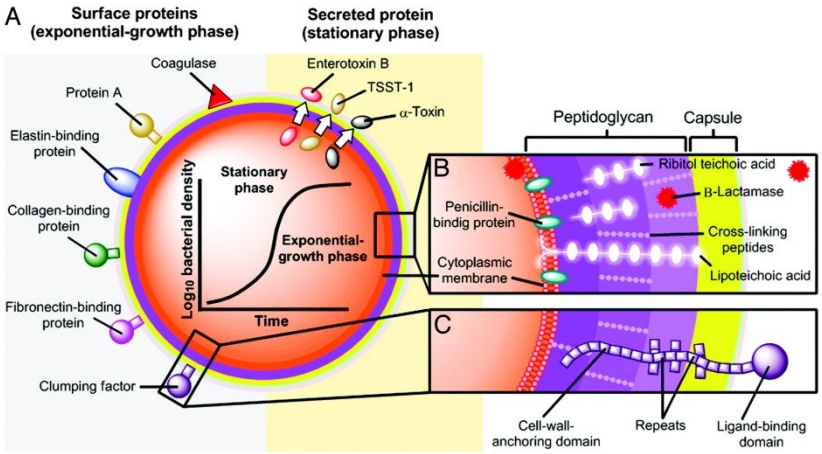

Figure 1 - Schematic representation of *S. aureus* virulence factors, showing both the structural and the secreted products, playing roles as virulence factors. (A) Surface and secreted proteins; (B and C) are cross-sections of the cell envelope. Reprinted from Vatansever *et al.* (2013).

The colonization and invasion processes are also enhanced by the production of a series of exotoxins and enzymes that convert local host tissue into nutrients required for bacterial growth, including exfoliative toxins (e.g. ETA, ETB), proteases, lipases, hyaluronidases, collagenases, and thermonucleases (Sandel & McKillip, 2004; Bukowski *et al.*, 2010).

The production of virulence factors is regulated by a series of global regulator molecules, whose activity is influenced by environmental signals – such as changes in nutrient availability, temperature, pH, osmolality, oxygen tension – (Cheung *et al.*, 2004; Bien *et al.*, 2011), including:

accessory gene regulator (agr). agr is a two-component system involved in the bacterial cell to cell communication mechanism of Quorum Sensing (QS). By its regulator activity on virulence factors production and biofilm formation, agr has a crucial role in pathogenesis (Singh & Ray, 2014; Kavanaugh & Horswill, 2016) .

staphylococcal accessory regulator (sarA). sarA is believed to contribute to the activation of *agr* expression. Furthermore, it promotes synthesis of some MSCRAMMS and toxins, and inhibit the expression of *spa* and proteases (Cheung *et al*., 2008)

sigma factor B (SigB). SigB is responsible for the transcription of genes that can confer resistance to heat, oxidative and antibiotic stresses (Bischoff *et al*., 2004; Hecker *et al*., 2009).

Among the repertoire of virulence factors, *S. aureus* toxins play an important role in the pathogenicity of this microorganism, since they are able to damage biological membranes, leading to cell death. These include α- and β- hemolysins (hla and hlb) and leukocidins (e.g., Panton-Valentine Leukocidin) (Otto, 2014). Some of the staphylococcal toxins, such as the staphylococcal enterotoxins (SEs) and the Toxic Shock Syndrome Toxin 1 (TSST-1), present also superantigenic activity, being able to stimulate the release of large amounts of cytokines (Dinges *et al*., 2000).

1.1.3 Staphylococcal Food Poisoning

S. aureus is a significant cause of food-borne diseases: as it multiplies in food, some *S. aureus* strains produce toxins (staphylococcal enterotoxins - SEs) that are responsible for food poisoning in humans (Le Loir *et al*., 2003; Hennekinne *et al*., 2012). Since the growth of microorganism in the host is not required, this kind of food poisoning is considered an intoxication.
The association between *S. aureus* and food poisoning was firstly described by V.C. Vaughan and J.M. Sternberg in 1884 in Michigan (USA), and confirmed 30 years later by M.A. Barber. He demonstrated that the consumption of milk from a cow suffering staphylococcal mastitis was able to cause poisoning (Barber, 1914). Finally, Dack et al. (1930) demonstrated that a heat-resistant toxin and not the microorganism itself was responsible for the poisoning.

Today, staphylococcal food poisoning (SFP) is one of the most common food-borne diseases worldwide, and remains a great social problem, causing frequent outbreaks and financial losses (Hennekinne, 2018).

Although *S. aureus* is not the only species within its genus to produce enterotoxins, it is the main source of SFP outbreaks (Seo & Bohach, 2013). Because of its short duration and usual full recovery of intoxicated people, SFP occurrence is probably under-reported. Most of the reported cases are related to highly publicized outbreaks involving several people, although SFP can also affect a single individual. Usually, SPF occurs cyclically, with the highest incidence of illness episodes in the late summer and in the last two months of the year, being mainly associated with leftover holiday food (Seo & Bohach, 2013).

European Food Safety Authority (EFSA) and European Center for Disease prevention and Control (ECDC) reported that 434 SFP outbreaks occurred in 2015 throughout 16 European member states, representing 9.9% of all European outbreaks (EFSA, 2016). A total of 39/434 were strong-evidence outbreaks, most of which were reported by France, and in many cases (13/39) were related to consumption of cheese. Other food vehicles were "mixed food" (6 outbreaks), whereas in 5 outbreaks no specific food category was provided (EFSA, 2016). A total of 14/39 outbreaks occurred after exposure in household, whereas in other cases the people was exposed at restaurant, cafe, pub, bar, hotel, catering service (EFSA, 2016).

Most of the weak evidence outbreaks were associated to the consumption of "mixed food" (75 outbreaks), meat and meat products (85 outbreaks), and other food vehicles such as fish and fish products, vegetables and juices, eggs and egg products, crustacean, shellfish, mollusks, and cheese (EFSA, 2016).

Foods most commonly involved in SFP episodes are rich in proteins, and some examples are meat and meat products, poultry and egg products, milk and dairy products, ready-to-eat (RTE) products, salads, and bakery products, reflecting the different food habits among countries (Seo & Bohach, 2013; EFSA, 2015, 2016,

2017). In particular, what poses the highest risk of SEs transmission is the consumption of food handled after been cooked. In fact, humans are frequently implicated in transmission of the pathogen to food during preparation (Seo & Bohach, 2013). The introduction of *S. aureus* into foods can also occurs by the usage of contaminated tools such as knives, utensils, cutting blocks, saw blades (Seo & Bohach, 2013). Another possible source of contamination is represented by the animals that are usually colonized with *S. aureus*. In addition, *S. aureus* is the most important etiological agent of sub-clinical mastitis in cows, representing a public health because it can results in contamination of food and milk before or during processing (Seo & Bohach, 2013).

SFPs have been frequently associated with improper handling of cooked or processed food, and with inadequate storage conditions that allow the growth of the pathogen and the production of SE(s), such as non-adequate refrigeration, preparation of foods too much in advance, poor hygiene and improper washing of hands and instruments, inadequate food cooking or heating, or foods served on warming plates for long time (Seo & Bohach, 2013).

In summary, the following conditions are necessary for inducing SFP:

- The presence of a source containing enterotoxin-producing staphylococci: raw materials, healthy or infected carrier;
- transfer of staphylococci from source to food, e.g., unclean food preparation tools because of poor hygiene practices;
- physicochemical characteristics of food composition that are favorable for *S. aureus* growth and toxins production;
- favorable conditions (e.g., temperature and time) for bacterial growth and toxinogenesis;
- ingestion of food containing sufficient amounts of toxin to provoke symptoms.

The production of SEs can occur at a wide range of temperatures (10-46ºC, optimal production at 34-45ºC), pH (4.0-9.6, optimum at 7-8), water activity (a_w = 0.85 ≥ 0.99, optimum at a_w ≥ 0.98) and salt content (< 12%) (Schelin *et al*., 2011; Hennekinne *et al*., 2012). Although heat-treatment (e.g., cooking) easily kill *S. aureus* bacteria, SEs are resistant to heat, freezing and irradiation (Hennekinne *et al*., 2012). Detectable levels of SEs are produced when the enterotoxigenic strain grows to levels >10^5 cfu/g. Foods involved in SFP are expected to be contaminated by SEs at levels of 0.5–10 μg per 100 mL or 100 g (Bergdoll & Wong, 2006).

To date, 24 different SEs have been described, based on sequence homology (Hennekinne, 2018). Five of them have been fully characterized: SEA, SEB, SEC (presenting 5 variants - C1, C2, C3, SEC ovine and SEC bovine), SED, and SEE.

These "classical" toxins are characterized by superantigenic and emetic activities (Hennekinne, 2018). In fact, SFP symptoms generally include vomiting, nausea, stomach cramps, and diarrhea, and occur within 30 minutes to 6 hours after ingestion of SEs, depending on the amount of toxin consumed and the sensitivity of the individuals involved (Pinchuk *et al*., 2010). Furthermore, SEs can induce life-threatening toxic shock syndrome, as a consequence of their superantigenic activity that stimulates massive proliferation of large population of T cells and uncontrolled release of proinflammatory cytokines (Dinges *et al*., 2000).

SEA – either alone or together with other SEs – is the toxin most commonly involved in SFP episodes worldwide, probably because of its remarkable resistance to the action of proteases (Holmberg & Blake, 1984; Argudín *et al*., 2010). SFP outbreaks involving SEB, SEC, or SED were also reported, whereas SEE was rarely associated to outbreaks (Argudín *et al*., 2010).

1.1.4 Methicillin-Resistant *Staphylococcus aureus*

Methicillin-Resistant *S. aureus* (MRSA) are highly virulent strains resistant to many antimicrobials. To date, MRSA are the most commonly identified antimicrobial-resistant pathogen in many part of the world (Taylor, 2013), e.g. more than 150,000 persons become ill annually in healthcare facilities in the EU (Köck *et al.*, 2010).

MRSA have been reported for the first time in 1961, and until last years of 1980s they have been associated with hospitals (Hospital-Associated, HA-MRSA) (Moellering, 2012). In fact, MRSA – in particular, five major epidemic clones – have been the cause of several nosocomial infections worldwide, above all in immunocompromised people (Oliveira *et al.*, 2002).

After 1990, different clones of MRSA emerged in the community, causing infections in young and healthy people outside of hospitals (Community-Associated, CA-MRSA) (DeLeo *et al.*, 2010). CA-MRSA are usually more virulent of HA-MRSA, frequently producing Panton-Valentine leucocidin, a toxin associated with severe skin infections. CA-MRSA can also cause nosocomial infections, making it difficult to distinguish between CA- and HA-MRSA isolates (David & Daum, 2010). Although the origin of these isolates is not clear, companion animals have been indicated as potential reservoirs of CA-MRSA, and the transmission between pets and their owners has been demonstrated many times (Baptiste *et al.*, 2005; Bramble *et al.*, 2011; Ferreira *et al.*, 2011).

In 1972, a clone of MRSA was isolated in Belgium from mastitis-affected bovine milk (Devriese & Hommez, 1975). Few cases of animal-isolated MRSA were reported until the first years of 2000s, when isolation became more frequent: a new zoonotic reservoir of MRSA was found in food producing animals. These strains – that was referred to Livestock-Associated MRSA (LA-MRSA) – have been reported to spread broadly in animals, showing a different profile compared to HA- and CA- MRSA (Cuny *et al.*, 2010). In particular, LA-MRSA has been identified as being less aggressive,

lacking many genes associated to virulence and colonization typical of *S. aureus* (Hallin *et al*., 2011). LA-MRSA have been isolated in pigs, cattle, poultry, domestic pets, wild-birds, and other animals, highlighting the adaptation of the species to diverse ecological niches (Cuny *et al*., 2010).

A specific clone of LA-MRSA, belonging to sequence type 398, showed a broader host range compared to other *S. aureus* lineages (Doulgeraki *et al*., 2016). Furthermore, MRSA ST398 was also found in retail meat and can transmit from animal to humans, posing a potential threat to public health. The (in)direct contact with MRSA-colonized animals seems to be the most probable transmission route, as already happened to people working in companion animals and equine clinics, and livestock production environments (**Figure 2**; Morgan, 2008). Transmission routes and dynamics between animals, between animals and humans, and between humans need to be further investigated anyway.

The origin of LA-MRSA is not clear, but it was hypothesized that they originated as methicillin-sensitive *S. aureus* in humans before being transferred to livestock, where the uptake of mobile genetic elements allowed ST398 to acquire methicillin resistance. Furthermore, the loss of phage-carried human virulence genes likely attenuated its zoonotic potential (Price *et al*., 2012).

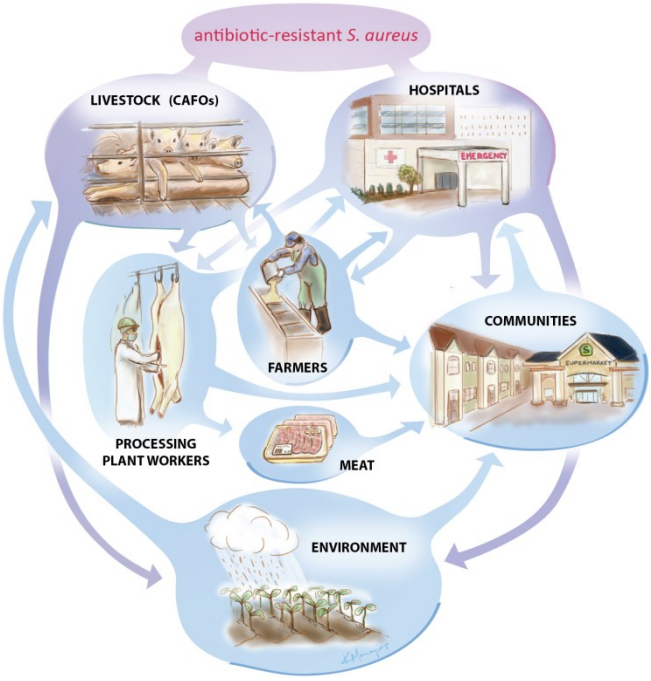

Figure 2 - Transmission route of MRSA. Reprinted from: Smith (2015).

MRSA in foodstuff – Food-producing animals colonized with MRSA have caused concern about the presence of MRSA in associated foodstuff. Different retailed meat products, in fact, have resulted positive to MRSA, with a prevalence that greatly depends on the animal's origin and country (Doulgeraki *et al.*, 2016). MRSA were isolated mainly from raw meat (including pork, beef, lamb, chicken, turkey and, more rarely, rabbit), and dairy products (milk and cheese) (Doulgeraki *et al.*, 2016). In particular, the highest contamination rate was found in pork – in the USA and Canada – and in poultry – in Denmark and the Netherlands – (Pu *et al.*, 2009; Weese *et al.*, 2010).

Some food-isolated MRSA were found to be HA- or CA- MRSA, indicating that food handlers were implicated in the transmission of the pathogen to foodstuff (Pu *et al.*, 2009; Hata *et al.*, 2010). On the other hand, MRSA-positive animals were at the root of foodstuff contamination by LA-MRSA in other surveys (de Boer *et al.*, 2009; Fessler *et al.*, 2011).

According to the last summary report by EFSA and ECDC on antimicrobial resistance in zoonotic and indicator bacteria from humans, animals and food, in 2015 a low number of member states (MSs) reported the monitoring of MRSA in food: Germany, Finland Slovakia, and Spain, as well as Switzerland (EFSA & ECDC, 2017). MRSA was detected in meat from rabbits and pigs in four of these countries. Finland and Switzerland also reported spa-type identification, with the presence of Spa types t034 and t2741, commonly associated to CC398 (EFSA & ECDC, 2017).

Genetic determinants of MRSA – The acquisition of the broad-spectrum β-lactam resistance in MRSA isolates is due to the presence in their genome of the Staphylococcal Cassette Chromosome *mec* (SCC*mec*; **Figure 3**).
SCC*mec* is a mobile genetic element composed of:

1) the *mec* gene complex, including:
   - the *mecA* gene, that encodes the penicillin-binding protein 2a (PBP2a), which shows low affinity for β-lactam antibiotics (e.g., penicillin and methicillin);
   - two regulation genes *mecR1* (encoding the signal transducer protein MecR1) and *mecI* (encoding the repressor protein MecI);
   - some associated insertion sequences (Ito *et al.*, 2001; Arias & Murray, 2009).
2) The cassette chromosome recombinases (*ccr*), a unique site-specific recombinases responsible for mobility of the elements (Katayama *et al.*, 2000; Ito *et al.*, 2001);
3) The J regions, that typically contain pseudogenes and truncated copies of transposons and insertion sequences, and encode important functions such as resistance to additional antibiotics and to heavy metals (Kondo *et al.*, 2007; IWG-SCC, 2009).

The integration of the SCC*mec* occurs through *ccr*-mediated recombination within a specific site, the *attB* integration site sequence (ISS) present at the 3' end of the *orfX* gene (IWG-SCC, 2009).

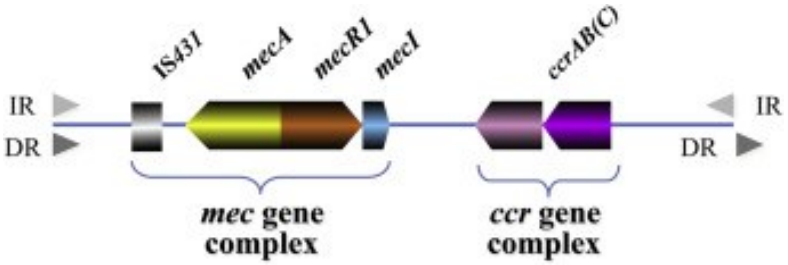

Figure 3. The structure of SCC*mec*. SCC*mec* is composed of two essential gene complexes. One is *mec*-gene complex, encoding methicillin resistance (*mecA* gene) and its regulators (*mecI* and *mecR1*), and the other is *ccr*-gene complex that encodes the movement, (integration to and precise excision form the chromosome), of the entire SCC element. Abbreviations: IR, inverted repeat; DR, direct repeat. Reprinted from: Hiramatsu *et al*. (2014)

SCC*mec* elements have been hierarchically classified into types and subtypes, highly differing in their structural organization and genetic content. The combination of the type of *ccr* gene complex and the class of the *mec* gene complex define the SCC*mec* types (IWG-SCC, 2009).

Ccr gene complex. Three phylogenetically distinct *ccr* genes with DNA sequence similarities below 50% have been so far identified: *ccrA*, *ccrB*, and *ccrC* (IWG-SCC, 2009). The *ccrA* and *ccrB* genes were in turn classified into different allotypes sharing 60 to 82 % nucleotide identities. The *S. aureus ccr* gene complex includes type 1 (carrying *ccrA1B1*), type 2 (carrying *ccrA2B2*), type 3 (carrying *ccrA3B3*), type 4 (carrying *ccrA4B4*), type 5 (carrying *ccrC*), type 6 (carrying *A5B3*), type 7 (carrying *A1B6*), and type 8 (carrying *A1B3*) (IWG-SCC, 2009, 2018).

Mec gene complex. The *mec* gene complex is classified into five classes (A to E). The prototype of mec gene complex is the class A *mec*, containing *mecA*, the complete *mecR1* and *mecI* regulator genes upstream of *mecA*, and the hypervariable region (HVR) and insertion sequence (IS) IS431 downstream of *mecA*. Insertion of IS1272 or IS431 within the *mecR1* gene and/or differences in spatial arrangement of the elements composing the complex define the other four classes (IWG-SCC, 2009).

SCCmec are also classified into subtypes, according to structural differences in J regions. J regions were firstly thought to be junk regions, but more recent findings showed that they are responsible for additional functions, such as resistance to other antibiotics or heavy metals. Three regions were identified: J1 – between right chromosome junction and the *ccr* complex, J2 – between the *ccr* and *mec* gene complexes, and J3 – between mec complex and left chromosome junction.

SCC*mec* also contains additional insertional sequences that may allow incorporation of additional antimicrobial resistance markers, giving reasons for the ability of different MRSA isolates to resist to antimicrobial compounds different from β-lactam antibiotics (Gorwitz, 2008). Furthermore, MRSA with a divergent *mecA* homologue – *mecC,* sharing the 70% nucleotide sequence – have been found in a range of Multi-Locus Sequence Type (MLST) lineages isolated from humans and other animal species (Cuny *et al*., 2010; García-Álvarez *et al*., 2011). Molecular confirmatory methods are not able to identify MRSA containing *mecC,* although they can be identified by routine culture and susceptibility testing (García-Álvarez *et al*., 2011).

A total of 11 SCC*mec* types (I to XI) have been described, to date. The most common ones are types I to V, whereas type XI comprises *mecC* containing MRSA.

1.1.5 *S. aureus* molecular typing

Different genotyping techniques have been developed for *S. aureus* isolates characterization.

Pulsed-Field Gel Electrophoresis (PFGE) – PFGE is considered the gold standard of epidemiological studies of different pathogens, including *S. aureus*. It is based on the application of an electric field that periodically changes direction. After being digested with restriction enzymes, the genomic bacterial DNA of the microorganism of interest is exposed to the electric field in an

agarose gel, generating a specific pattern (fingerprint) of the isolate (Wang *et al*., 2015).

This technique is characterized by high discriminatory power, a high between-lab reproducibility (thanks to standardized protocols), and the presence of international PFGE database (PulseNet International, n.d.) that can easily and quickly connect similar cases of foodborne diseases across states and countries, finding outbreaks. For this reason, PFGE is highly used for national surveillance and global tracking of *S. aureus* infection sources (Golding *et al*., 2015).

Multilocus Sequence Typing (MLST) – MLST is an unambiguous typing method based on the amplification and sequencing of internal fragments of seven housekeeping genes (Pérez-Losada *et al*., 2011). *S. aureus* MLST scheme was developed by Enright et al. (2000) and uses the following genes: carbamate kinase (*arcC*), shikimate dehydrogenase (*aroE*), glycerol kinase (*glpF*), guanylate kinase (*gmk*), phosphate acetyltransferase (*pta*), triosephosphate isomerase (*tpi*), acetyle coenzyme A acetyltransferase (*yqi*) (Saunders & Holmes, 2007). The analysis of the data is facilitated by the use of web-based tools.

***Spa* typing** – This single locus method consist in a variable number tandem repeat (VNTR) analysis. It is based on the amplification of a length polymorphic region located in the staphylococcal protein A (*spa*) gene. The *spa* type is determined by the variation in repeat succession (2 to 16 repeats) of specific regions generally composed of 24 bp, differing each other in at least one point mutation, and randomly designated with letters (A to Z, A2, B2, etc.) (Hallin *et al*., 2009). Rapidity, reproducibility, and portability are some of the advantages of this technique (Hallin *et al*., 2009).

Ribosomal Spacer PCR (RS-PCR) – RS-PCR is based on the analysis of the hypervariable 16S-23S rRNA intergenic spacer region (Hoffmann *et al*., 2010). The region is amplified and analyzed by a miniaturized electrophoresis system, which allow the separation of amplified DNA. Two patterns are considered

different if two or more peaks in the electropherogram differ in size (Kumari *et al*., 1997; Graber *et al*., 2009).

SCC*mec* typing – The type of SCC*mec* element is determined by the combination of the class of *mec* gene complex and the type of *ccr* gene complex. Multiplex PCR is the method most used for classifying SCCmec. It allows the simultaneous amplification of several genes or alleles by the use of many pairs of primers within the same reaction mixture. Kondo *et al*. (2007) developed a convenient system for SCC*mec* type assignment that consists of six multiplex PCR that allow the identification of the *ccr* gene complex, the *mec* gene complex, and specific structures in the J regions.

Whole genome sequencing (WGS). The use of next generation sequencing (NGS) approach allows to obtain all the genetic information of the typed isolate. Genetic variations, outbreak investigation, and global epidemiology are some of the variety of potential application of WGS data. (Hall, 2007; Zankari *et al*., 2012).

1.1.6 Legislation

Since the first years of 2000s, the European general principles and requirements of food are laid down by the Regulation (EC) No 178/2002. It established that all food business operators have a legal responsibility to produce safe food. This objective can be achieved by the implementation of Hazard Analysis and Critical Control Point (HACCP) -based food safety management systems and by the adoption and implementation of Good Hygiene Practices (GHP).

Hygiene requirements for foodstuff and for foods of animal origin are laid down by Regulations 852/2004 and 853/2004, respectively. Furthermore, Regulation (EC) 2073/2005 and its subsequent amendments lay down microbiological criteria for various combinations of food categories and microorganisms, their toxins or metabolites.

According to such Regulation, microbiological criteria are divided into:

1) Process hygiene criteria – indicating the hygienic conditions of the production process;
2) Food safety criteria – defining the acceptability of a foodstuff in terms of its microbiological safety, and are applicable to foodstuffs placed on the market during their shelf-life.

Coagulase positive Staphylococci are considered process hygiene criterion in dairy products – e.g. certain cheeses, milk power, and whey powder –, and in fishery products – shelled and shucked products of cooked crustaceans and molluscan shellfish. The criterion applies at the end of the manufacturing process, with exception of certain cheeses, in relation to which the criterion applies during the manufacturing process when the number of staphylococci is expected to be the highest (European Commision, 2005).

Regulation 2073/2005 and its subsequent amendments also consider staphylococcal enterotoxins as a food safety criterion in certain dairy products, i.e. cheeses, milk powder and whey powder. If coagulase-positive staphylococci are detected at levels $>10^5$ cfu/g in such products, they must be tested for SEs.

1.2 Biofilms in the Wonderland of Food Industry

1.2.1 Microbial biofilms: definition and development

Biofilms are communities of microbial cells strictly adhered each other and to a surface, embedded in a self-produced matrix of exopolymeric substances (EPS) (Donlan, 2002; Flemming & Wingender, 2010). A network of social and physical cell-cell interactions within a biofilm, together with the properties of the EPS matrix, result in a lifestyle completely different from that of planktonic (free-floating) cells and in the acquisition of emergent properties (Donlan, 2002; Flemming *et al.*, 2016).

It has been estimated that more than 99% of all bacteria live in a biofilm (Gilbert *et al.*, 2002), that may form on a wide variety of surfaces, both biotic (e.g., living tissues, bones, meat, vegetables, fruits, etc.) and abiotic (e.g., indwelling medical devices, food industry equipment, food industry surfaces, soil, etc.) (Donlan, 2002; Flemming *et al.*, 2016; Galié *et al.*, 2018).

Biofilm formation is a complex and dynamical process that comprise four steps: i) bacterial attachment to a surface, (ii) early development of biofilm architecture, (iii) maturation, and (iv) dispersion (**Figure 4**) (Srey *et al.*, 2013).

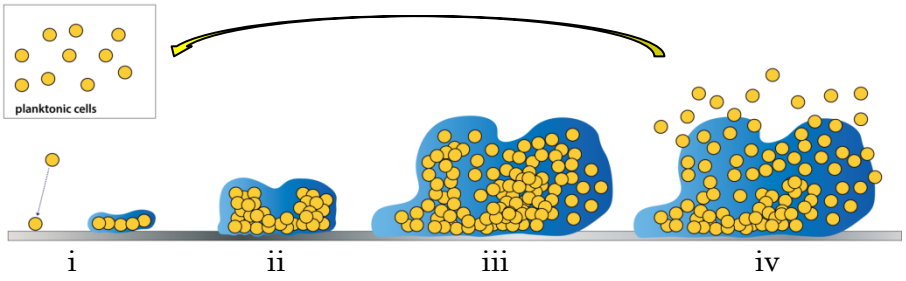

Figure 4 – Schematic representation of the biofilm formation process. i) bacterial attachment to a surface, (ii) early development of biofilm architecture, (iii) maturation, and (iv) dispersion.

Bacterial initial attachment. The attachment of bacteria to a surface is mediated by several forces, such as electrostatic and hydrophobic interactions, van der Waals forces and other

physiochemical parameters. The presence of fimbriae and flagella, as well as surface proteins (such as MSCRAMMs), provide mechanical attachment to the surface and has a pivotal role in the initial attachment.

A variety of surface properties greatly influence the initial adhesion: texture (roughness or smoothness), hydrophobicity (Donlan, 2002), surface charge (Abdallah *et al*., 2009), pH, temperature (Nilsson *et al*., 2011), etc. Furthermore, the attachment of bacterial cells is enhanced by the presence of a conditioning film overlaying the surfaces that comprise macromolecules such as organic substances (Tang *et al*., 2009).

At the beginning, the adhesion of the microorganisms is reversible, and many of them may detach from the surface returning to planktonic form, because cells are not yet committed to the differentiation process (Stoodley *et al*., 2002b). The production of EPS allows the shift from reversible to irreversible attachment of the bacteria, that interact with permanent bonding with the surface (Stoodley *et al*., 2002b).

Early development of biofilm architecture. The growth and accumulation of the cells attached to the surface led to the formation of microcolonies. In this step, the EPS produced by the cells have a pivotal role in strengthening the bond between the bacteria and the surface and among bacteria (Chmielewski & Frank, 2003). Furthermore, EPS matrix stabilizes the colonies from environmental stresses (Donlan, 2002). The recruitment of cells of the same species or other species from the surrounding environment can occur though cell-to-cell communication mechanism (Quorum Sensing – QS) (McLean *et al*., 1997; Pesci *et al*., 1999; Li *et al*., 2018). The coordination among microcolonies provides exchange and distribution of substrates and metabolites, as well as mutual end-product removal (Costerton & Lewandowski, 1995; Jamal *et al*., 2018).

Biofilm maturation. Biofilms continue to grow and develop into organized three-dimensional structures which can have different architectures, depending on a number of environmental and microbiological influence, e.g. flat, tower shaped, or mushroom shaped. Changes in gene expression are triggered by the contact of the cells with the surface. In particular, sessility favoring factors, such as those implicated in the formation of the extracellular matrix, result up-regulated. Hence, the cells within a mature biofilm are embedded in a complex EPS matrix, that form interstitial voids – a series of channels resembling a circulatory system – filled with water that distributes nutrients and removes end-products from the microcolonies communities (Flemming & Wingender, 2010). In a thick biofilm, the arrangement of bacteria follows their metabolism and aero tolerance, so anaerobic bacteria usually live in the deeper layers of the biofilm, where the oxygen concentration is lower (Rabin *et al*, 2015).

Biofilm dispersion. The last step of the biofilm lifecycle comprises the dispersion of sessile cells in the environment and their reversion into their planktonic form, and can involve the whole biofilm or a part of it.

Several factors could be responsible for the biofilm detachment, both external and internal to the biofilm. Lack of nutrients, intense competition, outgrown population, external perturbation – such as increased fluid shear (Stoodley *et al*., 2002a) –, internal biofilm processes – such as endogenous enzymatic degradation – are some of the causes of biofilm dispersion.

Biofilm dispersion seems to be an active process which allows for the colonization of new niches. In fact, the dispersed planktonic bacteria can search for a nutrient-rich environment and colonize other surfaces, promoting the formation of new biofilms.

1.2.2 The biofilm matrix

Biofilms have been metaphorically defined "smart cities of microbes" (Watnick & Kolter, 2000). If considering this image, the EPS matrix can be defined as "the house of biofilm cells" (Flemming *et al*., 2007). The cells within a biofilm usually account for less than 10% of the dry mass. On the contrary, the extracellular matrix in which the cells are embedded can account for over 90% (Flemming & Wingender, 2010). Many of the properties of a biofilm derive from its matrix. The biofilm matrix is composed by a conglomeration of different extracellular polymeric substances (EPS) that are responsible for cohesion in the biofilm and adhesion to the surfaces. The EPS matrix has a structural role, since it provides the three-dimensional architecture of mature biofilms forming a scaffold – that includes pores and channels – that immobilize biofilm cells keeping them in close proximity. For this reason, the matrix allows cell-to-cell communication and synergistic interactions among the microorganisms. It is involved in sorption of organic compounds and inorganic ions, and in retention of water, leading biofilm cells to the tolerance of the desiccation in water-deficient environments (Flemming & Wingender, 2010). The EPS matrix also acts as an external digestive system: it retains extracellular enzymes that metabolize the sequestered nutrients, as well as exogenous macromolecules, making them available as nutrient and energy sources. Furthermore, it keeps all of the components of lysed cells available, including DNA that can be involved in gene exchange processes such as horizontal gene transfer (HGT) (Flemming & Wingender, 2010).

The EPS matrix has also the important function of protecting the cells of a biofilm acting as a physical barrier. The complex network of exopolymers confers to the cells resistance to host defences during infections, as well as tolerance and/or resistance to various antimicrobial agents, such as disinfectants and antibiotics.

Table 1. Functions of extracellular polymeric substances in bacterial biofilms. Adapted by permission from Springer Nature: Springer *Nature Reviews Microbiology*, The biofilm matrix, Flemming HC & Wingender J, copyright (2010).

Function	Relevance for biofilms	EPS components involved
Adhesion	Allows the initial steps in the colonization of abiotic and biotic surfaces by planktonic cells, and the long-term attachment of whole biofilms to surfaces	Polysaccharides, proteins, DNA and amphiphilic molecules
Aggregation of bacterial cells	Enables bridging between cells, the temporary immobilization of bacterial populations, the development of high cell densities and cell–cell recognition	Polysaccharides, proteins and DNA
Cohesion of biofilms	Forms a hydrated polymer network (the biofilm matrix), mediating the mechanical stability of biofilms (often in conjunction with multivalent cations) and, through the EPS structure (capsule, slime or sheath), determining biofilm architecture, as well as allowing cell–cell communication	Neutral and charged polysaccharides, proteins (such as amyloids and lectins), and DNA
Retention of water	Maintains a highly hydrated microenvironment around biofilm organisms, leading to their tolerance of dessication in water-deficient environments	Hydrophilic polysaccharides and, possibly, proteins
Protective barrier	Confers resistance to nonspecific and specific host defences during infection, and confers tolerance to various antimicrobial agents (for example, disinfectants and antibiotics), as well as protecting cyanobacterial nitrogenase from the harmful effects of oxygen and protecting against some grazing protoza	Polysaccharides and proteins
Sorption of organic compounds	Allows the accumulation of nutrients from the environment and the sorption of xenobiotics (thus contributing to environmental detoxification)	Charged or hydrophobic polysaccharides and proteins

Sorption of inorganic ions	Promotes polysaccharide gel formation, ion exchange, mineral formation and the accumulation of toxic metal ions (thus contributing to environmental detoxification)	Charged polysaccharides and proteins, including inorganic substituents such as phosphate and sulphate
Enzymatic activity	Enables the digestion of exogenous macromolecules for nutrient acquisition and the degradation of structural EPS, allowing the release of cells from biofilms	Proteins
Nutrient source	Provides a source of carbon-, nitrogen- and phosphorus-containing compounds for utilization by the biofilm community	Potentially all EPS components
Exchange of genetic information	Faciliates horizontal gene transfer between biofilm cells	DNA
Electron donor or acceptor	Permits redox activity in the biofilm matrix	Proteins (for example, those forming pili and nanowires) and, possibly, humic substances
Export of cell components	Releases cellular material as a result of metabolic turnover	Membrane vesicles containing nucleic acids, enzymes, lipopolysaccharides and phospholipids
Sink for excess energy	Stores excess carbon under unbalanced carbon to nitrogen ratios	Polysaccharides
Binding of enzymes	Results in the accumulation, retention and stabilization of enzymes through their interaction with polysaccharides	Polysaccharides and enzymes

EPS, Extracellular Polymeric Substances

Because of the large range of exopolymers composing matrix and the difficulty to analyze them, the EPS have been called "the dark matter of biofilm" (Flemming *et al.*, 2007). The three-dimensional architecture of a biofilm is related to the composition of EPS matrix, that strongly depends on the microorganisms present in

the biofilm and physicochemical conditions of the environment, such as the shear forces experienced, the temperature and the presence of nutrients. It has been observed that mutants lacking components of EPS showed altered morphology of biofilms.

EPS matrix contain extracellular polysaccharides, proteins, glycoproteins, extracellular DNA (eDNA), lipids, and other biopolymers, as well as other extracellular structures, such as pili, flagella, and fimbriae (**Figure 5**; Zogaj *et al*., 2001).

Polysaccharides. Polysaccharides are a major fraction of the EPS matrix in many biofilms (Frølund *et al*., 1996; Wingender *et al*., 2001). Electron microscopy analysis of different biofilms showed polysaccharides attached to the cell surface and forming complex network. Exopolysaccharides have been found to be indispensable for biofilm formation in many bacteria. In fact, microorganisms mutated in the exopolysaccharides biosynthetic pathway were severely compromised or not able to form mature biofilms (Watnick & Kolter, 1999; Danese *et al*., 2000; Ma *et al*., 2009). In mixed-species biofilms, the presence of exopolysaccharide producing species allows the integration of other non-producing species (Sutherland, 2001).

Proteins. The biofilm matrix can contain considerable amounts of proteins including both enzymes and structural proteins. Extracellular enzymes can be involved in degradation of the matrix biopolymers, making of the matrix an external digestive system able to transform complex biopolymers in low-molecular-mass products that can be used as carbon and energy sources (Flemming & Wingender, 2010). Extracellular enzymes can also have a role in the detachment of the biofilms – favoring the degradation of structural EPS – or in the infectious processes – acting as virulence factors (Flemming & Wingender, 2010).

The structural proteins present in the matrix have a role in the formation and stabilization of the polysaccharide matrix as a link between the EPS and the bacterial surface. These proteins include the cell-surface associated and the extracellular carbohydrate-

binding proteins (known as lectins) (Flemming & Wingender, 2010).

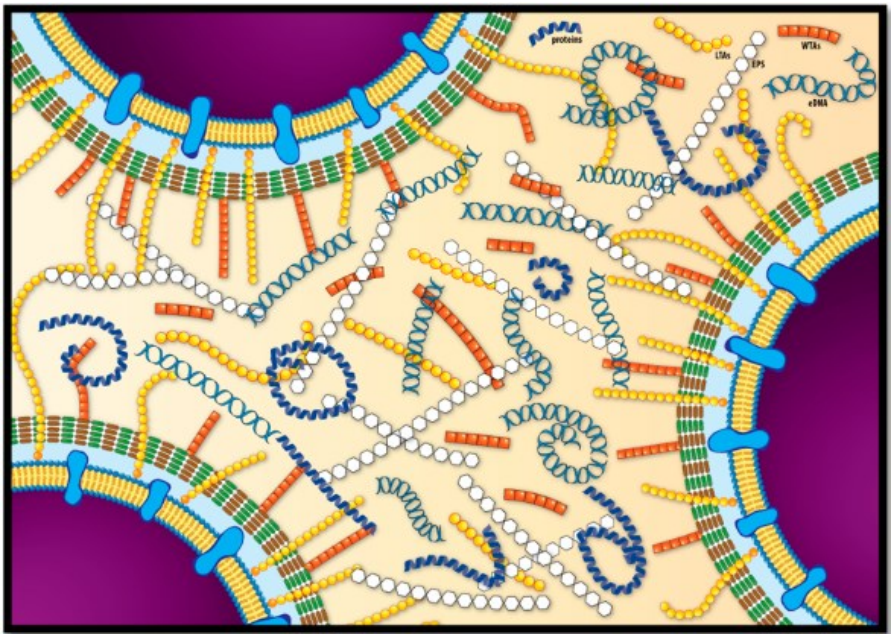

Figure 5 - Schematic representation of the extracellular matrix of a *Listeria monocytogenes* biofilm. The major components (exopolysaccharides, proteins, and eDNA) are distributed heterogeneously within the matrix. Reprinted from: Colagiorgi *et al.* (2016).

eNAs. The importance of extracellular nucleic acids (eNAs) has been underestimated at the beginning: although they have been found in EPS matrix for long time, it was thought that eNAs derived from the lysed cells within the biofilm (Molin & Tolker-Nielsen, 2003; Flemming & Wingender, 2010). Actually, lysed cells are not the only source of eDNA, and active excretion of DNA cannot be excluded (Flemming & Wingender, 2010). Further studies demonstrated that eNAs are required for the formation of the biofilms of several microorganismsm, such as *Pseudomonas aeruginosa*, *Listeria monocytogenes*, *Bacillus cereus*, etc. eDNA have a structural role in biofilm matrix, contributing also significantly to the mechanical stability of the EPS matrix (Molin & Tolker-Nielsen, 2003). Furthermore, it represent a source of genetic

information that can be involved in gene exchange processes among biofilm cells (Flemming & Wingender, 2010).

1.2.3 Microbial biofilms in Food Industry

The microorganisms present in food industry are able to grow on food matrixes and along industrial infrastructures potentially giving rise to biofilms. The biofilms formed on food processing surfaces have been found to be composed of a mix of bacteria, including pathogens and spoilers (Gounadaki *et al.*, 2008; Gutiérrez *et al.*, 2012).

Water, raw food, animals, and food handlers are some of the main route by which these microorganisms enter a food industry, depending on the food-processing industry (Gutiérrez *et al.*, 2016) (**Figure 6**). Biofilms can represent an important issue in dairy (Latorre *et al.*, 2010), meat (Giaouris *et al.*, 2014), poultry (Silagyi *et al.*, 2009), seafood (Thimothe *et al.*, 2014), and vegetable processing industries (Liu *et al.*, 2013), since their development can have different implication from both economic and hygienic point of view.

Food Hygiene. Many of the microorganisms present in food industry are human pathogens, that are able to develop biofilms on common surfaces present in food processing plants: stainless steel, plastics, rubber, wood, glass, etc (Di Ciccio *et al.*, 2015; Colagiorgi *et al.*, 2017; Galié *et al.*, 2018). Furthermore, microbial pathogens can also form biofilms on the biological surface of foods, such as vegetable, meat, bones, fruit, etc. (Srey *et al.*, 2013; Galié *et al.*, 2018). Biofilms formed on food surfaces or in processing environment can allow to microorganisms to persist, becoming reservoir of contamination (cross- and post-processing-contamination) for pathogens with the consequent risks to human health (Srey *et al.*, 2013; Colagiorgi *et al.*, 2017; Galié *et al.*, 2018).

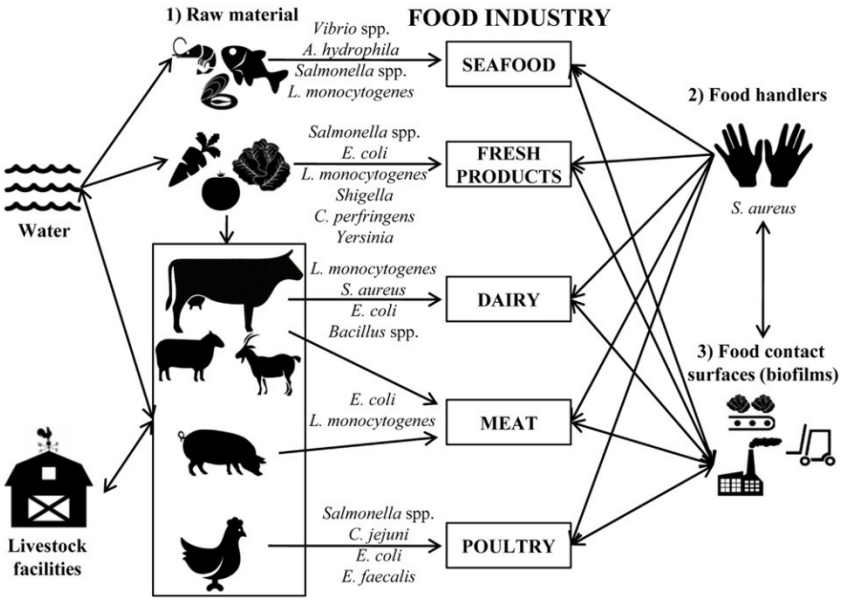

Figure 6 - Schematic representation of the main sources of contamination in food industries. Most common bacteria detected in each agri-food industry are indicated. Reprinted from Gutierrez *et al.* (2016).

Food spoilage. The formation of biofilms by spoiler microorganism can led to an alteration of organoleptic properties of food due to secretion of lipases or proteases, and to a reduction of the shelf-life of the products, with important economic losses (Srey *et al*., 2013; Mizan *et al*., 2015; Galié *et al*., 2018).

Biofouling. The formation of biofilms on surfaces in contact with liquid media (the so-called biofouling) is an important issue in food industry. In particular, biofouling can be responsible for the impediment of the flow of heat across the surface, and for the increase in the fluid frictional resistance at the surface and in the corrosion rate at the surface (Kumar & Anand, 1998; Galié *et al*., 2018). All these problems lead to energy and product losses.

Some important potential sources of contamination are represented by environmental surfaces such as walls and floor: people, air, and cleaning systems may serve as vectors for microorganisms transmission to food (Di Ciccio *et al*., 2012).

Floors, waste water pipes, bends in pipes, conveyor belts, rubber seals, and stainless steel surfaces are some of the most common sources involved in biofilm accumulation, as well as airborne microbiota. Properly cleaning and sanitation of surfaces are very important for preventing biofilm formation, since the presence of organic material on food-contact surfaces can represent a conditioning film for the initial steps of biofilm formation, allowing microbial adhesion. The Cleaning-in-Place (CIP) procedures employed in milk processing lines, for example, can lead to the accumulation of microorganisms on the equipment surfaces, that results in biofilm formation (Anand & Singh, 2013). Furthermore, the aerosols generated during the cleaning of food-processing surfaces can also be responsible of the transmission of microorganisms.

A great concern about biofilms is their resistance to a variety of commonly used disinfectants, including quaternary ammonium compounds (e.g. benzalkonium chloride - BAC), leading to a reduction of the efficacy of such compounds (Bridier *et al.*, 2011b; Ibusquiza *et al.*, 2011). This is due to the presence of the EPS matrix that acts as a physical barrier, but also to other biofilm properties such as reduced diffusion, physiological changes of cells, reduced growth rate, and the production of antimicrobials degrading enzymes (Bridier *et al.*, 2011b, 2015).

In summary, the ability of microorganisms to form biofilms in a food industry environment confers them many advantages, since they become more resistant to desiccation, starvation, mechanic stresses, disinfectants and antimicrobials commonly used in food industry (Bridier *et al.*, 2011b; Flemming *et al.*, 2016). This enable them to persist in the environment giving arise to contamination or cross-contamination of food products (including finished products).

1.2.4 *Staphylococcus aureus* biofilms: development, composition and regulation

Staphylococcus aureus can produce complex and multilayered biofilms encased in a matrix of exopolymeric substances. The staphylococcal biofilm matrix is mostly composed by a specific polysaccharide antigen named polysaccharide intercellular adhesin (PIA) (O'Gara, 2007; Archer *et al*., 2011). PIA is composed of β-1,6-linked N-acetylglucosamine polymer, and for this reason is also known as polymeric N-acetyl-glucosamine (PNAG) (O'Gara, 2007). The products of the *ica* locus – *icaR* (regulatory) and *icaADBC* (biosynthetic) genes – are responsible for the synthesis, export, and modification of PIA, whose role in the structural integrity of staphylococcal biofilms is very important. There are, nonetheless, *S. aureus* strains able to form biofilms in a PIA-independent way biofilms (Beenken *et al*., 2003; Fitzpatrick *et al*., 2005; Toledo-Arana *et al*., 2005; Brooks & Jefferson, 2014). Different studies observed that the matrix of such biofilms was composed by proteins and eDNA acting as intercellular adhesins (O'Neill *et al*., 2007, 2008; Rohde *et al*., 2007; Boles *et al*., 2010).

Proteins are important components of both PIA-dependent and PIA-independent *S. aureus* biofilms, since they are implicated in the attachment and the development of the matrix. Many of these are surface-associated proteins such as protein A (spa), fibrinogen-binding proteins (FnBPA and FnBPB), *S. aureus* surface protein (SasG), biofilm-associated protein (Bap), and clumping factors (ClfA and ClfB) (Cucarella *et al*., 2001; Corrigan *et al*., 2007; O'Neill *et al*., 2008; Merino *et al*., 2009; Geoghegan *et al*., 2010; Abraham & Jefferson, 2012). Other secreted proteins are also involved in biofilm maturation, such as extracellular adherence protein (Eap), and beta toxin (Hlb) (Huseby *et al*., 2010; Sugimoto *et al*., 2013). The importance of individual proteins is reported to greatly vary among strains, such in the case of Bap-dependent biofilms, that have been frequently reported in bovine mastitis but have never been identified in any human isolates (Lasa & Penadés, 2006; Artini *et al*., 2013).

The expression of the *icaADBC* operon products are regulated by the production of *tcaR* (transcriptional regulator of the teicoplanin-associated locus) and *icaR*, that are able to downregulate PIA and to inhibit biofilm formation (**Figure 7a**). The expression of *icaR*, in turn, is upregulated and downregulated by proteins Spx (global regulator of stress response genes) and Rbf (protein regulator of biofilm formation), respectively (Archer *et al*., 2011). *Ica* production is also regulated by a series of environmental factors, such as glucose, ethanol, osmolarity, temperature and antibiotics such as tetracycline. Anaerobic conditions, for example, induce the production of SrrAB (staphylococcal respiratory response regulator), which promotes the expression of the *icaADBC* gene cluster, leading to PIA production and biofilm formation (**Figure 7a**; Archer *et al*., 2011).

The eDNA has also an important role in staphylococcal biofilm development. The negative charge of eDNA potentially allow it to anchor cells to a surface, to host factors, and to each other, acting as an electrostatic polymer. It has been hypothesized that eDNA is important during attachment, since the treatment of early staphylococcal biofilms with DNAse is able to degrade them (Mann *et al*., 2009). The autolysis of a subpopulation of staphylococcal cells, mediated through the activity of murein hydrolases, encoded by the *atl* and *lytM* genes, allow the release of eDNA within the biofilm (Thomas & Hancock, 2009). The release of eDNA is regulated by *cidA* and *Irg* genes, two regulators of murein hydrolase activity and cell death (**Figure 7b**). In particular, *Irg* has a role in inhibiting the cellular lysis, DNA release and biofilm formation, that are, conversely, enhanced by *cidA* expression (Archer *et al*., 2011). The staphylococcal accessory regulator (*sarA*), the accessory gene regulator (*agr*), and the sigma B (sigB) protein have also a role in the regulation of biofilm formation (**Figure 7c**). In particular, the *sarA* gene is implicated in downregulating the expression of proteases and the thermostable nuclease, thus favouring the development of an early biofilm structure. The expression of sigB has a similar effect on the

proteases expression, but it also promotes the production of adherence factors that aid in the initial step of biofilm formation. The expression of the *agr* operon genes is induced when the biofilm is mature, since the Auto Inducing Peptides (AIPs), involved in the cell-to-cell communication process, reach a quorum sensing threshold. *Agr* is involved in the upregulation of detergent-like peptide, protease and thermostable nuclease, thus inducing the release of microbial cells from the biofilm matrix, a process called seeding dispersal (Archer *et al*., 2011).

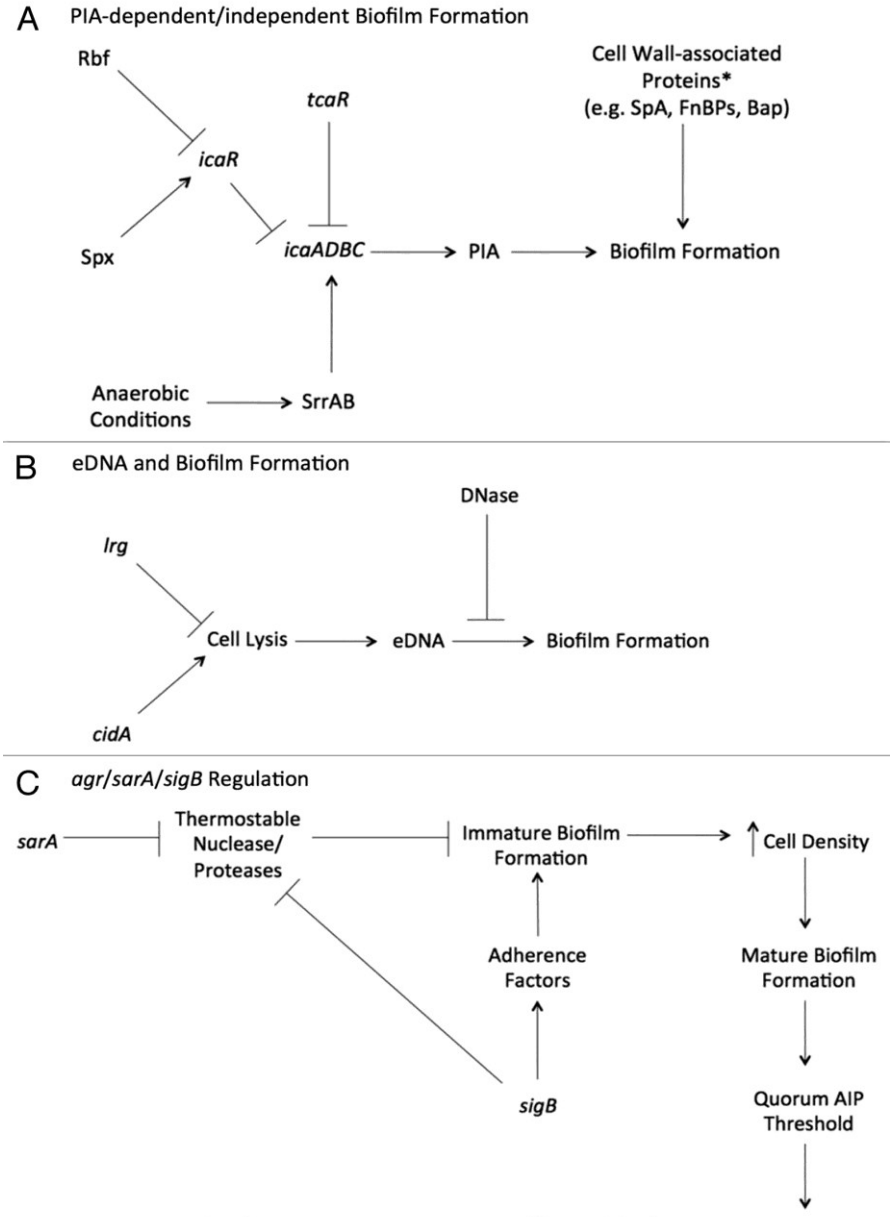

Figure 7 - Flowchart of regulatory factors involved in *S. aureus* biofilm formation. Reprinted from Archer *et al.* (2011)

1.2.5 Methods for studying bacterial biofilms

Because of the widespread presence of microbial biofilms in industrial settings or on medical devices, a variety of methods have been developed in order to better understand biofilm formation mechanisms as well as to study methods to control and eliminate them. The research on biofilms has started at the late seventies, when Bill Costerton and coworkers in 1978 gave the first definition of biofilm (Costerton *et al.*, 1978). Some of the main methods used all over the world are reported below.

Microtiter plates. It is the most commonly used method for studying biofilm formation. In the classical procedures cells are allowed to grow and to adhere and form biofilms in the wells of a polystyrene microtiter plate (usually 96-wells plate, but also 6, 12, 24 wells plates have been used). At different time points, the non-adhered planktonic cells are removed by empting and washing the wells. Then, the attached biomass is fixed and stained (usually with Crystal Violet – CV), or can be quantified by detachment and sub-sequent plating (Stepanović *et al.*, 2007; Di Ciccio *et al.*, 2015). This method could lead to overestimation of the biomass, since this is composed by biofilm cells as well as by other cells that have sedimented to the bottom of the wells and have been embedded by the biofilm matrix (Azeredo *et al.*, 2017).

Calgary Biofilm Device. The Calgary Biofilm Device (CBD) or Minimum Biofilm Eradication Concentration (MBEC) assay overcome the possible artifact of the biomass overestimation that can occur using microtiter plates. In fact, in the CBD the biofilm formation is assayed at the coverlid, composed of pegs that fit into the wells of the microtiter plate containing the growth medium and bacteria (Ceri *et al.*, 1999). Thus, the biomass formed on the pegs is formed only by sessile cells. The biomass is usually quantified after the recovery of sessile cells from the pegs by sonication (Azeredo *et al.*, 2017).

CDC biofilm reactor. In the CDC biofilm reactor (CBR) biofilm formation occurs on removable coupons housed on eight

independent rods (three coupons on each rod) – suspended from a polystyrene support – within a one-liter vessel in which the bulk fluid is continuously mixed by a magnetic stir bar. The coupons – that can be constructed by a variety of materials – experience a consistent high liquid surface shear from the rotation of the baffled stir bar that changes at different radial orbits. The CBR has been involved in studies related to biofilm resistance, to the development of biofilm control strategies, or to the evaluation of interactions among different species in mixed biofilms (Coenye & Nelis, 2010; Azeredo *et al*., 2017).

Biofilm microfluidic device. In microfluidic devices biofilms can interact with hydrodynamic environments within close systems, thus allowing the study of the effects of the hydrodynamic conditions on biofilm development. BioFlux by Fluxion Systems (South San Francisco, CA) is the most commonly used microfluidic device. It allows the study in real-time of the biofilm formation in microfluidic wells using light microscopy (Benoit *et al*., 2010). BioFlux device consists of a 48 wells plate with 24 independent channels connecting pairs of wells. A pneumatic pump, connected to the plate, create pneumatic pressure on the top of the inlet well pushing fresh medium through the microfluidic channel (containing the biofilm) and into the outlet well. The presence of a serpentine region in each channel provides sufficient back pressure. Furthermore, a dedicated chamber allows real-time microscopy observation. Due to the possibility to include up to 24 replicates and to the small applied media volume, this system is highly applicable for the screening of biofilm inhibitory agents, antibodies or other compounds.

Confocal Laser Scanning Microscopy. The Confocal Laser Scanning Microscopy (CLSM) is the microscopic technique that best allow the study of biofilms structure and architecture. The instrument is able to acquire several planes at different depths in fluorescently labeled biofilms, that can be combined with dedicated image analysis giving rise to a three-dimensional representation of the sample. Furthermore, specific software allow

the extraction of a variety of structural parameters, such as the bio-volume, thickness and roughness of the biofilms (Bridier *et al.*, 2010; Azeredo *et al.*, 2017). Depending on the fluorescent probes used to stain the samples the CLSM imaging can be used to analyze different properties. The permeant nucleic acid dyes, such as SYTO-9 and SYBR-Green, are the most widely used probe. The association of green SYTO-9 (cell permeant) and red propidium iodide (cell impermeant) – known as live/dead mixture – allow the distinction of bacteria with a compromised membrane (that appear yellow or red) from the live viable cells (appearing green). Live/dead staining is useful in analyzing the spatial distribution of viable bacteria. It is possible to observe, for example, the presence of cluster of dead bacteria (Hope *et al.*, 2002), or the effect of antimicrobials on the biofilm population (Bridier *et al.*, 2011a; b).

1.3 Prevention and Control of microbial biofilms

1.3.1 Prevention of biofilm formation

Due to the variety of issues related to biofilm formation in food industry, many researcher proposed different approaches in order to prevent or limit bacterial attachment on food processing surfaces. Antimicrobials, for example, have been incorporated within the surface materials themselves (Park *et al.*, 2004; Knetsch & Koole, 2011), and have been used to coat food contact surfaces (Gottenbos *et al.*, 2002; Thouvenin *et al.*, 2003; Knetsch & Koole, 2011). Other solutions include the modification of the physicochemical properties of surfaces (Chandra *et al.*, 2005; Rosmaninho *et al.*, 2007; Mauermann *et al.*, 2009) or the surface pre-conditioning with surfactants (Chen, 2003; Choi *et al.*, 2011).

The use of quorum sensing inhibitors (QSI) to prevent biofilm formation is an another effective strategy for their eradication. QSI are able to degrade the mediators of the QS system (quorum quenching) preventing their accumulation and the subsequent activation of the system (Brackman & Coenye, 2015; Galié *et al.*, 2018). Furthermore, bacteria do not develop resistance to such compounds because they cause less selection pressure than common bactericides (Galié *et al.*, 2018).

Biofilms formed on food processing surfaces and environments are not easy to detect. In fact, some foodborne pathogens form biofilms containing "viable but non-culturable" (VBNC) bacterial cells, characterized by a low metabolic activity, that cannot be detected by culture methods (Trevors, 2011). For this reason, the use of classical methods, such as agar plating, for the detection of biofilms in food industry could be not effective. Novel methods based on DNA analysis, such as qRT-PCR, metagenomics and metatranscriptomics, are required for the detection and the analysis of such microbial consortia, the effectiveness of which is limited by the high cost of the material required.

In this scenario, the development of new methodologies that allow the monitoring of biofilm formation on industrial surfaces and environments is pivotal in order to reduce the costs of cleaning and the production breaks for maintenance. The methods most commonly used to detect biofilms are based on the introduction of an external perturbation in the system, that can be measured by a suitable device. Some systems used in food or beverage industries are based on heat transfer and pressure measurements, or thermal pulse analysis that measures the local thermal conductivity and the heat variation due to biofilm formation, being able to detect few micrometers thick deposits (Pereira & Melo, 2009). The measurement of vibration signals can be also used for detecting biofilm formation on a surface. The Mechatronic Surface Sensor (MSS) is an instrument based on this technology, having two transducers that are attached to the industrial surface: one of them perform an excitation on the surface, whose wave is recorded by the second one, allowing the detection of the biofilm on the surface (Pereira & Melo, 2009).

1.3.2 Control of microbial biofilms

In order to control biofilm formation in the food industry a variety of methods are commonly used. The steps of cleaning and disinfection of the whole infrastructure are very important in order to remove residues that may have been produced during the processing and microorganisms, preventing the formation of biofilms. Chemical disinfectants are the most frequently used method for biofilm removal. These include a variety of disinfectants that can be grouped based on their mode of action: i) oxidizing agents, including chlorine-based compounds, ozone, hydrogen peroxide, and peracetic acid (PAA); ii) surface-active compounds, such as quaternary ammonium compounds (QACs) and acidic anionic compounds; iii) iodophores. In addition to chemical disinfectants, thermal and non-thermal physical methods have applied to the sanitation of surfaces, including hot stream, ultraviolet light, and electromagnetic radiations.

The elimination of bacterial biofilm can be very difficult in some cases, due to their complexity and emergent properties, e.g. tolerance or resistance to commonly used disinfectants, leading to the development of novel strategies, some of which are reported below.

Enzymatic disruption. Enzymes are important tools for biofilm control in food industry. Their targets are the components of the EPS matrix of biofilms and, furthermore, are biodegradable and have low toxicity. Enzymes such as proteases, glycosidases, and DNAses are commonly used in detergents for food industry applications in order to remove biofilms (Torres *et al*., 2011; Huang *et al*., 2014; Coughlan *et al*., 2016). For example, the enzyme pectin methylesterase is used in order to reduce biofilm formation in bioreactors, and can be also used to pretreat various machines and pipes (Torres *et al*., 2011). Enzymes are used also in combination with other chemical disinfectants, acting with a synergic action on microbial biofilms.

Biosurfactants. Biosurfactants are natural compounds, usually produced by microorganisms, used to treat food industry surfaces. Such compounds have the ability to modify the hydrophobicity of bacterial surface, altering their capacity to adhere and to bind any surface (Coronel-León *et al*., 2016; Zhao *et al*., 2017).

Bacteriophages. Bacteriophages are viruses able to specifically kill prokaryotic cells, being innocuous to humans, animals, and plants. Their use represent an attractive alternative to common antibiotics, being also able to eradicate biofilms, albeit with some limitation (Gutiérrez *et al*., 2016). Bacteriophages entrance within biofilm cells, for example, can be limited by the complex exopolymeric structure in which they are embedded. However, some phages possess enzymes (such as exopolysaccharide depolymerases, endolysins and virion-associated peptidoglycan hydrolases) that allow them to invade the biofilm leading to its dispersion (Gutiérrez *et al*., 2016, 2017). Another problem is related to the limited spectrum of infectivity of each bacteriophage,

making necessary the identification of the microorganisms (Lu & Koeris, 2011). Furthermore, some bacteria can become resistant to the phage action (Labrie *et al*., 2010). Their use in a food industry involves safety concerns, because a thorough genetic characterization is necessary in order to prevent the dissemination of undesirable traits, such as virulence and antibiotic resistance genes. In addition, their action on biofilm is influenced by different factors including their concentration, the complexity of the biofilms, and the environmental conditions (Galié *et al*., 2018).

Essential oils. The essential oils (EOs) are a variety of compounds derived from different plants, that showed anti-biofilm properties. EOs have antioxidant activity and their action is directed against a broad range of bacteria, parasites, insects, viruses, and fungi (Oussalah *et al*., 2007; George *et al*., 2009; Brenes & Roura, 2010; Nerio *et al*., 2010; Astani *et al*., 2011; Tserennadmid *et al*., 2011). The EOs effects include changes in the cell morphology, in the membrane physicochemical properties, and in the transcriptome, proteome and toxin production. Because of their strong flavour, poor solubility and partial volatility, the use of EOs in food industry has been limited (Delaquis *et al*., 2002; Kalemba & Kunicka, 2003).

Antimicrobial peptides. The antimicrobial peptides (AMPs) are natural molecules produced by both prokaryotes and eukaryotes involved in the innate host-defense mechanisms (Wang *et al*., 2016). AMPs are characterized by a series of interesting properties, such as a broad activity spectrum, a relative selectivity towards the bacterial membrane, a rapid mechanism of action and a low frequency in selecting resistant strains. AMPs have been used to contrast microbial biofilms, since several AMPs have the ability to permeabilize the microbial membranes, acting also against bacteria with a slow growth rate and low metabolic activity, typical of biofilms (Batoni *et al*., 2011).

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CHAPTER 2

Aim of the Thesis

AIM OF THE THESIS

The aim of this thesis was to study *Staphylococcus aureus* strains isolated from food, food environment and food handlers, through the analysis of their molecular characteristics in relationship with the ability to form biofilms on common food-contact surfaces. This thesis also aimed at developing new strategies to control staphylococcal biofilms in food industry, in order to prevent biofilm formation and to eradicate preformed biofilms.

The thesis is divided in two main parts: the first one concerning the characterization of food-related *S. aureus* isolates (chapters 3-5), and the second part (chapters 6-7) related to the study of antimicrobial resistance of MRSA in planktonic and sessile form, and to the development of a new strategy for the control of biofilms.

The third chapter of this thesis describes a study conducted in order to evaluate if the more virulent and persistent genotype B (GTB) *S. aureus* are more likely to be biofilm producer than other *S. aureus* genotypes, expanding the knowledge about different dairy-related *S. aureus* subtypes and indicating the benefit of genotyping when biofilms are studied.

The fourth chapter describes a study aimed at testing the ability of dairy isolated *S. aureus* strains to form biofilm, and at exploring their genetic diversity in terms of population structure and presence of genes involved in biofilm formation or enterotoxins production.

The study described in the fifth chapter concerns the investigation of the biofilm-forming ability of food-related MRSA strains, as well as the relationship between some molecular characteristics of MRSA and their ability to form biofilm. This study allowed a comparison between MRSA food isolates and MRSA clinical isolates in their ability to form biofilm.

The sixth chapter describes a study about antimicrobial resistance in MRSA isolated from pig production chain in relationship with antimicrobial consumption. Furthermore, a comparison between antimicrobial resistance of biofilm producing strains in planktonic and sessile form was performed. Results revealed the importance of finding and developing new antimicrobial molecules alternatives to common antibiotics.

Finally, in the seventh chapter, a new strategy to inhibit biofilm formation, as well as to eradicate preformed biofilms was tested. A new antimicrobial peptide was successfully tested *in vitro* against *S. aureus* biofilms, opening new scenarios on the development of biofilm control solutions based on this molecule.

PART 1

CHARACTERIZATION

CHAPTER 3

Biofilm formation of *Staphylococcus aureus* dairy isolates representing different genotypes

Adapted from:

Di Ciccio PA[1], Thiran E[2], Hummerjohann J[2], **Colagiorgi A**[1], Ianieri A[1]. Genotypic characterization and biofilm formation of food-related *Staphylococcus aureus* strains.

(**2016**) *Atti 70 Congresso Società Italiana Scienze Veterinarie – SISvet* 2016. Palermo. pp. 306-307

Thiran E[2], Di Ciccio PA[1], Graber HU[2], Zanardi E[1], Ianieri A[1], Hummerjohann J[2], Biofilm formation of *Staphylococcus aureus* dairy isolates representing different genotypes.

(**2018**) *Journal of Dairy Science.* 101. pp. 1000–1012

[1]University of Parma, Department of Food and Drug, Parma, Italy
[2]Agroscope, Institute for Food Sciences IFS, Bern (CH), Switzerland

ABSTRACT

The objective of this study was to compare the biofilm-forming capabilities of different genotypes of *Staphylococcus aureus* dairy isolates from Switzerland and northern Italy, including *Staph. aureus* genotype B (GTB) and methicillin-resistant *Staph. aureus* (MRSA). We hypothesized that biofilm formation might be more pronounced in the contagious GTB isolates compared with other genotypes affecting individual animals. Twenty-four dairy isolates, including 9 MRSA, were further characterized by genotyping by using ribosomal spacer PCR, *spa* typing, biofilm formation under static and dynamic conditions, and scanning electron microscopy. The GTB isolates (n = 6) were more able to form biofilms than other genotypes at 37°C and at 20°C after 48 and 72 h of incubation in the static assay using polystyrene microtiter plates. This result was supported by scanning electron micrographs showing a GTB isolate producing strong biofilm with extracellular matrix in contrast to a genotype C isolate. Furthermore, none of the MRSA isolates formed strong biofilms in the static assay. However, some MRSA produced low or moderate amounts of biofilm depending on the applied conditions. Under dynamic conditions, a much more diverse situation was observed. The ability of GTB isolates to be strong biofilm formers was not observed in all cases, emphasizing the importance of growth conditions for the expression of biofilm-related genes. No specific genotype, *spa* type, or MRSA isolate could be categorized significantly into one level of biofilm formation. Nineteen percent of isolates behaved similarly under static and dynamic conditions. The results of this study expand our knowledge of different dairy-related *Staph. aureus* subtypes and indicate the benefit of genotyping when biofilms are studied.

INTRODUCTION

Staphylococcus aureus is a foodborne pathogen considered the third most important causative bacterial agent of foodborne illnesses worldwide (Hennekinne et al., 2012); it is of great concern to the dairy industry (De Buyser et al., 2001; Oliver et al., 2009). In particular, dairy cow mastitis is the most important disease in the global dairy industry and *Staph. aureus* is one of the most important etiological agents of contagious mastitis (Silva et al., 2013; Voelk et al., 2014). Another major concern is that *Staph. aureus* can form biofilms (Santos et al., 2014). Biofilms are aggregates of microbial cells surrounded by a matrix of exopolymers (Costerton et al., 1999). Besides the production of exotoxins and surface proteins, the formation of these highly organized multicellular complexes is increasingly recognized as an important virulence factor in *Staph. aureus* (Tang et al., 2012; Lee et al., 2014). Biofilm formation can lead to persistent contamination or infection because the cells within the biofilm are very resistant to sanitation procedures and to the action of the host immune system and antimicrobial agents (Song et al., 2016). Different sources of *Staph. aureus* in the dairy cow environment have been described (Zadoks et al., 2002). Infected animals (cow-to-cow transmission), workers, and equipment and utensils used for milking are the main sources of the microorganism (Lee et al., 2014). Although some researchers have studied the ability of members of the *Staphylococcus* genus to adhere to surfaces and form biofilm, most studies have addressed the clinical aspects related to biofilm formation by Staphylococcus *intermedius* on medical implants and materials (Donlan and Costerton, 2002; de Souza et al., 2014). Moreover, few studies have reported biofilm formation by *Staph. aureus* isolated from ready-to-eat-foods (Oniciuc et al., 2016). Additionally, recent studies have identified several genotypes of *Staph. aureus* that differ in their contagiosity and pathogenicity (Fournier et al., 2008; Voelk et al., 2014; Cosandey et al., 2016). Graber et al. (2009) further demonstrated that genotype was highly associated with virulence gene pattern.

Among different genotypes, *Staph. aureus* genotype B (GTB) is associated with high within-herd prevalence, indicating an increased contagious and virulence potential compared with other genotypes (Graber et al., 2009; Voelk et al., 2014). In particular, *Staph. aureus* GTB, a major contaminant in Swiss raw milk cheese (Hummerjohann et al., 2014), was characterized by the presence of the enterotoxin genes *sea*, *sed*, and *sej*, and a SNP *lukE* gene (lukEB; Cosandey et al., 2016).

Genotype B has been found not only in Switzerland, but also in other countries of central Europe, including Italy, indicating that it is a relevant international problem in cow milk production (Cosandey et al., 2016). Regarding these aspects, the current study was carried out to compare the biofilm-forming capabilities of different genotypes of *Staph. aureus* dairy isolates, including *Staph. aureus* GTB and methicillin-resistant *Staph.aureus* (MRSA), because MRSA are a severe problem in the human population and have been isolated from milk, cheese, and other foodstuffs in different countries (Normanno et al., 2007; De Boer et al., 2009; Kav et al., 2011). We evaluated the ability of *Staph. aureus* dairy isolates to form biofilm under static and dynamic conditions and by using scanning electron microscopy. We hypothesized that biofilm formation might be more prevalent in the more contagious GTB strains compared with other genotypes (OGT).

MATERIALS AND METHODS

Bacterial Isolates

The experiment was conducted on 24 isolates (including 6 GTB strains) from milk and milk products. One isolate from poultry meat (PR 281), previously described as strong biofilm producer (Di Ciccio et al., 2015), and 3 strains from a culture collection (ATCC3556, ATCC12600, ATCC12228; American Type Culture Collection, Manassas, VA) were included as reference strains (Table 1). Stock cultures were stored at −80°C, and strains were incubated for 24 h at 37°C in tryptic soy broth (TSB, BBL Becton Dickinson, Le Pont de Claix, France) before experiments.

Table 1 - Optical density (OD) at 550 nm and biofilm production index (BPI) of reference strains on polystyrene

Reference strain	OD[1]	BPI
Staphylococcus aureus ATCC 35556 (positive control, strong biofilm producer)	0.756 ± 0.15	0.758
Staph. aureus ATCC 12600 (moderate biofilm producer)	0.450 ± 0.07	0.405
Staphylococcus epidermidis ATCC 12228 (negative control)	0.343 ± 0.05	0.294
Staph. aureus PR 281 (poultry isolate, very strong biofilm producer)	0.979 ± 0.255	1.09

[1] Values are expressed as OD mean ± SD.

Extraction of Nucleic Acids

A single colony of Staph. aureus was resuspended in 100 µL of Tris-EDTA buffer (10 mM Tris/HCl, 1 mM EDTA, pH 8.0), incubated at 95°C for 10 min, and immediately placed into ice. For PCR analysis, the lysate was diluted 1:100 in H_2O and directly used for amplification.

Genotyping

Genotyping of the strains was based on PCR amplification of the 16S-23S rRNA intergenic spacer region (RS-PCR) and was performed as described by Fournier et al. (2008). Briefly, the PCR

reaction mix (total volume of 25 µL) contained 1× HotStarTaq Master Mix (Qiagen AG, Hombrechtikon, Switzerland), 800 nmol of each primer G1 and L1 (Jensen et al., 1993), and 30 µg of the lysate nucleic acids. The PCR conditions were as follows: denaturation at 95°C for 15 min followed by 27 cycles of 94°C for 1 min, 2-min ramp time, annealing at 55°C for 7 min, 2-min ramp time, and extension for at 72°C for 2 min on a T-Professional thermal cycler (Biometra, Göttingen, Germany). The PCR products were analyzed by the miniaturized electrophoresis system DNA 7500 LabChip (Agilent Technologies, Basel, Switzerland). The resulting amplification patterns were interpreted according to Fournier et al. (2008), using a computer program developed in-house (Syring et al., 2012).

Spa Typing

The *spa* typing was based on the amplification of the spacer region of the spa gene of *Staph. aureus* which encodes staphylococcal protein A. It was performed according to the method described by Boss et al. (2016). Briefly, the PCR reaction mix (total volume of 25 µL) contained 300 nmol of each primer, 12.5 µL of of HotStarTaq Master Mix (Qiagen AG), and 2.5 µL of template DNA. The PCR cycles included a denaturation step at 95°C for 15 min, followed by 37 cycles of denaturation at 94°C for 60 s, annealing at 6The 0°C for 60 s, and a single extension step at 72°C for 10 min on a T-Professional thermal cycler (Biometra). PCR products were sent to Microsynth AG (Balgach, Switzerland) for purification and sequencing using the Sanger approach. The obtained sequences were then evaluated for corresponding spa type (t) using the Ridom server (http://www.spaserver.ridom.de/).

Detection of *nuc* and *mecA* Genes

All isolates were confirmed as MRSA by the detection of the methicillin resistance mecA gene and thermostable nuclease nuc gene. The DNA extracts were subjected to a duplex-PCR protocol for the detection of *mecA* and *nuc* (Virgin et al., 2009). A methicillin-susceptible *Staph. aureus* strain (ATCC 29213) was used

as a negative control and a MRSA strain (ATCC33591) as a positive control.

Biofilm Formation Under Static Conditions

All strains were tested in triplicate on polystyrene tissue culture plates at different temperatures (37°C, 20°C) and incubation times (24, 48, and 72 h) for biofilm production. For this purpose, 2 *Staph. aureus* and the *Staph. epidermidis* reference strains were used as control to define different categories of the *Staph. aureus* isolates to be studied. Biofilm formation, expressed as biofilm production index (BPI), was compared with reference strains: *Staph. aureus* ATCC35556 (strong biofilm producer; Cramton et al., 1999; Seidl et al., 2008) as positive control (BPI_{PC}); *Staph. aureus* ATCC12600 (moderate biofilm producer; Di Ciccio et al., 2015) (BPI12600); *Staph. epidermidis* 12228 (negative biofilm producer; Atshan et al., 2012; Lee et al., 2014) as negative control (BPI_{NC}) for each isolate (**Table 1**). The cutoff point for biofilm production was the BPI value obtained by BPI_{NC} on polystyrene (0.294). *Staphylococcus aureus* strains showing the ability to produce biofilms were classified as weak ($BPI_{NC} \leq$ *Staph. aureus* BPI < BPI12600), moderate (BPI12600 ≤ *Staph. aureus* BPI < BPI_{PC}), or strong (*Staph. aureus* BPI ≥ BPI_{PC}). Before conducting the experiments, *Staph. aureus* strains were activated by culturing twice in 10 mL of TSB (Oxoid S.p.A., Milan, Italy) at 37°C for 24 h following a previously described method (Di Ciccio et al., 2015). Cultures of *Staph. aureus*, from overnight tryptic soy agar (Oxoid) growth, were prepared in TSB by incubating at 37°C. Cultures were then washed 3 times with PBS (pH 7.3, Sigma-Aldrich S.r.l., Milan, Italy) and diluted with fresh TSB to reach a concentration of about 10^8 cfu/mL, which was assessed by reading the optical density (OD) at 550 nm using a Varian SII Scan Cary 100 spectrophotometer (Agilent Technologies, Santa Clara, CA). Three milliliters of the standardized inoculum was then added to polystyrene tissue culture plates (961 mm2, 35 mm in diameter). Samples were then incubated at 37°C (for 24 h) and 20°C (for 48 or 72 h). After incubation, nonadherent cells were removed by dipping each sample 3 times in sterile PBS. Samples were fixed at

60°C for 1 h and stained with 3 mL of 2% crystal violet solution in 95% ethanol for 15 min. After staining, samples were washed with distilled water. Negative controls underwent the same treatment, without inoculation. The quantitative analysis of biofilm production was performed by adding 3 mL of 33% acetic acid to destain the samples. From each sample, 200 μL was transferred to a microtiter plate and the OD level of the crystal violet solution present in the destaining solution was measured at 492 nm (Victor, Perkin Elmer, Waltham, MA). All results were expressed by calculating the BPI as follows: BPI = [ODmean biofilm surface (mm2)−1] × 1,000. Biofilm formation, expressed as BPI, was compared with reference strains for each isolate. Finally, all isolates were assigned to different categories based on their BPI values.

Biofilm Formation Under Dynamic Conditions

Biofilm formation under flow conditions was evaluated on 24 dairy isolates and 4 reference strains using a BioFlux 1000 microfluidic system (Fluxion Biosciences Inc., San Francisco, CA) as previously described (Moormeier et al., 2013) with some modifications. This device enables accurate control of fluid flow and permits simultaneous growth of multiple biofilms (Benoit et al., 2010). To grow biofilms in the BioFlux device, 48-well plates (Fluxion Biosciences Inc.) were used. The microfluidic channels (70 × 370 μm) were primed for 2 min with 200 μL of TSB at 2.0 dyn/cm2 (where 1 dyn = 10−5 N). After priming, the TSB was carefully removed from the outlet wells and replaced with 20 μL of fresh overnight culture of Staph. aureus adjusted to reach an inoculum concentration of 108 cfu/mL. The channels were seeded by pumping from the outlet wells to the inlet wells at 2.0 dyn/cm^2 for 5 s. Cells were then allowed to attach to the surface of the channels for 1 h at 37°C. Excess inoculum was aspirated from the outlet wells, and 1.2 mL of TSB was added to the inlet wells and pumped at 0.6 dyn/cm−2 for 17 h. For each isolate tested, one bright-field image per channel was acquired at 5-fold magnification in 30-min intervals for a total of 35 time points (17

h). Images were always taken at the middle of the channel (channel numbers and arrows on the plate were used as landmarks) with a digital camera, and gain, exposure, and magnification were kept constant for all images. Every isolate was tested in biological triplicates with 2 channels for each replicate. Biofilm-forming ability was evaluated by classifying isolates into 3 main phenotypical categories: biofilm, bacterial accumulation /aggregates, and non-biofilm. The biofilm phenotype included isolates forming dense, stable aggregates of bacteria sticking to the surface of the channel over time. Bacteria forming small, diffuse aggregates or bacterial smear were classified in the bacterial accumulation/aggregates phenotype. Finally, the non-biofilm phenotype includes all isolates presenting adherent bacteria only, with no accumulation abilities or forming unstable aggregates. All phenotypic observations were made on the entire time-lapse movie. The observation of a stable biofilm structure at least once during the time course of the experiment automatically classified the isolate in the biofilm-forming category. **Figure 1** illustrates the different phenotypes, and **Figure 2** summarizes the classification method. The 6 replicates of each isolate were classified into 1 of the 3 phenotypic categories. For each isolate, the total number of replicates belonging to each phenotype was calculated. Based on this, a first distinction was made regarding the isolates' behavior in terms of biofilm formation under flow conditions. Isolates were subcategorized into 3 groups: (1) the consistent group, including isolates presenting the same phenotype for each replicate; (2) the dominant group, including isolates presenting a dominant phenotype observed more often than the others among the replicates, and (3) the inconsistent group, including isolates that randomly showed different phenotypes. The isolates belonging to the consistent and dominant groups were further classified according to their biofilm formation potential following the method described before. For the dominant group, the dominant phenotype was chosen. This classification method gives information about biofilm formation phenotype and its reproducibility.

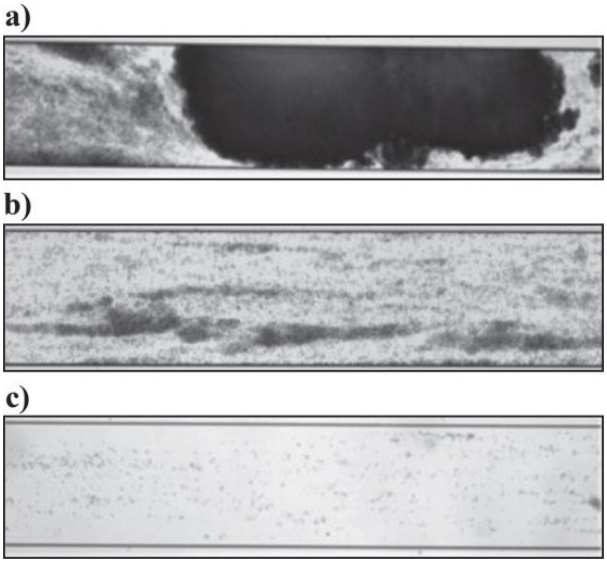

Figure 1. Different phenotypes obtained when growing Staphylococcus aureus under flow conditions (BioFlux, Fluxion Biosciences Inc., San Francisco, CA): (a) biofilm phenotype, (b) aggregates/accumulation phenotype, and (c) non-biofilm phenotype.

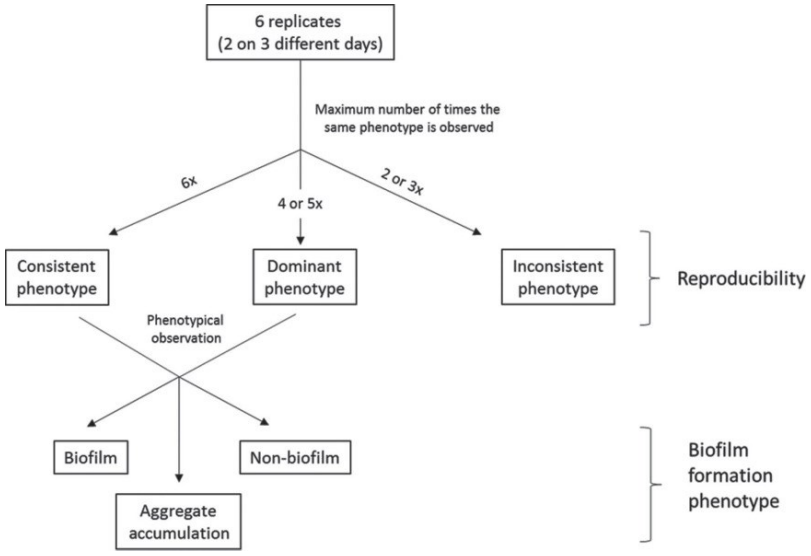

Figure 2. Classification method of the isolates analyzed under flow conditions.

Scanning Electron Microscopy of *Staph. aureus* Biofilm

Biofilm formation was further confirmed by scanning electron microscopy. Two isolates were selected: *Staph. aureus* 18 (GTB) and *Staph. aureus* 13 (genotype C, GTC), and biofilms were prepared as described above. The microbial cells were grown at 37°C for 24 h on polystyrene tissue plates and then washed by dipping 3 times in sterile PBS to remove nonadherent cells. Cells were fixed with 2.5% glutaraldehyde in 0.1 *M* sodium cacodylate buffer (pH 7.2) for 30 min at room temperature and then fixed in 1% osmium tetroxide (for 1 h). Samples were then washed with 0.1 *M* cacodylate buffer for 1 h to remove any unreacted glutaraldehyde before rinsing and dehydration. Samples were dehydrated through a series of alcohols and dried to critical point with liquid CO_2 (CPD 030 Baltec, Leica Microsystems GmbH, Wetzlar, Germany). Specimens were then sputter-coated with a gold-palladium layer using a SCD 040 coating device (Balzer Union, Liechtenstein). Samples were observed using a Zeiss DSM 950 scanning electron microscope at an accelerating voltage of 10 kV (Zeiss, Oberkochen, Germany). The images were processed for display using Photoshop (Adobe Systems Inc., San Jose, CA).

Statistics

All experiments were carried out in triplicate and repeated in 2 independent sets of experiments. Data are shown as mean ± standard deviation (SD), and IBM SPSS Statistics 23 (IBM Corp., Armonk, NY) was used for statistical analysis. The significance of differences in biofilm formation between GTB group and OGT group was assessed by one-way ANOVA, followed by Scheffé test. Differences were considered significant when $P < 0.05$.

RESULTS

Genotyping and *spa* Typing

The *Staph. aureus* dairy isolates of this study were genotyped and the results are summarized in Table 2. The RS-PCR analysis revealed 11 different genotypes with 3 genotypes carrying variants, and 18 *spa* types were detected, including a new one. Furthermore, 9 of the chosen isolates (7 strains from bovine milk, 1 strain from goat milk, 1 strain from sheep milk) carried the *mecA* gene and were thus categorized as MRSA (Table 2).

Table 2. *Staphylococcus aureus* dairy isolates (n = 24) used in this study and their corresponding genotype, *spa* type, and methicillin resistance status

Isolate no.	Origin (country)	Genotype	*Spa* type	MRSA (*mec*A PCR)
4	Cheese (Switzerland)	A	t2207	Negative
7	Cheese (Switzerland)	I^{I}	t524	Negative
8	Mastitis milk (Switzerland)	I	t524	Negative
12	Mastitis milk (Switzerland)	R	t267	Negative
13	Mastitis milk (Switzerland)	C	t529	Negative
15	Mastitis milk (Switzerland)	C	t529	Negative
18	Mastitis milk (Switzerland)	B	t024	Negative
19	Cheese (Switzerland)	B	t334	Negative
22	Cheese (Switzerland)	B	t2953	Negative
25	Mastitis milk (Switzerland)	B	t2953	Negative
27	Mastitis milk (Switzerland)	B	t5268	Negative
30	Cheese (Switzerland)	B	t2953	Negative
446	Bovine milk (Italy)	AN^{I}	t174	Positive
653	Bovine milk (Italy)	R^{VI}	t232	Negative
663	Mastitis milk (Italy)	F	t164	Negative
664	Bovine milk (Italy)	BS^{II}	t034	Negative
852	Bovine milk (Italy)	I	t688	Positive
916	Bovine milk (Italy)	AQ	New	Positive
1008	Bovine milk (Italy)	S	t524	Positive
1070	Bovine milk (Italy)	S	t899	Positive
1100	Bovine milk (Italy)	BN	t786	Positive
1140	Bovine milk (Italy)	AO	t1730	Positive
1234	Goat milk (Italy)	BS^{III}	t1255	Positive
1242	Sheep milk (Italy)	AN^{I}	t127	Positive

Biofilm Formation in the Static Model

Differences in biofilm formation were observed between the *Staph. aureus* isolates tested. **Figure 3** shows the ability of the 24 *Staph. aureus* dairy isolates and reference strains (ATCC35556, ATCC12600, ATCC12228, PR 281), to produce biofilms in polystyrene tissue culture plates. Results are summarized in **Table 3**.

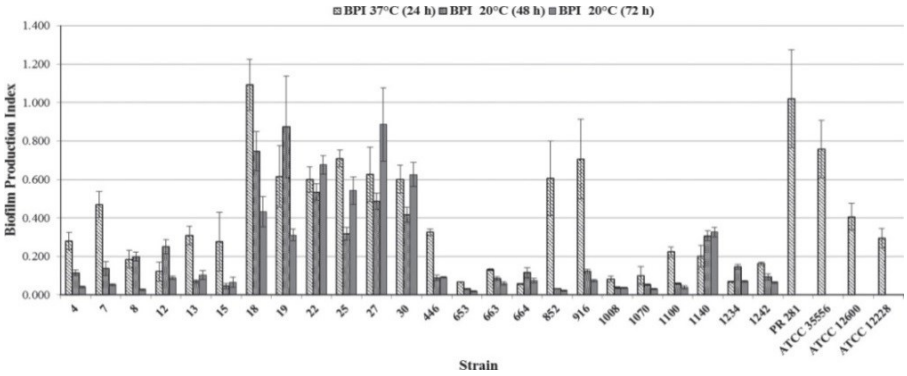

Figure 3. Biofilm formation of *Staphylococcus aureus* isolates at 37°C (24 h) and 20°C (48 and 72 h) on polystyrene. The results, expressed as biofilm production index (BPI), are means of 3 independent experiments. Error bars represent SD.

Table 3. Biofilm formation by *Staphylococcus aureus* dairy isolates (n = 24) on polystyrene (static conditions)

	Isolates	Biofilm producer		Weak producer		Moderate producer		Strong producer	
Genotype	**No.**	**No.**	**%**	**No.**	**%**	**No.**	**%**	**No.**	**%**
37°C, 24 h									
Genotype B	6	6	100	0	0	5	83.3	1	16.7
Other genotypes	18	5	27.8	2	40.0	3	60.0	0	0
Total	24	11	45.8	2	18.2	8	72.7	1	9.1
20°C, 48 h									
Genotype B	6	6	100	1	16.7	4	66.7	1	16.7
Other genotypes	18	1	5.5	1	100	0	0	0	0
Total	24	7	29.2	2	28.6	4	57.1	1	14.3
20°C, 72 h									
Genotype B	6	6	100	1	16.7	4	66.7	1	16.7
Other genotypes	18	1	5.5	1	100. 0	0	0	0	0
Total	24	7	29.2	2	28.6	4	57.1	1	14.3

At 37°C (24 h), out of 24 dairy isolates, 13 (54%) did not produce biofilm, whereas 11 (45.8%) were classified as weak (n = 2), moderate (n = 8), or strong (n = 1) biofilm producers, respectively. It was shown that none of the biofilm-negative strains were GTB. Among biofilm-positive strains, 6 (54.5%) GTB isolates had the ability to form moderate (n = 5) or strong (n = 1, isolate no. 18) biofilm, whereas 3 (25%) MRSA isolates had the ability to form moderate (n = 2) or weak (n = 1) biofilm.

At 20°C (48 h), out of 24 *Staph. aureus* dairy isolates, 17 (70.8%) strains did not produce biofilm, whereas 7 (29.2%) were classified as weak (n = 2), moderate (n = 4), or strong (n = 1) biofilm producers, respectively. It was shown that none of the biofilm-

negative (17) strains were GTB. Among the biofilm-positive strains, 6 (85.7%) GTB isolates had the ability to form weak (n = 1), moderate (n = 4), or strong (n = 1, isolate no. 19) biofilm, whereas 1 MRSA isolate (no. 1140) was a weak biofilm producer.

At 20°C (72 h), out of 24 *Staph. aureus* dairy isolates, 17 (70.8%) strains did not produce biofilm, whereas 7 (29.2%) strains were classified as weak (n = 2), moderate (n = 4), or strong (n = 1) biofilm producers. Only one MRSA strain (no. 1140) was classified as a weak biofilm producer. It was shown that none of the biofilm-negative strains (17) were GTB. Among the 7 biofilm-positive strains, 6 (85.7%) isolates belonging to GTB had the ability to form weak (n = 1), moderate (n = 4), or strong (n = 1, isolate no. 27) biofilm, respectively. Only 1 non-GTB isolate (no. 1140, MRSA) was biofilm positive (weak biofilm producer) at 20°C (48 to 72 h), although it was negative at 37°C (24 h). Interestingly, the dairy isolate *Staph. aureus* 18 (GTB strain) showed a higher BPI value (at 37°C on polystyrene) than the poultry isolate PR 281 (no GTB strain, *spa* type t002, non-MRSA) that was classified as a strong biofilm producer. Finally, none of the MRSA isolates (n = 9) was classified as a strong biofilm producer.

Correlations between GTB strains and biofilm formation were detected by statistical analysis. The GTB strains were statistically more able ($P < 0.05$) to form biofilm than OGT at 37°C and 20°C (at 48 and 72 h of incubation).

Scanning Electron Microscopy Analysis of *Staph. aureus* Biofilm

Two *Staph. aureus* genotypes were selected for scanning electron microscopy based on their different biofilm formation on polystyrene. In particular, *Staph. aureus* no. 18 (GTB) showed a BPI value higher than the strong biofilm producer, PR281 (Di Ciccio et al., 2015), whereas *Staph. aureus* no. 13 (GTC) showed a weak biofilm-producing ability. Representative micrographs of biofilms produced by 2 analyzed isolates are shown in **Figure 4**. In particular, one micrograph showed *Staph. aureus* no. 13 forming a rudimentary biofilm consisting of sparse aggregates of cells bound

by few or absent extracellular polymeric substances (**Figure 4 a,b**). In contrast, *Staph. aureus* no. 18 showed a complex 3-dimensional meshwork-like structure of cells at high density, embedded in a network of extracellular polymeric substances (**Figure 4 c,d**).

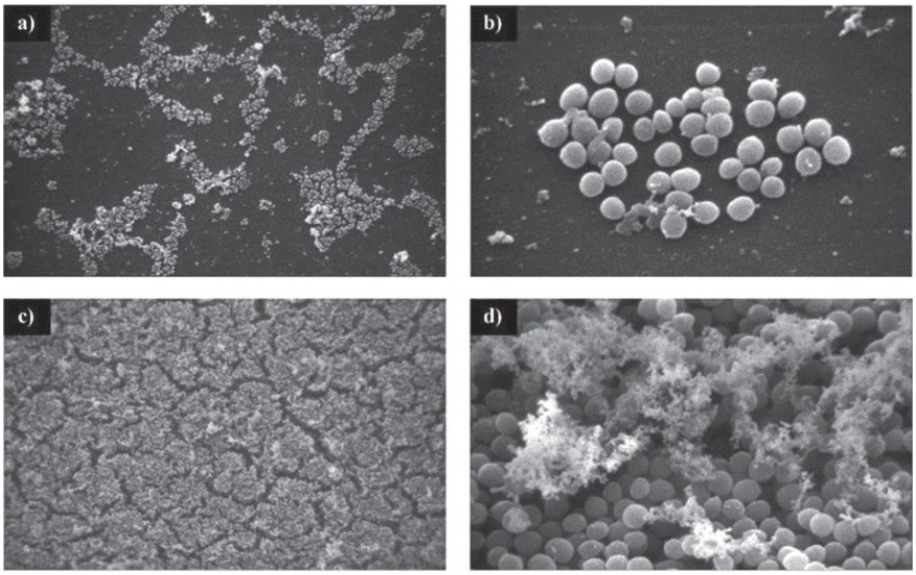

Figure 4. Scanning electron micrographs of biofilms formed by (a, b) weak biofilm producer *Staphylococcus aureus* isolate no. 13 (genotype C), and (c, d) strong biofilm producer *Staph. aureus* isolate no. 18 (genotype B). Magnification: 1,250× in panels a and c; 10,000× in panels b and d.

Biofilm Formation in the Dynamic Model

Isolates were first classified according to the reproducibility of their biofilm formation behavior under flow conditions using the BioFlux device. Out of the 28 tested isolates (24 dairy isolates, 1 poultry isolate, and 3 reference strains), 5 (17.9%) show a consistent phenotype: all 6 replicates of each isolate displayed the same phenotype. Sixteen (57.19%) of them displayed a dominant phenotype, with 4 or 5 replicates out of 6 presenting the same phenotype. Finally, for 7 (25%) of the isolates, including 2 MRSA, the phenotypes observed were not consistent from one replicate to another. Therefore, no biofilm phenotype was attributed to those isolates. The 21 (75%) isolates belonging to the consistent and

dominant groups were further characterized for their biofilm-forming potential. Of them, 14 isolates (66.7%), including 5 MRSA, presented biofilm structures, whereas only 3 (14.3%) showed the diffuse aggregate/accumulation phenotype and 3 (19.1%), including 1 MRSA, were unable to accumulate and remained as adherent bacteria (Table 4).

Table 4. Biofilm formation potential (no., with % in parentheses; isolates in brackets[1]) of *Staphylococcus aureus* isolates under dynamic conditions and its reproducibility

	Biofilm formation potential 37°C, 17 h, in flow cell		
Reproducibility	**Biofilm**	**Aggregate accumulation**	**Non-biofilm**
Consistency	4 (80); [15, PR 281, 446^{M}, 1070^{M}]	1 (20); [1100^{M}]	0 (0)
Dominancy	10 (62.5); [7, 8, 12, 18^{B}, 30^{B}, 653, 663, 916^{M}, 1234^{M}, 1242^{M}]	2 (12.5); [13, 664]	4 (25); [19^{B}, 25^{B}, 27^{B}, 1008^{M}]
Inconsistency	7 (25); [4, 22^{B}, 852^{M}, 1140^{M}, ATCC12600, ATCC29213, ATCC35556]		
Total, number (%)	14 (66.7)	3 (14.3)	4 (19.1)

[1] Superscript M = methicillin-resistant isolate (*mecA*-PCR+); superscript B = genotype B.

Out of the 6 GTB isolates, 3 were non-biofilm-formers (no. 19, 25, 27), 2 were biofilm formers (no. 18 and 30), and 1 could not be classified according to its biofilm-forming behavior because of inconsistency in reproducibility (no. 22).

Comparison of Biofilm Formation Between Static and Dynamic Conditions

The biofilm-forming potential of isolates was compared between static and dynamic conditions at 37°C (Table 5). Of 25 isolates, comparisons were performed for the 21 for which a phenotype could be attributed under dynamic conditions. Because the other 4 were unable to form reproducible structures under dynamic conditions, they were not included in the comparison.

Table 5. Comparison between biofilm formation under static and dynamic conditions in tryptic soy broth at 37°C[1]

Static conditions	Dynamic conditions	*S. aureus* isolates	Comparison static vs. dynamic conditions: no. (%)
-	-	1008[M]	Similar biofilm formation potential: 4 (19.1)
+	+	13	
++	++	18[B], PR 281	
-	+	664, 1100[M]	Stronger biofilm formation under dynamic conditions: 14 (66.7)
-	++	8, 12, 15, 653, 663, 1070[M], 1234[M], 1242[M]	
+	++	7, 30[B], 446[M], 916[M]	
+	-	19[B], 25[B], 27[B]	Stronger biofilm formation under static conditions: 3 (14.3)
-	/	4, 1140	No comparison possible: 4 (19.1)
+	/	22[B], 852	

[1]During static conditions, *Staph. aureus* biofilms were grown on polystyrene surfaces for 24 h; during dynamic conditions, they were grown in flow cells on glass surfaces for 17 h. ++ = strong biofilm formation, + = weak to moderate and bacterial aggregates/accumulation, − = no biofilm formation, / = inconsistent biofilm formation.

[2]Superscript M = methicillin-resistant isolate (*mecA*-PCR+); superscript B = genotype B.

Categorization under static conditions was based on 4 categories, whereas isolates were classified into 3 groups under dynamic conditions. We assumed that weak and moderate formation potential under static conditions could be compared with the aggregate phenotype observed under dynamic conditions. Four isolates (19.1%) displayed similar biofilm-forming potential under both static and dynamic conditions. One was a non-biofilm-former, 1 was a weak-to-moderate biofilm former, and 2 were strong biofilm formers. The majority of the isolates (66.7%) tended to form more biofilm under dynamic conditions, and 6 of the MRSA belong to these category. Finally, 3 isolates (14.3%) formed less biofilm under dynamic conditions compared with static conditions.

DISCUSSION

This is the first study on biofilm formation of *Staph. aureus* GTB compared with other genotypes of this species, including MRSA isolated from milk and milk products. *Staphylococcus aureus* isolated from bovine mastitis and cow milk is a genetically heterogeneous group (Cosandey et al., 2016). Among different genotypes, *Staph. aureus* GTB was found to be associated with high within-herd prevalence, indicating increased contagious and virulence potential compared with other genotypes (Graber et al., 2009; Voelk et al., 2014; van den Borne et al., 2017) and it has been described as a major contaminant in Swiss raw milk cheeses (Hummerjohann et al., 2014). Regarding these aspects, this study was carried out with the aim of evaluating the ability of *Staph. aureus* isolated from milk and milk products to form biofilm under static and dynamic conditions. Additionally, our aim was to test whether *Staph. aureus* GTB isolates were more likely to be biofilm producers than other *Staph. aureus* genotypes. The results of biofilm formation in the static model support this hypothesis, as GTB isolates produced significantly more biofilms than other genotypes under the several conditions tested. Furthermore, 1 GTB isolate was seen to be a better biofilm producer than GTC in the scanning electron microscopy analysis. Interestingly, the dairy isolate *Staph. aureus* 18 (GTB strain) showed a higher BPI value than the poultry isolate *Staph. aureus* PR 281 (not GTB), that was described as a strong biofilm producer by Di Ciccio et al. (2015). Further studies are needed to evaluate the contribution of biofilm formation to the persistence of *Staph. aureus* GTB in dairy, which has been observed for cheese-making facilities with duration of up to 27 wk (Hummerjohann et al., 2014).

Although the study presented here is one of the few reports on *Staph. aureus spa* types and biofilm formation in a dairy environment, it is generally believed that strong biofilm formation is linked to certain genetic lineages, as found by several clinical studies (Croes et al., 2009; Naicker et al., 2016). Application of

methods on the genomics, transcriptomics, and proteomics level of those different lineages could probably explain these observed phenotypes in the future.

Furthermore, because not all subtypes of *Staph. aureus* are distributed equally all over the world, application of subtyping is an important tool for local dairies. Veh et al. (2015) were able to characterize genotypic and phenotypic *Staph. aureus* causing persistent and nonpersistent subclinical bovine IMI in Canada. That study, where no GTB was detected, reported that t529 and t267 were the subtypes with the lowest biofilm production, which was confirmed by our study. This indicates the need for further regional studies on biofilm formation, including those genotypes that predominate in the milk production of certain specific regions.

Regarding the typing of our isolates, genotypes B, C, F, I, and R, are typically associated with bovine isolates and are the more frequently encountered genotypes when typing European *Staph. aureus* isolated from cow milk (Cosandey et al., 2016). In general, no correlation between genotype and *spa* type could be established except for *spa* type t529, which seemed to be associated with GTC, and t2953, which is the most frequently observed *spa* type of *Staph. aureus* GTB (Hummerjohann et al., 2014; Boss et al., 2016). In contrast, *spa* type t524 has been associated with 3 different genotypes. Several *spa* types described in this study (t524, t127, t267, t529, t204, t295) have previously been associated with bovine isolates in other countries (Hasman et al., 2010; Hwang et al., 2010; Sakwinska et al., 2011; Mitra et al., 2013; Boss et al., 2016).

Another aspect to note in our study was the presence of MRSA among our dairy isolates. Regarding their corresponding *spa* types, 2 isolates (t127 and t174) were of human origin (Grundmann et al., 2010; Lozano et al., 2011). Many reports have identified MRSA in bovine mastitis cases or in dairy products in several countries including Italy (Normanno et al., 2007; Kav et al., 2011; Kamal et al., 2013). In our study, 9 dairy isolates were classified as MRSA.

Among them, 55.5% form biofilms under dynamic conditions and 44.4% displayed weak to moderate biofilm-forming abilities under static conditions. Although the exact mechanisms and process of biofilm formation in MRSA are poorly understood, 2 studies performed by the same research group suggested that penicillin binding protein 2a (PBP2a) is also an important factor in biofilm accumulation (Pozzi et al., 2012; Rudkin et al., 2012). Other studies looked at dairy MRSA isolates and highlighted their high biofilm-forming potential. Bardiau et al. (2013) found that all MRSA isolated from bovine mastitis in Belgium were biofilm formers. Prenafeta et al. (2014) described a 50% prevalence of biofilm formation among MRSA isolated in bulk tank milk in Great Britain. It is well known that MRSA detected from milk and dairy products can be staphylococcal enterotoxinproducers (Normanno et al., 2007; Parisi et al., 2016). To date, the contribution of the contaminated environment to the spread of antimicrobial resistant microorganisms is not well understood. However, the biofilm-forming ability of MRSA that are potentially staphylococcal enterotoxin producers should be of concern for food safety, because they may colonize and spread in the dairy industry environment, leading to food contamination. Despite the importance of the *ica* gene locus in biofilm development, biofilms can occur in an *ica*-independent fashion. In a preliminary study, we discovered the presence of the *icaA* gene in some of the genomes of different genotypes. However, as the presence or absence of this gene is not correlated with a certain biofilm formation phenotype (H. U. Graber, unpublished data), further study on expression of the different genes is needed.

With regard to this, *ica*-independent biofilms appear to be the most important bacterial films produced by MRSA isolates (Vasudevan et al., 2003; Cucarella et al., 2004; O'Neill et al., 2007). The ability of dairy-isolated multidrug resistant bacteria to form biofilms in food processing facilities is of great concern for food safety. First, it contributes to the spread of antibiotic resistance along the food chain. Because biofilms confer an intrinsic

resistance to disinfection methods, they are very difficult to eliminate and contribute to bacterial persistence in food processing facilities. Moreover, the proximity of cells within biofilms favors horizontal gene transfer and risk for resistance transmission to pathogenic bacteria, leading to potential further treatment failure (Verraes et al., 2013).

When looking at the results of biofilm formation under dynamic conditions, the pronounced biofilm-forming potential of *Staph. aureus* GTB was not that obvious. Indeed, only half of the GTB isolates show biofilm structures and it was impossible to assign a biofilm formation potential for 1 isolate because of inconsistency in the reproducibility. However, the strong biofilm formation potential of *Staph. aureus* GTB isolates under static conditions reveals that this genotype possesses the genetic information necessary to form biofilms under certain conditions.

Only one strain (no. 1140, MRSA) was biofilm positive (weak biofilm producer) at 20°C (48 to 72 h), although it was negative for biofilm formation at 37°C (24 h). Rode et al. (2007), in contrast, noted the highest attachment capacity in *Staph. aureus* on polystyrene at suboptimal growth temperatures (20, 25, and 30°C).

Comparison of our data with reports from Pagedar et al. (2010), da Silva Meira et al. (2012), Lee at al. (2014) and Di Ciccio et al. (2015) on *Staph. aureus* dairy and other food isolates is rather limited because of the application of different methods of subtyping (if applied at all) and measurement or categorization of biofilm formation. The comparison between biofilm formation under static and dynamic conditions is even more complicated. In clinical isolates, only 19% of the isolates behaved similarly under both static and dynamic conditions (Vanhommerig et al., 2014), which is similar to our results (**Table 5**). Factors including incubation time, growth surface, and nutrients are thought to influence biofilm formation in staphylococci measured by static or dynamic model assays (Stepanović et al., 2001; Vanhommerig et al., 2014; Van Kerckhoven et al., 2016). Furthermore, we have

recently shown similar results on biofilm formation of *E. coli* dairy isolates, noting a lack of correlation between static and dynamic conditions (Marti et al., 2017). From our results, we cannot conclude that differences observed in terms of biofilm formation are due only to the growth under flow conditions because the growth surface was not the same (polystyrene for the static model; glass in the dynamic model), which can influence the adhesion process. Moreover, growth time was slightly different, with a longer incubation time under static conditions.

Finally, the evaluation process differed and could influence the conclusions. For the dynamic conditions, we had a time-lapse overview, whereas biofilm formation under static conditions was evaluated based on an end point state. Despite these differences, the fact that biofilm formation was observed in either condition indicates that the isolate possesses the genetic information necessary for biofilm formation. Further studies are needed to characterize the underlying mechanism of these phenotypes and to evaluate which of the different assays used for the measurement of biofilm formation best mimics the "real" situation in the dairy environment. Scanning electronic microscopy could be used as a semiquantitative technique, because it allows the observation of bacteria–surface interactions. Thus, in this study, scanning electron microscopy was used to confirm the biofilm formation of 2 differently categorized strains, and the images confirmed the results obtained from static biofilm assay, showing a strong difference in biofilm formation of 2 different genotypes.

CONCLUSIONS

All *Staph. aureus* GTB dairy isolates used in the present study showed significantly higher biofilm formation on polystyrene when exposed to different environmental conditions compared with most of the other genotypes, including MRSA. Under dynamic conditions, the ability of GTB strains to be good biofilm formers was not observed in all cases, emphasizing the importance of growth conditions for the expression of biofilm-related genes. In summary, the results of this study expand our knowledge of different *Staph. aureus* subtypes from the dairy field and show the benefit of genotyping when biofilm formation is studied.

ACKNOWLEDGMENTS

A. Ianieri, J. Hummerjohann, and P. A. Di Ciccio were members of the EU COST Action FA1202 "A European Network for Mitigating Bacterial Colonisation and Persistence on Foods and Food Processing Environments" (http://www.bacfoodnet.org/) and this action is acknowledged for a travel grant to P. A. Di Ciccio. We thank F. Ravanetti (Department of Veterinary Science, University of Parma, Italy) for her technical assistance. E. Thiran was supported by the Agroscope research programme REDYMO (Berne, Switzerland).

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CHAPTER 4

Molecular Characterization and Biofilm production in *Staphylococcus aureus* isolates from the Dairy production chain in Northern Italy

Filipello V[1], Di Ciccio PA[2,3], **Colagiorgi A**[2], Tilola M[1], Romanò A[4], Vitale N[1], Losio MN[1], Luini MV[4], Zanardi E[2], Ghidini S[2], Ianieri A[2], and Finazzi G[1]

[1] Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia Romagna – Headquarters. Brescia, Italy

[2] Food and Drug Department, University of Parma. Parma, Italy.

[3] Dipartimento di Scienze Veterinarie, University of Turin, Grugliasco, TO, Italy

[4] Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia Romagna – Lodi Chapter. Lodi, Italy.

International dairy journal (2018)

In press, accepted manuscript

doi: https://doi.org/10.1016/j.idairyj.2018.10.002

ABSTRACT

Staphylococcus aureus is able to produce enterotoxins causing staphylococcal food poisoning, and is frequently harboured by dairy products. Also, *S. aureus* is able to form biofilm in the production environment, enhancing the risk of food contamination. The ability of 49 *S. aureus* isolates from the dairy production chain to form biofilm was tested, and their genetic diversity in terms of population structure and presence of genes involved in biofilm formation or enterotoxins production was explored. The majority of the genotypes found were generally bovine associated; however, some have been also reported frequently in human clinical cases. Two isolates were methicillin-resistant. In total, 38.7% of the isolates were biofilm producers, and among them 47.3%, 42.1% and 10.5% exhibited weak, moderate, or strong biofilm-forming ability, respectively. In total 68% of the biofilm producing isolates were also positive for enterotoxins genes, raising concerns for consumer safety.

INTRODUCTION

Staphylococcus aureus (*S. aureus*) is a facultative anaerobic Gram-positive coccus that can be isolated from a wide range of food, animal, human, and medical environments (Lee, Bae, Lee, & Lee, 2015). *S. aureus* can produce staphylococcal enterotoxins (SEs) causing the most prevalent foodborne intoxication worldwide, namely, staphylococcal food poisoning (SFP) (Johler et al., 2015). In farms, *S. aureus* is the major mastitis pathogen, able to spread among cows through contact with contaminated equipment, and causing significant losses in the dairy industry (Fessler et al., 2010; Silva et al., 2013; Voelk et al., 2014).

Pulsed-field gel electrophoresis (PFGE) has long been regarded as the gold standard for *S. aureus* typing, however, the lack of an internationally shared nomenclature limits the use of this technique for epidemiology and population description (Chua, Howden, Jiang, Stinear, & Peleg, 2014). Multi-locus sequence typing (MLST), which involves sequencing of seven "housekeeping" genes present in all strains of *S. aureus*, and comparison with an online database to identify a sequence type (ST), is at present the most widely accepted typing method (Chua et al., 2014). A less laborious and costly sequence-based method is *spa* typing, based on the sequence variability in the polymorphic repeat region of the *S. aureus* protein A (*spa*) gene (Shopsin et al., 1999). Generally, *spa* typing is more discriminatory than MLST and the *spa* types broadly correspond to MLST based clonal complexes (Cookson et al., 2007). MLST and *spa* typing allow easy comparison of strains between laboratories through online databases, which can also be used to submit isolates sequences to designate new types (Enright, Day, Davies, Peacock, & Spratt, 2000; Harmsen et al., 2003). RS-PCR is another typing technique based on the PCR amplification of the ribosomal spacer (RS) (Graber et al., 2009). This technique has proved useful in discriminating *S. aureus* strains with different virulence potential (Cosandey et al., 2016).

Methicillin-resistant *S. aureus* (MRSA) harbor antibiotic-resistance genes, and are recognized through the detection of *mecA*, or its homologue *mecC*, a gene responsible for resistance to methicillin, (García-Álvarez et al., 2011). MRSA were historically related to the nosocomial environment, however, in the recent years they have been isolated from various foods of animal origin, including dairy products, and in bovine mastitis cases, raising alarms about possible dissemination throughout the food production chain (Kamal, Bayoumi, & Abd El Aal, 2013; Normanno et al., 2007). MRSA can cause severe infections to humans, and therefore, have been more thoroughly investigated, compared to methicillin-susceptible *S. aureus* (MSSA) (Chua et al., 2014). However, MSSA can still pose a risk, due to the production of SEs. Dairy products can be a vehicle of transmission of enterotoxigenic *S. aureus* strains, and are frequently involved in SFP outbreaks (EFSA, 2015; Hennekinne, De Buyser, & Dragacci, 2012).

Many SFPs are caused by food-handlers acting as carriers during food processing (Rola, Czubkowska, Korpysa- Dzirba, & Osek, 2016), but also the presence of mastitis in the herd or characteristics of the milking environment can be sources of contamination relevant to the dairy production chain (Kümmel et al., 2016). Indeed, *S. aureus* is a highly adaptable bacterium, which has the ability to form biofilms, microbial communities formed by cells that are strongly attached to a surface while embedded in a matrix of extracellular polymeric substances that they have produced, and exhibit an altered phenotype with respect to growth rate and gene transcription" (Donlan & Costerton, 2002). In this respect**,** *S. aureus* biofilms can be frequently found in food processing plants, and biofilm formation can protect the microorganism from sanitation procedures and environmental stresses (Abdallah, Benoliel, Drider, Dhulster, & Chihib, 2014).

Specifically related to the dairy industry, raw milk has a natural microbiota that can perform initial attachment to surfaces favoring the anchoring of contaminant bacteria to the biofilm, and raising concerns for food safety (Marchand et al., 2012). Many

studies have investigated the ability of MRSA from the dairy sector to form biofilm. However, most studies are focused on isolates collected from the herd, and not from the final products, which are more relevant in a food safety perspective. Moreover, since also MSSA can pose a risk for the consumer due to the production of SEs, it is important to investigate their ability to form biofilm and become contaminants (Bardiau et al., 2013; Prenafeta, Sitja, Holmes, & Paterson, 2014). Thus, the present study was carried out to i) explore the genetic diversity of *S. aureus* isolated from dairy products in Northern Italy through MLST, *spa* typing and RS-PCR to provide comparable results and hypothesis on the source of contamination, ii) detect the presence of SEs genes iii) detect relevant genes involved in biofilm formation, iv) determine the ability of isolates to produce biofilm. Finally, we attempted to evaluate the potential relationship among specific molecular types and biofilm phenotype of dairy isolates. Overall, no specific correlation between genotype and biofilm production was found. However, clones both from the animal and human reservoir have been found throughout the production chain, indicating multiple sources of contamination.

MATERIALS AND METHODS

Samples selection and *S. aureus* isolation and identification

S. aureus isolates were collected from products tested during routine surveillance carried out by the Regional Laboratory for Animal Heath and Food Safety (IZSLER), or through ad hoc sampling carried out by the Department of Food and Drug of the University of Parma. The samples were collected between 2014 and 2016 in 33 plants located in Northern Italy (Lombardy and Emilia Romagna Regions), of which were small scale artisanal dairies located in pastures, and five were industrial dairies. For *S. aureus* isolation, serial dilution of each sample homogenate were plated on Baird Parker agar + rabbit plasma fibrinogen (BP-RPF) (Biolife Italiana, Milano, Italy) and incubated at 37° C for 48 h. Up to 5 characteristic colonies for each sample were plated on blood agar to confirm *S. aureus* hemolytic property. A total of 49 representative isolates (n=13 from raw milk, n=5 from curd, n=25 from cheese, and n=6 from food contact surfaces), were selected for further characterization (**Table 1**). The species identification was confirmed with PCR of the *nuc* gene as described by Brakstad et al. (Brakstad, Aasbakk, & Maeland, 1992). DNA was obtained by boiling a suspension of one isolated colony from blood agar in 100 µL of demineralized water for 5 min at 99° C. The suspension was then centrifuged at 13,000 x *g* for 5 min and supernatant was used for all following PCR assays. The *S. aureus* isolates and their boiled extracts were then stored at -80° C.

Table 1. List of the *S. aureus* isolates analysed indicating the source and plant of production. [a]

ID	Source	Plant	ID	Source	Plant	ID	Source	Plant
A	Raw milk	1	B3	Cheese	a1	B19	Fresh cheese	a15
B	Vat	1	B4	Cheese	a1	B20	Fresh cheese	a16
C	Vat	1	B5	Curd	4	B21	Raw milk	a17
D	Drying plank	1	B6	Cheese	a2	B22	Fresh cheese	a18
E	Drying plank	1	B7	Cheese	a3	B23	Fresh cheese	a19
F	Drying plank	1	B8	Raw milk	a4	B24	Fresh cheese	a20
G	Drying plank	1	B9	Curd	a5	B25	Raw milk	a21
H	Cheese	1	B10	Fresh cheese	a6	B26	Fresh cheese	a22
I	Cheese	1	B11	Curd	a7	B27	Raw milk	a23
J	Cheese	1	B12	Fresh cheese	a8	B28	Raw milk	a24
K	Cheese	1	B13	Fresh cheese	a9	B29	Fresh cheese	a25
L	Cheese	1	B14	Raw milk	a10	B30	Curd	a26
M	Cheese	1	B15	Fresh cheese	a11	B31	Curd	a27
N	Cheese	1	B16	Raw milk	a12	B32	Raw milk	5
O	Cheese	1	B17	Raw milk	a13	B33	Fresh cheese	a10
B1	Raw milk	2	B18	Fresh cheese	a14	B34	Raw milk	a28
B2	Raw milk	3						

[a] Plants preceded by the "a" letter correspond to alpine artisanal dairies.

Molecular characterization

Multilocus Sequence Typing (MLST) and *spa* typing. All isolates were typed with MLST as described by Enright et al. (Enright et al., 2000); the Sequence Types (STs) were determined with the database available on the *Staphylococcus aureus* MLST website (https://pubmlst.org/saureus/) sited at the University of Oxford (Jolley & Maiden, 2010). For *spa* typing, the *spa* gene was amplified by PCR as described by Shopsin and colleagues (Shopsin 103 et al., 1999), and *spa* types were determined with the Ridom StaphType software (Ridom GmbH, Würzburg, Germany). All DNA sequences were obtained with a 3500xL Genetic Analyzer (Applied Biosystems, Foster City, CA, USA).

Novel MLST and *spa* sequences were submitted to the respective database for the designation of the new profile. MLST data were

analyzed with the goeBURST algorithm using the Phyloviz software (Francisco et al., 2012).

Detection of *mecA* and *mecC*. The detection of *mecA* and *mecC* (*mecA* homologue) was carried out by means of two PCR protocols using specific primers as reported by Pichon et al. (Pichon et al., 2012). Briefly, for both *mecA* and *mecC* the PCR reaction mix (final volume 20 μL) contained 1X HotStarTaq Master Mix (Qiagen INC, Hilden, Germany), 0.5 μM of each primer, and 1 μL boiled extract. The thermic profile was 95 °C for 15 min, followed by 35 cycles of 94 °C for 30 s, 58 °C for 40 s, and 72 °C for 1 min. The final elongation step was performed at 72 °C for 10 min.

Detection of staphylococcal enterotoxins. Two multiplex PCR protocols were used as described in Bianchi et al. (Bianchi et al., 2014) to detect *sea, seb, sec, sed, see, seg, seh, sei, sej, sep,* and *ser* SEs genes.

Detection of genes involved in biofilm production. Genotyping of the *S. aureus* accessory gene regulator (*agr*) was conducted by multiplex PCR amplification of the hypervariable domain of the *agr* locus using a single forward primer and 4 reverse primers specific for each of the 4 major specificity groups (*agr* I to IV), according to Shopsin and colleagues (Shopsin et al., 2003). PCR assays were conducted on genes encoding for intercellular adhesion (*icaA* and *icaD*), the collagen bindin protein (*cna*), the clumping factor A (*clfA*), the fibronectin binding proteins A and B (*fnbA* and *fnbB*), the α and β hemolysins (*hla* and *hlb*), and the regulator protein (*sarA*). Primers used in this work were synthesized by Integrated DNA Technologies (IDT, Coralville, Iowa, U.S.A.)(**Table 2**). Gene amplification was performed as described by Graber and others (Graber et al., 2009), with some modifications. Briefly, the PCR reaction mix (total volume of 25 μL) for amplification of the *icaA*, *icaD*, *cna*, *fnbA*, *fnbB*, *clfA*, 127 *hla*, *hlb*, and *sarA* genes contained 1X Maxima Hot Start PCR Master Mix (Thermo Fisher Scientific, Waltham, Mass., U.S.A.), 1 μM of each

primer, and 5 μL of boiled extract. For the *agr* genotyping, multiplex PCR assays were conducted using 0.3 μM of each primer. The PCR profile for *agr* genotyping was 95 °C for 5 min, followed by 35 cycles comprising 95 °C for 40 s, 50 °C for 40 s, and 72 °C for 40 s. The final elongation step was performed at 72 °C for 10 min. Then *icaA*, *icaD*, *cna*, *fnbA*, *hla*, and *sarA* were amplified using the following PCR cycle: 95 °C for 5 min, followed by 40 cycles comprising a denaturation step at 95 °C for 40 s, followed by the annealing step at 50 °C for 40 s and extension at 72 °C for 40 s. The final elongation step was performed at 72 °C for 10 min. The PCR conditions for *clfA*, *fnbB*, and *hlb* amplification were 95 °C for 5 min followed by 35 cycles of 95 °C for 35 s, 50 °C for 35 s, and 72 °C for 1 min. The final elongation step was performed at 72 °C for 10 min. Negative and positive controls were included in every run for all the different PCRs. Nucleic acid of *S. epidermidis* ATCC 12228 DNA was used for the negative control, and *S. aureus* ATTC 35556 DNA (Gene Bank; NCBI Reference Sequence: NC_007795.1) as the positive control. All PCR reactions for the detection of virulence genes were performed using a Techne TC- 412 thermal cycler (Bibby Scientific Limited, Staffordshire, U.K.). PCR assays were visualized by agarose electrophoresis (1% agarose gel in Tris-acetate-EDTA buffer) and GelRed (Biotium, Hayward, Calif., U.S.A.) staining.

Table 2. Oligonucleotide primers for the biofilm related genes analyzed in this study

Target gene	Forward primer (5'-3')	Reverse primer (5'-3')	Amplicon size (bp)	References
agrI	ATGCACATGGTG CACATGC	GTCACAAGTACTA TAAGCTGCGAT	441	(Shopsin et al., 2003)
agrII	ATGCACATGGTG CACATGC	TATTACTAATTGAA AAGTGGCCATAGC	575	(Shopsin et al., 2003)
agrIII	ATGCACATGGTG CACATGC	GTAATGTAATAGC TTGTATAATAATAC CCAG	323	(Shopsin et al., 2003)
agrIV	ATGCACATGGTG CACATGC	CGATAATGCCGTA ATACCCG	659	(Shopsin et al., 2003)

icaA	ACACTTGCTGGC GCAGTCAA	TCTGGAACCAACA TCCAACA	188	(Kouidhi, et al, 2010)
icaD	ATGGTCAAGCCC AGACAGAG	AGTATTTTCAATGT TTAAAGCAA	198	(Kouidhi, et al, 2010)
cna	AAAGCGTTGCCT AGTGGAGA	AGTGCCTTCCCAA ACCTTTT	192	(Montanaro, et al. 1999)
fnbA	GATACAAACCCA GGTGGTGG	TGTGCTTGACCAT GCTCTTC	191	(Arciola,et al, 2005)
fnbB	GGAGAAGGAATT AAGGCG	GCCGTCGCCTTGA GCGT	811	(Booth.et al, 2001)
clfA	CCGGATCCGTAG CTGCAGATGCAC C	GCTCTAGATCACT CATCAGGTTGTTC AGG	1000	(McDevitt, et al., 1995)
hla	CTGGCCTTCAGC CTTTAAGG	CTGTAGCGAAGTC TGGTGAAA	455	(Ando, et al., 2004)
hlb	GCCAAAGCCGAA TCTAAG	CGCATATACATCC CATGGC	845	(Ando, et al., 2004)
sarA	TTAGCTTTGAAGA ATTCGCTGT	TTCAATTTCGTTGT TTGCTTC	275	(Padmapriya, et al., 2003)

Biofilm formation assay. For the evaluation of biofilm production three strains from a culture collection (ATCC3556, ATCC12600, ATCC12228; American Type Culture Collection) were used as reference. All dairy isolates (n=49) and the reference strains were tested in triplicate on 6 wells Nunclon™ Delta Surface polystyrene tissue culture plates (Thermo Fisher Scientific, Waltham, MA, USA) at 37°C for biofilm production. Biofilm formation was evaluated following a previously described method (Di Ciccio et al., 2015). Before each experiment, *S. aureus* isolates from the frozen stocks (-80°C) were grown overnight at 37°C in 5 mL tryptic soy broth (TSB - Oxoid, S.p.A., Milan, Italy) and then plated on tryptic soy agar (TSA - Oxoid, S.p.A., Milan, Italy). Briefly, fresh cultures from TSA plates were suspended in TSB and incubated at 37°C overnight. Cultures were then washed three times with PBS (pH 7.3, Sigma-Aldrich S.r.l., Milan, Italy) and diluted to desired ino 152 culum concentration (108 cfu/mL) with fresh TSB based on the optical density (OD) at 550 nm (Varian SII Scan Cary 100 spectrophotometer - Agilent Technologies, Santa Clara, CA). Three

milliliters of the standardized inoculum was then added to polystyrene tissue culture plates (961 mm2155 , ⌀ 35 mm). Samples were then incubated at 37°C for 24 h. After incubation, non-adherent cells were removed by dipping each sample 3 times in sterile PBS. Samples were fixed at 60°C for 1 h and stained with 3 mL of 2% crystal violet solution in 95% ethanol for 15 min. After staining, samples were washed with distilled water. Negative controls underwent the same treatment, without inoculation. The quantitative analysis of biofilm production was performed by adding 3 mL of 33% acetic acid to de-stain the samples. From each sample, 200 μL was transferred to a microtiter plate and the OD level of the crystal violet solution present in the de-staining solution was measured at 492 nm (Victor, Perkin Elmer, Waltham, MA). All results were expressed by calculating the biofilm production index (BPI) as follows: BPI=[$OD_{meanbiofilm}$/surface (mm^2)] × 1,000. Finally, all isolates were assigned to different categories based on their BPI values. For this purpose, the BPI of the two *S. aureus* and the *S. epidermidis* reference strains were used as control. Briefly, BPI was compared with reference strains: *S. aureus* ATCC35556 (BPI_{PC}, strong biofilm producer; (Seidl et al., 2008) as positive control; *S. aureus* ATCC12600 (BPI12600, moderate biofilm producer; (Di Ciccio et al., 2015); *S. epidermidis* 12228 (BPI_{NC}, negative biofilm producer; (Lee et al., 2015) as negative control (**Table 3**). The cutoff point for biofilm production was the BPI value obtained by BPI_{NC} on polystyrene (0.294). *S. aureus* strains showing the ability to produce biofilms were classified as weak (BPI_{NC} ≤ *S. aureus* BPI < BPI12600), moderate (BPI12600 ≤ *S. aureus* BPI < BPI_{PC}), or strong (*S. aureus* BPI ≥ BPI_{PC}).

Table 3 - Biofilm formation, expressed as BPIs, by *S. aureus* and *S. epidermidis* reference strains on polystyrene (961 mm^2) at 37 °C. [a]

Reference strain	OD[1]	BPI
Staphylococcus aureus ATCC 35556 (positive control, strong biofilm producer)	0.756 ± 0.15	0.758
Staph. aureus ATCC 12600 (moderate biofilm producer)	0.450 ± 0.07	0.405
Staphylococcus epidermidis ATCC 12228 (negative control)	0.343 ± 0.05	0.294
Staph. aureus PR 281 (poultry isolate, very strong biofilm producer)	0.979 ± 0.255	1.09

[a] *S. aureus* ATCC 35556 and *S. epidermidis* ATCC 12228 were positive and negative controls, respectively. Values for $OD_{mean\ biofilm}$ polystyrene are expressed as OD mean ± standard deviation.

Statistical analysis

For each *S. aureus* isolate the relative frequencies (%) of genes involved in biofilm production, in antibiotic resistance and genes related to SEs were calculated, and the Clopper-Pearson method was used to compute 95% confidence interval. Correlation between genes involved in biofilm production, in antibiotic-resistance and genes related to SEs was calculate by Phi index. The association between *S. aureus* isolates and genes involved in biofilm production, in antibiotic-resistance and genes related to SEs was evaluated by a chi-square test ($\chi 2$) or Fisher exact test where appropriate. Statistically significant 177 associations were assessed by logistic regression model and estimated OR and 95% Wald's confidence interval (CI) were obtained as measures of predictor effect. The likelihood ratio test was used to assess the overall significance of the model (two-tailed significance level $p \leq 0.05$). All analyses were performed using R software (R Development Core Team).

RESULTS AND DISCUSSION

S. aureus is a pathogen that causes several serious diseases in both humans and animals worldwide. In dairy processing plants, *S. aureus* contamination could be due to various sources: raw milk, processing environment, and handlers (André et al., 2008; Arcuri et al., 2010). *S. aureus* is a major concern for the food processing industry due to its virulence factors and ability to form biofilm (Langsrud, 2009; Xing et al., 2016). Previous studies have investigated the biofilm formation of *S. aureus* isolated from clinical origin or food sector (de Souza et al., 2014; Rode, Langsrud, Holck, & Møretrø, 2007). However, data on the genetic diversity and on the biofilm forming capacity of *S. aureus* isolates collected throughout the dairy production chain are limited. In this study we examined the molecular diversity and the presence an array of virulence genes (i.e. enterotoxins, adhesins, and gene regulators) of 49 isolates. We also investigated whether the biofilm-forming capacity is affected by the presence of these genes or it is correlated with different *S. aureus* types.

Molecular typing

The MLST identified 14 different Sequence Types (STs) among the isolates tested (**Table 4**). We identified two novel STs, namely ST4104 and ST4162. The most commonly observed ST was ST8 (n=17, 35%), followed by ST71 and ST97 (n=8, 16%, and n=6, 12%, respectively). The BURST algorithm assigned the STs into ten groups, including one Clonal Complex (CC) with ST97 as founder (composed by 18 isolates; 37%), and 9 singletons (**Figure 1**). The *spa* typing identified 22 *spa* types, including 3 novel *spa* types, namely t16951, t16952 and t17143. The most commonly observed *spa* types were t2953, corresponding to ST8, and t254, corresponding to ST71 (9 isolates each, 18%; **Figure 1**).

Table 4. Results of the molecular typing and biofilm production assay for the 49 S. *aureus* isolates analysed in this study. [a]

ID	ST	CC	*spa* type	GT	BPI	Regulators		Adhesins						*Haem*	*MRSA*	*Enterotoxins*									
						*agr**	*sarA*	*icaA*	*icaD*	*cna*	*fnbA*	*fnbB*	*clfA*	*hla*	*mecA*	*sea*	*seb*	*sec*	*sed*	*see*	*ser*	*seg*	*seh*	*sei*	*sej*	*sep*
B2	1	1	t127	BJ	+	III	+	+	+	+	+	-	+	+	-	-	-	-	-	-	-	-	+	+	-	-
B4	1	1	t127	BJ	++	III	+	+	+	+	+	-	+	+	+	-	-	-	-	-	-	-	+	-	-	-
B26	1	1	t127	ND	+	III	+	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	+	-	-	-
E	8	8	t2953	B	-	I	+	+	+	+	+	+	+	-	-	-	-	-	+	-	+	-	-	-	+	-
F	8	8	t2953	B	-	I	+	+	+	+	+	+	+	+	-	+	-	-	-	-	+	-	-	-	+	-
H	8	8	t2953	B	-	I	+	+	+	+	+	+	+	-	-	-	-	-	+	-	+	-	-	-	-	-
B6	8	8	t2953	B	++	I	+	+	+	+	+	+	+	+	-	+	-	-	+	-	+	-	-	-	+	-
B7	8	8	t2935	B	+	I	+	+	+	+	+	+	+	+	-	+	-	-	+	-	+	-	-	-	+	-
B14	8	8	t2953	B	++	I	+	+	+	+	+	+	+	+	-	+	-	-	+	-	+	-	-	-	+	-
B16	8	8	t2953	B	-	I	+	+	+	-	+	+	+	+	-	+	-	-	+	-	+	-	-	-	+	-
B25	8	8	t2935	B	+	II	+	+	+	+	+	+	+	+	-	-	-	-	-	-	+	-	-	-	-	-
B33	8	8	t2953	B	-	I	+	+	+	+	+	+	+	-	-	+	-	-	+	-	+	-	-	-	+	-
B34	8	8	t2953	B	-	I	+	+	+	+	+	+	+	-	-	+	-	-	+	-	+	-	-	-	+	-
B17	8	8	t13265	B	+	I	+	+	+	+	+	+	+	+	-	-	-	-	+	-	+	-	-	-	+	-
B8	8	8	t024	B	+	I	+	+	+	+	+	+	+	+	-	+	-	-	-	-	-	-	-	-	-	-
B	8	8	t164	B	-	I	+	+	+	+	+	+	+	-	-	-	-	-	+	-	+	-	-	-	+	-
O	8	8	t3802	B	-	I	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-
B18	8	8	t3802	AA	+	I	+	+	+	+	+	+	+	+	-	+	-	-	+	-	+	-	-	-	+	-
B21	8	8	t16951	B_III	-	I	+	+	+	+	+	+	+	+	-	-	-	-	+	-	+	-	-	-	+	+
B24	8	8	t5429	AA	-	I	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-
B32	12	12	t16526	BN	-	IV	+	+	+	+	+	+	+	+	-	-	-	-	-	+	-	-	-	-	-	-
D	71	97	t524	BN	-	I	-	+	+	+	+	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-
G	71	97	t524	BN	-	I	-	-	-	+	+	+	+	-	-	-	-	-	+	-	-	-	-	-	-	-
I	71	97	t524	BN	-	I	-	-	-	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-
L	71	97	t524	I_I	-	I	-	-	-	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-
B9	71	97	t524	BN	++	I	+	-	-	+	+	-	+	+	-	-	-	-	+	-	-	-	-	-	-	-
B28	71	97	t524	BN	+	I	-	-	-	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-
B29	71	97	t524	BN	-	I	+	-	-	+	+	+	+	+	-	-	-	-	+	-	-	-	-	-	-	-
B30	71	97	t524	BN	-	II	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-
B15	4104	97	t524	BN	-	I	+	-	-	+	+	-	+	+	-	-	-	-	+	-	-	-	-	-	-	-
J	352	97	t521	AO	-	I	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-
B13	97	97	t521	R	-	I	+	+	+	+	+	-	+	+	-	-	-	-	+	-	-	-	-	-	-	-
K	97	97	t13277	R_I	-	I	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-
M	97	97	t13277	R_I	++	I	+	+	+	+	+	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-
B1	97	97	t3782	ND	-	I	-	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-
B22	97	97	t15786	R_IV	-	I	+	+	+	+	+	+	+	+	-	-	-	-	+	-	-	-	-	-	-	-
B27	97	97	t17143	R_I	-	I	-	+	+	+	+	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-
B12	3078	97	t359	R_VI	+	I	+	+	+	+	+	-	+	+	-	-	-	-	+	-	-	-	-	-	-	-
B20	3078	97	t359	R_VI	++	I	+	+	+	+	+	-	+	+	-	-	-	-	+	-	-	-	-	-	-	-
B11	4162	4162	t2420	R_I	-	I	+	+	+	+	+	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-
B10	130	130	t1773	ND	+++	III	+	+	+	+	+	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-

ID	ST	CC	*spa* type	GT	BPI	Regulators	Adhesins						*Haem*	*MRSA*	*Enterotoxins*										
						agr[a]	*sarA*	*icaA*	*icaD*	*cna*	*fnbA*	*fnbB*	*clfA*	*hla*	*mecA*	*sea*	*seb*	*sec*	*sed*	*see*	*ser*	*seg*	*seh*	*sei*	*sej*	*sep*
B10	130	130	t1773	ND	+++	III	+	+	+	+	+	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-
B5	188	188	t16952	S	++	I	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-
A	389	389	t164	F	-	I	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	+	-	-	-
C	389	389	t164	F_I	-	I	+	+	+	-	+	+	+	-	-	-	-	-	-	-	-	+	-	+	-	-
N	389	389	t164	F	-	I	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-
B3	504	504	t529	C	-	II	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-
B23	504	504	t529	C	-	II	+	+	+	+	+	+	-	+	-	-	-	-	+	-	-	+	-	+	-	-
B19	522	522	t5428	AA	++	I	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-
B31	522	522	t5428	AA	+++	III	-	+	+	+	+	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-

[a] Abbreviations are: ST, sequence type as defined by Multilocus Sequence Typing (MLST); CC, clonal complex as defined by MLST; GT, genotype as defined by RS-PCR; BPI, biofilm production index; Haem, haemolysin. Regulator gene *agr* is *agr* type. All strains tested typed positive for adhesin *fnba* and haemolysin *hlb* and typed negative for MRSA *mecC*, and enterotoxins *seb* and *sec*.

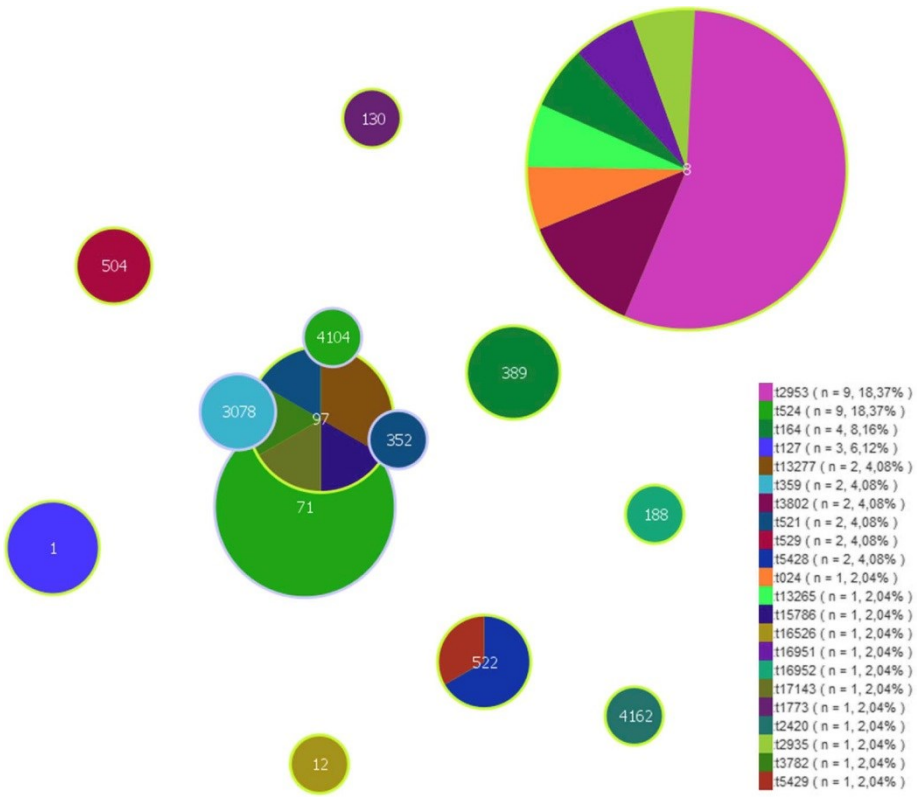

Figure 1. Minimum spanning tree of the 49 *S. aureus* isolates analysed with MLST. The minimum spanning tree was constructed by the goeBURST algorithm using the Phyloviz software v1.1 (http://www.phyloviz.net/). Each circle represents a single sequence type (ST). Yellow outlines indicate group founders, while grey outlines are single locus variants (n-1 loci in common). The colours of the circle filling correspond to the different *spa* types, and the size of the circles is proportional to the number of isolates belonging to the same ST.

The majority of the STs found, are previously reported to be bovine associated (https://pubmlst.org/saureus/, Feltrin et al., 2015). However, even if generally specific lineages are adapted to particular mammalian hosts, some lineages have a broad host range (i.e. ST1) and host shifts have 202 also been reported (i.e. CC97, ST8) (Budd et al., 2015; Feltrin et al., 2015; Lozano et al., 2011; Resch et al., 2013). As an example, CC97 is currently regarded as one of the major *S. aureus* CCs in bovines, and it has

been reported among pig and dairy cattle holdings also in Italy (Feltrin et al., 2015). Nevertheless, CC97 has been lately reported as the cause of human pandemics, demonstrating the ability to spread to the human host (Lozano et al., 2011; Spoor et al., 2013). Within CC97, it has been reported that ST71 evolved into a distinct subgroup, which may more efficiently infect the bovine udder due to the resistance acquired against the antibiotics most commonly used for mastitis treatment (Budd et al., 2015). The CC97 subgroup represented by ST71, and its double-locus variant (DLV) ST3078, correspond to genotype R (GTR) and its variants as defined by RS-PCR (**Table 4**). In a previous study, these genotypes were observed in several European countries with the exception of Italy (Cosandey et al., 2016).

The other major cluster of isolates was represented by ST8. Among ST8 isolates, 15 out of 17 (88%) were GTB or GTB variants (**Table 4**). GTB, which has been reported to be more virulent than the other GTs, is the most widespread bovine associated *S. aureus* type in Switzerland and central Europe countries (Cosandey et al., 2016). It is believed MSSA CC8 strains have adapted themselves to the bovine host following a human-to-cow shift, and the possibility for these strains of acquiring methicillin resistance is of concern for both the human and animal health (Resch et al., 2013).

Among the 49 isolates analyzed, two were MRSA (*mecA*+; **Table 4**), and both belonged to the ST1 cluster (2/3, 67%). CC1 MRSA are present worldwide and adapted to both the animal and the human host (Feltrin et al., 2015; Giuffrè et al., 2012).

Detection of enterotoxins

At least one SEs gene was found in 63% of the isolates (n=31) and 14 different SEs genes profiles, which were overall homogenous within the different STs, were distinguished (**Table 4; Table 5**). None of the isolates was positive for *seb* and *sec* genes, while *sed* gene, found in 22 (47%) of the isolates, was the most frequent, followed by *ser* (n=14; 29%) and *sej* (n=12; 24%) genes. In particular, almost all ST8-GTB isolates contained the SE gene pattern *sed*, *sej*,

ser, which are carried on the same plasmid (Bianchi e 227 t al., 2014), with about half of them (9/17) additionally carrying *sea,* as also reported in other studies (Hummerjohann, Naskova, Baumgartner, & Graber, 2014). Notably, it has been reported that CC97 (or GTR and its variants) isolates were characterized by the lack of SEs genes (Budd et al., 2015; Cosandey et al., 2016), while in our sample 50% of CC97 isolates (9/18), and 63% of GTR or variants isolates (5/8) were positive for *sed* (Table 4). *seh*, which has been responsible for milk-based SFP outbreaks (Bianchi et al., 2014), was detected in all MRSA ST1-t127 isolates (and in the ST1-t127 MSSA in the same cluster), consistently with the finding of Hummerjohann *et al*. (2014).

Table 5. Enterotoxin gene profiles.[a]

Number of isolates	***Enterotoxin gene***								
	sea	***sed***	***see***	***ser***	***seg***	***seh***	***sei***	***sej***	***sep***
1						+	+		
2						+			
3		+		+				+	
1	+			+				+	
1		+		+					
7	+	+		+				+	
1				+					
1	+								
1		+		+				+	+
1			+						
9		+							
1							+		
1					+		+		
1		+			+		+		

[a] No isolates tested were positive for enterotoxin genes *seb* or *sec*.

SEA and SED are the SEs most frequently associated with SFP (Hummerjohann et al., 2014; Sabike, Fujikawa, Sakha, & Edris, 2014). Improper handling and storage of raw milk and cheese in the early stages of processing contaminated with *S. aureus* can result in the production of SEs, which is also dependent on the initial dose of *S. aureus* contamination (Sabike et al., 2014). In our samples the contamination of raw milk averaged 10^2 cfu g^{-1}, while the average contamination of aged cheese was 10^4 cfu g^{-1} (data not shown), indicating that in the early phases of cheese-making the *S. aureus* contamination could have reached a concentration critical for the production of SEs (10^5 cfu g^{-1} ; Hummerjohann et al., 2014). Indeed, one of the samples (corresponding to B6 isolate) was referred to our laboratory for the suspect involvement in a SFP episode, and found positive for SEA (data not shown).

Biofilm formation

In food industries, biofilm is an important source of microbial contamination which can lead to spoilage and transmission of foodborne pathogens. In the present work, we studied biofilm forming ability of 49 dairy isolates by using polystyrene microtiter plates as this material is commonly used in food processing industry (Paz-Méndez et al., 2017). The biofilm phenotype was evaluated at 37° C which is the optimum growth temperature for *S. aureus* (Vázquez-Sánchez, Habimana, & Holck, 2013). Our results indicated that the isolates had different capability to form biofilm on the tested hydrophobic surface. Similar observations were also reported by a previous investigation of *S. aureus* isolated from food sector (Di Ciccio et al., 2015). **Figure 2** shows the ability of the 49 dairy isolates and reference strains (ATCC35556, 252 ATCC12600, ATCC12228), to produce biofilms in polystyrene tissue culture plates at 37° C for 24 h. Out of 49 dairy isolates, 30/49 (61.2%) did not produce biofilm, whereas 19/49 (38.7%) were biofilm producers. Among biofilm producers isolates, 9/19 (47.3%), 8/19 (42.1%) and 2/19, (10.5%) exhibited weak, moderate, or strong biofilm-forming ability, respectively. The highest amount of

biofilm was formed by two strains (B10 BPI=2.192; B31 BPI=2.538) isolated from cheese and curd, respectively, which were able to show a BPI greater than positive control (ATCC 35556) (**Figure 2**; **Table 3**). Remarkably, 5/19 (26.3%) and 8/19 (42.1 %) isolates classified as moderate and weak biofilm producer respectively, also harbored SEs genes (**Table 4**). The biofilm-forming ability of isolates that are also potential enterotoxins producers should be of concern for food safety, since they may colonize and spread in dairy producing plants and cause contamination of the dairy products (Vergara et al., 2017). The two MRSA ST1-t127-*seh* positive isolates were classified as moderate or weak biofilm producers.

Previous studies showed a correlation between GTB and biofilm formation, with 55% of the biofilm forming isolates belonging to GTB (Thiran et al., 2018). In this work, 40% of the ST8-GTB isolates were biofilm producers, and represent 30% of all biofilm producing isolates (**Table 4**). Given the wide prevalence of this genotype in the dairy production chain, further studies aimed at investigating the mechanism of biofilm formation specific to ST8-GTB *S. aureus* may be useful to better control the pathogen at herd and plant level.

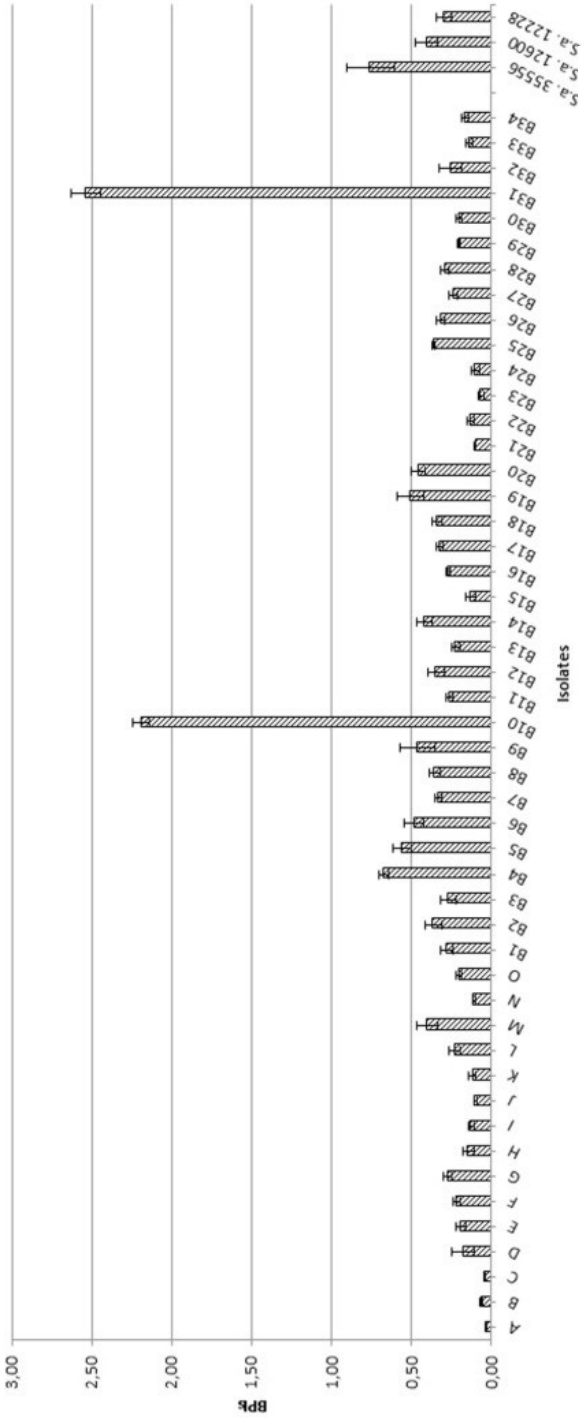

Figure 2. Biofilm production indices of the 49 *S. aureus* isolates and of the three reference strains.

Genes involved in biofilm production

All isolates were investigated for the presence of nine biofilm associated genes, encoding for adhesins (*icaA, icaD, cna, fnbA, fnbB, and clfA)*, toxins (*hla*, *hlb*), and regulators (*sarA*). Furthermore, the isolates were genotyped by the *agr* locus. Almost all *S. aureus* isolates were positive for at least five of the biofilm related genes investigated in this study (**Tables 4** and **6**). The most prevalent genes were *fnbA* gene and *hlb* gene which were detected in all of the isolates. The *icaA* gene was always associated with *icaD* gene, and they were found in 42 (85.7%) isolates. The prevalence rates of *cna*, *clfA sarA*, *hla*, and *fnbB* were 95.9%, 93.9%, 83.7%, 79.2%, and 73.6%, respectively. No correlation was found between the presence of the genes and biofilm production, as also reported in other studies (Tang, Chen, Li, Zeng, & Li, 2013).

Table 6. Patterns of the biofilm related genes and their associations with biofilm production and *agr* type.

Biofilm related genes patterns	**N. of isolates**	**Biofilm producing**	**Biofilm negative**	***agr* type**			
				I	**II**	**III**	**IV**
*sar*A-*ica*A-*ica*D-*cna*-*fnb*A-*fnb*B-*clf*A-*hla*-*hlb*	21	9	12	16	3	1	1
*sar*A-*ica*A-*ica*D-*cna*-*fnb*A-*fnb*B-*clf*A-*hlb*	5	0	5	5	-	-	-
*sar*A-*ica*A-*ica*D-*cna*-*fnb*A-*fnb*B-*hla*-*hlb*	1	0	1	-	1	-	-
*sar*A-*ica*A-*ica*D-*fnb*A-*fnb*B-*clf*A-*hla*-*hlb*	1	0	1	1	-	-	-
*sar*A-*ica*A-*ica*D-*fnb*A-*fnb*B-*clf*A-*hlb*	1	0	1	1	-	-	-
*sar*A-*ica*A-*ica*D-*cna*-*fnb*A-*clf*A-*hlb*	1	1	0	-	-	1	-
*sar*A-*ica*A-*ica*D-*cna*-*fnb*A-*fnb*B-*hlb*	1	1	0	1	-	-	-
*sar*A-*cna*-*fnb*A-*clf*A-*hla*-*hlb*	2	1	1	2	-	-	-
*sar*A-*cna*-*fnb*A-*fnb*B-*clf*A-*hla*-*hlb*	1	0	1	1	-	-	-
*sar*A-*ica*A-*ica*D-*cna*-*fnb*A-*clf*A-*hla*-*hlb*	7	5	2	5	-	2	-
*ica*A-*ica*D-*cna*-*fnb*A-*clf*A-*hla*-*hlb*	1	1	0	-	-	1	-
*ica*A-*ica*D-*cna*-*fnb*A-*clf*A-*hlb*	1	0	1	1	-	-	-

*ica*A-*ica*D-*cna*-*fnb*A-*fnb*B-*clf*A-*hla*-*hlb*	1	0	1	1	-	-
*ica*A-*ica*D-*cna*-*fnb*A-*hlb*	1	0	1	1	-	-
cna-*fnb*A-*fnb*B-*clf*A-*hla*-*hlb*	3	1	2	3	-	-
cna-*fnb*A-*fnb*B-*clf*A-*hlb*	1	0	1	1	-	-
Total	49	19	30	39	4	5

Agr type I was detected in most of the isolates (39/49; 79.6%), followed by *agr*III (5/277 49; 10.2%), *agr*II (4/49; 8.2%), and *agr*IV (1/49; 2%). Among the biofilm-negative isolates (n=30), 25 (83.3%) carried *agr*I, 3 (10%) carried *agr*II and one was *agr*IV. As for the biofilm producing isolates (n=19), the majority (13, 68.4%) carried *agr*I, 5 (26.3%) carried *agr*III and 1 carried *agr*II. Interestingly, all *agr*III isolates, which were positive for *ica*A, *ica*D, *cna*, *fnb*A, *clf*A, and *hlb* simultaneously, were able to form biofilm, and among them there were the isolates with the highest biofilm production (B10 BPI=2.192; B31 BPI=2.538, **Figure 2**). These findings suggest an association between this *agr* group and biofilm forming ability, which was also confirmed by the statistical analysis (OR 22.2; CI95% 2.21-2281). Similar results were reported in other studies (Fabres-Klein, Caizer Santos, Contelli Klein, Nunes de Souza, & de Oliveira Barros Ribon, 2015; Khoramrooz et al., 2016). However, these findings are in contrast with other authors that reported *agr*II and *agr*I as involved in higher biofilm production than other *agr* types in *S. aureus* isolated from bovine mastitis (Marjorie Bardiau, Detilleux, Farnir, Mainil, & Ote, 2014; Cafiso et al., 2007). The *agr* quorum sensing system controls the expression of virulence factors and is also involved in biofilm regulation at structuring and dispersal stages (Boles & Horswill, 2008). It has been observed that repression of *agr* is necessary for biofilm formation, while its activation is essential for the detachment of biofilm (Tan, Li, Jiang, Hu, & Li, 2018). The discrepancies among studies may be related to the lower prevalence of *agr* III in *S. aureus* isolated from bovine mastitis compared to *agr* I and II (Gilot & van Leeuwen, 2004; Melchior et al., 2009), for this reason it may be useful to extend the number of studied isolates to elucidate the results found to date.

CONCLUSIONS

We analyzed the clonal diversity of 49 *S. aureus* isolates from the dairy production chain in Northern Italy and determined their virulence gene profile and biofilm formation ability. A diversity of STs was observed among the *S. aureus* isolates, with STs common to multiple dairy facilities identified. The majority of the STs-*spa* types- GTs identified have been reported to be livestock-associated (Cosandey et al., 2016; Feltrin et al., 2015), however, it is not possible to exclude events of contamination originating from humans, due to the finding of genotypes common in the human population (e.g. ST1). Further, should be 301 of concern the capability of some strains, and in particular MRSA, to infect different hosts and become a possible public health threat. No correlation between presence of genes involved in biofilm formation and biofilm production was found. Based on statistical analysis, the *agr* type III seems to be related biofilm production at the conditions tested in this study. Extending the analysis to a bigger set of isolates could help to substantiate these findings. The biofilm-forming ability of dairy isolates that encode SEs genes should be of concern, since they may colonize and spread in dairy-producing plants leading to food contamination and causing food safety issues. Raw milk is commonly used for the production of many traditional cheeses because of the characteristic organoleptic features of the final product. As a consequence, the hygienic condition of the milk used for cheese-making is crucial for the final product quality and safety. Based on our results, the major source of *S. aureus* contamination seems to be the presence of infected animals in the herd. In this perspective, it would be crucial to implement at the farm level actions aimed at minimizing the risk of animal infection. However, attention should also be paid to the hygiene of environment, in particular to avoid the persistence of the bacterium in biofilms in cheese manufacturing environments.

ACKNOWLEDGMENTS

We would like to acknowledge Dr. M.L. Pacciarini (Genomics Department of the Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia Romagna) for her kind availability and for granting the access to the sequencing facilities of her laboratory. A. Ianieri and P.A. Di Ciccio were members of the EU COST Action FA1202 "A European Network for Mitigating Bacterial Colonisation and Persistence on Foods and Food Processing Environments" (http://www.bacfoodnet.org/) and are grateful to this Action for facilitating the collaborative networking that contributed to this study.

Conflict of interest: none.

FUNDING

This work was supported by the Italian Ministry of Health (grant number E88F15000120001).

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CHAPTER 5

Biofilm formation and its Relationship with the Molecular characteristics of food-related Methicillin-Resistant *Staphylococcus aureus* (MRSA)

Vergara A[1], Normanno G[2], Di Ciccio PA[3], Pedonese F[4], Nuvoloni R[4], Parisi A[5], Santagada G[5], **Colagiorgi A**[3], Zanardi E[3], Ghidini S[3], Ianieri A[3]

[1] Faculty of Veterinary Medicine, University of Teramo, Piano D'Accio, Teramo, Italy.

[2] Dept. of Science of Agriculture, Food and the Environment (SAFE), University of Foggia, via Napoli, 25 - 71121, Foggia, Italy.

[3] Food and Drug Department, University of Parma, via del Taglio, 10 - 43126, Parma, Italy.

[4] Department of Veterinary Sciences, University of Pisa, Viale delle Piagge 2 – 56124, Pisa, Italy.

[5] Experimental Zooprophylactic Institute of Apulia and Basilicata, via Manfredonia, 20 -71121, Foggia, Italy.

Journal of Food Science, **2017**. 82(10): 2364-2370

ABSTRACT

Abstract

The capability to produce biofilm is an important persistence and dissemination mechanism of some foodborne bacteria. This paper investigates the relationship between some molecular characteristics (*SCCmec*, ST, *spa*-type, *agr*-Type, *cna*, *sar*A, *ica*A, *ica*D, *clf*A, *fnb*A, *fnb*B, *hla*, *hlb*) of 22 food-related methicillin-resistant *Staphylococcus aureus* (MRSA) strains and their ability to form biofilm on stainless steel and polystyrene. Five (22.7%, 5/22) strains were able to synthesize biofilm on polystyrene, and one of these (4.5%, 1/22) strains was also able to synthesize biofilm on stainless steel. The largest amount of biofilm was formed on polystyrene by 2 MRSA strains isolated from cows' milk, thus raising concern about the dairy industry. The majority of MRSA biofilm producers carried SCC*mec* type IVa, suggesting that the presence of SCC*mec*IVa and/or *agr* type III could be related to the ability to form biofilm. In conclusion, in order to achieve an acceptable level of food safety, Good Hygiene Practices should be strictly implemented along the food chain to reduce the risk of colonization and dissemination of MRSA biofilm-producing strains in the food industry.

Practical Application

In this study, some assayed isolates of food-related MRSA demonstrated the capacity to form biofilm. Biofilm formation differed according to surface characteristics and MRSA strains. A relationship was observed between some molecular characteristics and the ability to form biofilms. Few studies have investigated the ability of MRSA to form biofilms, and the majority of these studies have investigated clinical aspects. This work was performed to investigate whether or not there is a difference between MRSA food isolates and MRSA clinical isolates in their ability to form biofilm. These initial findings could provide information that will contribute to a better understanding of these aspects.

INTRODUCTION

Methicillin-resistant *Staphylococcus aureus* (MRSA) is the most commonly identified antimicrobial-resistant pathogen in many parts of the world (Taylor 2013). While it has long been recognized as a hospital-related infection (Virgin and others 2009), MRSA epidemiology has changed in recent years with the emergence of community-acquired MRSA (Jones and others 2002). At present, new evidence suggests that domestic animals, including food animals, are capable of acting as reservoirs and MRSA shedders, and that transmission may be possible between host species (de Boer and others 2009). The emergence of MRSA in food-producing animals has caused great concern for the presence of MRSA in animal-derived foodstuffs; MRSA has been isolated from various foods of animal origin, giving cause for concern about possible dissemination throughout the food production chain (Crago and others 2012; Hiroi and others 2012; Caballero Gómez and others 2013; Normanno and others 2015; Parisi and others 2016).

Both methicillin-sensitive *S. aureus* (MSSA) and MRSA can form biofilms on various surfaces (Rode and others 2007; Scott and others 2008; Mirani and others 2013; Di Ciccio and others 2015). Biofilm is defined as a community of organisms encased in a protective and adhesive matrix that is a prevalent mode of growth for microorganisms (Mandlik and others 2008). Furthermore, staphylococcal biofilms allow MRSA strains to adhere to surfaces, including surgical implants and materials used in the food industry; Scott and others (2008) reported MRSA found on various household surfaces such as sponges/cloths, dish drainers, and work surfaces.

Biofilm formation in *S. aureus* is mediated by the intercellular adhesion operon (*ica*) formed by the genes *ica*A, *ica*B, *ica*C, and *ica*D and a regulator gene, *ica*R, which encodes the ICAA, ICAB, ICAC, and ICAD proteins (Gad and others 2009), but *agr*-locus and other genes have also been implicated in biofilm formation by *S. aureus* (Tsang and others 2008). Although the exact

mechanisms and process of biofilm formation in MRSA are poorly understood, 2 studies performed by the same research group suggested that PBP2a is also an important factor in biofilm accumulation (Pozzi and others 2012; Rudkin and others 2012).

The contribution of the contaminated environment to the spread of antimicrobial-resistant microorganisms is not well understood; to date, few studies have investigated the ability of MRSA to form biofilms, and the majority of these studies have investigated clinical aspects (Kwon and others 2008; Atshan and others 2013; Cha and others 2013). Here we studied the biofilm-forming ability of food-related MRSA strains and the relationship between some molecular characteristics of MRSA and their ability to form biofilm.

MATERIALS AND METHODS

Bacterial strains

The experiment was conducted on 22 MRSA strains isolated from milk and meat (n = 9 strains from cows' milk and n = 13 strains from pork). The strains were identified by phenotyping methods. Stock cultures were stored at −80 °C, and the strains were incubated for 24 h at 37 °C in Tryptone Soy Broth (TSB; Oxoid S.p.A., Milan, Italy) before testing.

MRSA characterization

All isolates were confirmed as MRSA by the detection of *mec*A and *nuc* genes, and were characterized by SCC*mec* typing, *spa*-typing, and multilocus sequence typing (MLST).

DNA extraction

The Genomic Prep DNA isolation Kit (Amersham Pharmacia Biotech, N.Y., New York, U.S.A.) was used to extract bacterial DNA from 1 mL of each culture broth, following the manufacturer's instructions. The extracts were tested for the detection of methicillin resistance *mec*A, thermostable nuclease *nuc* genes, and for some virulence factors, as reported below.

Detection of nuc and mecA genes

The DNA extracts were subjected to a duplex-PCR protocol for the detection of methicillin resistance *mec*A and thermostable nuclease *nuc* genes (Virgin and others 2009). A methicillin-susceptible *S. aureus* strain (ATCC 29213) was used as a negative control and an MRSA strain (ATCC 33591) as a positive control.

SCC-mec typing

Staphylococcal cassette chromosome *mec* element (SCC-*mec*) typing was carried out as described by Zhang and others (2005).

Spa-typing

The x region of the *spa* gene was amplified by PCR using primers reported in **Table 1**. DNA sequences were obtained using an ABI

3130 Genetic Analyzer (Applied Biosystems, Foster City, Calif., U.S.A.) and BigDye 3.1 Ready reaction mix (Applied Biosystems) according to the manufacturer's instructions. BioNumerics 7.1 (Applied Maths, Bruxelles, Belgium) software was used to determine *spa* types.

Table 1. Oligonucleotide primers used in this study

Target gene	Forward primer (5'-3')	Reverse primer (5'-3')	Amplicon size (bp)	References
nuc	GCGATTGATGGT GATACGGTT	AGCCAAGCCTTGAC GAACTAAAGC	270	Brakstad and others (1992)
mecA	AACAGGTGAATTA TTAGCACTTGTAA G	ATTGCTGTTAATAT TTTTTGAGTTGAA	174	Martineau and others (2000)
agrI	ATGCACATGGTGC ACATGC	GTCACAAGTACTAT AAGCTGCGAT	441	Shopsin and others (2003)
agrII	ATGCACATGGTGC ACATGC	TATTACTAATTGAA AAGTGGCCATAGC	575	Shopsin and others (2003)
agrIII	ATGCACATGGTGC ACATGC	GTAATGTAATAGCT TGTATAATAATACC CAG	323	Shopsin and others (2003)
agrIV	ATGCACATGGTGC ACATGC	CGATAATGCCGTAA TACCCG	659	Shopsin and others (2003)
icaA	ACACTTGCTGGCG CAGTCAA	TCTGGAACCAACAT CCAACA	188	Kouidhi and others (2010)
icaD	ATGGTCAAGCCCA GACAGAG	AGTATTTTCAATGT TTAAAGCAA	198	Kouidhi and others (2010)
cna	AAAGCGTTGCCTA GTGGAGA	AGTGCCTTCCCAAA CCTTTT	192	Montanaro and others (1999)
fnbA	GATACAAACCCAG GTGGTGG	TGTGCTTGACCATG CTCTTC	191	Arciola and others (2005)
fnbB	GGAGAAGGAATT AAGGCG	GCCGTCGCCTTGA GCGT	811	Booth and others (2001)
clfA	CCGGATCCGTAG CTGCAGATGCACC	GCTCTAGATCACTC ATCAGGTTGTTCAG G	1000	McDevitt and others (1995)
hla	CTGGCCTTCAGCC TTTAAGG	CTGTAGCGAAGTCT GGTGAAA	455	Ando and others (2004)
hlb	GCCAAAGCCGAA TCTAAG	CGCATATACATCCC ATGGC	845	Ando and others (2004)
sarA	TTAGCTTTGAAGA ATTCGCTGT	TTCAATTTCGTTGT TTGCTTC	275	Padmapriya and others (2003)
spa	TAAAGACGATCCT TCGGTGAGC	CAGCAGTAGTGCC GTTTGCTT		Strommenger and others (2006)

MLST

Alleles at the 7 loci, *arc*, *aro*E, *glp*F, *gmk*, *pta*, *tpi*, and *yqi*L, were assigned by comparing the sequences at each locus with those of the known alleles in the *S. aureus* MLST database. The allele numbers at each of the 7 loci define the allelic profile of each isolate, and an allelic profile is defined as a sequence type (ST). The eBURST program was used to determine the group of each ST based on the MLST database. Grouping was carried out using an analysis panel that selects 6 minimum numbers of identical loci out of 7 loci for group definition and 3 minimum single locus variant contents for subgroup definition (Kwon and others 2005).

Detection of virulence factors

The 22 food-related MRSA strains and 3 reference strains (*S. aureus* ATCC 35556, *S. aureus* ATCC 12600, *S. epidermidis* 12228) were tested for the detection of some virulence determinants. Genotyping of the *S. aureus* accessory gene regulator (*agr)* was conducted by multiplex PCR amplification of the hypervariable domain of the *agr* locus using a single forward primer and 4 reverse primers specific for each of the 4 major specificity groups (*agr I to IV*), according to Shopsin and others (2003). PCR assays were conducted on genes encoding for intercellular adhesion (*icaA* and *icaD*), the encoding collagen binding protein (*cna*), the encoding clumping factor A (*clfA*), the encoding fibronectin binding proteins A and B (*fnbA* and *fnbB*), the α and β hemolysins (*hla* and *hlb*), and the regulator protein (*sarA*). Primers used in this work were synthesized by Integrated DNA Technologies (IDT, Coralville, Iowa, U.S.A.), and are reported in Table 1. Gene amplification was performed as described by Graber and others (2009), with some modifications. Briefly, the PCR reaction mix (total volume of 25 μL) for amplification of the *icaA*, *icaD*, *cna*, *fnbA*, *fnbB*, *clfA*, *hla*, *hlb*, and *sarA* genes contained 1X Maxima Hot Start PCR Master Mix (Thermo Fisher Scientific, Waltham, Mass., U.S.A.), 1 μM of each primer, and 5 μL of the lysate nucleic acids. For the *agr* genotyping, multiplex PCR assays were conducted using 0.3 μM of each primer. The PCR profile for *agr* genotyping

was 95°C for 5 min, followed by 35 cycles comprising 95 °C for 40 s, 50 °C for 40 s, and 72 °C for 40 s. The final elongation step was performed at 72 °C for 10 min. Then *icaA*, *icaD, cna, fnbA, hla*, and *sarA* were amplified using the following PCR cycle: 95 °C for 5 min, followed by 40 cycles comprising a denaturation step at 95 °C for 40 s, followed by the annealing step at 50 °C for 40 s and extension at 72 °C for 40 s. The final elongation step was performed at 72 °C for 10 min. The PCR conditions for *clfA*, *fnbB*, and *hlb*amplification were 95 °C for 5 min followed by 35 cycles of 95 °C for 35 s, 50 °C for 35 s, and 72 °C for 1 min. The final elongation step was performed at 72 °C for 10 min. Negative and positive controls were included in every run for all the different PCRs. Nucleic acid of *S. epidermidis* ATCC 12228 DNA was used for the negative control, and *S. aureus* ATTC 35556 DNA (Gene Bank; NCBI Reference Sequence: NC_007795.1) as the positive control. All PCR reactions for the detection of virulence genes were performed using a Techne TC-412 thermal cycler (Bibby Scientific Limited, Staffordshire, U.K.). PCR assays were visualized by agarose electrophoresis (1% agarose gel in Tris-acetate-EDTA buffer) and GelRed (Biotium, Hayward, Calif., U.S.A.) staining.

Biofilm production assay

The 22 food-related MRSA strains were tested for biofilm production. For this purpose, the 2 *S. aureus* and the *S. epidermidis* reference strains (*S. aureus* ATCC 35556, *S. aureus* ATCC 12600, *S. epidermidis* 12228) were used as controls to classify the MRSA studied into different categories. Biofilm formation, expressed as Biofilm Production Index (BPI), was compared with reference strains: *S. aureus* ATCC 35556—strong biofilm producer (Cramton and others 1999; Seidl and others 2008) as positive control (BPI_{PC}); *S. aureus* ATCC 12600—moderate biofilm producer (Di Ciccio and others 2015) (BPI_{12600}); *S. epidermidis* 12228—negative biofilm producer (Atshan and others 2012; Lee and others 2014) as negative control (BPI_{NC}) for each isolate (**Table 2**). The cutoff point for biofilm production was the BPI value obtained by negative control on polystyrene (BPI_{NC} = 0.294) and stainless

steel (BPI_{NC} = 0.149). MRSA biofilm-producing strains were classified as weak ($BPI_{NC} \leq$ MRSA BPI < BPI_{12600}), moderate ($BPI_{12600} \leq$ MRSA BPI < BPI_{PC}), or strong (MRSA BPI ≥ BPI_{PC}). Before the experiments, all MRSA strains were activated by culturing twice in 10 mL TSB (Oxoid S.p.A.) at 37 °C for 24 h. A previously described method was used (Di Ciccio and others 2015). Polystyrene tissue culture plates (961 mm^2) and AISI 304 stainless steel chips (530 mm^2) were used for biofilm formation assays at 37 °C. Stainless steel chips were degreased before use by overnight immersion in ethanol, then rinsed thoroughly in distilled water and autoclaved for 15 min at 121 °C. *S. aureus* cultures were grown overnight on Tryptone Soy Agar (TSA, Oxoid, S.p.A.), and then incubated in TSB at the selected temperature of 37 °C. Cultures were then washed 3 times with phosphate-buffered saline (PBS; pH 7.3) (Sigma-Aldrich S.r.l., Milan, Italy) and diluted with fresh TSB to reach a concentration of about 10^8 CFU/mL by reading the optical density (OD) level at 550 nm (UV Mini-1240—Shimadsu, New York, U.S.A.). We then added 3 mL of the standardized *inocula* to polystyrene tissue culture plates (35 mm dia) and stainless steel chips. Samples were then incubated at 37 °C. After 24 h incubation, nonadherent cells were removed by dipping each sample 3 times in sterile PBS. Samples were fixed at 60 °C for 1 h and stained with 3 mL of 2% crystal violet solution in 95% ethanol for 15 min. After staining, samples were washed 3 times with distilled water. Negative controls underwent the same treatment, without inoculation. Quantitative analysis of biofilm production was performed by adding 3 ml of 33% acetic acid to destain the samples. Then 200 μL of each sample was transferred to a microtiter plate and the OD level of the crystal violet present in the destaining solution was measured at 492 nm (Varian SII Scan Cary 100, New York, U.S.A.). Considering the different growth areas of the tested surfaces (polystyrene = 961 mm^2 and stainless steel = 530 mm^2), results were normalized by calculating the BPI as follows: $BPI = [OD_{mean\ biofilm}\ \text{surface}\ (mm^2)^{-1}] \times 1000$. Two independent sets of all experiments were performed in triplicate. Biofilm formation, expressed as BPI, was compared with reference

strains for each isolate. Finally, all isolates were classified into different categories on the basis of their BPI values.

Table 2. Biofilm formation, expressed as BPIs, by *S. aureus* and *S. epidermidis* ATCC on polystyrene and stainless steel a 37 °C

Reference strains	$OD_{mean\ biofilm}$ Polystyrene[a]	BPI Polystyrene	$OD_{mean\ biofilm}$ Stainless steel[a]	BPI Stainless steel
S. aureus ATCC 35556 (positive control)	0.728 ± 0.15	0.758	0.424 ± 0.05	0.801
S. aureus ATCC 12600	0.389 ± 0.07	0.405	0.258 ± 0.02	0.486
S. epidermdis ATCC 12228 (negative control)	0.283 ± 0.05	0.294	0.079 ± 0.00	0.149

[a] Values are expressed as OD mean ± standard deviation.

Scanning electron microscopy (SEM) of MRSA biofilms

Biofilm formation was further confirmed by SEM. For SEM analysis, we selected one biofilm positive strain (MRSA 4) that was categorized as a strong biofilm producer on polystyrene. Biofilms were prepared as described above. The microbial cells were grown at 37 °C for 24 h on polystyrene tissue plates and then washed by dipping 3 times in sterile PBS to remove non-adherent cells. Samples were dehydrated in ethanol–water mixtures with increasing ethanol concentrations (65%, 75%, 85%, 95%, and 100%), and finally air-dried overnight.

Statistical analysis

Hierarchical cluster analysis was performed by the single Linkage method, in order to segment the microbial strains by using their ability to produce biofilm on polystyrene and stainless steel (STATISTICA ver. 10, StatSoft Inc., Tulsa, Okla., U.S.A.). BPI values of 0.294 and 0.149 were defined as lower limits for considering the sample as able to produce biofilms on polystyrene and stainless steel, respectively.

RESULTS AND DISCUSSION

MRSA characterization

All the strains were confirmed to be MRSA harboring the *nuc* and *mec*A genes; the molecular characteristics of the strains are reported in **Table 3**. Of the 22 strains analyzed, 13 *spa* types and 8 STs were detected, with a prevalence of ST 398 (12/22; 54.5%). All strains carried the SSC*mec* type IVa (36.4%) or V (50%) and showed the presence of the *ica*A, *ica*D, *fnb*A, and *hla*genes. All MRSA strains were found to be *fnbB* negative. The distribution of virulence-associated genes (adhesin encoding, toxin encoding, and gene regulators) detected in the 22 MRSA strains studied are shown in **Table 3**. With a range of over 90%, most isolates had a similar distribution of adhesion genes (*icaA, icaD, cna, fnbA*, and *fnbB*), toxin genes (*hla* and *hlb*), and staphylococcal regulators (*agr* and *sarA*).

Table 3. Results from genotyping of the 22 MRSA strains used in the study

Strain nr.	*Spa* type	ST	SCC*mec*	*Agr* type	*nuc*	*icaA*	*icaD*	*cna*	*fnbA*	*fnbB*	*clfA*	*hla*	*hlb*	*sarA*	Source
MRSA 1	t1255	398	V	1	+	+	+	+	+	−	+	+	+	+	Bovine milk
MRSA 2	t127	1	IVa	3	+	+	+	+	+	−	+	+	+	+	Bovine milk
MRSA 3	t174	1	IVa	3	+	+	+	+	+	−	+	+	+	+	Bovine milk
MRSA 4	new	8	IVa	3	+	+	+	+	+	−	+	+	+	+	Bovine milk
MRSA 5	t524	71	V	1	+	+	+	+	+	−	+	+	+	+	Bovine milk
MRSA 6	t899	398	IVa	1	+	+	+	+	+	−	+	+	+	+	Bovine milk
MRSA 7	t688	5	V	4	+	+	+	−	+	−	+	+	+	+	Bovine milk
MRSA 8	t786	88	IVa	3	+	+	+	−	+	−	+	+	+	+	Bovine milk
MRSA 9	t1730	2781	V	1	+	+	+	−	+	−	+	+	+	+	Bovine milk
MRSA 10	t011	398	IVa	1	+	+	+	+	+	−	+	+	+	+	Pork
MRSA 11	t127	1	IVa	3	+	+	+	+	+	−	+	+	+	+	Pork
MRSA 12	t899	398	V	1	+	+	+	+	+	−	+	+	+	+	Pork
MRSA 13	t1255	398	V	1	+	+	+	+	+	−	+	+	+	+	Pork
MRSA 14	t9301	398	ND	1	+	+	+	−	+	−	+	+	+	+	Pork
MRSA 15	t034	398	V	1	+	+	+	+	+	−	+	+	−	+	Pork
MRSA 16	t4474	9	ND	1	+	+	+	+	+	−	+	+	−	+	Pork
MRSA 17	t899	398	V	1	+	+	+	+	+	−	+	+	−	−	Pork
MRSA 18	t127	1	IVa	3	+	+	+	+	+	−	+	+	−	+	Pork
MRSA 19	t011	398	V	1	+	+	+	+	+	−	+	+	+	+	Pork
MRSA 20	t034	398	V	1	+	+	+	+	+	−	+	+	+	+	Pork
MRSA 21	t1255	398	V	1	+	+	+	+	+	−	+	+	+	+	Pork
MRSA 22	t899	398	V	1	+	+	+	+	+	−	−	+	+	+	Pork

Biofilm production assay

In this study, polystyrene and stainless steel were selected because they are the most widely used materials in medical devices and food processing equipment. Biofilm formation was observed in 5 (22.7%, 5/22) isolates that were able to synthesize biofilm on polystyrene; of these, one (4.5%, 1/22) strain was also able to synthesize biofilm on stainless steel (weak producer). Of the biofilm producers on polystyrene, one (20%, 1/5) strain produced moderate/strong biofilm, one (20%, 1/22) moderate biofilm, and 3 (60%, 3/5) strains were classified as weak biofilm producers (**Figure 1**). The highest amount of biofilm was formed on polystyrene by 2 MRSA strains (MRSA 7 – BPI = 0.61; MRSA 4 – BPI = 0.71) isolated from cows' milk, although they were either weak biofilm producers (MRSA 4) or nonproducers (MRSA 7) (MRSA 7 – BPI = 0.12; MRSA 4 – BPI = 0.15) on stainless steel (**Figure 1**). In agreement with the results of Pagedar and others (2010) and Di Ciccio and others (2015), our data demonstrated that biofilm formation occurred more on polystyrene (22.7 %) than on stainless steel (4.5 %). In particular, the strain (MRSA 4) that produced biofilm on stainless steel (weak producer) was also able to synthesize biofilm on polystyrene (moderate/strong producer).

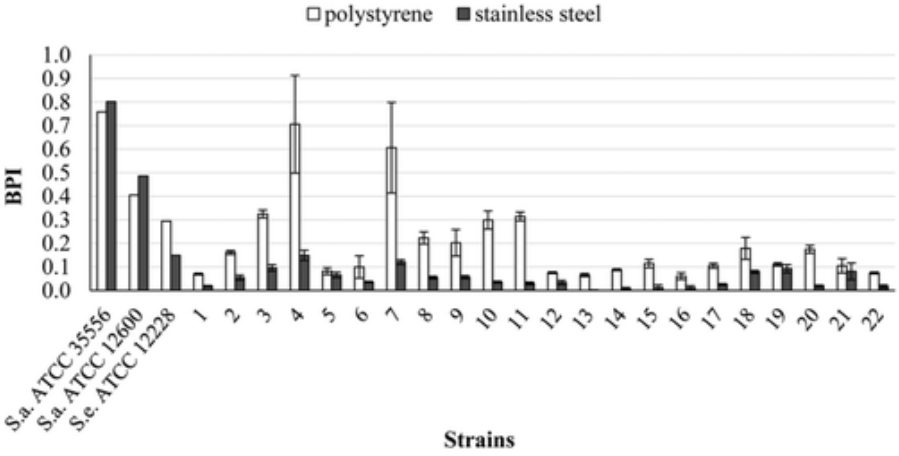

Figure 1. Biofilm formation of MRSA strains at 37 °C. The results are expressed as BPI. BPIs are the means of 2 independent sets of all experiments performed in triplicate.

Relationship between molecular characteristics and biofilm production

Of the biofilm-forming isolates, 4/5 (80%) showed SCC*mec* type IVa. Biofilm-forming isolates had the following genetic profiles: ST1/t174/IVa; ST1/t127/IVa; ST5/t688/V; ST8/t unknown/IVa; ST398/t011/IVa. The majority (11/17; 64.7%) of nonbiofilm-forming isolates contained ST398 clones. The majority (3/5, 60%) of biofilm positive isolates were found to carry *agr* type III; 1 isolate carried *agr* type I and 1 carried *agr* IV. Fourteen out of 17 (82.3%) of the biofilm-negative isolates were found to carry *agr* type I, and 3/17 (17.6%) carried *agr* type III. Of the biofilm-forming isolates, 1/5 (20 %) were *cna* negative.

The production of the *ica*ADBC operon encoded PIA by *S. aureus* is one of the most studied mechanisms of biofilm formation (O'Gara 2007; Joo and Otto 2012). However, biofilm formation independent of the *ica* operon has also been described in *S. aureus* (Geoghegan and others 2010). Expression of biofilm-associated genes is very complex and is influenced by a variety of factors, such as environmental conditions. In this study, all MRSA isolates (both biofilm positive or negative) were found to carry the *ica*A gene. However, as shown in the present study, and also reported elsewhere, individual strains are often found that are both *ica* positive and biofilm negative (Cha and others 2013). The *fnb*B gene was not found among biofilm-producing MRSA strains and nonproducing MRSA strains. This means that the *fnb*B gene may not be correlated with biofilm-forming ability, although a previous study suggests that *fnb*B mediated biofilm development is common (O'Neill and others 2008). Regarding the relationship between genetic characteristics (virulence factors and the accessory-gene-regulator) and the ability to form biofilm on both polystyrene and stainless steel of our strains, no clear relationship was observed between these strains and their genetic characteristics. However, considering only the biofilm-forming strains, SCC*mec*-typing performed in this study found that 4 (80%) of the 5 biofilm-

positive MRSA strains belong to SCC*mec* type IVa, suggesting that MRSA strains carrying SCC*mec* type IVa are more likely to form biofilm than those with SCC*mec* type V. Our results on polystyrene showed that the majority of MRSA biofilm-producers carried SCC*mec* type IVa; in contrast, the most common SCC*mec* type in non-biofilm-forming strains (17) was SCC*mec* typeV (11/17, 64.7%) (**Table 3**). Similarly, the results regarding stainless steel showed that one (4.5%) biofilm-producing strain (MRSA 4) carried SCC*mec*IVa (**Table 3**). This corroborates the hypothesis that the presence of SCC*mec*IVa may be related to the ability to form biofilm. Furthermore, a cluster analysis on the 5 biofilm-producing strains gave 2 groups. The 1st contained strains MRSA 7 and MRSA 4, and the 2nd contained strains MRSA 10, MRSA 11, and MRSA 3. These findings, limited to SCC*mec*-typing, agree with the results of Mirani and others (2013) that all the biofilm-positive isolates belonged to SCC*mec* type Iva, and the majority (91.8%) carried *agr* type II. Biofilm-negative isolates, on the other hand, belonged to SCC*mec* type V (Mirani and others 2013). Kwon and others (2013) also supported this finding, and reported that MRSA strains with SCC*mec* type IV are more likely to form biofilm than other types of SCC*mec*. Other authors also reported that strong biofilm-producing strains belong to SCC*mec* type IV and *agr* type II; these authors suggested that SCC*mec* type IV and *agr* type II are a good combination for biofilm formation in foodborne MRSA isolates (Manago and others 2006; Cafiso and others 2007). In contrast, our study showed that of the moderate/strong biofilm producers, one MRSA strain contained ST8/SCC*mec*IVa/*agr*III (MRSA 4) and one contained ST5/SCC*mec*V/*agr*IV (MRSA 7) genotypes, whereas the 2 weak biofilm producers contained ST1/SCC*mec*IVa/*agr*III genotype (MRSA 3, MRSA 11), and one weak biofilm producer contained ST398/SCC*mec*IVa/*agr*I genotype (MRSA 10). However, further studies involving a larger number of food-related MRSA strains are needed in order to confirm a relationship between the *SCCmec* ST and the ability to form biofilms. Interestingly, the highest amount of biofilm was formed on polystyrene by 2 MRSA

strains (MRSA 7: ST5/t688/SCC*mec*V/*agr*IV and MRSA 4 ST8/t-unknown/SCC*mec*IVa/*agr*III) isolated from cows' milk (**Table 3**). It is well known that MRSA detected from milk and dairy products can be staphylococcal enterotoxin(s) (SEs) producers (Parisi and others 2016). *S. aureus* has been described as forming biofilm on various materials commonly used in food processing plants (Lee and others 2014). The biofilm-forming ability of MRSA that are potentially SEs producers should be of concern for food safety, since they may colonize and spread in food-producing plants and cause food contamination.

Statistical analysis

In order to classify the MRSA strains according to their ability to produce biofilm, a hierarchical dendogram was obtained by considering all the data from both polystyrene and stainless steel. The similarities among the strains were evaluated using the Euclidean distances. Although some similarities were highlighted, such as a cluster consisting of strains MRSA 3, MRSA 10, and MRSA 11 and a cluster consisting of strains MRSA 7 and MRSA 4, no common genetic characteristics was observed among these strains. A 2nd series of cluster analysis was performed to separate the ability to produce biofilm on polystyrene and stainless steel. The results on polystyrene showed that 5 (22.7%) strains out of a total of 22 may be considered as biofilm-forming isolates. Moreover, 80% of these showed SCC*mec*type IVa, while the most common SCC*mec* type in the nonbiofilm-forming isolates was V. Similarly, the results regarding stainless steel showed that one (4.5%) strain (MRSA 4, SCC*mec* type IVa) of the 22 may be considered as biofilm forming.

SEM of MRSA biofilms

SEM analysis allows observation of bacteria/surface interaction and may be used as a semiquantitative technique; in our study, SEM proved to be a useful technique for confirming the presence of an extracellular polysaccharide and glycoprotein network layer, and also for better understanding of biofilm structures. As shown

in **Figure 2**, an MRSA isolate (MRSA 4) showed an extracellular product surrounding the cell aggregate on polystyrene tissue plates. In more detail, after 24 h of incubation, meshwork-like structures were observed around the cells at 37 °C and the surface tested was covered with dense cell clusters.

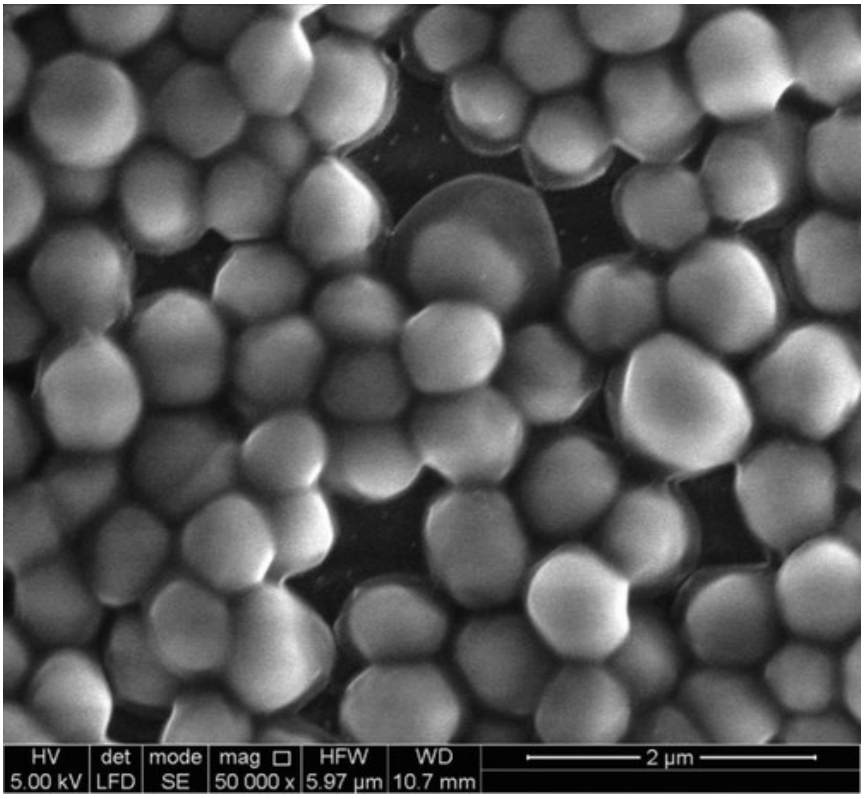

Figure 2. Scanning electron microscopy image of biofilm formed by an MRSA strain (MRSA 4) at 37 °C on polystyrene (BPI: 0.76). Magnification 50000×. After 24 h of incubation at 37 °C, the surface tested was covered with dense cell clusters.

CONCLUSION

As far as we know, few researchers have investigated the relationship of molecular characteristics to the ability of MRSA to form biofilm, and the majority of these studies have investigated biofilm formation by MRSA of clinical origin. Few works on biofilm have focused on MRSA isolated from food. In order to provide data on biofilm formation by food-related MRSA, we monitored the molecular characteristics, the presence of some genetic virulence markers, and of the accessory-gene-regulator *agr* and *sarA* among biofilm-forming and nonbiofilm-forming MRSA isolates from cows' milk and pork. We also investigated whether the presence of these genes affects biofilm formation. Briefly, we attempted to investigate the biofilm formation of food-related MRSA strains when they are exposed to conditions simulating those in food processing plants. The majority of biofilm studies, in fact, used microtiter plates in the 96 wells format, whereas in the present study we used microtiter plates in the 6 wells format. This system overcomes the limitation of the basic microtiter plate assay (96 wells format) concerning possible nutrient limitation and the inability to observe biofilm structure by direct microscopy. With regard to this, in order to show the presence of biofilm matrix, expressed as BPI, the SEM analysis was performed on a selected MRSA strain (MRSA 4 classified as a moderate/strong biofilm producer). Our findings have shown that genotypically different isolates of MRSA from food (milk and pork) have different abilities to produce biofilms on the materials commonly used for food processing equipment. As reported in literature, PBP2a protein is an important factor in biofilm accumulation (Pozzi and others 2012; Rudkinet and others 2012). However, in this survey there was no difference between the biofilm formation by food-related MRSA (expressed as BPI) compared to the biofilm formation (BPI values) by MSSA strains isolated from the food sector in a previous work (Di Ciccio and others 2015). Among the biofilm-forming strains it seems that SCC*mec*IVa could be a characteristic that better contributes to the

ability of MRSA isolated from the food sector to produce biofilm on polystyrene. In conclusion, our findings confirm for food isolates of MRSA what has been found previously for clinical MRSA. Further studies are needed in order to acquire better understanding as to whether the presence of SCC*mec* IVa is also related to the ability to form biofilm.

Acknowledgments

The following authors (Adriana Ianieri and Pierluigi Di Ciccio) are members of the EU COST Action FA1202 (CGA-FA1202): A European Network for Mitigating Bacterial Colonisation and Persistence on Foods and Food Processing Environments (http://www.bacfoodnet.org/) and are grateful to this action for facilitating the collaborative networking that contributed to this study. Authors do not have any conflict of interest.

Author Contributions

A. Vergara designed the study and drafted the paper. P. Di Ciccio carried out experiments in the lab and SEM imaging, and participated in drafting the paper. G. Normanno performed the statistical analysis and analyzed and interpreted the results. F. Pedonese and R. Nuvoloni carried out the phenotypic biofilm assays. A. Parisi and G. Santagada collected MRSA strains and performed molecular characterization studies. A. Colagiorgi carried out the detection of virulence factors. E. Zanardi and S. Ghidini participated in drafting the article. A. Ianieri helped in revising the paper and gave final approval of the last version.

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PART 2

CONTROL

CHAPTER 6

Antibiotic resistance of Methicillin-Resistant *Staphylococcus aureus* (MRSA) from Italian swine chain in planktonic and biofilm form

Colagiorgi A[1], Baldo V[2], Scali F[2], Bruini I[1], Di Ciccio PA[1,3], Zanardi E[1], Ghidini S[1], Alborali GL[2], and Ianieri A[1]

[1] Food and Drug Department, University of Parma. Parma, Italy.

[2] Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia Romagna – Headquarters. Brescia, Italy

[3] Dipartimento di Scienze Veterinarie, University of Turin, Grugliasco, TO, Italy

Manuscript in preparation

ABSTRACT

Pig herds are an important reservoir for Methicillin-Resistant *Staphylococcus aureus* (MRSA), a worldwide distributed antimicrobial-resistant pathogen.

This paper investigates the relationship between the antimicrobial usage (AMU) and the antimicrobial resistance (AMR) patterns of MRSA isolated from pig production chain in northern Italy, as well as the resistance profiles of biofilm-positive isolates in their planktonic and sessile form. A total of 37 strains was isolated from 50 pigs fattening herds in the Lombardy Region. Multidrug resistance was observed in 33/37 (89,1%) of isolates. Results did not showed a relationship between AMU and AMR, probably due to limited sample.

Antimicrobial resistance increased up to 500 folds in biofilm-organized bacteria with respect to their planktonic form, underlying the importance of developing alternatives to commonly used antibiotics to overcome the bacterial resistance issue.

INTRODUCTION

Methicillin-Resistant *Staphylococcus aureus* (MRSA) is the most commonly identified antimicrobial-resistant pathogen in many parts of the world (Taylor, 2013). MRSA clones have emerged in three separate settings: human hospitals (hospital-associated, HA-MRSA), human carriers outside the hospital (community-associated, CA-MRSA), and livestock animals (Livestock-associated, LA-MRSA) (Doulgeraki et al., 2016). In the case of food-producing animals, a specific clone (CC398) has been found in several countries, including Austria, Belgium, Canada, Denmark, France, Germany, The Netherlands and Italy (EFSA, 2009a).

Pig herds are an important reservoir for MRSA CC398, that has been frequently found also in cattle and poultry populations (Lassok & Tenhagen, 2013). On pig farms, various samples such as dust, and possibly even the air were positive for MRSA (Doulgeraki et al., 2016). Although CC398 has been found to colonize animals, only few isolated cases of clinical infections in animals have been reported (Aires-de-Sousa, 2017). MRSA can also occurs in slaughterhouses by entering in or on animals, where can become part of the endemic flora of the slaughterhouse (Van den Broek et al., 2009). Although it is not clear the reason for the colonization of CC398 clone of pigs and other food-producing animals, it has been hypothesized that the use of cephalosporins, tetracycline and other antibiotics may have a role in providing a niche for this clone (EFSA, 2009b).

Both methicillin -sensitive *S. aureus* (MSSA) and MRSA are able to produce multilayered biofilms on different surfaces (Di Ciccio et al., 2015; Doulgeraki et al., 2016; Vergara et al., 2017). Adhesion and biofilm formation are important virulence factors. In fact, biofilms formed on the surfaces could enhance the colonization and the persistence of *S. aureus* in the environment (Gutiérrez et al., 2012). Furthermore, it is generally accepted that cells within a biofilm are more resistant to antimicrobials than their planktonic counterparts (Van Houdt & Michiels, 2010; Bridier et al., 2015).

Antimicrobials are commonly used in livestock for therapeutic treatment, metaphylaxis, and prophylaxis. In addition, they are used in sub-therapeutic concentrations as growth promoters, with an high tendency to develop antimicrobial resistance (Jayaweera & Kumbukgolla, 2017). Such resistance can be passed to other microorganisms, including human pathogens, through e.g. horizontal gene transfer (HGT). In addition, MRSA can spread via environmental routes including air, water, or manure, and can lead to cross contamination occurrence (Jayaweera & Kumbukgolla, 2017).

This study is part of "Classyfarm", a national priority project chartered by the Italian Ministry of Health. *Classyfarm* is an integrated control system that allows to classify the risks on Italian farms in all the areas related to animal health and welfare (including veterinary drugs and biosecurity), based on the collection of data from multiple sources (official controls, databases and information from the veterinarians of the company) (classyfarm, n.d.).

This survey concerned the evaluation of the antimicrobial resistance (AMR) profiles of MRSA isolated from 50 pigs fattening herds in Lombardy region. In particular, fifteen different molecules belonging to twelve different classes of antibiotics were tested. The relationship between AMR profiles and antimicrobial usage (AMU) was studied. Furthermore, isolates were tested for their biofilm forming ability in microtiter plates. The biofilm-positive isolates were then tested for their resistance in their planktonic and biofilm form against two selected antibiotic (i.e. gentamicin and enrofloxacin), belonging to the list of critically important antibiotic of World Health Organization (WHO, 2017).

MATERIALS AND METHODS

MRSA sampling and isolation

A convenience sample of fifty finishing pigs herds located in Lombardy (Italy) was selected, i.e. 50 farms and two slaughterhouses. A total of 400 samples were collected. In details: three environmental sites at each farm, and five carcasses per farm at slaughterhouse were sampled.

Specimens were transferred into enrichment broth (1 ml; tryptic soy broth - Oxoid, Milan, Italy – supplemented with 7.5% NaCl) and incubated at 37°C, 24 h. Subsequently, they were subcultured on chromogenic agar medium (ChromID MRSA agar; bioMérieux, Marcy l'Etoile/France) at 37°C. Plates were read after 24 and 48 h of incubation, respectively. DNA was extracted from suspect colonies, and a multiplex PCR for the amplification of the 16S rRNA gene, the *nuc* gene (encoding the species-specific thermonuclease), and *mecA* gene (responsible for the resistance to methicillin) was performed, using primers listed in Table 1 (Louie *et al,* 2002). Thermocycling conditions were as follows: 94 °C for 2 min, followed by 25 cycles of 94 °C for 15 s, 55 °C for 30 s, and 72 °C for 30 s, with a final extension at 72 °C for 10 min.

Table 1. List of primers used in this study.

Primer	Sequence (5'-3')	Product size (bp)
16S-F	AGA GTT TGA TCA TGG CTC AG	798
16S-R	GGA CTA CCA GGG TAT CTA AT	
***mecA*-1**	AAA ATC GAT GGT AAA GGT TGG C	533
***mecA*-2**	AGT TCT GCA GTA CCG GAT TTG C	
***nuc*-1**	GCG ATT GAT GGT GAT ACG GTT	270
***nuc*-2**	AGC CAA GCC TTG ACG AAC TAA AGC	

Antimicrobial consumption data

Antimicrobial usage (AMU) was estimated, as days of treatments per bred pig, using defined daily dose animal for Italy (DDDAit) and an average weight at treatment of 100 kg. DDDAit were established according to Italian summaries of product characteristics. Data were collected retrospectively regarding consumption and pig population of 2016.

Antibiotic susceptibility tests in planktonic form

Antimicrobial susceptibility testing (disk diffusion test) was performed against MRSA isolates using kanamycin, amoxicillin/clavulanic acid, ampicillin, oxacillin, penicillin, cefalotin, ceftiofur, nalidixic acid, florfenicol, enrofloxacin, clindamycin, erythromycin, tiamulin, sulfamethoxazole-trimethoprim, and tetracycline. The disk diffusion test was conducted according to Clinical and Laboratory Standards Institute guidelines (CLSI, 2012) and CLSI breakpoints were taken to determine the antimicrobial resistance and susceptibility (CLSI, 2008, 2015b) (Table 2).

Table 2. List of antibiotics used in this study

CLASS	ACRONYM	ANTIBIOTIC	CONC.	REFERENCE
Aminoglycosides	K	Kanamycin	30 µg	CLSI M31-A3
ß-lactams	Aug	Amoxicillin/ Clavulanic acid	30 µg	CLSI VET 01-S2-2B
ß-lactams	Amp	Ampicillin	10 µg	CLSI VET 01-S2-2B
ß-lactams	Ox	Oxacillin	1 µg	CLSI M31-A3
ß-lactams	P	Penicillin G	10 IU	CLSI M31-A3
Cephalosporin 1st generation	Kf	Cefalotin	30 µg	CLSI VET 01-S2-2B
Cephalosporin 3rd generation	Eft	Ceftiofur	30 µg	CLSI VET 01-S2-2A
Quinolones	Na	Nalidixic acid	30 µg	CLSI M31-A3
Fenicols	Ffc	Florfenicol	30 µg	CLSI VET 01-S2-2A
Fluoroquinolones	Enr	Enrofloxacin	5 µg	CLSI VET 01-S2-2A

Lincosamides	Da	Clindamycin	2 µg	CLSI VET 01-S2-2A
Macrolides	E	Erythromycin	15 µg	CLSI VET 01-S2-2B
Pleuromutilins	T	Tiamulin	30 µg	CLSI VET 01-S2-2A
Folate inhibitor / Sulfonamides	Sxt	Trimethoprim / Sulfamethoxazole	25 µg	CLSI VET 01-S2-2B
Tetracycline	Te	Tetracycline	30 µg	CLSI VET 01-S2-2B

Biofilm formation assay

The ability of the isolates to form biofilm was assessed using a previously described colorimetric microtiter plate assay (Stepanović et al., 2007), with some modifications. Briefly, 16-24 hours old colonies were resuspended in Tryptic Soy Broth (TSB, Oxoid, Milan, Italy) at a concentration of 0.5 McFarland. Bacteria were diluted to a concentration of 10^6 cfu/ml, and 200 µl were transferred into the wells of a 96-wells microtiter plate (SPL life sciences, Naechon-Myeon, South Korea). TSB without bacteria served as negative control. Plates were incubated at 37°C for 24 h to allow bacterial adhesion and biofilm formation. Following incubation, the wells were emptied and rinsed with phosphate buffered saline (PBS, Oxoid, Milan, Italy). The biomass was stained with 2% Crystal Violet for 20 minutes. 95% ethanol was added to each well and plates were incubated at room temperature for 30 min. Finally, the OD of each well was measured at 540 nm using a microplate reader (Victor, Perkin Elmer, Waltham, MA). The results were collected from three independent experiments in which the biofilm formation of each culture tested was evaluated in eight plicate. The quantitative classification of biofilm production based on cut-off value (ODc) and average OD values was carried out leading to four categories of strains: no biofilm producer ($OD \leq ODc$); weak biofilm producer ($ODc < OD \leq 2 \times ODc$); moderate biofilm producer ($2 \times ODc < OD \leq 4 \times ODc$); strong biofilm producer ($4 \times ODc < OD$) (Stepanović et al., 2007).

Minimal inhibitory concentration determination

Antibiotic susceptibility of the biofilm-positive strains against gentamicin and enrofloxacin in planktonic form was determined

by the microtiter broth dilution method (CLSI, 2015a). Briefly, two-fold serial dilutions of gentamicin or enrofloxacin were prepared in MHB II in 96-well microtiter plates (Nunclon™ Delta Surface; Thermo Fisher Scientific, Denmark) (final volume: 100 µl). A bacterial suspension for each isolate was prepared in Muller Hinton Broth II (MHB II, Liofilchem, Roseto degli Abruzzi, Italy) with a visual turbidity comparable to 0.5 McFarland turbidity standard. The suspension was finally adjusted so that after inoculation, each well contains approximately 5×10^5 CFU/mL. Enrofloxacin concentrations of 0.125 – 64 µg/ml and gentamicin concentrations of 0.250 - 128µg/ml were used for testing. Plates were incubated at 37°C for 16 hours. MIC was defined as the lowest antibiotic concentration that prevented visible growth.

Antibiotic susceptibility evaluation in biofilm form

Minimum biofilm eradication concentration (MBEC) assay was used to test susceptibility of biofilm-positive MRSA isolates to gentamicin and enrofloxacin (Ceri et al., 2001). 96-wells flat-bottom microtiter plates (Nunc, Roskilde, Denmark) were inoculated with 150 µL of inoculum (10^5 CFU/mL) in each well. Then, plates were covered with a 96-peg lid (Immuno TSP lids; Nunc, Roskilde, Denmark), sealed with Parafilm®, and placed on an microtiter shaker (MTS 2/4 digital microtiter shaker, IKA-Works, Staufen im Breisgau, Germany), set to 150 revolutions per minute, in a humidified atmosphere, at 37 °C (G-Cell 115, Fratelli Galli, Milan, Italy), to allow biofilm formation on the pegs. Planktonic bacteria were then removed by rinsing the peg lid in 200 µl of sterile phosphate buffered saline (PBS; Oxoid, Milan, Italy) in a new plate. Biofilms on the pegs were exposed to gentamicin or enrofloxacin in 200 µL MHB II for 16 h at 37°C (challenge plate). After exposure, the peg lid was rinsed, as described previously, for 1 minute, and then transferred to a new microtiter plate (recovery plate) containing 200 µl/well of fresh TSB (Tryptic Soy Broth; Oxoid, Milan, Italy). The challenge plate was covered with a normal lid and incubated at 37°C, 24H, for evaluating the biofilm inhibiting concentration (BIC) on the cells

dispersed from the biofilm during the incubation. The recovery plate was sonicated for 15 minutes (Ultrasonik 57H; NEY, USA) in order to disperse the cells from the peg surface. After sonication, an aliquot (50 µl) was used for preparing serial dilutions that were spot plated (20 µl) on TSA (Oxoid, Milan, Italy). The peg lid was then discarded and the recovery plate was covered with a normal lid and incubated for 24h at 37 °C.

Enrofloxacin concentrations up to 512 µg/ml and gentamicin concentrations up to 1024 µg/ml were used for the assays. The MBEC was defined as the lowest concentration of gentamicin or enrofloxacin required to eradicate the biofilm of a selected isolate after a recovery period of 24 h at 37 °C. Six technical replicates were included for each group, and all experiments were repeated three times.

Statistical analysis

IBM SPSS Statistics 25 (IBM Corp., Armonk, NY) was used for statistical analysis. Comparison between antimicrobial resistance and antimicrobial usage was performed by one-way ANOVA. Differences were considered significant when $P < 0.05$.

RESULTS AND DISCUSSION

Prevalence of MRSA among pig herds

According to a baseline study of the European Food Safety Authority (EFSA), the MRSA prevalence among pig farms in the European Union was 14% (range, 0 to 46%) in breeding herds and 26.9% (range, 0 to 51%) in production herds. In this study, a total of 400 samples were collected from 50 pig finishing herds. 21 out of 50 (42%) pig herds were found positive for MRSA. 37 out of 400 (9.25%) samples were positive for MRSA. 13/37 (35.2%) MRSA strains were isolated from farms, and 24/37 (64.8%) from slaughterhouses.

Antibiotic susceptibility of the isolates and relationship with consumption

The isolates were tested against fifteen antibiotics belonging to twelve different classes. Results showed that 36/37 (97,2%) were resistant to at least 2 classes of antibiotics, 30/37 (81.1%) to at least 4 classes, and 8/37 (21.6%) to at least 8 different classes (**Figure 1**). Multidrug resistance (MDR) was defined as acquired non-susceptibility to at least one agent in three or more antimicrobial categories. In this study, 33/37 (89%) isolates showed multi-resistant phenotypes, with concern for farmers, veterinarians, and slaughterhouse employees. In fact, it has been reported MRSA nasal carriage in 77%–86% people with occupational exposure to pigs, and that the extension of colonization is related to the duration of exposure and to the intensity of animal contacts (Cuny et al., 2009, 2015; Graveland et al., 2011). In particular, it seems that the inhalation of MRSA-contaminated dust in farms is responsible for colonization of humans (Cuny et al., 2015; Bos et al., 2016). Furthermore, LA-MRSA nasal colonization was also reported in slaughterhouse workers (Mulders et al., 2010; Van Cleef et al., 2010).

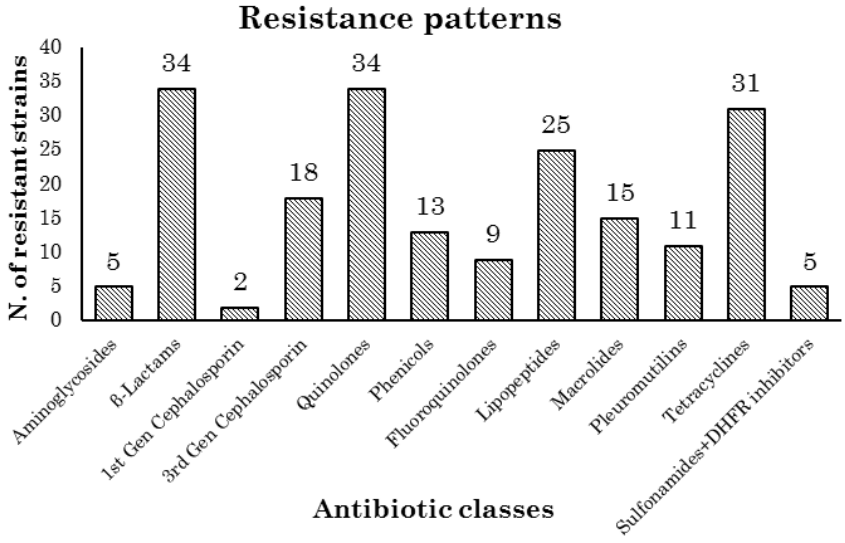

Figure 1. Schematic representation of resistance patterns of MRSA isolates.

MRSA isolates were grouped according to their resistance profiles in 3 groups: strains resistant to three or less, four to seven, and more than eight antibiotic classes. One-way ANOVA was used to compare DDDAit among the groups, and results did not showed significantly differences, suggesting that AMU did not affect AMR (Table 3).

Table 3. Antimicrobial consumption and resistance patterns in MRSA isolates.

N° **AMR classes**	**Medians of AM consumption (days/pig)**	**AM consumption ranges (days/pig)**
≤3	**22.84**	**0.68 - 77.03**
4-7	**19.53**	**0.68 - 178.8**
≥8	**18.31**	**12.25 - 89.18**

Biofilm forming ability

The 37 MRSA isolates were tested for their biofilm forming ability in the polystyrene wells of microtiter plates. 6 out of 37 (16.2%)

strains were able to produce biofilms. In particular, 1 moderate and 5 strong producers were found. These results are in according with other studies, where swine isolated MRSA were able to form robust biofilms (Nicholson et al., 2013). Interestingly, the highest OD value (corresponding to the greatest biomass) was detected in the strain MRSA 1, that has the ability to resist to 10 different antimicrobial classes in its planktonic form.

The moderate biofilm producer was isolated from carcasses at the slaughterhouse, as well as one of the five strong producers. The other biofilm-positive strains were isolated from farm environment (**Table 4**). It is possible that these biofilm forming isolates could survive in the environment for a long time, with concern to the health of livestock, as well as farm workers, since the transmission of LA-MRSA between pigs and pig farmers can be vehiculated by the dust.

Susceptibility of biofilm-positive isolates in planktonic form

The biofilm-positive isolates were tested for the sensitivity to enrofloxacin and gentamicin. Enrofloxacin is a broad spectrum antibiotic used in veterinary medicine to treat animals afflicted with certain bacterial infections, e.g. it is approved for the treatment and control of swine respiratory disease (SRD) associated with *Actinobacillus pleuropneumoniae*, *Pasteurella multocida*, *Haemophilus parasuis*, and *Streptococcus suis* (Papich MG, 2016a). Its mechanism of action is based on the inhibition of the process of DNA synthesis within the bacterial cells, which results in cell death. Enrofloxacin belongs to the class of antibiotics known as fluoroquinolones, which is included in the list of the critically important antibiotics of World Health Organization (WHO, 2017). Gentamicin is an aminoglycoside antibiotic used for the treatment of various bacterial infections both in human and in veterinary medicine. Similarly to other aminoglycosides, gentamicin is a bactericidal antibiotic that works by irreversibly binding the 30S subunit of the bacterial ribosome, interrupting protein synthesis. Gentamicin is used for pigs, cattle and horses (mainly through parenteral administration), and as an oral

solution for poultry (Papich, MG, 2016b). It is currently included in the list of essential medicines for human use of the WHO (WHO, 2017).

Results from MIC assays on the six biofilm-positive strains showed that 2/6 (33.3%) and 4/6 (66.6%) strains were resistant to gentamicin and to enrofloxacin in their planktonic form, respectively (Table 4). In particular, the moderate biofilm producer was sensitive to both antibiotics, whereas the strain that showed the highest biomass quantification was resistant to both gentamicin and enrofloxacin. Both the isolates resistant to gentamicin have been isolated from environmental samples, whereas the strains isolated from the samples collected from carcasses were all sensitive to gentamicin. On the contrary, enrofloxacin-resistant strains were isolated from both environment and carcasses.

Evaluation of antimicrobial profile in sessile form

The six isolates were tested for the susceptibility to enrofloxacin and gentamicin in their biofilm form. Results showed that all the strains were classified as resistant (Table 4). In particular, sensitive planktonic isolates were able to acquire resistance when embedded into a biofilm. A particular concern is that both gentamicin and enrofloxacin were unable to eradicate biofilm at high concentrations (independently from the mechanism of action), since biofilms were up to 512 fold more resistant than planktonic cells. The use of antibiotics that are generally able to eliminate planktonic staphylococcal cells is not effective against the same bacteria in their biofilm form. There are several factors that contribute to the acquisition or the increase of antibiotic resistance in biofilms. First of all, the biofilm exopolymeric matrix has an important role in shielding microorganisms from antimicrobials, due to the presence of a large number of extracellular products such as polysaccharides and proteins (Flemming & Wingender, 2010; Bridier et al., 2011). Furthermore, other biofilm properties such as reduced diffusion, physiological changes of cells, reduced growth rate, and the production of

antimicrobials degrading enzymes are also involved in the resistance mechanism (Bridier et al., 2011, 2015).

Antimicrobial resistance in biofilm-dispersed cells

Chua et al. (2014) found that the cells of *Pseudomonas aeruginosa* dispersed from biofilms showed a different physiology from their planktonic counterpart, e.g. showing higher virulence. For this reason, authors stated that the dispersed cells represent a distinct stage of bacterial life. In this work, we investigated the susceptibility to gentamicin and enrofloxacin of the planktonic cells dispersed from biofilms. Interestingly, we observed that such cells are generally more resistant than their planktonic counterpart. A higher concentration of antibiotic was necessary to inhibit the growth of the cells dispersed from all the biofilms classified as strong. In particular, the MIC values were 2 to 8 fold higher than the values of the planktonic counterpart. In some cases, the dispersed cells that were sensitive in their planktonic form were classified as resistant or intermediate (Table 4).

Table 4. Source, biofilm production, and sensitivity to antimicrobials of MRSA isolates

ISOLATE	SOURCE	BIOFILM	ANTIBIOTIC	MIC	BIC	MBEC
					(µg/ml)	
MRSA 1	Environment	Strong	Enrofloxacin	8 (R)	16 (R)	>512 (R)
			Gentamicin	128 (R)	256 (R)	>1024 (R)
MRSA 2	Carcasses	Moderate	Enrofloxacin	0.250 (S)	0.250 (S)	>32 (R)
			Gentamicin	0.125 (S)	0.125 (S)	>64 (R)
MRSA 3	Environment	Strong	Enrofloxacin	16 (R)	32 (R)	>512 (R)
			Gentamicin	0.250 (S)	1 (S)	>64 (R)

MRSA 4	Carcasses	Strong	Enrofloxacin	8 (R)	32 (R)	>512 (R)
			Gentamicin	4 (S)	32 (R)	>512 (R)
MRSA 5	Environment	Strong	Enrofloxacin	0.125 (S)	0.5 (S)	>64 (R)
			Gentamicin	32 (R)	128 (R)	>1024 (R)
MRSA 6	Carcasses	Strong	Enrofloxacin	16 (R)	32 (R)	>32 (R)
			Gentamicin	2 (S)	8 (M)	32 (R)

CONCLUSIONS

The present study investigated the presence of MRSA in pig production chain in northern Italy in relationship with antimicrobial usage. Multidrug resistance was observed in almost 90% of the isolates. Resistance patterns detected seem not related to drug consumption, maybe because of limited sampling.

Some of the isolates were able to produce moderate to strong biofilms. Results from antimicrobial challenge against biofilm-positive isolates in their planktonic and sessile form showed an increase in the resistance to biocides of bacteria within biofilms. These results suggest that biofilm could act as a mechanism for the tested swine-isolated MRSA strains to get a better survival, and stress once more the importance of finding and developing new alternatives to common antibiotics to overcome the bacterial resistance issue.

Further studies are needed to confirm these preliminary findings, which should encompass a larger sample and MRSA molecular and epidemiologic characterization.

FUNDINGS

This project has been chartered by Italian Ministry of Health, project “Classyfarm”.

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CHAPTER 7

New Perspectives towards Food Safety: anti-biofilm activity of a novel Antimicrobial Peptide (1018-K6) against *Staphylococcus aureus*

Colagiorgi A[1], Anastasio A[2], Di Ciccio PA[1,3], Marrone R[2], Balestrieri M[3], Gogliettino M[3], Ianieri A[1], and Palmieri G[3]

[1] Food and Drug Department, University of Parma. Parma, Italy

[2] Department of Veterinary Medicine and Animal Production, University of Naples Federico II, Naples, Italy

[3] Dipartimento di Scienze Veterinarie, University of Turin, Grugliasco, TO, Italy

[4] Institute of Biosciences and BioResources - UOS Naples, National Research Council, Naples, Italy

Manuscript in preparation

ABSTRACT

Foodborne diseases represent a growing public health problem worldwide. Among them, staphylococcal food poisoning – caused by enterotoxigenic *Staphylococcus aureus* strains – is one of the most common. Bacteria that form biofilms on food processing plant surfaces can represent a critical source of contamination, being more resistant to common antimicrobials and cleaning procedures. For this reason, it is pivotal to prevent and control biofilms in food facilities.

In the last years, antimicrobial peptides (AMPs) have emerged as tools able to contrast bacterial biofilms. The recently developed AMP 1018-K6, deriving from IDR-1018, has shown a significant bactericidal and anti-biofilm efficiency against food-isolated *Listeria monocytogenes* strains as well as a remarkable stability at different pH and temperature.

In this work, a kinetic of the action of the 1018-K6 peptide against the biofilm (classified as strong) of a *S. aureus* reference strain was performed. The peptide showed an impressive rapidity of action, being able to eradicate established biofilms within few minutes. Furthermore, antimicrobial activity against planktonic cells and inhibition of biofilm formation was also observed. The antimicrobial and anti-biofilm activity, together with its remarkable structural stability, make 1018-K6 a promising candidate for a variety of applications aimed at increasing the food safety.

INTRODUCTION

Food safety is a global concern with significant implications for human health. It has been estimated from world health organization that at least 2 billion people worldwide are subject to illnesses caused by unsafe food, annually (World Health Organization, 2015).

Staphylococcus aureus is a commensal and opportunistic pathogen that can cause contamination of food products during food preparation and processing (Grace & Fetsch, 2018). It is an ubiquitous microorganism occurring on the skin and the mucous membrane of many warm blooded animals, including human. For this reason, food handlers are frequently implicated in transmission of such bacteria to food (Gutiérrez et al., 2012). *S. aureus* is characterized by a high salt tolerance, so it can grow in ham and other meats, and in dairy products. Although heat-treatment (e.g., cooking) easily kill *S. aureus* bacteria, some strains are able to produce enterotoxins that are resistant to heat, freezing and irradiation (Hennekinne, De Buyser, & Dragacci, 2012). Detectable levels of the staphylococcal enterotoxins (SEs) are produced when the enterotoxigenic strain grow to levels $>10^5$ cfu/g, and their presence in the food is responsible for the staphylococcal food poisoning (SFP), one of the most common foodborne diseases worldwide (Hennekinne, 2018). SFP symptoms usually develop within 30 minutes to 6 hours, and generally include vomiting, nausea, stomach cramps, and diarrhea (Hennekinne, 2018).

The customer's awareness about chemical compounds used to contrast grow of spoiling and pathogenic microorganisms led to a growing demand for natural preservatives. In this scenario, antimicrobial peptides (AMPs) are assuming increasing interest because of their properties and their natural origin (Keymanesh, Soltani, & Sardari, 2009). AMPs are component of the innate immune system of both prokaryotes and eukaryotes, and are usually characterized by a broad antimicrobial

spectrum activity, a low propensity for developing bacterial resistance, and synergy with common antimicrobials (Palmieri et al., 2016, 2018; Wang, Zeng, Yang, & Qiao, 2016). The antimicrobial activity of AMPs is related to their physicochemical properties, e.g. amphiphilicity, net charge, hydrophobicity, and conformational flexibility (Bechinger & Gorr, 2017).

The innate defense regulator (IDR)-1018 is 12-amino-acid peptide (VRLIVAVRIWRR-NH2) developed by Hankcock and colleagues that displayed a broad-spectrum anti-biofilm activity (de la Fuente-Núñez et al., 2012; de la Fuente-Núñez, Reffuveille, Haney, Straus, & Hancock, 2014; Reffuveille, de la Fuente-Núñez, Mansour, & Hancock, 2014). IDR-1018 is a natural peptide deriving from bactenecin, the bovine host-defense peptide (HDP), which belongs to the cathelicidins family. Its antimicrobial activity was demonstrated against a variety of Gram-positive and –negative bacteria (de la Fuente-Núñez et al., 2014; Reffuveille et al., 2014). Recently, a novel antimicrobial peptide (1018-K6) deriving from IDR-1018 was developed (Palmieri et al., 2018). 1018-K6 was tested against *Listeria monocytogenes* showing an improved conformational stability and bactericidal/anti-biofilm activity compared to the parent IDR-1018 (Palmieri et al., 2018).

The present study aimed at evaluating the antimicrobial and anti-biofilm activity of 1018-K6 against the reference strain *S. aureus* ATCC 35556, known to be a strong biofilm producer (Di Ciccio et al., 2015; Thompson, Abraham, & Jefferson, 2010). The physicochemical characteristics of such molecule, together with its antimicrobial activity, allow 1018-K6 to completely eradicate preformed *S. aureus* biofilms within 10 minutes. Furthermore, a strong reduction in biofilm formation and antimicrobial activity against planktonic cells was also demonstrated.

MATERIALS AND METHODS

Materials

Staphylococcus aureus ATCC 35556 was used in this study because of its well-known ability to form biofilms on polystyrene (Di Ciccio et al., 2015; Thompson et al., 2010).

All culture media used in this study were purchased from Oxoid (Milan, Italy) unless otherwise specified. Phosphate buffered saline (PBS; Oxoid, Milan, Italy) was used for washing and rinsing bacteria.

The peptides used in this study were: the 12-amino-acid peptide IDR-1018 (VRLIVAVRIWRR-NH2) (de la Fuente-Núñez et al., 2014); and the 12-mer peptide 1018-K6 reproducing the IDR-1018 sequence in which a lysine replaced the alanine in position 6 (A6 → K6 mutation) (Palmieri et al., 2018).

Antibacterial activity assay

Minimum inhibitory concentrations (MICs) and minimum bactericidal concentrations (MBCs) were determined following the standard broth microdilution method of the Clinical & Laboratory Standards Institute (CLSI, 2015). Briefly, tested bacteria culture was diluted to 5×10^5 CFU/mL (CFU, colony forming units). The bacteria were treated with 1018-K6 (0.156 to 80 μM) or IDR-1018 in 100 μL culture medium (Muller Hinton II broth, Liofilchem, Italy) for 16 h. The cell growth was measured by the absorbance at 540 nm in a microplate reader (Victor, Perkin Elmer, Waltham, MA). MIC value is the lowest concentration of the peptide without any change in optical density. To measure the MBCs, an aliquot (50 μL) of the bacterial cell suspension was taken based on the MICs and cultivated on a TSA (Oxoid, Milan, Italy) plates. The bacterial cells were enumerated after incubation at 37 °C for 24-48h. MBCs were defined as the lowest concentration of 1018-K6 at which more than 99.9% of the cells were killed. Eight technical

replicates were included for each group, and all experiments were repeated three times.

Inhibition of biofilm formation assay

The ability of 1018-K6 and IDR-1018 to prevent biofilm formation was evaluated in 96-wells flat bottom microtiter plates (SPL Life Sciences, Naechon-Myeon, South Korea). Biofilms were allowed to form in absence or in presence of 1018-K6 or IDR-1018 [80μM], according to a previously described method (Stepanović et al., 2007), with slight modifications. Briefly, the wells of the microtiter plates were inoculated with 200 μl of bacteria culture diluted to 5×10^6 CFU/mL in TSB, and the peptide was added to wells in order to reach the concentration of 80μM. Plates were incubated at 37 °C, 24 h. After the incubation, the contents of the wells were decanted into a discard container and each well was washed three times with 300 μL of sterile PBS. The remaining bacteria were heat-fixed (60°C for 60 min) and stained with 150 μl of 2% crystal violet (Conda laboratories, Madrid, Spain) for 20 min. The microplate was air dried at room temperature, and the dye bound to the cells was resolubilized with 150 μL of 95% ethanol (Carlo Erba reagents, Milan, Italy) per well. The optical density (OD) of each well stained with crystal violet was measured at 540 nm using a microtiter-plate reader (Victor, Perkin Elmer, Waltham, MA). Eight technical replicates were included for each group, and all experiments were repeated three times. The cutoff value (ODc) was established as follow, according to the OD values of negative controls: OD_{mean} + 3 * St.deviation. The OD value of a tested condition (control, 1018-K6 treated, IDR-1018 treated) is expressed as OD mean value of the condition reduced by ODc value.

Minimum biofilm eradication concentration determination

Minimum biofilm eradication concentration (MBEC) assay was used to test susceptibility of *S. aureus* ATCC 35556 biofilms to 1018-K6 or IDR-1018 (Ceri et al., 2001). 96-wells flat-bottom

microtiter plates (Nunc, Roskilde, Denmark) were inoculated with 150 µL of inoculum (10^5 CFU/mL) in each well. Then, plates were covered with a 96-peg lid (Immuno TSP lids; Nunc, Roskilde, Denmark), sealed with Parafilm®, and placed on an microtiter shaker (MTS 2/4 digital microtiter shaker, IKA-Works, Staufen im Breisgau, Germany), set to 150 revolutions per minute, in a humidified atmosphere, at 37 °C (G-Cell 115, Fratelli Galli, Milan, Italy), to allow biofilm formation on the pegs. Planktonic bacteria were then removed by rinsing the peg lid in 200 µl of sterile phosphate buffered saline (PBS; Oxoid, Milan, Italy) in a new plate. Biofilms on the pegs were exposed to 1018-K6 or IDR1018 (0.25 to 80 µM) in 200 µL PBS (Oxoid, Milan, Italy) for 24 h at 37°C. After exposure, the peg lid was rinsed, as described previously, for 1 minute, and then transferred to a new microtiter plate containing 200 µl/well of fresh TSB (Tryptic Soy Broth; Oxoid, Milan, Italy) and sonicated for 15 minutes (Ultrasonik 57H; NEY, USA) in order to disperse the cells from the peg surface (recovery plate). After sonication, an aliquot (50 µl) was used for preparing serial dilutions that were spot plated (20 µl) on TSA (Oxoid, Milan, Italy). The peg lid was then discarded and the recovery plate was covered with a normal lid and incubated for 24h at 37 °C. The MBEC was defined as the lowest concentration of 1018-K6 or IDR-1018 required to eradicate the biofilm of a selected isolate after a recovery period of 24 h at 37 °C. Six technical replicates were included for each group, and all experiments were repeated three times.

Biofilm eradication assay

Biofilm eradication was assayed using the previously described protocol. Briefly, 150 µl inoculum were prepared at 10^3 and 10^5 CFU/ml concentration. Biofilms were grown for 24 h at 37°C, 150 rpm, on TSP peg lid. Then, cells were exposed to 1018-K6 or IDR-1018 [80 µM] at different times of contact: 1, 5, 10, 15, 30 minutes, and 1, 2, 4, 6, 8, 16, 24, 48, and 72 hours. Cells were recovered in fresh TSB by sonication, 15 min at room

temperature. An aliquot from each well was spot plated on TSA, then the microtiter plate was incubated at 37°C, 24 H. Following incubation, optical density was detected using a microtiter-plate reader (Victor, Perkin Elmer, Waltham, MA). Cells spot plated were counted after 24H incubation (37°C). Log_{10} CFU/peg was calculated as follow: $Log_{10}[(X \cdot B^{-1})(D)+1]$, where X is CFU counted on spot plate, B is the volume plated, and D is the dilution. Six technical replicates were included for each group, and all experiments were repeated three times.

Scanning Electron Microscopy

Biofilms exposed to 1018-K6 and positive control were prepared for analysis at scanning electron microscope (SEM). Biofilms were grown for 24 h and then exposed to 1018-K6 [80 μM], as previously described, for 16 h at 37°C. Pegs were removed from TSP lid using a flamed sterilized blade, and cells were fixed with 2.5% glutaraldehyde (Polysciences Inc., Warrington, PA, USA) in PBS at 4°C for 16h. Samples were washed three times with PBS (5 min each) and dehydrated at room temperature through a series of alcohol (50% to 99% ethanol; Carlo Erba reagents, Milan, Italy). Samples were left to dry in a fume hood for 72 hours, then were sputter-coated with a gold-palladium layer using a SCD 040 coating device (Balzer Union, Liechtenstein). Samples were observed using a Zeiss DSM 950 scanning electron microscope at an accelerating voltage of 10 kV (Zeiss, Oberkochen, Germany). The images were processed for display using Photoshop (Adobe Systems Inc., San Jose, CA).

Statistical analysis

Graphpad prism 7.04 (GraphPad Software, San Diego, CA, USA, 2017) was used for statistical elaboration of results. Outliers were identified by ROUT method (Q = 1 %).

RESULTS

Antimicrobial activity of AMPs

The antimicrobial activity against planktonic form of *S. aureus* ATCC 35556 was tested, and minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of 1018-K6 or IDR-1018 were determined (**Table 1**). 1018-K6 affected the bacterial growth at 20 μM. The same concentration is also required for killing 99.9% of bacterial cells, as resulted from MBC assay. As IDR-1018 concern, both MIC and MBC were 40 μM.

Table 1. Antimicrobial and anti-biofilm activity of 1018-K6 and IDR-1018. MIC: Minimum Inhibitory Concentration; MBC: Minimum Bactericidal Concentration; MBEC: Minimum Biofilm Eradication Concentration.

Peptide	MIC (μM)	MBC (μM)	MBEC (μM)
1018-K6	20	20	80
IDR-1018	40	40	80

Evaluation of the activity of AMPs against biofilm formation

The activity of the peptide on biofilm formation was assessed by the crystal violet assay. Planktonic cells were allowed to form biofilm in presence of 1018-K6 or IDR-1018, as well as without AMPs, in microtiter plates. The positive control (untreated bacteria) confirmed the ability of *S. aureus* ATCC 35556 to produce strong biofilms (OD=1.8). 1018-K6 and IDR-1018 treatments resulted in a 89.6% and 92.4% reduction in the biofilm biomass, respectively, in comparison to the untreated group. Significance was calculated by one-way ANOVA test (p=0.00). The OD value of 1018-K6 and IDR-1018 treated cells, obtained according to Stepanovic et al. (2007), resulted below

the ODc value, indicating that the bacteria were not able to form biofilms in presence of the peptides (**Figure 1**).

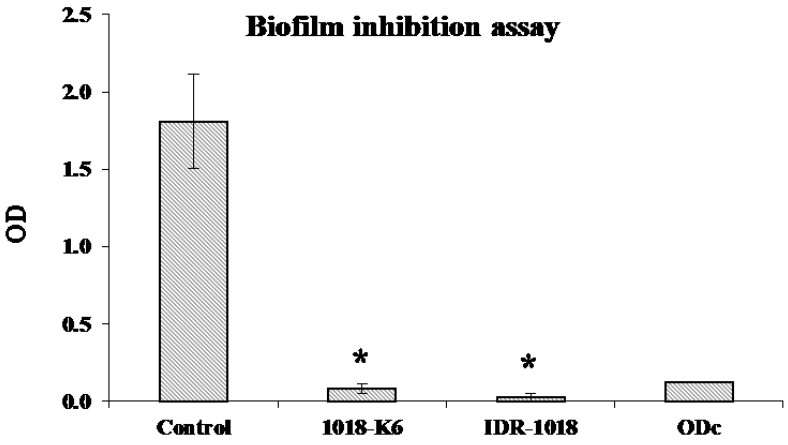

Figure 1. Inhibition of biofilm production of *S. aureus* ATCC 35556 by 1018-K6 and IDR-1018. Average OD measurements of crystal violet stained biofilms are shown with error bars representing standard deviation (n=3). "*" indicates significant difference between the treated group and the control group.

Minimum biofilm eradication concentration determination

24 hours old biofilms were exposed to scalar concentrations of 1018-K6 or IDR-1018 for 24 H at 37 °C. Results showed that the incubation with a concentration of 40 µM of 1018-K6 was able to affect the biomass of biofilms, since a reduction of 1.7 Log_{10} cfu/peg was achieved (data not shown). The same peptide was able to eradicate biofilms at the concentration of 80 µM (MBEC) (**table 1**). The MBEC of IDR-1018 was also 80 µM (**table 1**). Differently from 1018-K6, a higher concentration of peptide was necessary to reduce biomass of biofilms of at least 1 Log_{10} cfu/peg.

AMPs action against biofilms from different inoculum level

The action of 1018-K6 was evaluated against biofilms established starting from two different inoculum concentrations (i.e., 10^3 and 10^5 cfu/ml). Biofilms were exposed

to 1018-K6 at 24h, 48h, and 72h. Results showed a complete eradication ability of the peptide at both concentrations and times of exposure (Figure 2).

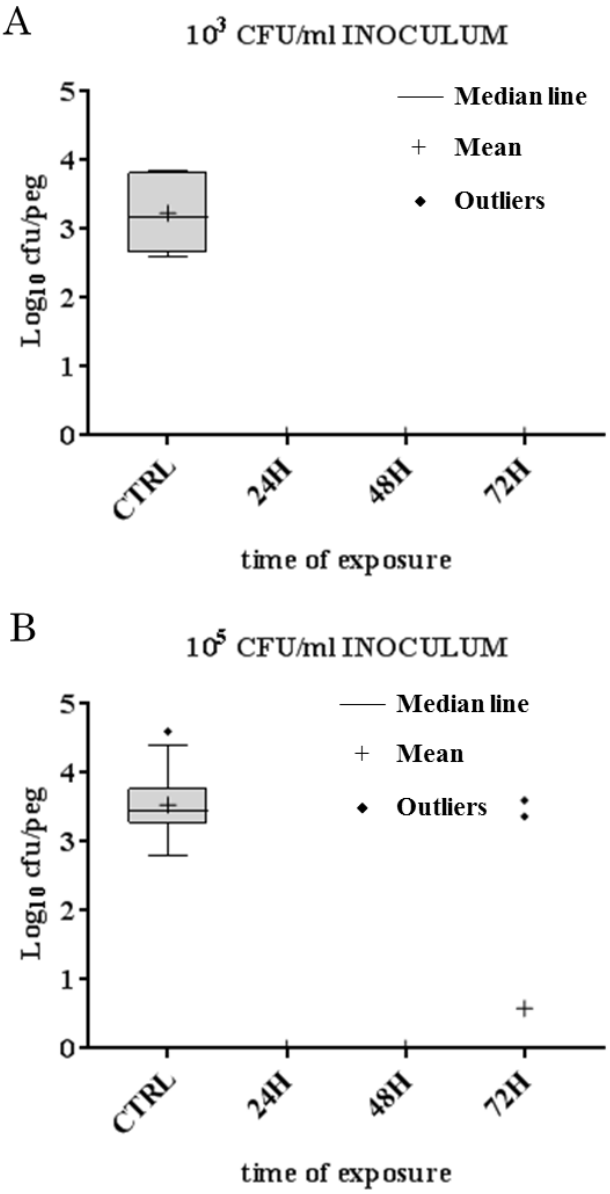

Figure 2. Boxplot of the eradication activity on *S. aureus* biofilms from starting inoculum concentrations of 10^3 (A) and 10^5 (B) cfu/ml (Tukey-style whiskers).

Kinetic of action of 1018-K6 against established biofilms

24 hours old biofilms (initial inoculum: 10^5 cfu/ml) were exposed to 1018-K6 [80 μM] for 1 minute to 72 hours. Results showed that the anti-biofilm activity of 1018-K6 started already after 1 minute of exposure (**Figure 3**). Interestingly, the complete eradication of biofilms was achieved after 5 minutes. In particular, a reduction of 3 Log_{10} cfu/peg was observed. No viable cells were detected after incubation from 10 minutes to 72 hours.

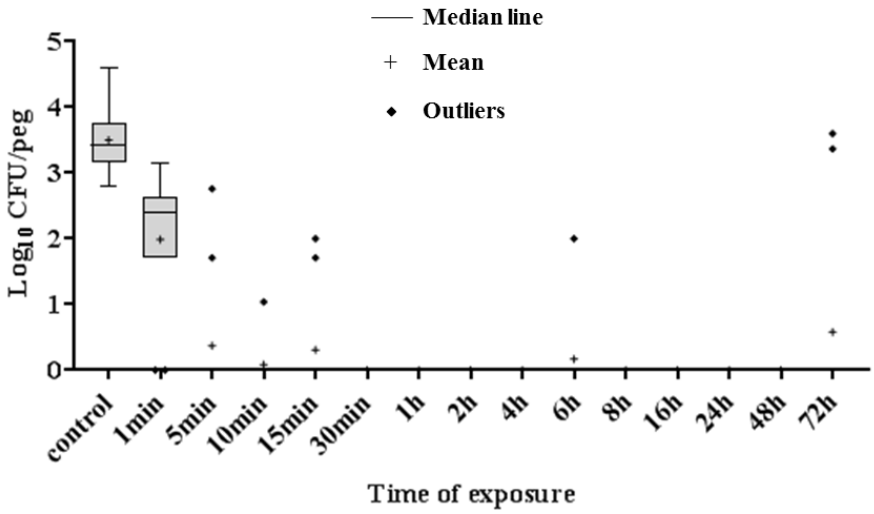

Figure 3. Box plot of eradication activity of 1018-K6 against 24h-old *S. aureus* biofilms at different time of exposure (Tukey-style whiskers).

1018-K6 *versus* IDR-1018

The action of 1018-K6 and IDR-1018 was tested on *S. aureus* biofilms at the same time. Biofilms were exposed to the peptides for 1, 5, 15, and 30 minutes. Results showed that both peptides were able to inhibit biofilm cells after 5 minute of exposure (**Supplementary Figure 1**). 1018-K6 showed a remarkable anti-biofilm activity in all the replicates. On the contrary, in some cases IDR-1018 was not able to completely

eradicate the staphylococcal biofilm (i.e, after 15 min. exposure, SF1).

Scanning electron microscopy analysis of treated and control *S. aureus* biofilms

SEM observations confirmed the anti-biofilm activity of 1018-K6, as shown in figure 5. A thick, complex, and multilayered biofilm, embedded in exopolymeric matrix was observed in the non-treated control sample (Figure 5 a, b, c). In details, pegs were covered by different clusters of bacterial cells all over the surface. On the contrary, only small aggregates of cells were observed in the 24 H 1018-K6 treated sample, and the presence of exopolysaccaride matrix residues on the wall of the analyzed pegs was detected (Figure 5 d, e, f). Furthermore, blebbing (extrusion) of bacterial membrane was observed, clearly indicating membrane damage (Figure 6).

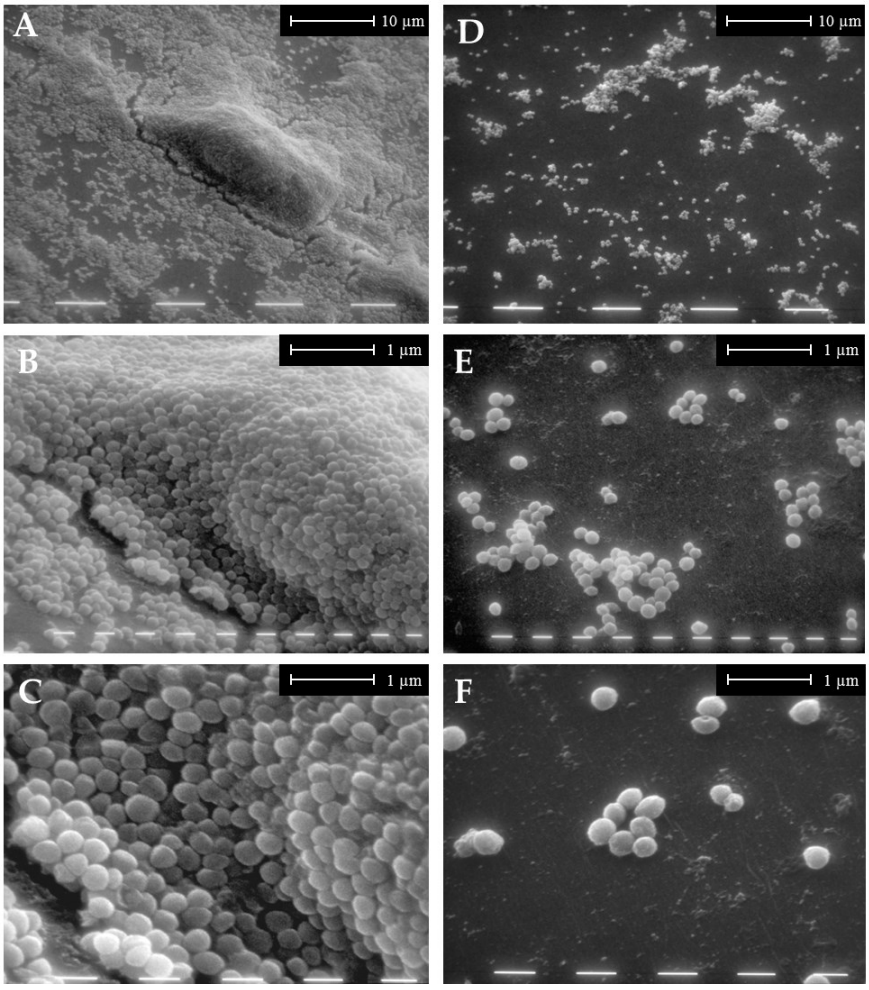

Figure 5. Scanning electron microscope micrographs of *S. aureus* ATCC 35556 biofilms in absence (A, B, C) and in presence (D, E, F) of 80 µM 1018-K6. Magnification: 1,250 x (A, D); 5,000 x (B, E); 10,000 x (C, F).

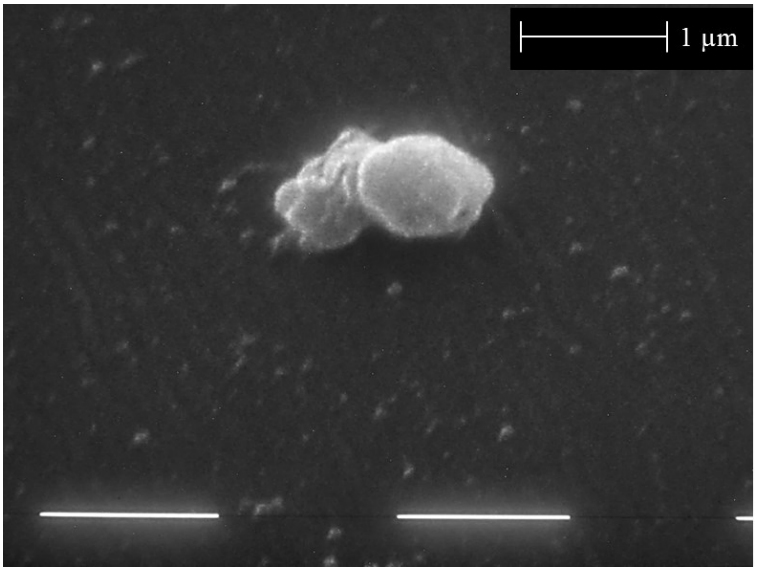

Figure 6. Scanning electron micrograph showing the blebbing of bacterial membrane of *S. aureus* ATCC 35556 after treatment with 1018-K6. Magnification: 20,000 X. Scale bar: 1 µm.

DISCUSSION

Planktonic cells are able to quickly proliferate and disseminate microorganisms to new surfaces. On the other hand, cells within a biofilm may represent critical source of contamination because of their ability to adhere to surfaces (Satpathy, Sen, Pattanaik, & Raut, 2016). Furthermore, biofilms constitutes a highly effective defense barrier that protects microorganisms from antimicrobial treatments (Flemming & Wingender, 2010).

In this work, it was investigated the antimicrobial activity of a novel antimicrobial peptide, 1018-K6, derived from IDR-1018, against both planktonic and sessile cells of *S. aureus* ATCC 35556. This strain was characterized by a remarkable ability to form biofilm (Cramton, Gerke, Schnell, Nichols, & Götz, 1999; Di Ciccio et al., 2015; Seidl et al., 2008; Thompson et al., 2010). It was shown to produce a complex three-dimensional structure embedded in considerable amount of exopolysaccharide matrix on different common food-contact surfaces, such as polystyrene and stainless steel (Di Ciccio et al., 2015).

IDR-1018 is a small cationic peptide developed based on bactenecin, a peptide antibiotic isolated from bovine neutrophil granules (de la Fuente-Núñez et al., 2014), whose antimicrobial and anti-biofilm activity has been well-studied in different works (Andresen, Tenson, & Hauryliuk, 2016; de la Fuente-Núñez et al., 2014; Reffuveille et al., 2014).

1018-K6 derives from IDR-1018, differing from the parent peptide in a single amino acid (A→K). This single point mutation has a strong impact on the conformational status of 1018-K6, that involve α-helix structures, differently from IDR-1018, characterized by a predominant β-sheet structure (Palmieri et al., 2018). Other findings showed a suitable propensity of 1018-K6 for helix formation only at the membrane surface, whereas it is present as unstructured, random coil form in buffer solutions (Palmieri et al., 2018). For

this reason it was hypothesized an inclusion of this peptide in the important class of α-helical AMPs (Zelezetsky & Tossi, 2006). Furthermore, 1018-K6 revealed a remarkable stability at different conditions of pH and temperatures. In particular, the peptide conserved its conformation after incubation for 2 h at 90°C (as well as IDR-1018), and appeared to better maintain its structural integrity over 24 h incubations in different buffers (pH range 1.0–11.0) with respect to IDR-1018.

The antimicrobial activity of 1018-K6 and IDR-1018 was tested against planktonic *S. aureus* ATCC 35556 cells. 1018-K6 showed the ability to inhibit the growth of planktonic cells at a concentration of 20 μM. The same concentration resulted also able to kill the 99.9% of free floating bacteria. On the other hand, a higher concentration of IDR-1018 (40 μM) was required to both inhibit and kill planktonic *S. aureus* bacteria. This value is the same of that reported by de la Fuente-Núñez and colleagues (2014), who tested IDR-1018 against a methicillin resistant *S. aureus* (MRSA) strain. Interestingly, modifications in 1018-K6 structure enable it to inhibit bacterial growth at a lower concentration (the half) of peptide than its parent.

In this study, a remarkable and significant (P=0.00) action of both 1018-K6 and IDR-1018 in inhibiting biofilm formation was observed. In particular, the peptides showed a reduction of ~90% in biomass with respect to the untreated control. Interestingly, planktonic cells treated with the peptides were not able to form biofilm, since the OD values were under the established OD cutoff value that define biofilm formation, according to Stepanović et al. (2007). The development of biofilms on surfaces present in food processing plants represent a serious issue for public health (Colagiorgi et al., 2017; Whitehead & Verran, 2015). In this scenario, the ability of 1018-K6 to inhibit biofilm formation represent a promising tool for preventing and controlling contaminations.

The ability of the peptides to eradicate established biofilms was also tested. MBEC assays showed that biofilm eradication by 1018-K6 was reached when the peptide is used at a concentration of 80 μM, even if a concentration equal to twice its MIC resulted in a 1.7 Log_{10} cfu/peg reduction of biofilm biomass. As IDR-1018 concerns, similarly to 1018-K6, the peptide showed a MBEC of 80 μM. This result differ from what reported by de la Fuente-Núñez and colleagues (2014), who found that IDR-1018 was able to kill an established biofilm at a concentration lower than the MIC. It is considered that sessile cells are usually more resistant to antimicrobials with respect to their planktonic counterpart (Høiby, Bjarnsholt, Givskov, Molin, & Ciofu, 2010), also because mature biofilms are surrounded and protected by the components of the extracellular matrix, such as DNA, polysaccharides, proteins, and others (Flemming & Wingender, 2010). For this reason the authors proposed that an unusual molecular mechanism of action of IDR-1018 was responsible for the peptide that specifically targets biofilms. An hypothesis about the discrepancies among results, suggested by Andresen et al. (2016) who also studied this molecule, is that differences are related to the technique used for evaluating peptide activity on biofilms. De la Fuente-Núñez and colleagues studied the effect of IDR-1018 on the glass of a flow cell chambers under conditions of constant medium flow, where the sorption and accumulation of the peptides on the cell surface over time could occur. On the other hand, the MIC was studied in a static system using a broth microdilution method. In this study, we used MBEC assay to study biofilm eradication. This technique, was developed by Ceri et al. (2001) in order to determine the efficacy of antimicrobials against biofilms of a variety of microorganisms. It consists in the establishment of biofilms on polystyrene pegs under batch conditions (no flow of nutrients into or out of an individual well) with gentle mixing, before being transferred to a new microtiter plate containing the antimicrobial molecules. Sonication allows the detachment of bacteria from pegs and their dispersion in the recovery

media. Differently from the classical microtiter plate assay using crystal violet, in the MBEC assay the biofilm formed on the pegs does not result from cell sedimentation but only from sessile development (Azeredo et al., 2017). Furthermore, there is a great reproducibility of thickness and architecture of biofilms formed from bacteria placed in the wells, allowing consistency and reproducibility of results (Rai, 2017).

Results from MBEC assays showed a remarkable anti-biofilm activity of both 1018-K6 and IDR-1018, since they was able to eradicate 24 h established *S. aureus* biofilms grown from both 10^3 and 10^5 cfu/ml initial inoculum concentration. In both cases the biofilms were completely eradicated after 24, 48, and 72 hours of exposure to 1018-K6 and IDR-1018.

For the first time, a kinetic of the action of 1018, as well as 1010-K6, against established biofilms was performed. Biofilms were established for 24 h starting from 10^5 cfu/ml inoculum, and then were exposed to each peptide for fifteen different time points (1 minute to 72 hours). Results showed a reduction in living bacteria already after 1 minute of exposure. Impressively, after only 10 minutes biofilms were completely eradicated by both peptides. When comparing 1018-K6 to his parent IDR-1018, it was possible to observe a major stability in antimicrobial activity during time (**Supplementary Figure 1**). The ability of the peptides to eradicate biofilms within few minutes indicate a very rapid mechanism of action. For this reason, we hypothesized that the peptides could kill bacteria in a way different from that indicated by de la Fuente-Nunez et al. (2014). In fact, they proposed a specific anti-biofilm effect based on the binding of the intracellular stringent response messenger (p)ppGpp – a highly complex and chiral molecule – and its targeting for degradation, allowing biofilm dispersal. This mechanism would require a time period longer than few minutes to work. Also Andresen and colleagues (2016) hypothesized that IDR-1018 does not specifically disrupt biofilms via a direct and specific interaction with (p)ppGpp.

An idea of the effect of the 1018-K6 peptide come from results from SEM observations, that revealed strong differences in the appearance of treated or untreated pegs (**Figures 5 and 6**). In particular, control pegs resulted covered by several cluster of thick, multilayered, three-dimensional structures of sessile cells. In contrast, microscopic observations of 1018-K6 treated biofilms revealed small aggregates of cells presenting blebbing on their surface (**Figure 6**). The modification of bacterial morphology suggest that the action of 1018-K6 could be directed to the cell-envelope, rather than to the intracellular messenger (p)ppGpp.

CONCLUSIONS

Staphylococcal food poisoning is still one of the most common foodborne diseases worldwide. Food contamination can occurs from bacteria sloughing from biofilms that have formed on surfaces of the processing plant. Preventing and controlling biofilms is a challenge because of their generally increased resistance to commons antimicrobials, e.g. commonly used disinfectants. In this scenario, AMPs are emerging as tools able to prevent biofilm formation and disrupt established biofilms.

In this work we found that the 1018-K6 peptide, deriving from IDR-1018, has a remarkable antimicrobial and anti-biofilm activity. In particular, it was able to eradicate staphylococcal biofilms within 10 minutes, as well as preventing biofilm formation, and killing planktonic cells. SEM observations revealed an effect on the bacterial envelope, showing blebbing on 1018-K6 treated cells. These results (rapid action and blebs formation) suggest a mechanism of action different to that proposed for IDR-1018 by de la Fuente-Nunez (2014) based on the interaction and inhibition of the intracellular messenger (p)ppGpp.

The antimicrobial and anti-biofilm properties, as well as its uncommon stability at different temperatures (i.e. 12 and 90 °C) and pHs, make 1018-K6 a good candidate for a wide range of application, e.g. antimicrobial packaging systems, solution for cleaning and disinfecting, both in food industry and in clinical applications.

Further studies are necessary to evaluate 1018-K6 activity, e.g. against wild isolates of pathogenic and spoiler bacteria, e.g. when coated on food packaging material.

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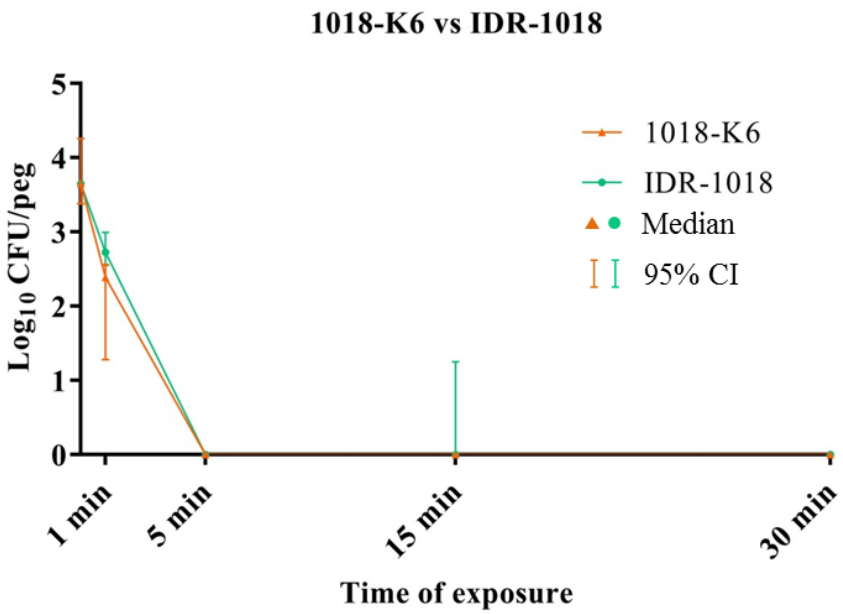
Supplementary Figure 1. Eradication activity of 1018-K6 or IDR-1018 against 24h-old *S. aureus* biofilms within 30 min of exposure.



CHAPTER 8

Summary and Conclusions

SUMMARY AND CONCLUSIONS

Many bacteria are able to adhere to surfaces and form biofilms. In the food industry, the presence of biofilms can be responsible for microbial contamination of the processed products, with economic (reduced shelf-life of products) and health (increased risk of food poisoning from pathogens) consequences. The biofilm formation is a complex and multifactorial process that is influenced by a variety of factors related to the properties of both the microorganisms and the surfaces. *Staphylococcus aureus* is an ubiquitous and opportunistic pathogen, as well as a well-known biofilm producer, although the molecular mechanisms that underline this process are not completely understood.

In this Ph.D. thesis, it has been analyzed the molecular characteristics of food-related *S. aureus* strains, as well as their biofilm forming ability. Both methicillin -sensitive and -resistant *S. aureus* (MSSA and MRSA) isolates have been molecular characterized and tested for their biofilm phenotype. Furthermore, resistance to antimicrobials in their planktonic and biofilm form has been surveyed, and a new strategy for staphylococcal biofilm prevention and control was developed.

Different studies have been conducted in order to investigate molecular mechanisms involved in *S. aureus* biofilm formation. A first study (in collaboration with Agroscope, the Swiss Confederation's center of excellence for agricultural research) was focused on the analysis of the biofilm formation of dairy isolated *S. aureus* strains belonging to different genotypes. It was observed a strain to strain variation in biofilm forming ability, as well as influence of growth conditions. In particular, all the strains belonging to GTB were able to form moderate to strong biofilm under static conditions, although the pronounced biofilm-forming potential of *S. aureus* GTB was not that obvious under dynamic conditions. Despite these differences, the fact that biofilm formation was observed in either condition indicates that the isolate possesses the genetic information necessary for biofilm

formation, with concern to food safety, because of toxin production ability of such strains. The results were also validated through Scanning electron microscope (SEM) observations.

A second study (in collaboration with "Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia Romagna") was aimed at testing the ability of *S. aureus* isolates from the dairy production chain in Northern Italy to form biofilm, and to explore their genetic diversity in terms of population structure and presence of genes involved in biofilm formation or enterotoxins production, that was scarcely investigated. In total, 19 of the 49 (38.7%) analyzed isolates were biofilm producers. No correlation between the distribution of adhesion genes and biofilm formation was found. Different Sequence Types (STs) were observed amongst the *S. aureus* isolates, with STs common to multiple dairy facilities identified. Two methicillin-resistant isolates were also found. The majority of the genotypes found are generally bovine associated, however they have been also reported frequently in human clinical cases (Feltrin *et al*., 2015). Although in this study the pronounced biofilm forming potential of *S. aureus* GTB was not showed as in the previous one, it was observed that GTB was the prevalent genotype among the biofilm forming strains (31%). Data in literature on biofilm formation by different genotypes of food-related *S. aureus* are limited, and biofilm formation by GTB strains in comparison with other genotypes was investigated only in these two studies. In this second work a greater number of *S. aureus* strains (included GTB) were tested. Anyway, further studies are needed to evaluate the contribution of biofilm formation to the persistence of *S. aureus* ST8-GTB in the dairy chain and show if the GTB possesses the genetic information necessary to form biofilms better than other genotypes.

The typing of the accessory gene regulator (*agr*), a well-defined quorum sensing system which plays an important role in promoting virulence and regulating biofilm (Shopsin *et al*., 2003), allowed the identification of an association between biofilm production and the *agr* type III.

A third study was conducted in collaboration with University of Foggia, University of Pisa, and "*Istituto Zooprofilattico Sperimentale di Puglia e Basilicata"* in order to investigate biofilm formation of MRSA strains isolated from dairy and meat industry on two commonly used food-contact surfaces, i.e. polystyrene and stainless steel. Results showed that genotypically different isolates of MRSA have different abilities to produce biofilm on food-contact surfaces, although no correlation was found between the presence of biofilm-associated factors and biofilm production, as also reported in other studies (Tang *et al.*, 2013). Interestingly, statistical analysis showed an association between a specific type of staphylococcal cassette chromosome *mec* (SCC*mec*, responsible for the resistance to β-lactams antibiotics), i.e. SCC*mec* IVa, and biofilm forming ability ($p \leq 0.05$).

The growing concern about antimicrobial resistance phenomenon drived us to explore antimicrobial resistance in MRSA isolated from the swine chain in Northern Italy, in collaboration with "Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia Romagna", as part of Classyfarm, a national priority project chartered by the Italian ministry of health. Isolates were screened for their sensitivity against a panel of 15 antibiotics in their planktonic form using disk diffusion assay, showing that almost the 90% of the strains were multidrug resistant. After been screened for their biofilm forming ability, the biofilm-positive isolates were tested in their planktonic and sessile form against the critically important antimicrobials gentamicin and enrofloxacin (WHO, 2017). Results showed that both antibiotics were unable to eradicate biofilms, and that isolates were up to 512-fold more resistant in their biofilm form.

Since biofilm could act as a mechanism for the tested swine-isolated MRSA strains to get a better survival, these results stress once more the importance of finding and developing new alternatives to common antibiotics to overcome the bacterial resistance issue. For this reason, in collaboration with University of Naples "Federico II" and the institute of Bioscience and

Bioresources - UOS Naples of the National Research Council (IBBR-CNR), it has been studied a strategy for the prevention and the eradication of staphylococcal biofilms. In particular, it has been evaluated the anti-biofilm activity against *S. aureus* of a novel antimicrobial peptide (named 1018-K6) designed starting from bactenecin sequences and already characterized in terms of bactericidal action against foodborne pathogens. 1018-K6 was tested against a reference strong biofilm producer *S. aureus* strain. The peptide showed a marked and uncommon structural stability under several environmental conditions as determined by different spectroscopic analyses. 1018-K6 was able to kill staphylococcal planktonic cells, as well as to prevent biofilm formation. Furthermore, eradication of 24 hours old biofilms was achieved. In particular, the *S. aureus* reference strains biofilm was eradicated within few minutes.

The works presented in this thesis have contributed to enhance the knowledge of the biofilm producing ability of food-related *S. aureus* strains and of the molecular mechanisms potentially involved in this process. The capacity of the staphylococcal isolates to produce biofilms on food contact surfaces was highlighted, with concern for public health since they can constitute a potential source for persistence of this pathogen in the food industry. We observed the influence of environmental conditions relevant for the food industry, e.g. temperature and surface properties, on the biofilm production. From a molecular point of view, the influence of the genotype on biofilm production is controversial and need to be further investigated, as well as the role of the *agr* type III in the sessile development of isolates. In this thesis, it has also been observed the presence and the spread of the multidrug resistance phenomenon in the Italian swine chain, as well as the role of biofilm formation in enhancing antimicrobial resistance. Results obtained from the evaluation of the activity of 1018-K6 against MSSA and MRSA represent a starting point for assessing the variety of potential applications of 1018-K6 in the food sector, such as active packaging for the

increase of shelf life and safety of products, as well as solutions for cleaning and disinfection of surfaces in food processing plants.

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ACKNOWLEDGMENTS

RINGRAZIAMENTI

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Come in ogni percorso che giunge a conclusione e che ci porta a voltarci indietro e a ripercorrere le tappe e i passi che ci hanno condotti fin lì, ripercorro con gratitudine questo scorcio di vita.

Il mio grazie va innanzi tutto alla prof.ssa Ianieri, perché con stima e fiducia mi ha sempre supportato nell'affrontare tutte le sfide di questo dottorato, e mi ha dato la possibilità di imparare, formarmi, e di crescere nella conoscenza. Grazie per aver condiviso con me il suo enorme bagaglio di conoscenze, e grazie per avermi dato la possibilità di "girare l'Italia e l'Europa", consentendomi così di conoscere ricercatori e scienziati di ogni parte del mondo, di poter imparare da loro, di vedere da vicino il loro lavoro.

Ringrazio Pierluigi, che è stato presenza importante durante l'intero percorso del dottorato. Grazie per la sua esperienza condivisa, per gli scambi di idee, le mediazioni, le chiacchierate... semplicemente per il suo esserci.

Ringrazio Emanuela, che è stata sempre un punto di riferimento importante, senza cui forse non sarei arrivato fin qui, e Sergio, che è stato un importante dispensatore di consigli e grande conoscitore della materia. Grazie anche a tutte le persone che hanno condiviso con me un pezzo di questo traguardo, professionalmente e non solo.

Questi tre anni mi hanno visto crescere professionalmente, ma al contempo hanno fatto da testimoni a cambiamenti profondi, con l'arrivo di Davide Michele che ha arricchito di gioia (ma anche di occhiaie e fatiche) le nostre giornate e la nostra famiglia, regalandoci una bellezza che non ha pari. E allora, il mio grazie di cuore va a te, amore mio, che con pazienza, docilità, e costanza mi sei sempre stata accanto, condividendo gioie, risa, amarezze, e

ogni tipo di fatica. Grazie, Anna Grazia, per l'amore con cui ogni giorno ti doni a me e alla nostra famiglia.

Un grande grazie ai miei genitori e alla mia famiglia d'origine, perché, ancora una volta a centinaia di chilometri di distanza, sono stati presenza e supporto.

Grazie ai miei fratelli e sorelle, di sangue e non solo, per il cammino di vita condiviso.

E grazie soprattutto a Colui, senza il quale nulla di tutto ciò sarebbe stato possibile.

Vostro,

Angelo

ABOUT THE AUTHOR

Angelo Colagiorgi was born on 4th June 1988 in the province of Lecce, in South Italy. In 2007, he was accepted to the degree programme in Biotechnology at the University of Parma. After completion of the bachelor, in 2013 he took his master degree, graduating summa cum laude, in Medical, Veterinary and Pharmaceutical Biotechnologies in the same university. The results of the project of his traineeship, supervised by prof. Gaetano Donofrio, are collected in the master thesis "Generation of recombinant BoHV-4 expressing Monkeypox virus antigens".

Shortly after graduating, he successfully applied for a fellowship at the *Probiogenomics Lab* of University of Parma, where he spent 20 months on the project "functional genomics of bifidobacteria" under the supervision of prof. Marco Ventura.

In September 2015, Angelo passed the selection for the Doctoral School in Food Science at University of Parma, under the supervision of prof. Adriana Ianieri. During his PhD, he has taken a strong interest in microbial biofilms, and he had the opportunity to present the results of his research at national and international scientific meeting. The results achieved during his PhD are described in this thesis.