UNIVERSITATEA „DUNĂREA DE JOS” DIN GALAȚI

Școala Doctorală de Inginerie

UNIVERSIDAD DE BURGOS

Avances en Ciencia y Biotecnología Alimentarias

TEZĂ DE DOCTORAT

Caracterizarea tulpinilor de Staphylococcus aureus meticilino-rezistente izolate din produsele alimentare

Characterization of Methicillin-Resistant Staphylococcus aureus Strains Isolated from Foods

Autor,

Drd. Ing. Elena-Alexandra ONICIUC

Conducător științific,

Prof. Dr. Ing. Anca Ioana NICOLAU

Conducător științific în cotutelă,

Prof. Dr. Vet. David RODRÍGUEZ-LÁZARO

Seria I4: Inginerie Industrială **Nr.** 47

Galați 2017

UNIVERSITATEA „DUNĂREA DE JOS” DIN GALAȚI

Școala Doctorală de Inginerie

UNIVERSIDAD DE BURGOS

Avances en Ciencia y Biotecnología Alimentarias

Nr. Decizie Senat ..... din .....

TEZĂ DE DOCTORAT

Caracterizarea tulpinilor de Staphylococcus aureus meticilino-rezistente izolate din produsele alimentare

Characterization of Methicillin-Resistant Staphylococcus aureus Strains Isolated from Foods

Autor,

Drd. Ing. Elena-Alexandra ONICIUC

COMISIA DE DOCTORAT

Președinte	Prof. Dr. Ing. Habil. Gabriela Râpeanu	Universitatea „Dunărea de Jos” din Galați
Conducător de doctorat	Prof. Dr. Ing. Anca Ioana Nicolau	Universitatea „Dunărea de Jos” din Galați
Conducător de doctorat în cotutelă	Prof. Dr.Vet. David Rodríguez-Lázaro	Universidad de Burgos
Referent	Conf. Dr. Vet. Marta Hernández Perez	Universidad de Valladolid
Referent	Prof. Dr. Biol. Mariana Carmen Chifiriuc	Universitatea din București
Referent	Prof. Dr. Ing. Daniela Borda	Universitatea „Dunărea de Jos” din Galați

Seria I4: Inginerie Industrială **Nr.** 47

Galați 2017

Seriile tezelor de doctorat susținute public în "Universitatea „Dunărea de Jos" din Galați, începând cu 1 Octombrie 2013 sunt:

Domeniul **ȘTIINȚE INGINEREȘTI**

Seria I 1: **Biotehnologii**

Seria I 2: **Calculatoare și tehnologia informației**

Seria I 3: **Inginerie electrică**

Seria I 4: **Inginerie industrială**

Seria I 5: **Ingineria materialelor**

Seria I 6: **Inginerie mecanică**

Seria I 7: **Ingineria produselor alimentare**

Seria I 8: **Ingineria sistemelor**

Domeniul **ȘTIINȚE ECONOMICE**

Seria E 1: **Economie**

Seria E 2: **Management**

Domeniul **ȘTIINȚE UMANISTE**

Seria U 1: **Filologie-Engleză**

Seria U 2: **Filologie-Română**

Seria U 3: **Istorie**

The research activities achieved in the present thesis took place in different national and international laboratories, in a stimulating environment for working, as following:

- Laboratory of Physico-chemical and Microbiological Analysis of Food from Faculty of Food Science and Engineering (Galati, Romania);
- Laboratory of Molecular Biology Techniques from Faculty of Food Science and Engineering (Galati, Romania);
- Laboratory of Microbiology from Faculty of Science (Burgos, Spain);
- Instituto Tecnológico Agrario de Castilla y León (Valladolid, Spain);
- Center of Biological Engineering from University of Minho (Braga, Portugal).

The work presented in this thesis has been partially financed from logistic point of view by the EU research project "*Protection of Consumers by Microbial Risk Mitigation Through Combating Segregation of Expertise*" (PROMISE- project number 265877), in which one objective of this project was to assess five significant foodborne pathogens for being introduced into EU *via* uncontrolled imports (www.promise-net.eu), of which *S. aureus* was investigated by all project partners in raw and ready-to-eat (RTE) food collected either from ports and airports or from terrestrial EU borders.

Moreover, the PhD student beneficiated of different European grants for performing the research activities, such as:

- COST Action "*A European Network for Mitigating Bacterial Colonisation and Persistence on Foods and Food Processing Environments*" grant (STSM-FA1202-011015-063402);
- FEMS Research Grant for Early Carrier Scientist (FEMS-RG-2015-0053);
- COST Action "*A European Network for Foodborne Parasites (Euro-FBP)*" grant (STSM-FA1408-33147);
- Erasmus+ mobility.

"We cannot fathom the marvelous complexity of an organic being; but on the hypothesis here advanced this complexity is much increased. Each living creature must be looked at as a microcosm- a little universe, formed of a host of self-propagating organisms, inconceivably minute and as numerous as the stars in heaven."

— Charles Darwin

TABLE OF CONTENTS

Key words:
Staphylococcus aureus, methicillin-resistant *Staphylococcus aureus*, antibiotics, food contamination, food safety, illegal import, food industry, biofilm, transmission, surveillance, EU.

Acknowledgements

Looking back, I cannot believe how time flies away, and at this stage in my life I cannot help but remember the special moments I lived and the good things which happened to me during my Master and Doctoral Studies. Being now in the position of presenting my PhD thesis, I would like to thank the people who supported me and significantly contributed to my professional development.

My first thoughts go to my beloved coordinators *Prof. Eng. Anca Ioana Nicolau*, PhD and *Prof. David Rodríguez-Lázaro*, PhD, who guided my research throughout this period. Professor *Anca Ioana Nicolau* 's precious help remains immeasurable. If it had not been for her professionalism, patience and constant scientific guidance, I would not have become what I am today. As regards Professor *David Rodríguez-Lázaro*, one of the top food microbiologists in Europe, who introduced me to the branch of antibiotic resistance, I will always be grateful to him for the motivation he gave me and the immense knowledge he passed to me. It was a great honor for me to work with Professor *David Rodríguez-Lázaro* and I am deeply grateful for the innovative approaches he suggested in my experimental research.

My very special thanks also go to *Prof. Marta Hernández,* PhD who facilitated my understanding of the molecular biology techniques used in the food microbiology and who made insightful comments which helped me a lot in my research.

I would like to express my sincere gratitude to the thesis committee: *Prof. Eng. Daniela Borda,* PhD, *Prof. Eng. Gabriela Bahrim,* PhD and *Assoc. Prof. Vasilica Barbu*, for all the advice they gave me during my PhD studies.

I would also like to thank the people whom I worked with at *Dunarea de Jos University of Galati* (Romania) and at *Universidad de Burgos* (Spain) for their professional support, guidance and precious advice, in particular. Among others, I am thanking *Lecturer Eng. Iulia Bleoancă* and *Secretary Eng. Gabriela Gavrilă*, for the suggestions and recommendations made throughout my studies and research.

Special thanks to all the people who offered me precious support in the research institutions from the Center of Biological Engineering (Portugal), Universidad de Burgos and Instituto Tecnológico Agrario de Castilla y León (Spain) during my mobility periods.

Thank you, my lab mates *Andrei, Narciso* and *Florentina*. We shared numerous sleepless nights and many fruitful working sessions. Thank you, *Bernar, David, Jaime, Lorena, Patricia, Julian, MariCruz* and *Raul* for the friendship you showed me at ITACyL, in my "second home". And thank you, *Monica*, *Nicole, Gislaine, Zoe, Mariela* and my Romanian friend, *Alina*, for constantly being by my side.

Finally, I would like to thank to my beloved family who have given me constant support over these years, whom I love unconditionally and who represent the most important people in my life: *my parents*, Laura Felicia and Eugen, *my sister*, Ana Maria, my *beloved grandparents* and *future parents*, *Andreea* and to *my other half*, Alexandru.

Elena-Alexandra Oniciuc

Mulțumiri

Nu îmi vine să cred cât de repede au trecut toți acești ani iar acum, uitându-mă înapoi, retrăiesc cu emoție toate momentele deosebite care mi s-au întâmplat pe toată perioada studenției, de la studiile de licență până la cele de doctorat. Fiind acum în poziția de a-mi prezenta teza de doctorat, îmi doresc să adresez câteva cuvinte de mulțumire celor care m-au îndrumat, m-au sprijinit și au contribuit în mod semnificativ la dezvoltarea mea profesională.

Primele gânduri se îndreaptă către coordonatorii mei științifici, doamna *prof. dr. ing. Anca Ioana Nicolau* și domnul *prof. dr. David Rodríguez-Lázaro*, cărora le mulțumesc pentru permanenta lor îndrumare, sprijinire și încurajare de-a lungul perioadei de pregătire a doctoratului și de elaborare a tezei. Doresc să îmi exprim recunoştinţa față de doamna profesor *Anca Ioana Nicolau*, care mă călăuzește încă din al doilea an de licență, pentru profesionalismul dumneai, pentru răbdarea și îndrumarea științifică constantă. Dacă nu ar fi fost dumneai, nu aș fi devenit ceea ce sunt astăzi.

În egală măsură, doresc să îi mulțumesc domnului prof. dr. *David Rodríguez-Lázaro*, unul dintre microbiologii de top din Europa, cel care m-a introdus în ramura legată de rezistența bacteriilor la antibiotice, pentru modul în care m-a motivat, pentru cunoștințele transmise și pentru abordările inovatoare sugerate în cercetarea mea îi sunt profund recunoscătoare. A fost o mare onoare pentru mine să lucrez cu domnia sa.

În aceeași măsură, adresez mulțumiri deosebite doamnei *prof. dr. Marta Hernández* pentru facilitarea înțelegerii tehnicilor de biologie moleculară utilizate în microbiologia alimentară și pentru toate sfaturile contructive oferite pe perioada cercetării mele.

Doresc să îmi exprim gratitudinea față de membrii comisiei de evaluare a lucrării: doamna *prof. dr. ing. Daniela Borda*, doamna *prof. dr. ing Gabriela Bahrim* și doamna *conf. biol. Vasilica Barbu*, pentru sfaturile și sugestiile oferite dar și pentru diferitele perspective de extindere a cercetării experimentale.

Aș dori, de asemenea, să le mulțumesc celor cu care am lucrat, atât la *Universitatea „Dunarea de Jos" din Galați* (România), cât și la *Universidad de Burgos* (Spania) pentru sfaturile și sprijinul acordat pe toată perioada cercetării. Mulțumesc în mod deosebit doamnei *lector dr. ing. Iulia Bleoancă* și doamnei *ing. Gabriela Gavrilă*, pentru sfaturile prietenoase pe care mi le-au oferit.

Tuturor colegilor mei din laborator: *Andrei, Narciso și Florentina*, le datorez mulțumiri, pentru nopțile albe petrecute înainte de orice deadline al vreunei lucrări. Mulțumesc lui *Bernar, David, Jaime, Lorena, Patricia, Julian, MariCruz și Raul* pentru prietenia arătată la ITACyL, „a doua mea casă". Mulțumesc *Monicăi, lui Nicole, Gislainei, lui Zoe, Marielei* și nu în ultimul rând, îi mulțumesc *Alinei*, pentru sprijinul moral acordat.

În încheiere, aș dori să mulțumesc în mod special familiei mele iubite, care m-a sprijinit necondiționat pe toată perioada studiilor doctorale și care a subliniat întotdeauna importanța unei bune educații. Părinților, *Laura Felicia și Eugen*, surorii mele, *Ana Maria*, *bunicilor* și *viitoriilor părinți*, *Andreei* și, nu în ultimul rând, celui care mă întregește, *Alexandru*.

Elena-Alexandra Oniciuc

Agradecimientos

Mirando hacia atrás, no puedo creer cómo el tiempo se aleja, y en esta etapa de mi vida no puedo evitar recordar los momentos especiales que viví y las cosas buenas que me sucedieron durante mis estudios de licenciatura y doctorado. Al estar ahora en la posición de presentar mi tesis de doctorado, quisiera agradecer a las personas que me apoyaron y contribuyeron significativamente a mi desarrollo profesional.

Mis primeros pensamientos van a mis queridos coordinadores *Prof. Eng. Anca Ioana Nicolau* y el *Prof. David Rodríguez-Lázaro*, quienes guiaron mi investigación a lo largo de este período. La preciosa ayuda del profesor *Anca Ioana Nicolau* sigue siendo inconmensurable. Si no hubiera sido por su profesionalismo, paciencia y constante orientación científica, no me habría convertido en lo que soy hoy. En cuanto al profesor *David Rodríguez-Lázaro*, uno de los mejores microbiólogos de Europa, que me ha presentado a la rama de la resistencia a los antibióticos, le agradeceré siempre por su motivación que me ha dado y el inmenso conocimiento que me ha transmitido. Fue un gran honor para mí trabajar con el profesor David Rodríguez-Lázaro y estoy profundamente agradecido por los enfoques innovadores que sugirió en mi investigación experimental.

Mi agradecimiento muy especial también para *Prof. Marta Hernández*, que me facilitó mi comprensión de las técnicas de biología molecular utilizadas en la microbiología alimentaria y que me hizo comentarios perspicaces que me ayudaron mucho en mi investigación.

Quisiera expresar mi sincera gratitud al comité de tesis: *Prof. Eng. Daniela Borda*, PhD, *Prof. Eng. Gabriela Bahrim*, PhD y *Asoc. Prof. Vasilica Barbu*, por todos los consejos que me dieron durante mis estudios de doctorado.

También quiero agradecer a las personas con las que trabajé en la *Universidad de Dunarea de Jos de Galati* (Rumania) y en la *Universidad de Burgos* (España) por su apoyo profesional, orientación y preciosos consejos, en particular. Entre otras cosas, estoy agradeciendo al *Prof. Eng. Iulia Bleoancă* y el *Eng. Gabriela Gavrilă*, por las sugerencias y recomendaciones hechas a lo largo de mis estudios e investigaciones.

A todas las personas que me ofrecieron un valioso apoyo en las instituciones de investigación del *Centro de Ingeniería Biológica* (Portugal), la *Universidad de Burgos* y el *Instituto Tecnológico Agrario de Castilla y León* durante mis periodos de movilidad.

Gracias, mis compañeros de laboratorio *Andrei, Narciso* y *Florentina*. Compartimos muchas noches sin dormir y muchas sesiones de trabajo fructíferas. Gracias, *Bernar, David, Jaime, Lorena, Patricia, Julián, MariCruz* y *Raúl* por la amistad que me mostraste en ITACyL, en mi "segunda casa". Y gracias, *Mónica, Nicole, Gislaine, Zoe, Mariela* y mi amiga rumana, *Alina*, por estar constantemente a mi lado.

Por último, quisiera dar las gracias a mi amada familia que me ha dado un apoyo constante durante estos años, a quienes amo incondicionalmente y que representan las personas más importantes de mi vida: mis padres, *Laura Felicia y Eugen*, mi hermana *Ana María*, mis amados *abuelos* y *futuros padres*, *Andreea* y mi otra mitad, *Alexandru*.

Elena-Alexandra Oniciuc

Background, Scope and Approach, Key Objectives

Staphylococcus aureus is an opportunistic bacterium which has drawn great interest for its high potential risks that may acquire as a clinical and epidemiological pathogen. Over the years, it has also been established that its potential pathogenic role as a foodborne pathogen should not be neglected.

Apart of its enterotoxigenic capacity and the leading cause of almost all food poisoning outbreaks worldwide, antimicrobial resistance is another major challenge of which methicillin-resistant *Staphylococcus aureus* (MRSA) is a particularly problematic nosocomial pathogen.

The first staphylococcal infections from clinical settings appeared in the late '50s, shortly after the introduction of antimicrobial drugs such as penicillins, so the urgent need of alternative ones was imperative. Soon, an increasing number of staphylococcal outbreaks demanded alternative semi-synthetic drugs of which methicillin and oxacillin, belonging to penicillin family group, started to be used. Not surprisingly, the first strains resistant to methicillin emerged and started to be associated with nosocomial infections. Resistance to methicillin is conferred by the acquisition of *mec*A or *mec*C genes, central elements of staphylococcal chromosomal cassettes, which are codifying a penicillin-binding protein designated as PBP2a or PBP2', with low affinity for β-lactam drugs. However, the resistance determinants are not yet clear since studies suggested that *mec* gene might be transmitted between *S. aureus* strains and other coagulase negative staphylococcal species. For example, the principal epidemic clones of MRSA might have, on its origins, a *mec*A-carrying SCC*mec* element coming from methicillin-susceptible *S. aureus* (MSSA) strains.

In the early '90s, MRSA started to be found in non-healthcare settings and, for differentiating community isolated strains from hospital strains, they were called community acquired methicillin-resistant *S. aureus* (CA-MRSA). More recently, other new MRSA strains emerged with a zoonotic potential being recognized and designated as livestock-associated MRSA (LA-MRSA).

Looking back to its origin and the high number of outbreaks caused by, we can assume that MRSA represents a relevant nosocomial and foodborne pathogen of which Public Health Systems and Food Safety Agencies, on a worldwide level, are nowadays not neglecting it. Likewise, until recently, the relevance of its emergency in the food chain has not been fully considered, as zoonotic transmission had not been yet demonstrated and food-related transmission was not evident.

More and more scientific studies document the involvement of such strains in the dissemination of different MRSA lineages among the food chain. The current knowledge about MRSA coming from food producing animals (raw materials for food industry) and associated foodstuffs demonstrate that antibiotic resistant strains can be transmitted to humans, along the food chain, by the consumption of such foods. Moreover, the overuse/or misuse of antibiotics in feed to promote growth, and in veterinary and human medicine could also be contributors to the emergence of MRSA resistance. Then, an array of questions may rise: Should population be aware of the possible emerging risks associated to MRSA in the food chain? What are the consequences, from a food safety perspective, if foods are not having any traceability in place? Should we expect other new mechanisms of resistance that can burst into new variants of MRSA, by enforcing developments in MRSA's epidemiology?

In the present thesis, which is entitled **"*Characterization of methicillin-resistant Staphylococcus aureus strains isolated from foods*"**, are discussed recent literature findings about developments in the epidemiology of MRSA, from hospital (human) settings and primary food production, to MRSA spread along the food chain. Devising this, the thesis approach was to investigate presence of MRSA in food samples confiscated from air and ground border traffic from different travelers entering to the European Union (EU), as nowadays is a growing concern regarding potential routes in which MRSA can be distributed.

In this context, the research activities during doctoral studies had the **main objective** focused on dissemination of MRSA from food illegally imported to EU from non-EU countries. High number of foreigners are illegally coming with food in their personal luggage, further being confiscated by the border inspection posts in different points of entering (ports, airports, terrestrial borders) to EU. By any carelessness, they declare that foods are brought by them for personal consumption and end up being illegally sold afterwards (known as "contraband" or "smuggled" food). As neither raw material origin and quality, nor technological process and hygienic conditions during food processing are known, illegally imported food poses a potential health risk. Additionally, the lack of refrigeration conditions and/or adequate packaging during transportation and sale might violate the safety rules. Usually little information is available regarding associated risks and prevalence of pathogens in these foods.

This retrospective thesis emphasizes the impact of such strains on public health, by discussing the potential routes of illegally food introduced into the EU space by travelers, either by air or ground border posts. A special situation exists at the Eastern EU border, ratified by Romanian Law 10/2010 (Monitorul Oficial), in which cross-border traffic between Romania and the Republic of Moldova is allowed based on an agreement. However, foods that are officially declared for personal use are legally brought into EU, but illegally sold in local Romanian markets organized to sell fresh fruits and vegetables. Even though selling food of animal origin is forbidden in these markets and that the local authorities are often checking such markets, animal origin foods are daily sold. A total of 210 food products have been analyzed at ground border traffic between Republic of Moldova and Romania (Giurgiulești-Galați).

Additionally, foods coming from passengers' luggage from non-EU countries has been confiscated by the border inspection posts in International Bilbao Airport (Spain) (269 food products) and Vienna International Airport (Austria) (600 food products).

In both cases, foods, either homemade or industrially produced, were kept at ambient temperature and often displayed out of the original package. Moreover, as these food products were transported, stored, and sold under conditions that facilitated the growth of pathogens, they might represent a potential threat to consumers' health. Besides investigating neglected routes of MRSA transmission to the EU, this thesis aims to analyze the routes of pathogenic genotypes involved in the illegally sold food.

The research activities carried out during the doctoral studies have been targeted the following **key scientific objectives**, presented below:

- Overview on the recent findings regarding the actual problematic of MRSA in the food chain, highlighting the need for adequate control and prevention programmes by providing current information from EU surveillance programmes;

- Focal point on neglected routes of transmission of MRSA *via* foods introduced from non-EU countries as personal goods but meant to be illegally sold to EU consumers, highlighting the role that food could play in the prevalence and dissemination of MRSA;
- Global results obtained regarding identification, isolation and characterization of MRSA strains following phenotypical and genotypical approaches;
- Correlation of genotypic aspects of MRSA strains and their biofilm formation and composition, by bringing improvements of better strategies for cleaning surfaces or cross-contamination events;
- Investigation based on whole genome sequencing (WGS) for identification of virulence factors and genes associated with antimicrobial resistance in an oxacillin-susceptible (OS)-MRSA strain;
- Evaluation of two commercially available chromogenic media for confirmation of MRSA from human, animal, and food samples;
- Integration of results obtained in the present thesis in the framework of the worldwide studies focused on dissemination of different lineages of MRSA together with the necessary information for understanding potential risks that *S. aureus* resistant to antimicrobials may represent.

The research provided in the present thesis would not have been possible without a strong communication between research institutions, from Romania, Spain, Portugal or Austria, both in the frame of FP7 PROMISE project and beyond.

Context actual și obiective cheie

Staphylococcus aureus este o bacterie oportunistă, căreia i se acordă un interes deosebit din cauza riscurilor de a produce infecții, a căror gravitate pot pune în pericol sănătatea omului. De-a lungul anilor, incidența acesteia a crescut în alimentele de origine animală, motiv pentru care a început să fie privită și ca agent patogen alimentar.

Pe lângă capacitatea acestei bacterii de a produce enterotoxine, motivul principal al apariției toxinfecțiilor alimentare la nivel mondial, rezistența la antibiotice constituie o altă problemă, prin tulpinile de *Staphylococcus aureus* meticilino-rezistent (MRSA).

Primele infecții stafilococice din mediul clinic au apărut la finele anilor '50, la scurt timp după introducerea medicamentelor antimicrobiene, cum ar fi penicilinele, astfel că nevoia urgentă de medicamente alternative a fost absolut necesară. La scurt timp, din cauza numărului mare de infecții stafilococice apărute, s-au introdus medicamente semisintetice alternative, cum sunt meticilina și oxacilina, ce aveau să fie utilizate la scară largă. Desigur, la scurt timp după introducerea lor în terapie, au început să apară primele tulpini rezistente la meticilină, acestea fiind asociate cu infecțiile nosocomiale.

Rezistența la meticilină este cauzată de prezența genelor *mec*A sau *mec*C, elemente centrale mobile ale casetelor cromozomiale stafilococice, ce codifică sinteza unei proteine denumită PBP2a sau PBP2', cu afinitate scăzută pentru medicamentele β-lactamice, protejând asftel sinteza peptidoglicanului și a peretelui celular bacterian față de acțiunea antibioticelor din această clasă. Cu toate acestea, determinanții ce codifică rezistența nu sunt încă clar identificați, deoarece studiile au sugerat că gena *mec* ar putea fi transmisă între tulpini de *S. aureus* și alte specii de stafilococi coagulazo-negativi. De exemplu, principalele clone epidemice de MRSA ar putea avea, la origine, un element SCC*mec*, *mec*A provenind de la tulpinile de *S. aureus* sensibile la meticilină (MSSA).

La începutul anilor '90, tulpinile de MRSA au început să fie izolate din comunități, iar pentru diferențierea acestora de cele clinice (HA-MRSA), au fost denumite community-acquired (CA-MRSA). Recent au apărut alte tulpini noi de MRSA, cu potențial zoonotic, denumite livestock-associated (LA-MRSA).

Privind originea sa și numărul mare de focare cauzate, putem admite faptul că MRSA reprezintă un patogen nosocomial și alimentar relevant, pe care sistemele de sănătate publică și agențiile de siguranță alimentară, la nivel mondial, îl iau în considerare. De asemenea, până în prezent, relevanța acestuia în lanțul alimentar nu a fost pe deplin analizată, deoarece transmisia zoonotică nu a fost încă demonstrată, iar transmisia prin intermediul alimentelor nu a fost încă evidențiată.

Din ce în ce mai multe studii științifice evidențiază implicarea unor astfel de tulpini în diseminarea diferitelor linii genealogice de MRSA în lanțul alimentar. Cunoștințele actuale despre tulpinile de MRSA izolate de la animalele de la care provin materiile prime utilizate în industria alimentară și produsele alimentare asociate demonstrează faptul că astfel de tulpini rezistente la antibiotice pot fi transmise oamenilor, de-a lungul lanțului alimentar, prin consumul alimentelor contaminate.

Mai mult decât atât, excesul/utilizarea abuzivă a antibioticelor în hrana pentru creșterea animalelor și în medicina veterinară și umană ar putea contribui, de asemenea, la apariția rezistenței. Apoi, o serie de

întrebări sunt adresate: Ar trebui populația să fie conștientă de posibilele riscuri emergente asociate tulpinilor de MRSA în lanțul alimentar? Care sunt consecințele, din perspectiva siguranței alimentare, în cazul în care alimentele nu au nicio trasabilitate? Ar trebui să ne așteptăm la noi mecanisme de rezistență care ar putea conduce la noi variante de MRSA?

În prezenta teză, intitulată "***Caracterizarea tulpinilor de Staphylococcus aureus meticilino-rezistente izolate din produsele alimentare***", se discută recentele descoperiri din literatura de specialitate cu privire la evoluția epidemiologică a tulpinilor de MRSA. În această teză, abordarea a avut ca scop investigarea prezenței MRSA în alimente confiscate de la diferiți călători care intră în Uniunea Europeană (UE) prin intermediul vamelor din aeroporturi sau a celor situate în punctele de trecere a frontierelor terestre.

În acest context, activitățile de cercetare desfășurate pe parcursul studiilor doctorale au avut ca **obiectiv principal** studiul MRSA din alimentele importate ilegal în UE. Un număr mare de călători traversează ilegal granița UE cu alimente ascunse în bagajele personale, acestea fiind ulterior confiscate de autorități în punctele de control la frontieră asociate porturilor, aeroporturilor sau frontierelor terestre.

Cu o oarecare indiferență, aceștia comunică agenților vamali că produsele aduse sunt pentru consum personal, însă ajung să fie vândute ilegal (fenomen cunoscut sub numele de "contrabandă"). Deoarece nu se cunoaște originea/calitatea materiilor prime, nici procesul tehnologic și nici condițiile de igienă din timpul procesării alimentelor, aceste produse alimentare importate ilegal prezintă un risc potențial pentru sănătate. În plus, lipsa condițiilor de refrigerare și/sau a ambalajului adecvat în timpul transportului și vânzării încalcă regulile de igienă. De obicei, puține informații sunt disponibile cu privire la riscurile asociate și la prevalența agenților patogeni în aceste alimente.

Teza pune accentul pe impactul acestor tulpini asupra sănătății publice, discutând posibilele căi de transmitere ca urmare a introducerii acestor alimente în mod ilegal în spațiul UE de către călători. O situație specială, ratificată prin Legea 10/2010 (Monitorul Oficial), există în România, unde este permis micul trafic la frontieră între România și Republica Moldova. Astfel, alimentele declarate oficial pentru consum propriu sunt introduse legal în UE, dar vândute ilegal pe piețele locale din România. Chiar dacă autoritățile locale verifică deseori astfel de piețe, produsele alimentare provenind din micul trafic de frontieră sunt vândute zilnic. În acest sens, au fost analizate 210 probe prelevate dintr-o astfel de piață și probe din alimentele găsite în bagajele pasagerilor călătorind din țările non-UE și confiscate la posturile de control la frontieră din Aeroportul Internațional Bilbao, Spania, (269 produse alimentare) și de la Aeroportul Internațional Viena, Austria, (600 de produse alimentare).

În ambele cazuri, aceste produse alimentare, fie produse în casă sau industriale, au fost păstrate la temperatura camerei și adesea scoase din pachetul original. În plus, deoarece acestea au fost transportate, depozitate și vândute în condiții care facilitează creșterea agenților patogeni, ar putea reprezenta un risc crescut la adresa sănătății consumatorilor. În afară de investigarea rutelor de transmitere a tulpinilor de MRSA către UE, această teză urmărește să analizeze liniile genealogice care ar putea fi implicate prin intermediul acestor produse alimentare.

Activitățile de cercetare desfășurate pe parcursul studiilor doctorale au vizat următoarele **obiective științifice cheie**:

- Prezentarea generală a descoperirilor privind situația actuală a tulpinilor de MRSA în lanțul alimentar, subliniind necesitatea unor programe adecvate de control și prevenire și furnizarea de informații actuale pentru programele de supraveghere ale UE;
- Controlul rutelor de transmitere a MRSA prin intermediul alimentelor introduse din țările din afara UE ca bunuri personale, dar care sunt ilegal vândute consumatorilor din UE, subliniind rolul pe care aceste alimente l-ar putea avea în prevalența și diseminarea MRSA;
- Identificarea, izolarea și caracterizarea tulpinilor de MRSA pe baza tehnicilor fenotipice și genotipice;
- Corelarea aspectelor genotipice ale tulpinilor de MRSA cu formarea și compoziția biofilmelor produse de acestea, pentru a putea îmbunătăți strategiile în ceea ce privește curățarea suprafețelor din fabricile de industrie alimentară sau a evita episoadele de contaminare încrucișată;
- Investigarea bazată pe secvențierea genomului complet (WGS) în vederea identificării factorilor de virulență și a genelor asociate cu rezistența la antibiotice a unei tulpini susceptibile la oxacilină (OS)-MRSA;
- Evaluarea mediilor cromogenice disponibile în comerț pentru confirmarea MRSA din probele provenite de la om, animal și aliment;
- Integrarea rezultatelor obținute în prezenta teză în cadrul studiilor la nivel mondial, axate pe diseminarea diferitelor linii genealogice ale MRSA, împreună cu informațiile necesare pentru înțelegerea riscurilor potențiale pe care MRSA le poate reprezenta.

Cercetarea oferită în teza de față nu ar fi fost posibilă fără o strânsă comunicare între instituțiile de cercetare din România, Spania, Portugalia sau Austria, atât în cadrul proiectului PROMISE FP7, cât și dincolo de acesta.

Enfoque

Staphylococcus aureus es una bacteria oportunista que ha despertado gran interés por sus riesgos de alto potencial que pueden adquirir como patógeno clínico y epidemiológico. A lo largo de los años, también se ha establecido que su posible desarrollo patogénico como uno alimentario no debe ser descuidado.

Aparte de su capacidad enterotoxigénica y la principala causa de casi todos los brotes de intoxicación alimentaria en todo el mundo, la resistencia a los antimicrobianos es otro de las rutas principales donde *Staphylococcus aureus* resistente a la meticilina (MRSA), es un patógeno nosocomial particularmente problemático.

Las primeras infecciones estafilocócicas en contexto clínico aparecieron a finales de los años cincuenta; poco después de la introducción de fármacos antimicrobianos como las penicilinas, la necesidad urgente de otras alternativas fuera necesaria. Después, un número mayor de brotes de estafilococos exigieron y por esto, fármacos semisintéticos empezaron a utilizarse como meticilina y oxacilina. No es sorprendente que las primeras cepas resistentes a la meticilina surgieran y comenzaran a asociarse con infecciones nosocomiales. La resistencia a meticilina se confiere mediante la adquisición de genes *mec*A o *mec*C, elementos centrales de las casetes cromosómicas estafilocócicas, que codifican una proteína de unión a la penicilina PBP2a o PBP2 ', con baja afinidad por los fármacos β-lactámicos. Sin embargo, los determinantes de la resistencia aún no están claros ya que los estudios sugieren que el gen *mec* podría transmitirse entre las cepas de *S. aureus* y otras especies estafilocócicas coagulasa negativas. Por ejemplo, los principales clones epidémicos de MRSA podrían tener, en sus orígenes, un elemento SCC*mec* portador de *mec*A procedente de cepas de *S. aureus* susceptibles a la meticilina (MSSA).

A principios de los años 90, el MRSA empezó a encontrarse en entornos no sanitarios y, para diferenciar las cepas aisladas de la comunidad de las cepas del hospital, les llamó *S. aureus* resistente a la meticilina adquirido en la comunidad (CA-MRSA). Más recientemente, surgieron otras nuevas cepas de MRSA con un potencial zoonótico reconocido y designado como MRSA asociado con el ganado (LA-MRSA).

Mirando hacia atrás a su origen y al elevado número de brotes causados por, podemos suponer que MRSA representa un patógeno nosocomial y de origen alimentario relevante de los cuales los Sistemas de Salud Pública y Agencias de Seguridad Alimentaria, a nivel mundial, no lo descuidan. Del mismo modo, hasta hace poco, la relevancia de su emergencia en la cadena alimentaria no se ha considerado plenamente, ya que la transmisión zoonótica aún no se había demostrado y la transmisión alimentaria no era evidente.

Más y más estudios científicos documentan la participación de estas cepas en la diseminación de diferentes linajes de MRSA entre la cadena alimentaria. Los conocimientos actuales sobre MRSA procedentes de animales productores de alimentos (materias primas para la industria alimentaria) y alimentos asociados demuestran que las cepas resistentes a los antibióticos pueden transmitirse a los humanos, a lo largo de la cadena alimentaria, por el consumo de estos alimentos. Además, el uso excesivo o abusivo de antibióticos para promover el crecimiento y en medicina veterinaria y humana también podría contribuir a la aparición de resistencia a MRSA. Entonces, puede surgir una serie de preguntas: ¿Debería la población ser consciente de los posibles riesgos emergentes asociados al MRSA en la cadena alimentaria? ¿Cuáles son las

consecuencias, desde el punto de vista de la inocuidad de los alimentos, de que los alimentos no tienen trazabilidad? ¿Debemos esperar otros nuevos mecanismos de resistencia que puedan irrumpir en nuevas variantes de MRSA, al hacer cumplir los desarrollos en la epidemiología de MRSA?

En esta tesis, titulada "***Caracterización de las cepas de Staphylococcus aureus resistentes a la meticilina aisladas de los alimentos***", se discuten los recientes resultados de la literatura acerca de los desarrollos en la epidemiología del MRSA, desde los entornos hospitalarios (humanos) a la cadena alimentaria. El enfoque de la tesis fue a investigar la presencia de MRSA en muestras de alimentos confiscados del tráfico aéreo y terrestre de diferentes viajeros que ingresan a la Unión Europea, ya que hoy en día es una preocupación creciente respecto a las posibles rutas en las que se puede distribuir MRSA.

En este contexto, las actividades de investigación durante los estudios de doctorado tuvieron como **objetivo principal** la difusión de MRSA de los alimentos importados ilegalmente a la UE procedentes de países no pertenecientes a la UE. Un número elevado de extranjeros cruce ilegalmente alimentos en su equipaje personal, además de ser confiscados por los puestos de inspección fronterizos en diferentes puntos de entrada (puertos, aeropuertos, fronteras terrestres) a la UE. Por cualquier descuido, dicen que los alimentos son traídos por ellos para consumo personal y terminan siendo vendidos ilegalmente después (conocidos como "contrabando").

Dado que no se conocen ni el origen ni la calidad de las materias primas, ni los procesos tecnológicos y las condiciones higiénicas durante el procesamiento de los alimentos, los alimentos importados ilegalmente representan un riesgo potencial para la salud. Además, la falta de condiciones de refrigeración y un embalaje adecuado durante el transporte y la venta podría violar las normas de seguridad. Por lo general, hay poca información disponible sobre los riesgos asociados y la prevalencia de patógenos en estos alimentos.

Esta tesis retrospectiva está centrada en el impacto de estas cepas en la salud pública, además debatir las rutas potenciales de los alimentos introducidos ilegalmente en el espacio de la UE por los viajeros, ya sea por vía aérea o terrestre. Existe una situación especial en la frontera sureste de la UE, ratificada por la Ley 10/2010 (Monitorul Oficial), en que se permite el tráfico transfronterizo entre Rumania y la República de Moldova sobre un acuerdo. Sin embargo, los alimentos oficialmente declarados para uso personal son legalmente introducidos en la UE, pero vendidos ilegalmente en los mercados locales rumanos organizados para vender frutas y hortalizas frescas. A pesar de que la venta de alimentos de origen animal está prohibida en estos mercados y que las autoridades locales están comprobando esos mercados, los alimentos de origen animal se venden diariamente. Un total de 210 productos se han analizado en el tráfico fronterizo terrestre entre la República de Moldova y Rumania (Giurgiuleşti-Galaţi). Además, los puestos de inspección fronterizos en el Aeropuerto Internacional de Bilbao (269 productos alimenticios) y el Aeropuerto Internacional de Viena (Austria) (600 productos alimenticios) han confiscado los alimentos procedentes de los países no pertenecientes a la UE.

En ambos casos, los alimentos, fabricados en casa o producidos industrialmente, se mantuvieron a temperatura ambiente y, a menudo, se presentaron fuera del envase original. Además, dado que estos productos se transportan y venden en condiciones que facilitan el crecimiento de patógenos, podrían representar una amenaza potencial para la salud de los consumidores. Además de investigar las rutas de transmisión de MRSA a la UE, esta tesis tiene, como objetivo, analizar las rutas de genotipos patógenos involucrados en la venta ilegal de alimentos.

Las actividades de investigación realizadas durante los estudios de doctorado se han centrado en los siguientes **objetivos científicos**, presentados a continuación:

- Visión general de las recientes conclusiones sobre la problemática real de MRSA en la cadena alimentaria, destacando la necesidad de programas adecuados de control y prevención, información actual de los programas de vigilancia de la UE;
- Punto focal sobre las vías de transmisión de MRSA desatendidas a través de alimentos introducidos de países no comunitarios como productos personales, pero destinados a ser vendidos ilegalmente a los consumidores de la UE, destacando las rutas que los alimentos pueden desempeñar en la prevalencia y diseminación de MRSA;
- Resultados globales obtenidos en relación con la identificación, aislamiento y caracterización de cepas de MRSA utilizando métodos fenotípicos y genotípicos;
- Correlación de los aspectos genotípicos de las cepas de MRSA y su formación y composición de biofilms, aportando para mejorar estrategias para limpiar superficies o eventos de contaminación cruzada;
- Investigación basada en la secuenciación completa del genoma (WGS) para la identificación de los factores de virulencia y los genes asociados con la resistencia a los antimicrobianos en una cepa susceptible a la oxacilina (OS)-MRSA;
- Evaluación de dos medios cromogénicos comercialmente disponibles para la confirmación de MRSA de muestras de humanos, animales y alimentos;
- Integración de los resultados obtenidos en la presente tesis en los estudios mundiales centrados en la diseminación de diferentes linajes de MRSA, junto con la información necesaria para la comprensión de los riesgos potenciales que *S. aureus* resistente a los antimicrobianos pueden representar.

La investigación realizada en la presente tesis no habría sido posible sin una fuerte comunicación entre instituciones de investigación de Rumania, España, Portugal o Austria, tanto en el marco del proyecto PROMISE FP7 y otros.

Summary

The present doctoral thesis comprises a total of 250 pages, including 28 figures and 20 tables. For a good managing and better representation, has been divided into five main parts, as following:

Part I speaks about *S. aureus* as foodborne pathogen in a general context. For this, Part I has been divided into two chapters. Chapter 1 entitled ***Staphylococcus aureus and Its Main Characteristics*** presents the recent literature about *S. aureus* whereas history, taxonomy, distribution and transmission, growth requirements and metabolism are given. Moreover, various factors associated with adherence-associated proteins, exotoxins and exoenzymes expressed or other factors associated with antimicrobial resistance in *S. aureus* are underlined. At the same time, characteristics of different lineages of MRSA isolated from farms, farm animals, food products and human carriers are presented, particularly considerable interest being focused on presence of MRSA in the food producing animals (raw materials for food industry) and associated foodstuff. Recent literature about surveillance programs in the EU and strategies of prevention and control are presented.

Chapter 2 describes ***Procedures Used for Detection and Identification of S. aureus*** beginning with conventional microbiological methods and ending with molecular biology techniques such as WGS. A special attention is conferred for decoding mechanisms involved in the phenotypic expression of methicillin resistance in *S. aureus* strains.

Part II points out the ***Materials, Equipments and Methods*** part. General information regarding strains used, bacterial culture media, enzymes, reagents, commercial kits, equipments and apparatus is presented in Chapter 3. Information regarding sequencing, bioinformatic tools or database used are also enumerated. Chapter 4 provides information about food sampling strategy adopted, about methods for isolation, detection and confirmation. Phenotypic and genotypic methods for characterization of MRSA strains collected are also detailed.

Part III discuss the original experimental results achieved during doctoral stage and is organized into six chapters, as followed:

Detection and Identification of Staphylococcus aureus in Food Isolated from Black Market is presented in Chapter 5, in which have been assessed presence of MRSA in foods illegally sold in a black market in Galati. This study highlights the presence of a livestock-associated (LA)-MRSA strain isolated from an animal origin food, constituting a neglected route of transmission to humans since such strains came into attention due to their rapid emergence and different epidemiology. This study is important especially for food safety authorities in designing their surveillance and control plans.

Chapter 6 entitled ***Compositional Analysis of Biofilms Formed by Staphylococcus aureus Isolated from Food Sources*** is focused on the capacity of such *S. aureus* strains to form biofilms as well as their biofilm composition. This study emphasizes the protein abundance in biofilms formed by *S. aureus* isolated from food sources, which is an important finding when designing solutions for fighting against biofilm both in food industry and medicine.

Tracking MRSA in food entering to the European Union via cross border traffic and international flights aim to highlight once again the potential risks for consumers on animal origin foods illegally introduced into the EU space (Chapter 7). The presence of enterotoxigenic lineages of MRSA identified in confiscated foods should not be neglected as can lead to possible outbreaks due to people's indifference. Additionally, isolation of a new variant OS-MRSA can be problematic as such strains show to have a different phenotype in comparison with the classical MRSA variants. This study justifies and encourages authorities to take adequate measures for food safety reasons at control borders.

Biofilm Formation by MRSA Isolates Recovered from Passenger's Luggage from Non-EU Flights is described in Chapter 8. By correlating information gathered in previous chapter and their biofilm capacity we can put into evidence if any interrelationship exists between biofilm formation and composition and their molecular features. Food safety managers, either working in food industry or industrial kitchens, can base their safety plans on such studies.

Case study- Oxacillin-Susceptible mecA-positive Staphylococcus aureus Associated to Processed Food in Europe is shown in Chapter 9. The certain problem of such strains has been described, for the first time, by WGS in which genetic factors critical in regulating the expression of methicillin resistance in *S. aureus* are examined, by identifying mechanisms which are conferring its oxacillin susceptibility.

Chapter 10 presents a ***Chromogenic Media Evaluation for Confirmation of MRSA Isolated from Humans, Animals and Food Samples***. Diagnostic performance of two commercially chromogenic media specific for confirmation of MRSA have been compared- Brilliance MRSA 2 agar (ThermoFisher Scientific) and ChromID MRSA agar (bioMérieux). Different *S. aureus* isolates from human, animal and food sources have been used in which lower diagnostic performance have been assessed for the food origin MRSA isolates. Such media are useful for food industry when microbiological food control is applied as they allowed rapid detection of presumptive MRSA.

Each chapter presented in the original experimental part has been mainly structured following subheadings such as *Introduction*, in which specific objectives and main characteristics were presented; *Materials and Methods*, where material, reagents, as well as procedures and data interpretation are given; *Results and Discussions*, in which original results accomplished and their comparison with similar data from literature are conferred; and *Conclusions*.

Part IV includes the ***General Discussion*** based on the results obtained and communicated to the international scientific community. Findings presented in the actual thesis highlight the potential risk that dissemination and prevalence of MRSA represents for consumers if hygienic and preventive measures are missing. New insights regarding MRSA transmission and epidemiology, in a food safety context, may provide a better understanding about neglected routes to Europe (international airports and markets close to EU borders), lowering the economic impact associated with health treatments on the EU community as well as on measures that food industry should take to avoid biofilm formation.

Part V synthetize the results of the entire research in ***Concluding Remarks*** part. Original contributions brought in the present thesis and future perspectives are also presented.

Sumar

Prezenta teză de doctorat cuprinde un total de 250 de pagini, inclusiv 28 figuri și 20 tabele. Pentru o bună gestionare și reprezentare a rezultatelor, a fost împărțită în cinci părți, după cum urmează:

În **Partea I** se discută despre *S. aureus* ca agent patogen alimentar într-un context general. Pentru aceasta, partea I a fost la rândul ei împărțită în două capitole. Capitolul 1 intitulat ***Staphylococcus aureus and Its Main Characteristics*** prezintă date recente din literatura de specialitate despre *S. aureus*, trecând de la istoric, taxonomie, distribuție și transmitere, până la cerințele necesare pentru creștere și metabolismul acestuia. Mai mult decât atât, în acest capitol sunt prezentați factorii asociați cu aderența, producerea de exotoxine și exoenzime sau alți factori asociați cu rezistența la antibiotice a bacteriei *S. aureus*. În același timp, sunt prezentate caracteristicile diferitelor linii genealogice de MRSA izolate din ferme, ferme de animale, produse alimentare și oameni, comentariile concentrându-se asupra prezenței MRSA în animalele de la care se obțin alimente (materii prime pentru industria alimentară) și produsele asociate. Sunt prezentate date din literatura de specialitate referitoare la programele de supraveghere din UE și strategiile de prevenire și control.

Capitolul 2 descrie ***Procedures Used for Detection and Identification of S. aureus*** începând cu metode convenționale și terminând cu tehnici de biologie moleculară, cum ar fi secvențierea totală a genomului. O atenție deosebită este acordată mecanismelor de decodare implicate în exprimarea fenotipică a rezistenței la meticilină în tulpinile de *S. aureus*.

Partea a II-a subliniază partea de ***Materials, Equipments and Methods***. În capitolul 3 sunt prezentate informații generale privind tulpinile utilizate, mediile de cultură, enzimele, reactivii, kiturile comerciale, echipamentele și aparatele. Sunt enumerate informații cu privirea la secvențierea, instrumentele bioinformatice sau baza de date utilizate. Capitolul 4 oferă informații despre strategia de prelevare a probelor de alimente, despre metodele de izolare, detectare și cele de confirmare a bacteriei *S. aureus*. Metodele fenotipice și genotipice utilizate pentru caracterizarea tulpinilor MRSA sunt, de asemenea, detaliate.

Partea a III-a discută rezultatele experimentale obținute în timpul stagiului doctoral și este organizată în șase capitole, după cum urmează:

Detection and Identification of Staphylococcus aureus in Food Isolated from Markets este prezentată în Capitolul 5. Acesta este capitolul în care este evaluată prezența MRSA în alimentele vândute ilegal pe piața din Galați. Acest studiu evidențiază prezența unei tulpini cu potențial zoonotic (LA)-MRSA izolată dintr-un produs de origine animală, ceea ce constituie o dovadă că alimentele pot reprezenta o cale de transmitere la oameni. Astfel de tulpini au intrat în atenția cercetătorilor datorită apariției lor rapide și epidemiologiei diferite. Acest studiu este important, în special, pentru autoritățile din domeniul siguranței alimentare, în vederea elaborării de planuri de supraveghere și control.

Capitolul 6 intitulat ***Compositional Analysis of Biofilms Formed by Staphylococcus aureus Isolated from Food Sources*** investighează capacitatea unor astfel de tulpini de a forma biofilme, precum și compoziția acestora din urmă. Acest studiu evidențiază abundența proteinelor ca fiind implicate în menținerea

structurii biofilmelor formate de *S. aureus* izolate din surse alimentare. Compoziția biofilmelor trebuie să fie cunoscută atunci când sunt dezvoltate noi soluții care sunt alese pentru combaterea biofilmelor, atât în industria alimentară, cât și în medicină.

Tracking MRSA in food entering to the European Union via cross border traffic and international flights este titlul Capitolului 7, cel care urmărește să sublinieze încă o dată potențialele riscuri pe care aceste alimente de origine animală introduse ilegal în spațiul UE le reprezintă pentru consumatorii finali. Prezența diferitelor linii genealogice de MRSA capabile să producă enterotoxine, identificate în alimentele confiscate, poate duce la apariția unor focare cauzate de inconștiența oamenilor. În plus, izolarea unei noi variante de OS-MRSA poate constitui o problemă de siguranță alimentară, deoarece astfel de tulpini sunt diferite din punct de vedere fenotipic față de variantele clasice de MRSA. Acest studiu justifică și încurajează autoritățile să ia măsuri adecvate din motive de siguranță alimentară.

Biofilm Formation by MRSA Isolates Recovered from Passenger's Luggage from Non-EU Flights este descrisă în Capitolul 8. Prin corelarea informațiilor adunate în capitolul precedent și a capacității acestora de a forma biofilme, putem pune în evidență dacă există o legătură între formarea biofilmului și compoziția acestuia cu caracteristicile sale moleculare. Managerii în siguranța alimentelor, care lucrează fie în industria alimentară, fie în bucătăriile industriale, își pot baza planurile de siguranță pe astfel de studii.

Case study- Oxacillin-Susceptible mecA-positive Staphylococcus aureus Associated to Processed Food in Europe este prezentat în Capitolul 9. Problema acestor tulpini a fost descrisă, pentru prima dată, prin secvențierea întregului genom, în care factorii genetici esențiali în vederea exprimării rezistenței la meticilină în *S. aureus* este examinată, prin identificarea mecanismelor care conferă susceptibilitatea la oxacilină.

Capitolul 10 prezintă ***Chromogenic Media Evaluation for Confirmation of MRSA Isolated from Humans, Animals and Food Samples***. Au fost comparate două medii cromogene pentru confirmarea MRSA-Brilliance MRSA 2 Agar (ThermoFisher Scientific) și ChromID MRSA (bioMérieux). Astfel de medii sunt utile în industria alimentară atunci când se realizează controlul microbiologic al alimentelor, deoarece acestea permit detectarea rapidă a tulpinilor prezumptive de MRSA.

Fiecare capitol prezentat în partea experimentală a fost structurat în subcapitole: *Introducere*, în care au fost prezentate obiectivele specifice și caracteristicile principale; *Materiale și Metode*, în care sunt prezentate materialele, reactivii, precum și procedurile folosite și interpretarea datelor; *Rezultate și Discuții*, în care sunt trecute rezultatele obținute și compararea acestora cu date similare din literatură; *Concluzii*.

Partea a IV-a cuprinde ***General Discussion***. Această parte este bazată pe rezultatele obținute și comunicate comunității științifice internaționale. Rezultatele prezentate în teza actuală evidențiază riscul potențial pe care diseminarea și prevalența MRSA îl reprezintă pentru consumatori dacă măsurile de igienă lipsesc.

Perspectivele viitoare privind transmiterea și epidemiologia MRSA, în contextul siguranței alimentare, pot oferi o mai bună înțelegere a rutelor de transmitere în Europa (aeroporturi internaționale și piețe apropiate granițelor UE), a modului în care se poate diminua impactul economic asociat tratamentelor medicale asupra comunității UE, precum și a măsurilor pe care industria alimentară ar trebui să le ia pentru a evita formarea biofilmelor.

Partea a V-a sintetizează rezultatele întregii cercetări în partea de ***Concluding Remarks***. Contribuțiile originale aduse de prezenta teza sunt subliniate, împreună cu perspectivele de continuare a cercetărilor.

Sumario

La tesis doctoral comprende un total de 250 páginas, incluyendo 28 figuras y 20 tablas. Para una buena gestión y mejor representación, se ha dividido en cinco partes principales, como sigue:

En la **Parte I** se habla de *S. aureus* como patógeno transmitido por los alimentos en un contexto general. Para esto, la Parte I se ha dividido en dos capítulos. El Capítulo 1 titulado ***Staphylococcus aureus and Its Main Characteristics*** presenta la reciente literatura sobre *S. aureus*, mientras la historia, taxonomía, distribución y transmisión, las necesidades de crecimiento y el metabolismo. Además, se subrayan varios factores asociados con las proteínas asociadas a la adherencia, exotoxinas y exoenzimas expresadas y otros factores asociados con la resistencia antimicrobiana en *S. aureus*. Al mismo tiempo, se presentan características de diferentes linajes de MRSA aislados de granjas, animales de granja, alimentos y humanos, particularmente con un interés considerable en la presencia de MRSA en los animales productores de alimentos (materias primas para la industria alimentaria) y productos alimentarios asociados. Se presentan publicaciones recientes sobre programas de vigilancia en la UE y estrategias de prevención y control.

El Capítulo 2 describe ***Procedures Used for Detection and Identification of S. aureus*** comenzando con métodos microbiológicos convencionales y terminando con técnicas de biología molecular. Una atención especial está dada a los mecanismos de decodificación implicados en la expresión fenotípica de la resistencia a la meticilina en cepas de *S. aureus*.

La **Parte II** señala la parte de ***Materials, Equipments and Methods***. En el Capítulo 3 se presenta información general sobre cepas utilizadas, medios de cultivo, enzimas, reactivos, kits comerciales, equipos y aparatos. El Capítulo 4 disponer de información sobre la estrategia de muestreo de alimentos adoptada, sobre métodos de aislamiento, detección y confirmación. También se detallan los métodos fenotípicos y genotípicos para la caracterización de las cepas de MRSA recogidas.

En la **Parte III** se analizan los resultados experimentales originales obtenidos durante la etapa de doctorado y se organiza en seis capítulos:

Detection and Identification of Staphylococcus aureus in Food Isolated from Black Market se presenta en el Capítulo 5, en el que se ha evaluado la presencia de MRSA en los alimentos vendidos ilegalmente en un mercado en Galati. Este estudio está hablando sobre la presencia de una cepa asociada al ganado (LA)-MRSA aislada de un alimento de origen animal, constituyéndose una vía de transmisión descuidada a los humanos, ya que estas cepas se han puesto de manifiesto por su rápida aparición y diferente epidemiología. Este estudio es importante especialmente para las autoridades de seguridad alimentaria en el diseño de sus planes de vigilancia y control.

El Capítulo 6 titulado ***Compositional Analysis of Biofilms Formed by Staphylococcus aureus Isolated from Food Sources*** se centra en la capacidad de cepas de *S. aureus* para formar biofilms y análisis de su composición. Este estudio muestra la abundancia de proteínas en los biofilms formados por aislados de *S. aureus*, que es un importante aspecto al diseñar soluciones para la lucha contra la biopelícula tanto en la industria alimentaria y medicina.

Tracking MRSA in food entering to the European Union via cross border traffic and international flights tienen como objetivo los riesgos potenciales para los consumidores sobre los alimentos de origen animales introducidas ilegalmente en el espacio de la UE (Capítulo 7). La presencia de los linajes de MRSA identificados en los alimentos confiscados puede despreciarse como posibles brotes debido a la indiferencia de la gente. Además, el aislamiento de una nueva variante OS-MRSA puede ser problemático para tener un fenotipo diferente en comparación con el fenotipo de clásico variantes de MRSA. En este estudio se justifica y estimula autoridades para tomar medidas adecuadas para la seguridad alimentaria en el control de fronteras.

Biofilm Formation by MRSA Isolates Recovered from Passenger's Luggage from Non-EU Flights se describe en el Capítulo 8. Al correlacionar la información recogida en el capítulo anterior y su capacidad de formar biofilms podemos evidenciar cualquier interrelación entre la formación de biopelículas y la composición y sus características moleculares. Los responsables de seguridad de los alimentos, ya sea trabajando en la industria alimentaria o en cocinas industriales, se pueden basar en estos ensayos.

Case study- Oxacillin-Susceptible mecA-positive Staphylococcus aureus Associated to Processed Food in Europe se muestra en el Capítulo 9. El problema de estas cepas se ha descrito, por primera vez por WGS en que los factores genéticos críticos por la expresión de resistencia a la meticilina en *S. aureus* se ha examinado, y la identificación de mecanismos que está confiriendo susceptibilidad a oxacilina.

Capítulo 10 presenta ***Chromogenic Media Evaluation for Confirmation of MRSA Isolated from Humans, Animals and Food Samples***. Diagnóstico de dos medios cromogénicos específicos para la confirmación de MRSA haber sido comparado- Brilliance MRSA 2 agar (ThermoFisher Scientific) y ChromID MRSA agar (bioMérieux). Diferentes *S. aureus* fueron aislados de humanos, animales y alimentos. Diagnóstico han sido más bajos para los MRSA aislado de los alimentos cuando hemos utilizado Brilliance. Estos medios son útiles para la industria alimentaria cuando un control de los alimentos microbiológico es necesario porque permite una detección rápida de MRSA presuntiva.

Cada capítulo presentado en la parte experimental original se ha estructurado principalmente en subtítulos como *Introduction*, donde los objetivos específicos y las principales características se presentaron; *Materials and Methods*, donde los materiales, reactivos, como los procedimientos y la interpretación de datos se da; *Results and Discussions*, en original, y su comparación con datos en literatura; y *Conclusions*.

Parte IV incluye ***General Discussion*** basadas en los resultados obtenidos y comunicados a la comunidad científica internacional. Los datos presentados en esta tesis informe sobre el riesgo potencial y la prevalencia de MRSA que representa para los consumidores si las medidas higiénicas y preventivas no se encuentra. Nuevos conocimientos en relación con la transmisión del MRSA y su epidemiología, en un contexto de seguridad de los alimentos, pueden proporcionar una mejor comprensión acerca de las rutas a Europa (aeropuertos internacionales y los mercados cerca de las fronteras de la UE), la reducción de los efectos económicos asociados con los tratamientos de salud en la comunidad de la UE, así como industria alimentaria en las medidas que debe tomar para evitar la formación de biopelículas.

Parte V sintetizo los resultados de la investigación en parte de ***Concluding Remarks***. Contribuciones originales en esta tesis y futuros perspectivas se presentan también.

Abbreviations

A	Adenine
AAD	Antibiotic Associated Diarrhea
AIV	Avian Influenza
AT	Austria
ATCC	American Type Culture Collection
Bap	Biofilm associated protein
BHIA	Brain Heart Infusion Agar
BHIB	Brain Heart Infusion Broth
BIOHAZ	EFSA's Panel on Biological Hazards
BLAST	Basic Local Alignment Search Tool
BORSA	Borderline Oxacillin-Resistant *S. aureus*
BP	Baird Parker
bp	Basepare
C	Citosine
CA-MRSA	Community-Associated MRSA
CC	Clonal Complex
CDC	Center for Disease and Control
CFU	Colony Forming Units
CHIPS	Chemotaxis Inhibitory Protein of Staphylococci
CIP	Clean-In-Place
ClfB	Clumping factor B
CLSI	Clinical and Laboratory Standards Institute
CLSM	Confocal Laser Scanning Microscopy
CO_2	Carbon dioxide
CoNS	Coagulase Negative Staphylococci
CPS	Coagulase Positive Staphylococci
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
CSF	Classical Swine Fever
CV	Crystal Violet
CWA	Cell Wall Adhesion
dATP	Deoxyadenosine Triphosphate
dCTP	Deoxycytosine Triphosphate
DEPC	Diethylpyrocarbonate
dGTP	Deoxyguanosine Triphosphate
DHFR	Dihydrofolate Reductase
DNA	Deoxyribonucleic Acid
dNTPs	Deoxynucleotides
dTTP	Deoxythymidine Triphosphate
DW	Dry Weight
Eap	Extracellular adherence protein
EBI	European Bioinformatics Institute
ECDC	European Center for Disease and Control

eDNA	Extracellular DNA
EEO	Electro-Endo-Osmosis
Efb	Extracellular fibrinogen binding protein
EFSA	European Food Safety Authority
ELISA	Enzyme-Linked Immunosorbent Assay
EMP	Embden-Meyerhof-Parnas
EPS	Extracellular Polymeric Substances
ES	Spain
EU	European Union
EUCAST	European Committee on Antimicrobial Susceptibility Testing
EY	Egg Yolk emulsion
F	Fast
FAO	Food and Agricultural Organization of the United Nations
FLIPr	Formyl peptide receptor-like inhibitory protein
FMD	Foot and Mouth Disease
FnBPA	Fibronectin/ fibrinogen Binding Protein A
FnBPB	Fibronectin/ fibrinogen Binding Protein B
G	Guanine
GHP	Good Hygiene Practices
GMP	Good Manufacturing Practices
H_2	Hydrogen
HA-MRSA	Hospital-Associated MRSA
HMP	Hexose Monophosphate
ICMSF	International Commission for the Microbiological Specifications of Foods
IS	Insertion Sequences
ISO	International Organization for Standardization
IUPAC	International Union of Pure and Applied Chemistry
IWG	International Working Group
kb	Kilobase
kbp	Kilobase pairs
kD	Kilodaltons
LA-MRSA	Livestock-Associated MRSA
M	Maximum
MBC	Minimum Bactericidal Concentration
MCM	Mauve Contig Mover
MEGA	Molecular Evolutionary Genetics Analysis
mg	Milligrams
MIC	Minimum Inhibitory Concentration
MLST	Multilocus Sequence Type
mM	Millimolar
MRSA	Methicillin Resistant *Staphylococcus aureus*
MSCRAMMs	Microbial Surface Components Recognizing Adhesive Matrix Molecules
MSSA	Methicillin-Sensitive *Staphylococcus aureus*
MST	Minnimum Spanning Tree
N_2	Nitrogen
NaCl	Sodium Chloride
NC	Negative Control
NCBI	National Center for Biotechnology Information

ng	Nanograms
NPV	Negative Predictive Value
NT-MRSA	Non-Typeable MRSA
NVI	Norwegian Veterinary Institute
OD	Optical Density
OS-MRSA	Oxacillin-Susceptible MRSA
PBP	Penicillin Binding Protein
PCR	Polymerase Chain Reaction
PED	Porcine Epidemic Diarrhea
PFGE	Pulsed Field Gel Electrophoresis
PIA	Polysaccharide Intercellular Adhesion
PNAG	Poly-*N*-Acetyl-1,6-β-Glucosamine
PPV	Positive Predictive Value
PT	Potassium Tellurite
PTSAgs	Pyrogenic Toxin Superantigens
PVL	Panton-Valentine Leukocidin
QRDR	Quinolone Resistance-Determining Region
RAST	Rapid Annotation using Subsystem Technology
RNA	Ribonucleic Acid
RO	Romania
RPF	Rabbit Plasma Fibrinogen
rRNA	Ribosomal RNA
RTE	Ready-To-Eat
RTi-PCR	Real Time PCR
S	Slow
SAM	Sequence Aligment/ Map
SasC, SasG	*S. aureus* surface proteins C and G
SCC*mec*	Staphylococcal Chromosomal Cassette *mec*
SCIN	Staphylococcal Complement Inhibitor
SD	Standard Deviation
SdrC	Serine aspartate repeat protein C
SEs	Staphylococcal Enterotoxins
SFP	Staphylococcal Food Poisoning
SSTI	Skin and Soft Tissue Infections
ST	Sequence Type
subsp.	Subspecie
T	Timine
tl	Thermolabile
Tm	Melting temperature
ts	Thermostable
TSB	Tryptic Soy Broth
TSBG	Tryptic Soy Broth supplemented with 0.4% glucose
TSBN	Tryptic Soy Broth supplemented with 4% NaCl
TSS	Toxic Shock Syndrome
TSST	Toxic Shock Syndrome Toxin
UPGMA	Unweighted Pair Group Mathematical Average
UV	Ultraviolet
v	Variable

VBNC	Viable But Non-Culturable Cells
VISA	Vancomycin-Intermediate *S. aureus*
VNTR	Variable Numbers of Tandem Repeat
VRSA	Vancomycin- Rresistant *S. aureus*
VTEC	Verocytotoxin- producing *Escherichia coli*
-w	Negative to weakly positive
WGA	Wheat Germ Agglutinin
WGS	Whole Genome Sequencing
WHO	World Health Organization
µg	Micrograms
SSSS	Staphylococcal Scalded Skin Syndrome

Antibiotics

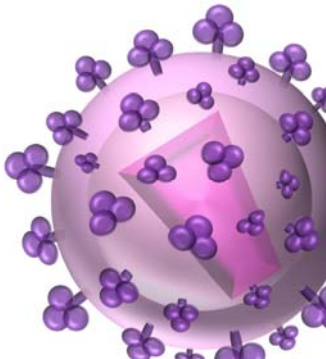
AMC	Amoxicillin/clavulanate
AMK	Amikacin
CIP	Ciprofloxacin
CLI	Clindamycin
DAP	Daptomycin
ERY	Erythromycin
FOF	Fosfomycin
FUS	Fusidic acid
GEN	Gentamicin
LVX	Levofloxacin
LZD	Linezolid
MUP	Mupirocin
OXA	Oxacillin
PEN	Penicillin
RIF	Rifampin
SXT	Trimethoprim sulfamethoxazole
TEC	Teicoplanin
TET	Tetracycline
TOB	Tobramicin
VAN	Vancomycin

Index of tables

Index of figures

PART 1

INTRODUCTION



CHAPTER 1

Staphylococcus aureus and Its Main Characteristics

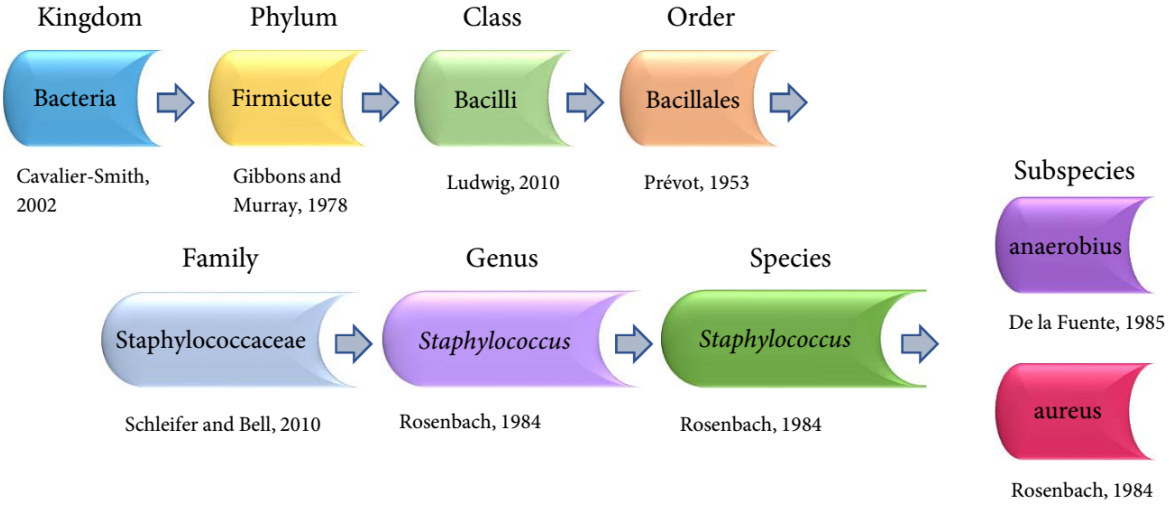
Results partially published

Oniciuc, E.-A., Nicolau, A.I., Hernández, M., Rodríguez-Lázaro, D. (2017). Presence of methicillin-resistant *Staphylococcus aureus* in the food chain. Trends in Food Science & Technology, doi: 10.1016/j.tifs.2016.12.002. Impact factor: 5.150

Staphylococcus aureus and Its Main Characteristics

1.1. History

Staphylococci were firstly identified in 1881 as a cause of surgical abscess in a knee joint by the Scottish surgeon, Sir Alexander Ogston (Orenstein, 1998). Four years later, *Staphylococcus aureus* and *Staphylococcus epidermidis* were isolated by the German scientist, Anton Julius Rosenbach who named them after the pigmented appearance of colonies: *S. aureus* (lat. *aurum* for gold), and *S. albus* (lat. *albus* for white). Later, *S. albus* was renamed due to its ubiquitous presence on human skin to *S. epidermidis* (Orenstein, 1998; Gilden, 2013). Current taxonomic hierarchy (www.itis.gov) of the *Staphylococcus* is as follows:



1.2. Taxonomy

Staphylococci are Gram positive, mesophilic bacteria, aerobic or facultatively anaerobic with a respiratory and fermentative metabolism (Foster, 1996; Batt and Tortorello, 2014). They gave rise to cocci-shaping forms, grouped in clusters similar to grapes or individual cells of maximum 1 μm in diameter, non-motile and non-spore forming (Foster, 1996; Le Loir *et al.*, 2003).

The genus *Staphylococcus* currently includes 48 species and 27 subspecies (http://www.bacterio.net/), of which only sixteen are having real and potential risk in foods (Table 1). Staphylococci differ in their potential to trigger human and animal health, ranging from non-pathogenic to medium and highly pathogenic species, with different grades of severity and showing resistance when commonly antibiotics for a certain treatment are applied (Dobrindt *et al.*, 2013).

Staphylococci are divided into two groups depending on coagulase activity, in which this extracellular product interacts with prothrombin, inducing transformation of fibrinogen into fibrin (Bodén and Flock, 1989; Ivana, 2011; Dobrindt *et al.*, 2013). There are seven staphylococcal species producing this enzyme– *S. aureus*, *S. intermedius*, *S. pseudintermedius, S. hyicus*, *S. delphini*, *S. schleiferi* spp. *coagulans*, and *S. lutrae*, from which *S. aureus* is the most studied of its group. The rest of the species are designed as coagulase negative staphylococci (CoNS), of which *S. epidermidis* is the one with major importance in human

medicine as many publications listed this pathogen as being related to indwelling devices or being involved in nosocomial diseases (John and Harvin, 2007; Otto, 2009). From their groups, *S. aureus* and *S. epidermidis* have the greatest pathogenic potential on humans and animal species (Foster, 1996).

Table 1. *Staphylococci known to be associated with food* (Jay *et al.*, 2005)

Specie	Coagulase	Nuclease	Enterotoxin	Hemolysin	Mannitol	G+C of DNA
S. aureus subsp.						
anaerobius	+	ts	−	+	−	31.7
aureus	+	ts	+	+	+	32-36
S. intermedius	+	ts	+	+	(+)	32-36
S. hyicus	(+)	ts	+	−	−	33-34
S. delphini	+	−		+	+	39
S. schleiferi subsp.						
coagulans	+	ts		+	(+)	35-37
schleiferi	−	ts		+	−	37
S. caprae	−	tl	+	(+)	−	36.1
S. chromogens	−	−w	+	−	v	33-34
S. cohnii	−	-	+	−	v	36-38
S. epidermidis	−	-	+	v	−	30-37
S. haemolyticus	−	tl	+	+	v	34-36
S. lentus	−		+	−	+	30-36
S. saprophyticus	−	-	+	−	+	31-36
S. sciuri	−		+	−	+	30-36
S. simulans	−	v		v	+	34-38
S. warneri	−	tl	+	−w	+	34-35
S. xylosus	−	-	+	+	v	30-36

Note: + positive; − negative; − w- negative to weakly positive; (+) weak reaction; v- variable; ts- thermostable; tl- thermolabile.

1.3. Distribution and Transmission

S. aureus is one of the most known and studied of its genus. It is an opportunistic bacterium which can be found in air, dust, sewage, water, environmental surfaces (Hennekinne *et al.*, 2012) but also colonizing skin and mucous membranes of humans or animal species, especially mammals (Kluytmans *et al.*, 1997; Dobrindt *et al.*, 2013; Brown *et al.*, 2014; Mustapha *et al.*, 2014; Grema *et al.*, 2015). The most common site of colonization remains the nasal cavities but may also be found on human epithelia, axillae (8%), chest/abdomen (15%), head, nares (nostrils), perineum (22%), pharynx, or intestinal tract (17-31%) (Foster, 1996; Kluytmans *et al.*, 1997; Frank *et al.*, 2010; Otto, 2010; Chen *et al.*, 2013; Sollid *et al.*, 2014; Grema *et al.*, 2015; van Belkum, 2016).

Healthy persistent carriers (20%) may be colonized with this pathogen, while 30% of the population carry *S. aureus* transiently and the rest (50%) are non-carriers (Otto, 2010). In persistent *S. aureus* carriers, the frequency of colonization is higher than the rest of the population, affecting children more frequently than adults (Otto, 2010; Blumental *et al.*, 2013), improved hygiene overtime explaining this.

Moreover, host-pathogen interactions evolved when humans started animal and plant breeding, favoring sharing bacterial species between (Pantosti, 2012). Companion animals such as dogs, cats, horses have been

also considered potential reservoirs (Mustapha *et al.*, 2014). Even though some studies revealed that certain clones of *S. aureus* were associated with animals such as goats, poultry or sheep, in fact were originated from human strains following profound genetic modifications (Lowder *et al.*, 2009; Lozano *et al.*, 2011; Price *et al.*, 2012; Pantosti, 2012). However, successful *S. aureus* colonization is the outcome of a process determined by multiple factors contributing to the host-pathogen interaction showing global variation among population (Sollid *et al.*, 2014).

In apparent contrast to its ability to colonize both humans and animals, can be a potential lethal pathogen (Brown *et al.*, 2014), colonization being a subsequent risk factor for acquiring infections (Gorwitz *et al.*, 2008; Brown *et al.*, 2014). This opportunistic pathogen can cause broad-spectrum infections (Deurenberg *et al.*, 2007; Kadariya *et al.*, 2014), ranging from diverse skin and soft tissue infections (SSTI) to severe and even fatal invasive disease, severe sepsis, toxic shock syndrome (TSST), endocarditis, pneumonia, meningitis, bacteremia or osteomyelitis (Monecke *et al.*, 2009; Frank *et al.*, 2010; Monecke *et al.*, 2011; Lozano *et al.*, 2011; Dobrindt *et al.*, 2013; Szczuka *et al.*, 2013; Brown *et al.*, 2014).

1.4. Growth Requirements and Metabolism

Staphylococci are multiplying when they encounter a proper environment with a certain chemical composition and a convenient substrate. However, their growth rate is proportionally with the substrate concentration, temperature, pH, and organic matter (Banville, 1964; Mah *et al.*, 1967).

S. aureus growth takes place between 7-47.8°C, having an optimum growth between 35-37°C. Although is a mesophilic bacterium, some *S. aureus* strains can grow as low as 6.7°C (Jay *et al.*, 2005). The pH interval varies between 4.0-10 with an optimum of 6.0-7.0 (Banville, 1964; Jay *et al.*, 2005). Moreover, *S. aureus* is halotolerant, resistant to chemical compounds *e.g.* mercuric chloride, polymyxin and capable to grow below $a_w < 0.83$. However, *S. aureus* can grow well in culture media without NaCl but it can grow readily in 7-10% concentration (Stone, 2017), and some strains even at 25% concentration of salts (Valero *et al.*, 2009). Its capacity to tolerate such huge amount of salts is shared by other bacteria such as *Micrococcus* and *Kocuria*, which are widely distributed in nature and occur in foods in larger numbers than staphylococci (Jay *et al.*, 2005).

S. aureus can tolerate chemical compounds such as tellurite, neomycin, polymyxin, mercuric chloride, and sodium azide, all of which can be found as selective agents in culture media used for their growth. However, other staphylococci can differentiate *S. aureus* due to its increased resistance to acriflavine (Jay *et al.*, 2005). With respect to a_w, *S. aureus* can grow at values lower than other nonhalophilic bacteria (Jay *et al.*, 2005).

Regarding its metabolism, *S. aureus* uses a variety of carbohydrates and obtain energy *via* glycolysis, utilizing the hexose monophosphate shunt and the tricarboxylic acids (Krebs cycle) (Vasu *et al.*, 2014; Rogatzki *et al.*, 2015). The glycolytic pathway (Embden-Meyerhof-Parnas; EMP) and the oxidative hexose monophosphate (HMP) are the two central pathways used for glucose metabolism. *S. aureus* metabolizes glucose mainly by glycolysis and to a limited extent by the HMP, resulting in lactate which is the major end product of anaerobic glucose metabolism (Sivakanesan and Dawes, 1980). Congruently, in the presence of adequate oxygenation, pyruvate has been viewed as the end product of glycolysis which can enters into Krebs cycle and regulates the energy levels linked to pathogenicity of organism (Vasu *et al.*, 2014; Rogatzki *et al.*, 2015).

1.5. Virulence Factors

S. aureus is the most opportunistic pathogen affecting human and animals and its virulence depends on multiple factors associated with extracellular proteins, contributing to skin infections, food poisoning or certain diseases (Haveri *et al.*, 2007). The pathogenicity of *S. aureus* (Figure 1) is characterized by the production of specific enzymes (coagulase, catalase, thermonuclease, hyaluronidase) and exotoxins (Table 2). *S. aureus* strains can harbor different virulence genes coding for staphylococcal enterotoxins (SEs), leukocidins, exfoliatins, toxic shock syndrome toxin 1 (TSST-1), accessory gene regulator alleles and antibiotic resistance (Spanu *et al.*, 2012). Moreover, cell wall adhesion (CWA) components (adhesins, protein A, teichoic acid, peptidoglycan) of *S. aureus* are also involved in virulence (Gordon and Lowy, 2008). This can be associated with the capacity of *S. aureus* to produce biofilms; once attached to tissue or matrix-covered devices, *S. aureus* biofilms starts to grow by proliferation and produces a scaffolding extracellular matrix (Speziale *et al.*, 2014). The expression of all these virulence factors favorize bacteria to adapt to hostile environmental conditions, allowing its survival and promoting infection by invading and destroying host tissues and metastasize to other sites (Gordon and Lowy, 2008). However, the presence or absence of these genes is essential to determine the potential virulence of *S. aureus* strains (Spanu *et al.*, 2012).

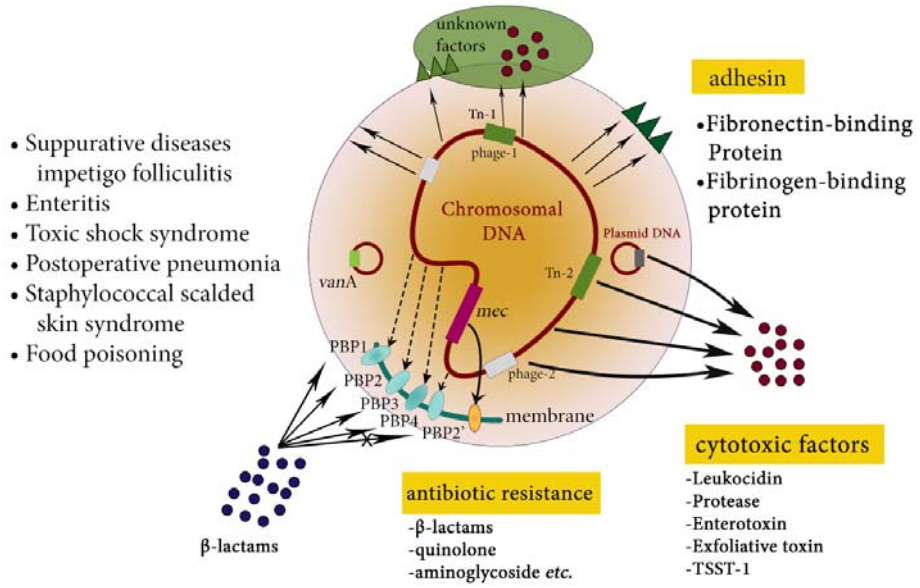


Figure 1. Virulence factors in *Staphylococcus aureus*

Exotoxins described so far to be involved in pathogenesis of *S. aureus* can be classified into three main categories: cytotoxins (leukocidins, hemolysins), exfoliatins and pyrogenic toxin superantigens (TSST, enterotoxins).

Leukocidins. One of the most virulent toxin of its group is represented by the Panton-Valentine leukocidin (PVL) produced by *S. aureus* causing leukocyte destruction and tissue necrosis (Lina *et al.*, 1999; Adler *et al.*, 2006). This cytotoxin is composed of two protein subunits, LukS-PV and LukF-PV respectively, which act together in order to damage membranes of host defense cells and erythrocytes (Lina *et al.*, 1999). Less than 5% of *S. aureus*- wild type isolates produce this pore-forming toxin (Löffler *et al.*, 2010).

PVL appears to be associated with necrotic lesions of the skin and subcutaneous tissues (*e.g.* furuncles) produced by community-acquired methicillin-resistant *S. aureus* (CA-MRSA) strains (Adler *et al.*, 2006). Moreover, it has been demonstrated its role as a major determinant of virulence in an acute pneumonia mouse model (Dumitrescu *et al.*, 2007). Meanwhile, *lukED* genes encodes the biocomponent leukotoxin LukE and LukD (Gharsa *et al.*, 2012) with a weak leukotoxic activity.

Hemolysins. To date, there are five hemolysins known to be involved as virulence factors in overcoming the host defenses. *S. aureus* lysis of red blood cells is mediated by the hemolysins known as alpha (α-hemolysin), beta (β-hemolysin), delta (δ-hemolysin), gamma (γ-hemolysin) and gamma variant encoding genes such as *hla*, *hlb*, *hld*, *hlg* and *hgv*, respectively (Burnside *et al.*, 2010). Alpha-hemolysin is by far the most studied of all *S. aureus* cytotoxins (Dinges *et al.*, 2000). Upon binding to the cell surface, α-hemolysin monomer assembles into a homoheptamer, forming a prepore and subsequently a mature β-barrel transmembrane pore (Vandenesch *et al.*, 2012). This pore allows the transport of molecules smaller than 2 kDa, such as K^+ and Ca^{2+} ions, leading to necrotic death of targeted cells (Vandenesch *et al.*, 2012).

Beta-hemolysin is an exotoxin with a molecular weight of 35 kDa (Wiseman, 1975; Dinges *et al.*, 2000) which does not form pores in the plasma cell membrane but instead is a neutral sphingomyelinase hydrolyzing sphingomyelin (Vandenesch *et al.*, 2012). However, sphingomyelin is enriched in lipid-ordered membrane microdomains with high content in cholesterol but the mechanism leading to cytotoxicity is poorly understood (Vandenesch *et al.*, 2012).

Delta-hemolysin is a small amphipathic peptide which can bind to the cell surface and aggregate to form transmembrane pores or to affect the membrane curvature, thus destabilizing the plasma membrane. Moreover, at higher concentration, could act as a detergent to solubilize the membrane (Vandenesch *et al.*, 2012). Delta-hemolysin is produced by 97% of *S. aureus* strains (Dinges *et al.*, 2000).

Gamma-hemolysin together with PVL represents two types of bicomponent toxins produced by *S. aureus* (Dinges *et al.*, 2000). In contrast with α-hemolysin, the formation of a mature pore involves two polypeptides which have been named S (slow) and F (fast), based on their electrophoretic activity. Later, the mature hetero-octamer forms a trans-membrane β-barrel pore across the plasma membrane, leading to host cell lysis (Vandenesch *et al.*, 2012). To note, *S. aureus* secretes numerous different types of hemolysins capable of damaging the host cell plasma membrane.

Exfoliatins. There are three types of exfoliative toxins most known to be implicated in humans such as exfoliative toxin A (*eta*), exfoliative toxin B (*etb*) and exfoliative toxin D (*etd*) (Yamaguchi *et al.*, 2002) and a later one, exfoliative toxin C (*etc*) with implications in animals, firstly being described in horses (Sato *et al.*, 1994). The exfoliatins are proteases which cleave the peptide bonds by inactivating the action of antibodies, *in vivo* and *in vitro*, and in the same time protects against antimicrobial peptides such as neutrophil defensing proteins and bactericidal platelet proteins (Mustapha *et al.*, 2014). However, they are exhibiting tissue specificity to different animal species (Harrison *et al.*, 2013). All these factors are contributing to tissue proteins destruction during invasion (Postier *et al.*, 2004; Mustapha *et al.*, 2014).

The first two *S. aureus* exfoliative toxin isoforms, exfoliative toxin A and B, are primarily responsible for the skin appearance of staphylococcal scalded skin syndrome and bullous impetigo (Yamaguchi *et al.*, 2002; Mustapha *et al.*, 2014). Only 5% of the clinical *S. aureus* isolates are responsible to produce these exfoliative

toxins, either A or B or both toxins. (Yamaguchi *et al*., 2002; Mustapha *et al*., 2014). It has been shown that CA-MRSA produce these types of toxins (Hososaka *et al*., 2007).

Pyrogenic toxin superantigens (PTSAgs). This group comprises the TSST and the staphylococcal enterotoxins of serotypes A-E (Carfora *et al*., 2015) and enterotoxin-like toxins G-U, IV (Hennekinne *et al*., 2012; Dobrindt *et al*., 2013). Besides their pyrogenic properties, they are also super antigenic and have the capacity to be enteropathogenic, thus their implications in staphylococcal toxic shock syndrome (TSS) and food poisoning (Dinges *et al*., 2000; Mustapha *et al*., 2014). The PTSAgs are exotoxins secreted by *S. aureus* or *Streptococcus pyogenes* disturbing the host defense reaction by immunosuppression and nonspecific T-cell proliferation (Haveri *et al*., 2007; Kadariya *et al*., 2014). As superantigens, can facilitate transcytosis allowing the toxin to enter the bloodstream, thus enabling it to interact with antigen-presenting cells and T cells (Ortega *et al*., 2010).

Enterotoxins are single-chain secreted proteins (Lauchlin *et al*., 2000), soluble in water and saline solution, and responsible for most of the food poisoning episodes registered so far due to their unique biochemical and structural properties and remarkable resistance to heat (Ortega *et al*., 2010). Heat stable SEs are resistant to most proteolytic enzymes, retaining their activity in the digestive tract after ingestion (Ortega *et al*., 2010). The predominant regulon in *S. aureus* for the expression of SE and TSST is represented by the *agr* system (accessory gene) whereas *in vitro*, upon activation, *agr* downregulates the gene expression for encoding surface proteins and upregulates genes encoding exoproteins such as SEB, SEC, SED or TSST (Ortega *et al*., 2010). However, this cannot apply for all types of SE because environmental factors may influence in triggering their expression (Tseng *et al*., 2004; Ortega *et al*., 2010).

Staphylococcal food poisoning (SFP) is characterized by intoxication resulting from the ingestion of food or beverages containing preformed SE (Lauchlin *et al*., 2000). The symptoms of a SFP episode usually is developed within four to six hours after the ingestion of contaminated food and generally lasts from twelve to forty-eight hours, characterized by sudden onset of nausea, vomiting, abdominal cramps, and diarrhea within two to six hours of ingestion of toxin (Jay *et al*., 2005; Lauchlin *et al*., 2000).

The **toxic shock syndrome** (TSS) represents a systemic illness associated with acute intoxication with the main causative agent which is the TSST (Mehrotra *et al*., 2000). The TSS pathophysiology consist in a capillary leak syndrome stemming from toxin- and cytokine-mediated endothelium damage (Ortega *et al*., 2010). Mainly has been associated with an acute onset of high fever, headache, desquamation of the skin, hypotension and organ system failure (Ortega *et al*., 2010).

Table 2. *Virulence factors involved in the pathogenesis of S. aureus (Gordon and Lowy, 2008; Costa et al., 2013)*

Name	Gene (s)	Function (s)
MSCRAMMs		
Clumping factor proteins	*clfA, clfB*	mediate clumping and adherence to fibrinogen in the presence of fibronectin
Fibronectin-binding proteins	*fnbA, fnbB*	attach to fibronectin and plasma clot
Collagen binding proteins	*cna*	adhere to collagenous tissue and cartilage
Surface-binding proteins	*spa*	bind to IgG interfering with opsinization and phagocytosis
Capsular polysaccharides		
Biofilm accumulation (*e.g.* PIA)	*ica* locus, *hemB* mutation	reduce phagocytosis by neutrophils; enhance bacterial colonization and persistence on mucosal surfaces

Cytotoxins		
α-hemolysin	*hla*	induce lysis on a wide spectrum of cells, mainly platelets and monocytes
β-hemolysin	*hlb*	hydrolyze of sphingomyelin of the plasmatic membrane of monocytes, erythrocytes, neutrophils, and lymphocytes, cells becoming sensitive to other lytic agents
Leukocidins E/D and M/F-PV	*lukE/D*	induce lysis on leukocytes
PVL	*lukS-PV, lukF-PV*	induce lysis on leukocytes
Pyrogenic toxin superantigens		
Enterotoxins	*sea-seq*	massively activate T cells and antibody presenting cells
TSST-1	*tst*	massively activate T cells and antibody presenting cells
Exoenzymes		
Proteases		
Serine (*e.g.* exfoliative toxins ETA and ETB)	*eta, etb*	inactivate neutrophil activity; activate T cells (in case of ETA and ETB)
Cysteine (*e.g.* staphopain)	*cysM*	block neutrophil activation and chemotaxis
Aureolysin	*aur*	inactivate antimicrobial peptides
Lipases	*geh*	inactivate fatty acids
Nucleases	*nuc*	cleave nucleic acids
Hyaluronidase	*hysA*	degrade hyaluronic acid
Staphylokinase	*sak*	activate plasminogen; inactivate antimicrobial peptides
Other proteins		
CHIPS	*chp*	inhibit chemotaxis and activation of neutrophils
Eap	*eap*	inhibit neutrophil migration
Efb	*efb*	inhibit complement activation
FLIPr		inhibit chemotaxis of neutrophils
SCIN	*scn*	inhibit complement activation

Note: CHIPS- chemotaxis inhibitory protein of staphylococci; Eap- extracellular adherence protein; Efb- extracellular fibrinogen binding protein; FLIPr- formyl peptide receptor-like inhibitory protein; MSCRAMMs- microbial surface components recognizing adhesive matrix molecules; SCIN- staphylococcal complement inhibitor.

1.6. Antimicrobial Resistance

The discovery of antibacterial agents revolutionized the management dealing with infections both in human and animal medicine, reducing drastically the mortality rates (ECDC, 2009). However, soon after their introduction, bacteria evolved and become resistant to antibiotics raising a global threat among public health (ECDC, 2009) (Table 3). Looking back in the past on their rapid adaptability and resistance capability, bacteria could endanger the efficacy of antibiotics and perhaps in the future we will be facing with ineffective antibiotics for several types of bacteria causing infections in humans.

The discovery of the first three antibiotics– Salvarsan, Prontosil and penicillin– set up the pillars for future drug research; later on, during the golden era of antimicrobials discovery (between 1950s and 1970s), several researchers developed other new antibiotics. However, the discovery rate stopped as bacteria were becoming resistant to antimicrobial agents and nowadays only modification of existing antibiotics are available (Aminov *et al.*, 2010; Ventola, 2015) (Figure 2).

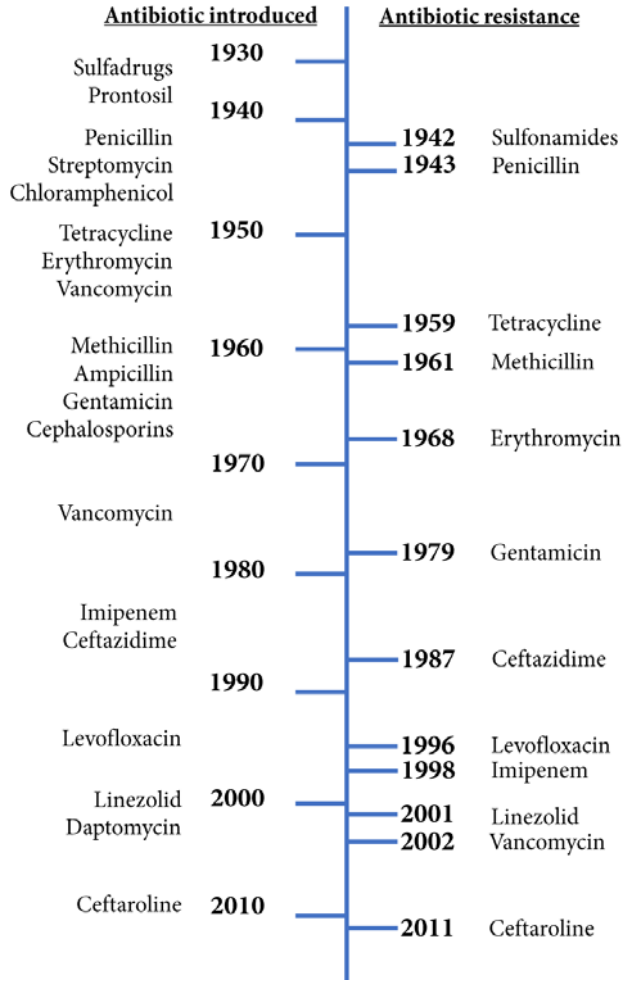


Figure 2. Timeline of antibiotic history and key events when resistance developed (adapted after Wright, 2010; Ventola, 2015)

The intensive use of antimicrobials in human and animal medicine led to different selective pressures on the bacterial populations favoring appearance of mutations corresponding to a level of antibiotic resistance (Davies and Davies, 2010). This may lead to life-threatening infections complicating treatment and, if drastic measures must to be taken, then it is possible to appeal for "last resort" antibiotics and use them in rational combinations to overcome multi-drug resistant bacterial infections (Yeh *et al.*, 2009; Davies and Davies, 2010).

***Table 3**. Mechanisms of S. aureus resistance to antimicrobials (Lowy, 2003; Reygaert, 2013)*

Antibiotic	Gene (s)	Gene product (s)	Function
β-lactams	*blaZ*	β-lactamase	enzymatic hydrolysis of β-lactam nucleus
	*mec*A, *mec*C	PBP2a	altered PBP2a targets
Glycopeptides	*unknown*	VISA-altered peptidoglycan	trapping of vancomycin in the cell wall
	vanA	VRSA-modified target	synthesis of dipeptide with reduced affinity for vancomycin
Quinolones	*parC*	ParC (or GrlA) component of topoisomerase IV	mutations in the QRDR region, reducing affinity of enzyme-DNA complex for quinolone
	gyrA, gyrB	GyrA or GyrB components of gyrase	

Aminoglycosides	*aac, aph, ant*	acetyltransferase, phosphotransferase	acetylating and/or phosphorylating enzymes modify aminoglycosides
Tetracyclines	*tetK, tetM*	active efflux	ribosomal protection- competitive binding
Chloramphenicol	*cat*		acetylation of drug- inactivation
Trimethoprim-sulfamethoxazole	*sulA*	dihydropteroate synthase	overproduction of *p*-aminobenzoic acid by enzyme
	dfrB	DHFR	reduced affinity for DHFR
Oxazolidinones	*rrn*	23S rRNA	mutations in domain V of 23S rRNA component of the 50S ribosome; interferes with ribosomal binding
Quinupristin-dalfopristin	*ermA, ermB, ermC*	ribosomal methylases	reduce binding to the 23S ribosomal subunit
	vat, vatB	acetyltransferase	enzymatic modification of dalfopristin

Note: DHFR- dihydrofolate reductase; PBP-penicillin binding protein; rRNA- ribosomal RNA; QRDR- quinolone resistance-determining region; VISA- vancomycin- intermediate *S. aureus*; VRSA- vancomycin- resistant *S. aureus*.

In this thesis, we are targeting methicillin resistance in *S. aureus*, for which, World Health Organization (WHO) listed MRSA as a superbug of high priority who urgently needs to be combated (WHO, 2017). Over the years, MRSA has shown outstanding versatility at thriving and spreading in different environments over time such as in hospitals, communities, and/ or animals (Ventola, 2015).

MRSA can easily adapt to the selective pressure of antibiotics by developing multiple mechanisms of resistance with implications in the bacterial cell wall structure alteration, production of enzymes which may modify antibiotic targets or active efflux of the antimicrobial agent from inside the cell (Wright, 2010). The evolution of different types of antimicrobial resistance elements in *S. aureus* generated numerous research studies with the purpose of defining the effects and which factors may contribute to its resistance (Cameron *et al.*, 2011). Although a variety of integrated genetic processes are known to be involved, little is known about phenotypic characteristics contributing too (Davies and Davies, 2010; Cameron *et al.*, 2011).

1.6.1. Methicillin-Resistant *Staphylococcus aureus*

Resistance to penicillinase-stable penicillins, also called "methicillin resistance" or "oxacillin resistance", in *S. aureus* is manifested as resistance to all β-lactam antimicrobial agents including cephalosporins and carbapenems and potential susceptibility to the newest class of MRSA-active cephalosporins (*e.g.* ceftaroline).

The appearance of methicillin-resistant *Staphylococcus aureus* (MRSA) dates back to 1961 in the United Kingdom (Petinaki and Spiliopoulou, 2012), one year after the first introduction of methicillin in clinical practice to treat infections with penicillin-resistant *S. aureus* (Jevons, 1961). MRSA became a serious threat in the early 90s in the USA and UK (Kluytmans, 2010). Resistance is conferred by a mobile genetic element, named the staphylococcal chromosomal cassette (SCC*mec*) (Milheiriço *et al.*, 2007), carrying *mec*A or *mec*C genes, encoding a penicillin-binding protein 2a (PBP2a) (Paterson *et al.*, 2014a; Petinaki and Spiliopoulou, 2012). Beta-lactam drugs bind to PBPs, essential for cell wall peptidoglycan synthesis, leading to bacterial cell lysis. However, PBP2a has low affinity for β-lactam agents, such that peptidoglycan synthesis can

continue in MRSA strains (Paterson *et al.*, 2014a) even in the presence of diverse β-lactam inhibitor combinations.

The *mec*A gene can be found on one of the eleven SCC*mec* types (I-XI) (Figure 3), which carry five different *mec* gene complexes, composed of *mec*A and its regulatory genes *mec*I and *mec*R1, and eight different *ccr* gene complexes, containing two different *ccr* recombinases responsible for the mobility of the element (Kondo *et al.*, 2007).

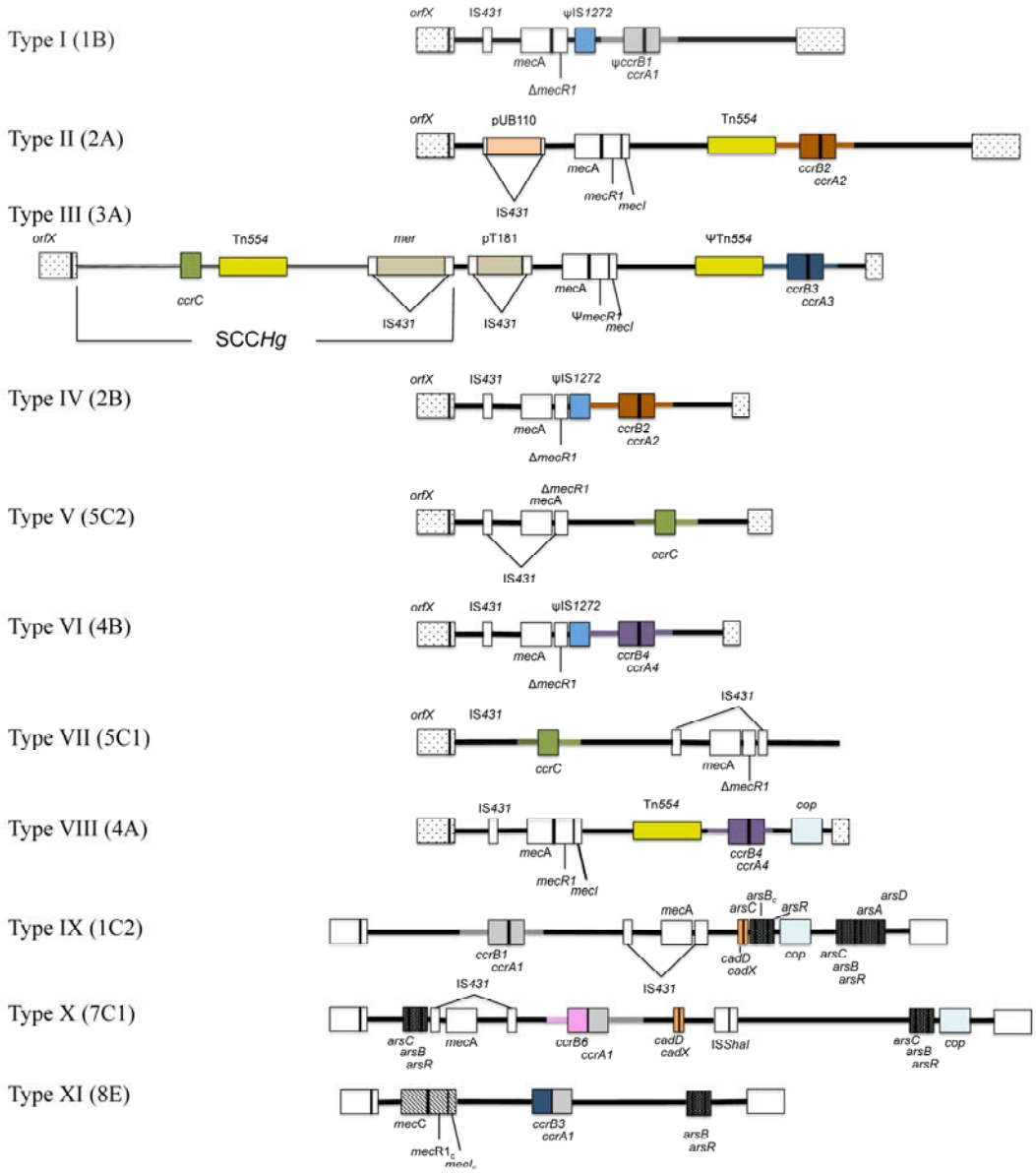


Figure 3. Features of the different types of the Staphylococcal Cassette Chromosome *mec* (SCC*mec*). The structures of SCC*mec* elements illustrated are based on the following published nucleotide sequences: AB033763 (Type I-1B); D86934 (Type II-2A); AB037671 (Type III-3A); AB063172 (Type IV-2B); WBG8318 (Type V-5C2); AF411935 (Type VI-4B); AB373032 (Type VII-5C1); FJ390057 (Type VIII-4A); AB505628 (Type IX-1C2); AB505630 (Type X-7C1); FR821779.1 (Type XI-8E)

The ancestry of *mec*A has been elucidated: MRSA is generated by the integration of a *mec*A-carrying SCC*mec* element into a methicillin-susceptible *S. aureus* (Enright *et al.*, 2002). A novel methicillin-

resistance gene, named *mec*C by the International Working Group (IWG) on the Classification of SCC*mec* elements (formerly *mec*A$_{LGA251}$), was first described in a *S. aureus* isolated from dairy cattle in England in 2011 (García-Álvarez *et al.*, 2011); it has since been isolated from clinical human samples in England, Scotland, and Denmark (van Duijkeren *et al.*, 2014). This *mec*C gene is located in a novel SCC*mec* element, type XI-SCC*mec*, and shares only 70% nucleotide sequence identity with *mec*A (Figure 4).

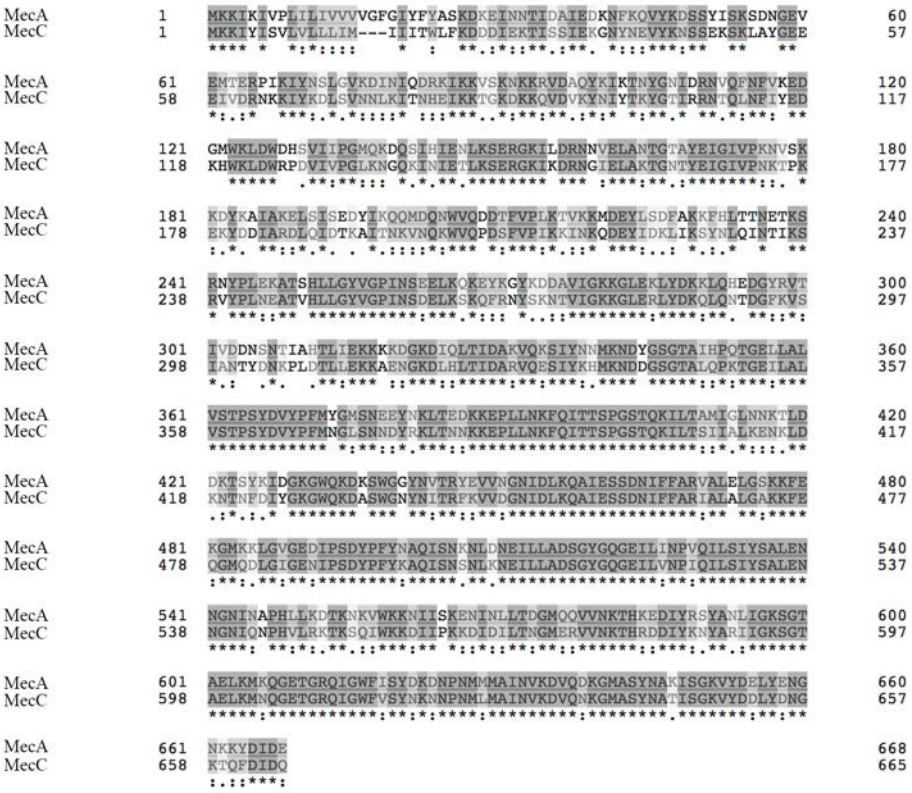


Figure 4. Comparison between amino acid sequences of MecA (Genbank accession number AGC51118.1) and MecC (Genbank accession number WP_000725529.1). Identity is 63.17% (422 identical positions and 187 similar positions). * (asterisk) indicates positions which have a single, fully conserved residue; : (colon) indicates residues with strongly similar properties- scoring > 0.5 in the Gonnet PAM 250 matrix; . (dot) indicates residues with weakly similar properties- scoring =< 0.5 in the Gonnet PAM 250 matrix. The online Clustal Omega tool, hosted at www.uniprot.org, was used

In addition to the MRSA strains' adaptation to selective beta-lactam antibiotic drugs pressure, their potential for production of enterotoxins has also been investigated. However, the prevalence of enterotoxigenic MRSA in food-producing facilities and associated foodstuffs is low and usually related to milk or dairy products. Three MRSA strains (1.2%) isolated from raw milk and dairy products were found to be enterotoxigenic in one study in Italy (Carfora *et al.*, 2015), and similarly 2 MRSA (1.33%) isolated from milk tanks were found to produce multiple staphylococcal enterotoxins in US (Haran *et al.*, 2012).

1.6.2. Epidemiology of MRSA

MRSA can be transmitted in several ways so the epidemiology is complicated; numerous and diverse stages of Public Health Systems and food production processes are implicated (Figure 5). MRSA has long been

considered a major hospital multidrug-resistant pathogen, and has become a serious threat in hospitals worldwide (Crombé *et al.*, 2013). Hospital-associated MRSA (HA-MRSA) has been extensively documented in industrialized countries and is associated with septicaemia, pneumonia, and surgical site infections (Diekema *et al.* 2001). In the USA, the Centers for Diseases Control and Prevention (CDC) reported that, compared to 2013, the incidence of HA-MRSA decreased by 5.36%, while the incidence of community-associated MRSA (CA-MRSA) increased by 1.57% (CDC, 2016). In Europe, countries with proactive "Search and Destroy" control programmes— Netherlands, Finland, Norway, Sweden, and Denmark —reported that MRSA incidence has now stabilized or decreased significantly over the last four years. However, MRSA remains a public health priority as its incidence is still above 25% in seven of 29 reporting countries, mainly in Southern and Eastern Europe (ECDC, 2015).

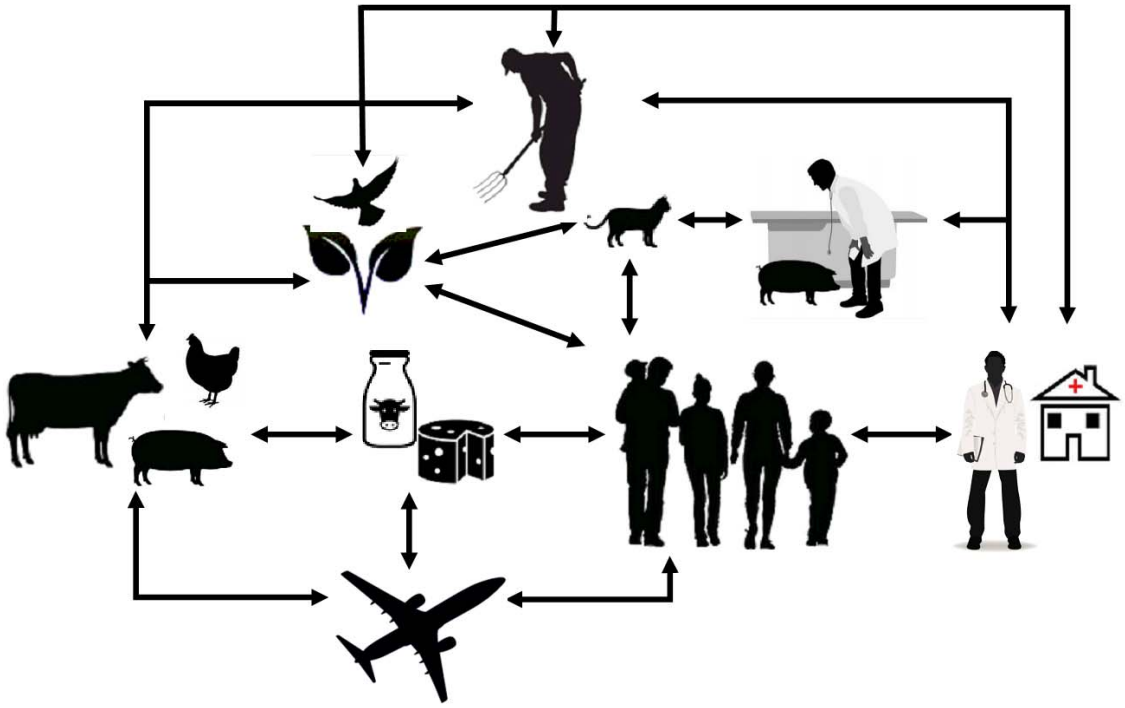


Figure 5. Potential routes of transmission of MRSA

MRSA is found in other, non-healthcare, ecological niches. Various MRSA lineages have been increasingly detected in community settings such as nursing homes and kindergartens, and are known as community-associated MRSA (CA-MRSA). CA-MRSA can cause infections with nonspecific virulence and spread behavior (Pantosti, 2012); many are susceptible to several narrow-spectrum antimicrobial agents (Bassetti *et al.*, 2009). CA-MRSA strains differ from HA-MRSA strains: they have a different accessory genome, which carries different SCC*mec* elements, and cause different clinical symptoms (Crombé *et al.*, 2013; Enright *et al.*, 2002).

A new MRSA lineage has emerged, the so-called livestock-associated MRSA (LA-MRSA) (Voss *et al.*, 2005), belonging to the Clonal Complex 398 (CC398) as determined by multi-locus sequence typing (MLST). This lineage arose as the result of a jump from humans to an animal host, associated with both the loss of phage-borne human virulence genes, which reduced its zoonotic potential, and the acquisition of tetracycline and methicillin resistance (Price *et al.*, 2012). Sequence type 398 (ST398) is the most prevalent LA-MRSA in Europe and North America (Pu *et al.*, 2009; Chuang and Huang, 2015); MRSA ST9 is the most prevalent in

Asian farms and animal-derived products (pork, chicken, and raw milk) (Wagenaar *et al.*, 2009; Chuang and Huang, 2015). A variant of ST9 has been identified indicating that the food chain allows continuous evolution and transmission of different MRSA lineages. ST9 and ST398 MRSA have become more widespread (Table 4); the prevalence of CC398 MRSA increased from 0.3% in 2004-2005 to 5.4% in 2010-2011 in Germany, suggesting that LA-MRSA could become a serious health risk for humans (Cuny *et al.*, 2013; Fromm *et al.*, 2014). There have been two human cases of *mec*C MRSA infection in Denmark, evidence that some *S. aureus* lineages are not strongly host-species restricted (García-Álvarez *et al.*, 2011; Petersen *et al.*, 2013; Angen *et al.* 2016).

Table 4. *Recently described prevalence and characteristics of MRSA isolated from farms, farm animals, food products, and human carriage, 2000-2013*

Year of study	Country/ State	No. of samples	Prevalence of MRSA, %	Major outlines	Reference
Animal carriage					
2004-2007	Germany	138	**43**	Most (57/60) of the MRSA isolates found in affected porcine tissue were spa-types associated with MRSA ST398. Three MRSA were ST97.	(Meemken *et al.*, 2010)
2005-2006	The Netherlands	540	**39**	MRSA isolated from pigs belonged to one clone, ST398 and closely related *spa* types t011, t108, t1254.	(de Neeling *et al.*, 2007)
2007-2008	The Netherlands	2151	**28**	Cross-sectional survey revealed lower risk of LA-MRSA in calves from rose veal farms than calves from white farms.	(Bos *et al.*, 2012)
2008	Italy	118	**38**	Eleven different *spa*-types were found among 102 MRSA pig isolates, clustering in lineages associated with farm animals (ST398, ST9, ST(CC)97 in 36 farms) and humans (ST1 in 7 farms).	(Battisti *et al.*, 2010)
2009	Denmark	789	**13**	MRSA was isolated from nasal swabs from pigs at the slaughter line. Ninety-three % were CC398 (*spa* t011, t034, t1451, t2876, t2974), 4% to CC30 (t1333) and one isolate to CC1 (t0127).	(Agersø *et al.*, 2012)
2010	Italy	461	**61**	Prevalence of MRSA carriage in humans significantly correlated with the % of positive cows on the farm, the number of livestock units and positive bulk tank milk samples.	(Antoci *et al.*, 2013)
2011-2012	The Netherlands	411	**3.9**	All MRSA isolated from healthy dairy cattle were CC398, with *spa* type t011 being predominant.	(van Duijkeren *et al.*, 2014)
2012-2013	Italy	82	**26***	First samples from rabbits and humans contained *spa* types t5210 and t034 belonging to MLST ST398	(Agnoletti *et al.*, 2014)
2013	Japan	100	**8**	Nasal swabs from pigs revealed ST97/*spa* t1236/SCC*mec* V and ST5/*spa* t002.	(Sato *et al.*, 2015)
Farm environment					
2008	China	46	**89**	From 4/9 pig farms the MRSA isolates of *spa* type t899 were assigned to ST9 whereas on one farm the MRSA *spa* type t899 isolates belonged to a single locus variant of MLST ST9 (ST1376).	(Wagenaar *et al.*, 2009)
2013	Meta-analysis	400	**53.5**	Pooled analysis of pig herds confirmed known risk factors (herd size, herd type) and identified other factors such as role of treatment of fattening pigs with antimicrobial drugs and effect of housing fattening pig herds.	(Fromm *et al.*, 2014)
Food products					

2003-2009	Japan	197	**1.5**	Seven isolates from three meat and four stool samples exhibited the same ST8, *spa* type 606 (t1767).	(Ogata *et al.*, 2012)
2007-2008	The Netherlands	2217	**11.9**	85% of isolates from raw meat samples belonged to *spa* types t011, t034, t108 corresponding to ST398.A few of these strains were found to be of other STs, possibly of human origin.	(de Boer *et al.*, 2009)
2007-2009	Spain	318	**1.6**	The two strains from pork and veal were to ST398-SCC*mec*V (*spa* type t011 and t1197, respectively), the two strains from chicken and rabbit were ST125-SCC*mec*IVa-t067, and the strain from a wild boar was ST217-SCC*mec*IVa-t032. All MRSA were PVL negative	(Lozano *et al.*, 2009)
2008	United States	120	**5**	Six retail meat samples (5%) contained MRSA strains, which were members of two unique human epidemic clones, USA100 and USA300.	(Pu *et al.*, 2009)
2008-2009	Germany	150	**6**	At the slaughter line, 6% of the 150 pig carcass samples were positive for MRSA. In most cases, only one sample was positive per carcass, but one carcass had two positive samples with MRSA of different *spa* types (t011, t034).	(Beneke *et al.*, 2011)
2008-2009	Spain	601*	**1.7**	A sample from one (0.44%) of 229 farms (1 of 601 *S. aureus* isolates) harboured *mec*C MRSA, and three (1.31%) farms (9 of 601 isolates) tested positive for *mec*A-MRSA.	(Ariza *et al.*, 2014a)
2009	Iowa	165	**1.2**	The MRSA strains from meat were t008/ST8 and t034/ST398, with the t008 isolate found to be SCC*mec* type IV and the t034 isolate SCC*mec* type V. The t008 MRSA isolate carried the *pvl* gene.	(Hanson *et al.*, 2011)
2009	Denmark	865	**18**	Imported broiler meat had the highest occurrence (18%) of MRSA, followed by imported pork (7.5%) and Danish pork (4.6%). MRSA ST398 was found for the first time in Danish beef (1.4%). The finding of MRSA CC30 (*spa* t1333) suggests possible spread of the SCC*mec* cassette associated with ST398 into another *S. aureus* lineage common in pigs.	(Agersø *et al.*, 2012)
2009	Georgia	100	**7**	One retail beef MRSA isolate belonged to ST8, and the other three were ST5. In retail pork MRSA ST5, ST9, and ST30 were also detected.	(Jackson *et al.*, 2013)
2010	Italy	48	**44**	MRSA carrier prevalence in humans significantly correlated with the % of positive cows on the farm, the number of livestock units and positive bulk tank milk samples.	(Antoci *et al.*, 2013)
2011-2012	England and Wales	465	**2.15**	*mec*C MRSA prevalence was 2.15% with seven isolates being ST425, and the other three being CC130. *mec*A MRSA was identified in a single farm in Worcestershire giving a prevalence of 0.27% (95% CI 0.05%-1.50%). This isolate was ST398.	(Paterson *et al.*, 2014a)
2012-2013	non-EU flights	195	**9.1**	Five MRSA food isolates were ST8 and harboured SCC*mec* type IVc and PVL genes. One isolate was ST1649, harbored SCC*mec* type IVc and tested negative for *pvl*.	(Rodríguez-Lázaro *et al.*, 2015)

2012-2013	non-EU flights	200	**0.5**	MRSA isolated from pork lard was ST398, harboured SCC*mec* type V, and tested negative for *pvl*.	(Oniciuc *et al*., 2015)
Human carriage					
2000-2011	Spain	164	**20**	Ten patients (30%) were infected; cancer was the most frequent underlying disease. In one case, death was caused by a MRSA-ST398-related infection.	(Camoez et *al*., 2013)
2005	UK	78	**17.9**	Evidence of EMRSA-15 mucosal carriage in veterinary staff and hospitalized dogs (9%).	(Loeffler *et al*., 2005)
2005-2011	Switzerland	1062	**7.5**	*mecC* MRSA was recovered from a 59-year-old man admitted to the ICU for community-acquired septic shock secondary to a perforated duodenal ulcer. The isolate belonged to ST130 (CC130) with *spa* type t11150.	(Basset *et al*., 2013)
2006	Denmark	272	**12.5**	Thirty-one veterinarians carried a PFGE non-typeable strain with *spa* types (t011, t034, t108, t571, t567, t899); s*pa* type t899 was found in Dutch pigs, pig farmers and/or vets.	(Wulf *et al*., 2008b)
2006-2008	Austria	1098	**1.9**	All 21 patients (14 males, 7 females; median age 58 years, range 1–83 years) harboured MRSA ST398. Only five patients were infected, and 15 were colonized. The prevalence of MRSA ST398 in Austria increased from 1.3% (2006) to 2.5% (2008).	(Krziwanek *et al*., 2009)
2007-2009	The Netherlands	117	**24**	Nasal swabs from human revealed *spa* types t108, t011, t567, t3934.	(Feßler *et al*., 2011)
2007-2008	Spain	70	**68***	ST5-MRSA-IV predominated (33.8%), followed by ST125-MRSA-IV (23.5%), ST8-MRSA-IV (23.5%), and ST125-MRSA-IV/VI (14.7%). ST228-MRSA-I and 2, novel STs, were each isolated once (1.5%). Neither CA-MRSA nor LA-MRSA isolates were observed.	(Ariza *et al*., 2014b)
2008	The Netherlands	155	**38**	The *spa*-types were heterogeneous; although 92.7% were LA-MRSA CC398, a surprisingly high proportion (7.3%) were not.	(Graveland *et al*., 2011)
2008-2010	USA	148	**20.9**	t034, a common *spa* type within the ST398 lineage of LA-MRSA was the predominant *spa* type found among human isolates.	(Smith *et al*., 2013)
2009	Australia	771	**5.9**	The prevalence was 11.8% among vets specializing on horses and 4.9% among those working with dogs and cats.	(Jordan *et al*. 2011)
2009-2010	Germany	341	**3.2**	Most nasal isolates (73%) from workers at Dutch pig slaughterhouses were the LA-MRSA clone ST398.	(Doyle *et al*., 2012)
2010	Italy	113	**36**	MRSA carriage prevalence in humans significantly correlated with the *per cent* of positive cows on the farm, the number of livestock units and positive bulk tank milk samples.	(Antoci *et al*., 2013)
2012	Taiwan	100	**13**	Pig workers carried LA-MRSA ST9 in nares.	(Fang *et al*., 2014)

**no of isolates*

Epidemiology of different variants of MRSA

Oxacillin-susceptible MRSA (OS-MRSA)

Oxacillin-susceptible strains carrying a *mec*A gene have been described by conventional phenotypic methods as oxacillin-susceptible *mec*A-positive (OS-MRSA). Saeed *et al.* (2014) demonstrated by the MicroScan method that such strains are fully susceptible to oxacillin, but show intermediate susceptibility by disk diffusion tests; chromogenic media detect them only poorly. Conventional microbiology tests can easily misidentify OS-MRSA strains, questioning the *mec*A gene's contribution to their level of resistance (or susceptibility) (Pournaras *et al.*, 2013). Based on pulsed field gel electrophoresis (PFGE), recent reports have revealed that OS-MRSA strains are genetically highly diverse and distinct from some common UK epidemic MRSA strains (Saeed *et al.*, 2014), while in OS-MRSA clonal spread in Brazil is related to epidemic clones such as USA400, USA100/New York/Japan, USA800/PC and BEC (Andrade-Figueiredo and Leal-Balbino, 2016). However, it is not known whether OS-MRSA strains could carry *mec*C genes.

OS-MRSA have been found among clinical isolates (Chen *et al.*, 2012; Conceiçao *et al.*, 2015), in animals and associated foodstuffs— bovine mastitis milk samples —(Pu *et al.*, 2014), and food imported into Europe illegally (Rodríguez-Lázaro *et al.*, unpublished data). Among 103 bovine mastitis milk isolates, 37 out of 49 *mec*A-positive isolates were OS-MRSA by conventional diagnostic tests, and belonged to different *spa* and SCC*mec* types (Pu *et al.*, 2014). Although rare, OS-MRSA should not be neglected because it may develop unusual resistance under antibiotic selection due to its *mec*A gene (Saeed *et al.*, 2014).

Borderline oxacillin-resistant S. aureus (BORSA)

S. aureus strains acquiring resistance through hyperproduction of β-lactamase are called borderline oxacillin-resistant *S. aureus* (BORSA); their minimum inhibitory concentration is near the oxacillin breakpoint (MIC >2 mg/L according to EUCAST). They are not resistant to multiple antimicrobial agents, and do not carry *mec*A or *mec*C genes (Dien Bard *et al.*, 2014). The clinical significance of this type of resistance is not yet known and laboratories should routinely test for both cefoxitin and oxacillin resistance, in addition to using PCR methods for *mec*A and *mec*C genes testing. Like the situation for OS-MRSA, chromogenic plates are of decreasing diagnostic value (Buchan and Ledeboer, 2010). BORSA has been also isolated from food products (minced pork meat), and is genetically closely related to human strains (Bystroń *et al.*, 2010).

1.7. Presence of *Staphylococcus aureus*, Particularly MRSA in The Food Chain

MRSA in food producing animals and associated foodstuff

MRSA has been isolated from food products, implicating food as a pathway for MRSA dissemination. Information about the potential zoonotic transmission of MRSA variants to humans is limited, but the issue is of public health interest because MRSA can cause life-threatening disease and cannot be detected by standard testing procedures (Ariza *et al.*, 2014a). MRSA has also been found in a wide range of companion animals, from dogs, cats, and horses to rabbits, guinea pigs, turtles, bats, and parrots, but the primary reservoir remains in food production animals (Loeffler *et al.*, 2005; EFSA, 2009a; Pantosti, 2012; Petinaki

and Spiliopoulou, 2012). An unexpectedly high prevalence was found in the Netherlands with 39% of screened pigs (229 out of 540) being positive for MRSA (de Neeling *et al.*, 2007).

The MRSA presence in pigs and workers at industrial abattoirs in southern Italy was also found to be as high as 37.6% (99 out of 215 pig nasal swabs) (Normanno *et al.*, 2015). Similarly, MRSA prevalence in milk and dairy products has been studied: 45 dairy farms from southeastern part of Sicily (Italy) were tested in 2010 for the presence of MRSA among farmers, cattle, and bulk tank milk samples, and a high MRSA rate was found (55%; 344 out of 622); 61% (283 out of 461) of bovine samples tested positive for MRSA, in comparison with 36% (40 out of 113) in human samples and 44% (21 out of 48) of bulk tank milk samples (Antoci *et al.*, 2013). In Germany, the highest fraction of MRSA positive samples was found in nasal swabs from veal calves in 2012 (45%; 144 out of 320) and the lowest rate in bulk tank milk in 2009 (4.1%; 14 out of 388) (Tenhagen *et al.*, 2014). In the Netherlands, van Duijkeren *et al.* (2014) found a MRSA prevalence of 3.9% (16 out of 411) in cattle, although none of the isolates harbored the *mec*C gene. Meanwhile, (Paterson *et al.*, 2014a) found a *mec*C MRSA prevalence rate of 2.15% among 465 bulk milk samples in England and Wales, but no *mec*C was detected in 625 farms sampled in Scotland.

Transmission of zoonotic MRSA to humans can occur *via* either animal contact or contaminated food (Schoder *et al.*, 2015). The first reported zoonotic transmission episodes from pig to human were early last decade in the Netherlands (Voss *et al.*, 2005): a 6-month-old child admitted to hospital for thoracic surgery was positive for MRSA, as well the parents who were pig farmers; all were colonized with the same identical MRSA isolate. Also, a veterinarian, his child, and the nurse who treated the child were all colonized with the same *spa* type 108 MRSA isolate. This was the first reported case of apparent zoonotic transmission from pigs to a vet, and from the vet to family members (Voss *et al.*, 2005).

The prevalence of CC398 MRSA in food-producing animals is increasing and people in direct contact with live animals are at higher risk of colonization or infection than the general population (EFSA, 2009a). In the Netherlands, Europe´s largest exporter of live pigs (Petinaki and Spiliopoulou, 2012), contact with pigs is recognized as a risk factor for MRSA nasal carriage (de Boer *et al.*, 2009). With an annual production of 25 million pigs, Denmark is also a large potential reservoir for MRSA ST398 (Lewis *et al.*, 2008). Ten per cent of Belgian farms had a MRSA problem originating from cases of subclinical or clinical mastitis (Vanderhaeghen *et al.*, 2010). Livestock-associated ST398 has been found in bulk tanks in dairy farms in the UK (Paterson *et al.*, 2014a), swine, and environmental holdings in Italy (Locatelli *et al.*, 2016), in veal calves (Tenhagen *et al.*, 2014) and poultry (Feßler *et al.*, 2011) in Germany, in raw food samples in Spain (Lozano *et al.*, 2009) and in food products illegally imported into the European Union (EU) (Oniciuc *et al.*, 2015). MRSA was recently isolated from retail pork meat sold in a British supermarket (Hadjirin *et al.*, 2015), and MRSA contamination of retail meat has been demonstrated in several studies.

The diversity of MRSA lineages, which varies not only with the animal species but within and between different farm units suggests the existence of an active and dynamic sharing of MRSA lineages among isolates (Locatelli *et al.*, 2016). However, only a small proportion of all MRSA in humans is MRSA ST398 (van Cleef *et al.*, 2011). Epidemiological data suggest that LA-MRSA have lower transfer rates, and are less virulent than other STs. Price *et al.* (2012) suggest that the low transmission rates of porcine CC398 strains to humans is due to poor adhesion of CC398 to human cells.

Although the origin of *mec*C in MRSA isolates from dairy cattle in England (García-Álvarez *et al.*, 2011) is not clear, it is obvious that contact with animals poses a zoonotic risk; *mec*C can be transmitted between species and therefore may be found in LA-MRSA (Paterson *et al.*, 2014b).

Human clones can colonize pigs, for example ST5 (USA100) was found in Canada, ST22 in Ireland (Pantosti, 2012), and variants of ST9 in Italy and China (Battisti *et al.*, 2010; Fang *et al.*, 2014). The presence of human clones in pigs can result from human-to-pig contamination, but some strains, such as the t127/ST1 clone, may be animal-adapted (Pantosti, 2012). MRSA strains with a "typical" human genetic background (ST5, ST8, ST22, ST30, and ST45) have been reported in pigs or pig farms in Europe, USA, and Africa (Crombé *et al.*, 2013). Ruminants (dairy cattle and sheep) can be healthy carriers of *mec*C MRSA CC130, evidence that they serve as a zoonotic reservoir allowing MRSA transmission to humans. Such transmission is increasing and now accounts for 2% of human MRSA cases annually in Denmark (Petersen *et al.*, 2013). OS-MRSA isolates have been identified in milk samples collected from cows with mastitis in four different regions in China (Pu *et al.*, 2014) and BORSA in pork meat (Bystroń *et al.*, 2010).

Incorrect handling during animal slaughter can lead to MRSA contamination of carcasses (Argudín *et al.*, 2015), and consequently a risk for human consumption and dissemination in the community. CA-MRSA is a public heath challenge because it spreads effectively in both urban and rural settlements, and meat may contribute to the spread of both CA-MRSA (Ogata *et al.*, 2012) and HA-MRSA (Feßler *et al.*, 2011). CA-MRSA clones have been also reported in farm animals and associated foodstuffs in the USA, and in foods confiscated from passengers on non-EU flights (Rodríguez-Lázaro *et al.*, 2015). Thus, MRSA contamination *via* food is associated not only with zoonotic transmission but also with unsafe food handling: CA-MRSA strains are by definition from a human source. Consequently, control and prevention measures are required throughout the entire meat chain, from primary production to retail (Normanno *et al.*, 2015).

Evolution and emergence of LA-MRSA

Intensive livestock farming has been developing since the mid-twentieth century in response to the demand for meat. This has included the overuse of antibiotics as growth promoters and as preventive therapy, which has facilitated the emergence of new pathogens, including those resistant to antibiotics. One consequence is the transmission of such pathogens to humans *via* food of animal origin. The food production chain, from farm to fork, may contribute to transmission of MRSA: MRSA has been already found in pigs (Fromm *et al.*, 2014) and workers in contact with them, particularly farmers, abattoir workers and vets (Cuny *et al.*, 2013).

CC398 has been found repeatedly in pigs since 2005 (Voss *et al.*, 2005; Lewis *et al.*, 2008) (Table 4). MRSA CC398 has been isolated in Germany from farmers (86%) and vets (45%), as well as a small proportion of their close relatives, evidencing inter-human transmission, albeit at a low rate (Cuny *et al.*, 2009). The prevalence is higher among people in contact with live animals (5.2%), especially those hanging broilers on the slaughter line (20.0%), than among other workers (1.9%) (Mulders *et al.*, 2010). MRSA prevalence is significantly higher among men than women (39.8% *vs.* 6.6%, $p<0.05$) (Antoci *et al.*, 2013).

MRSA isolates of ST2077 (Lozano *et al.*, 2012) and ST398 belong to CC398, and ST398 is the principal clone shared by animals and humans (Pantosti, 2012). Generally, MRSA CC398 emerged in the USA and Europe

(O'Brien *et al*., 2012; Buyukcangaz *et al*., 2013). Countries with a higher density of pig farms have a higher rate of MRSA (EFSA, 2009a). It was first reported in the Netherlands and confirmed elsewhere in Europe and beyond that living or working on a farm was a risk factor for acquiring LA-MRSA (Voss *et al*., 2005). ST398 is present on up to 70% of German pig farms, and includes more than 90% of all European porcine strains (Köck *et al*., 2012). MRSA ST398 has been found on cattle, turkey, rabbit, and poultry farms (Spohr *et al*., 2011; Richter *et al*., 2012; Agnoletti *et al*., 2014) and even in companion animals (Pantosti, 2012), leading to the recognition of a third epidemiological form of MRSA (Paterson et al. 2014a) that can cause severe human infections (Pan *et al*., 2009) and even death (Camoez *et al*., 2013).

Most LA-MRSA ST398 are susceptible to antibiotics other than β-lactams, but resistant to tetracycline; presumably the massive use of tetracycline in pig farming favored the emergence of this clone (Moodley *et al*., 2011). ST398 typically carries SCC*mec* type IV or, more frequently, type V (Monecke *et al*., 2011) or type VII (Price *et al*., 2012), and can be distinguished by its particular resistance to digestion by *Sma*I, the macro restriction enzyme most frequently used in *S. aureus* typing by PFGE (Chung *et al*., 2000). LA-MRSA ST398 classified as non-typeable (NT)-MRSA can however be typed by PFGE using the *Sma*I isoschizomer, *Cfr*91 (Molla *et al*., 2012). Although there is less nosocomial transmission of this LA-MRSA genotype than other genotypes (Cuny *et al*., 2013), its rapid emergence and epidemiological trajectory make it of public health relevance. MRSA ST398 has been increasingly frequently isolated from patients in the Netherlands since 2003 (Lewis *et al*., 2008) and a hospital outbreak has been reported (Wulf *et al*., 2008a).

Emergence of MRSA *via* neglected routes of transmission

Neglected pathways in terms of MRSA transmission and spread may occur. Few information is available regarding prevalence of enterotoxigenic antibiotic-resistant strains associated with food subjected to international trade. Moreover, illegal food transportation by travelers flying from different parts of the globe could lead to successful dissemination of MRSA lineages (Oniciuc *et al*., 2015; Rodríguez-Lázaro *et al*., 2015; Schoder *et al*., 2015). The USA custom office asked all passengers when arriving at the airport to fulfill a form saying if they have been in direct contact with animals (Category A referring to zoonotic disease transmissible through animal contact) or if they carry any animal food originated product which can potentially harm/ contaminate or animals infected with zoonotic agents (Category B) (Noordhuizen *et al*., 2013).

Referring to the EU, several regulations have been issued regarding the imports of animals and associated foodstuff. Most of these regulations refer to the importation of animals and products of animal origin, which were previously subjected to veterinary controls, however most of the directives mentions commercial trade, subsequently referring to large quantities which may be subjected to transportation (EC 275/2007; EC 206/2009). Therefore, small volumes of foods intended for personal consumption which are potentially present in traveler luggage are exempted from customs control (Schoder *et al*., 2015).

Unfortunately, few data are available concerning associated risks but also no attempts have been made to perform a meta-analysis on the actual prevalence of MRSA carried by travelers. In addition, travelers must understand and learn to accept the prohibition regarding the food traffic, consequently the risk of foodborne pathogens spreading. Unfortunately, the increased number of people travelling and the increased global trade will contribute to future outbreaks regardless the measures which will be taken.

1.8. Biofilm Formation and Composition Correlated with *S. aureus* Isolated From Food Sources

S. aureus has been considered as one of the world's leading cause of foodborne illnesses (Kim *et al.*, 2016), and the primary staphylococcal niche for human body colonization, subsequently being involved in appearance of infections ranging from folliculitis to septicemia, pneumonia, TSS, endocarditis, and other (Otto, 2010; Szczuka *et al.*, 2013; Brown *et al.*, 2014).

Nowadays, biofilm formation by this bacterium draws considerable attention since many infections are mediated by the ability of *S. aureus* to adhere to catheters or other medical devices (Szczuka *et al.*, 2013). *S. aureus*, together with *S. epidermidis* are commonly found on cardiovascular devices, with 40%-50% causing prosthetic heart valve infections and 50%-70% catheter biofilm infections, consequently increasing the health care associated costs (Chen *et al.*, 2013).

In addition, *S. aureus* biofilm formation (Figure 6) represents a hidden pathway for food processing plants and human handlers, for which has got increased attention in the last twenty years (Rode *et al.*, 2007; Gutiérrez *et al.*, 2012). Since then, many studies demonstrated *S. aureus* ability to adhere and form biofilms on food-contact surfaces of different kinds such as stainless steel, polystyrene or glass. However, the literature about biofilm formation by food-related *S. aureus* strains is still scarce as many environmental conditions and/or food handlers microbiota could interfere and contribute in their development (Vázquez-Sánchez *et al.*, 2013; Santos *et al.*, 2014; Di Ciccio *et al.*, 2015).

Moreover, biofilms came into attention as they have been shown increased tolerance to antimicrobial agents. MRSA strains adopt survival strategies by gaining several advantages when are disposed in biofilm state of growth, thus being protected from antibiotics, disinfectants, or various environmental conditions (Garrett *et al.*, 2008; Uršič *et al.*, 2008) which may be found in medical and food sectors. The gene expression is altered, in response to different environmental changes such as temperature, osmolarity, dryness, high pressure, high salt concentration, pH, oxygen supply, source of nutrients and other (Rode *et al.*, 2007; Szczuka *et al.*, 2013; Vázquez-Sánchez *et al.*, 2013; Kim *et al.*, 2016).

In other words, biofilm growing cells have distinct properties from their planktonic counterparts by adopting an altered phenotype (Garrett *et al.*, 2008). In a study conducted by Williams *et al.* (1997), ten times more the minimal bactericidal concentration (MBC) of vancomycin was necessary for a 3-log reduction for *S. aureus* biofilms. The metabolic rates may be explained by the capability of biofilms to act as a diffusion barrier to slow down the infiltration of some antimicrobial agents (Archer *et al.*, 2011). Another study conducted by Singh and coworkers (2010) showed diminished penetration of antibiotics (*e.g.* oxacillin, cefotaxime or vancomycin) against biofilms formed by *S. aureus* and *S. epidermidis*. However, *S. aureus* and *S. epidermidis* biofilms remained unaffected when amikacin and ciprofloxacin were introduced in the assay (Singh *et al.*, 2010).

Since first known use of the term "*biofilm*" in 1981, and Antoine van Leeuwenhoek's first discovery of microorganisms on tooth[1] surface in 1684, several general definitions of biofilms were adopted:

[1] "The number of these animicules in the scurf of a man's teeth are so many that I believe they exceed the number of men in a kingdom."

Donlan and Costerton (2002)– *"biofilm is a microbial derived sessile community characterized by cells that are irreversibly attached to a substratum or interface or to each other, are embedded in a matrix of extracellular polymeric substances (EPS) that they have produced, and exhibit an altered phenotype with respect to growth rate and gene transcription"*

Azeredo *et al.* (2014)– *"biofilms are microbial communities embedded in a self-produced polymeric matrix formed by EPS and present on the surface of biotic or abiotic materials or even in an air/ liquid interface"*

S. aureus biofilm can be viewed as a fortress that it is constructed gradually until it becomes an emergent form of microbial life. During biofilm growth, *S. aureus* adopts a biofilm-specific phenotype, completely different from their counterparts– planktonic cells. For this irreversible switch, *S. aureus* must go through different complex metabolic, phenotypic, and physiological transformations (Azeredo *et al.*, 2014).

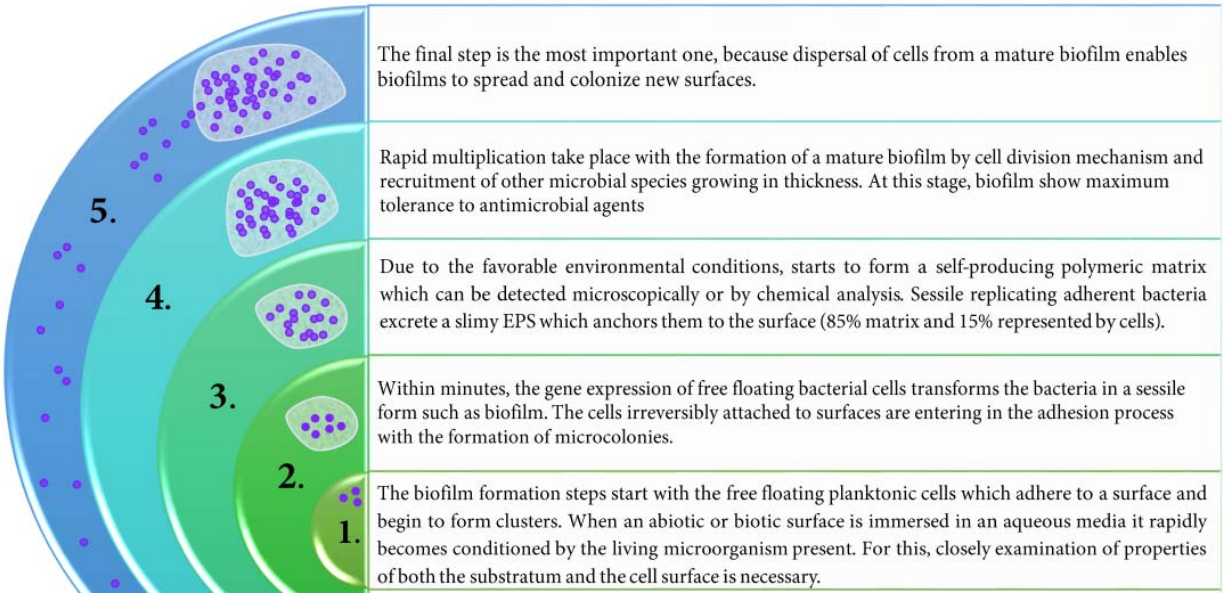


Figure 6. Biofilm formation steps (Donlan, 2001)

The development of a *S. aureus* biofilm is a complex, multifactorial process triggering a multitude of mechanisms (Speziale *et al.*, 2014) (Figure 7). Bacteria living in biofilms expresses a variety of macromolecular products, including exopolysaccharides, enzymes, proteins, extracellular DNA (eDNA), and other low mass solutes. All these components, known as EPS are responsible for the biofilm matrix forming, contributing to its architecture and stability (Flemming *et al.*, 2016). The EPS molecules can trap sources of nutrients and other molecules, enhancing biofilm's ability to survive in situations of starvation (Azeredo *et al.*, 2014; Flemming *et al.*, 2016).

Moreover, *S. aureus* biofilms undertake specific changes in protein secretions. The *S. aureus* surface proteins C and G (SasC and SasG), clumping factor B (ClfB), serine aspartate repeat protein (SdrC), the biofilm associated protein (Bap), and the fibronectin/ fibrinogen-binding proteins (FnBPA and FnBPB) are known to be individually implicated in the biofilm matrix formation (Speziale *et al.*, 2014). Its metabolic behavior may also change due to the altered phospholipids' synthesis and exopolysaccharide production (Azeredo *et al.*, 2014). These properties can trigger mechanisms related with virulence, resistance to antimicrobial agents and capacity for intracellular persistence, thus providing an ideal strategy for counteracting with external factors.

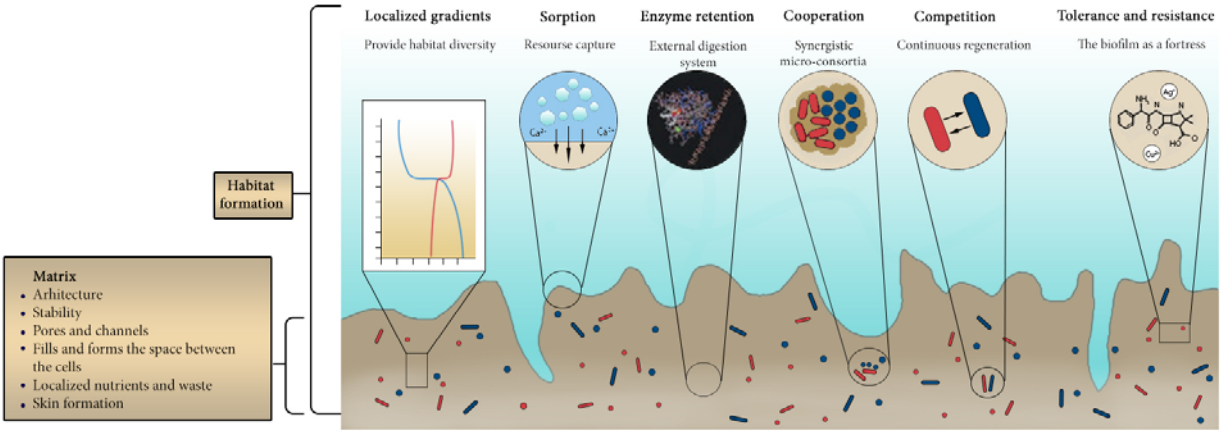


Figure 7. Emergent properties of biofilms and habitat formation

Many *in vitro* studies concluded that *Staphylococcus* spp. can produce strong biofilms. The effect of different incubation atmospheres using limited-nutrient media (Uršič *et al.*, 2008), different materials similarly to food processing surfaces (polystyrene and stainless steel) (Di Ciccio *et al.*, 2015), or the effect of NaCl, glucose addition to the original growing media– all of this proved to show the production of a significant higher biofilm biomass (Oniciuc *et al.*, 2016), or an improved medium for biofilm growing (Brain Heart Infusion-BHI plus 10% human plasma) (Chen *et al.*, 2012). However, low biomass has been observed in aerobic atmosphere rather than CO_2-rich environments (aerobic with 5% CO_2 or anaerobic using 10% CO_2, 10% H_2 and 80% N_2) (Chen *et al.*, 2012).

Knowing what drives a bacterium to produce a biofilm is the key element for applying proper and efficient hygienic measures for their removal. For example, the best strategy for improving the safety of meat products is by applying proper good manufacturing practices (GMP) and good hygiene practices (GHP) together with the application of antimicrobials at preharvest, postharvest, processing, storage, distribution, and consumption stages (Gutiérrez *et al.*, 2012). Moreover, milk processing equipments may get contaminated through water used for cleaning them. However, cleaning-in-place (CIP) procedures engaged in milk processing lines can have a limited effectiveness against residual microorganisms, which can regrow and form new biofilms (Bremer *et al.*, 2006).

To note, we are only beginning to scratch the surface regarding properties of the biofilm matrix in which their tolerance to antimicrobials sets the pilons on their lifestyle (Figure 8). Their biofilm-specific phenotype seems to be acquired by the hiding living cells in the matrix, in this way gaining protection from antimicrobials. However, these mechanisms are not fully understood as antimicrobial tolerance remains at concentrations of antimicrobials that are above the saturation point of diffusion–reaction inhibition (Flemming *et al.*, 2016).

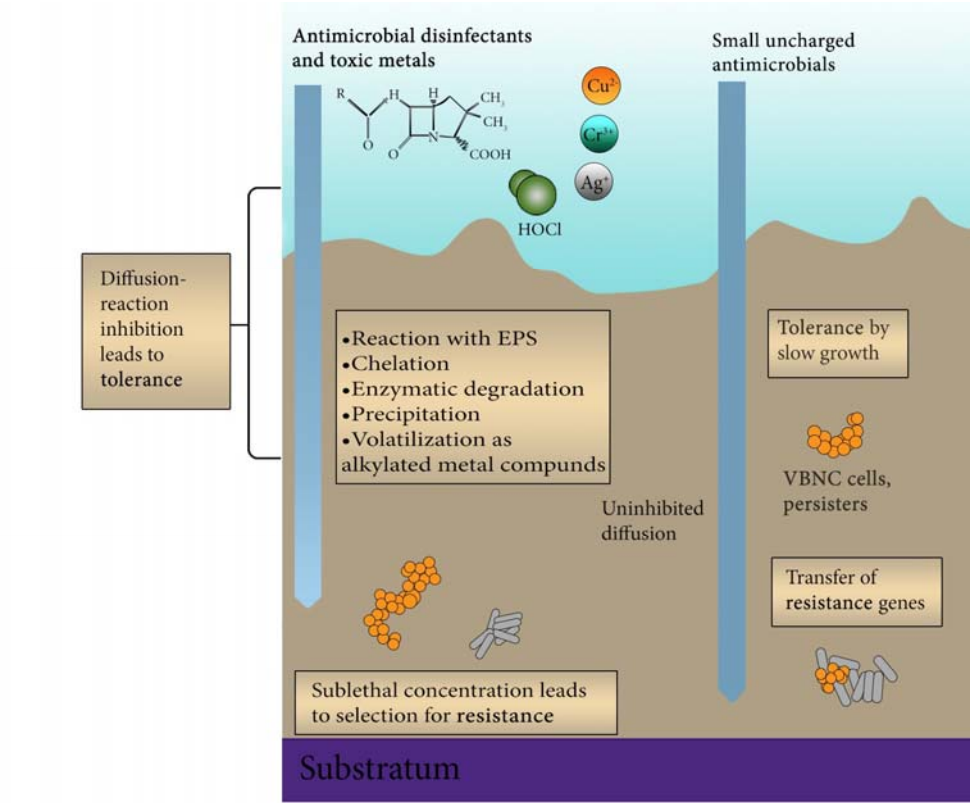


Figure 8. Tolerance of, and resistance to, antimicrobials

1.9. Strategies of Prevention and Control

MRSA is a problem worldwide. Antimicrobial agents have been used for intensive livestock production (as therapeutic and preventive treatment, and to promote growth), and for humans in the community and in hospital, and consequently the development of antibiotic-resistant strains became inevitable. The resulting risks to public health include food safety aspects, as many food products are ready-to-eat without subsequent cooking. LA-MRSA, CA-MRSA and even HA-MRSA can be present in food for human consumption. Food and feed safety is essential, and the presence of MRSA in the food chain may contribute to the increasing dissemination of MRSA worldwide. Moreover, their ability to form biofilms must be considered as well as it represents a big challenge for the food industry, as some strains in their sessile state may tolerate antimicrobial agents, making the bacterium extremely difficult to eradicate (Basanisi *et al.*, 2017).

Figure 5 shows MRSA dissemination pathways, from hospitals to the environment and livestock, and in the opposite direction from farms, farmers, people in contact with animals and associated foodstuffs along the food production chain to the community and hospital settings. Clearly, individuals in direct contact with MRSA-positive animals can acquire MRSA, and occupational livestock contact contains the risk of MRSA colonization for farmers, veterinarians, and abattoir staff: 87% of pig farmers in Denmark and Belgium were found to be colonized over a six-month monitoring period, 13% being intermittent carriers (Köck *et al.*, 2013). CC398 can be transmitted by direct exposure to animals but indirect exposure is also possible: 12.8% of household contacts of MRSA-positive veterinarians were also colonized with MRSA, and 77.8% of them with the same strain (Köck *et al.*, 2013). LA-MRSA (CC398) isolates are, however, increasingly colonizing

people with no contact with livestock, indicating that this lineage is successfully spreading into the community (Larsen *et al.*, 2015).

MRSA introduced into the slaughterhouse on animals or abattoir personnel can lead to contamination of raw meat. There can be cross contamination in the primary food production chain involving animal products or staff. Food contamination by human handling may contribute to the spread of MRSA in hospitals and other institutional environments. For food safety and consumer protection, it is important to determine the origin of MRSA, its dissemination in the food chain, and vectors (air, water) of transmission and spread. Various routes of MRSA transmission to community have been neglected, such as the illegal introduction and commerce of food contaminated with MRSA (Oniciuc *et al.*, 2015; Rodríguez-Lázaro *et al.*, 2015). Illegal commerce is by its nature difficult to assess, so the risk of MRSA transmission by this route is unknown but probably high.

Measures for controlling MRSA spread are necessary. LA-MRSA is a particular problem for the food sector related to the use of antimicrobials, but HA- and CA-MRSA must also be considered. Communication between medical and veterinary practitioners, the agro-food sector and consumers is insufficient, and a "one health" approach or holistic strategy is required to encompass all relevant aspects of the food chain in the community as a whole, from primary production to final consumers. Control and prevention strategies and monitoring programmes should be implemented from farm to hospital, in the same way as food safety applies from farm to fork. International action is needed to control cross-border MRSA spread. Monitoring and surveillance programmes should not be regarded as options but as essential for MRSA control. Prevention of zoonotic transmission from livestock to human requires biosecurity and hygiene control measures (Petersen *et al.*, 2013), and good farming practice involving in particular the rational use of antibiotics (Vázquez-Sánchez *et al.*, 2012).

Finally, the meat production chain should be subject to rigorous monitoring and preventive measures, including strict hygiene standards for staff in contact with meat carcasses and meat products.

1.10. Surveillance Programs in the European Union

MRSA is present all along the food chain in Europe and elsewhere, indicating the need for surveillance programmes. The incidence of HA-MRSA infections in many European countries has been decreasing or has remained low due to programmes aimed to control its spread. However, few European countries have national strategies for controlling CA- or LA-MRSA (Köck *et al.*, 2010). Although the prevalence of these MRSA is still low, such strains can be found in diverse sectors: home care, travel industries, leisure activities, food products, and livestock transport (Köck *et al.*, 2010). The prevalence of LA-MRSA in chicken and pork meat in Germany was reported to be 42% and 16% respectively, revealing the potential for human disease, and the need for surveillance programmes (Köck *et al.*, 2013). In Canada, health authorities consider that additional surveillance efforts are required to monitor the emergence and clinical relevance of LA-MRSA due to recently identification of LA-MRSA infections (Golding *et al.*, 2010). Collaboration between medical and veterinary practitioners is important for developing strategies to protect against LA-MRSA (EFSA, 2009a).

Surveillance along the food chain, from primary production until the final consumer, is necessary for traceability purposes, favoring detection and control of new types of MRSA. EFSA's Panel on Biological

Hazards (BIOHAZ) stated that both patients admitted to hospitals and people exposed to livestock should be systematically monitored for MRSA (EFSA, 2009b). Although the prevalence of CC398 is low in some countries, surveillance programmes are required to reveal the real prevalence and properties of LA-MRSA in livestock and foodstuffs (EFSA, 2009a). CC398 is the most prevalent lineage in Dutch pig farms, and consequently proactive screening, control and prevention have been implemented to hinder its dissemination: the incidence of LA-MRSA has subsequently been low (van Cleef *et al.*, 2013). The Norwegian Veterinary Institute (NVI) started the surveillance of pigs in 2014, following an increase in the prevalence of, and two outbreaks of, LA-MRSA in Norwegian pig herds (Urdahl *et al.* 2014). However, a low prevalence of MRSA due to consumption of foodstuff has been noted in the UK, compared with other routes of transmission (Stone, 2017).

Romania is a particular case since its MRSA rate is one of the highest worldwide; ranging from 30% to 70% (Nica *et al.*, 2010; Vremeră *et al.*, 2012). The most frequently isolated MRSA (41.2%) belonged to CC1, with only one LA-MRSA strain found in a blood culture from a chronic obstructive lung disease patient living in an urban area (Monecke *et al.*, 2014). However, there are no surveillance data available for CC398 in Romania, either for humans or livestock (Monecke *et al.*, 2014), although a *spa* type (t034) associated with this lineage has been found (Székely *et al.*, 2012). Romania is ranked fourth and ninth in the EU for sheep and swine densities, respectively, and MRSA colonization of farm animals and workers can be expected.

Indeed, LA-ST398 has been found both among swine workers and in food products illegally imported to Romania (Huang *et al.*, 2014; Oniciuc *et al.*, 2015). Surveillance programmes for the food chain in Romania would be valuable for early outbreak detection and assessing whether this clone is a major zoonotic foodborne pathogen. While in many developing countries surveillance systems have been implemented and developed with international support, this is not the case in Romania (Monecke *et al.*, 2014).

CHAPTER 2

Procedures Used for Detection and Identification of *Staphylococcus aureus*

Procedures Used for Detection and Identification of *Staphylococcus aureus*

2.1. Conventional Detection and Identification Methods

Over the years, several culture media have been used for detection and enumeration of coagulase-positive staphylococci. Of them, Baird Parker Agar media remains the commonly used culture media in Europe and USA for differentiation of staphylococci, especially *S. aureus* (Baird and Lee 1995; Hudson, 2010). However, this media is partially selective and it is primarily used in processing foods or environmental samples rather than clinical ones. Principle of action is based on the ability of staphylococci to reduce tellurite to tellurium and to put into evidence lecitinase from egg lecithin while other components such as pancreatic digest of casein, beef and yeast extracts provides sources of nitrogen, carbon, sulphur and vitamins (Hudson, 2010).

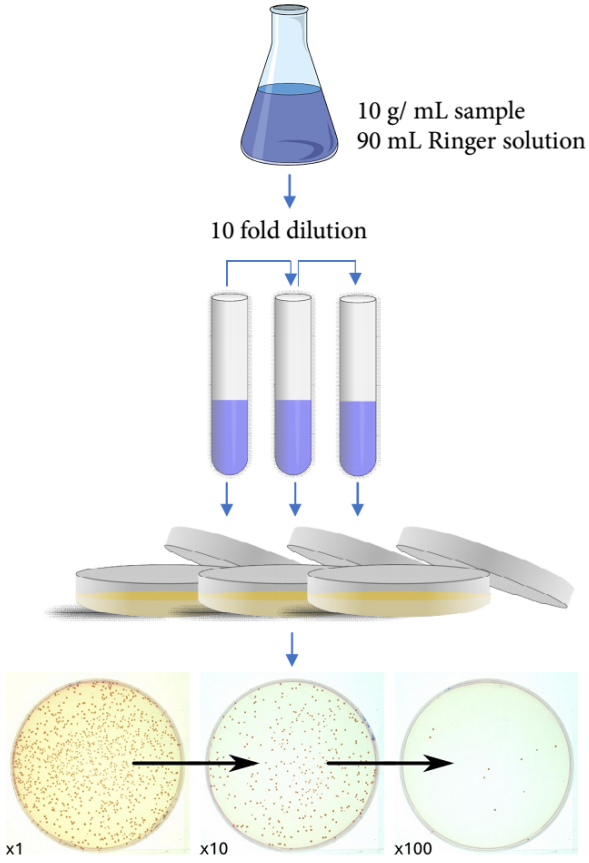


Figure 9. Horizontal method for the enumeration of coagulase-positive staphylococci (*S. aureus* and other species)

ISO 6888 describes two horizontal methods (part 1 and part 2) (Figure 9) for the enumeration of coagulase-positive staphylococci. In the general case, part 1 of ISO 6888 can be used but it is preferable to use the procedure described in part 2 (using rabbit plasma fibrinogen) in case of foodstuffs (such as cheeses made from raw milk and certain raw meat products) likely to be contaminated by staphylococci forming atypical colonies on a Baird-Parker agar medium or having a background flora which can obscure the colonies (ISO 6888).

Nowadays, rapid laboratory diagnosis is critical for treating, managing and preventing MRSA (Kumar *et al.*, 2013; Malhotra-Kumar *et al.*, 2010). Therefore, different chromogenic media for MRSA detection such as Brilliance MRSA agar (Oxoid), ChromID (bioMérieux), HardyCHROM™ MRSA (Hardy Diagnostics), MRSA*Select* (BioRad) or BBL-CHROMagar (BD Diagnostics) have appeared. However, even though it could provide good diagnostic performance for MRSA confirmation from clinical samples, in case of food samples have shown lower performance (Ariza *et al.*, 2015).

2.2. Molecular Amplification-Based Methods

Useful molecular methods to categorize isolates and compare the relevant genetic features of each clone are now available, such as pulsed-field gel electrophoresis (PFGE), multi-locus sequence typing (MLST), *spa* typing or SCC*mec* typing.

Polymerase Chain Reaction- Based Methods

Polymerase chain reaction (PCR)-based molecular methods have been developed and applied to study the population genetics and molecular epidemiology of foodborne pathogens for more than two decades (Oyarzabal and Kathariou, 2014). From chemical point of view, is based on successive cycles of *in vitro* DNA replication form the PCR reaction, in which two primers hybridize with the two strands of the original sequence. However, this reaction takes place by temperature steps and the only enzyme used in the reaction is a DNA-dependent DNA polymerase (replicase function).

Components: DNA sample, DNA polymerase, primers, deoxynucleotide triphosphates (dNTPs), PCR buffer, magnesium chloride.

PCR steps: initial denaturation→ denaturation→ primers annealing→ extension→final extension. Subsequently, analysis of the banding pattern in electrophoretic gel is required. DNA molecules are separated according to size when are subjected to an electric field. Since DNA is negatively charged when subjected to an electric current, they migrate through the gel to the positive pole. However, Real-Time PCR reactions eliminates the need for handling the product after amplification, reducing the risk of false positive results and cross-contamination.

Pulsed-Field Gel Electrophoresis

The most widely used molecular typing method of MRSA strains is PFGE, a technique based on the digestion of bacterial DNA by *Sma*I restriction enzyme, subsequently being separated into large fragments according with their size when are subjected to migration in an agarose gel. The orientation of electric field is changed periodically ("pulsed") (Szabo, 2014). A PFGE protocol for *S. aureus* was developed in 2003 to allow inter-laboratory comparisons. However, a new clonal lineage belonging to CC398 shows resistance to the *Sma*I digestion, in which methylation of adenine and cytosine residues prevents the *Sma*I cleavage. However, such isolates can be typed by PFGE using the *Sma*I isoschizomer, Cfr91 (Molla *et al*., 2012).

This method is often used for investigation of nosocomial outbreaks, to identify MRSA clones that have a particular ability to cause major outbreaks and to spread nationally and internationally (epidemic MRSA clones; EMRSA) (Szabo, 2014), for clustering and differentiation of pathogenic bacteria, being the reference method in epidemiological studies (Enright *et al*., 2000). Proved to be successfully used for comparing isolates coming from a known area, location, raw food, or surface from a food processing environment.

However, a national PFGE-based typing system for *S. aureus* would have to be validated with MLST and *spa* typing data to maintain continuity with the nomenclature already established in the literature (McDougal *et al*., 2003).

Multilocus sequence typing

MLST is a DNA sequence-based subtyping method developed in 2000 (Enright *et al*., 2000) for the unambiguous comparison of internal sequences (450-500 bp internal fragments) of seven housekeeping genes distributed in different loci around the *S. aureus* chromosome (www.mlst.net/). For each housekeeping gene, the different sequences present within a bacterial species are assigned as distinct alleles

and, for each isolate, the alleles at each of the seven loci define the allelic profile or sequence type (ST) (pubmlst.org/). As there are many alleles at each of seven loci, isolates are highly unlikely to have identical allelic profiles by chance, and isolates with the same allelic profile can be assigned as members of the same clone (Enright *et al*., 2000; Szabo, 2014). In the case of *S. aureus,* the following seven housekeeping genes are targeted: carbamate kinase (*arcC*), shikimate dehydrogenase (*aroE*), glycerol kinase (*glp*), guanylate kinase (*gmk*), phosphate acetyltransferase (pta), triosephosphate isomerase (*tpi*), and acetyl coenzyme A acetyltransferase (yqiL) (Enright *et al*., 2000). The sequences of these genes are compared with already known allele *via* mlst.net platform.

spa Typing

The method developed in 1996 for *S. aureus* is based on the detection of polymorphic X region of the gene encoding the surface protein A (*spa*) (Oyarzabal and Kathariou, 2014). Repeats are assigned a numerical code and the *spa*-type is deduced from the order of specific repeats (spaserver.ridom.de/). Analyzing the number of repetitions and their combination can be done by using Ridom Staphy-Type software. Grouping of related *spa*-types can be performed using Based Upon Repeat Pattern (BURP) algorithm implemented by the software, *spa* types with more than five repeats are clustered into different groups, with the calculated cost between members of a group being less than or equal to 6 (Strommenger *et al*., 2008). This method provides reliable, accurate and discriminatory power of MRSA typing in which a variety of *spa* types correspond to a single MLST. However, many of these genes reside on mobile genetic elements in which frequent exchange between different lineages might occur. Another disadvantage is that *spa* typing is less discriminatory than PFGE (Wiśniewska *et al*., 2012).

SCC*mec* Typing

MRSA strains are characterized by the presence of a large heterologous mobile genetic element called the SCC*mec*, carrying *mec*A or *mec*C genes, the central element of methicillin resistance (Milheiriço *et al*., 2007; Paterson *et al*., 2014a; Petinaki and Spiliopoulou, 2012). Besides the *mec* gene complex, SCC*mec* contains the *ccr* gene complex which encodes recombinases responsible for the mobility of SCC*mec* (Figure 3). The remaining parts of the gene complex are called J regions (regions J1, J2, and J3), which constitute nonessential components of the cassette (Oyarzabal and Kathariou, 2014; Szabo, 2014). Although, in some cases these regions harbor additional antibiotic resistance determinants.

To date, eleven SCC*mec* types have been identified so far based on the allotype of ccr gene and the class of mec gene complex, plus a variety of subtypes depending on variations in the joining regions (Table 5-7).

***Table 5**. Currently identified SCCmec types in S. aureus strains (www.sccmec.org)*

SCC*mec* types	*ccr* gene complexes	*mec* gene complexes	Strains
I	1 (A1B1)*	B	NCTC10442, COL
II	2 (A2B2)	A	N315, Mu50, Mu3, MRSA252, JH1, JH9
III	3 (A3B3)	A	85/2082
IV	2 (A2B2)	B	CA05, MW2, 8/6-3P, 81/108, 2314, cm11, JCSC4469, M03-68, E-MRSA-15, JCSC6668, JCSC6670
V	5 (C1)	C2	WIS(WBG8318), TSGH17, PM1,
VI	4 (A4B4)	B	HDE288
VII	5 (C1)	C1	JCSC6082
VIII	4 (A4B4)	A	C10682, BK20781
IX	1(A1B1)	C2	JCSC6943
X	7(A1B6)	C1	JCSC6945
XI	8(A1B3)	E	LGA251

Note: * A *ccr* gene or ccr genes in the gene complex are indicated in parenthesis.

***Table 6**. Currently identified ccr gene complexes in staphylococci (www.sccmec.org)*

ccr gene complexes	*ccr* genes	SCC*mec* types carrying the *ccr* gene complexes
Type 1	A1B1	I, IX
Type 2	A2B2	II, IV
Type 3	A3B3	III
Type 4	A4B4**	VI, VIII
Type 5	C1	V, VII
Type 6	A5B3	
Type 7	A1B6	X
Type 8	A1B3	XI

Note: **ccrA4B4 genes found in type VIII SCC*mec* were nearly identical to that in the *S. epidermidis* SCC-CI element and showed nucleotide identities to those found in type VI SCC*mec* of 89.6% and 94.5%, respectively.

***Table 7**. Currently identified mec gene complexes in staphylococci (www.sccmec.org)*

mec gene complexes		SCC*mec* types carrying the *mec* gene complexes
class A	IS*431*-*mec*A-*mec*R1-*mec*I	**II, III, VIII**
class B	IS*431*-*mec*A-*Δmec*R1-IS*1272*	**I, IV, VI**
class C1	IS*431*-*mec*A-*Δmec*R1-IS*431*(two IS*431*s were arranged in the same direction)	**VII,X**
class C2	IS*431*-*mec*A-*Δmec*R1-IS*431* (two IS*431*s were arranged in the opposite direction)	**V, IX**
class D	IS*431*-*mec*A-*Δmec*R1	
class E	blaZ-*mec*A*LGA251*-*mec*R1*LGA251*-*mec*I*LGA251*	**XI**

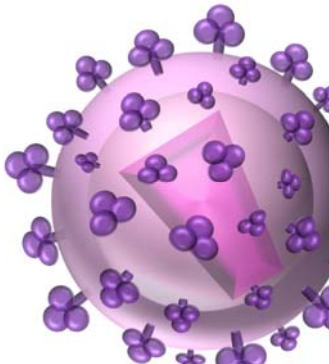
Whole Genome Sequencing

MRSA is of major concern worldwide and a serious threat for human and animal health. What in principle started to be a serious issue for healthcare settings, further studies elicited as well its presence in livestock. Comparative genomic analysis began in 2000 with the description of the complete genome of N315- MRSA strain isolated in 1982, and Mu50- a vancomycin-intermediate *S. aureus* (VISA) isolated in 1997, in which has been discovered a close relationship between strains (Kuroda *et al.*, 2001). Genome analysis led to identify mobile elements, of which some of them were carrying antibiotic resistance and virulence genes (Kuroda *et al.*, 2001). Nowadays, this tool proves to be invaluable not only for identifying horizontal gene transfer elements or other gene sequences regulating the expression of virulence factors (Alföldi *et al.*, 2013), but also helps to understand structural features which may contribute to the variations in the genomic rearrangement or changes in the gene repertory (Castillo *et al.*, 2016). Moreover, mechanisms leading to mutations that undergo to non-functional proteins (Chua *et al.*, 2013) are of great importance as can expose particularities on the evolution of such strains.

By WGS, genome comparison with other *S. aureus* strains already published in the literature can be done, that some are having distinct epidemiological and virulence properties that could lead to identification of virulence factors and genes associated with antimicrobial resistance. Moreover, by WGS, decoding the mechanisms involved in phenotypic expression of methicillin resistance can be performed.

PART 2

MATERIALS, EQUIPMENTS AND METHODS



CHAPTER 3

Materials and Equipments

Materials and Equipments

The materials used for the present thesis were provided by "Dunărea de Jos" University of Galați –(Romania), University of Burgos and Instituto Tecnológico Agrario de Castilla y León –(Spain), Institute of Milk Hygiene –(Austria) and Centro de Engenharia Biologica –(Braga, Portugal).

3.1. Bacterial Strains

Table 8. *Staphylococcus aureus strains used in the present thesis*

Isolate number	Isolation date	Origin (food item)	Control point	Country of origin
American Type Culture Collection (ATCC)				
ATCC®25923		Clinical isolate	-	-
Collection of Food Microbiology Department of the "Dunărea de Jos" University of Galați (Romania)				
E1	04/02/2013	Whey cheese	Galati market	Republic of Moldavia
E26	04/02/2013	Whey cheese	~	~
E1NC	04/02/2013	Whey cheese	~	~
E26NC	04/02/2013	Whey cheese	~	~
E5	07/02/2013	Poultry	~	~
E18	14/09/2012	Artificial black caviar	~	~
E10	14/09/2012	Artificial red caviar	~	~
E23	14/09/2012	Raw milk	~	~
E25	14/09/2012	Raw milk	~	~
E16	14/09/2012	Fresh cow cheese	~	~
E21	14/09/2012	Fresh cow cheese	~	~
E6	14/09/2012	Sheep cheese salted in brine	~	~
E4	14/09/2012	Unfermented goat cheese	~	~
E9	14/09/2012	Unfermented goat cheese	~	~
E12	14/09/2012	Unfermented goat cheese	~	~
E15	14/09/2012	Unfermented goat cheese	~	~
E20	14/09/2012	Unfermented goat cheese	~	~
E30	14/09/2012	Unfermented goat cheese	~	~
E2	14/09/2012	Smoked salmon	~	~
E22	06/11/2012	Pork lard	~	~
E19	06/11/2012	Raw milk	~	~
E8	06/11/2012	Poultry	~	~
E24	06/11/2012	Poultry	~	~
E13	06/11/2012	Non-fermented unsalted sheep cheese	~	~
E14	06/11/2012	Non-fermented unsalted sheep cheese	~	~
E29	06/11/2012	Non-fermented unsalted sheep cheese	~	~
E29NC	06/11/2012	Non-fermented unsalted sheep cheese	~	~
E7	06/11/2012	Smoked fish	~	~
E7NC	06/11/2012	Smoked fish	~	~
E3	06/11/2012	Fish canned in oil with herbs	~	~
E11	29/01/2013	Goat cheese	~	~

E17	29/01/2013	Goat cheese	~	~
01-02 a	13/07/2015	soft goat cheese	~	~
01-05 a	13/07/2015	ripened sheep cheese	~	~
01-05 b	13/07/2015	ripened sheep cheese	~	~
Collection of Food Microbiology Department of the University of Burgos and Instituto Tecnológico Agrario de Castilla y León (Spain)				
LBMM 820	19/05/2012	Cheese	Bilbao airport	Ecuador
LBMM 821	19/05/2012	Pork meat	~	Ecuador
LBMM 822	19/06/2012	Antelope	~	Unknown
LBMM 823	11/06/2012	Unknown meat	~	Ecuador
LBMM 824	11/06/2012	Unknown meat	~	Ecuador
LBMM 825	18/06/2012	Cheese	~	Ecuador
LBMM 826	18/06/2012	Cheese	~	Ecuador
LBMM 827	09/06/2012	Eggs	~	Ecuador
LBMM 828	09/06/2012	Guinea pig	~	Ecuador
LBMM 829	14/05/2012	Cheese	~	Perú
LBMM 830	12/07/2012	Pork meat	~	Argentina
LBMM 831	27/05/2012	Unknown meat	~	China
LBMM 832	27/05/2012	Duck meat	~	China
LBMM 833	03/08/2012	Cheese	~	Bolivia
LBMM 834	03/08/2012	Cheese	~	Bolivia
LBMM 835	03/08/2012	Cheese	~	Bolivia
LBMM 836	03/08/2012	Cheese	~	Bolivia
LBMM 837	15/05/2012	Cheese	~	Ecuador
LBMM 838	04/07/1905	Rodents	~	Guinea Equatorial
LBMM 839	30/08/2012	Cheese	~	Romania
LBMM 840	30/08/2012	Cheese	~	Romania
LBMM 841	30/08/2012	Unknown meat	~	Romania
LBMM 842	30/08/2012	Unknown meat	~	Romania
LBMM 843	11/09/2012	Duck meat	~	China
LBMM 844	13/08/2012	Pork meat	~	Bolivia
LBMM 845	30/05/2012	Unknown meat	~	Perú
LBMM 846	30/05/2012	Cheese	~	Perú
LBMM 847	30/05/2012	Cheese	~	Perú
LBMM 848	30/05/2012	Cheese	~	Perú
LBMM 849	30/05/2012	Cheese	~	Perú
LBMM 850	11/09/2012	Unknown meat	~	China
LBMM 851	09/02/2013	Beef meat	~	Bolivia
LBMM 852	09/02/2013	Beef meat	~	Bolivia
LBMM 853	09/02/2013	Cheese	~	Bolivia
LBMM 854	09/02/2013	Cheese	~	Bolivia
LBMM 855	24/01/2013	Unknown meat	~	Serbia
LBMM 856	04/01/2013	Cheese	~	Perú
LBMM 857	20/02/2013	Duck meat	~	China
LBMM 858	04/01/2013	Cheese	~	Perú
LBMM 859	04/01/2013	Cheese	~	Perú
LBMM 860	27/01/2013	Cheese	~	Perú
LBMM 861	17/02/2013	Cheese	~	Paraguay
LBMM 862	16/02/2013	Unknown meat	~	Bolivia

LBMM 863	16/02/2013	Unknown meat	~	Bolivia
LBMM 864	16/02/2013	Unknown meat	~	Bolivia
LBMM 865	16/02/2013	Unknown meat	~	Bolivia
LBMM 866	16/02/2013	Cheese	~	Bolivia
LBMM 867	16/02/2013	Cheese	~	Bolivia
LBMM 868	16/02/2013	Unknown meat	~	Bolivia
LBMM 869	18/04/2013	Cheese	~	Bolivia
LBMM 870	18/04/2013	Cheese	~	Bolivia
LBMM 871	28/03/2013	Cheese	~	Bolivia
LBMM 872	07/04/2013	Beef meat	~	Columbia
LBMM 873	11/04/2013	Cheese	~	Bolivia
LBMM 874	28/04/2013	Cheese	~	Perú
LBMM 875	27/04/2013	Cheese	~	Bolivia
LBMM 876	21/02/2013	Meat	~	Brazil
LBMM 877	16/05/2013	Cheese	~	Brazil
LBMM 878	27/05/2013	Cheese	~	Paraguay
LBMM 879	01/06/2013	Cheese	~	Bolivia
LBMM 880	01/06/2013	Cheese	~	Bolivia
LBMM 881	09/05/2013	Cheese	~	Columbia
LBMM 882	03/05/2013	Cheese	~	Brazil
LBMM 883	18/12/2012	Cheese	~	Bolivia
LBMM 884	11/03/2013	Cheese	~	Perú
LBMM 885	27/01/2013	Cheese	~	Perú
SA11	23/09/2012	Cheese	Land border Obrežje	Turkey
SA17	03/10/2012	Meat-salami (dry)	Brnik airport	Republic of Kosovo
SA21	08/11/2012	Cheese (green mold)	Land border Obrežje	Turkey
SA43	06/02/2013	Meat	Brnik airport	Republic of Kosovo
21a	13/08/2008	Sheep	Castile and Leon	Spain
125a	01/09/2008	Sheep	~	~
282b	28/10/2008	Sheep	~	~
355b	18/11/2008	Sheep	~	~
358c	18/11/2008	Sheep	~	~
620a	24/02/2009	Sheep	~	~
621a	24/02/2009	Sheep	~	~
622a	24/02/2009	Sheep	~	~
623a	24/02/2009	Sheep	~	~
625a	24/02/2009	Sheep	~	~
626a	24/02/2009	Sheep	~	~
627a	24/02/2009	Sheep	~	~
628a	24/02/2009	Sheep	~	~
629a	24/02/2009	Sheep	~	~
630a	24/02/2009	Sheep	~	~
631a	24/02/2009	Sheep	~	~
633a	24/02/2009	Sheep	~	~
681a	17/03/2009	Sheep	~	~
684a	17/03/2009	Sheep	~	~
686a	17/03/2009	Sheep	~	~
689a	17/03/2009	Sheep	~	~
690a	17/03/2009	Sheep	~	~

691a	17/03/2009	Sheep	~	~
694a	17/03/2009	Sheep	~	~
696a	17/03/2009	Sheep	~	~
697a	17/03/2009	Sheep	~	~
764a	14/04/2009	Sheep	~	~
769a	14/04/2009	Sheep	~	~
770a	14/04/2009	Sheep	~	~
771a	14/04/2009	Sheep	~	~
772a	14/04/2009	Sheep	~	~
773a	14/04/2009	Sheep	~	~
775a	14/04/2009	Sheep	~	~
776a	14/04/2009	Sheep	~	~
777b	14/04/2009	Sheep	~	~
778a	14/04/2009	Sheep	~	~
779a	14/04/2009	Sheep	~	~
924a	09/06/2009	Sheep	~	~
929a	09/06/2009	Sheep	~	~
943a	16/06/2009	Sheep	~	~
945a	16/06/2009	Sheep	~	~
947a	16/06/2009	Sheep	~	~
949a	16/06/2009	Sheep	~	~
1008a	14/07/2009	Sheep	~	~
648c	03/03/2009	Sheep	~	~
741a	07/04/2009	Sheep	~	~
1040a	28/07/2009	Sheep	~	~
1043a	28/07/2009	Sheep	~	~
SA 1	16/01/2008	Blood culture	Hospital of León	~
SA 2	28/01/2008	Blood culture	~	~
SA 3	31/01/2008	Blood culture	~	~
SA 4	06/02/2008	Central vascular catheter	~	~
SA 5	11/02/2008	Blood culture	~	~
SA 6	18/02/2008	Blood culture	~	~
SA 7	28/03/2008	Central vascular catheter	~	~
SA 8	05/05/2008	Blood culture	~	~
SA 9	05/05/2008	Central vascular catheter	~	~
SA 10	19/05/2008	Blood culture	~	~
SA 11	26/05/2008	Blood culture	~	~
SA 12	02/06/2008	Blood culture	~	~
SA 13	30/06/2008	Blood culture	~	~
SA 14	01/07/2008	Blood culture	~	~
SA 15	04/07/2008	Central vascular catheter	~	~
SA 16	04/07/2008	Central vascular catheter	~	~
SA 17	18/07/2008	Blood culture	~	~
SA 18	21/07/2008	Blood culture	~	~
SA 19	28/07/2008	Blood culture	~	~
SA 20	09/08/2008	Blood culture	~	~
SA 21	11/08/2008	Blood culture	~	~
SA 22	25/08/2008	Drainage	~	~
SA 23	26/08/2008	Central vascular catheter	~	~
SA 24	06/10/2008	Blood culture	~	~

SA 25	16/10/2008	Blood culture	~	~
SA 26	18/10/2008	Peripheral vascular catheter	~	~
SA 27	27/10/2008	Packed red blood cells	~	~
SA 28	28/10/2008	Ascitic fluid	~	~
SA 29	04/11/2008	Peripheral vascular catheter	~	~
SA 30	15/11/2008	Blood culture	~	~
SA 31	23/12/2008	Blood culture	~	~
SA 32	26/12/2008	Blood culture	~	~
SA 33	29/12/2008	Blood culture	~	~
SA 34	01/01/2007	Blood culture	~	~
SA 35	17/01/2007	Central vascular catheter	~	~
SA 36	22/01/2007	Peripheral vascular catheter	~	~
SA 37	22/01/2007	Central vascular catheter	~	~
SA 38	01/02/2007	Blood culture	~	~
SA 39	01/02/2007	Central vascular catheter	~	~
SA 40	06/02/2007	Blood culture	~	~
SA 41	27/02/2007	Blood culture	~	~
SA 42	05/03/2007	Blood culture	~	~
SA 44	12/03/2007	Blood culture	~	~
SA 45	26/03/2007	Blood culture	~	~
SA 46	26/03/2007	Blood culture	~	~
SA 47	07/04/2007	Blood culture	~	~
SA 48	07/04/2007	Blood culture	~	~
SA 49	12/04/2007	Blood culture	~	~
SA 50	24/04/2007	Blood culture	~	~
SA 51	30/04/2007	Blood culture	~	~
SA 52	02/05/2007	Blood culture	~	~
SA 53	16/05/2007	Blood culture	~	~
SA 54	17/05/2007	Blood culture	~	~
SA 55	28/05/2007	Central vascular catheter	~	~
SA 56	07/06/2007	Blood culture	~	~
SA 57	02/07/2007	Blood culture	~	~
SA 58	30/07/2007	Blood culture	~	~
SA 59	07/08/2007	Central vascular catheter	~	~
SA 60	24/08/2007	Blood culture	~	~
SA 61	18/09/2007	Blood culture	~	~
SA 62	21/09/2007	Central vascular catheter	~	~
SA 63	04/10/2007	Blood culture	~	~
SA 64	15/10/2007	Central vascular catheter	~	~
SA 65	18/10/2007	Central vascular catheter	~	~
SA 66	05/11/2007	Blood culture	~	~
SA 67	07/11/2007	Blood culture	~	~
SA 68	19/11/2007	Blood culture	~	~
SA 69	27/11/2007	Blood culture	~	~
SA 70	27/11/2007	Synovial joint fluid	~	~
SA 71	19/12/2007	Blood culture	~	~
LGA251	/05/2007	Bulk milk	Cattle farm	Southwest England
24.1	29/12/2014	sheep meat	Bilbao airport	Nigeria
24.2	29/12/2014	sheep meat	~	Nigeria
45-1.1	21/02/2015	cheese	~	Egypt

45-3.1	21/02/2015	fresh beef meat	~	Egypt
46-2.3	20/02/2015	cheese	~	Republic of Honduras
50-2.1	10/02/2015	dried meat	~	China
68.1	26/02/2015	cheese	~	Nicaragua
68.2	26/02/2015	cheese	~	Nicaragua
68.3	26/02/2015	cheese	~	Nicaragua
74.1	28/02/2015	cheese	~	Bolivia
74.2	28/02/2015	cheese	~	Bolivia
74.3	28/02/2015	cheese	~	Bolivia
80-2.1	26/02/2015	cheese	~	Nicaragua
80-2.2	26/02/2015	cheese	~	Nicaragua
80-2.3	26/02/2015	cheese	~	Nicaragua
115-1.1	09/06/2015	cheese	~	Bolivia
115-1.2	09/06/2015	cheese	~	Bolivia
115-1.3	09/06/2015	cheese	~	Bolivia
117.1	08/06/2015	cheese	~	Ecuador
117.2	08/06/2015	cheese	~	Ecuador
117.3	08/06/2015	cheese	~	Ecuador
122-2.1	13/05/2015	cheese	~	Perú
124.1	06/05/2015	cheese	~	Nicaragua
132.2	17/06/2015	cheese	~	Columbia
132.3	17/06/2015	cheese	~	Columbia
133-1.1	22/06/2015	cheese	~	Nicaragua
133-1.2	22/06/2015	cheese	~	Nicaragua
133-1.3	22/06/2015	cheese	~	Nicaragua
135.1	30/06/2015	cheese	~	Perú
135.2	30/06/2015	cheese	~	Perú
135.3	30/06/2015	cheese	~	Perú
137-2.1	17/02/2015	fresh meat	~	Republic of Serbia
137-2.2	17/02/2015	fresh meat	~	Republic of Serbia
138.1	23/06/2015	cheese	~	Nicaragua
138.2	23/06/2015	cheese	~	Nicaragua
151-1.1	08/06/2015	curd	~	Bolivia
151-1.2	08/06/2015	curd	~	Bolivia
151-1.3	08/06/2015	curd	~	Bolivia
153-1.1	22/06/2015	cheese	~	Bolivia
153-1.2	22/06/2015	cheese	~	Bolivia
153-1.3	22/06/2015	cheese	~	Bolivia
Collection of the Institute of Milk Hygiene (Austria)				
41	10/08/2012	Cheese	Vienna airport	Egypt
47	11/08/2012	Raw breaded meat	~	Egypt
50	11/08/2012	Meat	~	Egypt
140	19/09/2012	Cheese in brine	~	Turkey
153	19/09/2012	Pastrami, sliced	~	Turkey
165	25/09/2012	Lor, whey cheese	~	Turkey
176	28/09/2012	Cheese, ripened	~	Armenia
226	30/09/2012	Sausage	~	North Korea
247	25/10/2012	Raw muscle meat	~	Albania
249	25/10/2012	Head meat, cooked	~	Albania

294	08/11/2012	Cheese in brine with herbs	~	Republic of Kosovo
298	08/11/2012	Cheese	~	Turkey
364	29/11/2012	Sausage	~	Tunisia
476	26/01/2013	White cheese with spice sauce	~	Egypt
498	31/01/2013	Cheese, ripened	~	Egypt
519	09/02/2013	White cheese	~	Turkey
550	17/02/2013	White cheese	~	Tunisia
576	03/03/2013	Cheese, ripened	~	Egypt

3.2. Bacterial Culture Media

Baird Parker Agar (BP- Biolife Italiana srl., Milano, Italy)

Component	Quantity (g/L)
Pancreatic digest of casein	10
Beef extract	5
Yeast extract	1
Sodium pyruvate	10
Glycine	12
Lithium chloride	5
Agar	15

Basic medium recommended by ISO 6888 for the detection and enumeration of coagulase-positive staphylococci. ISO 6888-1 recommends the use of culture media in combination with egg yolk and potassium tellurite.

Directions: Suspend 58 g in 1 L of cold distilled water; heat to boiling and autoclave at 121°C for 15 min. Cool to about 50°C and, using aseptic conditions, add 50 ml of Egg Yolk Tellurite Emulsion 20%.

Final pH 7.2 ± 0.2, at temperature of 25°C after autoclaving (www. masciabrunelli.it)

Brain Heart Infusion Broth (BHIB- Biolife Italiana srl., Milano, Italy)

Component	Quantity (g/L)
Brain infusion solids	12.5
Beef heart infusion solids	5
Peptocomplex	10
Glucose	2
Sodium chloride	5
Disodium hydrogen phosphate	2.5

Nutrient medium for cultivation and isolation of a large range of microorganisms, including fastidious, yeasts and molds. It is used in order to prepare cultures of *S. aureus* for use in the coagulase test.

Directions: Suspend 37 g of BHIB in 1 L of cold distilled water. Heat to boiling, distribute and sterilise by autoclaving at 121°C for 15 min.

Final pH 7.4 ± 0.2, at temperature of 25°C after autoclaving (www. masciabrunelli.it)

Brain Heart Infusion Agar (BHIA- Biolife Italiana srl., Milano, Italy)

BHI supplemented with 15.0 g/L agar-agar (Biolife, Italy)

Brilliance MRSA 2 Agar (ThermoFisher Scientific, UK)

Component	Quantity (mg/L)
Peptone mix	20
Carbohydrates	4
Kaolin	15

Salts	5
Agar	13
Chromogen mix	0.2
Antibiotics mix	20 mL

Selective chromogenic medium for detection of MRSA, providing rapid results with high specificity and sensitivity.

Final pH 7.3 ± 0.2, at temperature of 25°C after autoclaving (www.oxoid.com/)

ChromID MRSA Agar (BioMérieux, France)

Selective chromogenic medium dedicated to the surveillance culture or screening of MRSA to identify hospitalized patients requiring isolation. The screening of MRSA carriers with surveillance cultures is a key step in the fight against nosocomial infections. ChromID MRSA has been developed to answer this major health concern. Identification of MRSA strains is based on the spontaneous green coloration of α- glycosidase- producing colonies and the presence of a mixture of antibiotic (www.biomerieux.com).

Tryptic Soy Broth (TSB- Liofilchem, Italy)

Component	Quantity (g/L)
Triptone	17.0
Soy peptone	3.0
Sodium chloride	5.0
Glucose	2.5
Dipotassium phosphate	2.5

Basic broth medium supporting the fastidious bacteria growth.

Directions: Suspend 30 g to 1 L of water, mix well and distribute and sterilise by autoclaving at 121°C for 15 min.

Final pH 7.3 ± 0.2, at temperature of 25°C after autoclaving (www.liofilchem.net)

3.3. Enzymes, Reagents and Materials

Enzymes

DNA Polymerase FastStart Taq*, 500 U* (Roche Molecular Systems Inc., Switzerland)

It is accompanied by reaction buffer (Roche, Switzerland) and $MgCl_2$ (Roche, Switzerland) which will form complexes with dNTPs for the polymerase to recognize them and to be added to the elongated strand (Barbu, 2008)

Lysostaphin from S. simulans*, 5 mg* (Sigma-Aldrich co., Saint Louis, USA)

Lysozyme (Sigma-Aldrich co., Saint Louis, USA)

Proteinase K*, 0.1 mg/mL* (Sigma-Aldrich co., Saint Louis, USA) *in 20 mM Tris-HCl (pH 7.5): 1 mM* $CaCl_2$

Proteinase K is an endopeptidase which hydrolyze the peptide bonds of the carboxylic groups of the aliphatic amino acids and aromatic, with the α-amino group blocking.

Restriction Enzyme SmaI*, 2000 U* (Biolabs, UK)

Restriction enzymes are molecular scissors capable to cut the bacterial DNA in specific areas, called restriction sites. They are used to generate small number of DNA fragments that can be separated based on the size. In general, these fragments are large and must be specially treated and separated in order to be able to generate a DNA fingerprint. Therefore, in a first phase, the bacteria is mixed with agarose gel and the cells are lysed to release DNA. Mixture of released DNA and the agarose forms a plug which is subsequently treated with the restriction enzymes (www.cdc.gov)

Restriction site used:
5'...CCC↓GGG...3'
3'...GGG↑CCC...5' (https://www.neb.com/products/r0141-smai)

Reagents

Agar-Agar (Biolife Italiana srl., Milano, Italy);
Agarose D1 Low EEO Molecular Biology Grade (Laboratorios Conda S.A., Madrid, Spain);
Calcium chloride anhydrous, 99.99% trace metals basis (Sigma-Aldrich co., Saint Louis, USA);
Certified Low Melt Agarose (BioRad, Munich, Germany);
Chelex® 100 (BioRad, Munich, Germany);
Crystal Violet (CV- Merck KGaA, Darmstadt, Germany);
Diethylpyrocarbonate (DEPC- ThermoFisher Scientific, Austria);
DNA Gel Loading Dye, 6× (ThermoFisher Scientific, Austria);
DNA Molecular Weight Marker 100 bp, 50-2000 bp (BioRad, Munich, Germany);
dATP, dCTP, dGTP, and dTTP, 10 mM/each (Roche Molecular Systems Inc., Switzerland);
EDTA Disodium Salt Dihydrate, 99⁺% (Sigma-Aldrich co., Saint Louis, USA);
EDTA for Molecular Biology, 0.5M (Sigma-Aldrich co., Saint Louis, USA);
Egg Yolk Tellurite Emulsion, 50% (EY- Biolife Italiana srl., Milano, Italy);
Ethanol (Sigma-Aldrich co., Saint Louis, USA);
Ethidium Bromide (Sigma-Aldrich co., Saint Louis, USA);
Film Tracer SYPRO Ruby Biofilm (Invitrogen, Paisley, UK); (ThermoFisher Scientific, Austria);
Formamide Hi-Di (Sigma-Aldrich co., Saint Louis, USA);
Glacial Acetic Acid, ≥99% (Sigma-Aldrich co., Saint Louis, USA);
Glucose (Sigma-Aldrich co., Saint Louis, USA); (B. Braun Melsungen AG, Melsungen, Germany);
Glycerol (Merck KGaA, Darmstadt, Germany);
Ladder DNA, 100 bp, 1 kbp (Biolabs, UK);
Lambda Ladder PFG, 48.5–1018 kb (Biolabs, UK);
Magnesium Chloride Hexahydrate, ≥ 99% (Sigma-Aldrich co., Saint Louis, USA);
Magnesium Chloride, 25 mM (Applied Biosystems, Warrington, UK);
McFarland 0.5 Equivalence Turbidity Standard (Remel, Lenexa, Kansas);
Methanol Anhydrous, 99.8% (Sigma-Aldrich co., Saint Louis, USA); Avantor Performance Materials, Norway);
N-lauroylsarcosine Sodium Salt, ≥ 97% (Sigma-Aldrich co., Saint Louis, USA);
Nucleic Acid Gel stain GelRed™, 10,000× in water (Biotium, ChemoMetec, Denmark);
Phosphate Buffer Saline Solution (PBS- Sigma-Aldrich co., Saint Louis, USA);
Pulsed Field Certified Agarose (UltraPure DNA grade agarose) (BioRad, Munich, Germany);
Rabbit Plasma Fibrinogen (RPF- Biolife Italiana srl., Milano, Italy);
Ringer Solution, sterile (Scharlau, Scharlab, Barcelona, Spain);
RNase, DNase-Free Water (Sigma-Aldrich co., Saint Louis, USA);
Sodium Acetate Anhydrous (Sigma-Aldrich co., Saint Louis, USA);

Sodium Chloride (Sigma-Aldrich co., Saint Louis, USA); (Liofilchem, Italy);

Sodium Periodate $NaIO_4$, 40 mM (Sigma-Aldrich co., Saint Louis, USA);

SYTO® 9 Green Fluorescent Nucleic Acid Stain (Invitrogen, Paisley, UK); (Molecular Probes, Inc., Eugene, USA);

TAE, 50× (Sigma-Aldrich co., Saint Louis, USA);

TBE (Tris/Borat acid/EDTA buffer) extended range, 10× (BioRad, Munich, Germany);

Tris-EDTA, 100× (Sigma-Aldrich co., Saint Louis, USA);

Trizma Base (Sigma-Aldrich co., Saint Louis, USA);

Wheat Germ Agglutinin, Oregon Green® 488 Conjugate (Invitrogen, Paisley, UK); (Molecular Probes, Inc., Eugene, USA);

Materials

96-well PCR Plates (Applied Biosystems);

96-well Plates Tissue Cultured (Orange Scientific, Braine-l'Alleud, Belgium);

96-well Plates Tissue Cultured (Nunc® MicroWell™, USA);

96-well Real Time PCR Plates (Applied Biosystems);

96-well Sensititre Plates (TREK Diagnostic Systems Inc., Cleveland);

Cryovials, 2 mL (CryoKING, Biologix Plastic Co., Ltd, China);

Cuvettes for Spectrophotometer 1.2 mL (AHN Biotechnology GmbH, Germany);

Erlenmeyer Flasks 150 mL, 500 mL, 1 L, 2 L;

Filters 0.22 µm (Minisart®, Sartorius Stedim Biotech, Germany); 0.45 µm (Teknokroma, Professionally Friendly, Spain);

Flasks of 100 mL, 250 mL, 500 mL, or 1 L;

Gloves, Nitril Material (AQL 1.5 EPI);

Micropipettes with Adjustable Volume: 1-10 µL, 0,5-20 µL, 20-200 µL, 100-1000 µL (AHN Biotechnology Gmbh, Germany); (Eppendorf Austria GmbH, Austria);

Parafilm M (Pechiney Plastic Packaging, Chicago);

Racks;

Reaction Plate, 0.1 mL (Applied Biosystems);

Petri Dishes, one use, d= 90 mm (ThermoFisher Scientific, Austria);

Spatulas Drigalsky, Metal or Glass (Ingenlaboratory, Romania);

Sterile Filter Bags (Stomacher Bags; Seward Ltd., Worthing West Sussex, UK)

Sterile Tips with Filter, RNase and DNase free (AHN Biotechnology GmbH, Germany);

Tubes of 1.5 mL, 2 mL, sterile (Eppendorf);

Tubes of 15 mL, 50 mL, sterile (Falcon);

Tubes PCR of 0.2 mL and strips (Applied Biosystems);

3.4. Oligonucleotides

The oligonucleotides used in the present thesis are shown in Table 9.

Table 9. *Oligonucleotides for performing conventional and Real Time PCRs*

Target	Gene	Primer name	Type	Primer sequence (5'→ 3')	Amplicon size (bp)	Reference
S. aureus	*nuc* *	fmhb4416F	F	5'-CTA GCT TTA TTT CAGCAG GTG ACG AT-3'	103	(Trnčíková *et al.*, 2008)
		aurR	R	5'-TCA ACA TCT TTC GCA TGA TTC AAC AC-3'		
		aurP	TaqMan® probe	5'-FAM-CTT GCT CCG TTT CAC CAG GCT TCG GTG-TAMRA -3'		
MRSA	*mec*A *	SAmecA P4	F	5'-TCC AGA TTA CAA CTT CAC CAG G-3'	162	(Stegger *et al.*, 2012)
		SAmecA P7	R	5'-CCA CTT CAT ATC TTG TAA CG-3'		
	*mec*C *	SAmecAlga251 F	F	5'-GAA AAA AAG GCT TAG AAC GCC TC-3'	138	
		SAmecAlga251 R	R	5'-GAA GAT CTT TTC CGT TTT CAG C-3'		
SCC*mec* *ccr* gene complex type	*mec*A *	mA1	F	5'-TGC TAT CCA CCC TCA AAC AGG-3'	286	(Kondo *et al.*, 2007)
		mA2	R	5'-AAC GTT GTA ACC ACC CCA AGA-3'		
	type 1 (A1B1) *	α1	F	5'-AAC CTA TAT CAT CAA TCA GTA CGT-3'	695	
		βc	R	5'-ATT GCC TTG ATA ATA GCC ITC T-3'		
	type 2 (A2B2) *	α2	F	5'-TAA AGG CAT CAA TGC ACA AAC ACT-3'	937	
		βc	R	5'-ATT GCC TTG ATA ATA GCC ITC T-3'		
	type 3 (A3B3) *	α3	F	5'-AGC TCA AAA GCA AGC AAT AGA AT-3'	1791	
		βc	R	5'-ATT GCC TTG ATA ATA GCC ITC T-3'		
	type 4 (A4B4) *	α4.2	F	5'-GTA TCA ATG CAC CAG AAC TT-3'	1287	
		β4.2	R	5'-TTG CGA CTC TCT TGG CGT TT-3'		
	type 5 (C1) *	γR	F	5'-CCT TTA TAG ACT GGA TTA TTC AAA ATA T-3'	518	
		γF	R	5'-CGT CTA TTA CAA GAT GTT AAG GAT AAT-3'		
	A *	mA7	F	5'-ATA TAC CAA ACC CGA CAA CTA CA-3'	1963	(Kondo *et al.*, 2007)
		mI6	R	5'-CAT AAC TTC CCA TTC TGC AGA TG-3'		
	B *	mA7	F	5'-ATA TAC CAA ACC CGA CAA CTA CA-3'	2827	

SCC*mec* *mec* gene complex class	C *	IS7	R	5'-ATG CTT AAT GAT AGC ATC CGA ATG-3'		
		mA7	F	5'-ATA TAC CAA ACC CGA CAA CTA CA-3'	804	
		IS2(iS-2)	R	5'-TGA GGT TAT TCA GAT ATT TCG ATG T-3'		
Subtyping SCC*mec* IV	*ccr*B2 *	ccrB2 F	F	5'-CGA ACG TAA TAA CAT TGT CG-3'	203	(Milheiriço *et al.*, 2007)
		ccrB2 R	R	5'-TTG GCW ATT TTA CGA TAG CC-3'		
	Type IVa *	J IVa F	F	5'-ATA AGA GAT CGA ACA GAA GC-3'	278	
		J IVa R	R	5'-TGA AGA AAT CAT GCC TAT CG-3'		
	Types IVb and IVF *	J IVb F	F	5'-TTG CTC ATT TCA GTC TTA CC-3'	336	
		J IVb R	R	5'-TTA CTT CAG CTG CAT TAA GC-3'		
	Types IVc and IVE *	J IVc F	F	5'-CCA TTG CAA ATT TCT CTT CC-3'	483	
		J IVc R	R	5'-ATA GAT TCT ACT GCA AGT CC-3'		
	Type IVd *	J IVd F	F	5'-TCT CGA CTG TTT GCA ATA GG-3'	575	
		J IVd R	R	5'-CAA TCA TCT AGT TGG ATA CG-3'		
	Type IVg *	J IVg F	F	5'-TGA TAG TCA AAG TAT GGT GG-3'	792	
		J IVg R	R	5'-GAA TAA TGC AAA GTG GAA CG-3'		
	Type IVh *	J IVh F	F	5'-TTC CTC GTT TTT TCT GAA CG-3'	663	
		J IVh R	R	5'-CAA ACA CTG ATA TTG TGT CG-3'		
Panton-Valentine leukocidin	*luk*-PV *	luk-PV Up	F	5'-ATC ATT AGG TAA AAT GTC TGG ACA TGA TCC A-3'	433	(Lina *et al.*, 1999)
		luk-PV Dn	R	5'-GCA TCA AST GTA TTG GAT AGC AAA AGC-3'		
S. aureus specific staphylococcal protein A	*spa* #	1095F	F	5'-AGA CGA TCC TTC GGT GAG C-3'	422	(Harmsen *et al.*, 2003)
		1517R	R	5'-GCT TTT GCA ATG TCA TTT ACT G-3'		
Staphylococcal enterotoxins	*sea* #	GSEAR1	F	5'-GGT TAT CAA TGT GCG GGT GG-3'	102	(Gonano *et al.*, 2009)
		GSEAR2	R	5'-CGG CAC TTT TTT CTC TTC GG-3'		
	seb #	GSEBR1	F	5'-GTA TGG TGG TGT AAC TGA GC-3'	164	
		GSEBR2	R	5'-CCA AAT AGT GAC GAG TTA GG-3'		
	sec #	GSECR1	F	5'-AGA TGA AGT AGT TGA TGT GTA TGG-3'	451	
		GSECR2	R	5'-CAC ACT TTT AGA ATC AAC CG-3'		
	sed #	GSEDR1	F	5'-CCA ATA ATA GGA GAA AAT AAA AG-3'	278	
		GSEDR2	R	5'-ATT GGT ATT TTT TTT CGT TC-3'		

	see #	GSEER1	F	5'-AGG TTT TTT CAC AGG TCA TCC-3'	209	
		GSEER2	R	5'-CTT TTT TTT CTT CGG TCA ATC-3'		
	seg #	SEG1	F	5'-TGC TAT CGA CAC ACT ACA ACC-3'	704	
		SEG2	R	5'-CCA GAT TCA AAT GCA GAA CC-3'		
	seh #	SEH1	F	5'-CGA AAG CAG AAG ATT TAC ACG-3'	495	
		SEH2	R	5'-GAC CTT TAC TTA TTT CGC TGT C-3'		
	sei #	SEI1	F	5'-GAC AAC AAA ACT GTC GAA ACT G-3'	630	
		SEI2	R	5'-CCA TAT TCT TTG CCT TTA CCA G-3'		
	sej	SEJF	F	5'-CAT CAG AAC TGT TGT TCC GCT AG-3'	142	
		SEJR	R	5'-CTG AAT TTT ACC ATC AAA GGT AC-3'		
		GTSSTR2	R	5'-TTT TCA GTA TTT GTA ACG CC-3'		
Multilocus Sequence Typing	*arcC* ^	arcCUp	F	5'-TTG ATT CAC CAG CGC GTA TTG TC-3'	456	(Enright *et al*., 2000)
		arcCDn	R	5'-AGG TAT CTG CTT CAA TCA GCG-3'		
	aroE ^	aroEUp	F	5'-ATC GGA AAT CCT ATT TCA CAT TC-3'	456	
		aroEDn	R	5'-GGT GTT GTA TTA ATA ACG ATA TC-3'		
	glp ^	glpUp	F	5'-CTA GGA ACT GCA ATC TTA ATC C-3'	465	
		glpDn	R	5'-TGG TAA AAT CGC ATG TCC AAT TC-3'		
	gmk ^	gmkUp	F	5'-ATC GTT TTA TCG GGA CCA TC-3'	417	
		gmkDn	R	5'-TCA TTA ACT ACA ACG TAA TCG TA-3'		
	pta ^	ptaUp	F	5'-GTT AAA ATC GTA TTA CCT GAA GG-3'	474	
		ptaDn	R	5'-GAC CCT TTT GTT GAA AAG CTT AA-3'		
	tpi ^	tpiUp	F	5'-TCG TTC ATT CTG AAC GTC GTG AA-3'	402	
		tpiDn	R	5'-TTT GCA CCT TCT AAC AAT TGT AC-3'		
	yqiL ^	yqiLUp	F	5'-CAG CAT ACA GGA CAC CTA TTG GC-3'	516	
		yqiLDn	R	5'-CGT TGA GGA ATC GAT ACT GGA AC-3'		

Note: *- Primers provided by MWG; F- forward primer; R- reverse primer.
- Primers provided by Eurofins; ^ - Primers provided by Metabion;

3.5. Commercial Kits

Big Dye Terminator Sequencing Kit (Applied Biosystems, Warrington, UK);
DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany);
QIAamp DNA Mini extraction kit (Qiagen, Hilden, Germany);
Master Mix PCR (Qiagen, Hilden, Germany);
NucleoSpin® Tissue kit for genomic DNA extraction (Macherey-Nagel, Germany);
QIAquick PCR Purification Kit (Qiagen, Hilden, Germany);
Rapid Latex Test Kit for *S. aureus* (Biolife Italiana srl., Milano, Italy);
TaqMan™ Universal PCR Master Mix (Applied Biosystems, Warrington, UK);

3.6. Equipments and Apparatus

Centrifuge with refrigeration 5424R (Eppendorf);
Centrifuge 5415D (Eppendorf);
Centrifuge 5804, for 96-well plates (Eppendorf);
Centrifuge rotors (Eppendorf);
Colony Counter 50971 (Bioblock Scientific);
Confocal laser scanning microscope FluoView FV1000 (Olympus);
Confocal laser scanning microscope LSM710 T-PMT (Zeiss);
Dry oven (Stericell);
Electrophoresis system Power PAC HC 250V/3.0A/300W (BioRad);
Equipment for ultrapure water MilliQ® MilliPore Synthesis (EMD Millipore);
Flow chamber BioUltra Class II Certified Cabinet (Telstar);
Flow chamber ABS 1000CLS Advanced Bio Safety Cabinet- Class II (Bioquell Bio);
Fluorometer Qubit 2.0 (Life Technologies);
Freezer -20°C (Liebherr); Freezer Hera -80°C (ThermoScientific);
Fridge (Liebherr);
Genetic analyzer Hitachi 3130 (Applied Biosystems);
Ice machine (ITV Gomar);
Image analyzer for agarose gel– Gel Doc 2000 (BioRad);
Incubator (Stericell);
Incubator Heraeus (ThermoScientific);
Magnetic stirrer with hot plate IKA® RCT Classic (IKA);
Magnetic stirrer 710/R (ASAL s.r.l, Cernusuco Sul Naviglio, Italy);
Microscan automated system (Beckman Coulter S.L.U, USA);
Microwave (Samsung) (SANYO);
MiniSpin Plus (Eppendorf);
Nanodrop Spectrophotometer ND 1000 (ThermoScientific);
Next generation sequencing MiSeq (Illumina);
Orbital shaker (Innova 42, New Brunswick Scientific);

Orbital shaker (ES-20/60 Environmental Shaker BIOSAN);
Pulsed Field Electrophoresis System CHEF-DR III (BioRad);
pH-meter GLP 21^{+} (Crison);
Scale, precision of 2 decimals (Kern, Germany) (TE612, Sartorius);
Scale, precision of 4 decimals XS205 Dual Range (Mettler Toledo);
Sonicator;
Spectrophotometer ELISA (Tecan 900 Pro);
Spectrophotometer UV 1800 (Shimadzu);
Stomacher (MiniMix® Interscience);
Steam sterilizer AE 75 DRY (Raypa Trade®, Espinar S.L);
Thermobloc for Eppendorf tubes, 1.5 mL, 2 mL (Eppendorf, Germany);
Thermocycler Eppendorf Master Cycler Gradient (Eppendorf, Germany);
Thermocycler 96-well GenAmp PCR System 9700 (Applied Biosystems);
Thermocycler 96-well VERITI PCR System (Applied Biosystems);
Themocycler ABI® 7500 96-well Real-Time PCR System (Applied Biosystems);
Thermomixer COMFORT (Eppendorf);
Tube-strip Picofuge (Stratagene®);
Ultraviolet (UV) lamp BIOCOMP J22;
Vitek II automated system (BioMérieux, France);
Vortex Eurolab (Merck);
Water bath (Precision™ Reciprocating Shaker Baths, ThermoScientific);

3.7. Sequencing, Bioinformatic Tools and Database Used

Sequencing tools

Basic Local Alignment Search Tool (BLAST)- Program dedicated for comparison of nucleotide/ protein sequences to sequence databases (https://blast.ncbi.nlm.nih.gov/Blast.cgi);

Chromas software- free trace viewer for simple DNA sequencing projects which do not require assembly of multiple sequences (http://technelysium.com.au/wp/chromas/);

Molecular Evolutionary Genetics Analysis (MEGA)- user-friendly software for analyzing DNA and protein sequence data from species and populations (http://www.megasoftware.net/);

Bioinformatic tools

Bionumerics platform- analysis of various genomic and phenotypic sources grouped into one global database (http://www.applied-maths.com/). Fingerprint data module (for electrophoresis gels) and Tree and Network Inference module (for constructing dendrograms, phylogenetic trees) respectively, have been used in the present thesis;

Bowtie 2 aligner; SAMtools- aligning sequencing reads to long reference sequences (http://bowtie-bio.sourceforge.net/bowtie2/index.shtml; http://samtools.sourceforge.net/);

FastQC- for quality control checks on raw sequence data coming from high throughput sequencing pipelines (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/);

Mauve Contig Mover (MCM)- ordering a draft genome against a reference one (http://darlinglab.org/mauve/user-guide/reordering.html);

Mass screening of contigs for antimicrobial and virulence genes (https://github.com/tseemann/abricate);

Prinseq- rapid quality control by filtering, reformatting or trimming genomic and metagenomic sequence data (http://prinseq.sourceforge.net/);

Rapid Annotations using Subsystems Technology (RAST)- annotating complete or nearly complete bacterial and archaeal genomes (http://rast.nmpdr.org/);

Scan contig files against PubMLST typing schemes (https://github.com/tseemann/mlst);

SPAdes- assembly toolkit containing various assembly pipelines (http://spades.bioinf.spbau.ru/release3.10.0/manual.html);

VarScan tool- variant detection in next-generation sequencing data (https://sourceforge.net/projects/varscan/files/);

Databases

CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats) Finder (http://crispr.i2bc.paris-saclay.fr/);
European Bioinformatics Institute (EBI) (http://www.ebi.ac.uk/);
Insertion Sequences (IS) Finder (https://www-is.biotoul.fr/about.php);
Multilocus Sequence Typing Database (http://saureus.mlst.net/);
National Center for Biotechnology Information (NCBI) (https://www.ncbi.nlm.nih.gov/);
Uniprot knowledgebase (www.uniprot.org);
spaTyper (Bartels *et al.*, 2014).

Others

COMSTAT 2- analysis of image stacks of biofilms recorded by CLSM (http://www.comstat.dk/)

CHAPTER 4

Methods

Methods

4.1. Food Sampling Strategy

A total of 1079 food products collected from August 2012 to July 2015 were tested for the presence of *S. aureus*, particularly MRSA. The food products were either confiscated by the Border Authorities at the Border Inspection Post at the International Bilbao Airport (Spain) (269 food products) and, Vienna International Airport (Austria) (600 food products) from luggage of passengers on flights from non-EU countries, or collected from a market in Galati, Romania, where goods from the EU border traffic between Republic of Moldavia and Romania (Giurgiulești-Galați) (210 food products) are sold.

Food samples included 519 (48.1%) milk and dairy products of diverse animal origin (cow, sheep or goat milk and cheese- either fresh, brined or with spices), 448 (41.52%) meat samples of diverse animal origin (including antelope, beef, chicken, duck, guinea pig, pork, rodents and turkey), fish and fish products (68, 6.3%), 9 eggs (0.83%), and other products such as pastry, alga, biscuits or dried fruits (35, 3.24%).

Both in international airports and EU border traffic between Republic of Moldavia and Romania randomized food sampling has been conducted. Immediately, food samples were transported in refrigeration conditions in suitable containers able to maintain the food samples at 0-4°C until arrival at the laboratory. Food samples were analyzed in the next 36 hours as recommended by FAO (1997).

4.2. Detection and Isolation Procedures for *S. aureus*

The detection of coagulase-positive staphylococci (CPS) was performed following ISO 6888-2 (ISO, 1999). Solid food samples (approx. 150-200 g) were aseptically cut in pieces and transfer in sterile filter bags. Subsamples, each of 10 g (tests for *S. aureus*) were transferred to sterile filter bags (Stomacher® Bags, UK), diluted 1:10 in sterile Ringer´s solution (Scharlau, Spain) and homogenized in a Stomacher lab blender for 180 sec to obtain the initial suspension. Further decimal dilutions (1:10) were prepared in sterile Ringer´s solution to achieve 10-300 colony forming units (CFU) when plated on each agar medium.

Up to five colonies from each BP+RPF agar plate were analyzed by qPCR targeting the *nuc* gene (Trnčíková *et al.*, 2008) in order to confirm *S. aureus* isolates. Positive colonies with correct morphology in BP+RPF plates and by qPCR confirmation were taken for further typing tests (MRSA biotype, antibiotic resistance, and genetic characterization).

Storage of Bacterial Strains

Confirmed *S. aureus* isolates have been preserved in 25% glycerol, at -80°C.

- Prepare 50% glycerol (Merck, Germany). Sterilize by autoclaving at 121°C for 15 min;
- Add 0.6 mL of sterile glycerol and 0.6 mL of bacterial culture into a labeled cryovial with a screw cap and air tight gasket;
- Mix by vortexing to ensure that the glycerol is evenly dispersed;

- Freeze at -80°C for long term storage.

Revival of Bacterial Strains

To revive the bacterial strains which have been subjected to long term storage at -80°C:

- Transfer the labeled cryovial onto special racks in order to facilitate opening the tubes and avoid any contamination;
- Scrape the frozen surface with a sterile inoculating loop and then immediately streak the bacteria onto the surface of BP agar plate, place the plate overnight at 37°C;
- Return the labeled cryovial at -80°C.

4.3. Isolation of MRSA Strains

Chromogenic culture media are commercially available in order to facilitate MRSA screening and already several researchers have implemented this (McElhinney *et al.*, 2013; Verkade *et al.*, 2011; Veenemans *et al.*, 2013). Chromogenic culture media such as Brilliance MRSA 2 Agar (ThermoFisher Scientific, USA) and ChromID MRSA Agar (BioMérieux, France) have been used in the present thesis for confirmation of staphylococcal isolates from different sources: clinical samples, food or animal derived.

Apart from this, confirmation by multiplex PCR targeting the *mec*A and *mec*C genes (Stegger *et al.*, 2012) has been performed as well.

4.4. Genomic DNA Extraction

a) Tris-HCl -based Method for DNA Extraction

Lysis by boiling is a conventional method used for DNA extraction.

Protocol:

- Transfer 1 mL of PBS 1× to a clean 1.5 mL micro-centrifuge tube;
- Carefully scrape one isolated colony from a BP+RPF agar plate and resuspend it in PBS 1×;
- Spin in a centrifuge at 12,400 rpm for 10 min at 4°C;
- Carefully discard the supernatant using a pipette;
- Resuspend the pellet in 100 µL of 10 mM Tris-HCl by vigorous vortexing for 10 sec and incubate at 95°C for 20 min;
- Spin in a centrifuge at 12,400 rpm for 5 min at 4°C;
- Transfer 70 µL of the supernatant into a clean 1.5 mL micro-centrifuge tube;
- Store the supernatant at 4°C up to one week or at -20°C for longer preservation.

b) Chelex® 100 resin -based DNA Extraction (BioRad, Germany)

The bottle contains 20 mL of 6% Chelex® 100 and a magnetic stirrer, the amount being sufficient for 100 DNA preparations. Prior to use, the Chelex® 100 suspension must be kept on a magnetic stirrer at moderate speed to maintain the matrix in suspension.

Protocol:

- Transfer 1 mL of autoclaved water to a clean 1.5 mL micro-centrifuge tube;
- Carefully pick one isolated colony from a BP+RPF agar plate and resuspend it in autoclaved water;
- Centrifuge at 10,000 x *g* for 5 min at 4°C;
- Carefully discard the supernatant using a pipette;
- Resuspend the pellet in 100 µL of Chelex® 100 suspension by vigorous vortexing for 10 sec and incubate at 56°C for 20 min (***Note****:* Pipet Chelex® 100 suspension using a pipette tip with a large bore, such as 1 mL pipette tip);
- Incubate at 100°C for 8 min;
- Vortex at high speed for 10 sec and immediately chill the tubes on ice;
- Transfer 70 µL of the supernatant into a clean 1.5 mL micro-centrifuge tube;
- Store the DNA samples at -20°C.

c) DNA Extraction Using QIAamp DNA Mini Extraction Kit (Qiagen, Germany)

The following protocol describes the isolation of genomic DNA using a commercial kit from bacterial plate cultures.

Protocol:

- Carefully pick one isolated colony from culture plate with an inoculation loop and suspend in 180 µL of buffer ATL by vigorous stirring;
- Add 20 µL of proteinase K (***Note:*** Qiagen protease has reduced activity in the presence of buffer ATL), mix by vortexing, and incubate at 56°C, 150 rpm, for 1 h 30 min in order to ensure that the lysis will be complete;
- Short spin the 1.5 mL micro-centrifuge tubes to remove drops from the inside of the lid;
- Add 4 µL of RNase A (100 mg/mL) (***Note:*** RNA may inhibit some downstream enzymatic reactions so RNA-free genomic DNA is required), mix by pulse-vortexing for 15 sec, and incubate for 2 min at room temperature;
- Short spin the 1.5 mL micro-centrifuge tubes to remove drops from the inside of the lid;
- Add 200 µL of buffer AL to the sample, mix again and incubate at 70°C for 10 min (***Note***: a white precipitate may form on addition of buffer AL which it will dissolve during incubation at 70°C);
- Short spin the 1.5 mL micro-centrifuge tubes to remove drops from the inside of the lid;
- Add 200 µL of ethanol (96-100%) to the sample and mix by pulse vortexing for 15 sec (***Note***: a white precipitate may form on addition of ethanol);
- Short spin the 1.5 mL micro-centrifuge tubes to remove drops from the inside of the lid;
- Carefully apply the mixture (including the precipitate from the previous step) to the QIAamp Mini spin column (in a 2 mL collection tube) without wetting the rim;
- Close the cap and centrifuge at 6000 *x g* for 1 min;
- Place the QIAamp Mini spin column in a clean 2 mL collection tube and discard the tube containing the filtrate (***Note***: close each spin column to avoid aerosol formation during centrifugation);
- Carefully open the QIAamp Mini spin column and add 500 µL buffer AW1 without wetting the rim;

- Close the cap and centrifuge at 6000 *x g* for 1 min;
- Place the QIAamp Mini spin column in a clean 2 mL collection tube and discard the tube containing the filtrate (***Note***: close each spin column to avoid aerosol formation during centrifugation);
- Carefully open the QIAamp Mini spin column and add 500 µL buffer AW2 without wetting the rim;
- Close the cap and centrifuge at full speed 20,000 *x g* for 3 min;
- Place the QIAamp Mini spin column in a clean 1.5 mL micro-centrifuge tube and discard the collection tube containing the filtrate;
- Carefully open the QIAamp Mini spin column and add 200 µL of buffer AE;
- Incubate at room temperature for 5 min and then centrifuge twice at 6000 *x g* for 1 min (***Note***: a 5 min incubation of the QIAamp Mini spin column loaded with buffer AE, before centrifugation, increase DNA yield);
- Store the DNA samples at -20°C.

4.5. Quantification of Nucleic Acids in Terms of Concentration, Yield and Purity

DNA quantification has been performed by measuring the absorbance at 260 and 280 nm using a nanodrop spectrophotometer. The reading at 260 nm gives the DNA yield which is determined from the DNA concentration in the eluate. The ratio of absorbance between readings at 260 nm and 280 nm gives the estimated purity of DNA. Pure DNA has an A_{260}/A_{280} ratio of 1.7-1.9. For example, in the table below, is the DNA yield purified from bacterial culture using QIAamp kit.

	Yield of nucleic acid with QIAamp kit	
	Nucleic acid yield (µg)	DNA yield (µg) with RNase
Cultured cells (5 x 10^6)	20-30	15-20

	Effect of elution volume on yield and concentration		
Elution volume	Yield (µg)	Yield (%)	DNA concentration (ng/ µL)
200	6.8	100	34
150	6.51	95	43.4
100	6.25	92	62.5
50	5.84	86	116.8

In most of the cases, sample dilution is required. For this, elution buffer or water free of DNase or RNase has been used to dilute DNA samples and to calibrate the spectrophotometer.

4.6. DNA Fragments Separation by Electrophoresis

a) Separation of Small DNA Fragments by Conventional Gel Electrophoresis

DNA fragments has been separated using horizontal agarose gels. Agarose gels were prepared in different concentrations depending on the DNA fragments needed to be separated. In most of the cases, a concentration of 1.5% agarose was used for DNA fragments separation higher than 1200 bp, and 2% for fragments for 500-1200 bp and 3% for fragments lower than 500 bp, respectively.

Before electrophoresis started (120 V; 1×TAE buffer), DNA samples were mixed 4:1 with 6× DNA gel loading dye (ThermoFisher Scientific). DNA molecular weight marker 100 bp, 50-2000 bp (BioRad) respectively were added in each run. After electrophoresis, the agarose gels were stained with GelRed™ (Biotium, ChemoMetec, Denmark) for 30 min and photographed under UV light (254 nm).

b) Separation of Genomic DNA by Pulsed Field Gel Electrophoresis

Genetic characterization of *S. aureus* isolates was carried out by PFGE as described by (McDougal *et al.*, 2003) (*See Appendix 11)*. Before starting, reagents preparation is necessary. This technique helps to analyze large DNA fragments generated by the *Sma*I restriction enzyme digestion, subsequently being subjected to an electric field with periodic changes of its orientation across the gel.

Reagents	Protocol
TE buffer (10 mM Tris : 1 mM EDTA, pH 8)	10 mL 1 M Tris, pH 8; 2 mL 0.5 M EDTA, pH 8; Up to 1000 mL with DEPC water*.
PIV saline solution (10 mM Tris-HCl (pH 8), 1 M NaCl)	1 mL 1M Tris-HCl; 20 mL 5 M NaCl; Up to 100 mL with DEPC water*.
Buffer EC (6 mM Tris-HCl pH 8, 1 M NaCl, 100 mM EDTA pH 8, 0.2% deoxycholate-Na, 0.5% sarkosyl, 100 mg/L lysozyme, 50 µg lysostaphin)	0.15 mL 1 M Tris- HCl; 5 mL 5 M NaCl; 5 mL 0.5 M EDTA; 0.5 mL 10% deoxycholate- Na; 1.25 mL 10% sarkosyl; Up to 25 mL with DEPC water*.
ESP solution (1% sarkosyl, 0.5 M EDTA pH 9)	2.5 mL 10% sarkosyl; 12.5 mL 1 M EDTA; Up to 25 mL with DEPC water*.
TBE buffer (0.5×)	0.5 L 1× TBE; Up to 1 L with MilliQ water.
*Sma*I restriction enzyme (30 U)	One unit is required to digest 1 µg of λ DNA in 1 hour at 25°C in a total reaction volume of 50 µl.
Ethidium bromide	40 µL of 10 mg/mL stock solution; Up to 400 mL of MilliQ water.

*Protocol for DEPC water preparation:

Total (mL)	1000	500	200	100
DEPC	1	0.5	0.2	0.1
Ethanol absolute	10	5	2	1
MilliQ water	989	494.5	197.8	98.9

First, mix DEPC with ethanol for better dissolving, then add the MilliQ water. Leave the bottle closed on a stirrer overnight, at RT. Autoclave twice at 121°C for 20 min (for ethanol evaporation).

Protocol for PFGE

Preparation of *S. aureus* cultures and agarose plugs

- Carefully pick one isolated colony from culture plate with an inoculation loop and put it into a tube containing 5 mL of BHIB (Biolife Italiana, Milano, Italy), after this incubate at 37°C, 200 rpm for 15-20 h;
- Switch on the spectrophotometer at least 30 min before starting to work. Put the 2% low melt certified agarose (BioRad, Munich, Germany) to melt using a thermomixer set at 95°C, 900 rpm. Once the temperature is right, low the temperature at 42°C, without any more agitation;
- Put 1 mL of culture in a cuvette and measure the absorbance at the spectrophotometer at 600 nm;
- Adjust the absorbance of the culture at OD=1, by using buffer PIV;
- Once this is settle, put 200 µL of culture in a centrifuge set it at 12,000 rpm, 2 min;
- Remove the supernatant, wash the pellet with 500 µL of buffer PIV and centrifuge again at 12,000 rpm for 4 min;
- Resuspend the pellet with 300 µL of buffer PIV and incubate for 10 min at 42°C, in order to equalize with the temperature of the agarose;
- Mix 100 µL of culture with 100 µL of 2% agarose and put them in molds;
- Leave it at RT for 10 min, and other 15 min at 4°C.

Cell lysis

- Put 1 mL of lysis buffer EC in 2 mL tubes;
- Unmold the agarose plugs and transfer in tubes containing the lysis buffer EC;
- Incubate at 37°C, 600 rpm for 5-6 h.;
- Put 1 mL of ESP solution in 2 mL tubes;
- Incubate at 56°C for 16-20 h.

Washing the agarose plugs

- Heat the TE buffer at 50°C in order to start the washing procedure;
- Put 1 mL of TE buffer in 2 mL tubes;
- Transfer the agarose plugs in tubes containing TE buffer, incubate at 50°C, 500 rpm for 30 min;
- Remove the supernatant and add another 1 mL of TE buffer;
- Repeat this procedure at least 5 times (***Note***: You may keep the agarose block at 4°C for maximum 6 months).

DNA digestion using *Sma*I restriction enzyme

- Carefully remove the plugs from TE buffer and put them in a Petri plate;
- Cut 1/3 of the agarose plug and incubate with 100 µL of restriction buffer using 1.5 mL tubes;
- Incubate at RT for 30 min;
- Prepare the restriction solution with *Sma*I enzyme (final concentration 1%, 30 U of enzyme, Biolabs, UK);
- Remove the restriction buffer from tubes;
- Put 100 µL of restriction solution and incubate at 25°C, overnight.

Electrophoresis

- Prepare 3 L of TBE extended range at 0.5× and add it in the electrophoresis cuvette (***Note***: Before this, check to see if the system is clean);
- Remove the restriction solution from tubes containing the plugs and add 100 μL of 0.5× TBE;
- Stabilize at 4°C for 30 min;
- Dissolve 1% low melt certified agarose (BioRad, Munich, Germany) and stabilize at 42°C;
- Prepare 1% pulsed field certified agarose (BioRad, Munich, Germany) gel in a final volume of 100 mL. Heat it for 3 min using the microwave, agitate and warm other 15" (***Note***: After the gel temperature stabilization is reached (54°C) you may poor it in the tray);
- Insert the plugs in the agarose gel (***Note***: Be careful not to form air bubbles);
- At both ends of the gel and in the center, include the molecular marker Lambda Ladder PFG, 48.5–1018 kb (Biolabs, UK);
- Seal the plugs using 1% agarose gel and leave to solidify it;
- Put the gel in the electrophoresis cuvette and set the conditions.

Pulse range: 5-15 sec x 10 h

15-60 sec x13 h;

Run time: 23 h;

Voltage: 6V/cm;

Angle: 120°;

Temperature: 14°C.

Image capture

- Submerge the agarose gel, containing the DNA fragments, into a cuvette with ethidium bromide (Sigma-Aldrich) for 15 min;
- Remove the ethidium bromide excess by putting the agarose gel in a cuvette with distilled water and leave it for 2 hours.

The image capture and gel analysis was done by using the fotodocumentation system Gel Doc 2000. The results interpretation was performed by drawing the dendrogram using Bionumerics programme v6.6. (www.appliedmaths.com).

4.7. DNA Genotyping by PCR Amplification

Alternative methods such as PCR -based methods are more sensitive than traditional methods for identification of *S. aureus*, made up of successive cycles of DNA replication *in vitro*, using two oligonucleotide primers that hybridize to the two strands of the original sequence (used as a template in the replication). The major difference between a reaction for replication and a process for DNA replication *in vivo*, is the fact that in the PCR reaction step, the attachment of the primers is not carried out enzymatically, but by temperature steps, and the only enzyme used in the reaction is a DNA polymerase (replicase function).

a) Conventional PCR

In the present thesis, DNA amplification took place by performing single plex and multiplex PCRs. In conventional single plex PCR a single target is amplified in a single reaction tube. In contrast, multiplex PCR uses specific primer sets for the simultaneous amplification of multiple target sequences in only one single tube (www.roche.com).

Reagents	Thermocycler conditions
1× Buffer Buffer (Roche) or 1× Multiplex PCR Master Mix (Qiagen)	95°C for 5 min (initial denaturation step allowing the activation of FastStart *Taq* DNA Polymerase)
2 mM $MgCl_2$ solution (Applied Biosystems)	30-35 cycles
0.2 mM dNTPs (Roche)	95°C for 1 min (denaturation)
1-2 U FastStart *Taq* DNA Polymerase (Roche)	48-60°C for 30 sec–1 min 30 sec (primer annealing)
0.2-1.8 µM each primer	72°C for 1–2 min (extension)
2-10 ng DNA sample	72°C for 7–10 min (final extension)
Final volume of 20-50 µL	

The thermocycler conditions and concentration of reagents has been adapted for each PCR analysis (See Appendix 1-9).

All the PCR reactions were run on a thermocycler 96-well VERITI PCR system (Applied Biosystems). Afterwards, PCR products were visualized by nucleic acid GelRed™ (Biotium, ChemoMetec, Denmark) staining after electrophoresis of agarose gels.

PCR Products Purification

For purifying DNA fragments from PCR products, several commercial kits are available. In the present thesis, we used the QIAquick PCR purification kit (Qiagen, Hilden, Germany) for purifying the PCR products obtained for MLST.

Protocol:

- Add 5 volumes of buffer PB to 1 volume of the PCR sample and mix (***Note***: for example, add 500 µL of buffer PB to 100 µL PCR sample);
- ***Note***: if pH indicator I has been added to the buffer PB, check that the color of the mixture is yellow. If the color of the mixture is orange/ violet, add 10 µL of 3M sodium acetate, pH 5.0 and mix;
- Place a QIAquick spin column in a 2 mL collection tube;
- To bind DNA, apply the sample to the QIAquick column and centrifuge for 30-60 sec;
- Discard the supernatant and place the QIAquick column back into the same tube;
- To wash, add 0.75 mL buffer PE to the QIAquick column and centrifuge for 30-60 sec;
- Discard the supernatant and place the QIAquick column back into the same tube;
- Centrifuge the column for an additional 1 min (***Note***: residual ethanol from buffer PE will not be completely removed if the supernatant is not completely discarded before this additional centrifugation);
- Place the QIAquick column in a clean 1.5 mL micro-centrifuge tube;
- To elute DNA, add 50 µL buffer EB (10 mM Tri-Cl, pH 8.5) to the center of the QIAquick membrane and centrifuge the column for 1 min;

- ***Note***: If the purified DNA is to be analyzed on a gel, add 1 volume of loading dye* to 5 volumes of purified DNA; mix the solution by pipetting up and down before loading the gel;
  *Loading dye contains three different markers (bromophenol blue, xylene cyanol, and orange G) that facilitate estimation of DNA migration distance and optimization of agarose gel run time.

b) Real Time PCR

The amplified DNA is measured based on the two primers, allowing amplification of the product in which a third dual-labelled fluorescent probe (TaqMan®, ThermoFisher Scientific, UK) will anneal. During amplification, the TaqMan probe relies on the 5'→3' exonuclease activity of *Taq* polymerase to release a 5' fluorescent (FAM) tag from the annealed TaqMan® probe, giving a real time measurable fluorescence emission. The increase in fluorescence is proportional to the concentration of DNA used as a target sequence.

Reagents	Thermocycler conditions
1× TaqMan® PCR Master Mix (ThermoFisher)	50°C for 2 min (activate the uracil *N*-glycosylase)
200 nM Taqman® probe aurP (FAM/BHQ) (ThermoFisher)	95°C for 10 min (initial denaturation step allowing the activation of *Taq* DNA Polymerase)
300 nM each primer	50 cycles
5 ng DNA sample	95°C for 15 sec (denaturation)
Final volume of 25 µL	60°C for 1 min (primer annealing and extension)

The thermocycler conditions and concentration of reagents has been adapted for qPCR analysis (See Appendix 10).

PCR reactions were run on a thermocycler ABI® 7500 96-well real time PCR system (Applied Biosystems). Negative values or lack of amplification has been established for C_T threshold values higher than 45.

4.8. DNA Sequencing

For DNA sequencing, three steps are required: DNA sequencing reaction by PCR, ethanol/EDTA precipitation and cycle sequencing of PCR products obtained.

a) PCR for sequencing

Reagents	Thermocycler conditions
2.5× Big Dye Ready Reaction Premix	96°C for 1 min (initial denaturation step)
5× Big Dye sequencing buffer	30 cycles
10 µM primer	96°C for 30 sec (denaturation)
10 ng/ µL DNA sample	55°C for 15 sec (hybridization)
Final volume of 10 µL	60°C for 4 min (elongation)

Before any PCR amplification took place, quantitating the amount of purified DNA has been measured using a nanodrop spectrophotometer set it at 260 nm. The necessary amount of DNA has been correlated with the amplicon size of each primer taken into analysis. All PCR reactions were run on a thermocycler 96-well GenAmp PCR System 9700 (Applied Biosystems).

PCR product	Quantity of DNA (ng)
100-200 bp	1-3
200-500 bp	3-10
500-1000 bp	5-20
1000-2000 bp	10-40
>2000 bp	20-50

Note: Higher DNA quantities gives higher signal intensities, however may also give shorter read lengths and top-heavy data. Also, the amount of PCR product to be used in sequencing may also depends on the length and purity of the PCR product.

b) Ethanol/EDTA Precipitation

To precipitate 10 µL sequencing reaction in 96-well reaction plate:

- Remove the 96-well reaction plate from the thermal cycler and briefly spin;
- Transfer the sequencing reaction product into a clean 1.5 mL micro-centrifuge tube;
- Add 5 µL of 125 mM EDTA to each well (***Note***: make sure that the EDTA reaches the bottom of the wells);
- Add 60 µL of 100% ethanol to each well and mix by inverting tubes four times;
- Incubate at room temperature for 15 min;
- Centrifuge at 13,000 rpm for 20 min, 4°C; afterwards remove the supernatant;
- Add 60 µL of 70% ethanol to each well;
- Centrifugate at 13,000 rpm for 15 min, 4°C; afterwards remove the supernatant (***Note***: make sure that the wells are dry and protected by light);
- Resuspend the samples in 20 µL of Hi-Di formamide and cover them with an aluminum foil in order to be protected by light;
- Store at -20°C.

c) Cycle Sequencing

- Incubate the samples at room temperature for 20 min;
- Vortex gently and centrifugate briefly;
- Denaturize the samples at 95°C for 5 min;
- Cool on ice for another 3 min;
- Vortex gently and centrifugate briefly in order to remove drops from the inside of the lid;
- Place the 96-well reaction plate into a Genetic analyzer Hitachi 3130 (Applied Biosystems);
- Set the parameters:

Fragment analysis run module	Array length	Polymer	Run time	24 h throughput†	Resolution	Performance SD‡
Fragment analysis 50_POP7	50 cm	POP-7™	50 min	2240	500 bp	0.15

† 20 genotypes/injection;

‡ Standard deviation: 1 bp resolution at 99.99% accuracy.

Whole Genome Sequencing

Genomic DNA of MRSA strains has been extracted using the DNeasy Blood and Tissue Kit (Qiagen) and purified DNA has been quantified with a Qubit 2.0 Fluorometer (Life Technologies). Whole genome sequencing (WGS) has been done by preparing libraries from the genomic DNA using tagmentation procedure and 300 bp-paired-end sequencing. A next-generation sequencing approach has been undertaken in a MiSeq device (Illumina).

4.9. Biofilm Formation Capacity

Culture Media screening

Media screening consisting in TSB with/ without addition of 0.4% glucose (TSBG) or 4% NaCl (TSBN) for supporting 24 h *S. aureus* biofilm formation was performed. Glucose sterilized by filtration (0.22 µm) (Minisart®, Sartorius) was added after autoclaving.

Biofilms were grown in 96-well plates tissue cultured (Nunc® MicroWell™, USA) with a total volume of 200 µL of TSB, TSBG and TSBN per well and a starting inoculum approximately equal to 10^6 CFU/mL. Only broth media were introduced in the assay as negative controls, and *S. aureus* ATCC® 25923 as positive control.

Protocol for biofilm quantification *(See Appendix 12)*:

- Inoculate one isolated colony into 5 mL TSB media and incubate at 37°C, 120 rpm for 18-24 h;
- Adjust the bacterial cultures to the same OD_{600} (***Note***: concentration $1x10^8$ CFU/mL);
- Add 2 µL of adjusted bacteria to a 96-well plate (***Note***: equivalent to $2x10^6$ CFU/mL);
- Incubate the plate aerobically on a horizontal shaker at 37°C, 120 rpm for 24 h;
- Wash the wells, containing the biofilm, to remove weakly adherent bacteria (once with 0.9% NaCl);
- Add 200 µL per well of pure methanol (Sigma-Aldrich, USA) for 15 min (fixation of biofilm);
- Withdraw the methanol and allow the wells to dry at room temperature (20 min);
- Add 200 µL of 1% CV (Merck, Germany) to each well and incubate for 5 min;
- Remove the CV solution;
- Wash three times with water and then allow the wells to dry at room temperature;
- Add 200 µL of 33% acetic acid (Sigma-Aldrich, USA) to each well to solubilize the stain;
- Read the absorbance at 570 nm.

Biofilm quantification was performed according to the procedure developed by Stepanović *et al.* (2000) with some modifications. As TSBG media gave the best results in terms of biofilm formation (Chapter 8), this culture media was used to test the MRSA biofilm ability, static conditions.

Biofilm Formation Overtime

This task referred to the formation of 48 h and 72 h *S. aureus* biofilms on 96-well microtiter plates. All the strains were included in biofilm formation overtime (Peeters *et al.*, 2008).

4.10. Biofilm Matrix Characterization by Chemical and Enzymatic Treatments

Sodium meta-periodate ($NaIO_4$) and proteinase K which targets biofilm matrix components as glucose-containing polysaccharides and proteins, respectively were tested for their ability to disrupt preformed *S. aureus* biofilms from polystyrene 96-plate wells. For this assay, were included only the strains capable to form stronger biofilms, after 48 h of growth. Biofilm detachment assays were carried out as described by (Kogan *et al.*, 2006) and (Fredheim *et al.*, 2009) with modifications.

Protocol:

- Biofilms were grown in the 96-well plates for 48 h following the conditions described above;
- After each biofilm formation period, the media and non-adherent cells were removed and adherent biofilm was washed once gently with 200 µL 0.9% NaCl;
- A volume of 200 µL of 40 mM $NaIO_4$ (Sigma-Aldrich co., Saint Louis, USA) or 0.1 mg/mL proteinase K (Sigma-Aldrich co., Saint Louis, USA) in 20 mM Tris-HCl (pH 7.5) : 1 mM $CaCl_2$ were carefully added to minimize mechanical detachment of biofilms (***Note***: Control wells received an equal volume of buffer without reagents);
- Plates were incubated for an extra 2 h at 37°C;
- The content of each well was discarded and washed once with 200 µL 0.9% NaCl and then resuspended into 200 µL of 0.9% NaCl;
- Biofilms were dislodged by scraping followed by sonication using a cycle of 1 s and an amplitude of 22%;
- Read the absorbance at 600 nm.

4.11. Biofilm Matrix Composition and Structural View by Confocal Laser Scanning Microscopy

CLSM was used to observe *S. aureus* biofilms with the selected fluorochromes accordingly to the laser lines for total biofilm cell analysis (laser lines accordingly to the excitation properties of the dye).

Protocol:

- Biofilms were grown for 48 h as described above using 24-well plates;
- Media was carefully removed from the wells and biofilms were rinsed with 300 µL of sterile water to remove loosely attached cells;
- The plate surface was cut with a heated metal cylinder;
- To visualize PNAG, biofilms were incubated in the dark for 15 min with 100 µL containing 0.01 mg/mL wheat germ agglutinin (WGA)-TRITC conjugated with Oregon Green488 (Molecular Probes, USA);
- Cells were stained with 100 µL of 5 µM of SYTO BC nucleic acid stain (Invitrogen, Paisley, UK)/ (Molecular Probes, Inc., Eugene, USA);
- Proteins were visualized with 100 µL undiluted of SYPRO Ruby biofilm matrix (Invitrogen, Paisley, UK)/ (ThermoFisher Scientific, Austria) by 30 min incubation;
- Stains were removed and wells were rinsed with sterile water between each stain and before imaging;

- Sample observation by CLSM (***Note***: particular care must be taken into consideration to properly fix the sample during manipulation and inspection of the samples; moreover, is important to avoid exposure to light, preventing the blanking of the samples)

The biofilm images were acquired in inverted FluoView FV 1000 (Olympus™) or LSM710 T-PMT (Zeiss) confocal laser microscopes and biofilms were observed using 40x fluar/ water-immersion objectives. The images were analyzed sequentially using two virtual channels. Up to three stacks of horizontal images were acquired for each biofilm at different areas in the well. Two surfaces of two independent replicates were observed in each CLSM experiment.

Labelled cells were detected using the following combination of laser excitation and emission band-pass wavelengths: (510-582 nm) for SYTO, (584-755 nm) for SYPRO and (406-540 nm) for WGA. The CLSMs were configured with two lasers (argon 458/488/514 nm and HeNe 613 nm).

Quantitative structural parameters of each MRSA-producing biofilm such as biovolume, area occupied, average diffusion distance, surface area and surface to biovolume ratio were calculated using the freely available COMSTAT v2.1 software.

4.12. Antimicrobial Resistance Profile

After the confirmatory identification by phenotypic and genotypic tests, *S. aureus* isolates were investigated for antimicrobial susceptibility patterns. Susceptibility to twenty antimicrobial agents was performed by the Microscan automated system (Beckman Coulter S.L.U, USA) for microdilution method for MICs interpretation and disk diffusion method for zone diameter measurements, following the recommendations of the European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines 2015 (www.eucast.org).

Antimicrobial agents tested were: penicillin (PEN), oxacillin (OXA), amoxicillin/clavulanate (AMC), daptomycin (DAP), erythromycin (ERY), clindamycin (CLI), teicoplanin (TEC), vancomycin (VAN), ciprofloxacin (CIP), levofloxacin (LVX), amikacin (AMK), gentamicin (GEN), tobramycin (TOB), mupirocin (MUP), rifampin (RIF), tetracycline (TET), fusidic acid (FUS), fosfomycin (FOF), linezolid (LZD) and trimethoprim sulfamethoxazole (SXT).

S. aureus isolates exhibiting resistance to at least two classes of antibiotics were considered multidrug-resistant.

4.13. Statistical Analysis

- Comparisons between both chromogenic media and *mec*A/*mec*C detection, which was considered the reference method, were performed in terms of sensitivity, specificity, Positive Predictive Value (PPV) and Negative Predictive Value (NPV). Differences of analytical performance between chromogenic media, and *mec*A/*mec*C detection, were analyzed using McNemar paired samples non-parametric test with an $\alpha = 0.01$.

Sensitivity measures the proportion of positives that are correctly identified as such.

$$\textbf{Sensitivity} = \frac{\mathbf{A}}{\mathbf{A+C}} \times \mathbf{100}, \% \quad (1)$$

Specificity measures the proportion of negatives that are correctly identified as such.

$$\textbf{Specificity} = \frac{\mathbf{D}}{\mathbf{D+B}} \times \mathbf{100}, \% \quad (2)$$

Positive Predictive Value (PPV)

$$\textbf{PPV} = \frac{\mathbf{A}}{\mathbf{A+B}} \times \mathbf{100}, \% \quad (3)$$

Negative Predictive Value (NPV)

$$\textbf{NPV} = \frac{\mathbf{D}}{\mathbf{D+C}} \times \mathbf{100}, \% \quad (4)$$

McNemar test is used to compare paired proportions. It can be used to analyze retrospective case-control studies, where each case is matched to a control (http://www.graphpad.com/quickcalcs/McNemar1.cfm)

$$\chi 2 = \frac{(\mathbf{b-c})^2}{(\mathbf{b+c})} \quad (5)$$

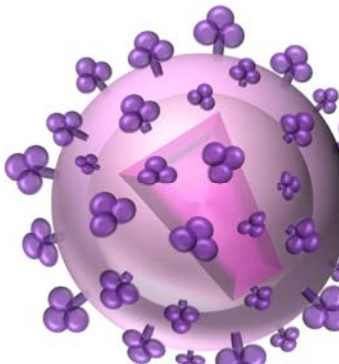
b, c- discordant cells because they represent pairs with a difference.

For a test with α= 0.05, the critical value for the McNemar statistic = 3.84. If the test statistic is >3.84, the p-value will be < 0.05 and the null hypothesis of equal proportions between pairs or over time will be rejected (http://www.biostat.umn.edu/~susant/Fall11ph6414/Section12_Part2.pdf).

- Statistical analysis of 24 h and 48 h MRSA biofilms have been performed using typical statistical parameters. Coefficient of variations (e%) was calculated in order to estimate the experimental errors.

PART 3

RESULTS AND DISCUSSIONS



CHAPTER 5

Detection and Identification of *Staphylococcus aureus* in Food Isolated from Markets

Results published

Oniciuc, E.A., Ariza-Miguel, J., Bolocan, A.S., Diez-Valcarce, M., Rovira, J., Hernández, M., Fernández-Natal, I., Nicolau, A.I., Rodríguez-Lázaro, D. (2015). Foods from black market at EU border as a neglected route of potential Methicillin-Resistant *Staphylococcus aureus* transmission. International Journal of Food Microbiology, 209: 34-38, doi:10.1016/j.ijfoodmicro.2014.11.015. Impact factor: 3.082

Ciolacu*, L., Stessl, B., Bolocan, A.S., **Oniciuc*, E.A.**, Wagner, M., Rychli, B., Nicolau, A.I. (2016). Tracking foodborne pathogenic bacteria in raw and ready-to-eat food illegally sold at the Eastern EU border. Foodborne Pathogens and Disease, doi:10.1089/fpd.2015.2057. Impact factor: 1.905

**those authors contributed equally to the current work*

Detection and Identification of *Staphylococcus aureus* in Food Isolated from Markets

It is estimated that *Staphylococcus aureus* produced an average of 241,148 episodes of domestically acquired foodborne illnesses caused in United States (Scallan *et al.*, 2011), representing an important cause of food poisoning. However, in Europe, only 21 domestic outbreaks have been reported in 2013 (EFSA and ECDC, 2015). In addition, this foodborne pathogen cause nosocomial invasive infections ranging from mild skin and soft tissue infections to life-threatening diseases such as septicemia, endocarditis and necrotizing pneumonia (Lowy, 1998). It has been estimated that around 30% of healthy human individuals are colonized by this opportunistic pathogen (Graveland *et al.*, 2011). *S. aureus* frequently harbor antibiotic resistance determinants which complicate treatment and significantly increase the associated costs.

Currently, MRSA is distributed worldwide and constitutes a major concern in human health because of its complex epidemiology and its ability to acquire novel antibiotic resistance mechanisms. MRSA was first described in 1960, within a year after the inclusion of methicillin in the clinical practice to treat infections caused by the emergence of penicillin-resistant *S. aureus* (Jevons, 1961). Its presence was restricted to the clinical environment initially, but at the end of the past decade first cases of MRSA infections in the community were reported affecting people who exhibited no typical risk factors of hospital acquisition (Otter and French, 2010). Until the beginning of this century, MRSA had been rarely reported in livestock. It was first described in 1975, and after that, only sporadic cases were reported in the following 25 years. From 2005 onwards, MRSA belonging to ST398 was observed to colonize pigs and people professionally exposed to pig farming in several European countries (Voss *et al.*, 2005; Witte *et al.*, 2007). Later studies revealed the presence of that lineage in other food producing animals, and therefore was designated LA-MRSA.

Emergence of MRSA in food-producing animals has provoked a great concern in the presence of MRSA in associated foodstuff due to the potential for dissemination in the population. EFSA considers that the role of food as vehicle of human MRSA dissemination is deemed to be low. However, foodborne MRSA infections have been formally demonstrated in several occasions (Kluytmans *et al.*, 1995; Jones *et al.*, 2002), so, food can be a successful route for transmission of MRSA lineages. In this study, we have evaluated the presence of MRSA in food (homemade and/or processed) illegally sold in a black market in Galati, Romania, a town situated in the South-East part of Romania, on the border with Republic of Moldavia. This information provides an overview on the potential risk introduced to European Union (EU) from non-EU countries *via* foods (foods introduced as personal goods, which are then illegally sold to EU consumers), consequently defining a neglected route of transmission, as well as to reveal the role that it could play in the prevalence and dissemination of MRSA.

Sampling and bacterial isolates

A total of 200 samples were taken from July 2012 to February 2013 in a black market in Galati, Romania, a place close to the border with Republic of Moldavia. The food samples were transported under refrigeration

to the laboratory and their microbiological analysis started in the same day they were collected. The food samples were classified into four categories: milk and dairy products (36%; n= 73; raw milk; cheeses made of cow, sheep, or goat raw or pasteurized milk, cream, and butter), fish and fish products (31%; n= 61; smoked or canned fish), meat and meat products (20%; n= 41, chicken carcasses, sausages, pork rind, and lard), and other food products (13%; spices, dried fruits, jellies, gingerbread, and candies). Sampling was done randomly and the solid food samples were aseptically cut into pieces, if necessary, and ground in a laboratory mill. Subsamples, 10 g each, were transferred to sterile filter bags (Stomacher Bags; Seward Ltd., Worthing West Sussex, UK), diluted 1:10 in sterile Ringer's solution (Scharlau; Scharlab, Barcelona, Spain) and homogenized in a Stomacher lab blender for 180 s.

The total number of coagulase-positive staphylococci (CPS) was enumerated on Baird Parker Agar with Rabbit Plasma Fibrinogen (Biolife Italiana srl.) following ISO 6888:2 (ISO, 1999). After 24–48 h of incubation at 37°C, agar plates yielding 10–300 colonies were included in the calculation of CPS (mean CFU/mL or g). Then, up to five colonies from each plate were analyzed by PCR targeting the *nuc* gene (Trnčíková *et al*., 2008) to confirm *S. aureus* isolates.

Screening for the presence of MRSA

We assessed the presence of *mec*A and *mec*C in all *S. aureus* isolates by multiplex PCR as previously described (Stegger *et al*., 2012). Genes *mec*C and *mec*A were used as positive controls, non-template controls were included in each run as well as.

Antimicrobial susceptibility testing

Antimicrobial susceptibility testing was performed by the microdilution method following the recommendations and MIC breakpoints of the CLSI guidelines (2012). Susceptibility to the following 21 antimicrobial agents was tested: penicillin (PEN), oxacillin (OXA), amoxicillin/clavulanate (AMC), daptomycin (DAP), cefazolin (CFZ), erythromycin (ERY), clindamycin (CLI), teicoplanin (TEC), vancomycin (VAN), ciprofloxacin (CIP), levofloxacin (LVX), amikacin (AMK), gentamicin (GEN), tobramycin (TOB), mupirocin (MUP), rifampin (RIF), tetracycline (TET), fusidic acid (FUS), fosfomycin (FOF), linezolid (LZD) and cotrimoxazole (CTX).

Genetic fingerprinting

Genetic characterization of all isolated *S. aureus* was carried out by PFGE as previously described (McDougal *et al*., 2003). PFGE patterns were analyzed with Bionumerics v.6.6 (Applied-Maths NV, Sint-Martens-Latem, Belgium) to describe genetic relationships among isolates. Dendograms were constructed using the Dice similarity coefficient and the unweighted pair group mathematical average (UPGMA) clustering algorithm with 1% in the tolerance and optimization values. The Simpson's index of diversity was calculated to assess the discriminative power of PFGE by using the Comparing Partitions website hosted at http://darwin.phyloviz.net/ComparingPartitions/index.php?link=Home. Multilocus sequence typing of MRSA was performed as described elsewhere (Enright *et al*., 2000). Allelic profiles were assigned

in the *S. aureus* MLST database hosted at http://saureus.mlst.net/. Information of the MRSA was submitted to that database.

The *S. aureus spa* typing using the sequence of a polymorphic VNTR in the 3' coding region of the *S. aureus*–specific staphylococcal protein A (*spa*) was determined as described by (Harmsen *et al.*, 2003; www.spaserver.ridom.de). *Spa* types were assigned according to the repeat succession in Bionumerics 6.6 software (Applied Math NV, Sint-Martens-Latem, Belgium).

Typing and subtyping of the SCC*mec* element

We determined the genetic structure of the SCC*mec* element by multiplex-PCR as previously described (Kondo *et al.*, 2007). This molecular method allows the discrimination of the SCC*mec* types I, II, III, IV, V and VI, as well as the variants IA & IIIA.

Detection of Panton–Valentine leukocidin (PVL) virulence factors

We investigated the presence of the PVL genes (l*ukS*-PV & *lukF*-PV) by conventional PCR as described by (Lina *et al.*, 1999). Reference strain ATCC 49775 was used as positive control.

Detection of enterotoxin genes

S. aureus strains were tested for the presence of *sea*, *seb*, *sec*, *sed*, *see*, *seg*, *seh*, *sei*, *sej* by PCR as published previously by Gonano *et al.* (2009), with modifications. Primers were combined in order to obtain two different sets of multiplex PCRs, in which one set for detection of *sea*, *seb*, *sec*, *sed*, and *see* genes (PCR 1) and the other one for *seg*, *seh*, *sei*, *sej* genes (PCR 2).

Results and discussion

The emergence of MRSA in food-producing animals has elicited a great concern in the last decade on the potential role of foods in the dissemination of MRSA lineages. Consequently, many studies have assessed the presence of this pathogen in food facilities and samples from different countries and animal origins. Prevalence of MRSA in foodstuff greatly varies depending on the animal and the country of origin. Thus, while pork showed the highest contamination rate in the USA and Canada, poultry did in the Netherlands and Denmark (Kluytmans, 2010; Bhargava *et al.*, 2011).

The 200 food samples, either homemade or processed ones, taken for the present study were assessed for the presence of *S. aureus*, MRSA respectively. Overall, 73% of the samples were homemade and sold in plastic bags or cartoon boxes, while 27% were produced at industrial level in which they were packed and labeled. In total, 80% of the foods originated from the Republic of Moldova, 17% from Ukraine, and 3% from Bulgaria.

Crossing the Romanian border with food is allowed just for low amounts as personal goods on distances that do not overpass 50 km, but very often these foods are illegally sold in black markets. There are 9 border crossings between Romania and Republic of Moldova and 5 Romanian cities situated at distances that are

in accordance with the Romanian law 10/2010 (Monitorul Oficial). In each of those cities, there are one or two markets where Moldavians are coming daily to sell foods. Those markets are organized to sell fresh fruits and vegetables and do not have facilities for refrigeration. Since refrigeration is not performed and hygiene standards are not met at the selling point, multiplication of pathogens to levels sufficient to cause illnesses in vulnerable populations can occur and isolates with increased virulence potential and antibiotic resistance can be transmitted. For example, Gwida *et al.* (2012) correlated the re-emergence of brucellosis in nonendemic regions of the EU with Turkish migrants, while Costard *et al.* (2013) estimated the risk of African swine fever introduced to the EU by illegally imported pork products respectively, in poultry carcasses from Romania in 2012–2013.

Even though local authorities installed adverts saying that food of animal origin is forbidden to be sold and make periodical controls of the activity taking place in these markets, eggs, fish and fish products, milk and dairy products, meat and meat products are sold daily. It is difficult to estimate the amounts of food from non-EU countries entering in that way, but the frequency of this phenomenon is constant and high. The type of food and the sampling period, which included the winter season with low temperatures, could affect the viability of pathogens. However, EFSA reports showed low occurrence of these pathogens in Romania (EFSA, 2013), and other studies reported low prevalence or absence of the mentioned pathogens in illegally imported foods into other European countries (Rodríguez-Lázaro *et al.*, 2015; Schoder *et al.*, 2015) Furthermore, Reg. (CE) 206/2009 regarding the introduction in the EC of personal foodstuff of animal origin bans the introduction of meat, milk and derived foodstuff if they are not from EU-countries or Croatia, Faeroe Islands, Greenland or Iceland.

Overall, thirty-two *S. aureus* isolates were recovered from sixteen confiscated food samples (8%): eight milk and dairy products, five fish products and three meat samples. Among them, one isolate (0.5%) recovered from pork lard sample was MRSA as harbored the *mec*A resistance determinant. None isolate harbored the *mec*C homologue. These results are consistent with other studies where isolation rates of *S. aureus* from food samples ranged from 10% to 40% (Normanno *et al.*, 2007; Pu *et al.*, 2009; Crago *et al.*, 2012). Storage time/ temperature abuses, and inadequate chilling or heat treatment of foodstuffs at restaurants, canteens, or private households were responsible for *S. aureus* outbreaks (EFSA and ECDC 2014; Hennekinne *et al.*, 2012). A recent study reported a 27% *S. aureus* prevalence at a RTE food-processing facility, where *S. aureus* has been isolated from pre- and post-cooked foods, surfaces, gloves of workers, and air (Syne *et al.*, 2013).

S. aureus could also be shed by ruminants affected by subclinical mastitis as an undetected stock problem (Voelk *et al.*, 2014; Walcher *et al.*, 2014). Especially homemade raw milk cheeses produced in small batches as investigated in this study are often affected (Rosengren *et al.*, 2010). Studies on the prevalence of *S. aureus* in fish products are rare, although 7% of all staphylococcal foodborne diseases are due to contaminated fish and fish products. Two recent studies reported an occurrence of *S. aureus* in 5–43% of fish and fish samples (Vázquez-Sánchez *et al.*, 2012; Zarei *et al.*, 2012).

S. aureus contamination levels from 10^4 to 10^5 CFU/g are sufficient to produce enterotoxin at a level that poses a risk to consumers' health (EC 2073/2005). In our study, only thirteen % of the *S. aureus*–positive samples harbored >10^5 CFU/g, while the majority was yielding between 10^2 and 10^4 CFU/g (Table 10). Eight *S. aureus* isolates harbored *sea* gene, while other five were tested positive for *seg* and *sei* genes. The rest of

the *S. aureus* isolates were tested negative for the presence of enterotoxins. However, the MRSA isolate recovered from pork lard was not enterotoxigenic. Occurrence of staphylococcal enterotoxins constitute a concern especially for milk and dairy products since they can be found in higher proportion than the rest of other animal origin food products (Carfora *et al.*, 2015). Interestingly, SE were predominant present in the analyzed milk and dairy samples, harboring one or two toxin genes.

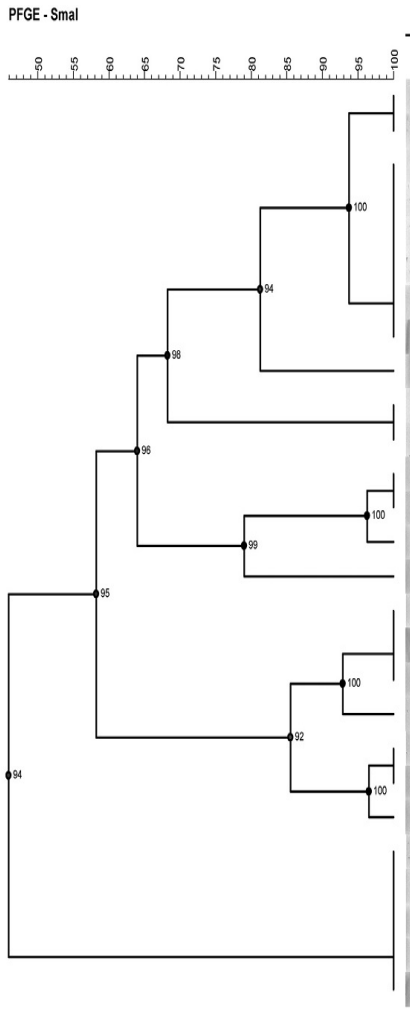
A further particular livestock-associated problem is the increasing number of MRSA (Haran *et al.*, 2012). Apart from dairy herds as a reservoir for MRSA, raw meat could also harbor higher loads of *S. aureus* (15–65%), among them 1–11% MRSA (Bhargava *et al.*, 2011; Jackson *et al.*, 2013).

***Table 10**. Staphylococcus aureus–positive ready-to-eat food illegally sold in a Romanian market*

Source	Isolation date	Amount (g)	ISO 6888-2
Artificial black caviar	14.09.2012	150	$1.1 \cdot 10^3$
Artificial red caviar	14.09.2012	150	$2.0 \cdot 10^2$
Fresh cow cheese	14.09.2012	1000	$1.5 \cdot 10^4$
Sheep cheese salted in brine	14.09.2012	1000	$1.4 \cdot 10^3$
Unfermented goat cheese	14.09.2012	500	$3.5 \cdot 10^3$
Raw milk	14.09.2012	2000	$1.1 \cdot 10^4$
Smoked salmon	14.09.2012	500	$1.0 \cdot 10^5$
Fish canned in oil with herbs	06.11.2012	1000	$1.0 \cdot 10^3$
Pork lard	06.11.2012	400	$2.3 \cdot 10$
Raw milk	06.11.2012	2000	$1.5 \cdot 10^2$
Non-fermented unsalted sheep cheese	06.11.2012	600	$1.6 \cdot 10^4$
Smoked fish	06.11.2012	500	$1.1 \cdot 10$
Poultry	06.11.2012	2400	$3.1 \cdot 10^3$
Goat cheese	29.01.2013	500	$2.6 \cdot 10^3$
Whey cheese	04.02.2013	250	$1.7 \cdot 10^5$
Poultry	07.02.2013	1100	$6.6 \cdot 10^3$

Genetic characterization of all 32 *S. aureus* isolates by *Sma*I-PFGE provided a fingerprint pattern consisting on 13–17 DNA fragments of 20–670 kbp, approximately. Twelve genotypes were observed resulting in a Simpson's Index of Diversity of 0.909 (CI 95% 0.854–0.963), but no relationship among the pulsotype and the sample type or the date of confiscation was observed.

Note that in some cases isolates obtained from the same sample showed different pulsotypes, though most of them were closely related (Figure 10). Five isolates, including the MRSA isolate, were not typeable by *Sma*I PFGE suggesting that they belonged to ST 398, since it has been previously demonstrated that this lineage shows an unusual resistance to digestion by *Sma*I (Chung *et al.*, 2000). Indeed, further characterization of MRSA isolate confirmed that it belonged to ST398, harbored SCC*mec* type V and tested negative for the presence of the PVL genes.



PFGE - SmaI

Code	Sample	Sample type	Date of confiscation
E19	22	Milk	6-11-2012
E20	17	Unfermented goat cheese	14-09.2012
E4	17	Unfermented goat cheese	14-09.2012
E9	17	Unfermented goat cheese	14-09.2012
E12	17	Unfermented goat cheese	14-09.2012
E30	17	Unfermented goat cheese	14-09.2012
E1	72	Whey cheese	04-02-2013
E15	17	Unfermented goat cheese	14-09.2012
E18	11	Artificial black caviar	14-09.2012
E8	23	Poultry	6-11-2012
E24	23	Poultry	6-11-2012
E23	13	Milk	14-09.2012
E25	13	Milk	14-09.2012
1NC	72	Whey cheese	04-02-2013
E11	63	Goat cheese	29-01-2013
E14	25	Non fermented unsalted sheep cheese	6-11-2012
E13	25	Non fermented unsalted sheep cheese	6-11-2012
29NC	25	Non fermented unsalted sheep cheese	6-11-2012
E29	25	Non fermented unsalted sheep cheese	6-11-2012
E17	63	Goat cheese	29-01-2013
26NC	72	Whey cheese	04-02-2013
E26	72	Whey cheese	04-02-2013
E2	18	Smoked salmon	14-09.2012
E6	16	Sheep cheese salted in brine	14-09.2012
E10	12	Artificial black caviar	14-09.2012
E21	15	Fresh cow cheese	14-09.2012
E16	15	Fresh cow cheese	14-09.2012

Figure 10. Genetic relationships among 27 *S. aureus* isolates based upon comparison of PFGE profiles obtained with the restriction enzyme *Sma*I. Isolates were observed among a total of 200 food samples confiscated in a black market in Romania, from July 2012 to March 2013. The dendrogram was produced by using a Dice similarity coefficient matrix with unweighted pair group method with arithmetic mean (UPGMA). The scale bar indicates similarity values

Spa typing of 16 *S. aureus* strains resulted in the following profiles presented in decreasing order according to the isolation frequency in this study: t449, t304, t1606, t524, t011, t91, t3625, and t803 (Table 11). Of these, t449, t304, and t524 were most often isolated from cow, sheep, and goat-milk cheeses contaminated with 10^3–10^5 CFU/g, indicating a contamination at herd level or unhygienic conditions during food processing and handling.

A strong indication of improper food handling at the market could be linked to the coincided isolation of *S. aureus* t449 at the same date of sampling from red caviar and different kinds of cheeses. The same observation could be made for *S. aureus* t1606 isolated from fish samples on the same day. *S. aureus* t011 and t3625, both related to the livestock-associated CC398, were isolated from pork lard and poultry meat. Another very frequently isolated *spa* type, t011, is often found to be methicillin resistant (www.spaserver.ridom.de). *S. aureus* t011, t304, t524, and t091 are all strongly related to human

colonization and infections. These data indicate the risk of selling food without hygiene precautions and unknown pathogen status and handling of unpackaged foodstuffs on an open market.

Table 11. *Spa typing of 16 Staphylococcus aureus isolated from ready-to-eat food illegally sold in a Romanian market*

Strain	Isolation code	*spa* type	Repeat succession	Source	Frequency [a]	Association
E10	14/09/2012	t449	26-23-13-23-31-05-05-17-25-17-25-16-28	Artificial red caviar	0.03%	MSSA/MRSA (colonization)
E16	14/09/2012			Fresh cow cheese		
E6	14/09/2012			Sheep cheese salted in brine		
E2	14/09/2012			Smoked salmon		
E4	14/09/2012	t304 (ST6, ST8)	11-10-21-17-34-24-34-22-25	Unfermented goat cheese	0.33%	MSSA/MRSA (colonization, infection)
E19	06/11/2012			Raw milk		
E1	04/02/2013			Whey cheese		
E3	06/11/2012	t1606	08-16-34-34-24-25	Fish canned in oil with herbs	0.01%	MRSA (colonization)
E7	06/11/2012			Smoked fish		
E13	06/11/2012	t524	04-17	Non-fermented unsalted sheep cheese	0.03%	MRSA (infection)
E11	29/01/2013			Goat cheese		
E22	06/11/2012	t011	08-16-02-25-34-24-25	Pork lard	3.28%	MRSA (colonization, infection), CC398
E18	14/09/2012	t091 (ST7)	07-23-21-17-34-12-23-02-12-23	Artificial black caviar	0.90%	MSSA/MRSA (colonization, infection)
E5	07/02/2013	t3625	08-16-34-25	Poultry	0.01%	MSSA, CC398
E8	06/11/2012	t803 (ST15)	07-23-02-12-23	Poultry	0.06%	MSSA/MRSA (colonization)
E23	14/09/2012	unknown	08-21-17-36-34-34-34-33-34	Raw milk	-	-

Note: [a] This information is based on the Ridom Spa Database (www.spaserver.ridom.de)

Further, antibiotic susceptibility testing revealed five resistance profiles (Table 12). Overall, 19 strains (59.4%) were fully susceptible to all antibiotics tested. However, the MRSA isolate was not only resistant to all β-lactams but also to ciprofloxacin, tetracycline and cefazolin. Among the methicillin-sensitive *S. aureus* (MSSA), 9 strains (28.1%) were resistant to penicillin, 3 strains (9.7%) to tetracycline and 1 strain (3.2%) to ciprofloxacin (Table 12).

Table 12. *Resistance profiles of 32 Staphylococcus aureus isolates recovered in food samples sold at a black market in the southeast border of Romania, 2012-2013*

Resistance profile	Antimicrobial agent [a]	Isolates	%
RP0	None	19	59.4
RP1	PEN	9	28.1
RP2	TET	2	6.3
RP3	TET, CIP	1	3.1
RP4	All β-lactams, TET, (CIP)	1[b]	3.1

Note: PEN, penicillin; TET, tetracycline; CIP, ciprofloxacin;
[a] parentheses indicate intermediate resistance;
[b] methicillin-resistant *S. aureus* isolate.

We found a relative low percentage of foods contaminated with *S. aureus* (8%), and only one isolate was MRSA (0.5%); ST398-MRSA-V. However, this isolate was multidrug resistant not only to β-lactams but also to other three antibiotics widely used in chemotherapy (Table 12). The MRSA recovered from food are not necessarily related to that present in the animal of origin, and two types of genetic backgrounds can be found in foods: community-associated MRSA (CA-MRSA) present in food due to a human source of contamination by inappropriate handling, or LA-MRSA *via* contamination of carcasses during slaughtering of MRSA-positive animals. Interestingly, while most European studies have reported the presence of LA-MRSA clone ST398 in food of various animal origins (de Boer *et al*., 2009; Lozano *et al*., 2009) as in the case of our study, it seems that the presence of this clone in the USA and Canada is still scarce, and successful CA-MRSA clones are frequently reported instead (Bhargava *et al*., 2011). The results of a recent study monitoring the presence of MRSA in illegally imported food confiscated to passengers of non-EU flights in a Spanish Airport, which also represents a neglected route of transmission of MRSA to EU, corroborated that scenario; the MRSA obtained were from the American continent (Bolivia) and belonged to two successful clones of CA-MRSA (ST8 and ST1649) (Rodríguez-Lázaro *et al*., 2015). In both cases, it seems clear that food can play a role in the dissemination of successful CA- or LA-MRSA into general population. Indeed, foodborne outbreaks of MRSA infection have been reported (Kluytmans *et al*., 1995; Jones *et al*., 2002), and the role of food in the prevalence of MRSA has been recently demonstrated (Ogata *et al*., 2012). In this sense, there is a growing general consensus that the transmission route from environment to hospital involves not only humans and environmental bacteria, but also animals and food products (González-Zorn and Escudero, 2012; Spanu *et al*., 2012).

In conclusion, this study investigated for the first time the pathogens' presence in food legally brought by Moldavian citizens into the European Union as personal goods, but illegally sold in Romania, and revealed that contamination occurs at levels like those usually reported by (EFSA, 2013) for foods produced and sold under official control. Moreover, the results obtained in our study confirm the potential role of food in the dissemination of successful MRSA lineages and define illegally introduced and sold food as a neglected route of MRSA dissemination, which can play a role in the prevalence and evolution of MRSA clones in the community. More than that, some *S. aureus* isolates were harboring one or more than one toxin gene, underlying the need of standardized diagnostic methods to be considered for possible food poisoning episodes. Moreover, food distribution to a certain limited number of consumers can most likely lead to sporadic or family-associated cases of diseases.

CHAPTER 6

Compositional Analysis of Biofilms Formed by *Staphylococcus aureus* Isolated from Food Sources

Results published

Oniciuc, E.A., Cerca, N., Nicolau, A.I. (2016). Compositional analysis of biofilms formed by *Staphylococcus aureus* isolated from food sources. Frontiers in Microbiology, 7:390, doi:10.3389/fmicb.2016.00390. Impact factor: 3.989

Compositional Analysis of Biofilms Formed by *Staphylococcus aureus* Isolated from Food Sources

Few studies have been reported so far regarding the biofilm formation by *S. aureus* isolated from foods (Di Ciccio *et al.*, 2015) and the impact of the environmental factors encountered in food processing plants on the adherence and biofilm formation (Vázquez-Sánchez *et al.*, 2013; Santos *et al.*, 2014). In food industry it is important to know the conditions under which *S. aureus* is able to survive, adhere to surfaces and form biofilms (Futagawa-Saito *et al.*, 2006), leading to contamination of food products. In planktonic form, *S. aureus* does not appear resistant to disinfectants, compared to other bacteria, but it may be among the most resistant ones when is attached to a surface (Fratamico *et al.*, 2009).

S. aureus can produce a multilayered biofilm embedded within a glycocalix with heterogeneous protein expression throughout, forming at least two types of biofilms: *ica*-dependent, mediated by polysaccharide intercellular adhesin (PIA)/poly-*N*-acetyl-1,6-β-glucosamine (PNAG), and *ica*-independent, mediated by proteins (Beloin and Ghigo, 2005). Biofilm-associated protein (Bap), which shows global organizational similarities to surface proteins of Gram-negative (*Pseudomonas aeruginosa* and *Salmonella enterica* serovar *Typhi*) and Gram-positive (*Enteroccocus faecalis*) bacteria (Cucarella *et al.*, 2001), was the first protein that has been found to be involved in biofilm formation by staphylococcal strains isolated from mammary glands in ruminants suffering from mastitis (Speziale *et al.*, 2014). Meanwhile, Foulston *et al.* (2014) discovered that the extracellular matrix of clinical *S. aureus* biofilms comprises cytoplasmic proteins that associate with the cell surface in response to decreasing pH. Regarding the capacity to form biofilms, Bridier *et al.* (2010) demonstrated that *S. aureus* strains from different sources (five clinical, two originating from water, two unknown, and one milk isolate from ewes with mastitis) produce biofilms with high bio volumes and high substratum coverage.

Having in view the significant damages caused by biofilms in food industry in general, more studies should be conducted to elucidate formation of such biofilms and to develop countermeasures for their removal from food contact surfaces (Marques *et al.*, 2007). This study was carried out to evaluate the ability of *S. aureus* strains isolated from food products to form biofilms on hydrophobic surfaces at 37°C, followed by biofilm matrix characterization. The composition of the biofilms formed by *S. aureus* strains on polystyrene surfaces was first inferred using enzymatic and chemical treatments and later confirmed by confocal laser scanning microscopy (CLSM).

Bacterial strains

Sixteen *S. aureus* strains isolated from food products of animal origin (8 from dairy products, 5 from fish and fish products and 3 from meat and meat products) (Oniciuc *et al.*, 2015) were tested to show their ability to form biofilms. Prior to inoculation, all strains were transferred from the stock cultures (preserved in 25% glycerol at −80°C) to Baird Parker (BP) (Biolife Italiana srl., Milano, Italy) and incubated aerobically at 37°C for 24 h. For biofilm assays we used overnight precultures in Tryptic Soy Broth (TSB) (Liofilchem srl., Roseto degli Abruzzi, Italy) incubated aerobically at 37°C, with shaking.

Media screening and biofilm formation overtime

Media screening consisting in TSB with/ without addition of 0.4% glucose (TSBG) or 4% NaCl (TSBN) (Liofilchem srl.) for supporting 24 h biofilm formation was performed *(See Appendix 12)*. Glucose (B. Braun Melsungen AG, Melsungen, Germany) sterilized by filtration (0.22 μm) was added after autoclaving. Prolonged incubation time (48, 72 h) was also performed (Peeters *et al.*, 2008). Biofilms were grown in 96-well plates tissue cultured (Orange Scientific, Braine-l'Alleud, Belgium) with a total volume of 200 μL of TSB, TSBG and TSBN per well and a starting inoculum approximately equal to 10^6 CFU/mL. Only broth media were introduced in the assay as negative controls, and *S. aureus* ATCC 25923® as positive control (clinical isolate). The plates were incubated aerobically at 37°C, on an orbital shaker (ES-20/60 Environmental Shaker BIOSAN) set at 120 rpm.

Biofilm quantification was performed according to the procedure developed by Stepanović *et al.* (2000), by using 1% crystal violet (CV) (Merck KGaA, Darmstadt, Germany). Biofilm formation in the microplates was measured in an ELISA reader set at 570 nm, and values were expressed in optical density (OD) values.

Matrix characterization

Biofilm detachment assays were carried out as described by Kogan *et al.* (2006) and Fredheim *et al.* (2009) with slight modifications, for six strains capable to form strong biofilms with an OD> 4 × OD_{NC}. Biofilms were washed twice with 200 μL of 0.9% NaCl and then treated for 2 h at 37°C without shaking, with 200 μL of 40 mM of sodium periodate ($NaIO_4$), or 200 μL proteinase K (0.1 mg/mL in 20 mM Tris-HCl:1 mM $CaCl_2$). Control wells were filled with 0.9% NaCl. After treatment, the biofilms were washed once with 200 μL of 0.9% NaCl, and then resuspended into 200 μL of 0.9% NaCl and dislodged by scraping followed by sonication using a cycle of 5 s and an amplitude of 22%. Biomass quantification was performed by measuring the OD at 600 nm of each sonicated cell suspension. Measuring the OD of sonicated cell suspensions was preferred for this assay as we observed that $NaIO_4$ used to assess polysaccharides reacts unspecific with CV therefore yielding false positive results.

Biofilm composition by CLSM

The composition of 48 h biofilms was observed by CLSM, exposed to three types of dyes: (i) SYTO dye that stains nucleic acids; (ii) FilmTracer SYPRO Ruby Biofilm Matrix stain (Invitrogen, Paisley, UK), which labels most classes of proteins (Berggren *et al.*, 2000); (iii) wheat germ agglutinin (WGA) conjugated with Oregon Green (Invitrogen), which stains *N*-acetyl-D-glucosamine residues (Wright, 1984). The fluorescence of dyes was detected using the following combination of laser excitation and emission band-pass wavelengths: 476 nm/500–520 nm for SYTO, 405 nm/655–755 nm for SYPRO and 459 nm/505–540 for WGA.

After each staining step, the biofilms were gently rinsed with sterile water. The biofilm images were acquired in an Olympus™ FluoView FV1000 confocal laser microscope and biofilms were observed using 40x water-immersion objective. The images were analyzed sequentially using two virtual channels. Three stacks of

horizontal images (640 × 640 pixels) were acquired for each biofilm at different areas in the well. Two surfaces of two independent replicates were observed in each CLSM experiment.

Results and discussions

Glucose and NaCl have been previously shown to induce biofilm formation in clinical strains of *S. aureus* (Fratamico *et al.*, 2009). Measuring the effect of 0.4% glucose and 4% NaCl on biofilm formation enabled us to determine the conditions necessary for *S. aureus* strains isolated from food to form biofilms. For most strains, there was not a significant difference within the media used showing a small degree of variability regarding the amount of biomass produced, but overall, six strains (E2, E6, E8, E10, E16, E23; 37.5%) with OD > 0.4 were distinguished for higher biofilm formation with TSBG (Figure 11, left graphic). As the determination of the total biomass over a specific period of time is a common practice for the characterization of biofilms and *S. aureus* biofilms are growing slowly, prolonged incubation times were used in our experiment too. Not surprisingly, quantification of biofilm proved a progressive accumulation of biomass during the analyzed time course (Figure 11, right graphic). Based on these findings we further characterized *S. aureus* biofilms after 48 h of incubation.

In order to reveal the molecules behind biofilm accumulation, the biofilm chemical compositions were assessed by measuring the ability of $NaIO_4$ or proteinase K to disperse *S. aureus* biofilms.

Although both ATCC and food isolates have PNAG and proteins in the matrix, proteins prevail on PNAG, thus having a relevant role in maintaining biofilm structure. In this sense, biomass formed by *S. aureus* strains isolated from foods was reduced by 60–70% when anti-protein agents were used, while a reduction of 20–49% was obtained in the presence of the anti-polysaccharide agent (Table 13). Proteinase K treatment enhanced dispersion of Bap-positive *S. aureus* biofilms as demonstrated by Shukla and Rao (2013). The disruption effects observed on 48 h biofilms were similar for all isolates originating from food sources.

***Table 13**. Biomass reduction of S. aureus biofilms when using metaperiodate or proteinase K*

***S. aureus* strains**	**Biomass reduction, %**	
	with $NaIO_4$	with proteinase K
E2	23 ± 10.34	71 ± 4.1
E6	34 ± 2.74	71 ± 0.74
E8	46 ± 11.07	69 ± 0.63
E10	20 ± 6.51	66 ± 3.5
E16	25 ± 0.71	64 ± 1.75
E23	49 ± 3.71	67 ± 6.05
ATCC 25923	28 ± 5.25	9 ± 1.9

***Note:** Preformed biofilms were treated with $NaIO_4$ or proteinase K for 2 h at 37°C. Control wells were filled with 0.9% NaCl. Average results ± SD of eight wells for each strain are shown. The experiments were performed in triplicate. Values of negative controls have been subtracted from the shown values.*

Differences were observed in the biofilm disruption pattern when comparing results obtained for biofilms formed by *S. aureus* isolated from food sources with those developed by the clinical isolate *S. aureus* ATCC® 25923, presenting a high density of cell clusters embedded in polysaccharides. At present, there are no references to composition of biofilms formed by *S. aureus* isolated from food sources. Literature mentions

only biofilms produced by strains of *Staphylococcus* spp. Isolated from a poultry processing plant, which have been described by (Ferreira *et al.*, 2014), as containing a significant amount of exopolysaccharides (EPS).

CLSM in conjugation with three different fluorescent dyes was used to differentiate bacterial cells from PNAG and proteins within the biofilm matrix (Figures 12-13). Qualitative approach was preferred as biofilms obtained were heterogeneous and more than three sections per each biofilm were needed for a meaningful quantification. Biofilm matrices of E8 and E10 formed by *S. aureus* strains isolated from food are represented in Figure 12 in comparison with those formed by the reference strain. These experiments confirmed that proteins are of prime importance for the structure of biofilms formed by *S. aureus* strains isolated from food sources as revealed by the quantitative approach from biofilm disruption assays.

Conclusions and perspectives

Phenotypic production of EPS by *S. aureus* strains used in the present study suggests that staphylococcal biofilm development may have occurred *via* an *ica*-independent pathway. Clearly, in our population of bacteria, PIA independent biofilm formation was more prevalent. Nevertheless, to determine if this characteristic is in fact a key difference between foodborne *S. aureus* and clinical isolates or food processing environment isolates, future research is needed to include a broader range of foodborne isolates. Presence of biofilm forming strains of *S. aureus* in food and food processing environments is equally important as for the medical sector. Besides causing serious engineering problems as described by Garrett *et al.* (2008), biofilms are involved in cross contamination events. The proteic extracellular matrix developed by *S. aureus* isolates of food origin can behave in a similar way that the one developed by clinical isolates of *S. aureus* allowing enhanced flexibility and adaptability for this bacterium in forming biofilms and supporting the formation of mixed species biofilms either with spoilage or pathogenic bacteria as demonstrated by (Foulston *et al.*, 2014). Composition of biofilms must be known to provide a basis for the development of better strategies for cleaning surfaces and cross contamination avoidance.

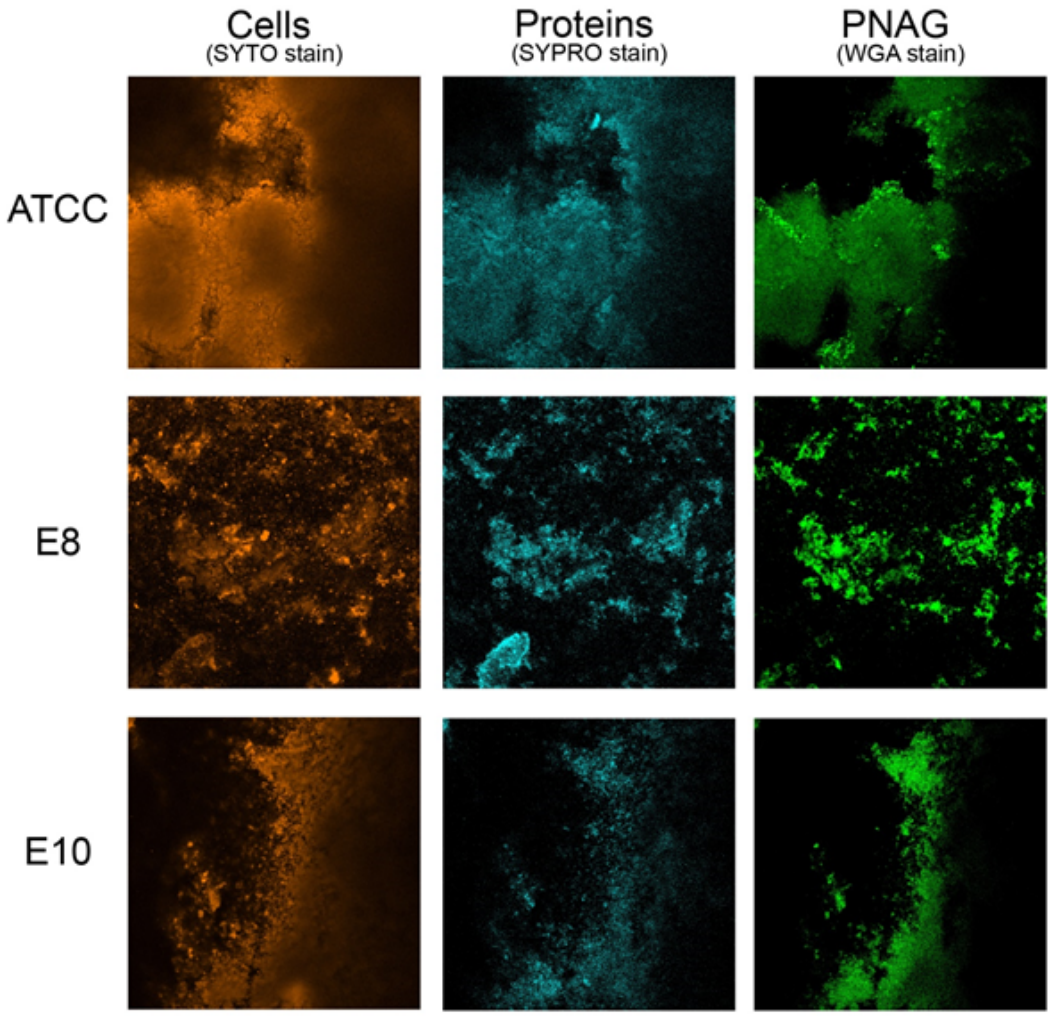


Figure 12. Biofilm matrix structure obtained from confocal microscopy observations of *S. aureus* ATCC® 25923, E8 isolated from poultry, and E10 isolated from artificial red caviar. One z-stack is represented for each biofilm

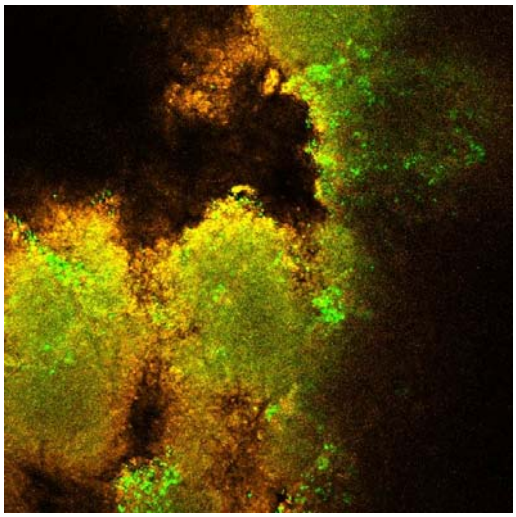


Figure 13. Biofilm structure obtained from confocal microscopy observation of *S. aureus* ATCC® 25923, exposed to three types of dyes: SYTO dye for cells, (WGA)-TRITC conjugate for polysaccharides visualization, and SYPRO Ruby for extracellular proteins. One z-stack is represented

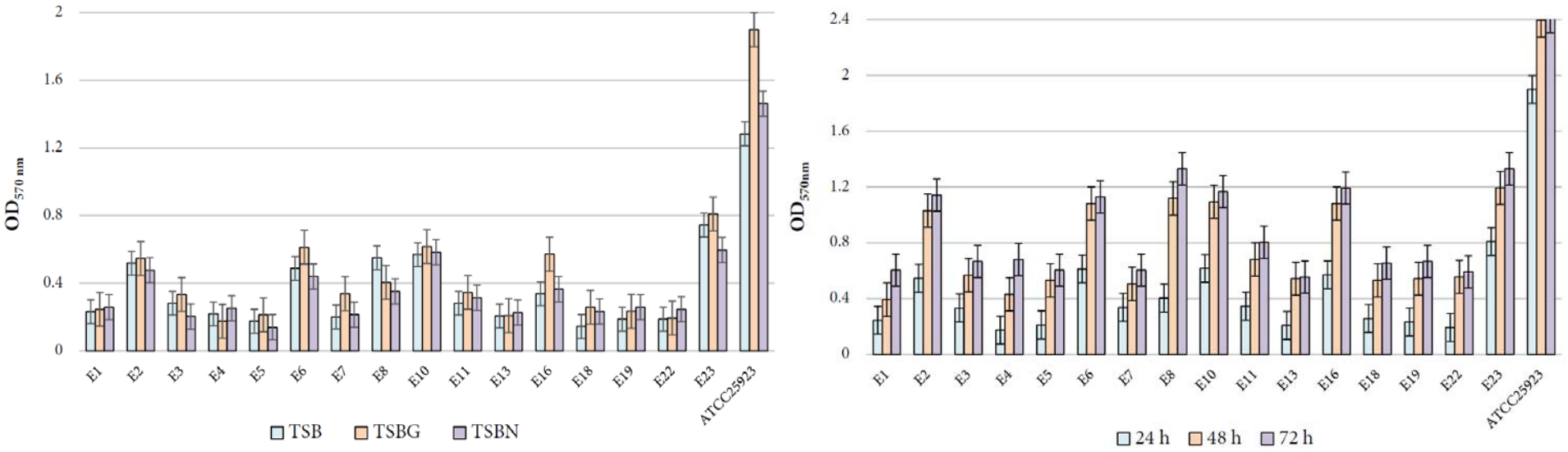


Figure 11. *Staphylococcus aureus* biofilm development. Biomass accumulation when using 0.4% glucose and 4% NaCl to the standard TSB (left). Biofilm formation overtime using TSBG (right). Bars represent the means of the OD value ± standard deviation (SD) evaluated in three independent measures obtained upon different treatments tested, as indicated. Values of negative controls have been subtracted from the shown values

CHAPTER 7

Tracking MRSA in food entering to the European Union *via* cross border traffic and international flights

Results accepted for publishing

Rodríguez-Lázaro*, D., **Oniciuc*, E.A.**, González García, P., Gallego, D., Fernández-Natal, I. Dominguez-Gil, M., Eiros-Bouza, J.S., Wagner, M., Nicolau, A.I., Hernández, M. (*manuscript submitted*). Methicillin-resistant *Staphylococcus aureus* crosses EU borders *via* uncontrolled imported food. Frontiers in Microbiology. Impact factor: 4.165

**those authors contributed equally to the current work*

Tracking MRSA in food entering to the European Union *via* cross border traffic and international flights

Staphylococcus aureus, a well-known opportunistic foodborne pathogen, is involved in numerous outbreaks worldwide, both nosocomial and community associated infections or common foodborne cases (Paterson *et al.*, 2014). The widespread use of antibiotics, and particularly their incorrect or overuse, has facilitated the emergence of new pathogens, including those resistant to antibiotics, such as MRSA. In addition to its use for human therapy, antibiotics are also used in veterinary medicine or animal feeding, contributing to the vast appearance of antibiotic resistant strains (Valsangiacomo *et al.*, 2000; Grema *et al.*, 2015). After the first nosocomial episode in 1960, MRSA became an emergent pathogen (Bonten and Weinstein, 2016), affecting not only patients in hospitals but people in community settings such as nursing homes, nurseries, *etc.* (Lo *et al.*, 2007; Murphy *et al.*, 2012; Blumental *et al.*, 2013). More recently, MRSA was found in livestocks, being clearly linked to a jump from humans to the animal host (Voss *et al.*, 2005; Price *et al.*, 2012). However, the prevalence of LA-MRSA is increasing in farm animals as it could represent important ecological niches, affecting the associated foodstuff and stimulating the evolution of this lineage (Yan *et al.*, 2014; Verhegghe *et al.*, 2016), and producing human outbreaks probably as a consequence of that increasing prevalence (Grøntvedt *et al.*, 2016).

Besides LA-MRSA, other lineages can occur in food intended for human consumption. Food of animal origin could gain a relevant role in the prevalence of CA-MRSA (Ogata *et al.*, 2012; Rodríguez-Lázaro *et al.*, 2015) or HA-MRSA (Pu *et al.*, 2009; Weese *et al.*, 2010). Recently, we have demonstrated the presence of CA-MRSA in processed foods confiscated from passengers from international flights originated mostly from Central and South America (Rodríguez-Lázaro *et al.*, 2015). This is extremely relevant from a food safety perspective as these lineages may occur because of the incorrect food handling, and not being associated to a zoonotic transmission.

The presence of staphylococcal enterotoxins (SEs, *se* genes) have been reported in numerous studies causing staphylococcal food poisoning, toxic shock and other allergic and autoimmune reactions (Gonano *et al.*, 2009). Staphylococcal enterotoxins are divided into five "classical types" (SEA, SEB, SEC, SED, and SEE) (Carfora *et al.*, 2015; Hennekinne *et al.*, 2012). However, little is known on the prevalence of MRSA in foods (homemade or processed) associated to international trade combined with their ability to produce enterotoxins, and the role that confiscated food transported in luggage of passengers flying from different parts of the globe could play. Due to the MRSA complex epidemiology, we have conducted a study to evaluate if the illegal entrance of foods to Europe through international airports or open markets close to EU borders can constitute a neglected pathway of transmission of enterotoxigenic antibiotic-resistant strains, particularly MRSA. Information gathered from this study will reveal lineages involved in MRSA contaminated food correlated with their enterotoxigenicity, which represents a major concern for human health.

Food sample collection

A total of 868 animal derived foods collected from August 2012 to July 2015 were tested for the presence of *S. aureus* and MRSA: foods confiscated from luggage of passengers on flights from non-EU countries by the Border Authorities at the International Bilbao Airport (Spain) (263 food products) and Vienna International Airport (Austria) (595 food products), and foods collected from EU border traffic between Republic of Moldova and Romania (Giurgiulești- Galați) (10 food products). Food samples included 408 (47%) meat samples of diverse animal origin (including antelope, beef, chicken, duck, guinea pig, pork, rodents and turkey), 447 (51.5%) milk and dairy products and 7 eggs (0.8%), and fish and fish products (6, 0.7%).

Geographical origin of the food samples collected at airports was wide: Albania, Argentina, Armenia, Azerbaijan, Republic of Serbia, Bolivia, Bosnia and Herzegovina, Brazil, China, Columbia, Côte d'Ivoire, Cuba, Ecuador, Egypt, Ethiopia, Former Republic of Macedonia, Honduras, India, Iran, Israel, Jordan, Kazakhstan, North Korea, Republic of Kosovo, Latvia, Mexico, Nicaragua, Moldova, Mongolia, Montenegro, Russia, Niger, Panama, Paraguay, Peru, Philippines, Qatar, Dominican Republic, Romania, South Africa, South Korea, Thailand, Tunisia, Turkey, Ukraine, United Arab Emirates, and Vietnam.

Detection and isolation of *S. aureus*

The detection of *S. aureus* was performed following ISO 6888-2 (ISO, 1999). Real-time PCR further confirmed *S. aureus* isolates as previously described (Trnčíková *et al*., 2008). Positive colonies with correct morphology in BP agar plates were taken for further typing tests (MRSA biotype, antibiotic resistance, and genetic characterization).

Screening for the presence of MRSA

The presence of *mec*A and *mec*C in *S. aureus* isolates was tested by multiplex PCR as previously described (Stegger *et al*., 2012).

Antibiotic susceptibility testing

Susceptibility to antimicrobials was performed by the microdilution method following the recommendations and MIC breakpoints of the European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines 2015 (www.eucast.org). Susceptibility to twenty antimicrobial agents was tested: penicillin (PEN), oxacillin (OXA), amoxicillin/clavulanate (AMC), daptomycin (DAP), erythromycin (ERY), clindamycin (CLI), teicoplanin (TEC), vancomycin (VAN), ciprofloxacin (CIP), levofloxacin (LVX), amikacin (AMK), gentamicin (GEN), tobramycin (TOB), mupirocin (MUP), rifampin (RIF), tetracycline (TET), fusidic acid (FUS), fosfomycin (FOF), linezolid (LZD) and trimethoprim sulfamethoxazole (SXT).

Characterization of the genetic background

Pulsed Field Gel Electrophoresis (PFGE) (McDougal *et al*., 2003), Multi Locus Sequence Typing (MLST) (Enright *et al*., 2000) and typing and subtyping of the SCC*mec* element (Kondo *et al*., 2007; Milheiriço *et al*., 2007) of all MRSA isolates were performed. PFGE patterns were analyzed with Bionumerics software v6.6 (Applied-Maths NV, Sint-Martens-Latern, Belgium), and dendrograms were constructed using the Dice similarity coefficient and the unweighted pair group mathematical average (UPGMA) clustering algorithm with 1% in the tolerance and optimization values. The allelic profiles obtained by MLST were assigned by comparing the results obtained with data available *(See Appendix 13)* in the *S. aureus* MLST database hosted at saureus.mlst.net. Information of MRSA strains was submitted to that database.

To define the relationships among MRSA strains at the micro evolutionary level, an allelic profile-based comparison applying a minimum spanning tree (MST) was performed applying the Bionumerics software v6.6. A distinct sequence type (ST) number was attributed to each distinct combination of alleles at the seven genes (Ragon *et al*., 2008).

Detection of Panton-Valentine leukocidin virulence factors

The presence of the PVL genes (*luk*S-PV & *luk*F-PV) was tested by conventional PCR in all MRSA strains as described by (Lina *et al*., 1999). Reference strain ATCC 49775 was used as positive control.

Enterotoxin profiling

MRSA isolates were tested by a multiplex PCR targeting *sea*, *seb*, *sec*, *sed*, *see*, *seg*, *seh*, *sei*, *sej* genes as previously described by Gonano *et al.* (2009).

Results

Tracking MRSA in food samples confiscated by the border authorities

Microbiological tests revealed that 15.7% of foods were positive for *S. aureus* (Figures 14-15), from which 3% (26/868) were MRSA-positive (49 isolates) harboring the *mec*A gene. The *mec*C homologue has not been identified. However, the mean count for *S. aureus* was established to 2.9 x 10^6 CFU/g, with a minimum value of 1.00 x 10^1 CFU/g in a raw pork meat confiscated by the border control in Bilbao airport from a passenger flying from Moscow, whereas the *S. aureus* maximum value count was 2.45 x 10^8 CFU/g in an unknown cheese type confiscated by authorities in Vienna airport from a passenger flying from Turkey (Figure 16).

All MRSA isolates were represented by 21 milk and dairy products (cow, sheep or goat milk and cheese-either fresh, brined or with spices), and 5 meat and meat products (raw and cooked meat). The MRSA strains recovered from positive *S. aureus* samples confiscated at the International Bilbao Airport originated from flights from Nigeria (1), Egypt (2), Republic of Honduras (1), China (1), Nicaragua (5), Bolivia (4), Ecuador (1), Peru (2), Columbia (1), and Republic of Serbia (1). At the Vienna International Airport, MRSA contaminated food originated from flights from Egypt (3) and Turkey (2). Two food samples were coming from Republic of Moldova and were the object of border traffic to Romania.

Antibiotic profile of the MRSA isolates

Antibiotic susceptibility testing reported 14 resistance profiles (Table 14). From all the MRSA strains studied, 16 of them were multiresistant. Moreover, MRSA isolates were sensitive to all non β-lactam antibiotics tested.

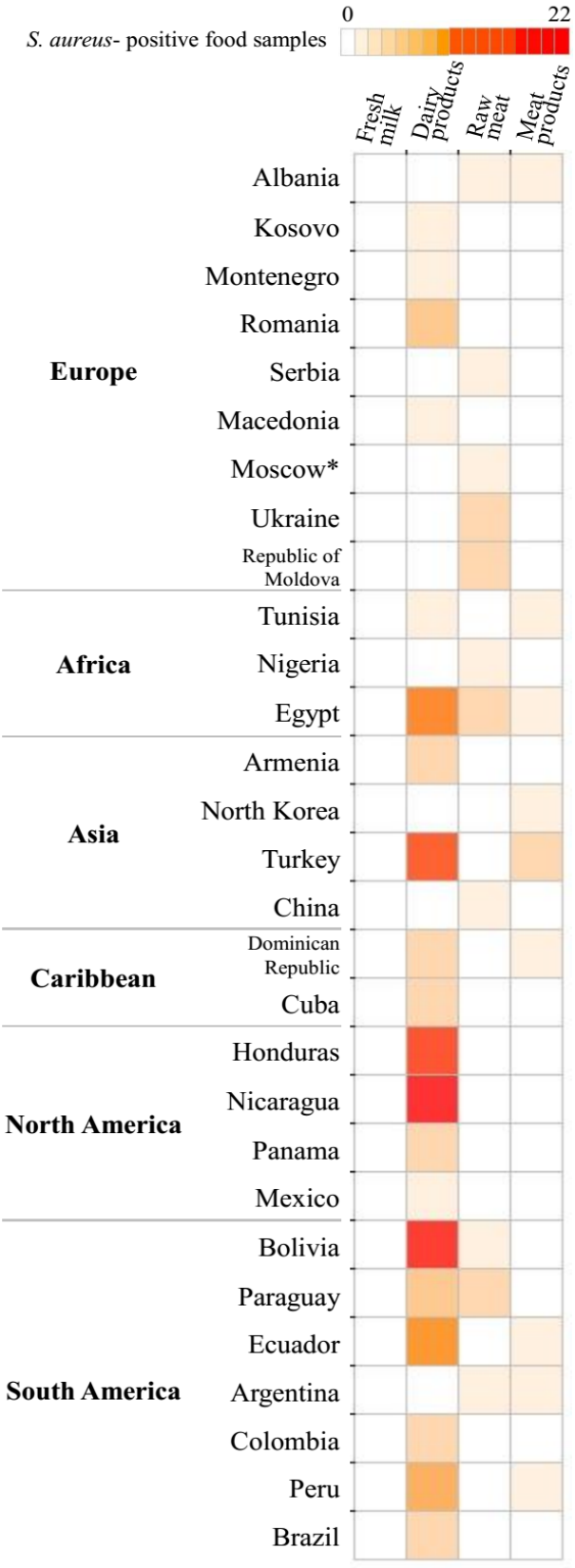


Figure 14. Heat map regarding the origin and distribution of *S. aureus*-positive food samples

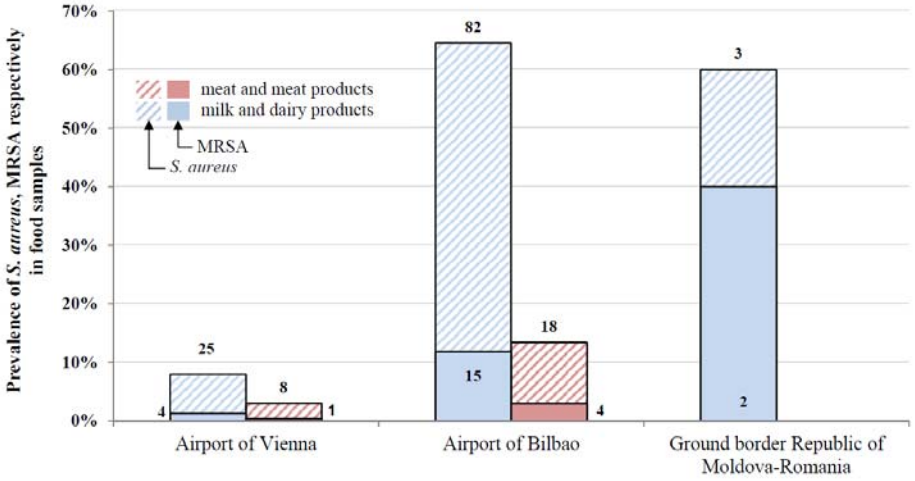


Figure 15. Prevalence (%) of *S. aureus* in food samples analyzed. The solid columns represent the number of MRSA positive samples, while the columns with stripes represent the number of *S. aureus* positive isolates

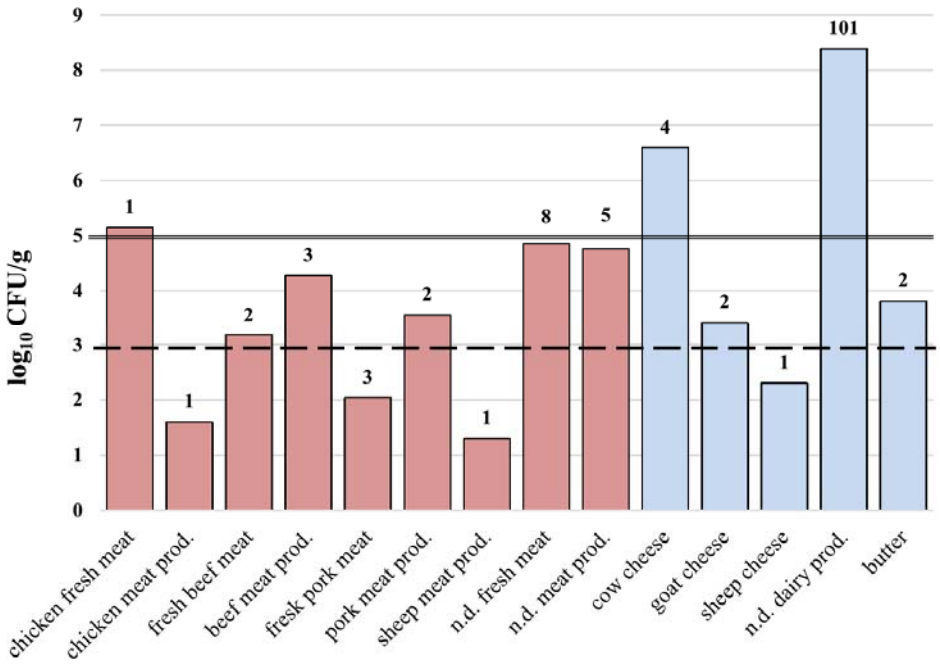


Figure 16. *Staphylococcus aureus* counts (log_{10}CFU/g) per food category, type and origin. The number of food samples analyzed per food type are displayed above each column. Lines passing through the columns (- -) and (−) represent the maximum (M) value in the microbiological criteria for raw milk intended for processing and in powdered milk, and M value for cheeses made from raw milk respectively, according to EC 2073/2005. International Commission on Microbiological Specifications for Foods (ICMSF) recommends 10^3 CFU/g for meat and poultry cooked products as M value (ICMSF, 2011)

Genetic characterization of MRSA isolates

All MRSA isolates harbored *mec*A gene by Multiplex PCR and none isolate harbored the *mec*C homologue. Further characterization of MRSA isolates regarding the SCC*mec* revealed that 37 isolates (75.5%) belong to SCC*mec* type IV, whereas the last 12 isolates (24.5%) belong to SCC*mec* type V. Furthermore, for subtyping the SCC*mec* IV, 48.9% were represented by IVc and IVe, 22.4% to IVa, and 4.1% to IVh.

Interestingly, SCC*mec* typing of three isolates was not possible: the multiplex PCR-2, which types the *mec*A complex class, amplified the 804 bp DNA fragment, consistent with type C, but the multiplex PCR-1 providing the *ccr* gene complex did not amplify. The same situation happened for another isolate whereas the multiplex PCR-1 amplified a 937 bp DNA fragment consistent with *ccr* type 2 (A2B2), the multiplex PCR-2 did not amplify. Moreover, seven isolates were tested positive for *luk*-PVL genes (SCC*mec* IV-subtypes IVc and IVe).

***Table 14**. Antibiotic resistance profiles of MRSA strains from confiscated foods from passengers of non-EU-flights or ground borders, 2012-2015*

Resistance profile	Antibiotics [a]	Number of strains	Percentage (%)
RP0	β-lactams	6	22.2
RP1	PEN, TET, ERY	5	18.5
RP2	PEN, ERY	3	11.1
RP3	PEN, FUS, TET, TOB, GEN	2	7.4
RP4	PEN, TET, TOB	2	7.4
RP5	PEN, TET, SXT	1	3.7
RP6	PEN, TET, FUS	1	3.7
RP7	PEN, FOF	1	3.7
RP8	PEN, LVX	1	3.7
RP9	PEN, LVX, SXT	1	3.7
RP10	PEN, LVX, FOF, RIF	1	3.7
RP11	PEN, TET, ERY, CLI	1	3.7
RP12	PEN, TET, ERY, [OXA][b]	1	3.7
RP13	PEN, TET, CIP, LVX, ERY, CLI	1	3.7

Note: PEN, penicillin; FOF, fosfomycin; TET, tetracycline; SXT, trimethoprim sulfamethoxazole; FUS, fusidic acid; ERY, erythromycin; LVX, levofloxacin; TOB, tobramycin; RIF, rifampin; GEN, gentamicin; CLI, clindamycin; CIP, ciprofloxacin; MUP, mupirocin.
[a]The MIC breaking points used were those indicated in the EUCAST guidelines (2015);
[b] Parentheses indicates susceptibility.

Enterotoxin profiles of MRSA isolates were determined. The majority of isolates were positive for the tested enterotoxin genes A, B, C, D, G, H, I, J. None isolate tested positive for enterotoxin E. Overall, 73% (19 out of 26 MRSA strains) tested positive for one or more *se* genes (Table 15). Four (15.4%) strains harbored only one kind of *se* gene, the remaining 15 (57.6%) of them harbored more than one type of *se* gene. Most of them synthetized *seg/sei* genes accounting 6 strains from the total of *se* positive genes. Interestingly, MRSA isolates tested positive for *luk*-PVL genes were not enterotoxigenic.

***Table 15**. Enterotoxin profiles of MRSA strains*

	Number (%) of MRSA strains	
Type of *se* gene	Milk and dairy products	Meat and meat products
se- negative	5 (19.2)	2 (7.7)
se- positive	16 (61.6)	3 (11.5)
sea	1 (3.85)	-
seg	1 (3.85)	-
seh	2 (7.7)	-
sea/seb	3 (11.5)	1 (3.8)

sea/seh	1 (3.85)	-
seg/sei	4 (15.4)	2 (7.7)
sec/seg/sei	1 (3.85)	-
sed/seg/sej	1 (3.85)	-
sed/seg/sei/sej	2 (7.7)	-

To achieve further insights into the molecular characterization of the MRSA isolates recovered in this study, PFGE patterns and ST types of the selected strains were determined. Genetic characterization of MRSA isolates by *Sma*I-PFGE provided a fingerprint pattern consisting on 13-17 DNA fragments of 20-670 kbp, approximately (Figure 17). Two isolates were not typeable by *Sma*I-PFGE suggesting it might belong to ST398 since this lineage manifests an unusual resistance to digestion by *Sma*I (Chung *et al.*, 2000).

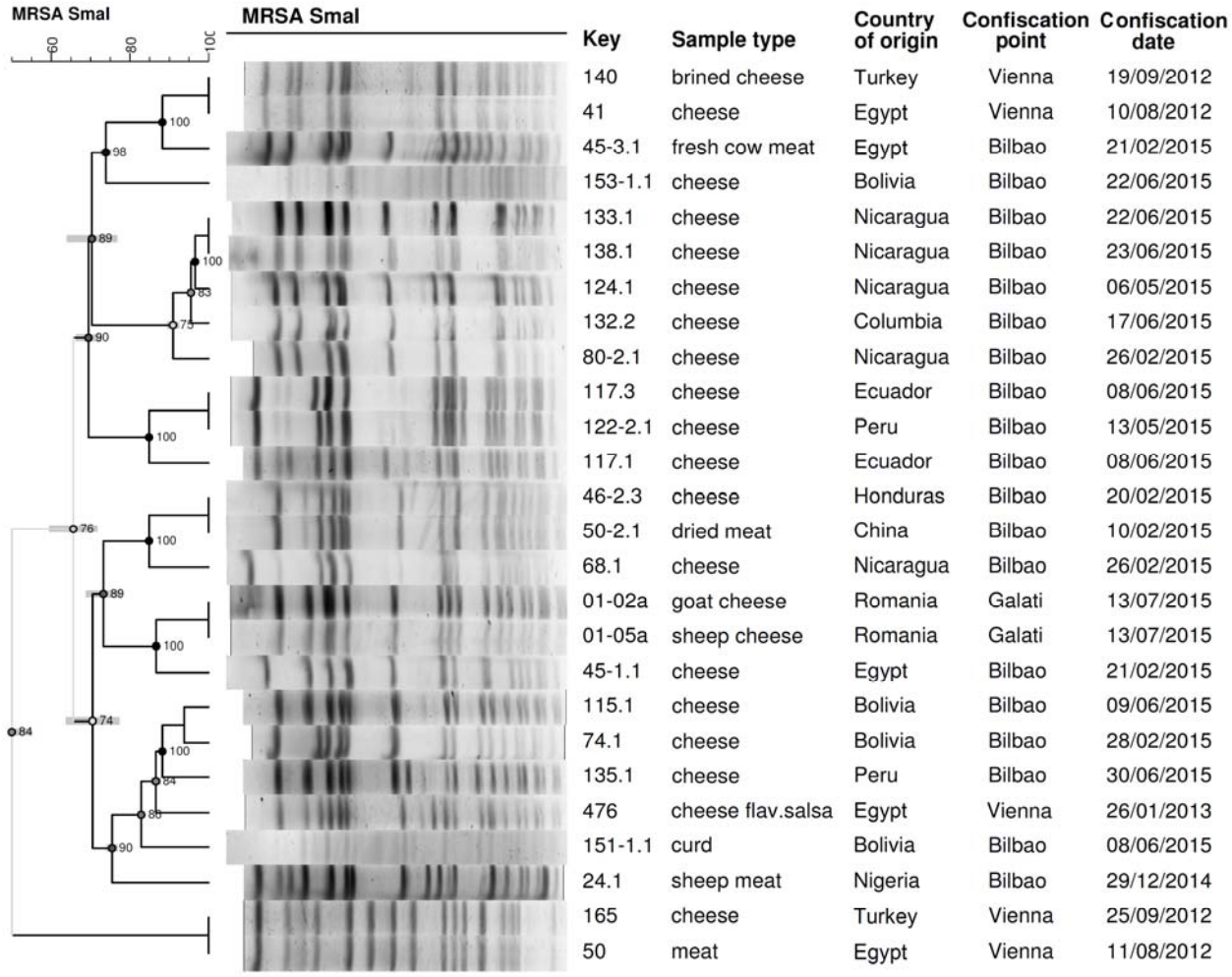


Figure 17. Genetic relationship among 26 methicillin-resistant *Staphylococcus aureus* (MRSA) isolates obtained by comparison of pulsed-field gel electrophoresis profiles, using the restriction enzyme *Sma*I. MRSA isolates were observed among a total of 136 food samples positive to *S. aureus*, confiscated from passengers on flights or ground border close to EU countries, from August 2012 to July 2015. Dendrogram was done by using the Dice similarity coefficient with the unweighted pair group mathematical average (UPGMA) clustering algorithm with 1% in the tolerance and optimization values. The scale indicates similarity values

Further characterization of MRSA strains by MLST revealed nine ST. The most common allelic profile was represented by ST5 (30.8%). These strains were recovered from seven cheese products coming from passengers from Nicaragua, Columbia, Egypt, and Turkey and one fresh beef product from a passenger flying from Egypt. Moreover, all strains showed related genotypes: three strains showed the same fingerprint pattern, harboring SCC*mec* type V, whereas the last five strains were harboring SCC*mec* type IV. Interestingly, all eight food samples were not confiscated on the same date of sampling, neither from the same airport so the fact that a possible cross-contamination occurred during handling the original package is not realistic. Another important fact is the cheese sample recovered from a passenger flying from Turkey, from which has been isolated an oxacillin-susceptible *mec*A-positive *S. aureus* (OS-MRSA) strain. To our knowledge, this is the first presence of OS-MRSA on foods from illegal routes of entrance to Europe.

This fact draws attention on the potential circulation of OS-MRSA in Europe as consequence of illegal entrance of food *via* international flights. All other MRSA strains were overspread among other lineages ST1649 (15.4%) and ST8 (15.4%), the last lineage revealing 3 out of 4 strains harboring PVL genes as well. Furthermore ST1, ST22, ST72 and ST97 displayed in the MST (Figure 18) were prevalent as well in different hard and semi-hard cheese products. A further particular livestock clone ST398 have been found in a fresh meat sample confiscated from a passenger travelling from Republic of Serbia towards Bilbao.

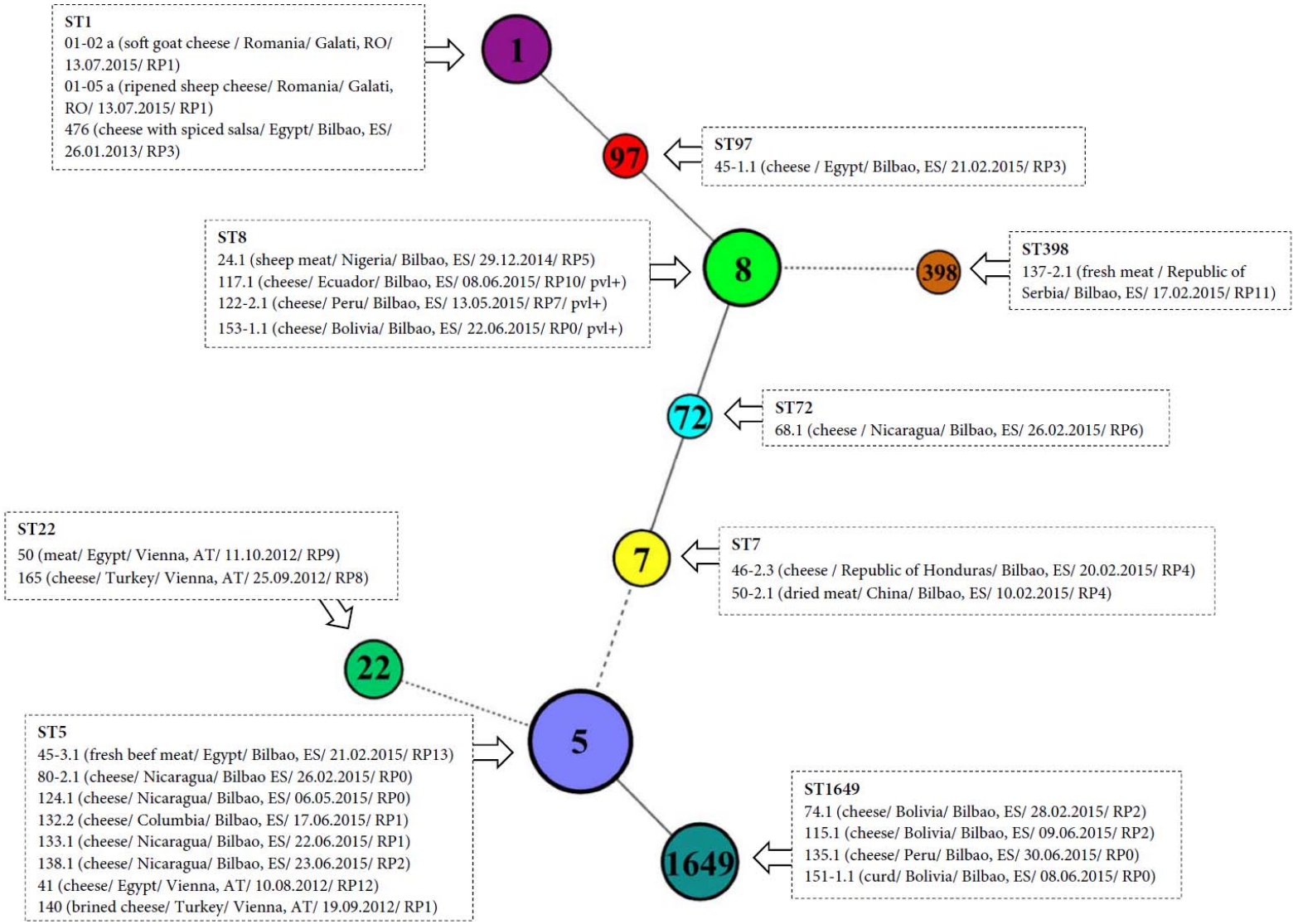


Figure 18. Multi-Locus Sequence Typing of 26 MRSA strains isolated from illegally introduced food into the EU. The STs were clustered according to the seven housekeeping genes using a minimum spanning tree (MST). A different randomized color was attributed to each ST. MRSA strains belonging to the same ST are displayed surrounded by dotted boxes. Information referring to sample type/ country of origin/ confiscation point/ date of confiscation: DD.MM.YYYY/ resistance profile is given in brackets for each MRSA strain, following the strain code. Abbreviations used: AT (Austria); ES (Spain); RO (Romania)

Discussions

This study highlights a major issue due to the MRSA spread *via* illegal entrance of foods to Europe through international flights and open markets close to EU ground borders. Approximately 1/6 of the total sample collection (136 out of 868 food samples confiscated; 15.7%) were confirmed as being positive for the presence of *S. aureus* from which 3% were represented by MRSA-positive strains exhibiting *mec*A resistance mechanism. From all isolates, a high number of them showed to be multiresistant to three or more antimicrobial agents (Table 14). Both facts outline the important role of illegally trespassing animal origin food in passengers' luggage since multiresistant strains could freely be distributed worldwide either by flights, ground borders or other ways of transmission. For example, USA custom officers ask all passengers from outside the USA to fill a form regarding if they have been in direct contact with animals (Category A referring to zoonotic disease transmissible through animal contact) or if they carry any animal food originated product which can potentially harm/ contaminate or animals infected with zoonotic agents (Category B) (Noordhuizen *et al.*, 2013).

Another issue highlighted in our study is the great amount of food samples contaminated with *S. aureus* (Figure 15), in which *S. aureus* exceeded the EU microbiological criteria (Figure 16) (EC 2073/2005) posing a serious concern for public health as elevated amount of it is sufficient to produce preformed enterotoxins in food products. These results are consistent with other studies in which prevalence of *S. aureus* contamination ranked from 13% to 60% (Crago *et al.*, 2012; Rodríguez-Lázaro *et al.*, 2015; Sun *et al.*, 2015; Ciolacu *et al.*, 2016) in processed food products of animal origin. We reported a prevalence of 64.6% in *S. aureus*-positive milk and dairy food samples confiscated in International airport of Bilbao, and the highest value (11.8%) registered for MRSA-positive samples (Figure 16). Interestingly, more than 3.17 $\log_{10}$ over the established limit according to EC 2073/2005 has been found in our study for different types of cheeses such as soft, semi hard, or hard cheeses. The elevated amount of food contaminated with *S. aureus* may be linked with improper storage and poorly hygienic conditions in which bacterial transfer could range from 0.0005 to 100% according to Chen *et al.* (2001) depending on the nature of surfaces and number of people handling the food. As a dynamic process, bidirectional bacterial transfer between clothes and food preparation surface may occur further (Bloomfield *et al.*, 2011). Moreover, 26 strains were MRSA (3%) of which 19 of them were enterotoxigenic. The people's disregard on the risk associated with the illegally animal food origin transportation combined with failing border controls could lead to emergence of foodborne outbreaks (Noordhuizen *et al.*, 2013). Under improper food transportation conditions, heat-stable enterotoxins could emerge leading to appearance of gastroenteritis outbreaks. Many of the MRSA strains yielded one or more *se* genes, this being in accordance with another study published by Carfora *et al.* (2015) in which presence of "classical enterotoxin types" in milk and dairy products is confirmed. Moreover, presence of *S. aureus* in different food origins showed that 19% of them were both enterotoxigenic and oxacillin positives (Pereira *et al.*, 2009). It seems that contaminated milk and dairy products are of prime importance for acquiring enterotoxins but, however, the link between source of food contamination and transfer of antibiotic resistance determinants remains unclear since only several reports describe the presence and possible origin of MRSA in foods (Ortega *et al.*, 2010).

More recently, EU has issued several regulations regarding animals and food products of animal origin imports. However, these regulations often refer to commercial trade and big amounts of food products (EC

275/2007; EC 206/2009), leaving small volumes of food products superficially trespassing during the border control if are intended for personal consumption.

The confiscated foods were coming from passengers with a very diverse geographical origin: South and Central America, Europe, Africa, or Asia (Figure 14), becoming a serious concern for public health due to the appearance of food poisoning episodes since raw food products have been found in passenger's luggage, products which have not suffered a thermal treatment previously conducting to the spread of multidrug resistant and enterotoxigenic strains such as MRSA. On top of that, EC forbids the introduction of personal foodstuff of animal origin such as *e.g.* meat, milk and derived foodstuff, if people are not from EU-countries or Croatia (joined EU on the 1st of July 2013), Faeroe Islands, Greenland or Iceland (EC 206/2009). Road entry station represents another important route for enterotoxigenic MRSA transmission as people can come to a neighbor country and illegally sell foods intended for personal consumption. The prevalence of MRSA in food depends on animal's origin and country of provenience, therefore occurrence of MRSA in associated foodstuff have been already addressed in several reports (Rodríguez-Lázaro *et al.*, 2015; Oniciuc *et al.*, 2015). Moreover, foodborne outbreaks of MRSA infection have already been reported (Jones *et al.*, 2002). This proved that the emergence of this resistant pathogen in associated food products has raised the query about hidden role of food as a pathway for MRSA transmission (Oniciuc *et al.*, 2017).

Remarkably, 26 MRSA-positive food samples have been identified in this study, isolated from foods coming from travelers distributed worldwide. The predominant lineages found in our study are represented by ST5, ST8, ST1649, ST1 and other lineages locally distributed such as ST7, ST22, ST72, ST97 and ST398 (Figure 18). The most widely genetic sequence spread was represented by ST5 (30.8%), considered as a host jump followed by adaption of strain to the new host (Lowder *et al.*, 2009). Despite the fact that ST5 has been predominant found among poultry isolates (Lowder *et al.*, 2009), in our study was mostly implicated in successful isolation from dairy products. Moreover, ST5 lineage has been considered a major component of MRSA and MSSA hospital and community associated worldwide (Miko *et al.*, 2013). Other MRSA strains identified in our study have been associated with ST8-MRSA-IV/V and ST1649-MRSA-IV, which belong to successful clones of CA-MRSA. One case of community-acquired foodborne illness caused by SEC-producing MRSA (Jones *et al.*, 2002) have already occurred in USA, and production of staphylococcal enterotoxin types SEB, SEC, SED, and SEE in two MRSA strains of milk origin from Minnesota farms (Haran *et al.*, 2012).

The presence of PVL genes and different antimicrobial susceptibility patterns linked to ST8-MRSA may cause concern as it is not clear whether human handlers played any role in the preliminary post slaughter process. Surprisingly, although many European studies have reported the presence of ST398 lineage in food with a slightly high prevalence accounting, in our study the isolation of this clone was currently limited only to one dairy product found in a passenger luggage coming from Egypt. Of note is that in The Netherlands this clone emerged rapidly and now accounts for 20% of human MRSA cases and for 42% of newly detected MRSA, indicating that animals are important reservoirs for human MRSA infection (Kadariya *et al.*, 2014). Moreover, outbreaks due to LA-MRSA ST398 have already occurred (Wulf *et al.*, 2008b; Verkade *et al.*, 2012). However, in our study strains harboring the *luk*-PVL genes and the ones associated with LA-MRSA, respectively were not enterotoxigenic. It seems that low levels of ST398 isolates carrying SE have been found (Argudín *et al.*, 2011), despite the fact that this lineage is widely spread in European countries (Oniciuc *et al.*, 2017).

Uncommonly and yet problematic is the successful isolation of an OS-MRSA-positive strain recovered from a cheese product illegally transported by a passenger from Turkey towards Vienna. Such phenotype is considered of prime importance since may misidentify the presence of OS-MRSA (Ariza-Miguel *et al.*, 2015) resulting in the development of highly resistant MRSA under treatment with β-lactam antibiotics.

Besides, this strain could synthetize three types of SE such as D, G, J. It could be considered of major concern as a prove for demonstrating a potential route of illegal entrance of food to Europe.

In conclusion, this study shows presence of enterotoxigenic HA-, CA-, and LA- MRSA identified in food confiscated from passengers from non-EU flights, for which its potentially pathogenic role as a foodborne pathogen should not be neglected. This study stresses the illegally introduced processed food in luggage as an important and alarming pathway of enterotoxigenic MRSA transmission and spread. Efficient control measurements must be taken for avoiding antibiotic resistant strains transmission to humans by the consumption of such foods. In the same time, travelers must understand and learn to accept the prohibition regarding food traffic, consequently the risk of foodborne pathogens spreading. Unfortunately, the increased number of people travelling and the increased global trade will contribute to future outbreaks regardless the measures which are to be taken.

CHAPTER 8

Biofilm Formation by MRSA Isolates Recovered from Passenger's Luggage from Non-EU Flights

Biofilm Formation by MRSA Isolates Recovered from Passenger's Luggage from Non-EU Flights

Methicillin-resistant *Staphylococcus aureus* (MRSA) has begun to emerge ever since antimicrobial therapy was introduced in hospitals, showing the ability to gain resistance to almost all classes of antibiotics (Lowy, 2003). As its epidemiology is so diverse and its ability to acquire novel antibiotic resistance mechanisms is high, MRSA started to appear in communities and more recently in food and associated foodstuff (Rodríguez-Lázaro *et al*., 2015), whereas, from a food perspective, great significance for the public health has been conferred as MRSA presence might be disseminated throughout the food chain (Doulgeraki *et al*., 2016).

Likewise, MRSA strains have shown ability to form biofilms serving as a virulence factor allowing them to adhere to abiotic and biotic surfaces (Sadekuzzaman *et al*., 2015) (*e.g*. indwelling medical devices). Recent studies indicate that MRSA biofilm formation may represent a hidden pathway for food contamination in food processing plants and human handlers, by colonizing equipments and other materials and posing serious safety concern (Rode *et al*., 2007; Gutiérrez *et al*., 2012).

Gaining resistance to antimicrobial agents is similar as adopting survival strategies (Uršič *et al*., 2008) of which MRSA biofilms can benefit of. Gene expression is altered when MRSA adopts the biofilm mode of growth (Archer *et al*., 2011; Foulston *et al*. 2014) as in response to different environmental changes such as temperature, osmolarity, pH, oxygen supply, source of nutrients and other factors that might appear (Costa *et al*., 2013; Srey *et al*., 2013). The metabolic rates may explain the capability of biofilms to act as a diffusion barrier to slow down the infiltration of some antimicrobial agents (Archer *et al*., 2011).

S. aureus can evolve as a multi-layered biofilm embedded within a glycocalyx with heterogenous protein expression throughout (Campoccia *et al*., 2013; Drago *et al*., 2016; Oniciuc *et al*., 2016), arbitrating biofilm formation proteins such as SpA, FnBPs and Bap (Merino *et al*., 2009; Cucarella *et al*., 2001; Houston *et al*., 2011). Singh and coworkers (2010) showed diminished penetration of antibiotics such as oxacillin, cefotaxime or vancomycin against biofilms formed by *S. aureus* and *S. epidermidis*. However, *S. aureus* and *S. epidermidis* biofilms remained unaffected when amikacin and ciprofloxacin were introduced in the assay. Due to this reason, the potential of *S. aureus*, especially MRSA, to cause infections in people with indwelling medical devices is increasingly high and for this reason many studies have been addressed to this topic; yet, the literature in the food industry sector is still scarce and remains an important niche that must be investigated. Biofilm formed by *S. aureus* isolated from foods (Di Ciccio *et al*., 2015) and the impact of the environmental factors encountered in food processing plants on the adherence and biofilm formation (Vázquez-Sánchez *et al*., 2013; Santos *et al*., 2014) have been addressed so far.

From a food safety perspective, different MRSA lineages can be acquired with different responses in attachment and MRSA biofilm formation *via* food manipulation and/or consumption. This study aim to evaluate the biofilm forming ability of MRSA isolated from food products. A proper *in vitro* approach has been adopted. A correlation between biofilm formation and composition and molecular aspects of MRSA isolates has been put into evidence.

MRSA collection and their characteristics

Forty-nine well-characterized MRSA isolates coming from animal food sources (21 milk and dairy products and 5 meat and meat products) were selected to be used for their biofilm formation capacity. Previously, their genetic background has been characterized based on MLST, SCC*mec* typing and PFGE typing by *Sma*I. Moreover, antimicrobial susceptibility testing to 20 antimicrobial agents has been performed following the recommendations of EUCAST guidelines 2015, using a Microscan automated system.

Prior to biofilm assays, MRSA isolates were transferred from the freeze-dried culture state (preserved in 25% glycerol, -80°C) to Baird Parker (BP- Biolife Italiana, Milano, Italy) agar plates, followed by their aerobic incubation for 24 h at 37°C. Reference strain ATCC 25923 (Stepanović *et al.*, 2000; Bauer *et al.*, 2013) was introduced in all assays.

Biofilm formation and quantification

Broth subcultures were prepared by inoculating one single colony of MRSA from the BP plate into a test tube containing 10 mL of Tryptic Soy Broth (TSB-HiMedia, Mumbai, India) media, then incubated overnight at 37°C±1°C. Inoculum of each MRSA isolate has been adjusted to OD_{600} 1, later being confirmed by plate counts of ten-fold dilutions of each standardized inoculum. Experiments were performed in 96-well polystyrene microtiter plates (Nunc® MicroWell™, Saint Louis, Missouri, USA) using TSB enriched with 0.4% (w/v) glucose (TSBG, Sigma Aldrich, Saint Louis, Missouri, USA), glucose supplement being sterilized by 0.22 µm (Minisart®, Sartorius Stedim Biotech, Germany) filtration and added to the broth media after autoclavation. A total volume of 0.2 mL of TSBG and a starting inoculum approximately equal to 10^6 CFU/mL were added into each well, followed by their 24 h aerobic incubation at 37°C, without shaking.

Crystal violet (CV- Merck KGaA, Darmstadt, Germany) staining was used to quantify the total amount of biofilm biomass attached on the 96-well hydrophobic surfaces following the procedure developed by Stepanović *et al.* (2000). After carefully removing broth media from wells, biofilms were rinsed twice with 0.9% NaCl (Liofilchem, Roseto degli Abruzzi, Italy) to remove weakly adherent cells. Pure methanol (15 min) (Sigma Aldrich, Saint Louis, Missouri, USA) has been used for biofilm fixation, then the supernatants were discarded allowing the wells to dry at room temperature.

Gram staining has been achieved with 1% CV, the stain excess being removed after 5 min by gently wash with tap water. Absorbance was determined at 570 nm using an ELISA reader (Tecan 900 Pro), after CV bound being released in 0.2 mL of 33% acetic acid (Sigma Aldrich, Saint Louis, Missouri, USA).

S. aureus biofilm-forming strain ATCC 25923 (positive control) and broth medium (negative control) were used as controls during all biofilm assays. All assays were carried out three times and results were averaged. Prolonged incubation time (48 h) was performed (Peeters *et al.*, 2008) for those MRSA isolates exhibiting a biofilm forming capacity in accordance with OD_{570} higher than 3, in order for the cells located in the deeper layers of biofilms (adhesion surface) to overcome and stimulate their metabolic activity as well.

Cell viability by CFU and dry weight determination

Viable cell quantification was performed by CFU counting using nutrient agar media (Biolife Italiana, Milano, Italy). After 48 h incubation, non-adherent cells were removed from the 24-well plates and rinsed twice with 0.9% NaCl. Then, each biofilm was resuspended in 1 mL of 0.9% saline solution and dislodged by vigorous scraping and vortexing. Three replicates of ten-fold dilutions were made and the plates were incubated at 37°C for 24 h, before counting the number of grown colonies using the following equation:

$$CFU/mL = \frac{\sum CFU}{(n_1+0.1n_2)\cdot d} \quad (6)$$

whereas: $\sum CFU$- total number of colonies from plates containing 10-150 colonies;

n_1- number of plates containing 10-150 colonies each;

n_2- number of plates from the following dilution;

d- dilution factor corresponding to the plates containing 10-150 colonies.

Similarly to viable cell quantification, the amount of each biofilm growing on the well surface of the 24-well plate was scraped into 1 mL of 0.9% saline solution. Resuspended biofilm cells were filtered through a preweighted filter (0.45 µm) (Teknokroma, Professionally Friendly, Spain) and air dried in the oven for 24 h at 105°C. The dry weight (DW) of each biofilm was calculated by differences between weights. Moreover, the coefficient of variation (e%) was used to estimate the experimental error using the following formula:

$$e\% = \frac{s}{m} \cdot 100 \quad (7)$$

whereas: $\overline{m}$- average of n values;

s- standard deviation of these n values.

Structural and matrix composition evidenced by CLSM

Biofilm composition (48 h) of MRSA isolates with the OD_{570} > 3 was evidenced by confocal laser scanning microscopy (CLSM) after exposure to three types of dyes: 5 µM SYTO 9 green fluorescent nucleic acid stain (Molecular Probes, Eugene, Oregon, USA); 0.01 mg/mL wheat germ agglutinin (WGA) conjugated with Oregon Green488 (Molecular Probes, Eugene, Oregon, USA) for *N*-acetyl-D-glucosamine residues staining (Wright, 1984); undiluted Film Tracer SYPRO Ruby biofilm matrix for protein staining (ThermoFisher Scientific, Carlsbad, California, USA) (Berggren *et al.*, 2000). Prior to the CLSM assay, the supernatant and non-adherent cells were removed, rinsed with sterile water, then biofilms were treated with the above mentioned fluorescent dyes. The excess of each stain was gently removed, then MRSA biofilms were automatically scanned with an inverted Zeiss LSM710 T-PMT CLSM, which has been configured with three lasers (Argon 458/488/514 nm, Diode 405 and HeNe 633 nm). Confocal images (2048 x 2048 pixels) of each MRSA-producing biofilm were captured using a 63X oil immersion objective with minimum 1.4 numerical aperture and a working distance of 0.19 mm. Labelled cells were detected using the following combination of laser excitation and emission band-pass wavelengths: SYTO 9 (514-582 nm); WGA (406-511 nm) and SYPRO (584-700 nm). Quantitative structural parameters of MRSA-producing biofilms were calculated using the freely available COMSTAT v2.1 software.

Statistical analysis

All experiments were done in triplicates. Statistical analysis of 24 h and 48 h MRSA biofilms have been performed using classical statistical parameters. Coefficient of variations (e%) was calculated in order to estimate the experimental errors.

Results

Biofilm forming ability of MRSA isolates

Isolates were categorized based on their ability to produce biofilms (Figure 19). The cut-off points based on OD values separate MRSA biofilm producing ability into weak ($OD_{NC} \leq OD < OD_C$), moderate ($OD_{NC} < OD \leq 3$) and strong ($OD > 3$) biofilm formers. The cut-off OD values for weak (OD_{570} 1.03, SD 0.03), moderate (OD_{570}, 1.03 − 3), and strong (OD_{570} 3.82, SD 0.12) biofilm formers were defined based on the averaged OD_C obtained (OD_{570}, 3.65, SD 0.07) after the correction of the blank sample (OD_{570} 0.16, SD 0.03). Forty-one (83.7%) of the 49 MRSA tested isolates showed moderate biofilm formation, whereas the remaining 8 (16.3%) were strong biofilm producers. Table 16 describes the summarized results of MRSA isolates from different food sources based of their ability to produce biofilms.

Table 16. *Biofilm formation by MRSA isolates on hydrophobic 96-well microtiter plates at 37°C, static conditions*

Source	Biofilm producer		Moderate biofilm producer		Strong biofilm producer	
	n	%	n	%	n	%
Milk and dairy products	42	85.7	37	75.5	5	10.2
Meat and meat products	7	14.3	4	8.17	3	6.13

Molecular aspects and biofilm formation pattern of MRSA isolates have been evidenced (Table 17), whereas, overall, a higher biofilm biomass has been put into evidence for those harboring SCC*mec* type IV. However, MRSA isolates analyzed in this study harbored SCC*mec* types IV or V, being in accordance that such strains are having greater probability to produce higher biofilm biomasses in comparison with those carrying SCC*mec* types I-III (Vanhommerig *et al.*, 2014).

Table 17. *Molecular aspects of MRSA and its 24 h biofilm formation pattern, static conditions*

Isolate	Sample type	Country of origin	SCC*mec*	ST	Biofilm formation	
					Moderate	Strong
50	meat	Egypt	IV	22	+	
151-1.1	cheese	Bolivia	IV	1649	+	
151-1.2						
151-1.3						
153-1.1*	cheese	Bolivia	IV	8	+	
153-1.2*						
153-1.3*						
137-2.1	fresh meat	Republic of Serbia	V	398	+	
137-2.2						
140	brined cheese	Turkey	V	5	+	
132.2	cheese	Columbia	IV	5	+	

132.3						
165	cheese	Turkey	IV	22	+	
41	cheese	Egypt	V	5	+	
80-2.1						
80-2.2	cheese	Nicaragua	IV	5	+	
80-2.3						
138.1						
138.2	cheese	Nicaragua	IV	5	+	
476	cheese in spicy sauce	Egypt	V	1	+	
124.1	cheese	Nicaragua	IV	5	+	
68.1						
68.2	cheese	Nicaragua	V	72	+	
68.3						
133-1.1						
133-1.2	cheese	Nicaragua	IV	5	+	
133-1.3						
115-1.1						
115-1.2	cheese	Bolivia	IV	1649	+	
115-1.3						
01-05a	maturated sheep cheese	Romania	IV	1	+	
01-05b						
01-02a	soft goat cheese	Romania	IV	1	+	
135.1						
135.2	cheese	Peru	IV	1649	+	
135.3						
45-3.1	fresh beef meat	Egypt	V	5	+	
117.1*					+	
117.2*	cheese	Ecuador	IV	8		+
117.3*						
122-2.1*	cheese	Peru	IV	8	+	
45-1.1	cheese	Egypt	V	97	+	
50-2.1	dried meat	China	IV	7		+
74.1						
74.2	cheese	Bolivia	IV	1649		+
74.3					+	
24.1	sheep meat	Nigeria	V	8		+
24.2						
46-2.3	cheese	Republic of Honduras	IV	7		+

Note: *-presence of *pvl* gene

Prolonged incubation period (48 h) has been applied for those eight MRSA isolates having an OD_{570} higher than 3, proving an increased biofilm biomass accumulation during the analyzed time course (Figure 20). Based on these findings, we further characterize MRSA isolates after 48 h of incubation.

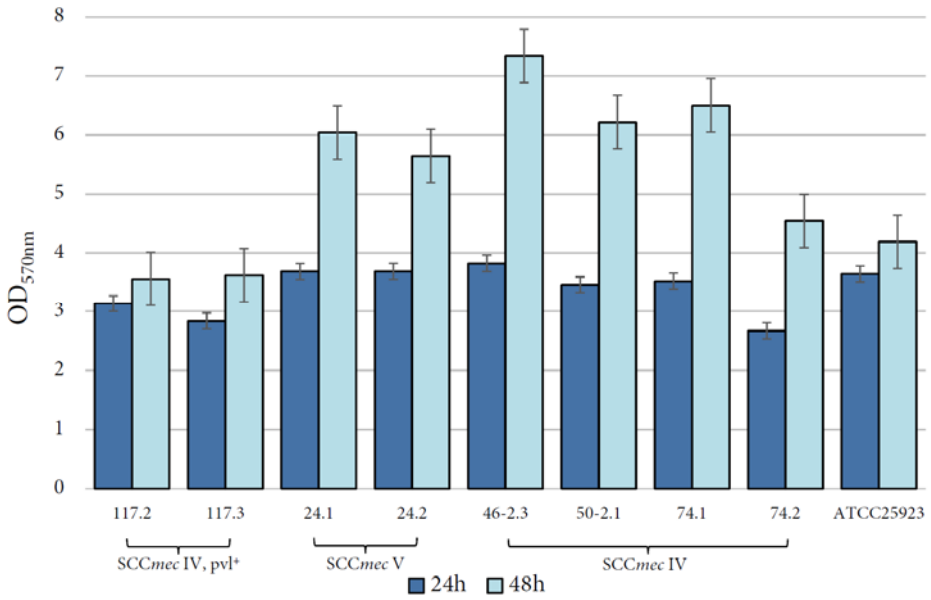


Figure 20. Quantification of 24- and 48 h MRSA biofilm biomasses using TSBG. Bars indicate the average of the OD value ± standard deviation (SD) from three independent experiments. Negative samples have been extracted from the shown values

Similarly to the OD measurements, significant differences in viable cell quantification and dry weight calculations of MRSA isolates were observed for the older 48 h MRSA biofilms (Figure 21). As can be seen, the number of viable cells within a MRSA biofilm decreases as its biomass increases. This can be explained by the fact that metabolic activity differs as MRSA biofilms are competing for nutrients available in a delimited space. The remaining viable cells may show different metabolic states dependent of the total biofilm biomass accumulated during 48 h. Although *Staphylococcus* spp. are known to produce strong biofilms (Bridier *et al.*, 2010), this is not the case for biofilm mode of growth of isolates 117.2 and 117.3 in which cell viability was extremely higher, explained by the lack of cells adhesion to the polystyrene surfaces.

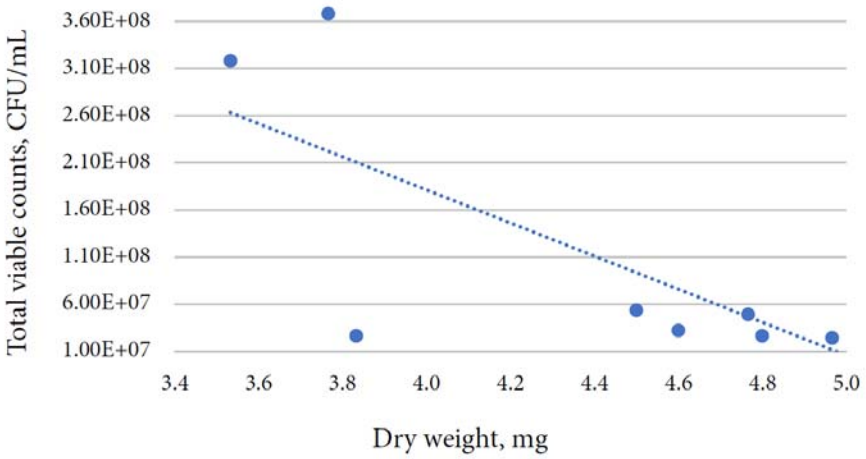


Figure 21. Biomass and viable cell quantification of 48 h MRSA biofilms. Values indicate the average ± standard deviation from 3 independent experiments

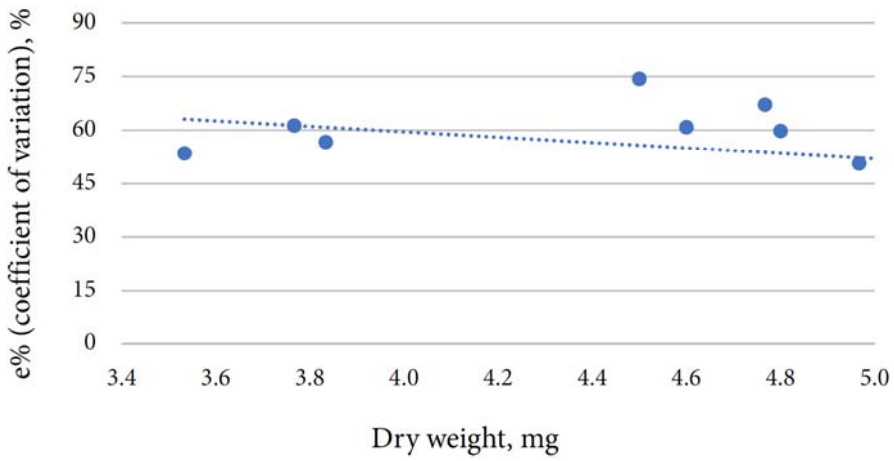


Figure 22. Coefficient of variation as a function of biofilm biomass in MRSA isolates

In Figure 22 is represented the coefficient of variation calculated for different biomasses belonging to the 48 h MRSA biofilms. No more than 15% error was observed between different plotted biomasses.

CLSM analysis of MRSA isolates

Representative 48 h MRSA-producing biofilm structures by CLSM in conjugation with different fluorescent dyes are presented in Figure 23. CLSM images differentiate bacterial cells (green) from proteins (red) and PNAG (blue) within the biofilm matrix. MRSA isolates formed flat and compact structures, while some of them developed slightly three-dimensional structures covered by highly fluorescent cell aggregates areas within. For example, MRSA isolate 117.2 had a biomass accumulation of 0.06481 $\mu m^3/\mu m^2$ represented by proteins; 0.0792 $\mu m^3/\mu m^2$ for PNAG while cells covered only 0.05938 $\mu m^3/\mu m^2$ (Figure 24). The biofilm-producing strain of *S. aureus* ATCC 25923 used in this study as reference apparently formed biofilms mainly composed of PNAG, data confirmed also by other studies (Skogman *et al.*, 2012; Oniciuc *et al.*, 2016). This is not the case of isolate MRSA 24.1 where it had a biomass accumulation of 0.15277 $\mu m^3/\mu m^2$ covered by proteins; 0.08279 $\mu m^3/\mu m^2$ of poly-*N*-acetylglucosamine residues and 0.10528 $\mu m^3/\mu m^2$ of viable cells. It seems that, in this particular case, protein content is responsible for the structure of this biofilm. However, biofilm matrices of the analyzed MRSA biofilms had similar amounts of polysaccharides, proteins and DNA in their matrix.

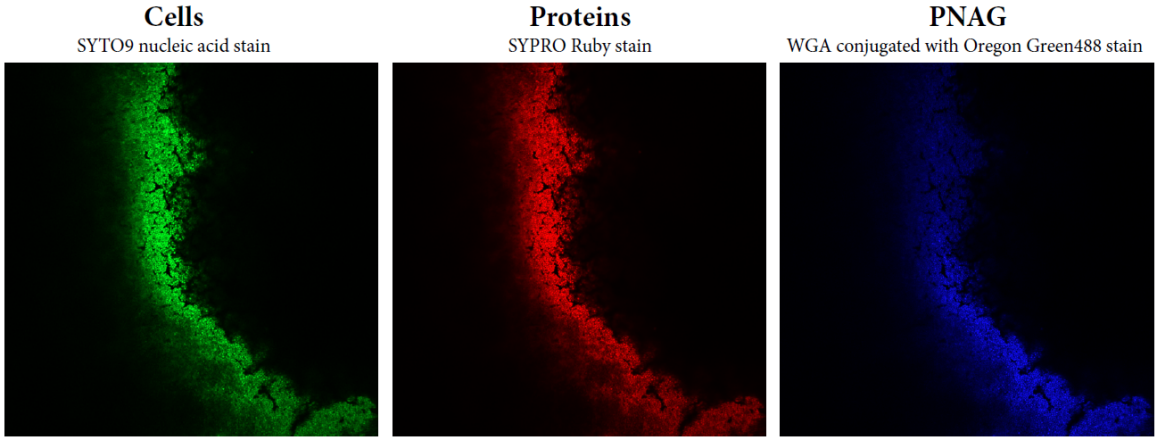


Figure 23. Biofilm imaging obtained from confocal observations of MRSA isolate 117.2, with different biofilm components differentiated (cells- left; proteins- center; polysaccharides- right). One z-stack is represented

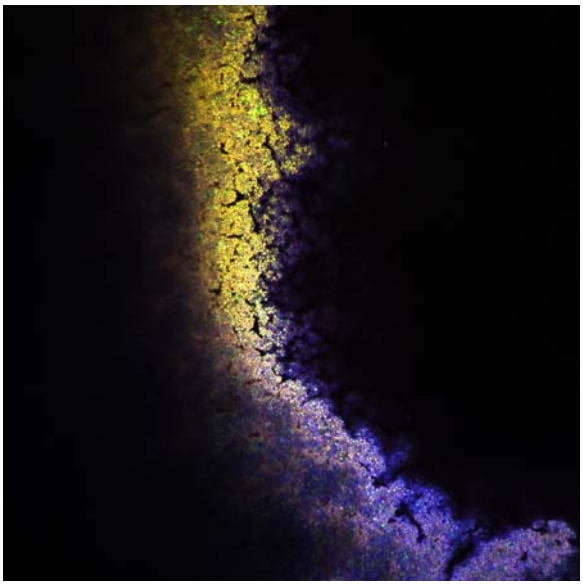


Figure 24. Biofilm imaging obtained from 63X confocal observations of MRSA isolate 117.2 stained with all three types of dyes

Discussions

Bacterial ability to form biofilms is of great importance and represents a big challenge for the food industry, as some strains in their sessile state may tolerate antimicrobial agents, making the bacterium extremely difficult to eradicate (Basanisi *et al*., 2017). The emergence of *S. aureus* resistant to antimicrobial agents, such as methicillin resistance has provoked a great concern due to its presence in associated foodstuff (Rodríguez-Lázaro *et al*., 2015). However, the risk of MRSA to produce biofilms may occur by contamination from human handlers rather than food itself (Doulgeraki *et al*., 2016). On the other hand, MRSA biofilm mode of growth has been deeply studied in clinical branch, as being one of the important pathogens associated with indwelling medical devices, catheters, or surface colonization of such equipments, besides *S. epidermidis* (Ferreira *et al*., 2012; Prakash *et al*., 2016). Likewise, MRSA can adhere

and form biofilms on different surfaces mostly used in food sector such as polystyrene, stainless steel, glass, ceramic and others (Oulahal *et al.*, 2008; Mirani *et al.*, 2013; Di Ciccio *et al.*, 2015).

In the present study, forty-nine MRSA isolates recovered from food sources were tested regarding their biofilm formation ability using TSBG media at 37°C, the temperature relevant for infectious disease's appearance (EC 853/2004). TSB plus glucose or NaCl have been shown to improve the biofilm formation on microtiter (static well) plates as suggested by some researchers in their attempt to find the best culture media whereas *S. aureus* may be able to form reproducible and robust biofilms (Luong *et al.*, 2009; Merino *et al.*, 2009; Chen *et al.*, 2012). However, Stepanović *et al.* (2007) conducted a study where he demonstrated that some *S. aureus* strains may have greater adherence in TSB without glucose than in TSBG, but may not support the *S. aureus* biofilm mode of growth.

Based on results obtained, a variation in the ability to form biofilms based on OD measurements has been observed. Our data showed that most of the MRSA isolates had the ability to accumulate moderate (83.7%) but there are also strong biofilm formers (16.3%). Hydrophobicity seems to be an important factor contributing to the biofilm formation capacity of MRSA isolates. Similar results are in accordance with previous studies (Pagedar *et al.*, 2010).

Evaluation of 48 h MRSA biofilms has been achieved by correlating the number of viable cells within the total amount of biofilm biomass. Based on DW measurements, MRSA isolate 74.1 had a significantly higher biomass than the biofilm-producing model ATCC25923, but those differences were not correlated with the CFU's counting after 48 h of growth. These may be explained by the fact that those biofilms were composed of bacterial cells and extracellular polymeric substances, suggesting that 74.1 accumulated a denser biofilm matrix, the remaining cells competing for their survival. However, older biofilms may expect to have stable cell clusters which can interfere with the quantification of sessile bacteria by plate-counting (Freitas *et al.*, 2014). Later, CLSM allowed us to perform a visual analysis of the concurrent distribution of polysaccharides, nucleic acids, and proteins components within the biofilms. Similar distribution in cell density as well as regarding the self-produced exopolymeric matrix has been noticed. However, higher content of proteins rather than PNAG within the biofilm matrices related to food sources has been previously observed (Ferreira *et al.*, 2012; Oniciuc *et al.*, 2016).

In this study, different biofilm patterns related to MRSA clonal lineages were observed, especially for those MRSA harboring SCC*mec* type IV and V, these data being in accordance with other studies. For example, Mirani *et al.* (2013) found that 98.3% of MRSA isolates harbored SCC*mec* type IV being related further with their biofilm ability. Moreover, different biofilm patterns related to MRSA clonal lineages are presented in a work study performed by Vanhommerig *et al.* (2014), suggesting that MRSA harboring SCC*mec* type IV produce significantly more biomass under static conditions than SCC*mec* type I-III. But better biofilm formers are corresponding to SCC*mec* type I-III rather than to SCC*mec* type IV, when dynamic conditions are used (Vanhommerig *et al.*, 2014). However, Parisi *et al.* (2016) noticed an association between SCC*mec* type IV or V and biofilm formation, whereas the high prevalence of such staphylococcal cassettes promotes the *S. aureus* biofilm producing ability, thus allowing the bacteria to persist in the environment.

Conclusions

Since many studies confirmed the potential role of food in the successful dissemination of MRSA lineages, it is important, as well, to take into consideration their capacity to form biofilms. Nowadays, there is a

growing concern regarding potential routes of MRSA vehiculating from passengers' luggage in the EU space as these strains can form biofilms, which may act as survival strategists against harsh environmental conditions that may encounter. Results obtained so far gave us new insights that MRSA strains isolated from food of animal origin are capable of forming biofilms. By knowing the main matrix components within biofilms, we can counteract the mechanisms involved in biofilm resistance by applying proper control strategies with a great focus, currently, on alternative ones such as biofilm degrading enzymes, quorum sensing inhibitors or the use of bacteriophages. Thus, efforts to combine conventional solution-based targeting different biofilm constitutes should be done. Moreover, a considerable need for routine surveillance and control regarding foods introduced in the EU is necessary as foodborne pathogens can be freely distributed and promote biofilm formation.

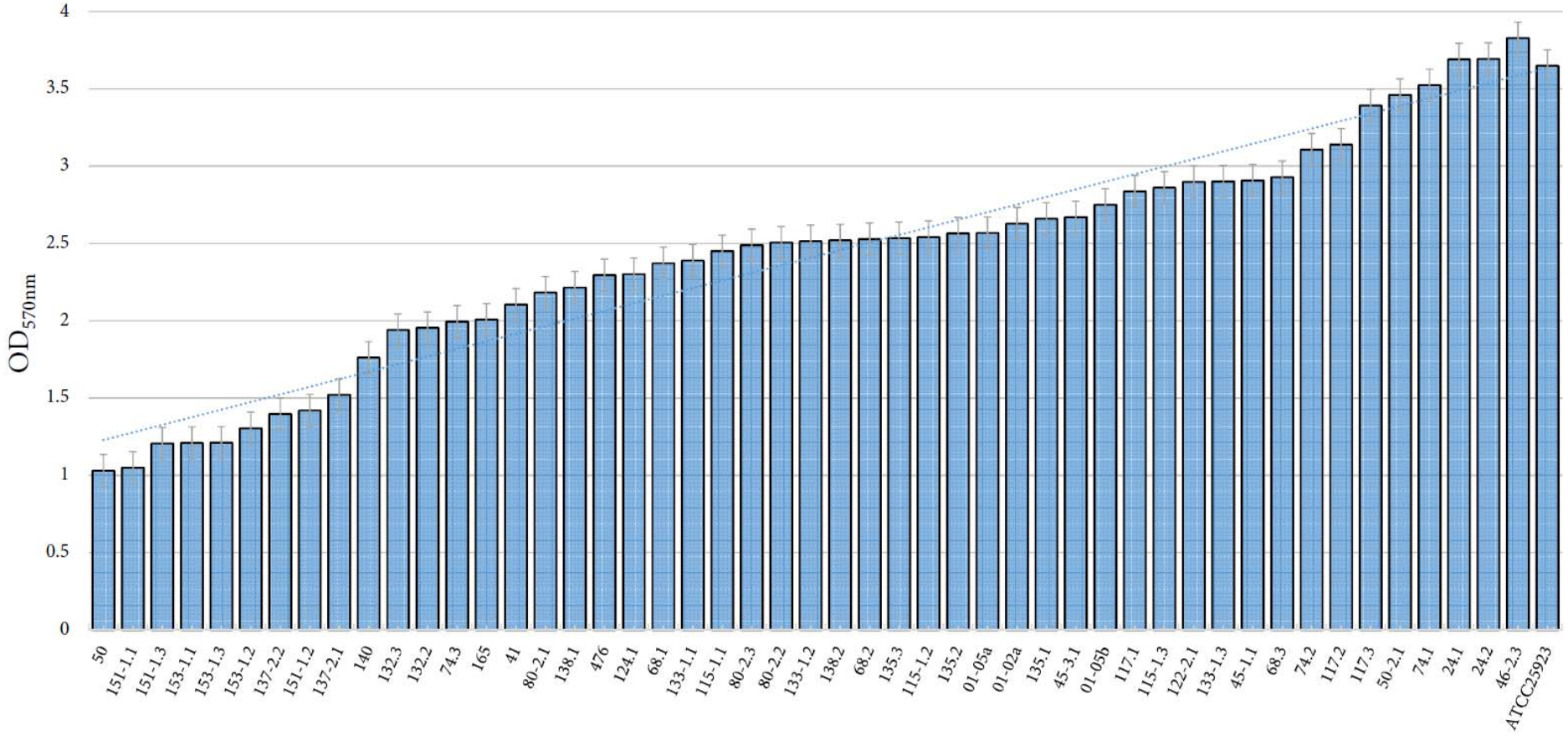


Figure 19. Biofilm forming ability (arranged in an increasing order) of MRSA isolated from food animal sources using TSBG. Bars indicate the average of the OD value ± standard deviation (SD) from three independent experiments. Blank sample represented by TSBG media has been extracted from the shown values

CHAPTER 9

Case study- Oxacillin-Susceptible *mec*A-positive *Staphylococcus aureus* Associated to Processed Food in Europe

Case study- Oxacillin-Susceptible *mec*A-positive *Staphylococcus aureus* Associated to Processed Food in Europe

Methicillin-resistant *Staphylococcus aureus* (MRSA) is an opportunistic pathogen associated with nosocomial and community acquired infections and recently being found in food producing animals and associated processed food (Rodríguez-Lázaro *et al.*, 2015; Oniciuc *et al.*, 2015). MRSA is mediated by the expression of *mec*A or *mec*C genes, encoding a modified penicillin-binding protein (PBP) designated as PBP2a with reduced affinity for β-lactams and phenotypically having a minimum inhibitory concentration (MIC) of oxacillin higher than 4 μg/mL. Moreover, expression of *mec*A or *mec*C can be additionally regulated by the *bla* system (Sabat *et al.*, 2015). Beta-lactamase regulates the synthesis of *blaZ* gene (Sabat *et al.*, 2015), while regulation of *mec*A gene is predicted by the production of PBP2a located in the mobile genetic element, named as staphylococcal chromosomal cassette (SCC*mec*) (Milheiriço *et al.*, 2007; Peacock and Paterson, 2015). Later on, transcription of *blaZ* and *mec*A genes is driven by their genetic organization of *blaR1-blaI* genes, and *mecR1* (sensor/inducer)-*mecI* (repressor), respectively (Milheiriço *et al.*, 2007; Peacock and Paterson, 2015), in which higher similarity has been noticed between *blaZ* and *mecA* inducers (61% amino acid similarity) and repressors (44%) (Oliveira and de Lencastre, 2011), allowing to cross-talk between their regulatory systems (Sabat *et al.*, 2015). However, it is not necessary for both systems to be present in a *S. aureus* strain as each one alone can control the transcription of *mec*A and *blaZ* genes (Ryffel *et al.*, 1992; Mckinney *et al.*, 2001). This is the case of SCC*mec* types IV and V in which the *mecI-mecR1* regulatory elements are absent or truncated resulted from the insertion of IS*1272* and IS*431*, respectively (http://www.sccmec.org/Pages/SCC_ClassificationEN.html).

Some studies reported cefoxitin or methicillin-sensitive *S. aureus* (MSSA) strains classified by conventional phenotypic laboratory testing, but genotypically carrying *mec*A gene. These strains have been defined as oxacillin-susceptible *mec*A-positive (OS-MRSA), also known as cefoxitin-sensitive MRSA. Such strains are exhibiting oxacillin MIC in the susceptible range (≤ 2 mg/L) (EUCAST, 2015), showing an intermediate susceptibility by disk diffusion tests and low sensitivity of chromogenic media for their detection (Saeed *et al.*, 2014). Due to misinterpretation of oxacillin or cefoxitin phenotypic studies, such strains can easily be misdiagnosed, potentially triggering the development of highly new resistant MRSA variants under antibiotic selection due to the possession of *mec*A.

OS-MRSA has been reported in clinical isolates (Hososaka *et al.*, 2007; Chen *et al.*, 2012; Conceiçao *et al.*, 2015), in animals (Pu *et al.*, 2014) and recently in meat (Raji *et al.*, 2016), posing a serious challenge for routine conventional diagnostic tests (Malhotra-Kumar *et al.*, 2010; Ariza-Miguel *et al.*, 2015) and for possible associated treating infections due to oxacillin-susceptible phenotype of such strains. OS-MRSA strains seems to be genetically diverse in which regulatory systems mentioned above are of prime importance in regulating the phenotypic expression of methicillin resistance (Sabat *et al.*, 2015). This study aimed to examine the whole genome sequence of an oxacillin-susceptible *mec*A *S. aureus* isolated from a processed food, harboring a SCC*mec* type V and belonging to the MLST sequence type 5.

Bacterial strain

An oxacillin-susceptible *mec*A positive strain has been previously isolated from a dairy product among 600 foods confiscated from August 2012 to March 2013 at International Vienna airport from passengers of international flights from non-EU countries. This strain, designated OS-MRSA 41, has been genetically characterized and demonstrated that it belong to MLST ST5, SCC*mec* type V.

Phenotypic testing of *mec*A positive isolate

The OS-MRSA 41 was tested to 20 antimicrobials agents by the Microscan (Beckman Coulter S.L.U, USA) and Vitek II (BioMérieux, France) automated systems for MICs interpretation and disk diffusion method for zone diameter measurements, following the recommendations of EUCAST guidelines, 2015 v5.0. The Sensititre Gram Positive All-in-One Plate system (TREK Diagnostic Systems Inc., Cleveland) was also used.

Whole genome sequencing

Genomic DNA of OS-MRSA 41 strain was extracted using the DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany) and the purified DNA was quantified with a Fluorometer Qubit 2.0 (Life Technologies). Whole genome sequencing (WGS) approach was undertaken by preparing libraries from the genomic DNA using tagmentation procedure and 300 bp-paired-end sequencing. A next-generation sequencing approach was launched in a MiSeq device (Illumina).

Data analysis

Raw reads quality was evaluated by using FastQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/). Bases with a Phred score below 25 within a 20-bp-long window were trimmed and reads shorter than 100 bp were discarded by using Prinseq (http://prinseq.sourceforge.net/). Filtered reads were assembled by using SPAdes version 3.10.0 (option "--careful" enabled) (Bankevich *et al.*, 2012) and contigs shorter than 200 bp were discarded. The resulting 190 contigs were ordered against *S. aureus* isolate JS395 (Larsen *et al.*, 2017) by using Mauve (Darling *et al.*, 2004). Ordered contigs annotation was performed using the RAST annotation server (Aziz *et al.*, 2008). MLST and SPA typing were performed by using mlst v2.6. (T. Seemann; https://github.com/tseemann/mlst) and spaTyper v1.0 (Bartels *et al.*, 2014), respectively. BLASTn (Altschul *et al.*, 1990) searches were undertaken on the draft genome against a custom gene database. Insertion and CRISPR sequences were predicted by using ISFinder (Siguier *et al.*, 2006) and CRISPRFinder (Grissa *et al.*, 2007), respectively. Whole genome SNPs were identified by mapping the sequence reads to *S. aureus* isolate JS395 genome using Bowtie2 v2.2.6 aligner (Langmead and Salzberg, 2012) and generating a Variant Call Format with SAMtools v1.3.1 (Li *et al.*, 2009) and VarScan v2.3.9 (Koboldt *et al.*, 2012). Antimicrobial resistance genes were predicted by using abricate v0.3 (T. Seemann; https://github.com/tseemann/abricate) against the ResFinder database (https://cge.cbs.dtu.dk/services/ResFinder/). Plasmids presence was evaluated by assembling the reads by using PlasmidSpades (Antipov *et al.*, 2016), which uses reads coverage to infer the difference between chromosomic and plasmidic reads.

Results and discussions

Phenotypic features

The MIC for oxacillin of the OS-MRSA 41 was in the susceptibility range by Microscan (Beckman Coulter S.L.U) and Vitek II (BioMérieux, France) automated systems, confirmed also by Sensititre Gram Positive All-in-One Plate system (TREK Diagnostic Systems Inc., Cleveland). Further screening for zone diameter interpretation showed that the studied strain was susceptible to cefoxitin (30 µg/disk, Oxoid) by disk diffusion method. Moreover, the antibiotic susceptibility pattern showed to be positive for inducible clindamycin resistance and tetracycline, resistant to penicillin and exhibiting intermediary resistance to erythromycin. The characteristics of the OS-MRSA isolate are detailed in the following table (Table 18).

Table 18. *Phenotypic and genotypic characterization of the OS-MRSA 41 strain*

Test	OS-MRSA 41
Phenotypic screening	
Coagulase production	positive
MIC for oxacillin (µg/mL)	0.5
MIC for tetracycline (µg/mL)	>8
MIC for erythromycin (µg/mL)	>4
MIC for penicillin (µg/mL)	>0.25
Cefoxitin screening	
-Disk diffusion (mm)	sensitive (24.5)
-Brilliance MRSA 2 Agar	sensitive
Genotypic screening	
*mec*A	positive
SCC*mec* type	V
lukS-PV & *lukF*	negative
Sequence Type	ST5

Genome features

The genome sequence of OS-MRSA 41 was compared with the sequence of a *mec*A-positive *S. aureus* isolate harboring SCC*mec* V, recovered from a patient in Switzerland in 2008, designated as JS395 (CC395-V) (Larsen *et al.*, 2017). MLST and SPA typing were performed by using mlst v2.6. (T. Seemann; https://github.com/tseemann/mlst) and spaTyper v1.0 (Bartels *et al.*, 2014), respectively, showing that OS-MRSA 41 have a *spa*-type t688 usually associated with MRSA ST5/SCC*mec* V (Basanisi *et al.*, 2017). The genome of OS-MRSA 41 strain consisted of a 2,819,217 bp chromosome size while the JS395 complete genome was composed of 2,846,866 bp (GenBank/ DDBJ/ENA; accession number CP012756). Ordered contigs were annotated using the RAST platform determining the number of subsystems automatically determined and being present in the both genomes taken into analysis. Detailed estimation regarding various subsystems present in both genomes are illustrated in Figure 25.

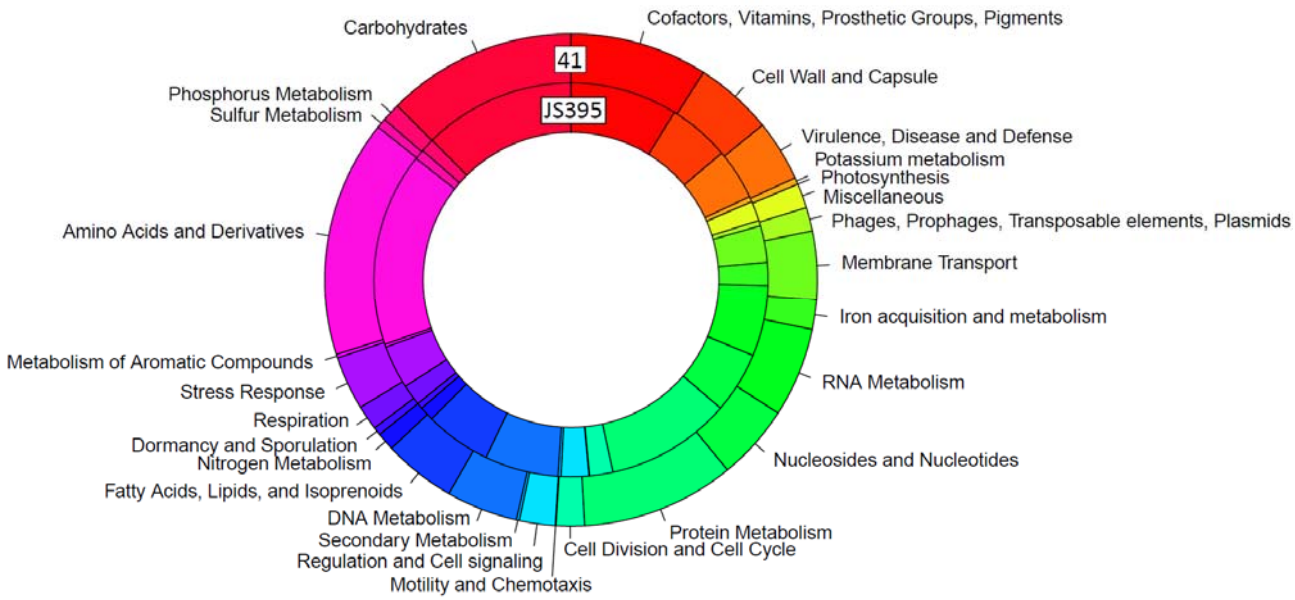


Figure 25. Comparison between various subsystems present in the OS-MRSA 41 and JS295 genomes

As can be seen, gene content belonging to each subsystem present in both genomes are slightly different. Distribution of genes related to phages, prophages, transposable elements, or plasmids present in the OS-MRSA 41 seems to be higher in comparison with the one belonging to JS395-related subsystem group. Thirty-two phage and transposable elements, represented by 1.57% of its total genome content were present in the OS-MRSA 41. However, less variants were present in the JS395, amounting only 0.44% (no. 9) of its total DNA content.

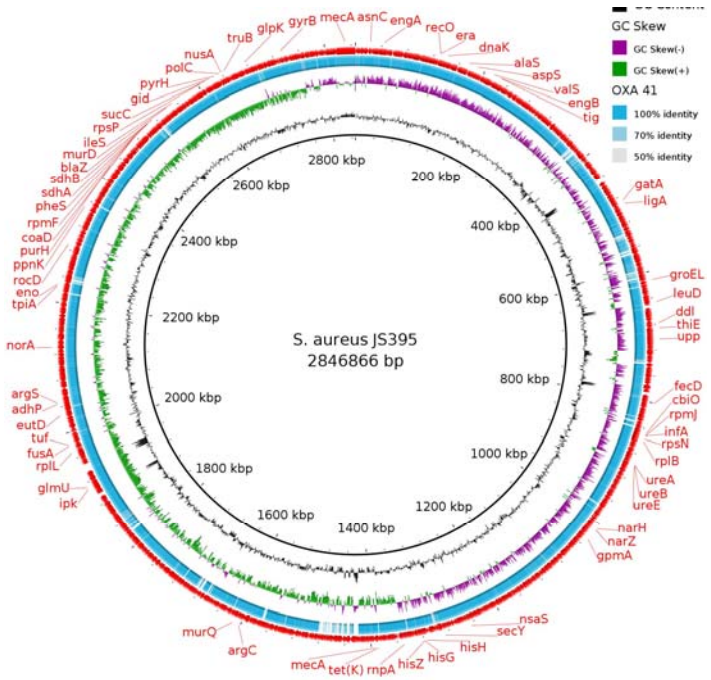


Figure 26. Chromosome of OS-MRSA 41

The OS-MRSA 41 genome consists of a circular 2,819,217 bp chromosome size, with a GC content of 32.8% (Figure 26). The unique features of this genome were compared with the genome of JS395 *S. aureus* strain (black inner circle). OS-MRSA 41 genome represents the blue inner circle. The red outer circle predicted the coding sequences (putative genes) on the plus and minus strands, respectively. Both strains carried SCC*mec* type V, whereas detailed analysis showed that the structure of J1 region and *mec*A and *ccrC* genes are nearly identical, but differing between them in the J3 region (Figure 27). In the OS-MRSA 41 SCC*mec* element, a tetracycline resistance gene, *tet(K)*, caused by the integration of IS*431* is to be found in the J2 region. Of note, CRISPRFinder (Grissa *et al.*, 2007) identified 11 CRISPR spacers in the whole genome of the studied strain, whereas a CRISPR locus of 10,687 bp size has been found in the J1 region.

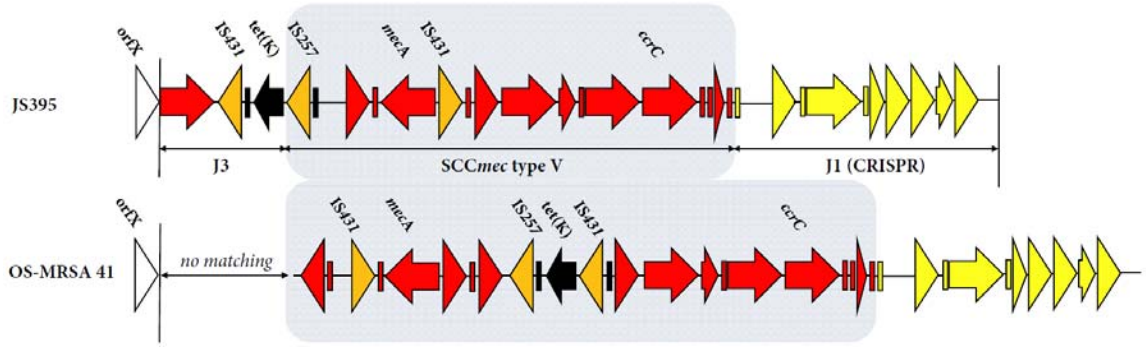


Figure 27. Comparative structure analysis of the SCC*mec* element in the *S. aureus* JS395 (DDBJ/ENA/GenBank accession number CP012756) and OS-MRSA 41 strain

Sequence analysis of present genes

Antimicrobial resistance genes prediction performed by using abricate v0.3 (T. Seemann; https://github.com/tseemann/abricate) revealed the presence of *blaZ, erm(C), fexA, mecA, norA, tet(K)* and *tet(M)* (Table 19). In addition, were observed to have mobile genetic elements such as insertion sequences (IS)*30*, IS*256*, IS*431*, IS*1181*, IS*1182*(*ISSau3*), ISL*3* (*ISSau8*); transposon (Tn)*3*; tyrosinase recombinase *XerD*. The genome analysis showed resistance genes that should have conferred phenotypic resistance to β-lactams, macrolides, tetracyclines and aminoglycosides.

Sequence analysis of mec gene complex. SCC*mec* type V possesses the class C *mec* gene complex and the *mecI-mecR1* regulatory elements are absent or truncated resulted from the insertion of IS*431*. The 1904 bp *mec* gene was almost identical to the JS395, for which *mec*A was found with 100% gene coverage but 99.95% gene identity. Variant calling revealed a SNP (A → G) in *mec*A position 1770 that is translated in an amino acid variation in the trans peptidase domain (position 589) of the forming MecA protein (S → P).

Sequence analysis of blaZ system. The β-lactamase gene *blaZ* and its regulatory genes *blaI* and *blaR1* were present in the OS-MRSA 41 strain, with 100% coverage but 99.054% identity, revealing seven SNPs. Even though OS-MRSA 41 strain showed a penicillin resistance level (MIC >0.25 µg/mL), several SNPs in the sequence of the *blaZ* gene could contribute to the phenotypical susceptibility to oxacillin of the *mec*A-positive strain studied.

Sequence analysis of other genes. BLASTn (Altschul *et al*., 1990) against a custom gene database was performed. Table 19 describe virulence factors associated with adherence-associated proteins, exotoxins and exoenzymes expressed in the OS-MRSA 41. Many secreted exoenzymes found in the OS-MRSA 41 were represented by proteases, lipases (such as glycerol ester hydrolase) and nucleases, with their specific role on pathogenesis. OS-MRSA 41 produces several major proteases, including a metalloproteinase (Aur, aureolysin), two cysteine proteases (SspB, staphopain B and SspC, staphostatin B) and a serine protease (SspA, staphopain A). The biological function of such proteases can be regarded as potential complement inhibitors (Jusko *et al*., 2014), by limiting the capacity of the host to fight against bacterial pathogens. For example, cysteine proteases expressed by OS-MRSA 41 could degrade elastin, collagen and fibrinogen (Ohbayashi *et al*., 2011), most important affecting tissues, leading to destruction and ulceration; while degradation of human immunoglobulins could be produced by the serine protease V8 (Prokešová *et al*., 1992). Other studies showed the implications of metalloproteinase Aur in the formation of a mature serine protease (Rice *et al*., 2001). Moreover, inactivation of antimicrobial peptides can be assessed by the metalloproteinase Aur, which have already been shown to have a great impact on the pathogenesis of osteomyelitis (Cassat *et al*., 2013). Other exoproteins found in the OS-MRSA 41 were represented by staphylokinase and staphylococcal complement inhibitor (SCIN) implicated in the degradation of fibrin clots, due to their activation of plasminogen into plasmin (Jusko *et al*., 2014). Finally, other exoenzymes encoding potential virulence factors are represented by lipases and nucleases which can inactivate fatty acids and decrease the antibacterial activity of neutrophils, respectively (Otto, 2014).

Moreover, virulence of OS-MRSA 41 could be characterized by the secretion of several exotoxins, in which its potential to cause possible diseases is greater as can interfere directly with the host (Otto, 2014). Several γ-hemolysin variants encoding *hlgA*, *hlgB* and *hlgC* genes were found in OS-MRSA 41, with their presumptive role in damaging the host cell plasma membrane (Vandenesch *et al*., 2012). Later on, although enterotoxigenic MRSA in foods have been found sporadically and typically associated with dairy products (Haran *et al*., 2012; Carfora *et al*., 2015), OS-MRSA 41 harbored not one but several types of enterotoxins, fact confirmed by PCR when testing for classical enterotoxins (types A-E). Such enterotoxins may be resistant to most proteolytic enzymes, by retaining their activity in the digestive tract after ingestion (Ortega *et al*., 2010), for this reason, their presence and possible activation should not be disregarded. However, the pyrogenic toxin superantigen such as toxic shock syndrome toxin (TSST), exfoliatins and leukocidins such as Panton Valentine leukocidin (PVL) were not detected. The fact that PVL was not detected was not surprising as all the environmental tested OS-MRSA isolates published so far have been negative for this virulence factor, instead *lukED* genes encoding the biocomponent leukotoxin LukE and LukD (Gharsa *et al*., 2012) with weak leukotoxic activity was detected. In addition, cell wall adhesion (CWA) components found in OS-MRSA 41 may have a role in virulence (Gordon and Lowy, 2008), favorizing bacteria to adapt to hostile environmental conditions, allowing its survival and promoting infection by invading and destroying host tissues and metastasize to other sites. For all this to happen, the predominant *agr* regulon in OS-MRSA 41 needs to be expressed. Has been demonstrated that the *agr* system is needed for the expression of staphylococcal enterotoxins (Ortega *et al*., 2010). Moreover, upon activation, *agr* system can regulate the synthesis of extracellular toxins and enzymes (Ortega *et al*., 2010); however, *Bap* gene presence

is lacking so proliferation and production of a scaffolding extracellular matrix (Speziale *et al.*, 2014) once attached to tissue or matrix-covered devices might be missing.

Conclusions

Numerous studies have been reported during the last decade regarding detection of OS-MRSA isolates from very distinct geographical countries (Hososaka *et al.*, 2007; Kumar *et al.*, 2013; Pu *et al.*, 2014; Andrade-Figueiredo and Leal-Balbino, 2016; Sabat *et al.*, 2015) but, to our knowledge, this is the first report on the presence of oxacillin-susceptible *mec*A-positive *S. aureus* on processed foods in Europe. This finding together with previous results obtained in our group (Rodríguez-Lázaro *et al.*, 2015; Oniciuc *et al.*, 2015) draw attention on a neglected dissemination route of MRSA *via* the entrance of illegal food in Europe.

The OS-MRSA isolate was found in a cheese confiscated to an air passenger traveling from Cairo, Egypt. It has an oxacillin MIC in the susceptible range of the EUCAST breakpoint (< 2µg/mL), indicating that presence of *mec*A gene does not confer a high-level resistance to oxacillin. It was also resistant to penicillin, tetracycline and exhibiting intermediary resistance to erythromycin. Without testing *mec*A gene by PCR, the isolate could have been misinterpreted as MSSA based only on the result of conventional antimicrobial susceptibility tests such as Microscan, VITEK, or Sensititre screening. Studies reported that routine clinical laboratories could misidentify such OS-MRSA isolates, by applying only conventional phenotypic methods, *e.g.* chromogenic media, antimicrobial susceptibility testing or detection of PBP2a by latex agglutination tests. In contrast, using molecular detection method alone, targeting *mec*A gene, could lead to false positively interpretation of such strains as MRSA. This is why culture-based and molecular detection methods must be done in parallel. Since few information is available regarding the biology of such isolates, they should be regarded as MRSA with more safety measures that needs to be taken.

The results of WGS analysis indicated that several mutations in the promoter and operator regions of the resistance genes *mec*A and *blaZ* drove to the low-level oxacillin MIC in the genetic background of OS-MRSA 41 strain. Moreover, other genetic factors involved contributed as well to the phenotypic oxacillin susceptibility. One reason could be related to the SNP located in the *mec*A gene that led to an amino acid change from serine to proline in the position 589. Moreover, amino acid substitutions in the *bla* system are also factors involved in the oxacillin-susceptible phenotype. Sabat *et al.* (2015) demonstrated that truncated *blaR1* gene was responsible for the susceptibility of GR2 isolate since, after removing the *bla* operon, has regained resistance to penicillin and oxacillin. Likewise, accumulation of amino acid mutations in Fem proteins (responsible for the stabilization of the staphylococcal cell wall), may contribute to the atypical oxacillin responsiveness and may be correlated with the oxacillin susceptibility of OS-MRSA (Giannouli *et al.*, 2010; Pournaras *et al.*, 2013).

The detection of OS-MRSA is quite recent. Reports of OS-MRSA isolates in hospital settings have increasingly appeared in the recent years with a wide geographical distribution; from Asian counties such as Taiwan, Japan or China to European (UK) and African countries (Pournaras et al. 2013). Although OS-MRSA has been mainly circumscribed to medical settings, a recent study demonstrated the presence of OS-MRSA isolates in livestock associated to bovine mastitis in four different regions in China (Pu et al. 2014) and camel meat samples from a neighborhood meat shop in Riyadh, Saudi Arabia (Raji et al. 2016). Since very few information is available about their ability to regain full gene regulatory capacity (Sabat et al. 2015),

such isolates should not be neglected because it may develop unusual resistance under antibiotic selection due to its *mec*A gene (Saeed et al. 2014) and instead should be regarded of prime importance concerning clinical settings and livestock.

However, until this current study, OS-MRSA had not been reported in processed foodstuff. Interestingly, the OS-MRSA obtained in this study was classified into MRSA-ST5-V, which has been also identified previously in environmental OS-MRSA isolates (Pu *et al*., 2014; Raji *et al*., 2016). MRSA STs and SCC*mec* types identified in isolates from non-clinical settings are not identical to the most recurrent ones isolated in clinical environments. This could suggest that a significant dissemination from medical settings to the environment has not occurred yet as environmental OS-MRSA strains show a distinctive genetic profile. Clinical OS-MRSA strains isolated so far in medical settings have shown a variable ST and SCC*mec* types, irrespective of the geographic origin from which they were recovered.

In conclusion, we report, for the first time, the presence of OS-MRSA in a processed food in Europe. Although this MRSA variant seems to be rare, it is of particular public health relevance because of its potential of develop highly resistant MRSA under treatment with β-lactam antibiotics, and as it might not have been detected by standard test procedures. The demonstration that new emerging MRSA variant, OS-MRSA, is already present in food in Europe, highlights the need for not underestimating food as neglected route of MRSA transmission as well as the need for monitoring the presence and evolution of OS-MRSA in food and environmental reservoirs. However, further studies are necessary to identify additional genetic factors and mechanisms within such isolates.

***Table 19**. Antimicrobial resistance and virulence genes found in the OS-MRSA 41*

Gene	Identity (%)	Coverage	Contig	Position in contig	Predicted phenotype	Function	Acc. number comparison
Antimicrobial resistance genes							
blaZ	99.054	846/846	NODE_37	15953...16798	Beta-lactam resistance	Beta-lactamase	AJ302698
erm(C)	100.000	735/735	NODE_119	875...1609	Macrolide resistance	rRNA adenine N-6-methyltransferase	M13761
fexA	99.720	1428/1428	NODE_25	14056…15483	Phenicol resistance	Drug resistance transporter, EmrB/QacA family	AJ549214
mecA	99.950	2010/2010	NODE_68	1797...3806	Beta-lactam resistance	Penicillin-binding protein PBP2a, methicillin resistance determinant MecA, transpeptidase	AB512767
norA	91.252	1165/1167	NODE_23	25843...27008	Fluoroquinolone resistance	Quinolone resistance protein norA	M97169
tet(K)	99.928	1380/1380	NODE_68	10135...11514	Tetracycline resistance	Tetracycline resistence protein, metal-tetracycline/H+ antiporter	U38428
tet(M)	99.583	1920/1920	NODE_21	33371...35290	Tetracycline resistance	Tetracycline resistance protein TetM	X92947
***S. aureus* exoenzyme associated genes**							
aur	99.935	1530/1530	NODE_7	32516...34045	Aureolysin	Zinc metalloproteinase precursor, inactivate antimicrobial peptides	EF070223.1
cysM	100.000	906/906	NODE_12	31135...32040	Cysteine (e.g. staphopain)	Cystathionine beta-synthase, block neutrophil activation and chemotaxis	AKA98565.1
femA	98.892	1262/1263	NODE_48	1481...2743	Methicillin resistance	Cell wal synthesis, formation of the pentaglycine bridges	GQ284643.1
femB	99.841	1259/1260	NODE_48	203...1462	Methicillin resistance	Cell wal synthesis, formation of the pentaglycine bridges	GQ284646.1
femX	99.052	1265/1266	NODE_27	3709...4974	Methicillin resistance	Cell wal synthesis, formation of the pentaglycine bridges	KXA35239.1
geh	97.447	2076/2076	NODE_9	46461...48536	Glycerol ester hydrolase	Triacylglycerol lipase, inactivate fatty acids	SHC05250.1
nuc	99.237	655/655	NODE_36	26091...26745	Nuclease	Phage-encoded chromosome degrading nuclease YokF	DQ507377.1
sak	99.797	492/492	NODE_14	35818...36309	Staphylokinase	Cleave nucleic acids	SHC59485.1
scn	100.000	351/351	NODE_14	37806...38156	SCIN	Involved in expression of fibrinogen binding protein, phage associated	BAV01400.1
sspA	98.084	1095/1167	NODE_145	342...1437	Serine V8 protease	Staphopain A precursor, inhibit complement activation	AJ538362.1
sspB	99.323	1182/1182	NODE_73	4941...6122	Cisteine protease	Staphopain B precursor, inhibit complement activation	SHB67173.1
sspC	99.394	330/330	NODE_73	4574...4903	Cisteine protease	Staphostatin B	AAG45845.1

S. *aureus* toxin genes							
hlgA	99.896	966/966	NODE_2	35824...36789	Gamma-hemolysin chain II precursor	Gamma-hemolysin component A	AKB00487.1
hlgB	100.000	978/978	NODE_2	38305...39282	Gamma-hemolysin component B precursor	Gamma-hemolysin component B	AKB00489.1
hlgC	100.000	948/948	NODE_2	37356...38303	Gamma-hemolysin component C precursor	Gamma-hemolysin component C	AKB00488.1
lukD	98.171	984/984	NODE_116	934...1917	Leukocidins D	Leukotoxin LukD, induce lysis on leukocytes	SHC70465.1
lukE	100.000	936/936	NODE_116	1919...2854	Leukocidins E	Leukotoxin LukE, induce lysis on leukocytes	BAU35396.1
sed	100.000	777/777	NODE_37	6380...7156	Enterotoxin D	Enterotoxin, phage associated	EUR29576.1
sej	100.000	807/807	NODE_37	8051...8857	Enterotoxin J	Enterotoxin, phage associated	AAC78590.1
sep	99.872	783/783	NODE_14	33233...34015	Enterotoxin P	Enterotoxin, phage associated	SAO38424.1
ser	99.359	780/780	NODE_37	8947...9726	Enterotoxin R	Enterotoxin, phage associated	BAC97795.1
set3	98.723	705/705	NODE_31	30207...30911	Superantigen-like protein	Exotoxin	AMV89422.1
set5	90.831	697/699	NODE_12	417...1114	Superantigen-like protein	Exotoxin	CXU96712.1
set6	100.000	681/681	NODE_31	25579...26259	Superantigen-like protein	Exotoxin	BAU33973.1
S. *aureus* adherence associated genes							
agr	98.728	786/786	NODE_123	1092...1877	Accessory regulator	Hydrolase in agr operon	CRI15192.1
atl	99.600	3747/3747	NODE_85	993...4739	Bifunctional autolysin Atl	Phage lysin, N-acetylmuramoyl-L-alanine amidase	AMR00583.1
clfB	99.383	648/648	NODE_7	42873...43520	Clumping factor protein B	Mediate clumping and adherence to fibrinogen in the presence of fibronectin	AJ744763.1
eap	100.000	435/435	NODE_107	114...548	Extracelullar adherence protein	Similar to cell surface protein Map-w, inhibit neutrophil migration	AKA98985.1
eap	99.656	291/291	NODE_14	41361...41651	Extracelullar adherence protein	Extracellular adherence protein of broad specificity Eap/Map, inhibit neutrophil migration	KZS28310.1
ebpS	99.247	1461/1461	NODE_44	8057...9517	Cell surface elastin-binding protein	Elastin binding protein EbpS	SGV08663.1
efb	99.509	611/611	NODE_152	374...984	Extracelullar fibrinogen-binding protein	Extracellular fibrinogen-binding protein Efb, inhibit complement activation	AJ306909.1
icaA	100.000	1239/1239	NODE_90	405...1643	Intercelullar adhesion protein A	Polysaccharide intercellular adhesin (PIA) biosynthesis N-glycosyltransferase IcaA	SGU84486.1

icaB	99.885	873/873	NODE_90	1909...2781	Intercelullar adhesion protein B	PIA biosynthesis deacetylase IcaB	CUD59794.1
icaC	98.865	1053/1053	NODE_90	2768...3824	Intercelullar adhesion protein C	PIA biosynthesis protein IcaC	AAD52058.1
sdrE	96.852	3462/3462	NODE_19	7836...11297	Ser-Asp rich fibrinogen-binding protein E	Adhesin of unknown specificity SdrE, similar to bone sialoprotein-binding protein Bbp	CYC72419.1
sdrH	99.365	1260/1260	NODE_70	9433...10692	Ser-Asp rich fibrinogen-binding protein H	Membrane anchored protein	BAS52105.1

CHAPTER 10

Chromogenic Media Evaluation for Confirmation of MRSA Isolated from Humans, Animals and Food Samples

Results published

Ariza-Miguel*, J., **Oniciuc*, E.A.**, Sanz, I., Fernández-Natal, I., Hernández, M., Rodríguez-Lázaro, D. (2015). Evaluation of two commercially available chromogenic media for confirmation of methicillin-resistant *Staphylococcus aureus* from human, animal, and food samples. International Journal of Food Microbiology, 209: 26-28, doi:10.1016/j.ijfoodmicro.2015.05.004. Impact factor: 3.082

**those authors contributed equally to the current work*

Chromogenic Media Evaluation for Confirmation of MRSA Isolated from Humans, Animals and Food Samples

Dissemination of MRSA from the healthcare system to community and animal settings in the last two decades has elicited a great concern (Okuma *et al*., 2002; Graveland *et al*., 2011). Furthermore, the emergence of MRSA in food-producing animals and derived food products has raised the question on the potential role of food as a route for transmission of successful livestock and community associated MRSA lineages (Oniciuc *et al*., 2015; Rodríguez-Lázaro *et al*., 2015). Therefore, monitoring the presence and genetic features of MRSA in all environments and potential reservoirs is required to better understand the dissemination, genetic evolution and evolutionary success of epidemic MRSA lineages.

Several commercially available chromogenic media have been developed to facilitate the screening of MRSA, and some studies have assessed their diagnostic performance (Verkade *et al*., 2011; Veenemans *et al*., 2013; McElhinney *et al*., 2013). However, they have been mainly focused on human clinical samples, and there is a knowledge gap regarding MRSA from animal and food samples. Therefore, in this study we evaluated the performance of Brilliance MRSA 2 Agar (ThermoFisher Scientific, Waltham, MA, USA) and ChromID MRSA Agar (bioMérieux, France) (Figure 28) as rapid MRSA confirmation screening assays for *S. aureus* isolates from a wide range of origins: clinical, animal and food samples. We assessed, by using the McNemar's test for paired samples, if there are statistically significant differences among a reference method, the molecular detection of resistance genes *mec*A and *mec*C, and MRSA confirmation by using both chromogenic media.

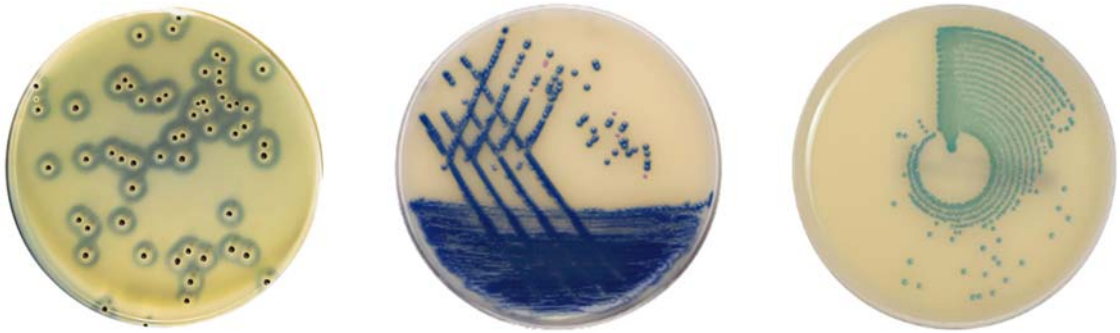


Figure 28. Evaluation of chromogenic media for MRSA detection: Baird Parker media (left side), Brilliance MRSA 2 Agar (center) and ChromID MRSA Agar (right side) (www.oxoid.com; www.biomerieux.com)

Bacterial isolates

A collection of 239 *S. aureus* isolates, comprising 154 methicillin-sensitive *S. aureus* isolates (MSSA), 83 MRSA isolates harbouring *mec*A, and two MRSA isolates harbouring *mec*C, were selected to perform this study *(Appendix 14)*. Isolates were collected from clinical (70 isolates), animal (48 isolates) and food samples (121 isolates) of very diverse geographical origin (Table 20) (Ariza *et al*., 2014a; Ariza-Miguel *et al*. 2014b; Oniciuc *et al*., 2015; Rodríguez-Lázaro *et al*., 2015).

Species confirmation was done by real-time PCR (Trnčíková *et al*., 2008). Further characterization of the *S. aureus* isolates was performed (Ariza *et al*., 2014a; Ariza-Miguel *et al*. 2014b; Oniciuc *et al*., 2015; Rodríguez-Lázaro *et al*., 2015): presence of the β-lactam resistance genes *mec*A and *mec*C by multiplex PCR (Stegger *et al*., 2012), characterization of the SCC*mec* (Kondo *et al*., 2007; Milheiriço *et al*., 2007; Shore *et al*., 2011), the presence of PVL genes (Lina *et al*., 1999), and the antimicrobial susceptibility testing using the microdilution method following the recommendations and MIC breakpoints of the CLSI guidelines (2012) to 20 antimicrobial agents: PEN, OXA, AMC, DAP, ERY, CLI, TEC, VAN, CIP, LVX, AMK, GEN, TOB, MUP, rifampin (RMP), TET, FUS, FOF, LZD and CTX.

Growth of bacterial isolates

Isolated colonies of *S. aureus* isolates on BP agar (ThermoFisher Scientific, Waltham, MA, USA) were streaked onto two chromogenic selective media, Brilliance MRSA 2 Agar (ThermoFisher Scientific) and ChromID MRSA Agar (bioMérieux). Growth and characteristic morphology was assessed after 24 h of incubation at 37°C, as recommended by the manufacturers. Presumptive MRSA strains form blue denim or green colonies on Brilliance MRSA 2 and ChromID MRSA Agars, respectively. Experiments were performed in duplicate, and inconsistencies in the results among the methods were repeated in triplicate.

Statistical analysis

Comparisons between both chromogenic media and *mec*A/*mec*C detection, which was considered the reference method, were performed in terms of sensitivity, specificity, Positive Predictive Value (PPV) and Negative Predictive value (NPV). Differences of analytical performance between chromogenic media, and *mec*A/*mec*C detection, were analyzed using McNemar paired samples non-parametric test with an $\alpha = 0.01$.

Results and discussion

Overall, whereas statistically significant differences were not observed between MRSA confirmation by *mec*A/*mec*C PCR, and by culture in both chromogenic media ($p = 0.013$ and $p = 1.000$ for Brilliance MRSA 2 agar and ChromID MRSA agar, respectively), a statistically significant difference was observed between the results obtained by both chromogenic media ($p = 0.003$). ChromID MRSA agar showed better overall performance values (*i.e.* sensitivity and specificity) than Brilliant MRSA 2 agar (Table 20): 83 and 84 out of 85 *mec*A/*mec*C positive MRSA were detected by Brilliance MRSA 2 agar and ChromID MRSA agar, respectively, corresponding to a sensitivity of 97.7% and 98.8%. The general specificity was also higher for the ChromID MRSA agar (100% *vs* 92.9%) (Table 20). Remarkably, both chromogenic media were capable of detecting *mec*C-positive MRSA.

Segregated analysis of the results depending on the origin of the isolates (clinical, animal, and food) revealed that performance for clinical and animal isolates was excellent regardless the chromogenic media used (*i.e.*, 100% specificity and sensitivity in animal samples, and 100% specificity and sensitivity or 100% and 98.5% in clinical samples by ChromID MRSA agar and Brilliant MRSA 2 agar, respectively). These results are similar to

those obtained previously in human clinical samples (McElhinney *et al.*, 2013; Veenemans *et al.*, 2013). However, a significantly lower performance was observed in the MRSA confirmation of food-derived isolates by using Brilliance MRSA 2 agar in comparison to PCR-based MRSA confirmation (p= 0.003) or ChromID MRSA agar (p= 0.001). In addition, the results obtained by using Brilliance MRSA 2 agar in food-derived isolates differed significantly from those obtained in human and animal isolates (p= 0.0001).

Interestingly, most of the false positives by using Brilliance MRSA 2 agar were detected in milk and cheese samples regardless the time of isolation or the geographical origin (10 out of 12 false negatives; 83.3%). A remarkable finding is that the sensitivity obtained in food samples by both chromogenic media was not 100% as in both cases it was 1 false negative. This particular isolate belongs to a novel emergent MRSA type: OS-MRSA, that harbours *mec*A but it is sensitive to both cefoxitin and oxacillin antibiotics.

In conclusion, the use of chromogenic agar plates for MRSA confirmation of *S. aureus* isolates can provide a good diagnostic performance regardless of the type of chromogenic media used or the origin of the *S. aureus* isolates. However, our results revealed a lower diagnostic performance for MRSA confirmation of *S. aureus* isolates from food samples by using Brilliance MRSA 2 agar. This fact should be taken into account when designing MRSA screening in food samples and food processing facilities-associated isolates.

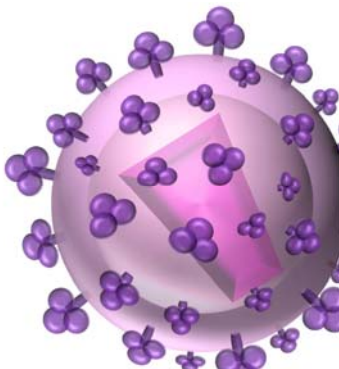
Table 20. *Comparative diagnostic performance of Brilliance MRSA 2 Agar and Chrom ID MRSA Agar for detection of animal, human and food isolates of MRSA*

Performance*	**No. of isolates (n=239)**		**Clinical isolates (n=70)**		**Animal isolates (n=48)**		**Food isolates (n=121)**	
	Brilliance	*Chrom ID*	*Brilliance*	*Chrom ID*	*Brilliance*	*Chrom ID*	*Brilliance*	*Chrom ID*
Positive	83	84	67	68	4	4	12	12
False negative	2	1	1	0	0	0	1	1
Negative	142	154	2	2	44	44	96	108
False positive	12	0	0	0	0	0	12	0
Sensitivity	97.7 (91.7-99.7)	98.8 (93.6-99.8)	98.5 (92.1-99.8)	100 (94.7-100)	100 (40.2-100)	100 (40.2-100)	92.3 (63.9-98.7)	92.3 (63.9-98.7)
Specificity	92.2 (86.8-95.9)	100 (97.6-100)	100 (19.3-100)	100 (19.3-100)	100 (91.9-100)	100 (91.9-100)	88.9 (81.4-94.1)	100 (96.6-100)
PPV	87.4 (79.0-93.3)	100 (95.7-100)	100 (94.6-100)	100 (94.7-100)	100 (40.2-100)	100 (40.2-100)	50.0 (29.2-70.9)	100 (73.4-100)
NPV	98.6 (95.1-99.8)	99.4 (96.4-99.9)	66.7 (11.5-94.5)	100 (19.3-100)	100 (91.9-100)	100 (91.9-100)	99.0 (94.4-99.8)	99.1 (95.0-99.9)

Note: *PPV- positive predictive value; NPV- negative predictive value.

PART 4

GENERAL DISCUSSION



General Discussion

Food safety is an important concept aiming to protect consumers' health. In EU, the General Food Law Regulation must guarantee a high level of protection for consumers' life and health, and fair practices in food trade, for animal health and welfare, plant health and environment.

Moreover, the Treaty of Rome signed in 1957 (EC, 1957) and its revisions stipulates the movement of food and feed manufactured and marketed in the Union as being free, facilitating trade of safe food and feed between member states, in accordance with standards and criteria established by the EU legislation. To achieve these objectives, the EU established and implemented control standards measures related to food and feed hygiene in which all member states are involved.

However, some member states are having border inspection posts mainly for travelers and commercial imports coming from third countries. It is known that high number of travelers coming from the non-EU space are entering to EU with food, either raw or cooked, without any food hygiene control based on the EU legislation regarding food safety.

Illegally imported food represents a worldwide problem nowadays as traceability of such items cannot be performed, in terms of raw material origin and quality, or technological process and hygienic conditions during food processing being known. Food illegally brought can be carried by people through different points of entering the EU such as airports, ports or major railways, post and road entry stations, facilitating the widespread of food- or airborne pathogens. Largest amounts of food items have been confiscated in Spain, the United Kingdom, and Germany (EC 206/2009); in these cases food was mainly carried in the personal luggage of travelers and primarily designated for personal consumption, and secondly for illegal sale (Noordhuizen *et al*., 2013; Beutlich *et al*., 2015; de Melo *et al*., 2015; Rodríguez-Lázaro *et al*., 2015; Schoder *et al*., 2015). A better situation exists in the United States, where some of such foods are microbiologically examined. For example, cheese smuggled into the country by Mexican citizens has been tested for presence of pathogens and has been found that such foods were harboring *Salmonella* (13%), *Listeria* subsp. (4%), and *Mycobacterium* subsp. (Kinde *et al*., 2007). Moreover, given the major ways of pathogens' transmission, the USA custom officers oblige all passengers coming from outside the USA to fulfill a form in which they declare if, previously, they have visited farms and were in contact with animals (Category A) or if they are transporting food products of animal origin or animals infected with zoonotic agents (Category B) (www.cbp.gov). In this way, USA customs have a better control regarding introduction and possible spread of infectious diseases for humans and animals. Similar situation exists in Australia in which import permissions are necessary prior to importing animal origin foods, regulated by the Biosecurity Act (2015).

Furthermore, travelers are not aware about associated risks and incidence of possible pathogens in illegally introduced foods and that the conditions during food transportation and sale might violate the safety rules, as refrigeration and adequate packaging are lacking (Ciolacu *et al*., 2016).

These contribute to possible food-derived diseases in which not only the public health and well-being is affected, but also economic loses are taking place for individuals, families, communities, or at larger scale, countries. Moreover, failure at border inspection posts might facilitate entrance of pathogens by citizens

which have been in contact with apparently healthy animals. Furthermore, a single failure at border control is sufficient to waste decades of financial efforts supported by the farming communities or national authorities (Noordhuizen *et al*., 2013). For example, in case of highly contagious diseases such as classical swine fever (CSF), avian influenza (AIV), porcine epidemic diarrhea (PED), or foot and mouth disease (FMD), many animals must be killed, thus going to huge economic losses.

Foodborne and waterborne diarrheal diseases taken together kill almost two millions of people annually (Food Standards Agency, 2011). By these alarming statistics, outbreaks of foodborne diseases attract social media attention and raise consumers' concern. Despite strong efforts made by all involved agencies for controlling these outbreaks, foodborne pathogens play their role since they can enter the food chain at different steps and can adapt to any kind of environment. In 2013, a total of 5196 foodborne and waterborne outbreaks were reported in the EU causing 43,183 human cases, 5946 hospitalizations, and 11 deaths. Most of them were caused by *Salmonella* (22.5%), viruses (18.1%), bacterial toxins (16.1%), and *Campylobacter* (8.0%) (EFSA and ECDC, 2015). Of great concern is that in 28.9% of all outbreaks, the causative agent was unknown (EFSA and ECDC, 2015).

Nowadays, a dynamic and complex relationship exists between human, animal and environment since globalization, food trade and increased urbanization hit, influencing the organization of food supply chain. The highly demand of food made the global food supply undergo dramatic changes in the last twenty years, forcing the food products to arrive as fast as possible on the consumer's table (Noordhuizen *et al*., 2013). For this, intensive livestock farming has been developed for satisfying consumers but this speed came together with emergence of zoonotic pathogens resistant to antimicrobials through live animals or goods of animal origin (www.cbp.gov).

The purpose of this thesis was to assess presence of *S. aureus*, especially MRSA isolated from foods being introduced into EU *via* uncontrolled imports from terrestrial EU borders and airports. Nevertheless, the thesis treats the actual problematic of this emergent pathogen and its tolerance to antimicrobial agents in which food- security, food safety, food hygiene, and biological risks associated are detailed and the overall findings are discussed.

Food security concept comes into our attention as food availability at all times is necessary to sustain a steady expansion of food consumption. In 2015, the global community proposed to improve people's lives by making great progress in reducing hunger by 2030, according to FAO. However, by 2050 at least 50% of food needs to be produced in order for all population to be fed (www.worldbank.org). In this regard, the top priority of the World Bank Group is focused on investment in agriculture and rural development to boost food production and nutrition (www.worldbank.org). To date, all this comes with a price: higher demand of food needs intensive production for a global food system sustaining conjoined with possible appearance of drug-resistant infections causing global economic damages (www.worldbank.org).

Food safety aspect represents the gateway in which a high level of human' health protection is achieved, however, forgetting about it, is an assuring recipe for disaster. Nowadays, resistance to antimicrobials has become a worldwide problem for both human and animal health sector. The massive use of antibiotics in feed to promote growth, and the inappropriate use of antimicrobial agents in veterinary and human medicine are considered to be major contributors to the emergence of resistance (Oniciuc *et al*., 2017). In addition, overuse of antimicrobial agents has been adopted in animal husbandry for disease prevention, apart to the veterinary use for treatment of a certain disease. In USA, for example, up to 70% of antibiotics

goes to animals raised on industrial farms that are not sick, in order to eradicate the effects of crowding and poor sanitation conditions (www.collective-evolution.com).

Since 1960s, the massive use of antimicrobial agents has facilitated intensive farming, contributing further to increasing outputs together with meat prices lowering. In this way, such amounts of antimicrobials used in livestock led to propagation of resistant bacteria in the animal reservoir (Choffnes *et al.*, 2012), further having consequences on the animal and human health. It has been stated that almost 700,000 people died in 2014 due to antimicrobial resistant bacteria and could increase up to 10 million by 2050, with almost 100 trillion of dollars in economic losses (www.worldbank.org). Further, transmission from animals to humans can take place by different routes in which bacterial movements could play an important role as variants of bacterial clones between animals might appear.

In this case, developments in the epidemiology of MRSA, in hospital (human) settings and primary food production are taking place. Furthermore, MRSA spread along the food chain could be enhanced by an expansion in the international movement of people, animals and food (Oniciuc *et al.*, 2015; Rodríguez-Lázaro *et al.*, 2015). For this, relevance of MRSA in the food safety context remains a public health priority as its incidence is still above 25% in seven of 29 reporting countries, according to ECDC (ECDC, 2015).

Moreover, ***food hygiene*** plays its role for delivering safe foods to consumers. By applying GMP and GHP along the food chain, it is certain at least, that food does not pose any possible threat for consumer's health. However, this thesis has been focused on food samples that are not having any traceability in place, in which we may know exactly the food origin, conditions of transport, storage or other important information related with.

In this thesis, a total of 1079 food products were analyzed, prior being confiscated from luggage of passengers on flights from non-EU countries by border authorities at the border inspection posts in International Bilbao Airport (Spain) (269 food products) and Vienna International Airport (Austria) (600 food products). Moreover, others (210 food products) were collected at terrestrial border traffic between Republic of Moldova and Romania (Giurgiulești-Galați).

The great diversity of food highlights the **biological risks associated**, leading to presumptive foodborne outbreaks due to people's ignorance. Randomized food sampling (August 2012 to July 2015) applied led to investigation of 519 (48.1%) milk and dairy products of diverse animal origin (cow, sheep or goat milk and cheese- either fresh, brined or with spices), 448 (41.52%) meat samples of diverse animal origin (including antelope, beef, chicken, duck, guinea pig, pork, rodents and turkey), fish and fish products (68, 6.3%), 9 eggs (0.83%), and other products such as pastry, alga, biscuits or dried fruits (35, 3.24%).

Regarding foods collected from black market at EU border, presence of MRSA in foods sold in Galati (the South-East part of Romania, on the border with Republic of Moldova) has been assessed, as illegal entrance of foods to EU through black markets at the EU borders can constitute a neglected route of dissemination of foodborne pathogens, and in particular of MRSA. Food illegally brought into the EU, mainly in the personal luggage of travelers, represents a potential threat to consumers' health.

However, at the Eastern EU border, the Romanian Law 10/2010 (Monitorul Oficial) ratifies the agreement on cross-border traffic between Romania and the Republic of Moldova. Among other goods, foods that are officially declared for personal use are legally brought into EU, but illegally sold in local Romanian markets organized to sell fresh fruits and vegetables. Even though selling food of animal origin is forbidden in these

markets and that the local authorities are regularly performing controls and post informative advertisements, food products such as eggs, fish and fish products, milk and dairy products, or meat and meat products are daily sold. Foods, either homemade or industrially produced, are kept at ambient temperatures and displayed out of the original package.

This study investigated 200 food samples collected from 2012 to 2013, searching for MRSA presence in the analyzed food samples introduced into the EU. All *S. aureus* were studied by PFGE, SE profile and antimicrobial susceptibility testing. MRSA isolates were further characterized by MLST and SCC*mec* typing, and tested for the presence of PVL virulence factors. Overall, 32 *S. aureus* isolates were recovered from 16 food samples (8%). One isolate detected in a pork lard sample was MRSA (0.5%), however not enterotoxigenic. Among *S. aureus* isolates, eight of them harbored *sea* gene, while *seg* and *sei* genes were found only in five *S. aureus* isolates.

PFGE with the restriction enzyme *Sma*I revealed 12 genotypes among the 32 *S. aureus* isolates. The MRSA isolate belonged to ST398, harbored SCC*mec* type V, tested negative for the presence of the PVL genes and was resistant to ciprofloxacin, tetracycline and cefazolin, besides all β-lactams. Among 31 MSSA, 29% were resistant to penicillin, 9.7% to tetracycline and 3.2% to ciprofloxacin.

S. aureus spa types t449, t304, and t524 were most often isolated from raw-milk cheeses contaminated with 10^3–10^5 CFU per gram, evidencing a contamination at herd level or unhygienic conditions during processing. *S. aureus* t011 and t3625 were isolated from pork lard and poultry meat. Finally, we reported the presence of LA-MRSA (ST398-MRSA-V) in foods brought by Moldavian citizens but illegally sold in a black market, evidencing that cross-border trade from non-member states represents a neglected route of transmission of foodborne pathogens such as MRSA into the EU, that could lead to sporadic or family-associated cases of disease.

Other studies focused on identification of foodborne pathogens by illegal foods introduced into the EU has been reported so far. For example, Beutlich *et al.* (2015) analyzed a total of 663 confiscated food products from passengers arriving in Germany in which most of the contaminated food was represented by meat (33%), meat products (42%) and milk products (21%). However, their objective was to identify foodborne zoonotic bacteria such as *Salmonella* subsp., *Listeria* subsp., *Campylobacter* subsp., *Yersinia* subsp., verocytotoxin-producing *Escherichia coli* (VTEC) and *Brucella* subsp. but not *S. aureus* resistant to antimicrobials. Later on, Rodríguez-Lázaro *et al.* (2015) performed a study as a concern on the amount of foods which people bring with them in their luggage, confirming presence of foodborne pathogens in such items and more important, presence of CA-MRSA strains in such foods being reported.

Regarding biofilm formation, we further analyzed composition of biofilms formed by ***S. aureus isolated from food sources*** illegally sold in a local Romanian market (Oniciuc *et al.*, 2015)**.** For this, 16 *S. aureus* isolates originating from foods (eight from dairy products, five from fish and fish products and three from meat and meat products) recovered from foods collected from black market at EU border were evaluated regarding their biofilms formation ability. Six strains (E2, E6, E8, E10, E16, and E23) were distinguished as strong biofilm formers, either in standard Tryptic Soy Broth, Tryptic Soy Broth supplemented with 0.4% glucose or with 4% NaCl. The biofilms composition formed by these *S. aureus* strains on polystyrene surfaces was first inferred using enzymatic and chemical treatments. Later on, biofilms were characterized by CLSM.

Our experiments proved that protein-based matrices are of prime importance for the structure of biofilms formed by *S. aureus* strains isolated from food sources. These biofilm matrix compositions are similar to those put into evidence for coagulase negative staphylococci. However, other studies mention abundance of exopolysaccharides in biofilms produced by *S. aureus* isolated from poultry processing plant, proteins occupying only a small fraction of their matrices (Ferreira *et al.*, 2014).

As discussed before, we have detected presence of *S. aureus*, particularly MRSA in food brought into the EU by Moldavian travelers, in which under improper food transportation conditions, heat-stable enterotoxins may develop in such food items, leading to possible foodborne outbreaks. Although in our case, MRSA isolate was not enterotoxigenic, several studies have demonstrated that such MRSA strains isolated from food sources could yield SE types. Furthermore, *S. aureus* isolates have demonstrated to have capacity to form biofilm which is of great concern as it can adhere to different surfaces, leading to serious engineering associated problems. This is a new finding having in view that scientific literature mentions exopolysaccharide abundance in biofilms produced by clinical isolates and food processing environment isolates of *S. aureus*.

Later on, this thesis intended to expand its research by ***tracking MRSA in food entering to the EU via cross border traffic and international flights***. It is already known that MRSA represents the major causative agent of severe and non-severe infections in humans, which in principle started to be a problem for healthcare settings, further reports elicited its presence in livestock.

This study aimed to determine the prevalence and characteristics of MRSA isolated from animal origin foods illegally brought by travelers in their luggage, coming from non-EU countries through international flights and markets close to EU ground borders. Out of 868 food samples confiscated (meat samples of diverse animal origin such as antelope, duck, guinea pig, pork, rodents, turkey; milk and dairy products or eggs), 136 (15.7%) were positive for *S. aureus*, in which 3% were represented by MRSA-positive strains exhibiting *mec*A resistance mechanism. None isolate harbored the *mec*C homologue.

To date, we found a prevalence of 64.6% in *S. aureus*-positive milk and dairy food samples confiscated in International Bilbao Airport (Spain), and the highest value (11.8%) registered for MRSA-positive samples. Such values have been reported for food-producing animals and retail meat in North Dakota, USA, accounting the highest prevalence in chicken (67.6% out of 37) (Buyukcangaz *et al.*, 2013). Furthermore, de Neeling *et al.* (2007) reported an unexpected high prevalence of MRSA (39% out of 540) associated with pigs and people in contact with such animals.

Furthermore, MRSA isolates were studied by PFGE and antimicrobial susceptibility testing, later characterized by MLST, SCC*mec* typing and tested for the presence of SEs genes and Panton-Valentine leukocidin virulence factors.

Fourteen resistance profiles resulted, out of which a high number of MRSA isolates showed to be multiresistant to three or more antimicrobial agents and sensitive to all non β-lactams tested. *Sma*I-PFGE provided a fingerprint pattern consisting of 13-17 DNA fragments of 20-670 kbp, approximately. The predominant genetic spread belonged to ST5 (30.8%), followed by ST8 (15.4%), ST1649 (15.4%), ST1 (11.5%) and other lineages locally distributed such as ST7 (7.7%), ST22 (7.7%), ST72 (3.8%), ST97 (3.8%) and ST398 (3.8%).

Seven isolates were tested positive for *luk*-PVL genes (SCC*mec* IV- subtypes IVc and IVe). Enterotoxin profile revealed that 19 MRSA strains were enterotoxigenic, harboring one or more *se* genes. Interestingly, MRSA isolates tested positive for *luk*-PVL genes were not enterotoxigenic, as well as none of the isolates of the total of MRSA tested positive for enterotoxin E. Of great importance is the isolation of an OS-MRSA strain harboring SCC*mec* type V, positive for *sed*, *seg*, and *sej* genes but negative for PVL virulence factors.

Besides, MRSA strains recovered from foods illegally transported by travelers *via* ground border and international flights were tested to observe if there is any ***correlation between biofilm formation and composition and their molecular aspects***.

As evidenced before, there is a growing concern regarding potential transmission of MRSA by illegal entrance of food to Europe. From a food safety perspective, different MRSA lineages can be acquired *via* food manipulation and/or consumption, and consequently circulation of multidrug resistant strains may arise.

In this assay, our objective was focused on obtaining further information regarding MRSA strains capacity to build biofilms. Likewise, matrix composition involved in the attachment and colonization of food-contact surfaces has been evaluated.

Forty-nine MRSA isolates recovered from animal food origin, previously confiscated from travelers' luggage coming from non-EU countries, were evaluated for their biofilm formation (24 h) ability using TSB supplemented with 0.4% glucose in which MRSA isolates had the capacity to accumulate moderate (83.7%) to strong (16.3%) amounts of biofilm biomasses on polystyrene surfaces. Resistance to antimicrobial agents can be also related to different responses in attachment and biofilm formation.

Further, different biofilm patterns related to MRSA lineages have been observed, in which those harboring SCC*mec* type IV produced more biomass after 48 h incubation. This is in accordance with Vanhommerig *et al.* (2014) in which MRSA strains harboring smaller cassettes (types IV, V) have been shown to produce higher biofilm biomasses than those carrying types I-III, this fact being later confirmed by Parisi and coworkers (2016).

Besides, composition of biofilms with an OD_{570} higher than 3 was evidenced by CLSM after exposure to three types of dyes: SYTO9 (nucleic acids), SYPRO Ruby (proteins), and WGA-TRITC (*N*-acetyl-D-glucosamine residues). Half of the isolates formed flat and compact structures, while the rest of them developed highly fluorescent cell aggregates areas within the three-dimensional structures.

In conclusion, this study emphasizes the possible risks among population associated with presence of multidrug resistant and enterotoxigenic strains such as MRSA, additionally capable of forming biofilms, in food products illegally introduced into EU by citizens coming from America (Dominican Republic, Cuba, Honduras, Nicaragua, Panama, Mexico, Bolivia, Paraguay, Ecuador, Argentina, Colombia, Peru, Brazil), Europe (Albania, Kosovo, Montenegro, Romania, Serbia, Macedonia, Russian Federation (Moscow), Ukraine, Republic of Moldova), Africa (Tunisia, Egypt, Nigeria) or Asia (Armenia, North Korea, Turkey, China). In addition, this could become a serious concern as most of foods encountered in passenger's luggage were not previously suffered a thermal treatment. Further, routine surveillance and control is needed regarding foods illegally introduced in the EU space as MRSA strains are freely being distributed and promote biofilm formation.

Of great interest for this thesis, from a food safety point of view, is the successful isolation of an ***OS-MRSA associated with a processed food product***, highlighting the potential role of food brought by passengers *via* international flights as a neglected route of dissemination of new emergent MRSA variants. Although such isolates have been reported among clinical ones, isolation from animals and food products has been described quite recently. Such MRSA variants are representing a challenge because routine microbiological testing cannot identify them due to an oxacillin susceptible phenotype.

In this study, OS-MRSA 41 has been found in a dairy product (unknown cheese origin) confiscated from a passenger travelling from Cairo, Egypt towards Vienna, Austria. Antimicrobial susceptibility testing showed to be resistant to penicillin, tetracycline, erythromycin but susceptible to oxacillin (<2 μg/mL). Further testing showed to harbor the *mec*A gene by PCR. WGS analysis revealed SNPs located in the *mec*A and *blaZ* genes, which could perhaps have influenced the low level of oxacillin in OS-MRSA 41. Before, such investigation led in describing an oxacillin-susceptible CC80 *mec*A-positive *S. aureus* in a clinical isolate (Sabat *et al*., 2015), but is the first time such an analysis is performed for an OS-MRSA (MRSA-ST5-V) isolated from a food product.

Interestingly, until now, no OS-MRSA strains have been reported in associated foods, mostly being described in medical settings and rarely in livestock. However, it seems to be widely distributed, from Asia to Europe and Africa continents (Pournaras *et al*., 2013). A recent study highlighted the successful isolation of an OS-MRSA associated to bovine mastitis in China (Pu *et al*., 2014) and camel meat samples in Saudi Arabia (Malhotra-Kumar *et al*., 2010).

Even though 0.17% of food samples (no. samples 600) tested in this study was represented by one MRSA variant, it should not be neglected due to its particular relevance for public health. It can develop highly resistant MRSA variants under treatment with β-lactams. Once more, we are highlighting the potential role of food as neglected route of transmission of MRSA and other MRSA variants, stressing the need for monitoring and controlling possible spread of such foodborne pathogens. Close attention shall be conferred to new emerging MRSA variants as additional genetic factors and mechanisms may appear, crypting the methicillin resistance.

The last part of this thesis is proposing to ***evaluate two commercially available chromogenic media for confirmation of MRSA from human, animal, and food samples.*** We have compared the diagnostic performance of two chromogenic media, Brilliance MRSA 2 agar (ThermoFisher Scientific) and ChromID MRSA agar (bioMérieux), for MRSA confirmation of 239 *S. aureus* isolates from clinical (70 isolates), animal (48 isolates) and food samples (121 isolates) of very diverse geographical origin. Statistically, significant differences were not observed between MRSA confirmation by *mec*A/*mec*C PCR, and by culture in both chromogenic media.

However, a statistically significant difference was observed between the results obtained by both chromogenic media (p= 0.003). Segregated analysis of the results depending on the origin of the isolates (clinical, animal, and food) revealed a significant lower performance in the MRSA confirmation of food-derived isolates by using Brilliance MRSA 2 agar in comparison to PCR confirmation (p= 0.003) or ChromID MRSA agar (p= 0.001). Our results are in accordance to those obtained previously by McElhinney *et al.* (2013) and Veenemans *et al.* (2013) in testing clinical samples.

Both chromogenic media provided a good diagnostic performance for detection of MRSA isolates of human and animal origin. In conclusion, the use of chromogenic agar plates for MRSA confirmation of *S. aureus* isolates can provide a good diagnostic performance (sensitivity > 92% and specificity > 89%) regardless of the type of chromogenic media used or the origin of the *S. aureus* isolates. However, our results revealed a lower diagnostic performance for MRSA confirmation of *S. aureus* isolates from food samples by using Brilliance MRSA 2 agar, fact which should be considered when designing chromogenic media especially for MRSA confirmation in food samples.

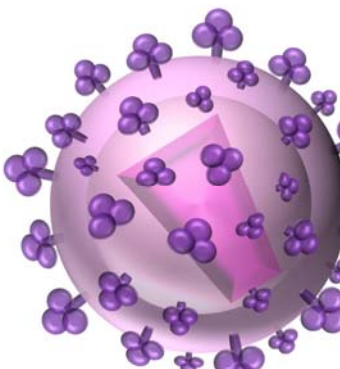
Overall, these findings highlighted in the present thesis confirm once again the potential role of food in the dissemination of MRSA lineages among population, and the potential role of illegally introduced food into EU by road and air entries. Confiscated foods were coming from travelers with a diverse geographical origin such as South, Central and North America, Europe, Africa, or Asia accounting a wide dissemination of MRSA lineages.

We have demonstrated that foods, without being able to trace back to its origin, not knowing how they were transported or manipulated, either being processed or homemade food, may lead to appearance of foodborne outbreaks since raw food products that have been found in passenger's luggage, products which have not suffered previously a thermal treatment, could lead to the spread of multidrug resistant and enterotoxigenic strains such as MRSA.

It is known that EU has issued several regulations regarding introduction of animal origin foods into the EU space but, however, people's disregard is still of concern as they are trespassing with food saying that is intended for personal use but ending by selling them in markets. Therefore, the purpose of this thesis regarding MRSA dissemination has been attained, stressing the need of an adequate control and prevention programmes to avoid food safety issues together with the protection of human life and health.

PART 5

CONCLUDING REMARKS



Concluding Remarks

Research activities accomplished in the current doctoral thesis have been focused on analysis of MRSA strains isolated from animal origin foods, either homemade or industrially produced, illegally introduced to EU from non-EU countries. Based on the results obtained, several concluding remarks have been formulated:

- Elevated prevalence of *Staphylococcus aureus* (14.1%) and MRSA (2.5%) in foods highlights the potential risk generated at public health level by foods illegally entered to EU through different neglected routes as airports or terrestrial borders;
- Presence of enterotoxigenic HA-, CA- and LA-MRSA strains identified in animal origin foods illegally introduced to EU should not be neglected as their potential pathogenic role is yet unknown;
- Activation of classical enterotoxins and their potential presence in diverse food products brought by travelers in their luggage should not be disregarded;
- Detection of distinct genetic lineages associated to livestock (ST398-MRSA-V) and community settings (ST8-MRSA-IV/V and ST1649-MRSA-IV) emphasizes that illegal importation of animal origin foods constitutes routes of dissemination of *S. aureus* resistant to antimicrobials;
- Successful isolation, for the first time, of a ST5-OS-MRSA-V (OS-MRSA 41) strain associated to a processed food illegally transported by a passenger from Turkey towards Vienna. WGS analysis showed that several mutations in the *mec*A and *blaZ* resistance genes could be responsible for the low-level oxacillin MIC in the genetic background of OS-MRSA 41 strain;
- Biofilm formation assays evidenced the capacity of *S. aureus* and MRSA strains to build moderate to strong biofilms;
- Different biofilm patterns related to distinct MRSA lineages have been noticed, greater production of biofilm biomasses being evidenced for those harboring SCC*mec* type IV;
- Use of conventional microbiological methods have been succeeded by the molecular detection techniques, more sensitive, for validating and/or evidencing genetic patterns among analyzed isolates;
- Poor diagnostic performance of chromogenic agar plates, such as Brilliance MRSA 2 agar, for MRSA confirmation of *S. aureus* isolated from food samples, revealed that the exclusive use of conventional methods could lead to false positive results;
- Need for efficient control measures at the border inspection posts is imperative for lowering down the prevalence and dissemination of MRSA related with food producing animals (raw materials for food industry) and associated foodstuff.

Summarizing, outbreaks could be detected earlier if the holistic strategies could undergo, encompassing all relevant aspects of the food chain in the community as a whole, from primary production to final consumers.

By the attained research, we have demonstrated the necessity of conducting such studies to characterize MRSA strains isolated from foods from a phenotypical and genotypical point of view, in order to improve the prevention/control and surveillance programmes. Moreover, the research opens new directions for food processing facilities, contributing to the improvements of food safety and hygiene.

Beyond scientific aspects, this thesis had contributed to the overall database regarding the epidemiology of *Staphylococcus aureus* resistant to antimicrobials.

Concluzii Generale

Activitățile de cercetare realizate în această teză de doctorat s-au axat pe analiza tulpinilor de MRSA izolate din alimente de origine animală, produse în casă sau la nivel industrial, introduse ilegal în UE din țări care nu au aderat la această comunitate. Pe baza rezultatelor obținute, au fost formulate mai multe concluzii:

- Prevalența crescută a bacteriei *Staphylococcus aureus* (14,1%) și MRSA (2,5%) în alimente evidențiază riscul potențial generat asupra consumatorilor, cauzat de introducerea în UE a produselor alimentare, pe cale ilegală, prin diferite rute, cum ar fi aeroporturile sau frontierele terestre;
- Prezența tulpinilor enterotoxigenice HA-, CA- și LA-MRSA identificate în alimentele de origine animală introduse ilegal în UE nu trebuie neglijată, deoarece rolul lor patogen nu este cunoscut;
- Activarea enterotoxinelor clasice și prezența lor potențială în produsele transportate de călători în bagajele lor nu ar trebui ignorate;
- Detectarea liniilor genealogice distincte asociate animalelor (ST398-MRSA-V) sau comunităților (ST8-MRSA-IV/V și ST1649-MRSA-IV) subliniază faptul că importul ilegal de alimente de origine animală constituie căi de transmisie a bacteriei *S. aureus* rezistentă la antibiotice;
- A fost izolată pentru prima oară o tulpină ST5-OS-MRSA-V (OS-MRSA 41) asociată cu un produs procesat de origine animală, transportat ilegal de către un pasager din Turcia către aeroportul din Viena. Analiza WGS a arătat că mai multe mutații în genele de rezistență *mec*A și *blaZ* ar putea fi responsabile pentru nivelul scăzut de concentrație minimă inhibitorie a oxacilinei în background-ul genetic al acestei tulpini;
- Evaluarea capacității tulpinilor de *S. aureus* și MRSA de a forma biofilme, evidențiind faptul că acestea pot adera moderat sau puternic pe suprafețe;
- S-au observat diferite capacități de aderare a biofilmelor funcție de liniile genealogice ale tulpinilor de MRSA analizate, unde producție mai mare de biomasă s-a observat în cazul acelor tulpini care posedă SCC*mec* type IV;
- Utilizarea metodelor microbiologice convenționale a fost urmată de tehnicile de detecție moleculară, mai sensibile, pentru validarea și/sau evidențierea tiparelor genetice în rândul tulpinilor analizate;
- Performanță scăzută a mediului cromogen Brilliance MRSA 2 în ceea ce privește confirmarea tulpinilor de MRSA izolate din produsele alimentare, indicând faptul că utilizarea exclusivă a metodelor convenționale ar putea duce la rezultate fals pozitive;
- Necesitatea unor măsuri eficiente la punctele de control la frontieră este imperativă pentru reducerea prevalenței și diseminării MRSA legate de materiile prime provenite de la animale și alimentele asociate.

Rezumând, epidemiile ar putea fi detectate din timp dacă s-ar aplica strategii holistice, care să cuprindă toate aspectele relevante ale lanțului alimentar în ansamblu, de la producția primară la consumatorii finali.

Prin cercetarea realizată, am demonstrat necesitatea efectuării unor astfel de studii pentru a caracteriza tulpinile de MRSA izolate din alimente din punct de vedere fenotipic și genotipic, pentru a îmbunătăți programele de prevenire/control și supraveghere. Mai mult, cercetarea deschide noi direcții pentru fabricile de procesare a alimentelor, contribuind la îmbunătățirea siguranței și igienei alimentare.

Dincolo de aspectele științifice, această teză contribuie la baza de date globală privind epidemiologia lui *Staphylococcus aureus* rezistent la antimicrobiene.

Conclusiones

Las actividades de investigación llevadas a través de la tesis doctoral se han centrado en el análisis de cepas de MRSA aisladas de alimentos de origen animal, caseras o producidas industrialmente, introducidas ilegalmente en la UE de países no pertenecientes a la UE. Sobre la base de los resultados obtenidos, se han formulado varias observaciones finales:

- Prevalencia de *Staphylococcus aureus* (14.1%) y MRSA (2.5%) en los alimentos constituye un riesgo potencial generado a nivel de salud pública, por los alimentos ingresados ilegalmente en la UE a través de diferentes rutas descuidadas como aeropuertos o fronteras terrestres;
- No debe descuidarse la presencia de cepas enterotoxigénica de HA, CA y LA-MRSA identificadas en alimentos de origen animal introducidos ilegalmente en la UE, ya que aún no se conoce su posible función patogénica;
- No debe descartarse la activación de las enterotoxinas clásicas y su presencia potencial en diversos productos traídos por los viajeros en su equipaje;
- La detección de distintos linajes genéticos asociados al ganado (ST398-MRSA-V) y la comunidad (ST8-MRSA-IV/V y ST1649-MRSA-IV) enfatiza que la importación ilegal de alimentos de origen animal constituye rutas de diseminación de *S. aureus* resistente a los antimicrobianos;
- El aislamiento, por primera vez, de una cepa ST5-OS-MRSA-V (OS-MRSA 41) asociada a un alimento procesado ilegalmente transportado por un pasajero de Turquía hacia Viena. El análisis WGS mostró que varias mutaciones en los genes de resistencia *mec*A y *blaZ* podrían ser responsables de la MIC de oxacilina, de bajo nivel, en el fondo genético de la cepa OS-MRSA 41;
- Los ensayos de formación de biofilm demostraron la capacidad de las cepas de *S. aureus* y MRSA para construir moderada y fuerte biofilms;
- Se han observado diferentes patrones de biofilm relacionados con distintos linajes de MRSA, y se evidencia una mayor producción de biomasa de biofilms para los que tiene SCC*mec* tipo IV;
- El uso de métodos microbiológicos convencionales ha sido seguido por las técnicas de detección molecular, más sensibles, para validar y/o evidenciar patrones genéticos entre los aislamientos analizados;
- El diagnóstico de bajo nivel de las placas de agar cromogénico, como el Brilliance MRSA 2, para la confirmación de cepas de *S. aureus* aislada de muestras de alimentos, reveló que el uso exclusivo de métodos convencionales podría conducir a resultados falsos;
- La necesidad de eficaces métodos de control en los puestos de inspección fronterizos es imprescindible para reducir la prevalencia y la diseminación de MRSA relacionados con los animales productores de alimentos (materias primas para la industria alimentaria) y los productos asociados.

Resumiendo, los brotes se podrían detectar más temprano si la estrategia holística podría ser seguido, abarcando todos los aspectos relevantes de la cadena alimentaria en la comunidad en su conjunto, desde la producción primaria hasta los consumidores finales.

Por la investigación obtenida, hemos demostrado la necesidad de llevar a cabo estos estudios para caracterizar las cepas de MRSA aisladas de los alimentos desde un punto de vista fenotípico y genotípico, mejorar los programas de prevención/control y vigilancia. Además, la investigación abre nuevas direcciones para las instalaciones de procesamiento de alimentos, contribuyendo a mejorar la seguridad alimentaria.

Más de los aspectos científicos, esta tesis ha contribuido a la base de datos global sobre la epidemiología de *Staphylococcus aureus* resistente a los antimicrobianos.

Original contributions

By overall results obtained in the present thesis, I have contributed to the extension of the current knowledge related to *Staphylococcus aureus* and its antimicrobial resistance, by the following key elements:

- Assessing for the first time *S. aureus* for being introduced into EU *via* uncontrolled imports (such as raw and RTE food collected either from airports or from terrestrial EU borders);
- Reporting for the first time presence of HA-, CA-, and LA- MRSA strains in food confiscated from non-EU flights and ground borders;
- Reporting for the first time ST5-OS-MRSA-V associated to a processed food product. This finding highlights the potential role of food as a neglected route of dissemination of this new emerging MRSA variant;
- Comparative genome analysis of OS-MRSA strain with other *S. aureus* strain already published in the literature;
- Characterization of biofilm matrices of *S. aureus* strains by chemical and enzymatic tests, reporting that proteins were the main source for maintaining the structure of biofilms formed by *S. aureus* strains isolated from food sources (confirmed by CLSM assays). Such studies are necessary as better strategies may be developed for solution-based cleaning surfaces;
- Comparing the genotypic features of MRSA isolated strains with their biofilm capacity, in which we have confirmed that those harboring smaller SCC*mec* cassettes are showing greater capacity of forming biofilms;
- Proving the necessity of using molecular biology techniques such as PFGE or MLST for evidencing genetic relationships that might exist among analyzed MRSA isolates. Results obtained helped on improving the database regarding this foodborne pathogen, the existing genetic relationship among isolates but also comparing allelic profiles with data available in the *S. aureus* MLST database for traceability purposes;
- Managing different sequencing and bioinformatic tools for analyzing and interpreting data obtained in the present thesis;
- Confirmation of the potential role of food in the prevalence and dissemination of successful MRSA lineages among illegally introduced and sold food into the EU;
- Implementation of surveillance programmes for the food chain in Romania in case of an early outbreak caused by livestock associated clones;
- Research achieved in the current doctoral thesis opens new directions on how MRSA should be regarded from a food safety perspective, whereas monitoring and surveillance programmes should not be watched as options but as fundamental for MRSA control.

Future research perspectives

- Correlating genotypic features of the analyzed MRSA isolates with their susceptibility to essential oils and plant extracts;
- Evaluating the antibiofilm activity of such alternative compounds (essential oils and different solvent extracts) against MRSA biofilms;
- Evaluating alternative coatings (based on zinc oxide nanoparticles) on inhibiting *S. aureus* to adhere and form biofilms;
- Risk assessment of *S. aureus* and its antimicrobial resistance along the food chain in Romania;
- Contributing/ or coming with improvements of the overall food safety in Romania.

APPENDIXES

Protocol for detection of *mec*A and *mec*C by multiplex PCR (Stegger *et al.*, 2012)

	Reagents's Traceability				PCR				
REACTIVES	**Supplier**	**Reference**	**Stock conc.**	**Units**	**Final conc.**	**Units**	**µL/ reaction**	**MIX**	**SUPERMIX**
Primer Mix	MWG	(Stegger *et al.* 2012)	10	µM/each	0.4	µM/each	1	1	--
PCR Buffer	Roche	12 161 559	10	×	1	×	2.5		2.5
$MgCl_2$ Solution	Applied	58002032-1	25	mM	2	mM	2		2
PCR Nucleotide Mix	Roche	11 581 295 001	10	mM/each	0.2	mM	0.5	22	0.5
FastStart Taq DNA Polymerase	Roche	04 659 163	5	U/µL	1	U	0.2		0.2
Water Molecular Biology Reagent	Sigma	W4502	--	--	--	--	16.8		16.8
DNA	--	--	1	ng/µL	2	ng	2	--	--
							25	***23***	***22***

Number Total reactions per MIX 1
Number Total reactions 1

Thermocycler conditions:

1 cycle	95°C	5 min
35 cycles	95°C	1 min
	59°C	1 min
	72°C	1 min
1 cycle	72°C	7 min

Genes to amplify:

	Primer sequence (5'→3')		Product size (bp)	Tm supplier(°C)	Tm used(°C)
*mec*A	mecA P4	TCCAGATTACAACTTCACCAGG	162	58.4	59
	mecA P7	CCACTTCATATCTTGTAACG		53.2	
*mec*C	mecAlga251 MultiFP	GAAAAAAAGGCTTAGAACGCCTC	138	58.9	
	mecAlga251 MultiRP	GAAGATCTTTTCCGTTTTCAGC		56.5	

Gel electrophoresis in 2% low EEO agarose (10 µL PCR product)

Protocol for detection of SCC*mec* (*ccr* gene complex type) by multiplex PCR (Kondo *et al.*, 2007)

	Reagents's Traceability				PCR			
REACTIVES	**Supplier**	**Reference**	**Stock conc.**	**Units**	**Final conc.**	**Units**	**µL/ reaction**	**MIX**
Primer Mix	MWG	(Kondo *et al.*, 2007)	2	µM/each	0.2	µM/each	2.5	2.5
Multiplex PCR Master Mix	QIAGEN	206143	2	×	1	×	12.5	12.5
Water Molecular Biology Reagent	Sigma	W4502	--	--	--	--	5	5
DNA	--	--	1	ng/µL	5	ng	5	--
							25	***20***
Number Total reactions per MIX	1							
Number Total reactions	1							

Thermocycler conditions:

1 cycle	95°C	15 min
30 cycles	94°C	30 sec
	57°C	1 min 30 sec
	72°C	2 min
1 cycle	72°C	10 min

Genes to amplify:

		Primer sequence (5'→3')	Product size (bp)	Tm supplier(°C)	Tm used(°C)
*mec*A	mA1	TGCTATCCACCCTCAAACAGG	286	59.8	57
	mA2	AACGTTGTAACCACCCCAAGA		57.9	
type 1 (A1B1)	α1	AACCTATATCATCAATCAGTACGT	695	55.9	
	βc	ATTGCCTTGATAATAGCCITCT		54.7	
type 2 (A2B2)	α2	TAAAGGCATCAATGCACAAACACT	937	57.6	
	βc	ATTGCCTTGATAATAGCCITCT		54.7	
type 3 (A3B3)	α3	AGCTCAAAAGCAAGCAATAGAAT	1791	55.3	
	βc	ATTGCCTTGATAATAGCCITCT		54.7	
type 4 (A4B4)	α4.2	GTATCAATGCACCAGAACTT	1287	53.2	

	β4.2	TTGCGACTCTCTTGGCGTTT		57.3
type 5 (C1)	γR	CCTTTATAGACTGGATTATTCAAAATAT	518	56.3
	γF	CGTCTATTACAAGATGTTAAGGATAAT		57.4

Gel electrophoresis in 1.5% agarose gels (25 µL PCR product

Protocol for detection of SCC*mec* (*mec* gene complex class) by multiplex PCR (Kondo *et al.*, 2007)

	Reagents's Traceability				PCR			
REACTIVES	**Supplier**	**Reference**	**Stock conc.**	**Units**	**Final conc.**	**Units**	**µL/ reaction**	**MIX**
Primer Mix	MWG	(Kondo *et al.*, 2007)	2	µM/each	0.2	µM/each	2.5	2.5
Multiplex PCR Master Mix	QIAGEN	206143	2	×	1	×	12.5	12.5
Water Molecular Biology Reagent	Sigma	W4502	--	--	--	--	5	5
DNA	--	--	1	ng/µL	10	ng	5	--
							25	***20***
Number Total reactions per MIX	1							
Number Total reactions	1							

Thermocycler conditions:

1 cycle	95°C	15 min
30 cycles	94°C	30 sec
	60°C	1 min 30 sec
	72°C	2 min
1 cycle	72°C	10 min

Genes to amplify:

		Primer sequence (5'→3')	Product size (bp)	Tm supplier(°C)	Tm used(°C)
A	mA7	ATATACCAAACCCGACAACTACA	1963	57.1	60
	mI6	CATAACTTCCCATTCTGCAGATG		58.9	
B	mA7	ATATACCAAACCCGACAACTACA	2827	57.1	
	IS7	ATGCTTAATGATAGCATCCGAATG		57.6	
C	mA7	ATATACCAAACCCGACAACTACA	804	57.1	
	IS2(iS-2)	TGAGGTTATTCAGATATTTCGATGT		56.4	

Gel electrophoresis in 1.5% agarose gels (25 µL PCR product)

Protocol for subtyping the SCC*mec* type IV by multiplex PCR (Milheiriço *et al.*, 2007)

	Reagents's Traceability				PCR				
REACTIVES	**Supplier**	**Reference**	**Stock conc.**	**Units**	**Final conc.**	**Units**	**µL/ reaction**	**MIX**	**SUPERMIX**
Primer Mix 1	MWG		20	µM	0.2	µM	0,5	0.5	--
Primer Mix 2	MWG		20	µM	0.4	µM	1	1	--
Primer Mix 3	MWG	(Milheiriço *et al.*, 2007)	20	µM	0.8	µM	2	2	--
Primer Mix 4	MWG		20	µM	0.9	µM	2.25	2.25	--
Primer Mix 5	MWG		20	µM	1.8	µM	4.5	4.5	--
PCR Buffer	Roche	12 161 559	10	×	1	×	5		5
$MgCl_2$ Solution	Applied	58002032-1	25	mM	2	mM	4		4
PCR Nucleotide Mix	Roche	11 581 295 001	10	mM/each	0.2	mM	1	34.75	1
FastStart Taq DNA Polymerase	Roche	04 659 163	5	U/µL	2	U	0.4		0.4
Water Molecular Biology Reagent	Sigma	W4502	--	--	--	--	24.35		24.35
DNA	--	--	1	ng/µL	5	ng	5	--	--
							50	***45***	***34.75***

Number Total reactions per MIX 1
Number Total reactions 1

Thermocycler conditions:

1 cycle	95°C	5 min
35 cycles	95°C	1 min
	48°C	30 sec
	72°C	2 min
1 cycle	72°C	7 min

Genes to amplify:

		Primer sequence (5'→3')	Product size (bp)	Tm supplier(°C)	Tm used (°C)
ccrB2 (internal positive control)	ccrB2 F	CGAACGTAATAACATTGTCG	203	53.2	48
	ccrB2 R	TTGGCWATTTTACGATAGCC		53.2	
Type IVa	J IVa F	ATAAGAGATCGAACAGAAGC	278	53.2	
	J IVa R	TGAAGAAATCATGCCTATCG		53.2	
Types IVb and IVF	J IVb F	TTGCTCATTTCAGTCTTACC	336	53.2	
	J IVb R	TTACTTCAGCTGCATTAAGC		53.2	
Types IVc and IVE	J IVc F	CCATTGCAAATTTCTCTTCC	483	53.2	
	J IVc R	ATAGATTCTACTGCAAGTCC		53.2	
Type IVd	J IVd F	TCTCGACTGTTTGCAATAGG	575	53.2	
	J IVd R	CAATCATCTAGTTGGATACG		53.2	
Type IVg	J IVg F	TGATAGTCAAAGTATGGTGG	792	53.2	
	J IVg R	GAATAATGCAAAGTGGAACG		53.2	
Type IVh	J IVh F	TTCCTCGTTTTTTCTGAACG	663	53.2	
	J IVh R	CAAACACTGATATTGTGTCG		53.2	

Gel electrophoresis in 2% low EEO agarose (20 µL PCR product)

Protocol for Panton-Valentine Leukocidin gene detection by conventional PCR (Lina *et al.*, 1999)

	Reagents's Traceability				PCR				
REACTIVES	**Supplier**	**Reference**	**Stock conc.**	**Units**	**Final conc.**	**Units**	**µL/ reaction**	**MIX**	**SUPERMIX**
Primer Forward	MWG	(Lina *et al.*, 1999)	10	µM	0.5	µM	1	1	--
Primer Reverse	MWG		10	µM	0.5	µM	1	1	--
PCR Buffer	Roche	12 161 559	10	×	1	×	2		2
$MgCl_2$ Solution	Applied	58002032-1	25	mM	2	mM	1.6		1.6
PCR Nucleotide Mix	Roche	11 581 295 001	10	mM/each	0.2	mM	0.4	16	0.4
FastStart Taq DNA Polymerase	Roche	04 659 163	5	U/µL	0.5	U	0.1		0.1
Water Molecular Biology Reagent	Sigma	W4502	--	--	--	--	11.9		11.9
DNA	--	--	1	ng/µL	2	ng	2	--	--
							20	***18***	***16***

Number Total reactions per MIX 1
Number Total reactions 1

Thermocycler conditions:

1 cycle	95°C	5 min
30 cycles	95°C	30 sec
	55°C	30 sec
	72°C	1 min
1 cycle	72°C	7 min

Gene to amplify:

	Primer sequence (5'→3')		**Product size (bp)**	**Tm supplier (°C)**	**Tm used (°C)**
luk-PV	luk-PV Up	ATCATTAGGTAAAATGTCTGGACATGATCCA	433	62.9	55
	luk-PV Dn	GCATCAASTGTATTGGATAGCAAAAGC		61.9	

Protocol for staphylococcal enterotoxin genes (*sea-see*) detection by multiplex PCR (Gonano *et al.*, 2009)

	Reagents's Traceability		PCR						
REACTIVES	**Supplier**	**Reference**	**Stock conc.**	**Units**	**Final conc.**	**Units**	**µL/ reaction**	**MIX**	**SUPERMIX**
Primer Mix 1	Eurofins		10	µM	0.4	µM	1	**1**	--
Primer Mix 2	Eurofins		10	µM	0.4	µM	1	**1**	--
Primer Mix 3	Eurofins	(Gonano *et al.*, 2009)	10	µM	0.4	µM	1	**1**	--
Primer Mix 4	Eurofins		10	µM	0.4	µM	1	**1**	--
Primer Mix 5	Eurofins		10	µM	0.4	µM	1	**1**	--
PCR Buffer	Roche	12 161 559	10	×	1	×	2.5		2.5
$MgCl_2$ Solution	Applied	58002032-1	25	mM	2	mM	2		2
PCR Nucleotide Mix	Roche	11 581 295 001	10	mM/each	0.2	mM	0.5	**18**	0.5
FastStart Taq DNA Polymerase	Roche	04 659 163	5	U/µL	2	U	0.4		0.4
Water Molecular Biology Reagent	Sigma	W4502	--	--	--	--	12.6		12.6
DNA	--	--	1	ng/µL	2	ng	2	--	--
							25	***23***	***18***

Number Total reactions per MIX 1
Number Total reactions 1

Thermocycler conditions:

1 cycle	95°C	5 min
30 cycles	95°C	1 min
	55°C	1 min
	72°C	1 min
1 cycle	72°C	7 min

Genes to amplify:

		Primer sequence (5'→3')	Product size (bp)	Tm supplier (°C)	Tm used (°C)
sea	GSEAR1	GGTTATCAATGTGCGGGTGG	102	59.4	**55**
	GSEAR2	CGGCACTTTTTTCTCTTCGG		57.3	
seb	GSEBR1	GTATGGTGGTGTAACTGAGC	164	57.3	
	GSEBR2	CCAAATAGTGACGAGTTAGG		55.3	
sec	GSECR1	AGATGAAGTAGTTGATGTGTATGG	451	57.6	
	GSECR2	CACACTTTTAGAATCAACCG		53.2	
sed	GSEDR1	CCAATAATAGGAGAAAATAAAAG	278	51.7	
	GSEDR2	ATTGGTATTTTTTTTCGTTC		47.0	
see	GSEER1	AGGTTTTTTCACAGGTCATCC	209	55.9	
	GSEER2	CTTTTTTTTCTTCGGTCAATC		52.0	

Gel electrophoresis in 3% low EEO agarose (10 µL PCR product)

Protocol for staphylococcal enterotoxin genes (*seg-sej*) detection by multiplex PCR (Gonano *et al.*, 2009)

	Reagents's Traceability				PCR				
REACTIVES	**Supplier**	**Reference**	**Stock conc.**	**Units**	**Final conc.**	**Units**	**µL/ reaction**	**MIX**	**SUPERMIX**
Primer Mix 1	Eurofins		10	µM	0.4	µM	1	**1**	--
Primer Mix 2	Eurofins	(Gonano *et al.*, 2009)	10	µM	0.4	µM	1	**1**	--
Primer Mix 3	Eurofins		10	µM	0.4	µM	1	**1**	--
Primer Mix 4	Eurofins		10	µM	0.4	µM	1	**1**	--
PCR Buffer	Roche	12 161 559	10	×	1	×	2.5		2.5
$MgCl_2$ Solution	Applied	58002032-1	25	mM	2	mM	2		2
PCR Nucleotide Mix	Roche	11 581 295 001	10	mM/each	0.2	mM	0.5	**19**	0.5
FastStart Taq DNA Polymerase	Roche	04 659 163	5	U/µL	2	U	0.4		0.4
Water Molecular Biology Reagent	Sigma	W4502	--	--	--	--	13.6		13.6
DNA	--	--	1	ng/µL	2	ng	2	--	--
							25	***23***	***19***

Number Total reactions per MIX 1
Number Total reactions 1

Thermocycler conditions:

1 cycle	95°C	5 min
30 cycles	95°C	1 min
	55°C	1 min
	72°C	1 min
1 cycle	72°C	7 min

Genes to amplify:

		Primer sequence (5'→3')	Product size (bp)	Tm supplier (°C)	Tm used (°C)
seg	SEG1	TGCTATCGACACACTACAACC	704	57.9	**55**
	SEG2	CCAGATTCAAATGCAGAACC		55.3	
seh	SEH1	CGAAAGCAGAAGATTTACACG	495	55.9	
	SEH2	GACCTTTACTTATTTCGCTGTC		56.5	
sei	SEI1	GACAACAAAACTGTCGAAACTG	630	56.5	
	SEI2	CCATATTCTTTGCCTTTACCAG		56.5	
sej	SEJF	CATCAGAACTGTTGTTCCGCTAG	142	60.6	
	SEJR	CTGAATTTTACCATCAAAGGTAC		55.3	

Gel electrophoresis in 3% low EEO agarose (10 µL PCR product)

Protocol for *S. aureus* specific staphylococcal protein A detection by conventional PCR (Harmsen *et al.*, 2003)

	Reagents's Traceability				PCR				
REACTIVES	**Supplier**	**Reference**	**Stock conc.**	**Units**	**Final conc.**	**Units**	**µL/ reaction**	**MIX**	**SUPERMIX**
Primer Forward	Eurofins	(Harmsen *et al.*, 2003)	10	µM	0.5	µM	1	1	--
Primer Reverse	Eurofins		10	µM	0.5	µM	1	1	--
PCR Buffer	Roche	12 161 559	10	×	1	×	2		2
$MgCl_2$ Solution	Applied	58002032-1	25	mM	2	mM	1.6		1.6
PCR Nucleotide Mix	Roche	11 581 295 001	10	mM/each	0.2	mM	0.4	16	0.4
FastStart Taq DNA Polymerase	Roche	04 659 163	5	U/µL	0.5	U	0.1		0.1
Water Molecular Biology Reagent	Sigma	W4502	--	--	--	--	11.9		11.9
DNA	--	--	1	ng/µL	2	ng	2	--	--
							20	***18***	***16***

Number Total reactions per MIX 1
Number Total reactions 1

Thermocycler conditions:

1 cycle	95°C	5 min
30 cycles	95°C	30 sec
	55°C	30 sec
	72°C	1 min
1 cycle	72°C	7 min

Gene to amplify:

		Primer sequence (5'→3')	Product size (bp)	Tm supplier (°C)	Tm used (°C)
spa	1095F	AGACGATCCTTCGGTGAGC	422	58.8	57
	1517R	GCTTTTGCAATGTCATTTACTG		54.7	

Protocol for MultiLocus Sequence Typing by multiplex PCR (Enright *et al.*, 2000)

	Reagents's Traceability				PCR				
REACTIVES	**Supplier**	**Reference**	**Stock conc.**	**Units**	**Final conc.**	**Units**	**µL/ reaction**	**MIX**	**SUPERMIX**
Primer Forward	Metabion	(Enright *et al.*, 2000)	10	µM	0.5	µM	2.5	**2.5**	--
Primer Reverse	Metabion		10	µM	0.5	µM	2.5	**2.5**	--
PCR Buffer	Roche	12 161 559	10	×	1	×	5		5
$MgCl_2$ Solution	Applied	58002032-1	25	mM	2	mM	4		4
PCR Nucleotide Mix	Roche	11 581 295 001	10	mM/each	0.2	mM	1	**5.7**	1
FastStart Taq DNA Polymerase	Roche	04 659 163	5	U/µL	2	U	0.4		0.4
Water Molecular Biology Reagent	Sigma	W4502	--	--	--	--	29.6		29.6
DNA	--	--	1	ng/µL	5	ng	5	--	--
							50	***10.7***	***40***

Number Total reactions per MIX 1
Number Total reactions 1

Thermocycler conditions:

1 cycle	95°C	5 min
35 cycles	95°C	1 min
	55°C	1 min
	72°C	1 min
1 cycle	72°C	10 min

Genes to amplify:

	Primer sequence (5'→3')	Product size (bp)	Tm supplier (°C)	Tm used (°C)
arcC	TTGATTCACCAGCGCGTATTGTC	456	63.0	55
	AGGTATCTGCTTCAATCAGCG		59.0	
aroE	ATCGGAAATCCTATTTCACATTC	456	58.0	
	GGTGTTGTATTAATAACGATATC		56.0	
glpF	CTAGGAACTGCAATCTTAATCC	465	58.0	
	TGGTAAAATCGCATGTCCAATTC		59.0	
gmk	ATCGTTTTATCGGGACCATC	417	56.0	
	TCATTAACTACAACGTAATCGTA		56.0	
pta	GTTAAAATCGTATTACCTGAAGG	474	58.0	
	GACCCTTTTGTTGAAAAGCTTAA		58.0	
tpi	TCGTTCATTCTGAACGTCGTGAA	402	61.0	
	TTTGCACCTTCTAACAATTGTAC		58.0	
yqiL	CAGCATACAGGACACCTATTGGC	516	65.0	
	CGTTGAGGAATCGATACTGGAAC		63.0	

Protocol for Real Time PCR (Trnčíková *et al.*, 2008)

	Reagents's Traceability				PCR				
REACTIVES	**Supplier**	**Reference**	**Stock conc.**	**Units**	**Final conc.**	**Units**	**µL/ reaction**	**MIX**	**SUPERMIX**
Primer Forward	MWG	(Trnčíková *et al.*, 2008)	10	µM	0.3	µM	0.6	**0.6**	--
Primer Reverse	MWG		10	µM	0.3	µM	0.6	**0.6**	--
Taqman PCR Master Mix	ThermoFisher	4304437	2	×	1	×	10		10
Taqman probe aurP (FAM/BHQ)	ThermoFisher	4304437	10	µM	0.2	µM	0.4	**18.8**	0.4
Water Molecular Biology Reagent	Sigma	W4502	--	--	--	--	8.4		8.4
DNA	--	--	1	ng/µL	5	ng	5	--	--
							25	***20***	***18.8***

Number Total reactions per MIX 1
Number Total reactions 1

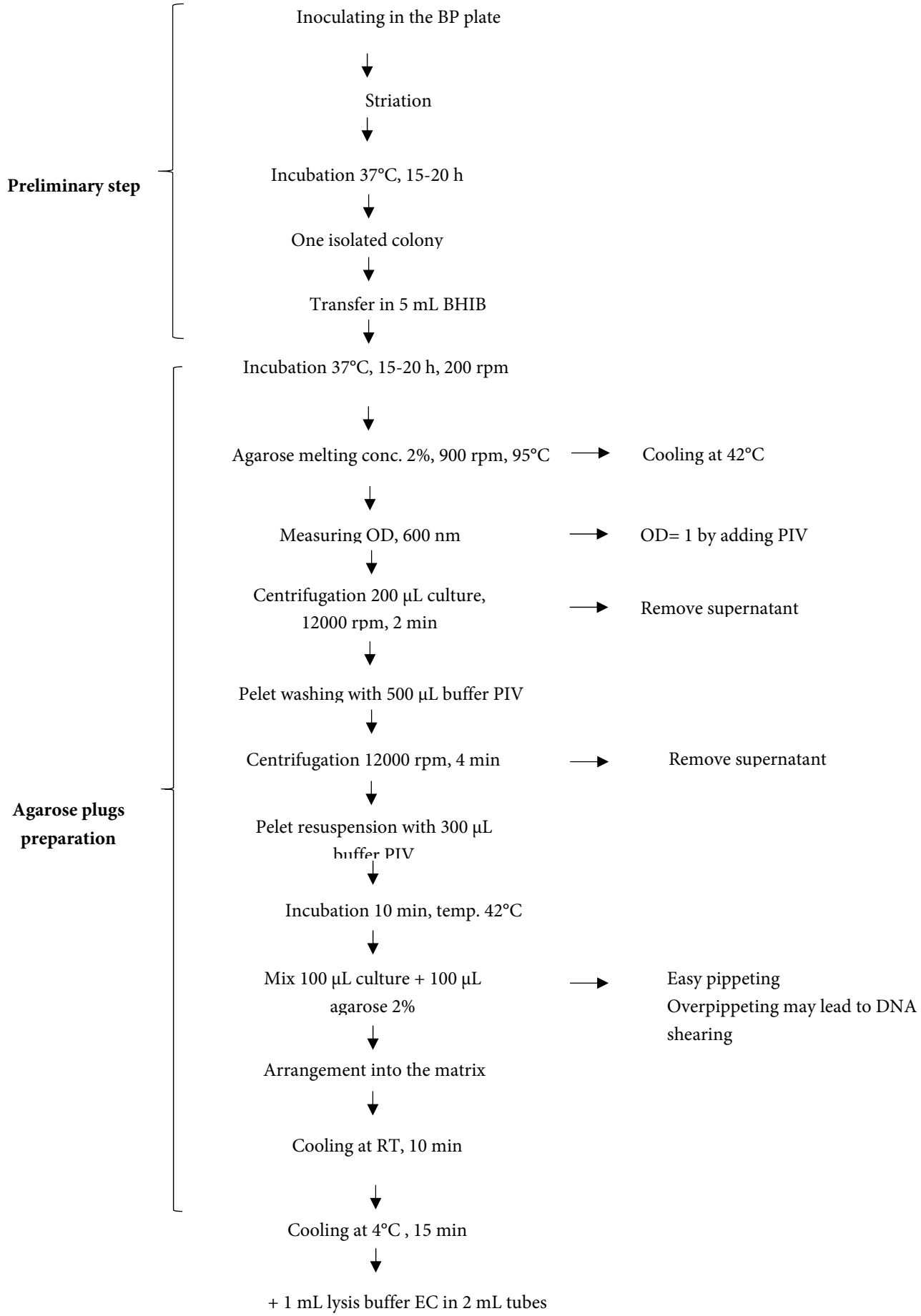
Thermocycler conditions:

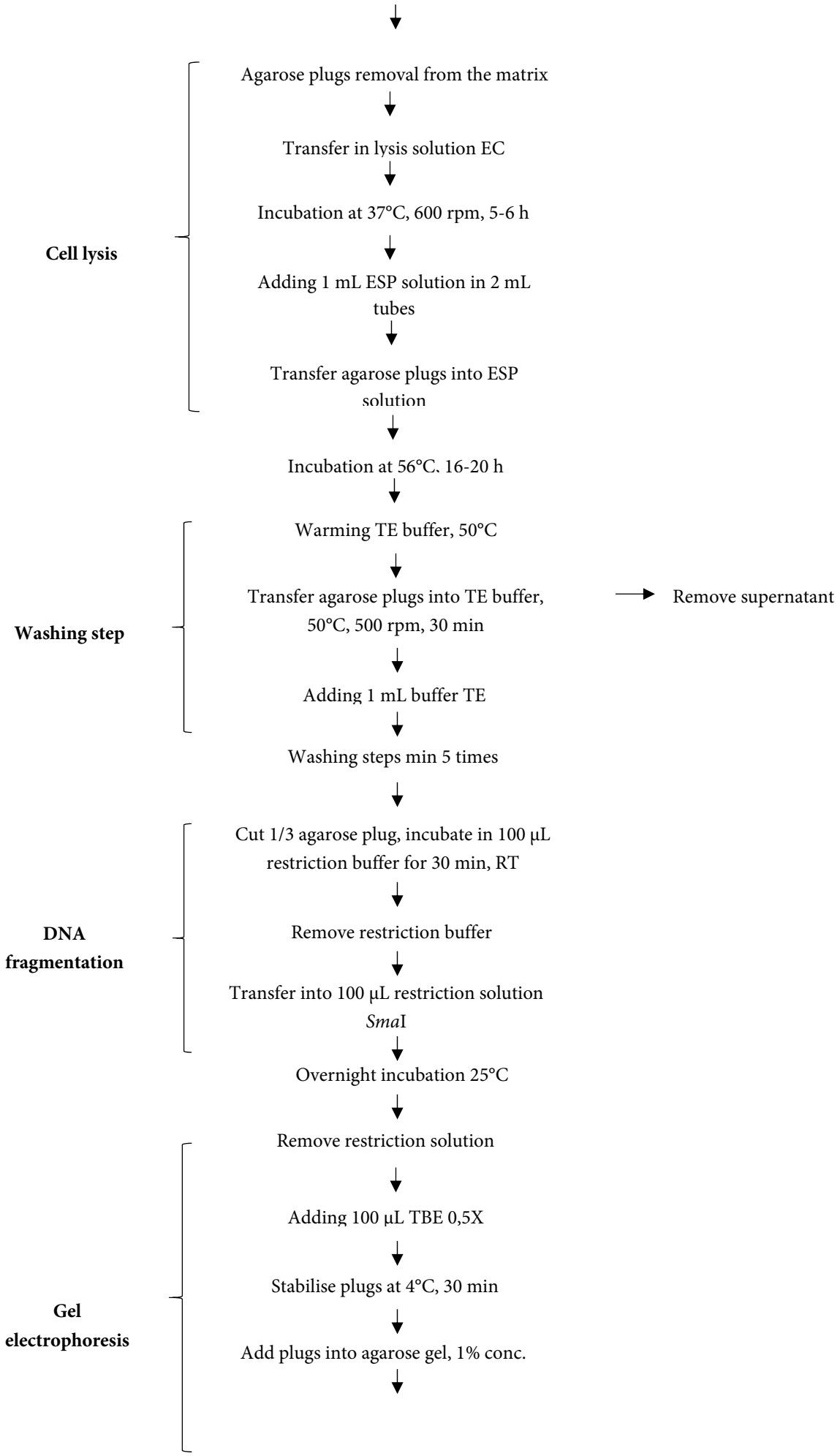
1 cycle	50°C	2 min
1 cycle	95°C	10 min
50 cycles	95°C	15 sec
	60°C	1 min

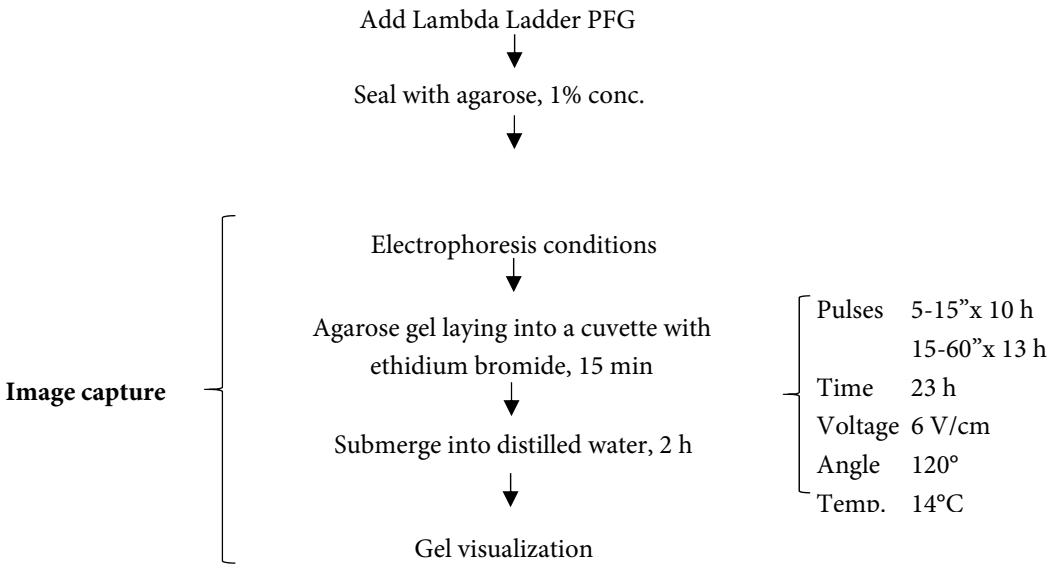
Genes to amplify:

	Primer sequence (5'→3')		**Product size (bp)**	**Tm used (°C)**
	fmhb4416F	CTAGCTTTATTTCAGCAGGTGACGAT		
nuc	aurR	TCAACATCTTTCGCATGATTCAACAC	102	60
	aurP	FAM-CTTGCTCCGTTTCACCAGGCTTCGGTG-TAMRA		

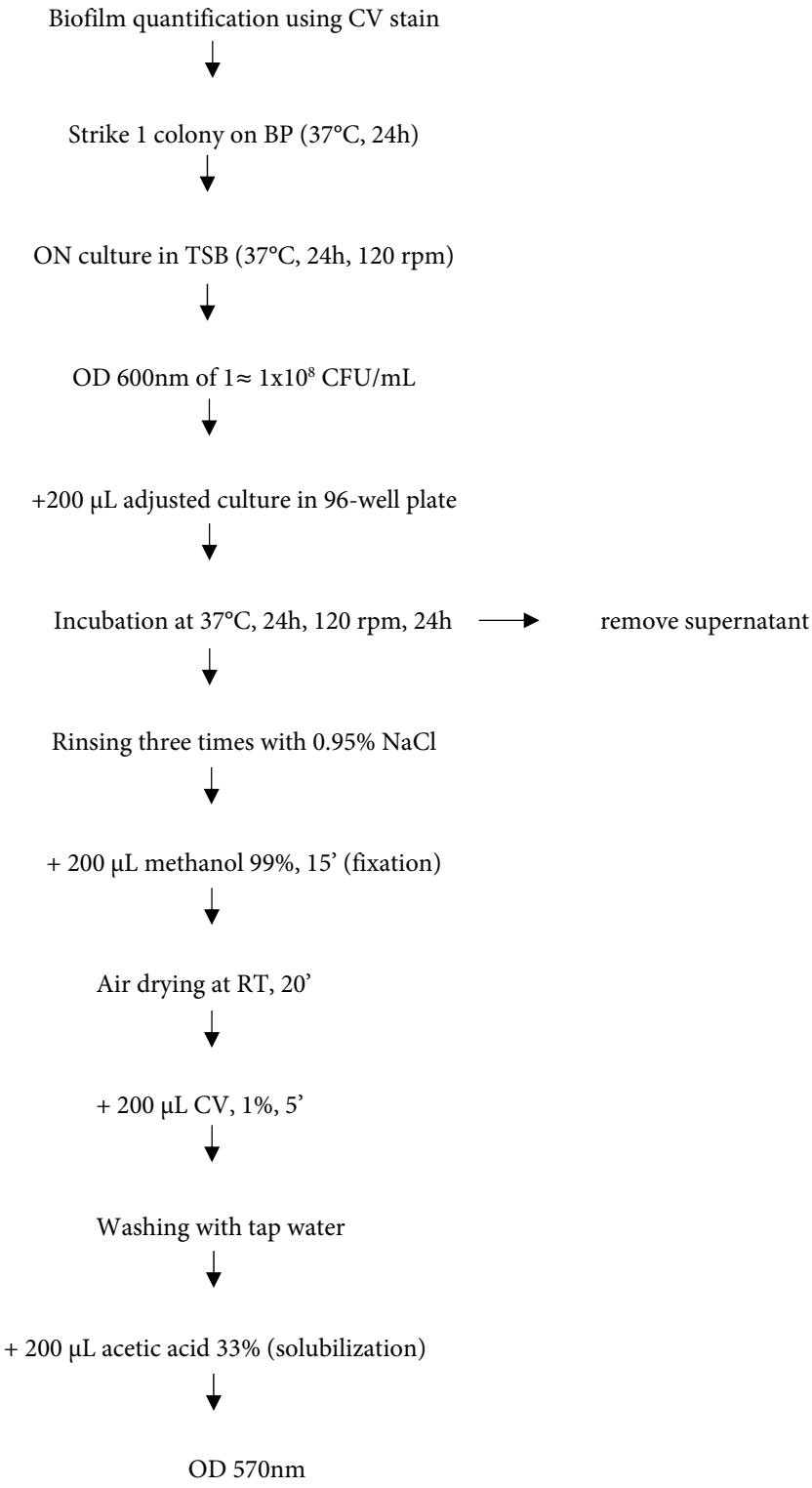
PFGE working protocol for *S. aureus* DNA fragments, by using *Sma*I restriction enzyme




Agarose plugs removal from the matrix
Transfer in lysis solution EC
Incubation at 37°C, 600 rpm, 5-6 h
Cell lysis
Adding 1 mL ESP solution in 2 mL tubes
Transfer agarose plugs into ESP solution
Incubation at 56°C, 16-20 h
Warming TE buffer, 50°C
Transfer agarose plugs into TE buffer, 50°C, 500 rpm, 30 min
Remove supernatant
Washing step
Adding 1 mL buffer TE
Washing steps min 5 times
Cut 1/3 agarose plug, incubate in 100 µL restriction buffer for 30 min, RT
DNA fragmentation
Remove restriction buffer
Transfer into 100 µL restriction solution SmaI
Overnight incubation 25°C
Remove restriction solution
Adding 100 µL TBE 0,5X
Stabilise plugs at 4°C, 30 min
Gel electrophoresis
Add plugs into agarose gel, 1% conc.


Add Lambda Ladder PFG
Seal with agarose, 1% conc.
Image capture
Electrophoresis conditions
Agarose gel laying into a cuvette with ethidium bromide, 15 min
Submerge into distilled water, 2 h
Gel visualization
Pulses 5-15"x 10 h
15-60"x 13 h
Time 23 h
Voltage 6 V/cm
Angle 120°
Temp. 14°C

Biofilm quantification procedure using 96-well plate



Staphylococcus aureus- Allelic profile of C38-ST 1 (Saureus.mlst.net)

arcC

TTATTAATCCAACAAGCTAAATCGAACAGTGACACAACGCCGGCAATGCCATTGGATACTTGTGGTGCAATGTCACA
GGGTATGATAGGCTATTGGTTGGAAACTGAAATCAATCGCATTTTAACTGAAATGAATAGTGATAGAACTGTAGGCA
CAATCGTTACACGTGTGGAAGTAGATAAAGATGATCCACGATTCAATAACCCAACCAAACCAATTGGTCCTTTTTAT
ACGAAAGAAGAAGTTGAAGAATTACAAAAAGAACAGCCAGACTCAGTCTTTAAAGAAGATGCAGGACGTGGTTATA
GAAAAGTAGTTGCGTCACCACTACCTCAATCTATACTAGAACACCAGTTAATTCGAACTTTAGCAGACGGTAAAAAT
ATTGTCATTGCATGCGGTGGTGGCGGTATTCCAGTTATAAAAAAAGAAAATACCTATGAAGGTGTTGAAGCG

aroE

AATTTTAATTCTTTAGGATTAGATGATACTTATGAAGCTTTAAATATTCCAATTGAAGATTTTCATTTAATTAAAGAA
ATTATTTCGAAAAAAGAATTAGATGGCTTTAATATCACAATTCCTCATAAAGAACGTATCATACCGTATTTAGATCAT
GTTGATGAACAAGCGATTAATGCAGGTGCAGTTAACACTGTTTTGATAAAAGATGACAAGTGGATAGGGTATAATAC
AGATGGTATTGGTTATGTTAAAGGATTGCACAGCGTTTATCCAGATTTAGAAAATGCATACATTTTAATTTTGGGCG
CAGGTGGTGCAAGTAAAGGTATTGCTTATGAATTAGCAAAATTTGTAAAGCCCAAATTAACTGTTGCGAATAGAACG
ATGGCTCGTTTTGAATCTTGGAATTTAAATATAAACCAAATTTCATTAGCAGATGCTGAAAAGTATTTA

glpF

GGTGCTGATTGGATTGTCATCACAGCTGGATGGGGATTAGCGGTTACAATGGGTGTGTTTGCTGTCGGTCAATTCTC
AGGTGCACATTTAAACCCAGCGGTGTCTTTAGCTCTTGCATTAGACGGAAGTTTTGATTGGTCATTAGTTCCTGGTTA
TATTGTTGCTCAAATGTTAGGTGCAATTGTCGGAGCAACAATTGTATGGTTAATGTACTTGCCACATTGGAAAGCGA
CAGAAGAAGCTGGCGCGAAATTAGGTGTTTTCTCTACAGCACCGGCTATTAAGAATTACTTTGCCAACTTTTTAAGT
GAGATTATCGGAACAATGGCATTAACTTTAGGTATTTTATTTATCGGTGTAAACAAAATTGCCGATGGTTTAAATCCT
TTAATTGTCGGAGCATTAATTGTTGCAATCGGATTAAGTTTAGGCGGTGCTACTGGTTATGCAATCAACCCAGCACG
T

gmk

CGAATATTTGAAGATCCAAGTACATCATATAAGTATTCTATTTCAATGACAACACGTCAAATGCGTGAAGGTGAAGT
TGATGGCGTAGATTACTTTTTTAAAACTAGGGATGCGTTTGAAGCTTTAATCAAAGATGACCAATTTATAGAATATG
CTGAATATGTAGGCAACTATTATGGTACACCAGTTCAATATGTTAAAGATACAATGGACGAAGGTCATGATGTATTT
TTAGAAATTGAAGTAGAAGGTGCAAAGCAAGTTAGAAAGAAATTTCCAGATGCGCTATTTATTTTCTTAGCACCTCC
AAGTTTAGAACACTTGAGAGAGCGATTAGTAGGTAGAGGAACAGAATCTGATGAGAAAATACAAAGTCGTATTAAC
GAAGCGCGTAAAGAAGTTGAAATGATGAATTTA

pta

GCAACACAATTACAAGCAACAGATTATGTTACACCAATCGTGTTAGGTGATGAGACTAAGGTTCAATCTTTAGCGCA
AAAACTTGATCTTGATATTTCTAATATTGAATTAATTAATCCTGCGACAAGTGAATTGAAAGCTGAATTAGTTCAATC
ATTTGTTGAACGACGTAAAGGTAAAGCGACTGAAGAACAAGCACAAGAATTATTAAACAATGTGAACTACTTCGGT
ACAATGCTTGTTTATGCTGGTAAAGCAGATGGTTTAGTTAGTGGTGCAGCACATTCAACAGGAGACACTGTGCGTCC
AGCTTTACAAATCATCAAAACGAAACCAGGTGTATCAAGAACATCAGGTATCTTCTTTATGATTAAAGGTGATGTAC
AATACATCTTTGGTGATTGTGCAATCAATCCAGAACTTGATTCACAAGGACTTGCAGAAATTGCAGTAGAAAGTGCA
AAATCAGCATTA

tpi

CACGAAACAGATGAAGAAATTAACAAAAAAGCGCACGCTATTTTCAAACATGGAATGACTCCAATTATTTGTGTTGG
TGAAACAGACGAAGAGCGTGAAAGTGGTAAAGCTAACGATGTTGTAGGTGAGCAAGTTAAGAAAGCTGTTGCAGGT
TTATCTGAAGATCAACTTAAATCAGTTGTAATTGCTTATGAGCCAATCTGGGCAATCGGAACTGGTAAATCATCAAC
ATCTGAAGATGCAAATGAAATGTGTGCATTTGTACGTCAAACTATTGCTGACTTATCAAGCAAAGAAGTATCAGAAG
CAACTCGTATTCAATATGGTGGTAGTGTTAAACCTAACAACATTAAAGAATACATGGCACAAACTGATATTGATGGG
GCATTAGTAGGTGGCGCA

yqiL

GCGTTTAAAGACGTGCCAGCCTATGATTTAGGTGCGACTTTAATAGAACATATTATTAAAGAGACGGGTTTGAATCC
AAGTGAGATTGATGAAGTTATCATCGGTAACGTACTACAAGCAGGACAAGGACAAAATCCAGCACGAATTGCTGCT
ATGAAAGGTGGCTTGCCAGAAACAGTACCTGCATTTACAGTGAATAAAGTATGTGGTTCTGGGTTAAAGTCGATTCA
ATTAGCATATCAATCTATTGTGACTGGTGAAAATGACATCGTGCTAGCTGGCGGTATGGAGAATATGTCTCAGTCAC
CAATGCTTGTCAACAACAGTCGCTTCGGTTTTAAAATGGGACATCAATCAATGGTTGATAGCATGGTATATGATGGT
TTAACAGATGTATTTAATCAATATCATATGGGTATTACTGCTGAAAATTTAGTGGAGCAATATGGTATTTCAAGAGA
AGAACAAGATACATTTGCTGTAAACTCACAACAAAAAGCAGTACGTGCACAGCAA

Table S1. Information of the *S. aureus* isolates analyzed in this study.

Pathogen	Species	Sample-Nr.	Isolate-Nr.	Isol. Date	Origin (food item)	Control point	Country of origin	mecA/mecC	Antibiotic resistance by susceptibility testing[a]
Staphylococcus	S. aureus	6	LBMM 820	19/05/2012	Cheese	Airport Bilbao	Ecuador	—	None
Staphylococcus	S. aureus	10	LBMM 821	19/05/2012	Pork meat	Airport Bilbao	Ecuador	—	PEN, ERY, TET
Staphylococcus	S. aureus	13	LBMM 822	19/06/2012	Antelope	Airport Bilbao	Unknown	—	PEN, AMI, TOB, (TET)
Staphylococcus	S. aureus	15	LBMM 823	11/06/2012	Unknown meat	Airport Bilbao	Ecuador	—	PEN, AMI, TOB, (TET)
Staphylococcus	S. aureus	16	LBMM 824	11/06/2012	Unknown meat	Airport Bilbao	Ecuador	—	PEN, (CIP)
Staphylococcus	S. aureus	18	LBMM 825	18/06/2012	Cheese	Airport Bilbao	Ecuador	—	None
Staphylococcus	S. aureus	19	LBMM 826	18/06/2012	Cheese	Airport Bilbao	Ecuador	—	TET
Staphylococcus	S. aureus	23	LBMM 827	09/06/2012	Eggs	Airport Bilbao	Ecuador	—	PEN, ERY, (TET)
Staphylococcus	S. aureus	24	LBMM 828	09/06/2012	Guinea pig	Airport Bilbao	Ecuador	—	None
Staphylococcus	S. aureus	28	LBMM 829	14/05/2012	Cheese	Airport Bilbao	Perú	—	PEN
Staphylococcus	S. aureus	30	LBMM 830	12/07/2012	Pork meat	Airport Bilbao	Argentina	—	PEN, TET
Staphylococcus	S. aureus	35	LBMM 831	27/05/2012	Unknown meat	Airport Bilbao	China	—	PEN
Staphylococcus	S. aureus	36	LBMM 832	27/05/2012	Duck meat	Airport Bilbao	China	—	None
Staphylococcus	S. aureus	50	LBMM 833	03/08/2012	Cheese	Airport Bilbao	Bolivia	—	None
Staphylococcus	S. aureus	51	LBMM 834	03/08/2012	Cheese	Airport Bilbao	Bolivia	—	None
Staphylococcus	S. aureus	53	LBMM 835	03/08/2012	Cheese	Airport Bilbao	Bolivia	—	None
Staphylococcus	S. aureus	54	LBMM 836	03/08/2012	Cheese	Airport Bilbao	Bolivia	—	PEN, AMI, TOB, TET
Staphylococcus	S. aureus	55	LBMM 837	15/05/2012	Cheese	Airport Bilbao	Ecuador	—	PEN
Staphylococcus	S. aureus	58	LBMM 838	04/07/1905	Rodents	Airport Bilbao	Guinea ecuatorial	—	PEN
Staphylococcus	S. aureus	71	LBMM 839	30/08/2012	Cheese	Airport Bilbao	Romania	—	None
Staphylococcus	S. aureus	72	LBMM 840	30/08/2012	Cheese	Airport Bilbao	Romania	—	None
Staphylococcus	S. aureus	74	LBMM 841	30/08/2012	Unknown meat	Airport Bilbao	Romania	—	None
Staphylococcus	S. aureus	75	LBMM 842	30/08/2012	Unknown meat	Airport Bilbao	Romania	—	PEN
Staphylococcus	S. aureus	77	LBMM 843	11/09/2012	Duck meat	Airport Bilbao	China	—	PEN
Staphylococcus	S. aureus	81	LBMM 844	13/08/2012	Pork	Airport Bilbao	Bolivia	—	PEN
Staphylococcus	S. aureus	85	LBMM 845	30/05/2012	Unknown meat	Airport Bilbao	Perú	—	PEN
Staphylococcus	S. aureus	87	LBMM 846	30/05/2012	Cheese	Airport Bilbao	Perú	—	None
Staphylococcus	S. aureus	89	LBMM 847	30/05/2012	Cheese	Airport Bilbao	Perú	—	None
Staphylococcus	S. aureus	91	LBMM 848	30/05/2012	Cheese	Airport Bilbao	Perú	—	None
Staphylococcus	S. aureus	92	LBMM 849	30/05/2012	Cheese	Airport Bilbao	Perú	—	None
Staphylococcus	S. aureus	99	LBMM 850	11/09/2012	Unknown meat	Airport Bilbao	China	—	None
Staphylococcus	S. aureus	103	LBMM 851	09/02/2013	Beef meat	Airport Bilbao	Bolivia	—	PEN
Staphylococcus	S. aureus	104	LBMM 852	09/02/2013	Beef meat	Airport Bilbao	Bolivia	—	None
Staphylococcus	S. aureus	105	LBMM 853	09/02/2013	Cheese	Airport Bilbao	Bolivia	—	None
Staphylococcus	S. aureus	106	LBMM 854	09/02/2013	Cheese	Airport Bilbao	Bolivia	—	PEN, AMI, TOB, TET
Staphylococcus	S. aureus	109	LBMM 855	24/01/2013	Unknown meat	Airport Bilbao	Serbia	—	PEN
Staphylococcus	S. aureus	111	LBMM 856	04/01/2013	Cheese	Airport Bilbao	Peru	—	None
Staphylococcus	S. aureus	112	LBMM 857	20/02/2013	Duck meat	Airport Bilbao	China	—	PEN, (CIP)
Staphylococcus	S. aureus	118	LBMM 858	04/01/2013	Cheese	Airport Bilbao	Peru	—	None
Staphylococcus	S. aureus	119	LBMM 859	04/01/2013	Cheese	Airport Bilbao	Peru	—	None
Staphylococcus	S. aureus	120	LBMM 860	27/01/2013	Cheese	Airport Bilbao	Peru	—	None
Staphylococcus	S. aureus	125	LBMM 861	17/02/2013	Cheese	Airport Bilbao	Paraguay	—	None
Staphylococcus	S. aureus	132	LBMM 862	16/02/2013	Unknown meat	Airport Bilbao	Bolivia	mecA	β-lactams
Staphylococcus	S. aureus	133	LBMM 863	16/02/2013	Unknown meat	Airport Bilbao	Bolivia	mecA	β-lactams
Staphylococcus	S. aureus	134	LBMM 864	16/02/2013	Unknown meat	Airport Bilbao	Bolivia	mecA	β-lactams
Staphylococcus	S. aureus	135	LBMM 865	16/02/2013	Unknown meat	Airport Bilbao	Bolivia	mecA	β-lactams
Staphylococcus	S. aureus	137	LBMM 866	16/02/2013	Cheese	Airport Bilbao	Bolivia	—	None
Staphylococcus	S. aureus	138	LBMM 867	16/02/2013	Cheese	Airport Bilbao	Bolivia	—	None
Staphylococcus	S. aureus	139	LBMM 868	16/02/2013	Unknown meat	Airport Bilbao	Bolivia	mecA	β-lactams
Staphylococcus	S. aureus	144	LBMM 869	18/04/2013	Cheese	Airport Bilbao	Bolivia	—	None
Staphylococcus	S. aureus	145	LBMM 870	18/04/2013	Cheese	Airport Bilbao	Bolivia	—	PEN, AMI, TOB, TET
Staphylococcus	S. aureus	152	LBMM 871	28/03/2013	Cheese	Airport Bilbao	Bolivia	—	PEN
Staphylococcus	S. aureus	153	LBMM 872	07/04/2013	Beef meat	Airport Bilbao	Colombia	—	None
Staphylococcus	S. aureus	157	LBMM 873	11/04/2013	Cheese	Airport Bilbao	Bolivia	—	None
Staphylococcus	S. aureus	168	LBMM 874	28/04/2013	Cheese	Airport Bilbao	Peru	—	None
Staphylococcus	S. aureus	179	LBMM 875	27/04/2013	Cheese	Airport Bilbao	Bolivia	mecA	β-lactams
Staphylococcus	S. aureus	180	LBMM 876	21/02/2013	Meat	Airport Bilbao	Brazil	—	PEN
Staphylococcus	S. aureus	181	LBMM 877	16/05/2013	Cheese	Airport Bilbao	Brazil	—	None
Staphylococcus	S. aureus	182	LBMM 878	27/05/2013	Cheese	Airport Bilbao	Paraguay	—	None
Staphylococcus	S. aureus	185	LBMM 879	01/06/2013	Cheese	Airport Bilbao	Bolivia	—	None
Staphylococcus	S. aureus	186	LBMM 880	01/06/2013	Cheese	Airport Bilbao	Bolivia	—	None
Staphylococcus	S. aureus	188	LBMM 881	09/05/2013	Cheese	Airport Bilbao	Colombia	—	PEN
Staphylococcus	S. aureus	190	LBMM 882	03/05/2013	Cheese	Airport Bilbao	Brazil	—	PEN
Staphylococcus	S. aureus	208	LBMM 883	18/12/2012	Cheese	Airport Bilbao	Bolivia	—	PEN
Staphylococcus	S. aureus	209	LBMM 884	11/03/2013	Cheese	Airport Bilbao	Peru	—	None
Staphylococcus	S. aureus	213	LBMM 885	27/01/2013	Cheese	Airport Bilbao	Peru	—	PEN
Staphylococcus	S. aureus	72	E1	04/02/2013	Whey cheese	Market Galati	Rep. Moldova	—	None
Staphylococcus	S. aureus	18	E2	14/09/2012	Smoked salmon	Market Galati	Rep. Moldova	—	None
Staphylococcus	S. aureus	31	E3	06/11/2012	Fish in oil with herbs	Market Galati	Rep. Moldova	—	TET
Staphylococcus	S. aureus	17	E4	14/09/2012	Unfermented goat cheese	Market Galati	Rep. Moldova	—	None
Staphylococcus	S. aureus	92	E5	07/02/2013	Poultry	Market Galati	Rep. Moldova	—	None
Staphylococcus	S. aureus	16	E6	14/09/2012	Sheep cheese salted in brine	Market Galati	Rep. Moldova	—	None
Staphylococcus	S. aureus	30	E7	06/11/2012	Smoked fish (savorian)	Market Galati	Rep. Moldova	—	TET, CIP
Staphylococcus	S. aureus	23	E8	06/11/2012	Poultry	Market Galati	Rep. Moldova	—	None
Staphylococcus	S. aureus	17	E9	14/09/2012	Unfermented goat cheese	Market Galati	Rep. Moldova	—	PEN
Staphylococcus	S. aureus	12	E10	14/09/2012	Artificial red caviar	Market Galati	Rep. Moldova	—	None
Staphylococcus	S. aureus	63	E11	29/01/2013	Goat cheese	Market Galati	Rep. Moldova	—	None
Staphylococcus	S. aureus	17	E12	14/09/2012	Unfermented goat cheese	Market Galati	Rep. Moldova	—	PEN
Staphylococcus	S. aureus	25	E13	06/11/2012	Non fermented unsalted sheep cheese	Market Galati	Rep. Moldova	—	None
Staphylococcus	S. aureus	25	E14	06/11/2012	Non fermented unsalted sheep cheese	Market Galati	Rep. Moldova	—	None
Staphylococcus	S. aureus	17	E15	14/09/2012	Unfermented goat cheese	Market Galati	Rep. Moldova	—	PEN
Staphylococcus	S. aureus	15	E16	14/09/2012	Fresh cow cheese (cottage cheese)	Market Galati	Rep. Moldova	—	None
Staphylococcus	S. aureus	63	E17	29/01/2013	Goat cheese	Market Galati	Rep. Moldova	—	None
Staphylococcus	S. aureus	11	E18	14/09/2012	Artificial black caviar	Market Galati	Rep. Moldova	—	PEN
Staphylococcus	S. aureus	22	E19	06/11/2012	Milk	Market Galati	Rep. Moldova	—	PEN
Staphylococcus	S. aureus	17	E20	14/09/2012	Unfermented goat cheese	Market Galati	Rep. Moldova	—	PEN
Staphylococcus	S. aureus	15	E21	14/09/2012	Fresh cow cheese (cottage cheese)	Market Galati	Rep. Moldova	—	None
Staphylococcus	S. aureus	21	E22	06/11/2012	Pork lard	Market Galati	Rep. Moldova	mecA	β-lactams, TET, (CIP)
Staphylococcus	S. aureus	13	E23	14/09/2012	Milk	Market Galati	Rep. Moldova	—	PEN
Staphylococcus	S. aureus	23	E24	06/11/2012	Poultry	Market Galati	Rep. Moldova	—	None
Staphylococcus	S. aureus	13	E25	14/09/2012	Milk	Market Galati	Rep. Moldova	—	PEN
Staphylococcus	S. aureus	72	E26	04/02/2013	Whey cheese	Market Galati	Rep. Moldova	—	None
Staphylococcus	S. aureus	25	E29	06/11/2012	Non fermented unsalted sheep cheese	Market Galati	Rep. Moldova	—	None
Staphylococcus	S. aureus	17	E30	14/09/2012	Unfermented goat cheese	Market Galati	Rep. Moldova	—	None
Staphylococcus	S. aureus	72	1NC	04/02/2013	Whey cheese	Market Galati	Rep. Moldova	—	PEN
Staphylococcus	S. aureus	30	7NC	06/11/2012	Smoked fish (savorian)	Market Galati	Rep. Moldova	—	TET
Staphylococcus	S. aureus	72	26NC	04/02/2013	Whey cheese	Market Galati	Rep. Moldova	—	None
Staphylococcus	S. aureus	25	29NC	06/11/2012	Non fermented unsalted sheep cheese	Market Galati	Rep. Moldova	—	None
Staphylococcus	S. aureus	--	SA11	23/09/2012	Cheese	Land border Obrežje	Turkey	—	None
Staphylococcus	S. aureus	--	SA17	03/10/2012	Meat-salami (dry)	Brnik airport	Republic of Kosovo	—	None

Staphylococcus	S. aureus	--	SA21	08/11/2012	Cheese (green mold)	Land border Obrežje	Turkey	—	None
Staphylococcus	S. aureus	--	SA43	06/02/2013	Meat	Brnik airport	Republic of Kosovo	—	None
Staphylococcus	S. aureus	21	21a	13/08/2008	Sheep	Castile and Leon	Spain	—	(ERY)
Staphylococcus	S. aureus	125	125a	01/09/2008	Sheep	Castile and Leon	Spain	—	None
Staphylococcus	S. aureus	282	282b	28/10/2008	Sheep	Castile and Leon	Spain	—	(CLI)
Staphylococcus	S. aureus	355	355b	18/11/2008	Sheep	Castile and Leon	Spain	—	PEN
Staphylococcus	S. aureus	358	358c	18/11/2008	Sheep	Castile and Leon	Spain	—	(ERY), TET
Staphylococcus	S. aureus	620	620a	24/02/2009	Sheep	Castile and Leon	Spain	—	PEN
Staphylococcus	S. aureus	621	621a	24/02/2009	Sheep	Castile and Leon	Spain	—	None
Staphylococcus	S. aureus	622	622a	24/02/2009	Sheep	Castile and Leon	Spain	—	None
Staphylococcus	S. aureus	623	623a	24/02/2009	Sheep	Castile and Leon	Spain	—	None
Staphylococcus	S. aureus	625	625a	24/02/2009	Sheep	Castile and Leon	Spain	—	None
Staphylococcus	S. aureus	626	626a	24/02/2009	Sheep	Castile and Leon	Spain	—	None
Staphylococcus	S. aureus	627	627a	24/02/2009	Sheep	Castile and Leon	Spain	—	None
Staphylococcus	S. aureus	628	628a	24/02/2009	Sheep	Castile and Leon	Spain	—	PEN
Staphylococcus	S. aureus	629	629a	24/02/2009	Sheep	Castile and Leon	Spain	—	PEN
Staphylococcus	S. aureus	630	630a	24/02/2009	Sheep	Castile and Leon	Spain	—	None
Staphylococcus	S. aureus	631	631a	24/02/2009	Sheep	Castile and Leon	Spain	—	None
Staphylococcus	S. aureus	633	633a	24/02/2009	Sheep	Castile and Leon	Spain	—	None
Staphylococcus	S. aureus	681	681a	17/03/2009	Sheep	Castile and Leon	Spain	—	None
Staphylococcus	S. aureus	684	684a	17/03/2009	Sheep	Castile and Leon	Spain	—	None
Staphylococcus	S. aureus	686	686a	17/03/2009	Sheep	Castile and Leon	Spain	—	None
Staphylococcus	S. aureus	689	689a	17/03/2009	Sheep	Castile and Leon	Spain	—	None
Staphylococcus	S. aureus	690	690a	17/03/2009	Sheep	Castile and Leon	Spain	—	None
Staphylococcus	S. aureus	691	691a	17/03/2009	Sheep	Castile and Leon	Spain	—	None
Staphylococcus	S. aureus	694	694a	17/03/2009	Sheep	Castile and Leon	Spain	—	None
Staphylococcus	S. aureus	696	696a	17/03/2009	Sheep	Castile and Leon	Spain	—	FOS
Staphylococcus	S. aureus	697	697a	17/03/2009	Sheep	Castile and Leon	Spain	—	None
Staphylococcus	S. aureus	764	764a	14/04/2009	Sheep	Castile and Leon	Spain	—	PEN
Staphylococcus	S. aureus	769	769a	14/04/2009	Sheep	Castile and Leon	Spain	—	None
Staphylococcus	S. aureus	770	770a	14/04/2009	Sheep	Castile and Leon	Spain	—	None
Staphylococcus	S. aureus	771	771a	14/04/2009	Sheep	Castile and Leon	Spain	—	None
Staphylococcus	S. aureus	772	772a	14/04/2009	Sheep	Castile and Leon	Spain	—	None
Staphylococcus	S. aureus	773	773a	14/04/2009	Sheep	Castile and Leon	Spain	—	None
Staphylococcus	S. aureus	775	775a	14/04/2009	Sheep	Castile and Leon	Spain	—	None
Staphylococcus	S. aureus	776	776a	14/04/2009	Sheep	Castile and Leon	Spain	—	None
Staphylococcus	S. aureus	777	777b	14/04/2009	Sheep	Castile and Leon	Spain	—	None
Staphylococcus	S. aureus	778	778a	14/04/2009	Sheep	Castile and Leon	Spain	—	TET
Staphylococcus	S. aureus	779	779a	14/04/2009	Sheep	Castile and Leon	Spain	—	None
Staphylococcus	S. aureus	924	924a	09/06/2009	Sheep	Castile and Leon	Spain	—	None
Staphylococcus	S. aureus	929	929a	09/06/2009	Sheep	Castile and Leon	Spain	—	TET
Staphylococcus	S. aureus	943	943a	16/06/2009	Sheep	Castile and Leon	Spain	—	None
Staphylococcus	S. aureus	945	945a	16/06/2009	Sheep	Castile and Leon	Spain	—	None
Staphylococcus	S. aureus	947	947a	16/06/2009	Sheep	Castile and Leon	Spain	—	None
Staphylococcus	S. aureus	949	949a	16/06/2009	Sheep	Castile and Leon	Spain	—	None
Staphylococcus	S. aureus	1008	1008a	14/07/2009	Sheep	Castile and Leon	Spain	—	None
Staphylococcus	S. aureus	648	648c	03/03/2009	Sheep	Castile and Leon	Spain	mecC	β-lactams
Staphylococcus	S. aureus	741	741a	07/04/2009	Sheep	Castile and Leon	Spain	mecA	β-lactams, TET
Staphylococcus	S. aureus	1040	1040a	28/07/2009	Sheep	Castile and Leon	Spain	mecA	β-lactams, TET
Staphylococcus	S. aureus	1043	1043a	28/07/2009	Sheep	Castile and Leon	Spain	mecA	β-lactams, TET
Staphylococcus	S. aureus	--	SA 1	16/01/2008	Blood culture	Hospital of León	Spain	mecA	β-lactams, CIP
Staphylococcus	S. aureus	--	SA 2	28/01/2008	Blood culture	Hospital of León	Spain	mecA	β-lactams, CIP
Staphylococcus	S. aureus	--	SA 3	31/01/2008	Blood culture	Hospital of León	Spain	mecA	β-lactams, ERY, CIP
Staphylococcus	S. aureus	--	SA 4	06/02/2008	Central vascular catheter	Hospital of León	Spain	mecA	β-lactams, ERY, CIP, COT
Staphylococcus	S. aureus	--	SA 5	11/02/2008	Blood culture	Hospital of León	Spain	mecA	β-lactams, CIP
Staphylococcus	S. aureus	--	SA 6	18/02/2008	Blood culture	Hospital of León	Spain	mecA	β-lactams, CIP
Staphylococcus	S. aureus	--	SA 7	28/03/2008	Central vascular catheter	Hospital of León	Spain	mecA	β-lactams, CIP
Staphylococcus	S. aureus	--	SA 8	05/05/2008	Blood culture	Hospital of León	Spain	mecA	β-lactams, CIP
Staphylococcus	S. aureus	--	SA 9	05/05/2008	Central vascular catheter	Hospital of León	Spain	mecA	β-lactams, ERY, CIP
Staphylococcus	S. aureus	--	SA 10	19/05/2008	Blood culture	Hospital of León	Spain	mecA	β-lactams, CIP
Staphylococcus	S. aureus	--	SA 11	26/05/2008	Blood culture	Hospital of León	Spain	mecA	β-lactams, ERY, CIP
Staphylococcus	S. aureus	--	SA 12	02/06/2008	Blood culture	Hospital of León	Spain	mecA	β-lactams, ERY, CIP
Staphylococcus	S. aureus	--	SA 13	30/06/2008	Blood culture	Hospital of León	Spain	—	PEN
Staphylococcus	S. aureus	--	SA 14	01/07/2008	Blood culture	Hospital of León	Spain	mecA	β-lactams, CIP
Staphylococcus	S. aureus	--	SA 15	04/07/2008	Central vascular catheter	Hospital of León	Spain	mecA	β-lactams, CIP
Staphylococcus	S. aureus	--	SA 16	04/07/2008	Central vascular catheter	Hospital of León	Spain	mecA	β-lactams, ERY, CIP
Staphylococcus	S. aureus	--	SA 17	18/07/2008	Blood culture	Hospital of León	Spain	mecA	β-lactams, CIP
Staphylococcus	S. aureus	--	SA 18	21/07/2008	Blood culture	Hospital of León	Spain	mecA	β-lactams, ERY, CIP
Staphylococcus	S. aureus	--	SA 19	28/07/2008	Blood culture	Hospital of León	Spain	mecA	β-lactams, CIP
Staphylococcus	S. aureus	--	SA 20	09/08/2008	Blood culture	Hospital of León	Spain	mecA	β-lactams, ERY, CIP, RIF
Staphylococcus	S. aureus	--	SA 21	11/08/2008	Blood culture/bile	Hospital of León	Spain	mecA	β-lactams, ERY, CIP
Staphylococcus	S. aureus	--	SA 22	25/08/2008	Drainage	Hospital of León	Spain	mecA	β-lactams, ERY, CIP
Staphylococcus	S. aureus	--	SA 23	26/08/2008	Central vascular catheter	Hospital of León	Spain	mecA	β-lactams, ERY, CIP
Staphylococcus	S. aureus	--	SA 24	06/10/2008	Blood culture	Hospital of León	Spain	mecA	β-lactams, CIP
Staphylococcus	S. aureus	--	SA 25	16/10/2008	Blood culture	Hospital of León	Spain	mecA	β-lactams, ERY, CIP
Staphylococcus	S. aureus	--	SA 26	18/10/2008	Peripheral vascular catheter	Hospital of León	Spain	mecA	β-lactams, ERY, CIP
Staphylococcus	S. aureus	--	SA 27	27/10/2008	Packed red blood cells	Hospital of León	Spain	mecA	β-lactams, CIP
Staphylococcus	S. aureus	--	SA 28	28/10/2008	Ascitic fluid	Hospital of León	Spain	mecA	β-lactams, CIP
Staphylococcus	S. aureus	--	SA 29	04/11/2008	Peripheral vascular catheter	Hospital of León	Spain	mecA	β-lactams, CIP
Staphylococcus	S. aureus	--	SA 30	15/11/2008	Blood culture	Hospital of León	Spain	mecA	β-lactams, ERY, CIP
Staphylococcus	S. aureus	--	SA 31	23/12/2008	Blood culture	Hospital of León	Spain	mecA	β-lactams, CIP
Staphylococcus	S. aureus	--	SA 32	26/12/2008	Blood culture	Hospital of León	Spain	mecA	β-lactams, CIP
Staphylococcus	S. aureus	--	SA 33	29/12/2008	Blood culture	Hospital of León	Spain	mecA	β-lactams, CIP, RIF, TET
Staphylococcus	S. aureus	--	SA 34	01/01/2007	Blood culture	Hospital of León	Spain	mecA	β-lactams, CIP
Staphylococcus	S. aureus	--	SA 35	17/01/2007	Central vascular catheter	Hospital of León	Spain	mecA	β-lactams, CIP
Staphylococcus	S. aureus	--	SA 36	22/01/2007	Peripheral vascular catheter	Hospital of León	Spain	mecA	β-lactams, CIP
Staphylococcus	S. aureus	--	SA 37	22/01/2007	Central vascular catheter	Hospital of León	Spain	mecA	β-lactams, CIP
Staphylococcus	S. aureus	--	SA 38	01/02/2007	Blood culture	Hospital of León	Spain	mecA	β-lactams, CIP
Staphylococcus	S. aureus	--	SA 39	01/02/2007	Central vascular catheter	Hospital of León	Spain	mecA	β-lactams, CIP
Staphylococcus	S. aureus	--	SA 40	06/02/2007	Blood culture	Hospital of León	Spain	mecA	β-lactams, CIP
Staphylococcus	S. aureus	--	SA 41	27/02/2007	Blood culture	Hospital of León	Spain	mecA	β-lactams, ERY, CIP
Staphylococcus	S. aureus	--	SA 42	05/03/2007	Blood culture	Hospital of León	Spain	mecA	β-lactams, ERY, CIP
Staphylococcus	S. aureus	--	SA 44	12/03/2007	Blood culture	Hospital of León	Spain	mecA	β-lactams, ERY, CIP
Staphylococcus	S. aureus	--	SA 45	26/03/2007	Blood culture	Hospital of León	Spain	mecA	β-lactams, ERY, CIP
Staphylococcus	S. aureus	--	SA 46	26/03/2007	Blood culture	Hospital of León	Spain	mecA	β-lactams, ERY, CIP
Staphylococcus	S. aureus	--	SA 47	07/04/2007	Blood culture	Hospital of León	Spain	mecA	β-lactams, CIP
Staphylococcus	S. aureus	--	SA 48	07/04/2007	Blood culture	Hospital of León	Spain	mecA	β-lactams, ERY, CIP
Staphylococcus	S. aureus	--	SA 49	12/04/2007	Blood culture	Hospital of León	Spain	mecA	β-lactams, CIP
Staphylococcus	S. aureus	--	SA 50	24/04/2007	Blood culture	Hospital of León	Spain	mecA	β-lactams, ERY, CIP, RIF
Staphylococcus	S. aureus	--	SA 51	30/04/2007	Blood culture	Hospital of León	Spain	mecA	β-lactams, ERY, CIP
Staphylococcus	S. aureus	--	SA 52	02/05/2007	Blood culture	Hospital of León	Spain	mecA	β-lactams, CIP
Staphylococcus	S. aureus	--	SA 53	16/05/2007	Blood culture	Hospital of León	Spain	mecA	β-lactams, CIP
Staphylococcus	S. aureus	--	SA 54	17/05/2007	Blood culture	Hospital of León	Spain	mecA	β-lactams, CIP

Staphylococcus	S. aureus	--	SA 55	28/05/2007	Central vascular catheter	Hospital of León	Spain	mecA	β-lactams, CIP
Staphylococcus	S. aureus	--	SA 56	07/06/2007	Blood culture	Hospital of León	Spain	mecA	β-lactams, CIP
Staphylococcus	S. aureus	--	SA 57	02/07/2007	Blood culture	Hospital of León	Spain	mecA	β-lactams, ERY, CIP
Staphylococcus	S. aureus	--	SA 58	30/07/2007	Blood culture	Hospital of León	Spain	mecA	β-lactams, ERY, CIP
Staphylococcus	S. aureus	--	SA 59	07/08/2007	Central vascular catheter	Hospital of León	Spain	mecA	β-lactams, ERY, CIP
Staphylococcus	S. aureus	--	SA 60	24/08/2007	Blood culture	Hospital of León	Spain	mecA	β-lactams, CIP
Staphylococcus	S. aureus	--	SA 61	18/09/2007	Blood culture	Hospital of León	Spain	mecA	β-lactams, ERY, CIP
Staphylococcus	S. aureus	--	SA 62	21/09/2007	Central vascular catheter	Hospital of León	Spain	mecA	β-lactams, CIP
Staphylococcus	S. aureus	--	SA 63	04/10/2007	Blood culture	Hospital of León	Spain	mecA	β-lactams, CIP
Staphylococcus	S. aureus	--	SA 64	15/10/2007	Central vascular catheter	Hospital of León	Spain	mecA	β-lactams, ERY, CIP
Staphylococcus	S. aureus	--	SA 65	18/10/2007	Central vascular catheter	Hospital of León	Spain	—	PEN
Staphylococcus	S. aureus	--	SA 66	05/11/2007	Blood culture	Hospital of León	Spain	mecA	β-lactams, CIP
Staphylococcus	S. aureus	--	SA 67	07/11/2007	Blood culture	Hospital of León	Spain	mecA	β-lactams, CIP
Staphylococcus	S. aureus	--	SA 68	19/11/2007	Blood culture	Hospital of León	Spain	mecA	β-lactams, CIP
Staphylococcus	S. aureus	--	SA 69	27/11/2007	Blood culture	Hospital of León	Spain	mecA	β-lactams, ERY, CIP
Staphylococcus	S. aureus	--	SA 70	27/11/2007	Synovial joint fluid	Hospital of León	Spain	mecA	β-lactams, CIP
Staphylococcus	S. aureus	--	SA 71	19/12/2007	Blood culture	Hospital of León	Spain	mecA	β-lactams, ERY, CIP
Staphylococcus	S. aureus	--	41	10/08/2012	Cheese	Airport Vienna	Egypt	mecA	β-lactams, TET, ERY, CLI
Staphylococcus	S. aureus	--	47	11/08/2012	Raw breaded meat	Airport Vienna	Egypt	—	CIP, AMI, GEN, TOB
Staphylococcus	S. aureus	--	50	11/08/2012	Meat	Airport Vienna	Egypt	mecA	β-lactams
Staphylococcus	S. aureus	--	140	19/09/2012	Cheese in brine	Airport Vienna	Turkey	mecA	CIP, GEN, TET, TOB
Staphylococcus	S. aureus	--	153	19/09/2012	Pastrami, sliced	Airport Vienna	Turkey	—	None
Staphylococcus	S. aureus	--	165	25/09/2012	Lor cheese	Airport Vienna	Turkey	mecA	β-lactams
Staphylococcus	S. aureus	--	176	28/09/2012	Cheese, ripened	Airport Vienna	Armenia	—	None
Staphylococcus	S. aureus	--	226	30/09/2012	Sausage	Airport Vienna	North Korea	—	TET
Staphylococcus	S. aureus	--	247	25/10/2012	Raw muscle meat	Airport Vienna	Albannia	—	None
Staphylococcus	S. aureus	--	249	25/10/2012	Head meat, cooked	Airport Vienna	Albannia	—	ERY
Staphylococcus	S. aureus	--	294	08/11/2012	Cheese in brine with herbs	Airport Vienna	Kosovo	—	None
Staphylococcus	S. aureus	--	298	08/11/2012	Cheese	Airport Vienna	Turkey	—	None
Staphylococcus	S. aureus	--	364	29/11/2012	Sausage	Airport Vienna	Tunisia	—	None
Staphylococcus	S. aureus	--	476	26/01/2013	White cheese with spice sauce	Airport Vienna	Egypt	mecA	β-lactams, TET, AMI, TOB, GEN
Staphylococcus	S. aureus	--	498	31/01/2013	Cheese, ripened	Airport Vienna	Egypt	—	None
Staphylococcus	S. aureus	--	519	09/02/2013	White cheese	Airport Vienna	Turkey	—	None
Staphylococcus	S. aureus	--	550	17/02/2013	White cheese	Airport Vienna	Tunisia	—	None
Staphylococcus	S. aureus	--	576	03/03/2013	Cheese, ripened, sliced	Airport Vienna	Egypt	—	ERY
Staphylococcus	S. aureus	--	LGA251	05/2007	Bulk milk	Cattle farm	Southwest England	mecC	β-lactams

[a]PEN, penicillin; ERY, erythromycin; TET, tetracycline; AMI, amikacin; TOB, tobramycin; CIP, ciprofloxacin; FOS, fosfomycin; COT, cotrimoxazole; RIF, rifampicin; CLI, clindamycin; GEN, gentamicin
Brackets mean intermediate resistance

REFERENCES

References

A

1. Adler, A., Temper, V., Block, C. S., Abramson, N., & Moses, A. E. (2006). Panton-Valentine Leukocidin-Producing *Staphylococcus aureus*. *Emerging Infectious Diseases*, **12**(11), 1789–1790. https://doi.org/10.3201/eid1211.060726

2. Agersø, Y., Hasman, H., Cavaco, L. M., Pedersen, K., & Aarestrup, F. M. (2012). Study of methicillin resistant *Staphylococcus aureus* (MRSA) in Danish pigs at slaughter and in imported retail meat reveals a novel MRSA type in slaughter pigs. *Veterinary Microbiology*, **157**(1–2), 246–250. https://doi.org/10.1016/j.vetmic.2011.12.023

3. Agnoletti, F., Mazzolini, E., Bacchin, C., Bano, L., Berto, G., Rigoli, R., Drigo, I. (2014). First Reporting of Methicillin-Resistant *Staphylococcus aureus* (MRSA) ST398 in an Industrial Rabbit Holding and in Farm-Related People. *Veterinary Microbiology*, **170**(1–2), 172–177. https://doi.org/10.1016/j.vetmic.2014.01.035

4. Alföldi, J., Lindblad-toh, K., & Alfo, J. (2013). Comparative Genomics as a Tool to Understand Evolution and Disease. *Genome Research*, **23**, 1063–1068. https://doi.org/10.1101/gr.157503.113

5. Altschul, S. F., Gish, W., Miller, W., Myers, E. W., & Lipman, D. J. (1990). Basic Local Alignment Search Tool. Journal of Molecular Biology, **215**(3), 403–10. https://doi.org/10.1016/S0022-2836(05)80360-2

6. Aminov, R. I., Otto, M., & Sommer, A. (2010). A Brief History of the Antibiotic Era: Lessons Learned and Challenges for The Future. *Frontiers in Microbiology*, **1**(December), 1–7. https://doi.org/10.3389/fmicb.2010.00134

7. Andrade-Figueiredo, M., & Leal-Balbino, T. C. (2016). Clonal Diversity and Epidemiological Characteristics of *Staphylococcus aureus*: High Prevalence of Oxacillin-Susceptible *mec*A-Positive Staphylococcus aureus (OS-MRSA) Associated with Clinical Isolates in Brazil. *BMC Microbiology*, **16**(1), 1–9. https://doi.org/10.1186/s12866-016-0733-4

8. Andrews, W. (1997). *Manuals of food quality control*, Chapter 4 - Microbiological analysis.

9. Angen, Ø., Stegger, M., Larsen, J., Lilje, B., Kaya, H., Pedersen, K. S., Larsen, A. R. (2016). Report of *mec*C-Carrying MRSA in Domestic Swine. *Journal of Antimicrobial Chemotherapy*, 1–4. https://doi.org/10.1093/jac/dkw389

10. Antipov, D., Hartwick, N., Shen, M., Raiko, M., Lapidus, A., & Pevzner, P. A. (2016). plasmidSPAdes: Assembling Plasmids from Whole Genome Sequencing Data. *Bioinformatics*, **32**(22), 3380–3387. https://doi.org/10.1093/bioinformatics/btw493

11. Antoci, E., Pinzone, M. R., Nunnari, G., Stefani, S., & Cacopardo, B. (2013). Prevalence and Molecular Characteristics of Methicillin-Resistant *Staphylococcus aureus* (MRSA) Among Subjects Working on Bovine Dairy Farms. *Le Infezioni in Medicina*, **2**, 125–129.

12. Archer, N. K., Mazaitis, M. J., Costerton, J. W., Leid, J. G., Powers, M. E., & Shirtliff, M. E. (2011). *Staphylococcus aureus* Biofilms: Properties, Regulation and Roles in Human Disease. *Virulence*, **2**(5), 445–459. https://doi.org/10.4161/viru.2.5.17724

13. Argudín, M. A., Tenhagen, B. A., Fetsch, A., Sachsenröder, J., Käsbohrer, A., Schroeter, A., Guerra, B. (2011). Virulence and Resistance Determinants of German *Staphylococcus aureus* ST398 Isolates from Nonhuman Sources. *Applied and Environmental Microbiology*, **77**(9), 3052–3060. https://doi.org/10.1128/AEM.02260-10

14. Argudín, M. A., Vanderhaeghen, W., Vandendriessche, S., Vandecandelaere, I., André, F. X., Denis, O., Butaye, P. (2015). Antimicrobial Resistance and Population Structure of *Staphylococcus epidermidis* Recovered from Animals and Humans. *Veterinary Microbiology*, **178**(1–2), 105–113. https://doi.org/10.1016/j.vetmic.2015.04.019
15. Ariza-Miguel, J., Hernández, M., Fernández-Natal, I., & Rodríguez-Lázaro, D. (2014a). Methicillin-Resistant *Staphylococcus aureus* Harboring *mec*C in Livestock in Spain. *Journal of Clinical Microbiology*, **52**(11), 4067–4069. https://doi.org/10.1128/JCM.01815-14

16. Ariza-Miguel, J., Hernández, M., Fernández-Natal, I., & Rodríguez-Lázaro, D. (2014b). Molecular Epidemiology of Methicillin-Resistant *Staphylococcus aureus* in a University Hospital in Northwestern Spain. *International Microbiology*, **17**, 149–157. https://doi.org/10.2436/20.1501.01.217

17. Ariza-Miguel, J., Oniciuc, E.-A., Sanz, I., Fernández-Natal, I., Hernández, M., & Rodríguez-Lázaro, D. (2015). Evaluation of Two Commercially Available Chromogenic Media for Confirmation of Methicillin-Resistant *Staphylococcus aureus* from Human, Animal, and Food Samples. *International Journal of Food Microbiology*, **209**. https://doi.org/10.1016/j.ijfoodmicro.2015.05.004

18. Azeredo, J., Cerca, N., Henriques, M., & Pereira, M. O. (2014). *Advanced Techniques in Biofilms*.

19. Aziz, R. K., Bartels, D., Best, A. A., DeJongh, M., Disz, T., Edwards, R. A., Palsson, B. (2008). The RAST Server: Rapid Annotations using Subsystems Technology. *BMC Genomics*, **9**(75), 1–15. https://doi.org/10.1186/1471-2164-9-75

B

20. Babraham Bioinformatics. (n.d.). Retrieved from http://www.bioinformatics.babraham.ac.uk/projects/fastqc/

21. Baird, R. M., & Lee, W. H. (1995). Media Used in The Detection and Enumeration of *Staphylococcus aureus*. *International Journal of Food Microbiology*, **26**, 15–24.

22. Bankevich, A., Nurk, S., Antipov, D., Gurevich, A. A., Dvorkin, M., Kulikov, A. S., Pevzner, P. a. (2012). SPAdes: A New Genome Assembly Algorithm and Its Applications to Single-Cell Sequencing. *Journal of Computational Biology*, **19**(5), 455–477. https://doi.org/10.1089/cmb.2012.0021

23. Banville, R. R. (1964). Factors Affecting Growth of *Staphylococcus aureus* Forms on Semi defined Medium. *Journal of Bacteriology*, **87**(5), 1192–1197.

24. Barbu, V. (2008). *Principii de genetica*. Galati University Press.

25. Bartels, M. D., Petersen, A., Worning, P., Nielsen, J. B., Larner-Svensson, H., Johansen, H. K., Westh, H. (2014). *spa*-Typing of Methicillin-Resistant *Staphylococcus aureus* Comparing Whole Genome Sequencing with Sanger Sequencing. *Journal of Clinical Microbiology*, **52**(12), 4305–4308. https://doi.org/10.1128/JCM.01979-14

26. Basanisi, M. G., La Bella, G., Nobili, G., Franconieri, I., & La Salandra, G. (2017). Genotyping of Methicillin-Resistant *Staphylococcus aureus* (MRSA) Isolated from Milk and Dairy Products in South Italy. *Food Microbiology*, **62**, 141–146. https://doi.org/10.1016/j.fm.2016.10.020

27. Basset, P., Prod'hom, G., Senn, L., Greub, G., & Blanc, D. S. (2013). Very Low Prevalence of Methicillin-Resistant *Staphylococcus aureus* Carrying the *mec*C Gene in Western Switzerland. *Journal of Hospital Infection*, **83**(3), 257–259. https://doi.org/10.1016/j.jhin.2012.12.004

28. Bassetti, M., Nicco, E., & Mikulska, M. (2009). Why is Community-Associated MRSA Spreading Across the World and How Will It Change Clinical Practice? *International Journal of Antimicrobial Agents*, **34**(S1), S15–S19. https://doi.org/10.1016/S0924-8579(09)70544-8

29. Batt, C. A., & Tortorello, M.-L. (2014). *Encyclopedia of Food Microbiology*. In Encyclopedia of Food Microbiology (Second edi). Elsevier.

30. Battisti, A., Franco, A., Merialdi, G., Hasman, H., Iurescia, M., Lorenzetti, R., Aarestrup, F. M. (2010). Heterogeneity Among Methicillin-Resistant *Staphylococcus aureus* from Italian Pig Finishing Holdings. *Veterinary Microbiology*, **142**(3–4), 361–366. https://doi.org/10.1016/j.vetmic.2009.10.008

31. Bauer, J., Siala, W., Tulkens, P. M., & Van Bambeke, F. (2013). A Combined Pharmacodynamic Quantitative and Qualitative Model Reveals the Potent Activity of Daptomycin and Delafloxacin Against *Staphylococcus aureus* Biofilms. *Antimicrobial Agents and Chemotherapy*, **57**(6), 2726–2737. https://doi.org/10.1128/AAC.00181-13

32. Beloin, C., & Ghigo, J. M. (2005). Finding Gene-Expression Patterns in Bacterial Biofilms. *Trends in Microbiology*, **13**(1), 16–19. https://doi.org/10.1016/j.tim.2004.11.008

33. Beneke, B., Klees, S., Stührenberg, B., Fetsch, A., Kraushaar, B., & Tenhagen, B.-A. (2011). Prevalence of Methicillin-Resistant *Staphylococcus aureus* in a Fresh Meat Pork Production Chain. *Journal of Food Protection*, **74**(1), 126–129. https://doi.org/10.4315/0362-028X.JFP-10-250

34. Berggren, K., Chernokalskaya, E., Steinberg, T. H., Kemper, C., Lopez, M. F., Diwu, Z., Patton, W. F. (2000). Background-Free, High Sensitivity Staining of Proteins in One- and Two-Dimensional Sodium Dodecyl Sulfate-Polyacrylamide Gels Using a Luminescent Ruthenium Complex. *Electrophoresis*, **21**(12),2509–2521.https://doi.org/10.1002/1522-2683(20000701)21:12<2509::AID-ELPS2509>3.0.CO;2-9

35. Beutlich, J., Hammerl, J. A., Appel, B., Nöckler, K., Helmuth, R., Jöst, K., Mayer-Scholl, A. (2015). Characterization of Illegal Food Items and Identification of Foodborne Pathogens Brought into the European Union *via* Two Major German Airports. *International Journal of Food Microbiology*, **209**, 13–19. https://doi.org/10.1016/j.ijfoodmicro.2014.10.017

36. Bhargava, K., Wang, X., Donabedian, S., Zervos, M., da Rocha, L., & Zhang, Y. (2011). Methicillin-resistant *Staphylococcus aureus* in retail meat, Detroit, Michigan, USA. *Emerging Infectious Diseases*, **17**(6), 1135–1137. https://doi.org/10.3201/eid1706.101095

37. bioMérieux. (n.d.). Get a Culture Ahead. Retrieved from http://www.biomerieux-diagnostics.com/sites/clinic/files/catalogue-ppm-final_0.pdf

38. Biosecurity Act. (2015), 61.

39. Bloomfield, S. F., Exner, M., Signorelli, C., Nath, K. J., & Scott, E. A. (2011). The Infection Risks Associated with Clothing and Household Linens in Home and Everyday Life Settings, and the Role of Laundry. *International Scientific Forum on Home Hygiene*, **(April)**, 1–43. Retrieved from http://europeantissue.com/wp-content/uploads/The-infection-risks-associated-with-clothing-and-household-linens.pdf

40. Blumental, S., Deplano, A., Jourdain, S., De Mendonça, R., Hallin, M., Nonhoff, C., Denis, O. (2013). Dynamic Pattern and Genotypic Diversity of *Staphylococcus aureus* Nasopharyngeal Carriage in Healthy Pre-School Children. *Journal of Antimicrobial Chemotherapy*, **68**(7), 1517–1523. https://doi.org/10.1093/jac/dkt080

41. Bodén, M. K., & Flock, J.-I. (1989). Fibrinogen-Binding Protein/Clumping Factor from *Staphylococcus aureus*. *Infection and Immunity*, **57**(8), 2358–2363.

42. Bonten, M. J. M., & Weinstein, R. A. (2016). Making sense of universal screening for MRSA. *The Lancet Infectious Diseases*, **16**(3), 272–273. https://doi.org/10.1016/S1473-3099(15)00435-1

43. Bos, M. E. H., Graveland, H., Portengen, L., Wagenaar, J. A., & Heederik, D. J. J. (2012). Livestock-Associated MRSA Prevalence in Veal Calf Production is Associated with Farm Hygiene, Use of Antimicrobials, and Age of The Calves. *Preventive Veterinary Medicine*, **105**(1–2), 155–159. https://doi.org/10.1016/j.prevetmed.2012.01.002

44. Bremer, P. J., Fillery, S., & McQuillan, A. J. (2006). Laboratory Scale Clean-In-Place (CIP) Studies on the Effectiveness of Different Caustic and Acid Wash Steps on the Removal of Dairy Biofilms. *International Journal of Food Microbiology*, **106**(3), 254–262. https://doi.org/10.1016/j.ijfoodmicro.2005.07.004

45. Bridier, A., Dubois-Brissonnet, F., Boubetra, A., Thomas, V., & Briandet, R. (2010). The Biofilm Architecture of Sixty Opportunistic Pathogens Deciphered Using a High Throughput CLSM Method. *Journal of Microbiological Methods*, **82**(1), 64–70. https://doi.org/10.1016/j.mimet.2010.04.006

46. Brown, A. F., Leech, J. M., Rogers, T. R., & McLoughlin, R. M. (2014). *Staphylococcus aureus* Colonization: Modulation of Host Immune Response and Impact on Human Vaccine Design. *Frontiers in Immunology*, **4**, 1–20. https://doi.org/10.3389/fimmu.2013.00507

47. Buchan, B. W., & Ledeboer, N. A. (2010). Identification of Two Borderline Oxacillin-Resistant Strains of *Staphylococcus aureus* from Routine Nares Swab Specimens by One of Three Chromogenic Agars Evaluated for the Detection of MRSA. *American Journal of Clinical Pathology*, **134**(6), 921–927. https://doi.org/10.1309/AJCPO9TOID1EPUIM

48. Burnside, K., Lembo, A., de los Reyes, M., Iliuk, A., BinhTran, N. T., Connelly, J. E., Rajagopal, L. (2010). Regulation of Hemolysin Expression and Virulence of *Staphylococcus aureus* by a Serine/Threonine Kinase and Phosphatase. *PLoS ONE*, **5**(6), 1–16. https://doi.org/10.1371/journal.pone.0011071

49. Buyukcangaz, E., Velasco, V., Sherwood, J. S., Stepan, R. M., Koslofsky, R. J., & Logue, C. M. (2013). Molecular Typing of *Staphylococcus aureus* and Methicillin-Resistant *S. aureus* (MRSA) Isolated from Animals and Retail Meat in North Dakota, United States. *Foodborne Pathogens and Disease*, **10**(7), 608–617. https://doi.org/10.1089/fpd.2012.1427

50. Bystroń, J., Podkowik, M., Korzekwa, K., Lis, E., Molenda, J., & Bania, J. (2010). Characterization of Borderline Oxacillin-Resistant *Staphylococcus aureus* Isolated from Food of Animal Origin. *Journal of Food Protection*, **73**(7), 1325–1327. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/20615346

C

51. Cameron, D. R., Howden, B. P., & Peleg, A. Y. (2011). The Interface Between Antibiotic Resistance and Virulence in *Staphylococcus aureus* and Its Impact Upon Clinical Outcomes. *Antimicrobial Resistance*, **53**, 576–582. https://doi.org/10.1093/cid/cir473

52. Camoez, M., Sierra, J. M., Pujol, M., Hornero, A., Martin, R., & Domínguez, M. A. (2013). Prevalence and Molecular Characterization of Methicillin-Resistant *Staphylococcus aureus* ST398 Resistant to Tetracycline at a Spanish Hospital over 12 Years. *PLoS ONE*, **8**(9), 1–6. https://doi.org/10.1371/journal.pone.0072828

53. Campoccia, D., Montanaro, L., & Arciola, C. R. (2013). A Review of the Biomaterials Technologies for Infection-Resistant Surfaces. *Biomaterials*, **34**(34), 8533–8554. https://doi.org/10.1016/j.biomaterials.2013.07.089

54. Carfora, V., Caprioli, A., Marri, N., Sagrafoli, D., Boselli, C., Giacinti, G., Amatiste, S. (2015). Enterotoxin Genes, Enterotoxin Production, and Methicillin Resistance in *Staphylococcus aureus* Isolated from Milk and Dairy Products in Central Italy. *International Dairy Journal*, **42**, 12–15. https://doi.org/10.1016/j.idairyj.2014.10.009

55. Cassat, J. E., Hammer, N. D., Campbell, P. J., Benson, M. A., Perrien, D. S., Mrak, L. N., Skaar, E. P. (2013). A Secreted Bacterial Protease Tailors the *Staphylococcus aureus* Virulence Repertoire to Modulate Bone Remodeling during Osteomyelitis. *Cell Host Microbe*, **13**(6), 759–772. https://doi.org/10.1038/nm1636.Mutational

56. Castillo, D., Christiansen, R. H., Dalsgaard, I., & Madsen, L. (2016). Insights Into the Pathogenicity of *Flavobacterium psychrophilum*. *PLoS ONE*, **11**(4), 1–18. https://doi.org/10.1371/journal.pone.0152515

57. Center for Genomic Epidemiology. (n.d.). Acquired antimicrobial resistance genes in total or partial sequenced isolates of bacteria. Retrieved from https://cge.cbs.dtu.dk/services/ResFinder/

58. Centers for Disease Control and Prevention. (2016). Active Bacterial Core Surveillance Report, Emerging Infections Program Network, Methicillin-Resistant *Staphylococcus aureus*, **5**, 1–3. https://doi.org/10.1086/659760.Deriving

59. Chen, F. J., Huang, I. W., Wang, C. H., Chen, P., Wang, H. Y., Lai, J. F., Lauderdale, T. L. Y. (2012). *mec*A-Positive *Staphylococcus aureus* with Low-Level Oxacillin MIC in Taiwan. *Journal of Clinical Microbiology*, **50**(5), 1679–1683. https://doi.org/10.1128/JCM.06711-11

60. Chen, M., Yu, Q., & Sun, H. (2013). Novel Strategies for the Prevention and Treatment of Biofilm Related Infections. *International Journal of Molecular Sciences*, **14**(9), 18488–18501. https://doi.org/10.3390/ijms140918488

61. Chen, P., Abercrombie, J. J., Jeffrey, N. R., & Leung, K. P. (2012). An Improved Medium for Growing *Staphylococcus aureus* Biofilm. *Journal of Microbiological Methods*, **90**(2), 115–118. https://doi.org/10.1016/j.mimet.2012.04.009

62. Chen, Y., Jackson, K. M., Chea, F. P., & Schaffner, D. W. (2001). Quantification and Variability Analysis of Bacterial Cross-Contamination Rates in Common Food Service Tasks. *Journal of Food Protection*, **64**(1), 72–80.

63. Choffnes, E. R., Relman, D. A., Olsen, L., Hutton, R., & Mack, A. (2012). *Improving Food Safety Through a One Health Approach*. Nature (Vol. 447). The National Academies Press.

64. Chua, K. Y. L., Stinear, T. P., & Howden, B. P. (2013). Functional Genomics of *Staphylococcus aureus*. *Briefings in Functional Genomics*, **12**(4), 305–315. https://doi.org/10.1093/bfgp/elt006

65. Chuang, Y. Y., & Huang, Y. C. (2015). Livestock-Associated Methicillin-Resistant *Staphylococcus aureus* in Asia: An Emerging Issue? *International Journal of Antimicrobial Agents*, **45**(4), 334–340. https://doi.org/10.1016/j.ijantimicag.2014.12.007

66. Chung, M., de Lencastre, H., Matthews, P., Tomasz, A., and Collaborators from the Multi Laboratory project, Adamsson, I., Villari, P. (2000). Molecular Typing of Methicillin-Resistant *Staphylococcus aureus* by Pulsed-Field Gel Electrophoresis: Comparison of Results Obtained in a Multi Laboratory Effort Using Identical Protocols and MRSA Strains. *Microbial Drug Resistance*, **6**(3), 189–198.

67. Ciolacu, L., Stessl, B., Bolocan, A. S., & Oniciuc, E.-A. (2016). Tracking Foodborne Pathogenic Bacteria in Raw and Ready-to-Eat Food Illegally Sold at the Eastern EU Border. F*oodborne Pathogens and Disease*, **13**(3), 148–155. https://doi.org/10.1089/fpd.2015.2057

68. Clinical and Laboratory Standards Institute. (2012). A*nalysis and Presentation of Cumulative Antimicrobial Susceptibility Test Data*; Approved Guideline. Third Edition., M29(6).

69. Collective Evolution. (n.d.). Retrieved from www.collective-evolution.com

70. Committee, T. E., Testing, A. S., Changes, N., & Pseudomonas, E. (2015). European Committee on Antimicrobial Susceptibility Testing Breakpoint tables for interpretation of MICs and zone diameters European Committee on Antimicrobial Susceptibility Testing Breakpoint tables for interpretation of MICs and zone diameters.

71. Comparing Partitions website. (n.d.). Retrieved from http://darwin.phyloviz.net/ComparingPartitions/index.php?link=Home

72. Conceiçao, T., Coelho, C., Lencastre, H. de, & Aires-De-Sousa, M. (2015). Frequent Occurrence of Oxacillin-Susceptible *mec*A-Positive *Staphylococcus aureus* (OS-MRSA) Strains in Two African Countries. *Journal of Antimicrobial Chemotherapy*, **70**(August), 3200–3204. https://doi.org/10.1093/jac/dkv261

73. Costa, A. R., Batistão, D. W. F., Ribas, R. M., Sousa, A. M., Pereira, O., & Botelho, C. M. (2013). *Staphylococcus aureus* Virulence Factors and Disease. *Microbial Pathogens and Strategies for Combating Them: Science, Technology and Education*, 702–710.

74. Costard, S., Jones, B. A., Martínez-López, B., Mur, L., de la Torre, A., Martínez, M., Wieland, B. (2013). Introduction of African Swine Fever into the European Union through Illegal Importation of Pork and Pork Products. *PLoS ONE*, **8**(4), 1–12. https://doi.org/10.1371/journal.pone.0061104

75. Crago, B., Ferrato, C., Drews, S. J., Svenson, L. W., Tyrrell, G., & Louie, M. (2012). Prevalence of *Staphylococcus aureus* and Methicillin-Resistant *S. aureus* (MRSA) in Food Samples Associated with Foodborne Illness in Alberta, Canada from 2007 to 2010. *Food Microbiology*, **32**(1), 202–205. https://doi.org/10.1016/j.fm.2012.04.012

76. Crombé, F., Argudín, M. A., Vanderhaeghen, W., Hermans, K., Haesebrouck, F., & Butaye, P. (2013). Transmission Dynamics of Methicillin-Resistant *Staphylococcus aureus* in Pigs. *Frontiers in Microbiology*, **4**(57), 1–21. https://doi.org/10.3389/fmicb.2013.00057

77. Cucarella, C., Solano, C., & Valle, J. (2001). Bap, a *Staphylococcus aureus* Surface Protein Involved in Biofilm Formation. *Journal of Bacteriology*, **183**(9), 2888–2896. https://doi.org/10.1128/JB.183.9.2888

78. Cuny, C., Köck, R., & Witte, W. (2013). Livestock Associated MRSA (LA-MRSA) and Its Relevance for Humans in Germany. *International Journal of Medical Microbiology*, **303**(6–7), 331–337. https://doi.org/10.1016/j.ijmm.2013.02.010

79. Cuny, C., Nathaus, R., Layer, F., Strommenger, B., Altmann, D., & Witte, W. (2009). Nasal Colonization of Humans with Methicillin-Resistant *Staphylococcus aureus* (MRSA) CC398 With and Without Exposure to Pigs. *PLoS ONE*, **4**(8), 1–6. https://doi.org/10.1371/journal.pone.0006800

80. Current Taxonomic Hierarchy. (n.d.). Retrieved from www.itis.gov

D

81. Darling, A. C. E., Mau, B., Blattner, F. R., & Perna, N. T. (2004). Mauve: Multiple Alignment of Conserved Genomic Sequence With Rearrangements. *Genome Research*, **14**, 1394–1403. https://doi.org/10.1101/gr.2289704

82. Davies, J., & Davies, D. (2010). Origins and Evolution of Antibiotic Resistance. *Microbiology and Molecular Biology Reviews*, **74**(3), 417–433. https://doi.org/10.1128/MMBR.00016-10

83. de Boer, E., Zwartkruis-Nahuis, J. T. M., Wit, B., Huijsdens, X. W., de Neeling, A. J., Bosch, T., Heuvelink, A. E. (2009). Prevalence of Methicillin-Resistant *Staphylococcus aureus* in Meat. *International Journal of Food Microbiology*, **134**(1–2), 52–56. https://doi.org/10.1016/j.ijfoodmicro.2008.12.007

84. de Melo, C. B., de Sá, M. E. P., Sabino, V. M., de Fatima Boechat-Fernandes, M., Santiago, M. T., Schwingel, F. F., Seixas, L. (2015). Microbiological Detection of Bacteria in Animal Products Seized in Baggage of International Air Passengers to Brazil. *Preventive Veterinary Medicine*, **118**(1), 22–27. https://doi.org/10.1016/j.prevetmed.2014.11.011

85. de Neeling, A. J., van den Broek, M. J. M., Spalburg, E. C., van Santen-Verheuvel, M. G., Dam-Deisz, W. D. C., Boshuizen, H. C., Huijsdens, X. W. (2007). High Prevalence of Methicillin Resistant *Staphylococcus aureus* in Pigs. *Veterinary Microbiology*, **122**(3–4), 366–372. https://doi.org/10.1016/j.vetmic.2007.01.027

86. Deurenberg, R. H., Kalenic, S., Friedrich, A. W., van Tiel, F. H., & Stobberingh, E. E. (2007). Molecular Epidemiology of Methicillin-Resistant *Staphylococcus aureus*. *Communicating Current Research and Educational Topics and Trends in Applied Microbiology*, 766–777. https://doi.org/10.1111/j.1469-0691.2006.01672.x

87. Di Ciccio, P., Vergara, A., Festino, A. R., Paludi, D., Zanardi, E., Ghidini, S., & Ianieri, A. (2015). Biofilm Formation by *Staphylococcus aureus* on Food Contact Surfaces: Relationship with Temperature and Cell Surface Hydrophobicity. *Food Control*, **50**, 930–936. https://doi.org/10.1016/j.foodcont.2014.10.048

88. Diekema, D. J., Pfaller, M. A., Schmitz, F. J., Smayevsky, J., Bell, J., Jones, R. N., Diekema, D. J. (2001). Survey of Infectious Due to *Staphylococcus* Species: Frequency of Occurrence and Antimicrobial Susceptibility of Isolates Collected in the United States, Canada, Latin America, Europe, and the Western Pacific Region for the SENTRY Antimicrobial Surveillance. *Clinical Infectious Diseases*, **32**(2), S114–S132.

89. Dien Bard, J., Hindler, J. A., Gold, H. S., & Limbago, B. (2014). Rationale for Eliminating *Staphylococcus* Breakpoints for ß-lactam Agents Other Than Penicillin, Oxacillin or Cefoxitin, and Ceftaroline. *Clinical Infectious Diseases*, **58**(9), 1287–1296. https://doi.org/10.1093/cid/ciu043

90. Dinges, M. M., Orwin, P. M., Schlievert, P. M., Dinges, M. M., & Orwin, P. M. (2000). Exotoxins of *Staphylococcus aureus*. *Clinical Microbiology Reviews*, **13**(1), 16–34. https://doi.org/10.1128/CMR.13.1.16-34.2000.Updated

91. Dobrindt, U., Hacker, J. H., & Svanborg, C. (2013). *Current Topics in Microbiology and Immunology. In Between Pathogenicity and Commensalism*. Springer.

92. Donlan, R. M. (2001). Biofilm Formation: A Clinically Relevant Microbiological Process. *Clinical Infectious Diseases*, **33**(8), 1387–1392. https://doi.org/10.1086/322972

93. Donlan, R. M., & Costerton, J. W. (2002). Biofilms: Survival Mechanisms of Clinically Relevant Microorganisms. *Clinical Microbiology Reviews*, **15**(2), 167–193. https://doi.org/10.1128/CMR.15.2.167

94. Doulgeraki, A. I., Ciccio, P. Di, Ianieri, A., & Nychas, G.-J. E. (2016). Methicillin-Resistant Food-Related *Staphylococcus aureus*: A Review of Current Knowledge and Biofilm Formation for Future Studies and Applications. *Research in Microbiology*, **(August)**, 1–15. https://doi.org/10.1016/j.resmic.2016.08.001

95. Doyle, M. E., Hartmann, F. a, & Lee Wong, A. C. (2012). Methicillin-Resistant Staphylococci: Implications for Our Food Supply? *Animal Health Research Reviews / Conference of Research Workers in Animal Diseases*, **13**(2), 157–80. https://doi.org/10.1017/S1466252312000187

96. Drago, L., Agrappi, S., Bortolin, M., Toscano, M., Romanò, C. L., & De Vecchi, E. (2016). How to study biofilms after microbial colonization of materials used in orthopedic implants. *International Journal of Molecular Sciences*, **17**(3). https://doi.org/10.3390/ijms17030293

97. Dumitrescu, O., Boisset, S., Badiou, C., Bes, M., Benito, Y., Reverdy, M. E., Lina, G. (2007). Effect of Antibiotics on *Staphylococcus aureus* Producing Panton-Valentine Leukocidin. *Antimicrobial Agents and Chemotherapy*, **51**(4), 1515–1519. https://doi.org/10.1128/AAC.01201-06

E

98. EC 206/2009 on the introduction into the Community of personal consignments of products of animal origin and amending Regulation (EC) No 136/2004. (2009). *Official Journal of the European Union*.

99. EC 275/2007 concerning lists of animals and products to be subject to controls at border inspection posts under Council Directives 91/496/EEC and 97/78/EC. (2007). *Official Journal of the European Union*, **2007**(301), 9–33.

100. ECDC. (2009). *The Bacterial Challenge: Time to React*. EMEA. https://doi.org/10.2900/2518

101. EFSA. (2005). Commission Regulation (EC) No 2073/2005 of 15th November 2005 on microbiological criteria for foodstuffs. *Official Journal of the European Union*, **L338**, 1–26.

102. EFSA. (2009). Scientific Opinion of the Panel on Biological Hazards on a request from the European Commission on Assessment of the Public Health significance of methicillin resistant *Staphylococcus aureus* (MRSA) in animals and foods. *The EFSA Journal*, **993**, 1–73.

103. EFSA & ECDPC. (2014). The European Union Summary Report on Trends and Sources of Zoonoses, Zoonotic Agents and Food-borne Outbreaks in 2012. *EFSA Journal*, **12**(2), 1–312. https://doi.org/doi:10.2903/j.efsa.2012.2597

104. Enright, M. C., Day, N. P. J., Davies, C. E., Peacock, S. J., & Spratt, B. G. (2000). Multilocus Sequence Typing for Characterization of Methicillin-Resistant and Methicillin-Susceptible Clones of *Staphylococcus aureus*. *Journal of Clinical Microbiology*, **38**(3), 1008–1015.

105. Enright, M. C., Robinson, D. A., Randle, G., Feil, E. J., Grundmann, H., & Spratt, B. G. (2002). The Evolutionary History of Methicillin-Resistant *Staphylococcus aureus* (MRSA). *Proc. Natl. Acad. Sci. USA*, **99**(11), 7687–92.

106. European Centre for Disease Prevention and Control. (2015). Annual Epidemiological Report 2014. *Antimicrobial Resistance and Healthcare-Associated Infections*, 1–28.

107. European Commission. (1957). *The Treaty of Rome*. The Oxford Companion to the Politics of the World, 39(March), 1–80. Retrieved from http://ec.europa.eu/economy_finance/emu_history/documents/treaties/rometreaty2.pdf

108. European Food Safety Authority (EFSA). (2013). Annual Epidemiological Report 2013: Reporting on 2011 Surveillance Data and 2012 Epidemic Intelligence Data. https://doi.org/10.2900/76137

109. European Food Safety Authority (EFSA) & European Centre for Disease Prevention and Control (ECDC). (2015). The European Union Summary Report on Trends and Sources of Zoonoses, Zoonotic Agents and Food-Borne Outbreaks in 2013. *EFSA Journal*, **13**(1), 1–165. https://doi.org/10.2903/j.efsa.2015.3991

F

110. Fang, H. W., Chiang, P. H., & Huang, Y. C. (2014). Livestock-Associated Methicillin-Resistant *Staphylococcus aureus* ST9 in Pigs and Related Personnel in Taiwan. *PLoS ONE*, **9**(2), 1–7. https://doi.org/10.1371/journal.pone.0088826

111. Ferreira, A. A., Tette, P. A. S., Mendonça, R. C. S., Soares, A. D. S., & Carvalho, M. M. De. (2014). Detection of Exopolysaccharide Production and Biofilm-Related Genes in *Staphylococcus* spp. Isolated from a Poultry Processing Plant. *Food Science and Technology*, **34**(4), 710–716. https://doi.org/10.1590/1678-457X.6446

112. Ferreira, F. A., Souza, R. R., Bonelli, R. R., Américo, M. A., Fracalanzza, S. E. L., & Figueiredo, A. M. S. (2012). Comparison of *in vitro* and *in vivo* Systems to Study *ica*-Independent *Staphylococcus aureus* Biofilms. *Journal of Microbiological Methods*, **88**(3), 393–398. https://doi.org/10.1016/j.mimet.2012.01.007

113. Feßler, A. T., Kadlec, K., Hassel, M., Hauschild, T., Eidam, C., Ehricht, R., Schwarz, S. (2011). Characterization of Methicillin-Resistant *Staphylococcus aureus* Isolates From Food and Food Products of Poultry Origin in Germany. *Applied and Environmental Microbiology*, **77**(20), 7151–7157. https://doi.org/10.1128/AEM.00561-11

114. Filtering reformating or trimming genomic and metagenomic sequence data. (n.d.). *PRINSEQ*. Retrieved from http://prinseq.sourceforge.net/

115. Flemming, H.-C., Wingender, J., Szewzyk, U., Steinberg, P., Rice, S. A., & Kjelleberg, S. (2016). Biofilms: An Emergent Form of Bacterial Life. *Nature Reviews Microbiology*, **14**(9), 563–575. https://doi.org/10.1038/nrmicro.2016.94

116. Food and Agricultural Organization of the United Nations. (n.d.). Retrieved from www.fao.org

117. Food Standards Agency. (2011). Foodborne Disease Strategy for the Reduction of Foodborne Disease in the UK, **(May)**, 1–24.

118. Foster, T. (1996). *Chapter 12. Staphylococcus Identification of Staphylococci in the Clinical Laboratory*. In Medical Microbiology (pp. 1–12).

119. Foulston, L., Elsholz, A. K. W., DeFrancesco, A. S., & Losick, R. (2014). The Extracellular Matrix of *Staphylococcus aureus* Biofilms Comprises Cytoplasmic Proteins That Associate with the Cell Surface in Response to Decreasing pH. *mBio*, **5**(5), 1–9. https://doi.org/10.1128/mBio.01667-14

120. Frank, D. N., Feazel, L. M., Bessesen, M. T., Price, C. S., Janoff, E. N., & Pace, N. R. (2010). The Human Nasal Microbiota and *Staphylococcus aureus*. *PLoS ONE*, **5**(5), 1–15. https://doi.org/10.1371/journal.pone.0010598

121. Fratamico, P. M., Annous, B. A., & Guenther, N. W. (2009). *Biofilms in the Food and Beverage Industries (1st Editio)*. Oxford; Cambridge; Philadelphia; New Delhi: Woodhead Publishing Limited. Retrieved from https://www.elsevier.com/books/biofilms-in-the-food-and-beverage-industries/fratamico/978-1-84569-477-7

122. Fredheim, E. G. A., Klingenberg, C., Rohde, H., Frankenberger, S., Gaustad, P., Flægstad, T., & Sollid, J. E. (2009). Biofilm Formation by *Staphylococcus haemolyticus*. *Journal of Clinical Microbiology*, **47**(4), 1172–1180. https://doi.org/10.1128/JCM.01891-08

123. Freitas, A. I., Vasconcelos, C., Vilanova, M., & Cerca, N. (2014). Optimization of An Automatic Counting System for The Quantification of *Staphylococcus epidermidis* Cells in Biofilms. *Journal of Basic Microbiology*, **54**(7), 750–757. https://doi.org/10.1002/jobm.201200603

124. Fromm, S., Beißwanger, E., Käsbohrer, A., & Tenhagen, B. A. (2014). Risk Factors for MRSA in Fattening Pig Herds - A Meta-Analysis Using Pooled Data. *Preventive Veterinary Medicine*, **117**(1), 180–188. https://doi.org/10.1016/j.prevetmed.2014.08.014

125. Futagawa-Saito, K., Ba-Thein, W., Sakurai, N., & Fukuyasu, T. (2006). Prevalence of Virulence Factors in *Staphylococcus intermedius* Isolates from Dogs and Pigeons. *BMC Veterinary Research*, **2**(4), 1–5. https://doi.org/10.1186/1746-6148-2-4

G

126. García-Álvarez, L., Holden, M. T. G., Lindsay, H., Webb, C. R., Brown, D. F. J., Curran, M. D., Holmes, M. A. (2011). Methicillin-Resistant *Staphylococcus aureus* With a Novel mecA Homologue in Human and Bovine Populations in the UK and Denmark: A Descriptive Study. *The Lancet Infectious Diseases*, **11**(8), 595–603. https://doi.org/10.1016/S1473-3099(11)70126-8

127. Garrett, T. R., Bhakoo, M., & Zhang, Z. (2008). Bacterial Adhesion and Biofilms on Surfaces. P*rogress in Natural Science*, **18**(9), 1049–1056. https://doi.org/10.1016/j.pnsc.2008.04.001

128. Gharsa, H., Ben Sallem, R., Ben Slama, K., Gomez-Sanz, E., Lozano, C., Jouini, A., Torres, C. (2012). High Diversity of Genetic Lineages and Virulence Genes in Nasal *Staphylococcus aureus* Isolates from Donkeys Destined to Food Consumption in Tunisia with Predominance of the Ruminant Associated CC133 Lineage. *BMC Veterinary Research*, **8**, 203. https://doi.org/10.1186/1746-6148-8-203

129. Giannouli, S., Labrou, M., Kyritsis, A., Ikonomidis, A., Pournaras, S., Stathopoulos, C., & Tsakris, A. (2010). Detection of Mutations in the FemXAB Protein Family in Oxacillin-Susceptible *mec*A-Positive *Staphylococcus aureus* Clinical Isolates. *Journal of Antimicrobial Chemotherapy*, **65**(4), 626–633. https://doi.org/10.1093/jac/dkq039

130. Gilden, D. (2013). *Neuroinfections (What Do I Do Now?)*. Book Press- Center for Disease Control and Prevention, **19**(9), 1553. Retrieved from https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3810938/pdf/ET-1909.pdf

131. Golding, G. R., Bryden, L., Levett, P. N., McDonald, R. R., Wong, A., Wylie, J., Mulvey, M. R. (2010). Livestock-Associated Methicillin-Resistant *Staphylococcus aureus* Sequence Type 398 in Humans, Canada. *Emerging Infectious Diseases*, **16**(4), 587–594. https://doi.org/10.3201/eid1604.091435

132. Gonano, M., Hein, I., Zangerl, P., Rammelmayr, A., & Wagner, M. (2009). Phenotypic and molecular characterization of *Staphylococcus aureus* strains of veterinary, dairy and human origin. *Epidemiology and Infection*, **137**(5), 688–99. https://doi.org/10.1017/S0950268808001453

133. González-Zorn, B., & Escudero, J. A. (2012). Ecology of Antimicrobial Resistance: Humans, Animals, Food and Environment. *International Microbiology*, **15**(3), 101–109. https://doi.org/10.2436/20.1501.01.163

134. Gordon, R. J., & Lowy, F. D. (2008). Pathogenesis of Methicillin-Resistant *Staphylococcus aureus* Infection. *Clinical Infectious Diseases*, **46**(5), S350–S359. https://doi.org/10.1086/533591

135. Gorwitz, R. J., Kruszon-Moran, D., McAllister, S. K., McQuillan, G., McDougal, L. K., Fosheim, G. E., Kuehnert, M. J. (2008). Changes in the Prevalence of Nasal Colonization with *Staphylococcus aureus* in the United States, 2001-2004. *Journal of Infectious Diseases*, **197**(9), 1226–1234. https://doi.org/10.1086/533494

136. Graveland, H., Duim, B., van Duijkeren, E., Heederik, D., & Wagenaar, J. A. (2011). Livestock-Associated Methicillin-Resistant *Staphylococcus aureus* in Animals and Humans. *International Journal of Medical Microbiology*, **301**(8), 630–634. https://doi.org/10.1016/j.ijmm.2011.09.004

137. Grema, H. A., Geidam, Y. A., Gadzama, G. B., Ameh, J. A., & Suleiman, A. (2015). Methicillin Resistant *Staphylococcus aureus* (MRSA): A Review. *Advances in Animal and Veterinary Sciences*, **3**(2), 79–98. https://doi.org/10.14737/journal.aavs/2015/3.2.79.98

138. Grissa, I., Vergnaud, G., & Pourcel, C. (2007). CRISPRcompar: A Web Tool to Identify Clustered Regularly Interspaced Short Palindromic Repeats. *Nucleic Acids Research*, **36**(Web Server issue), 52–57. https://doi.org/10.1093/nar/gkn228

139. Grøntvedt, C. A., Elstrøm, P., Stegger, M., Skov, R. L., Skytt Andersen, P., Larssen, K. W., Bjørnholt, J. V. (2016). Methicillin-Resistant *Staphylococcus aureus* CC398 in Humans and Pigs in Norway: A One Health" Perspective on Introduction and Transmission. *Clinical Infectious Diseases*, **63**(11), 1431–8. https://doi.org/10.1093/cid/ciw552

140. Gutiérrez, D., Delgado, S., Vázquez-Sánchez, D., Martínez, B., Cabo, M. L., Rodríguez, A., García, P. (2012). Incidence of *Staphylococcus aureus* and Analysis of Associated Bacterial Communities on Food Industry Surfaces. *Applied and Environmental Microbiology*, **78**(24), 8547–8554. https://doi.org/10.1128/AEM.02045-12

141. Gwida, M., Neubauer, H., Ilhan, Z., Schmoock, G., Melzer, F., Nöckler, K., Al Dahouk, S. (2012). Cross-Border Molecular Tracing of Brucellosis in Europe. *Comparative Immunology, Microbiology and Infectious Diseases*, **35**(2), 181–185. https://doi.org/10.1016/j.cimid.2011.12.012

H

142. Hadjirin, N. F., Lay, E. M., Paterson, G. K., Harrison, E. M., Peacock, S. J., Parkhill, J., Holmes, M. A. (2015). Detection of Livestock-Associated Methicillin-Resistant *Staphylococcus aureus* CC398 in Retail Pork, United Kingdom, February 2015. *Eurosurveillance*, **20**(24), 1–4.

143. Hanson, B. M., Dressler, A. E., Harper, A. L., Scheibel, R. P., Wardyn, S. E., Roberts, L. K., Smith, T. C. (2011). Prevalence of *Staphylococcus aureus* and Methicillin-Resistant *Staphylococcus aureus* (MRSA) on Retail Meat in Iowa. *Journal of Infection and Public Health*, **4**(4), 169–174. https://doi.org/10.1016/j.jiph.2011.06.001

144. Haran, K. P., Godden, S. M., Boxrud, D., Jawahir, S., Bender, J. B., & Sreevatsan, S. (2012). Prevalence and Characterization of *Staphylococcus aureus*, including Methicillin-resistant *Staphylococcus aureus*, Isolated from Bulk Tank Milk from Minnesota Dairy Farms. *Journal of Clinical Microbiology*, **50**(3), 688–695. https://doi.org/10.1128/JCM.05214-11

145. Harmsen, D., Claus, H., Witte, W., Rothgänger, J., Claus, H., Turnwald, D., & Vogel, U. (2003). Typing of methicillin-resistant *Staphylococcus aureus* in a university hospital setting by using novel software for *spa* repeat determination and database management. *Journal of Clinical Microbiology*, **41**(12), 5442–5448. https://doi.org/10.1128/JCM.41.12.5442

146. Harrison, E. M., Paterson, G. K., Holden, M. T. G., Larsen, J., Stegger, M., Larsen, A. R., Holmes, M. A. (2013). Whole Genome Sequencing Identifies Zoonotic Transmission of MRSA Isolates with the Novel mecA Homologue *mec*C. *EMBO Molecular Medicine*, **5**(4), 509–515. https://doi.org/10.1002/emmm.201202413

147. Haveri, M., Roslöf, A., Rantala, L., & Pyörälä, S. (2007). Virulence Genes of Bovine *Staphylococcus aureus* from Persistent and Nonpersistent Intramammary Infections with Different Clinical Characteristics. *Journal of Applied Microbiology*, **103**(4), 993–1000. https://doi.org/10.1111/j.1365-2672.2007.03356.x

148. Hennekinne, J. A., De Buyser, M. L., & Dragacci, S. (2012). *Staphylococcus aureus* and Its Food Poisoning Toxins: Characterization and Outbreak Investigation. *FEMS Microbiology Reviews*, **36**(4), 815–836. https://doi.org/10.1111/j.1574-6976.2011.00311.x

149. Homepage of the Bioinformatics Group at the Babraham Institute. (n.d.). *Babraham Bioinformatics*. Retrieved from http://www.bioinformatics.babraham.ac.uk/projects/fastqc/

150. Hososaka, Y., Hanaki, H., Endo, H., Suzuki, Y., Nagasawa, Z., Otsuka, Y., Sunakawa, K. (2007). Characterization of Oxacillin-Susceptible *mec*A-Positive *Staphylococcus aureus*: A New Type of MRSA. *Journal of Infection and Chemotherapy*, **13**(2), 79–86. https://doi.org/10.1007/s10156-006-0502-7

151. Houston, P., Rowe, S. E., Pozzi, C., Waters, E. M., & O'Gara, J. P. (2011). Essential Role for the Major Autolysin in the Fibronectin-Binding Protein-Mediated *Staphylococcus aureus* Biofilm Phenotype. *Infection and Immunity*, **79**(3), 1153–1165. https://doi.org/10.1128/IAI.00364-10

152. Huang, E., Gurzau, A. E., Hanson, B. M., Kates, A. E., Smith, T. C., Pettigrew, M. M., Rabinowitz, P. M. (2014). Detection of Livestock-Associated Methicillin-Resistant *Staphylococcus aureus* Among Swine Workers in Romania. *Journal of Infection and Public Health*, **7**(4), 323–332. https://doi.org/10.1016/j.jiph.2014.03.008

153. Hudson, J. A. (2010). Evaluation of Methods for Detection of Coagulase Positive *Staphylococcus* and *Staphylococcal* Toxin in Milk.

I

154. ICMSF. (2011). http://www.icmsf.org/

155. International Working Group on the Staphylococcal Cassette Chromosome elements. (n.d.). Staphylococcal Cassette Chromosome *mec*. Retrieved from www.sccmec.org

156. ISO. (1999). ISO 6888-2:1999. In Microbiology of food and animal feeding stuffs- horizontal method for the enumeration of coagulase-positive staphylococci (*Staphylococcus aureus* and other species)- Part 2: technique using rabbit plasma fibrinogen agar medium.

157. Ivana, S., Bogdan, A. T., Țogoe, I., Câmpeanu, G., Enache, T., Bărăităreanu, S., Popescu, A. (2011). *Microbiologia Alimentelor - Patogeni alimentari*. Asclepius.

J

158. Jackson, C. R., Davis, J. A., & Barrett, J. B. (2013). Prevalence and Characterization of Methicillin-Resistant *Staphylococcus aureus* Isolates from Retail Meat and Humans in Georgia. *Journal of Clinical Microbiology*, **51**(4), 1199–1207. https://doi.org/10.1128/JCM.03166-12

159. Jay, J. M., Loessner, M. J., & Golden, D. A. (2005). *Modern Food Microbiology*. In Modern Food Microbiology (pp. 1–782). Springer.

160. Jevons, M. P. (1961). "Celbenin"- Resistant Staphylococci. *British Medical Journal*, **1**, 124–125. https://doi.org/10.1136/bmj.1.5219.124-a

161. John, J. F., & Harvin, A. M. (2007). History and Evolution of Antibiotic Resistance in Coagulase-Negative Staphylococci: Susceptibility Profiles of New Anti-Staphylococcal Agents. *Therapeutics and Clinical Risk Management*, **3**(6), 1143–1152.

162. Jones, T. F., Kellum, M. E., Porter, S. S., Bell, M., & Schaffner, W. (2002). An Outbreak of Community-Acquired Foodborne Illness Caused by Methicillin-Resistant *Staphylococcus aureus*. E*merging Infectious Diseases*, **8**(1), 82–84. https://doi.org/10.3201/eid0801.010174

163. Jordan, D., Simon, J., Fury, S., Moss, S., Giffard, P., Maiwald, M., Trott, D. J. (2011). Carriage of Methicillin-Resistant *Staphylococcus aureus* by Veterinarians in Australia. *Australian Veterinary Journal*, **89**(5), 152–159. https://doi.org/10.1111/j.1751-0813.2011.00710.x

164. Jusko, M., Potempa, J., Kantyka, T., Bielecka, E., Miller, H., & Kalinska, M. (2014). Staphylococcal Proteases Air in Evasion of the Human Complement System. *Journal of Innate Immunity*, **6**(1), 31–46. https://doi.org/10.1016/j.biotechadv.2011.08.021.Secreted

K

165. Kadariya, J., Smith, T. C., & Thapaliya, D. (2014). *Staphylococcus aureus* and Staphylococcal Food-Borne Disease: An Ongoing Challenge in Public Health. *BioMed Research International*, **2014**, 1–9. https://doi.org/10.1155/2014/827965

166. Kim, B.-R., Bae, Y.-M., Hwang, J.-H., & Lee, S.-Y. (2016). Biofilm Formation and Cell Surface Properties of *Staphylococcus aureus* Isolates from Various Sources. *Food Science and Biotechnology*, **25**(2), 643–648. https://doi.org/10.1007/s10068-016-0090-y

167. Kinde, H., Mikolon, A., Rodriguez-Lainz, A., Adams, C., Walker, R. L., Cernek-Hoskins, S., Ardans, A. (2007). Recovery of *Salmonella*, *Listeria monocytogenes*, and *Mycobacterium bovis* from Cheese Entering the United States through a Noncommercial Land Port of Entry. *Journal of Food Protection*, **70**(1), 47–52. https://doi.org/10.4315/0362-028X-70.1.47

168. Kluytmans, J. (2010). MRSA in Food Products: Cause for Concern or Case for Complacency? *Clinical Microbiology and Infection*, **16**, 11–15.

169. Kluytmans, J., Leeuwen, W. Van, Goessens, W., Hollis, R., Messer, S., Kluytmans, J. a N., Belkum, A. Verbruch, H. (1995). Food-Initiated Outbreak of Methicillin-Resistant *Staphylococcus aureus* Analyzed by Pheno- and Genotyping, *Journal of Clinical Microbiology*, **33**(5), 1121–1128.

170. Kluytmans, J., van Belkum, A., & Verbrugh, H. (1997). Nasal Carriage of *Staphylococcus aureus*: Epidemiology, Underlying Mechanisms, and Associated Risks. *Clinical Microbiology Reviews*, **10**(3), 505–520. https://doi.org/.doi:10.1016/S0006-3207(03)00146-0

171. Koboldt, D. C., Zhang, Q., Larson, D. E., Shen, D., McLellan, M. D., Lin, L., Wilson, R. K. (2012). VarScan 2: Somatic Mutation and Copy Number Alteration Discovery in Cancer by Exome Sequencing. *Genome Research*, **22**(3), 568–576. https://doi.org/10.1101/gr.129684.111

172. Köck, R., Becker, K., Cookson, B., van Gemert-Pijnen, J. E., Harbarth, S., Kluytmans, J., Friedrich, A. W. (2010). Methicillin-Resistant *Staphylococcus aureus* (MRSA): Burden of Disease and Control Challenges in Europe. *Euro Surveillance*, **15**, 1–9.

173. Köck, R., Loth, B., Köksal, M., Schulte-Wülwer, J., Harlizius, J., & Friedrich, A. W. (2012). Persistence of Nasal Colonization with Livestock-Associated Methicillin-Resistant *Staphylococcus aureus* in Pig Farmers After Holidays from Pig Exposure. *Applied and Environmental Microbiology*, **78**(11), 4046–4047. https://doi.org/10.1128/AEM.00212-12

174. Köck, R., Schaumburg, F., Mellmann, A., Köksal, M., Jurke, A., Becker, K., & Friedrich, A. W. (2013). Livestock-Associated Methicillin-Resistant *Staphylococcus aureus* (MRSA) as Causes of Human Infection and Colonization in Germany. *PLoS ONE*, **8**(2). https://doi.org/10.1371/journal.pone.0055040

175. Kogan, G., Sadovskaya, I., Chaignon, P., Chokr, A., & Jabbouri, S. (2006). Biofilms of Clinical Strains of *Staphylococcus* That Do Not Contain Polysaccharide Intercellular Adhesin. *FEMS Microbiology Letters*, **255**(1), 11–16. https://doi.org/10.1111/j.1574-6968.2005.00043.x

176. Kondo, Y., Ito, T., Ma, X. X., Watanabe, S., Kreiswirth, B. N., Etienne, J., & Hiramatsu, K. (2007). Combination of Multiplex PCRs for Staphylococcal Cassette Chromosome *mec* Type Assignment: Rapid Identification System for *mec*, *ccr*, and Major Differences in Junkyard Regions. *Antimicrobial Agents and Chemotherapy*, **51**(1), 264–274. https://doi.org/10.1128/AAC.00165-06

177. Krziwanek, K., Metz-Gercek, S., & Mittermayer, H. (2009). Methicillin-Resistant *Staphylococcus aureus* ST398 from Human Patients, upper Austria. *Emerging Infectious Diseases*, **15**(5), 766–769. https://doi.org/10.3201/eid1505.080326

178. Kumar, V. A., Steffy, K., Chatterjee, M., Sugumar, M., Dinesh, K. R., Manoharan, A., Biswas, R. (2013). Detection of Oxacillin-Susceptible *mec*A-Positive *Staphylococcus aureus* Isolates by Use of Chromogenic Medium MRSA ID. *Journal of Clinical Microbiology*, **51**(1), 318–319. https://doi.org/10.1128/JCM.01040-12

179. Kuroda, M., Ohta, T., Uchiyama, I., Baba, T., Yuzawa, H., Kobayashi, I., Hiramatsu, K. (2001). Whole Genome Sequencing of Methicillin-Resistant *Staphylococcus aureus*. *Lancet*, **357**(9264), 1225–40. https://doi.org/10.1016/S0140-6736(00)04403-2

L

180. Langmead, B., & Salzberg, S. L. (2012). Fast Gapped-Read Alignment with Bowtie 2. *Nature Methods*, **9**(4), 357–359. https://doi.org/10.1038/nmeth.1923

181. Larsen, J., Andersen, P. S., Winstel, V., & Peschel, A. (2017). *Staphylococcus aureus* CC395 Harbours a Novel Composite Staphylococcal Cassette Chromosome *mec* Element. *The Journal of Antimicrobial Chemotherapy*, **72**(January), 1002–1005. https://doi.org/10.1093/jac/dkw544

182. Larsen, J., Petersen, A., Sørum, M., Stegger, M., Van Alphen, L., Valentiner-Branth, P., Skov, R. (2015). Methicillin-Resistant *Staphylococcus aureus* CC398 is An Increasing Cause of Disease in People with No Livestock Contact in Denmark, 1999 to 2011. *Euro Surveillance*, **20**(37), 1–17. https://doi.org/10.2807/1560-7917.ES.2015.20.37.30021

183. Lauchlin, J. M. C., Narayanan, G. L., & Mithani, V. (2000). The Detection of Enterotoxins and Toxic Shock Syndrome Toxin Genes in *Staphylococcus aureus* by Polymerase Chain Reaction. *Journal of Food Protection*, **63**(4), 479–488.

184. Le Loir, Y., Baron, F., & Gautier, M. (2003). *Staphylococcus aureus* and Food Poisoning. *Genetics and Molecular Research*, **2**(1), 63–76. https://doi.org/S02 [pii]

185. Lewis, H. C., Mølbak, K., Reese, C., Aarestrup, F. M., Selchau, M., Sørum, M., & Skov, R. L. (2008). Pigs as Source of Methicillin-Resistant *Staphylococcus aureus* CC398 Infections in Humans, Denmark. *Emerging Infectious Diseases*, **14**(9), 1383–1389. https://doi.org/10.32007-1576Research

186. Li, H., Handsaker, B., Wysoker, A., Fennell, T., Ruan, J., Homer, N., Durbin, R. (2009). The Sequence Alignment/Map format and SAMtools. *Bioinformatics*, **25**(16), 2078–2079. https://doi.org/10.1093/bioinformatics/btp352

187. Lina, G., Piémont, Y., Godail-Gamot, F., Bes, M., Peter, M.-O., Gauduchon, V., Etienne, J. (1999). Involvement of Panton-Valentine Leukocidin-Producing *Staphylococcus aureus* in Primary Skin Infections and Pneumonia. *Clinical Infectious Diseases*, **29**(5), 1128–1132. https://doi.org/10.1086/313461

188. Lo, W.-T., Lin, W.-J., Tseng, M.-H., Lu, J.-J., Lee, S.-Y., Chu, M.-L., & Wang, C.-C. (2007). Nasal carriage of a single clone of community-acquired methicillin-resistant *Staphylococcus aureus* among kindergarten attendees in northern Taiwan. *BMC Infectious Diseases*, **7**(1), 51. https://doi.org/10.1186/1471-2334-7-51

189. Locatelli, C., Cremonesi, P., Caprioli, A., Carfora, V., Ianzano, A., Barberio, A., Moroni, P. (2016). Occurrence of Methicillin-Resistant *Staphylococcus aureus* in Dairy Cattle Herds, Related Swine Farms, and Humans in Contact with Herds. *Journal of Dairy Science*, 1–12. https://doi.org/10.3168/jds.2016-11797

190. Loeffler, A., Boag, A. K., Sung, J., Lindsay, J. A., Guardabassi, L., Dalsgaard, A., Lloyd, D. H. (2005). Prevalence of Methicillin-Resistant *Staphylococcus aureus* Among Staff and Pets in a Small Animal Referral Hospital in the UK. *Journal of Antimicrobial Chemotherapy*, **56**(4), 692–697. https://doi.org/10.1093/jac/dki312

191. Löffler, B., Hussain, M., Grundmeier, M., Brück, M., Holzinger, D., Varga, G., Peters, G. (2010). *Staphylococcus aureus* Panton-Valentine Leukocidin is a Very Potent Cytotoxic Factor for Human Neutrophils. *PLoS Pathogens*, **6**(1), 1–12. https://doi.org/10.1371/journal.ppat.1000715

192. Lowder, B. V, Guinane, C. M., Ben Zakour, N. L., Weinert, L. A., Conway-Morris, A., Cartwright, R. A., Fitzgerald, J. R. (2009). Recent Human-to-Poultry Host Jump, Adaptation, and Pandemic Spread of *Staphylococcus aureus*. *Proc.Natl.Acad.Sci.U.S.A*, **106**(46), 19545–19550. https://doi.org/10.1073/pnas.0909285106

193. Lowy, F. (2003). Antimicrobial Resistance: The Example of *Staphylococcus aureus*. *Journal of Clinical Investigation*, **111**(9), 1265–1273. https://doi.org/10.1172/JCI200318535.In

194. Lowy, F. D. (1998). *Staphylococcus aureus* Infections. *New England Journal of Medicine*, **339**, 520–532. https://doi.org/10.1056/NEJM199808203390806

195. Lozano, C., Gómez-Sanz, E., Benito, D., Aspiroz, C., Zarazaga, M., & Torres, C. (2011). *Staphylococcus aureus* Nasal Carriage, Virulence Traits, Antibiotic Resistance Mechanisms, and Genetic Lineages in Healthy Humans in Spain, with Detection of CC398 and CC97 Strains. *International Journal of Medical Microbiology*, **301**(6), 500–505. https://doi.org/10.1016/j.ijmm.2011.02.004

196. Lozano, C., López, M., Gómez-Sanz, E., Ruiz-Larrea, F., Torres, C., & Zarazaga, M. (2009). Detection of Methicillin-Resistant *Staphylococcus aureus* ST398 in Food Samples of Animal Origin in Spain. *Journal of Antimicrobial Chemotherapy*, **64**(6), 1325–1326. https://doi.org/10.1093/jac/dkp378

197. Lozano, C., Rezusta, A., Gómez, P., Gómez-sanz, E., Báez, N., Martin-saco, G., Torres, C. (2012). High Prevalence of *spa* Types Associated with the Clonal Lineage CC398 Among Tetracycline-Resistant Methicillin-Resistant *Staphylococcus aureus* Strains in a Spanish Hospital. *Journal of Antimicrobial Chemotherapy*, **67**(2), 330–334. https://doi.org/10.1093/jac/dkr497

198. Luong, T. T., Lei, M. G., & Lee, C. Y. (2009). *Staphylococcus aureus* Rbf Activates Biofilm Formation in vitro and Promotes Virulence in a Murine Foreign Body Infection Model. *Infection and Immunity*, **77**(1), 335–340. https://doi.org/10.1128/IAI.00872-08

M

199. Mah, R. A., Fung, D. Y. & Morse, S. A. (1967). Nutritional Requirements of *Staphylococcus aureus* S-6. *Applied Microbiology*, **15**(4), 866–870. Retrieved from http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=547084&tool=pmcentrez&rendertype=abstract

200. Malhotra-Kumar, S., Abrahantes, C., Sabiiti, W., Lammens, C., Vercauteren, G., Ieven, M., Aerts, M. (2010). Evaluation of Chromogenic Media for Detection of Methicillin-Resistant *Staphylococcus aureus*. *Journal of Clinical Microbiology*, **48**(4), 1040–1046. https://doi.org/10.1128/JCM.01745-09

201. Marques, S. C., Rezende, J. D. G. O. S., Alves, L. A. D. F., Silva, B. C., Alves, E., De Abreu, L. R., & Piccoli, R. H. (2007). Formation of Biofilms by *Staphylococcus aureus* on Stainless Steel and Glass Surfaces and Its Resistance to Some Selected Chemical Sanitizers. *Brazilian Journal of Microbiology*, **38**(3), 538–543. https://doi.org/10.1590/S1517-83822007000300029

202. McDougal, L. K., Steward, C. D., George, E., Chaitram, J. M., Mcallister, S. K., Tenover, C., Tenover, F. C. (2003). Pulsed-Field Gel Electrophoresis Typing of Oxacillin-Resistant *Staphylococcus aureus* Isolates from the United States: Establishing a National Database. *Journal of Clinical Microbiology*, **41**(11), 5113–5120. https://doi.org/10.1128/JCM.41.11.5113

203. McElhinney, R., Millar, C., & Scopes, E. (2013). A Comparative Evaluation of ChromID MRSA agar and Brilliance 2 MRSA Agar for Detection of MRSA in Clinical Samples. *British Journal of Biomedical Science*, **70**(1), 41–43.

204. Mckinney, T. K., Sharma, V. K., Craig, W. a, Archer, G. L., & Kinney, T. K. M. C. (2001). Transcription of the Gene Mediating Methicillin Resistance in *Staphylococcus aureus* (*mec*A) Is Corepressed but Not Coinduced by Cognate *mec*A and β Lactamase Regulators. *Journal of Bacteriology*, **183**(23), 6862–6868. https://doi.org/10.1128/JB.183.23.6862

205. Media, C. (n.d.). Brilliance MRSA. Retrieved from http://www.oxoid.com/pdf/mrsa/27659-Data-MRSA.pdf

206. Meemken, D., Blaha, T., Tegeler, R., Tenhagen, B. A., Guerra, B., Hammerl, J. A., Fetsch, A. (2010). Livestock Associated Methicillin-Resistant *Staphylococcus aureus* (LA-MRSA) Isolated from Lesions of Pigs at Necropsy in Northwest Germany Between 2004 and 2007. *Zoonoses and Public Health*, **57**, e143–e148. https://doi.org/10.1111/j.1863-2378.2009.01313.x

207. Mehrotra, M., Wang, G., & Johnson, W. M. (2000). Multiplex PCR for Detection of Genes for *Staphylococcus aureus* Enterotoxins, Exfoliative Toxins, Toxic Shock Syndrome Toxin 1, and Methicillin Resistance. *Journal of Clinical Microbiology*, **38**(3), 1032–1035.

208. Merino, N., Toledo-Arana, A., Vergara-Irigaray, M., Valle, J., Solano, C., Calvo, E., Lasa, I. (2009). Protein A-Mediated Multicellular Behavior in *Staphylococcus aureus*. *Journal of Bacteriology*, **191**(3), 832–843. https://doi.org/10.1128/JB.01222-08

209. Miko, B. A., Hafer, C. A., Lee, C. J., Sullivan, S. B., Hackel, M. A. M., Johnson, B. M., Lowy, F. D. (2013). Molecular Characterization of Methicillin-Susceptible *Staphylococcus aureus* Clinical Isolates in the United States, 2004 to 2010. *Journal of Clinical Microbiology*, **51**(3), 874–879. https://doi.org/10.1128/JCM.00923-12

210. Milheiriço, C., Oliveira, D. C., & de Lencastre, H. (2007). Multiplex PCR Strategy for Subtyping the Staphylococcal Cassette Chromosome *mec* Type IV in Methicillin-Resistant *Staphylococcus aureus*: "SCC*mec* IV Multiplex." *Journal of Antimicrobial Chemotherapy*, **60**(1), 42–48. https://doi.org/10.1093/jac/dkm112

211. Mirani, Z. A., Aziz, M., Khan, M. N., Lal, I., Hassan, N. ul, & Khan, S. I. (2013). Biofilm Formation and Dispersal of *Staphylococcus aureus* Under the Influence of Oxacillin. *Microbial Pathogenesis*, **61–62**, 66–72. https://doi.org/10.1016/j.micpath.2013.05.002

212. Molla, B., Byrne, M., Abley, M., Mathews, J., Jackson, C. R., Fedorka-Cray, P., Gebreyes, W. A. (2012). Epidemiology and genotypic characteristics of methicillin-resistant *Staphylococcus aureus* strains of porcine origin. *Journal of Clinical Microbiology*, **50**(11), 3687–3693. https://doi.org/10.1128/JCM.01971-12

213. Monecke, S., Coombs, G., Shore, A. C., Coleman, D. C., Akpaka, P., Borg, M., Ehricht, R. (2011). A Field Guide to Pandemic, Epidemic and Sporadic Clones of Methicillin-Resistant *Staphylococcus aureus*. *PLoS ONE*, **6**(4), 1–24. https://doi.org/10.1371/journal.pone.0017936

214. Monecke, S., Müller, E., Dorneanu, O. S., Vremeră, T., & Ehricht, R. (2014). Molecular Typing of MRSA and of Clinical *Staphylococcus aureus* Isolates from Iași, Romania. *PLoS ONE*, **9**(5), 1–9. https://doi.org/10.1371/journal.pone.0097833

215. Moodley, A., Nielsen, S. S., & Guardabassi, L. (2011). Effects of Tetracycline and Zinc on Selection of Methicillin-Resistant *Staphylococcus aureus* (MRSA) Sequence Type 398 in Pigs. *Veterinary Microbiology*, **152**, 420–423. https://doi.org/10.1016/j.vetmic.2014.08.021

216. Mulders, M. N., Haenen, P. J., Geenen, P. L., Vesseur, P. C., Poldervaart, E. S., Bosch, T., Van De Giessen, W. (2010). Prevalence of Livestock-Associated MRSA in Broiler Flocks and Risk Factors for Slaughterhouse Personnel in The Netherlands. *Epidemiology and Infection*, **138**, 743–755. https://doi.org/10.1017/S0950268810000075

217. Murphy, C. R., Quan, V., Kim, D., Peterson, E., Whealon, M., Tan, G., McGeer, A. (2012). Nursing home characteristics associated with methicillin-resistant *Staphylococcus aureus* (MRSA) Burden and Transmission. *BMC Infectious Diseases*, **12**:269(1), 1–7. https://doi.org/10.1186/1471-2334-12-269

218. Mustapha, M., Bukar-kolo, Y. M., Geidam, Y. A., & Gulani, I. A. (2014). Review on Methicillin-Resistant *Staphylococcus aureus* (MRSA) in Dogs and Cats. *International Journal of Animal and Veterinary Advances*, **6**(2), 61–73.

N

219. Nica, M., Biolan, T., Dascalu, A., Mozes, E., Toderan, A., Calistru, P., & Al., E. (2010). Bacterial Strains Isolated from Systemic Infections and Reported for Evaluation and Antibiotic Resistance Surveillance by the "Dr. Victor Babeș" Clinical Hospital for Infectious and Tropical Diseases, Bucharest. *Bacteriologia, Virusologia, Parazitologia, Epidemiologia*, **55**, 161–168.

220. Noordhuizen, J., Surborg, H., & Smulders, F. J. M. (2013). On the Efficacy of Current Biosecurity Measures at EU Borders to Prevent the Transfer of Zoonotic and Livestock Diseases by Travelers. *Veterinary Quarterly*, **33**(3), 161–71. https://doi.org/10.1080/01652176.2013.826883

221. Normanno, G., Corrente, M., La Salandra, G., Dambrosio, A., Quaglia, N. C., Parisi, A., Celano, G. V. (2007). Methicillin-resistant *Staphylococcus aureus* (MRSA) in foods of animal origin product in Italy. *International Journal of Food Microbiology*, **117**(2), 219–222. https://doi.org/10.1016/j.ijfoodmicro.2007.04.006

222. Normanno, G., Dambrosio, A., Lorusso, V., Samoilis, G., Di Taranto, P., & Parisi, A. (2015). Methicillin-Resistant *Staphylococcus aureus* (MRSA) in Slaughtered Pigs and Abattoir Workers in Italy. *Food Microbiology*, **51**, 51–56. https://doi.org/10.1016/j.fm.2015.04.007

O

223. O'Brien, A. M., Hanson, B. M., Farina, S. A., Wu, J. Y., Simmering, J. E., Wardyn, S. E., Smith, T. C. (2012). MRSA in Conventional and Alternative Retail Pork Products. *PLoS ONE*, **7**(1), 1–6. https://doi.org/10.1371/journal.pone.0030092

224. Monitorul Oficial. Legea 10/2010 pentru ratificarea Acordului dintre Guvernul Romaniei si Guvernul Republicii Moldova privind micul trafic de frontiera, semnat la Bucuresti la 13 noiembrie 2009. Retrieved from http://www.dreptonline.ro/legislatie/lege_ratificare_acord_romania_moldova_micul_trafic_frontiera_10_2010.php

225. Ogata, K., Narimatsu, H., Suzuki, M., Higuchi, W., Yamamoto, T., & Taniguchi, H. (2012). Commercially Distributed Meat as a Potential Vehicle for Community-Acquired Methicillin-Resistant *Staphylococcus aureus*. *Applied and Environmental Microbiology*, **78**(8), 2797–2802. https://doi.org/10.1128/AEM.07470-11

226. Ohbayashi, T., Irie, A., Murakami, Y., Nowak, M., Potempa, J., Nishimura, Y., Imamura, T. (2011). Degradation of Fibrinogen and Collagen by Staphopains, Cysteine Proteases Released from *Staphylococcus aureus*. *Microbiology*, **157**(3), 786–792. https://doi.org/10.1099/mic.0.044503-0

227. Okuma, K., Iwakawa, K., Turnidge, J. D., Grubb, W. B., Bell, J. M., O'Brien, F. G., Hiramatsu, K. (2002). Dissemination of New Methicillin-Resistant *Staphylococcus aureus* Clones in the Community. *Journal of Clinical Microbiology*, **40**(11), 4289–4294. https://doi.org/10.1128/JCM.40.11.4289

228. Oliveira, D. C., & de Lencastre, H. (2011). Methicillin-Resistance in *Staphylococcus aureus* Is Not Affected by The Overexpression in Trans of The *mec*A Gene Repressor: A Surprising Observation. *PLoS ONE*, 6(8), 1–9. https://doi.org/10.1371/journal.pone.0023287

229. Olivier, A., Budka, H., Buncic, S., Colin, P., Collins, J., De Koeijer, A., Griffin, J., Havelaar, A., Hope, J., Klein, G., Kruse, H., Magnino, S., Martínez López, A., McLauchlin, J., Nguyen, C., Karsten, E. V. (2009). Assessment of the Public Health Significance of Methicillin Resistant *Staphylococcus aureus* (MRSA) in Animals and Foods. *EFSA Journal*, **993**, 1–73. https://doi.org/10.2903/j.efsa.2009.993

230. Oniciuc, E.-A., Ariza-Miguel, J., Bolocan, A.-S., Diez-Valcarce, M., Rovira, J., Hernández, M., Rodríguez-Lázaro, D. (2015). Foods from Black Market at EU Border as a Neglected Route of Potential Methicillin-Resistant *Staphylococcus aureus* Transmission. *International Journal of Food Microbiology*, **209**, 34-38. https://doi.org/10.1016/j.ijfoodmicro.2014.11.015

231. Oniciuc, E.-A., Nicolau, A. I., Hernández, M., & Rodríguez-Lázaro, D. (2017). Presence of Methicillin-Resistant *Staphylococcus aureus* in the Food Chain. *Trends in Food Science & Technology*, **61**, 49–59. https://doi.org/10.1016/j.tifs.2016.12.002

232. Oniciuc, E. A., Cerca, N., & Nicolau, A. I. (2016). Compositional Analysis of Biofilms Formed by *Staphylococcus aureus* Isolated from Food Sources. *Frontiers in Microbiology*, **7**:390, 2014–2017. https://doi.org/10.3389/fmicb.2016.00390

233. Orenstein, A. (1998). The Discovery and Naming of *Staphylococcus aureus*. *Infectious Diseases & Antimicrobial Agents*, 1880–1881. https://doi.org/10.1021/bc049951i

234. Ortega, E., Abriouel, H., Lucas, R., & Galvez, A. (2010). Multiple Roles of *Staphylococcus aureus* Enterotoxins: Pathogenicity, Superantigenic Activity, and Correlation to Antibiotic Resistance. *Toxins*, **2**(8), 2117–2131. https://doi.org/10.3390/toxins2082117

235. Otter, J. A., & French, G. L. (2010). Molecular epidemiology of community-associated methicillin-resistant *Staphylococcus aureus* in Europe. *The Lancet Infectious Diseases*, **10**(4), 227–239. https://doi.org/10.1016/S1473-3099(10)70053-0

236. Otto, M. (2009). *Staphylococcus epidermidis*— the "accidental" pathogen. *Nature Reviews Microbiology*, **7**(8), 555–567. https://doi.org/10.1038/nrmicro2182

237. Otto, M. (2010). *Staphylococcus* Colonization of the Skin and Antimicrobial peptides. *Expert Review of Dermatology*, **5**(2), 183–195. https://doi.org/10.1586/edm.10.6

238. Otto, M. (2014). *Staphylococcus aureus* Toxins. *Current Opinion in Microbiology*, **17**(1), 32–37. https://doi.org/10.1016/j.mib.2013.11.004

239. Oulahal, N., Brice, W., Martial, A., & Degraeve, P. (2008). Quantitative Analysis of Survival of *Staphylococcus aureus* or *Listeria innocua* on Two Types of Surfaces: Polypropylene and Stainless Steel in Contact with Three Different Dairy Products. *Food Control*, **19**(2), 178–185. https://doi.org/10.1016/j.foodcont.2007.03.006

240. Oyarzabal, O. A., & Kathariou, S. (2014). *DNA Methods in Food Safety*. Wiley Blackwell

P

241. Pagedar, A., Singh, J., & Batish, V. K. (2010). Surface Hydrophobicity, Nutritional Contents Affect *Staphylococcus aureus* Biofilms and Temperature Influences Its Survival in Preformed Biofilms. *Journal of Basic Microbiology*, **50**(SUPPL. 1), 98–106. https://doi.org/10.1002/jobm.201000034

242. Pan, A., Battisti, A., Zoncada, A., Bernieri, F., Boldini, M., Franco, A., Pantosti, A. (2009). Community-Acquired Methicillin-Resistant *Staphylococcus aureus* ST398 Infection, Italy. *Emerging Infectious Diseases*, **15**(5), 846–847. https://doi.org/10.1007/BF01309849

243. Pantosti, A. (2012). Methicillin-Resistant *Staphylococcus aureus* Associated with Animals and Its Relevance to Human Health. *Frontiers in Microbiology*, **3**(127), 1–12. https://doi.org/10.3389/fmicb.2012.00127

244. Parisi, A., Caruso, M., Normanno, G., Latorre, L., Sottili, R., Miccolupo, A., Santagada, G. (2016). Prevalence, Antimicrobial Susceptibility and Molecular typing of Methicillin-Resistant *Staphylococcus aureus* (MRSA) in Bulk Tank Milk from Southern Italy. *Food Microbiology*, **58**, 36–42. https://doi.org/10.1016/j.fm.2016.03.004

245. Paterson, G. K., Harrison, E. M., & Holmes, M. A. (2014a). The Emergence of *mec*C Methicillin-Resistant *Staphylococcus aureus*. *Trends in Microbiology*, **22**(1), 42–47. https://doi.org/10.1016/j.tim.2013.11.003

246. Paterson, G. K., Morgan, F. J. E., Harrison, E. M., Cartwright, E. J. P., Török, M. E., Zadoks, R. N., Holmes, M. A. (2014b). Prevalence and Characterization of Human *mec*C Methicillin-Resistant *Staphylococcus aureus* Isolates in England. Journal of Antimicrobial Chemotherapy, 69(4), 907–910. https://doi.org/10.1093/jac/dkt462

247. Paterson, G. K., Morgan, F. J. E., Harrison, E. M., Peacock, S. J., Parkhill, J., Zadoks, R. N., & Holmes, M. A. (2014c). Prevalence and Properties of *mec*C Methicillin-Resistant *Staphylococcus aureus* (MRSA) in Bovine Bulk Tank Milk in Great Britain. *Journal of Antimicrobial Chemotherapy*, **69**(3), 598–602. https://doi.org/10.1093/jac/dkt417

248. Peacock, S. J., & Paterson, G. K. (2015). Mechanisms of Methicillin Resistance in *Staphylococcus aureus*. *Annual Review of Biochemistry*, **84**, 577–601. https://doi.org/10.1146/annurev-biochem-060614-034516

249. Peeters, E., Nelis, H. J., & Coenye, T. (2008). Comparison of Multiple Methods for Quantification of Microbial Biofilms Grown in Microtiter Plates. *Journal of Microbiological Methods*, **72**(2), 157–165. https://doi.org/10.1016/j.mimet.2007.11.010

250. Pereira, V., Lopes, C., Castro, A., Silva, J., Gibbs, P., & Teixeira, P. (2009). Characterization for Enterotoxin Production, Virulence Factors, and Antibiotic Susceptibility of *Staphylococcus aureus* Isolates from Various Foods in Portugal. *Food Microbiology*, **26**(3), 278–282. https://doi.org/10.1016/j.fm.2008.12.008

251. Petersen, A., Stegger, M., Heltberg, O., Christensen, J., Zeuthen, A., Knudsen, L. K., Larsen, A. R. (2013). Epidemiology of Methicillin-Resistant *Staphylococcus aureus* Carrying the Novel *mec*C Gene in Denmark Corroborates a Zoonotic Reservoir with Transmission to Humans. *Clinical Microbiology and Infection*, **19**(1), E16–E22. https://doi.org/10.1111/1469-0691.12036

252. Petinaki, E., & Spiliopoulou, I. (2012). Methicillin-resistant *Staphylococcus aureus* Among Companion and Food Chain Animals: Impact in Human Contacts. *Clinical Microbiology and Infection*, **(18)**, 626–634. https://doi.org/10.1111/j.1469-0691.2012.03881.x

253. Pournaras, S., Stathopoulos, C., & Tsakris, A. (2013). Oxacillin-Susceptible MRSA: Could It Become a Successful MRSA Type? *Future Microbiology*, **8**(11), 1365–1367. https://doi.org/10.2217/fmb.13.118

254. Prakash, P. H., Rajan, V., & Gopal, S. (2016). Predominance of SCC*mec* type IV and V Among Biofilm Producing Device-Associated *Staphylococcus aureus* Strains Isolated from Tertiary Care Hospitals in Mysuru, India. *Enfermedades Infecciosas Y Microbiología Clínica*, **5**.

255. Price, L. B., Stegger, M., Hasman, H., Aziz, M., Larsen, J., Andersen, S., & Pearson, T. (2012). *Staphylococcus aureus* CC398: Host Adaptation and Emergence of Methicillin Resistance in Livestock. *mBio*, **3**(1), 1–6. https://doi.org/10.1128/mBio.00305-11.Editor

256. Prokešová, L., Potužníková, B., Potempa, J., Zikán, J., Radl, J., Hachová, L., John, C. (1992). Cleavage of Human Immunoglobulins by Serine Proteinase from *Staphylococcus aureus*. *Immunology Letters*, **31**(3), 259–265. https://doi.org/10.1016/0165-2478(92)90124-7

257. Pu, S., Han, F., & Ge, B. (2009). Isolation and Characterization of Methicillin-Resistant *Staphylococcus aureus* Strains from Louisiana Retail Meats. *Applied and Environmental Microbiology*, **75**(1), 265–267. https://doi.org/10.1128/AEM.01110-08

258. Pu, W., Su, Y., Li, J., Li, C., Yang, Z., Deng, H., & Ni, C. (2014). High Incidence of Oxacillin-Susceptible *mec*A-Positive *Staphylococcus aureus* (OS-MRSA) Associated with Bovine Mastitis in China. *PLoS ONE*, **9**(2), 1–9. https://doi.org/10.1371/journal.pone.0088134

R

259. Ragon, M., Wirth, T., Hollandt, F., Lavenir, R., Lecuit, M., Monnier, A. Le, & Brisse, S. (2008). A New Perspective on *Listeria monocytogenes* Evolution. *PLoS Pathogens*, **4**(9), e1000146. https://doi.org/10.1371/journal.ppat.1000146

260. Raji, M. A., Garaween, G., Ehricht, R., Monecke, S., Shibl, A. M., & Senok, A. (2016). Genetic Characterization of *Staphylococcus aureus* Isolated from Retail Meat in Riyadh, Saudi Arabia. *Frontiers in Microbiology*, **7**(JUN), 1–7. https://doi.org/10.3389/fmicb.2016.00911

261. Reygaert, W. C. (2013). Antimicrobial Resistance Mechanisms of *Staphylococcus aureus*. *Microbial Pathogens and Strategies for Combating Them: Science, Technology and Education*, 297–305.

262. Rice, K., Peralta, R., Bast, D., Azavedo, J. De, & Mcgavin, M. J. (2001). Description of *Staphylococcus* Serine Protease (ssp) Operon in *Staphylococcus aureus* and Nonpolar Inactivation of sspA- Encoded Serine Protease. *Infection and Immunitymunity*, **69**(1), 159–169. https://doi.org/10.1128/IAI.69.1.159

263. Richter, A., Sting, R., Popp, C., Rau, J., Tenhagen, B. A., Guerra, B., Fetsch, A. (2012). Prevalence of Types of Methicillin-Resistant *Staphylococcus aureus* in Turkey Flocks and Personnel Attending the Animals. *Epidemiology and Infection*, **(1469–4409)**, 1–10. https://doi.org/10.1017/S095026881200009X

264. Rode, T. M., Langsrud, S., Holck, A., & Møretrø, T. (2007). Different Patterns of Biofilm Formation in *Staphylococcus aureus* Under Food-Related Stress Conditions. *International Journal of Food Microbiology*, **116**(3), 372–383. https://doi.org/10.1016/j.ijfoodmicro.2007.02.017

265. Rodríguez-Lázaro, D., Ariza-Miguel, J., Diez-Valcarce, M., Fernandez-Natal, I., Hernandez, M., & Rovira, J. (2015). Foods Confiscated from Non-EU Flights as a Neglected Route of Potential Methicillin-Resistant *Staphylococcus aureus* Transmission. *International Journal of Food Microbiology*, **209**, 29–33. https://doi.org/10.1016/j.ijfoodmicro.2014.08.016

266. Rogatzki, M. J., Ferguson, B. S., Goodwin, M. L., & Gladden, L. B. (2015). Lactate is Always the End Product of Glycolysis. *Frontiers in Neuroscience*, **9**(FEB), 1–7. https://doi.org/10.3389/fnins.2015.00022

267. Rosengren, Å., Fabricius, A., Guss, B., Sylvén, S., & Lindqvist, R. (2010). Occurrence of Foodborne Pathogens and Characterization of *Staphylococcus aureus* in Cheese Produced on Farm-Dairies. International *Journal of Food Microbiology*, **144**(2), 263–269. https://doi.org/10.1016/j.ijfoodmicro.2010.10.004

268. Ryffel, C., Kayser, F. H., & Berger-Bachi, B. (1992). Correlation Between Regulation of mecA Transcription and Expression of Methicillin Resistance in Staphylococci. *Antimicrobial Agents and Chemotherapy*, **36**(1), 25–31. https://doi.org/10.1128/AAC.36.1.25.

S

269. Sabat, A. J., Pournaras, S., Akkerboom, V., Tsakris, A., Grundmann, H., & Friedrich, A. W. (2015). Whole-Genome Analysis of An Oxacillin-Susceptible CC80 *mec*A-Positive *Staphylococcus aureus* Clinical Isolate: Insights

Into The Mechanisms of Cryptic Methicillin Resistance. *Journal of Antimicrobial Chemotherapy*, **70**(11), 2956–2964. https://doi.org/10.1093/jac/dkv210

270. Sadekuzzaman, M., Yang, S., Mizan, M. F. R., & Ha, S. D. (2015). Current and Recent Advanced Strategies for Combating Biofilms. *Comprehensive Reviews in Food Science and Food Safety*, **14**(4), 491–509. https://doi.org/10.1111/1541-4337.12144

271. Saeed, K., Ahmad, N., Dryden, M., Cortes, N., Marsh, P., Sitjar, A., Green, S. (2014). Oxacillin-Susceptible Methicillin-Resistant *Staphylococcus aureus* (OS-MRSA), a Hidden Resistant Mechanism Among Clinically Significant Isolates in the Wessex Region/UK. *Infection*, **42**(5), 843–847. https://doi.org/10.1007/s15010-014-0641-1

272. Santos, V. M., Martins, H. B., Rezende, I. S., Barbosa, M. S., Andrade, E. F., Souza, S. G., Marques, L. M. (2014). Virulence Factor Profile of *Staphylococcus aureus* Isolated from Bovine Milk from Brazil. *Food and Nutrition Sciences*, **5**(15), 1496–1505. https://doi.org/10.4236/fns.2014.515162

273. Sato, H., Matsumori, Y., Tanabe, T., Saito, H., Shimizu, A., & Kawano, J. (1994). A New Type of Staphylococcal Exfoliative Toxin from a *Staphylococcus aureus* Strain Isolated from a Horse with Phlegmon. *Infection and Immunity*, **62**(9), 3780–3785.

274. Sato, T., Usui, M., Motoya, T., Sugiyama, T., & Tamura, Y. (2015). Characterisation of Methicillin-Resistant *Staphylococcus aureus* ST97 and ST5 Isolated from Pigs in Japan. *Journal of Global Antimicrobial Resistance*, **3**(4), 283–285. https://doi.org/10.1016/j.jgar.2015.07.009

275. Scallan, E., Hoekstra, R. M., Angulo, F. J., Tauxe, R. V., Widdowson, M. A., Roy, S. L., Griffin, P. M. (2011). Foodborne Illness Acquired in the United States-Major Pathogens. *Emerging Infectious Diseases*, **17**(1), 7–15. https://doi.org/10.3201/eid1701.P11101

276. Schoder, D., Strauß, A., Szakmary-Brändle, K., Stessl, B., Schlager, S., & Wagner, M. (2015). Prevalence of Major Foodborne Pathogens in Food Confiscated from Air Passenger Luggage. *International Journal of Food Microbiology*, **209**, 3–12. https://doi.org/10.1016/j.ijfoodmicro.2014.08.010

277. Shore, A. C., Deasy, E. C., Slickers, P., Brennan, G., O'Connell, B., Monecke, S., Coleman, D. C. (2011). Detection of staphylococcal cassette chromosome *mec* type XI carrying highly divergent *mec*A, *mecI*, *mecR1*, *blaZ*, and *ccr* genes in human clinical isolates of clonal complex 130 methicillin-resistant *Staphylococcus aureus*. *Antimicrobial Agents and Chemotherapy*, **55**(8), 3765–3773. https://doi.org/10.1128/AAC.00187-11

278. Shukla, S. K., & Rao, T. S. (2013). Dispersal of Bap-Mediated *Staphylococcus aureus* Biofilm by Proteinase K. *The Journal of Antibiotics*, **66**(2), 55–60. https://doi.org/10.1038/ja.2012.98

279. Siguier, P., Perochon, J., Lestrade, L., Mahillon, J., & Chandler, M. (2006). ISfinder: The Reference Centre for Bacterial Insertion Sequences. *Nucleic Acids Research*, **34**(Database issue), D32-6. https://doi.org/10.1093/nar/gkj014

280. Singh, R., Ray, P., Das, A., & Sharma, M. (2010). Penetration of Antibiotics Through *Staphylococcus aureus* and *Staphylococcus epidermidis* Biofilms. *Journal of Antimicrobial Chemotherapy*, **65**(9), 1955–1958. https://doi.org/10.1093/jac/dkq257

281. Sivakanesan, R., & Dawes, E. a. (1980). Anaerobic Glucose and Serine Metabolism in *Staphylococcus epidermidis*. *Journal of General Microbiology*, **118**, 143–157. https://doi.org/10.1099/00221287-118-1-143

282. Skogman, M. E., Vuorela, P. M., & Fallarero, A. (2012). Combining Biofilm Matrix Measurements with Biomass and Viability Assays in Susceptibility Assessments of Antimicrobials Against *Staphylococcus aureus* Biofilms. *The Journal of Antibiotics*, **65**(9), 453–459. https://doi.org/10.1038/ja.2012.49

283. Smith, T. C., Gebreyes, W. A., Abley, M. J., Harper, A. L., Forshey, B. M., Male, M. J., Davies, P. R. (2013). Methicillin-Resistant *Staphylococcus aureus* in Pigs and Farm Workers on Conventional and Antibiotic-Free Swine Farms in the USA. *PLoS ONE*, **8**(5), 1–5. https://doi.org/10.1371/journal.pone.0063704

284. Sollid, J. U. E., Furberg, A. S., Hanssen, A. M., & Johannessen, M. (2014). *Staphylococcus aureus*: Determinants of Human Carriage. *Infection, Genetics and Evolution*, **21**, 531–541. https://doi.org/10.1016/j.meegid.2013.03.020

285. Spanu, V., Spanu, C., Virdis, S., Cossu, F., Scarano, C., & De Santis, E. P. L. (2012). Virulence Factors and Genetic Variability of *Staphylococcus aureus* Strains Isolated From Raw Sheep's Milk Cheese. *International Journal of Food Microbiology*, **153**(1–2), 53–57. https://doi.org/10.1016/j.ijfoodmicro.2011.10.015

286. Speziale, P., Pietrocola, G., Foster, T. J., & Geoghegan, J. A. (2014). Protein-Based Biofilm Matrices in Staphylococci. *Frontiers in Cellular and Infection Microbiology*, **4**:171, 1–10. https://doi.org/10.3389/fcimb.2014.00171

287. Spohr, M., Rau, J., Friedrich, A., Klittich, G., Fetsch, A., Guerra, B., … Tenhagen, B. A. (2011). Methicillin-Resistant *Staphylococcus aureus* (MRSA) in Three Dairy Herds in Southwest Germany. *Zoonoses and Public Health*, **58**(4), 252–261. https://doi.org/10.1111/j.1863-2378.2010.01344.x

288. Srey, S., Jahid, I. K., & Ha, S. Do. (2013). Biofilm Formation in Food Industries: A food safety concern. *Food Control*, **31**(2), 572–585. https://doi.org/10.1016/j.foodcont.2012.12.001

289. Standard, I. (2003). ISO 6888-2: 1999/Amd. 1:2003- Microbiology of Food and Animal Feeding Stuffs-Horizontal Method for The Enumeration of Coagulase-Positive Staphylococci (*Staphylococcus aureus* and Other Species), 2003.

290. Stegger, M., Andersen, P. S., Kearns, A., Pichon, B., Holmes, M. A., Edwards, G., Larsen, A. R. (2012). Rapid Detection, Differentiation and Typing of Methicillin-Resistant *Staphylococcus aureus* Harbouring Either *mec*A or the new *mec*A Homologue mecALGA251. *Clinical Microbiology and Infection*, **18**(4), 395–400. https://doi.org/10.1111/j.1469-0691.2011.03715.x

291. Stepanović, S., Vuković, D., Dakić, I., Savić, B., & Švabić-Vlahović, M. (2000). A Modified Microtiter-Plate Test for Quantification of Staphylococcal Biofilm Formation. *Journal of Microbiological Methods*, **40**(2), 175–179. https://doi.org/10.1016/S0167-7012(00)00122-6

292. Stepanović, S., Vuković, D., Hola, V., Di Bonaventura, G., Djukić, S., Ćirković, I., & Ruzicka, F. (2007). Quantification of Biofilm in Microtiter Plates: Overview of Testing Conditions and Practical Recommendations for Assessment of Biofilm Production by Staphylococci. *Apmis*, **115**(8), 891–899. https://doi.org/10.1111/j.1600-0463.2007.apm_630.x

293. Stone, K. (2017). Risk Assessment on Methicillin-Resistant *Staphylococcus aureus* (MRSA), with a Focus on Livestock-Associated MRSA, in the UK Food Chain. *Food Standards Agency*, (February).

294. Strommenger, B., Braulke, C., Heuck, D., Schmidt, C., Pasemann, B., Nübel, U., & Witte, W. (2008). spa typing of *Staphylococcus aureus* as a frontline tool in epidemiological typing. *Journal of Clinical Microbiology*, **46**(2), 574–581. https://doi.org/10.1128/JCM.01599-07

295. Sun, J., Yang, M., Sreevatsan, S., & Davies, P. R. (2015). Prevalence and Characterization of *Staphylococcus aureus* in Growing Pigs in the USA. *PLoS ONE*, **10**(11), 1–14. https://doi.org/10.1371/journal.pone.0143670

296. Syne, S.-M., Ramsubhag, A., & Adesiyun, A. (2013). Microbiological Hazard Analysis of Ready-To-Eat Meats Processed at a Food Plant in Trinidad, West Indies. *Infection Ecology and Epidemiology*, **3**, 1–12. https://doi.org/10.3402/iee.v3i0.20450

297. Szabo, J. (2014). Molecular Methods in Epidemiology of Methicillin Resistant *Staphylococcus aureus* (MRSA): Advantages, Disadvantages of Different Techniques. *Journal of Medical Microbiology & Diagnosis*, **3**(3). https://doi.org/10.4172/2161-0703.1000147

298. Szczuka, E., Urbańska, K., Pietryka, M., & Kaznowski, A. (2013). Biofilm Density and Detection of Biofilm-Producing Genes in Methicillin-Resistant *Staphylococcus aureus* Strains. *Folia Microbiologica*, **58**(1), 47–52. https://doi.org/10.1007/s12223-012-0175-9

299. Székely, E., Man, A., Mare, A., Vas, K. E., Molnár, S., Bilca, D., Lorinczi, L. (2012). Molecular Epidemiology and Virulence Factors of Methicillin-Resistant *Staphylococcus aureus* Strains in a Romanian University Hospital. *Revista Romană de Medicină de Laborator*, 20(4), 371–382.

T

300. T. Seemann. (n.d.-a). Mass screening of contigs for antimicrobial and virulence genes. Retrieved from https://github.com/tseemann/abricate

301. T. Seemann. (n.d.-b). Scan contig files against traditional PubMLST typing schemes. Retrieved from https://github.com/tseemann/mlst

302. Tenhagen, B. A., Vossenkuhl, B., Käsbohrer, A., Alt, K., Kraushaar, B., Guerra, B., Fetsch, A. (2014). Methicillin-Resistant *Staphylococcus aureus* in Cattle Food Chains- Prevalence, Diversity, and Antimicrobial Resistance in Germany. *Journal of Animal Science*, **92**(6), 2741–2751. https://doi.org/10.2527/jas2014-7665

303. Trnčíková, T., Hrušková, V., Oravcová, K., Pangallo, D., & Kaclíková, E. (2008). Rapid and Sensitive Detection of *Staphylococcus aureus* in Food Using Selective Enrichment and Real-Time PCR Targeting a New Gene Marker. *Food Analytical Methods*, **2**(4), 241–250. https://doi.org/10.1007/s12161-008-9056-x

304. Tseng, C. W., Zhang, S., & Stewart, G. C. (2004). Accessory Gene Regulator Control of Staphylococcal Enterotoxin D Gene Expression. *Journal of Bacteriology*, **186**(6), 1793–1801. https://doi.org/10.1128/JB.186.6.1793

U

305. Union, O. J. of the E. (2004). Regulation (EC) No. 853/2004 of the European Parliament and of the Council LAying Down Specific Hygiene Rules for on the Hygiene of Foodstuffs.

306. Urdahl, A. M., BergsjØ, B., Hofshagen, M., Nordström, M., & Lium, B. (2014). The Surveillance Programmes for Methicillin Resistant Staphylococcus aureus in Pigs in Norway 2014. Surveillance Programmes for Terrestrial and Aquatic Animals in Norway. Annual Report 2014.

307. Uršič, V., Tomič, V., & Košnik, M. (2008). Effect of Different Incubation Atmospheres on the Production of Biofilm in Methicillin-Resistant Staphylococcus aureus (MRSA) Grown in Nutrient-Limited Medium. Current Microbiology, 57(4), 386–390. https://doi.org/10.1007/s00284-008-9211-z

V

308. Valero, A., Pérez-Rodríguez, F., Carrasco, E., Fuentes-Alventosa, J. M., García-Gimeno, R. M., & Zurera, G. (2009). Modelling the Growth Boundaries of *Staphylococcus aureus*: Effect of Temperature, pH and Water Activity. *International Journal of Food Microbiology*, **133**(1–2), 186–194. https://doi.org/10.1016/j.ijfoodmicro.2009.05.023

309. Valsangiacomo, C., Dolina, M., Peduzzi, R., & Jäggli, M. (2000). Antimicrobial susceptibility of *Staphylococcus aureus* Isolates from Hospitalized Patients and Dairy Food (Fresh Cheese): A Survey Over a Decade in Southern Switzerland. *Clinical Microbiology and Infection*, **6**(7), 393–394. https://doi.org/10.1046/j.1469-0691.2000.00080.x

310. van Cleef, B. A. G. L., Monnet, D. L., Voss, A., Krziwanek, K., Allerberger, F., Struelens, M., Kluytmans, J. A. J. W. (2011). Livestock-Associated Methicillin-Resistant *Staphylococcus aureus* in Humans, Europe. *Emerging Infectious Diseases*, **17**(3), 502–505. https://doi.org/10.3201/eid1703.101036

311. van Cleef, B. A. G. L., van Benthem, B. H. B., Haenen, A. P. J., Bosch, T., Monen, J., & Kluytmans, J. A. J. W. (2013). Low Incidence of Livestock-Associated Methicillin-Resistant *Staphylococcus aureus* Bacteraemia in The Netherlands in 2009. *PLoS ONE*, **8**(8), 1–6. https://doi.org/10.1371/journal.pone.0073096

312. van Duijkeren, E., Hengeveld, P. D., Albers, M., Pluister, G., Jacobs, P., Heres, L., & van de Giessen, A. W. (2014). Prevalence of Methicillin-Resistant *Staphylococcus aureus* Carrying *mec*A or *mec*C in Dairy Cattle. *Veterinary Microbiology*, **171**(3–4), 364–367. https://doi.org/10.1016/j.vetmic.2013.12.024

313. Vandenesch, F., Lina, G., & Henry, T. (2012). *Staphylococcus aureus* Hemolysins, Bi-Component Leukocidins, and Cytolytic Peptides: A Redundant Arsenal of Membrane-Damaging Virulence Factors? *Frontiers in Cellular and Infection Microbiology*, **2**(February), 1–15. https://doi.org/10.3389/fcimb.2012.00012

314. Vanderhaeghen, W., Cerpentier, T., Adriaensen, C., Vicca, J., Hermans, K., & Butaye, P. (2010). Methicillin-Resistant *Staphylococcus aureus* (MRSA) ST398 Associated with Clinical and Subclinical Mastitis in Belgian Cows. *Veterinary Microbiology*, **144**(1–2), 166–171. https://doi.org/10.1016/j.vetmic.2009.12.044

315. Vanhommerig, E., Moons, P., Pirici, D., Lammens, C., Hernalsteens, J. P., De Greve, H., Malhotra-Kumar, S. (2014). Comparison of Biofilm Formation Between Major Clonal Lineages of Methicillin Resistant *Staphylococcus aureus*. *PLoS ONE*, **9**(8), 1–8. https://doi.org/10.1371/journal.pone.0104561

316. Vasu, D., Sunitha, M. M., Srikanth, L., Swarupa, V., Prasad, U. V., Sireesha, K., Sarma, P. V. G. K. (2014). In *Staphylococcus aureus* the Regulation of Pyruvate Kinase Activity by Serine/Threonine Protein Kinase Favors Biofilm Formation. *3 Biotech*, **5**, 505–512. https://doi.org/10.1007/s13205-014-0248-3

317. Vázquez-Sánchez, D., Habimana, O., & Holck, A. (2013). Impact of Food-Related Environmental Factors on the Adherence and Biofilm Formation of Natural *Staphylococcus aureus* Isolates. *Current Microbiology*, **66**(2), 110–121. https://doi.org/10.1007/s00284-012-0247-8

318. Vázquez-Sánchez, D., López-Cabo, M., Saá-Ibusquiza, P., & Rodríguez-Herrera, J. J. (2012). Incidence and Characterization of *Staphylococcus aureus* in Fishery Products Marketed in Galicia (Northwest Spain). *International Journal of Food Microbiology*, **157**(2), 286–296. https://doi.org/10.1016/j.ijfoodmicro.2012.05.021

319. Veenemans, J., Verhulst, C., Punselie, R., Van Keulen, P. H. J., & Kluytmans, J. A. J. W. (2013). Evaluation of Brilliance MRSA 2 Agar for Detection of Methicillin-resistant *Staphylococcus aureus* in Clinical Samples. *Journal of Clinical Microbiology*, **51**(3), 1026–1027. https://doi.org/10.1128/JCM.02995-12

320. Ventola, C. L. (2015). The Antibiotic Resistance Crisis. *Pharmacy and Therapeutics*, **40**(4), 277–283.

321. Verhegghe, M., Crombé, F., Luyckx, K., Haesebrouck, F., Butaye, P., Herman, L., Rasschaert, G. (2016). Prevalence and Genetic Diversity of Livestock-Associated Methicillin-Resistant *Staphylococcus aureus* on Belgian Pork. *Journal of Food Protection*, **79**(1), 82–89. https://doi.org/10.4315/0362-028X.JFP-15-266

322. Verkade, E., Bosch, T., Hendriks, Y., & Kluytmans, J. (2012). Outbreak of Methicillin-Resistant *Staphylococcus aureus* ST398 in a Dutch Nursing Home. *Infection Control and Hospital Epidemiology*, **33**(6), 624–626. https://doi.org/10.1086/665726

323. Verkade, E., Ferket, M., & Kluytmans, J. (2011). Clinical Evaluation of Oxoid Brilliance MRSA Agar in Comparison with bioMérieux MRSA ID Medium for Detection of Livestock-Associated Methicillin-resistant *Staphylococcus aureus*. *Journal of Medical Microbiology*, **60**(7), 905–908. https://doi.org/10.1099/jmm.0.021964-0

324. Voelk, V., Graber, H. U., van den Borne, B. H. P., Sartori, C., Steiner, A., Bodmer, M., & Haerdi-Landerer, M. C. (2014). A Longitudinal Study Investigating the Prevalence of *Staphylococcus aureus* Genotype B in Seasonally Communal Dairy Herds. *Journal of Dairy Science*, **97**:1-9, 4184–4192. https://doi.org/10.3168/jds.2013-7291

325. Voss, A., Loeffen, F., Bakker, J., Klaassen, C., & Wulf, M. (2005). Methicillin-Resistant *Staphylococcus aureus* in Pig Farming. *Emerging Infectious Diseases*, **11**(12), 1965–1966. https://doi.org/10.3201/eid1112.050428

326. Vremeră, T., Iancu, L. S., Nastase, E. V, Logigan, C., Miftode, E. G., Mereuță, A. I., Dorneanu, O. S. (2012). Triplex Real Time PCR for Detection of Methicillin-Resistant *Staphylococcus aureus* and Panton-Valentine Leukocidin in North-East Romania. *Revista Medico-Chirurgicală a Societății de Medici și Naturaliști din Iași*, **116**(4), 1171–1176.

W

327. Wagenaar, J. A., Yue, H., Pritchard, J., Broekhuizen-Stins, M., Huijsdens, X., Mevius, D. J., Van Duijkeren, E. (2009). Unexpected Sequence Types in Livestock Associated Methicillin-Resistant *Staphylococcus aureus* (MRSA): MRSA ST9 and a Single Locus Variant of ST9 in Pig Farming in China. *Veterinary Microbiology*, **139**(3–4), 405–409. https://doi.org/10.1016/j.vetmic.2009.06.014

328. Walcher, G., Gonano, M., Kümmel, J., Barker, G. C., Lebl, K., Bereuter, O., Stessl, B. (2014). *Staphylococcus aureus* Reservoirs During Traditional Austrian Raw Milk Cheese Production. *The Journal of Dairy Research*, **81**(4), 462–470. https://doi.org/10.1017/S0022029914000417

329. Weese, J. S., Avery, B. P., & Reid-Smith, R. J. (2010). Detection and Quantification of Methicillin-Resistant *Staphylococcus aureus* (MRSA) Clones in Retail Meat Products. *Letters in Applied Microbiology*, **51**(3), 338–342. https://doi.org/10.1111/j.1472-765X.2010.02901.x

330. Williams, I., Venables, W. A., Lloyd, D., Paul, F., & Critchley, I. (1997). The Effects of Adherence to Silicone Surfaces on Antibiotic Susceptibility in *Staphylococcus aureus*. *Microbiology*, **143**(7), 2407–2413. https://doi.org/10.1099/00221287-143-7-2407

331. Wiseman, G. M. (1975). The Hemolysins of *Staphylococcus aureus*. *Bacteriological Reviews*, **39**(4), 317–344.

332. Wiśniewska, K., Szewczyk, A., Piechowicz, L., Bronk, M., Samet, A., & Świeć, K. (2012). The Use of *spa* and Phage Typing for Characterization of Clinical Isolates of Methicillin-Resistant *Staphylococcus aureus* in the University Clinical Center in Gdańsk, Poland. *Folia Microbiologica*, **57**(3), 243–249. https://doi.org/10.1007/s12223-012-0148-z

333. Witte, W., Pasemann, B., & Cuny, C. (2007). Detection of low-level oxacillin resistance in *mec*A-positive *Staphylococcus aureus*. *Clinical Microbiology and Infection*, **13**(4), 408–412. https://doi.org/10.1111/j.1469-0691.2006.01671.x

334. Wright, C. S. (1984). Structural Comparison of the Two Distinct Sugar Binding Sites in Wheat Germ Agglutinin Isolectin II. *Journal of Molecular Biology*, **178**(1), 91–104. https://doi.org/10.1016/0022-2836(84)90232-8

335. Wright, G. D. (2010). Q & A: Antibiotic Resistance: Where Does it Come From and What Can We Do About It ? *BMC Biology*, **8**(123), 1–6.

336. Wulf, M. W. H., Markestein, A., van der Linden, F., Voss, A., Klaassen, C., & Verduin, C. (2008a). First Outbreak of Methicillin-Resistant *Staphylococcus aureus* ST398 in a Dutch Hospital, June 2007. *Euro Surveillance*, **13**(1–3), 1–2. https://doi.org/10.1111/j.1469-0691.2007.01927.x

337. Wulf, M. W. H., Sørum, M., van Nes, A., Skov, R., Melchers, W. J. G., Klaassen, C. H. W., & Voss, A. (2008b). Prevalence of Methicillin-Resistant *Staphylococcus aureus* Among Veterinarians: An International Study. *Clinical Microbiology and Infection*, **14**(1), 29–34.

Y

338. Yamaguchi, T., Nishifuji, K., Sasaki, M., Fudaba, Y., Aepfelbacher, M., Takata, T., Sugai, M. (2002). Identification of the *Staphylococcus aureus* and Pathogenicity Island Which Encodes a Novel Exfoliative Toxin, ETD, and EDIN-B. *Infection and Immunity*, **70**(10), 5835–5845. https://doi.org/10.1128/IAI.70.10.5835

339. Yan, X., Yu, X., Tao, X., Zhang, J., Zhang, B., Dong, R., Zhang, J. (2014). *Staphylococcus aureus* ST398 from slaughter pigs in northeast China. *International Journal of Medical Microbiology*, **304**, 379–383. https://doi.org/10.1016/j.ijmm.2013.12.003

340. Yeh, P. J., Hegreness, M. J., Aiden, A. P., & Kishony, R. (2009). Drug Interactions and The Evolution of Antibiotic Resistance. *Nature*, **7**, 460–466.

Z

341. Zarei, M., Maktabi, S., & Ghorbanpour, M. (2012). Prevalence of *Listeria monocytogenes*, *Vibrio parahaemolyticus*, *Staphylococcus aureus*, and *Salmonella* spp. in Seafood Products using Multiplex Polymerase Chain Reaction. *Foodborne Pathogens and Disease*, **9**(2), 108–112. https://doi.org/10.1089/fpd.2011.0989

Website

342. www.masciabrunelli.it/
343. www.oxoid.com/
344. www.liofilchem.net
345. www.cdc.gov
346. https://blast.ncbi.nlm.nih.gov/Blast.cgi
347. http://technelysium.com.au/wp/chromas/
348. http://www.megasoftware.net/
349. http://www.applied-maths.com/
350. http://bowtie-bio.sourceforge.net/bowtie2/index.shtml
351. http://samtools.sourceforge.net/
352. http://darlinglab.org/mauve/user-guide/reordering.html
353. https://github.com/tseemann/abricate
354. http://prinseq.sourceforge.net/
355. http://rast.nmpdr.org/
356. https://github.com/tseemann/mlst
357. http://spades.bioinf.spbau.ru/release3.10.0/manual.html
358. https://sourceforge.net/projects/varscan/files/
359. http://crispr.i2bc.paris-saclay.fr/
360. http://www.ebi.ac.uk/
361. https://www-is.biotoul.fr/about.php
362. http://saureus.mlst.net/
363. https://www.ncbi.nlm.nih.gov/
364. http://www.comstat.dk/
365. www.roche.com
366. http://www.graphpad.com/quickcalcs/McNemar1.cfm
367. http://www.biostat.umn.edu/~susant/Fall11ph6414/Section12_Part2.pdf
368. www.spaserver.ridom.de
369. www.uniprot.org
370. Saureus.mlst.net.

371. http://www.abc.net.au
372. www.worldbank.org
373. www.cbp.gov
374. www.eucast.org.

Results publication during the doctoral studies

Articles

Rodríguez-Lázaro, D., **Oniciuc, E.-A.**, González García, P., Gallego, D., Fernández-Natal, I. Dominguez-Gil, M., Eiros-Bouza, J.S., Wagner, M., Nicolau, A.I., Hernández, M. Methicillin-resistant *Staphylococcus aureus* crosses EU borders *via* uncontrolled imported food. Frontiers in Microbiology. Impact factor: 4.165, *accepted for publishing*

Oniciuc, E.-A., Nicolau, A.I., Hernández, M., Rodríguez-Lázaro, D. (2017). Presence of methicillin-resistant *Staphylococcus aureus* in the food chain. Trends in Food Science & Technology, doi: 10.1016/j.tifs.2016.12.002. Impact factor: 5.150

Oniciuc, E.A., Cerca, N., Nicolau, A.I. (2016). Compositional analysis of biofilms formed by *Staphylococcus aureus* isolated from food sources. Frontiers in Microbiology, 7:390, doi:10.3389/fmicb.2016.00390. Impact factor: 3.989

Ciolacu, L., Stessl, B., Bolocan, A.S., **Oniciuc, E.A.**, Wagner, M., Rychli, B., Nicolau, A.I. (2016). Tracking foodborne pathogenic bacteria in raw and ready-to-eat food illegally sold at the Eastern EU border. Foodborne Pathogens and Disease, doi:10.1089/fpd.2015.2057. Impact factor: 1.905

Ariza-Miguel, J., **Oniciuc, E.A.**, Sanz, I., Fernández-Natal, I., Hernández, M., Rodríguez-Lázaro, D. (2015). Evaluation of two commercially available chromogenic media for confirmation of methicillin-resistant *Staphylococcus aureus* from human, animal, and food samples. International Journal of Food Microbiology, 209: 26-28, doi:10.1016/j.ijfoodmicro.2015.05.004. Impact factor: 3.082

Oniciuc, E.A., Ariza-Miguel, J., Bolocan, A.S., Diez-Valcarce, M., Rovira, J., Hernández, M., Fernández-Natal, I., Nicolau, A.I., Rodríguez-Lázaro, D. (2015). Foods from black market at EU border as a neglected route of potential Methicillin-Resistant *Staphylococcus aureus* transmission. International Journal of Food Microbiology, 209: 34-38, doi:10.1016/j.ijfoodmicro.2014.11.015. Impact factor: 3.082

International conferences

Oniciuc, E.A., Bucur, F.I., Rodríguez Lázaro, D., Barbu, V., Hernandez, M., Nicolau, A.I.- Correlation between biofilm formation and composition and molecular aspects of methicillin-resistant *Staphylococcus aureus*. **FEMS Congress**, 09-13/07/2017, Valencia, Spain

Rodríguez Lázaro, D., Ariza-Miguel, J., **Oniciuc, E.A.**, Nicolau, A.I., Rovira, J., Wagner, M., Fernández Natal, I., Hernandez, M.- Evidence on an emerging risk associated with the methicillin resistant *Staphylococcus aureus* (MRSA) in the food chain. **Food Micro Conference**, 19-22/07/2016, Dublin, Ireland

Oniciuc, E.A., Bolocan, A.S., Fotin, A.A., Dajbog, A., Nicolau, A.- Comparative assessment of disk diffusion and micro dilution methods using CLSI guidelines for antimicrobial susceptibility testing of *Staphylococcus aureus* isolated from foods. **Food Safety Congress**, 07-08/05/2015, Istanbul, Turkey

National conferences

Oniciuc, E.A., Martín Quijada, N., Nicolau, A.I., Hernández, M., Rodríguez-Lázaro, D.- Comparative genomic analysis for understanding evolution of MRSA Strains. Scientific **Conference of Doctoral Schools from UDJ Galati**, 5th edition, 08-09/06/2017, Galati, Romania

Oniciuc, E.A., Nicolau, A.I.- Protein based matrices evidenced in biofilm structure of *Staphylococcus aureus* isolated from food sources. Scientific **Conference of Doctoral Schools from UDJ Galati**, 4th edition, 02-03/06/2016, Galati, Romania

Fernández Natal, I., Ariza Miguel, J., **Oniciuc, E.A**., Nicolau, A.I., Rovira Carballido, J., Wagner, M., Hernandez, M., Rodríguez Lázaro, D.- Descripción del primer caso de la variante OXA sensible de una cepa de MRSA aislada en alimentos. **XX Congreso Nacional de la Sociedad Española de Enfermedades Infecciosas y Microbiología Clínica**, 26-28/05/2016, Barcelona, Spain

Fotin, A.A., **Oniciuc, E.A**., Nicolau, A.- Antibiotic susceptibility of *Staphylococcus aureus* strains isolated from animal origin foods sold in an open market. **Scientific Session for Students**, Faculty of Food Science and Engineering, 22/05/2015, Galati, Romania

Oniciuc, E.A., Ariza-Miguel, J., Bolocan, A.S., Diez-Valcarce, M., Rovira, J., Hernández, M., Fernández-Natal, I., Nicolau, A.I., Rodríguez-Lázaro, D.- Incidence of *Staphylococcus aureus* in food products illegally sold in a Romanian market and characterization of the isolated strains. **Scientific Conference of Doctoral Schools from UDJ Galati**, 3rd edition, 04-05/06/2015, Galati, Romania

Projects

2015-2016- COST FA1202 project- A European Network For Mitigating Bacterial Colonisation and Persistence On Foods and Food Processing Environments

01/06/2014-31/12/2014- FP7th PROMISE project no 265877- Protection of consumers through mitigation of segregation of expertise (Dunarea de Jos University of Galati, Galati, Romania)

Internships

16/01/2017-15/04/2017- Erasmus internship- Whole genome sequencing analysis of an OS-MRSA strain associated with food (ITACyL and Universidad de Burgos, Spain)

01/12/2015-28/02/2016- Doctoral internship- Characterization of methicillin resistant *Staphylococcus aureus* strains from different neglected routes to Europe (FEMS grant- ITACyL and Universidad de Burgos, Spain)

01/10/2015-30/11/2015- Doctoral internship- Characterization of methicillin resistant *Staphylococcus aureus* strains from different neglected routes to Europe (international airports and open market close to EU borders) (COST FA1202 grant- ITACyL and Universidad de Burgos, Spain)

10/03/2015-30/04/2015- Doctoral internship- Characterization of *Staphylococcus aureus* biofilms by CLSM (COST FA1202 grant- Center of Biological Engineering, University of Minho, Braga, Portugal)

06/10/2014-12/12/2014- Doctoral internship- Characterization of *Staphylococcus aureus* isolates by PFGE (PROMISE grant- Instituto Tecnológico Agrario de Castilla y León, Valladolid, Spain)

Workshops

09/09/2016- One day Summer School- *In vitro/ In silico* approaches for food science (EFSA, Parma, Italy)

18/12/2014-19/12/2014- Workshop on sensible data produced by the PROMISE consortium with involved research partners and Food Safety Agencies (PROMISE consortium and AGES, Vienna, Austria)

Honours and awards

Oniciuc, E.A., Martín Quijada, N., Nicolau, A.I., Hernández, M., Rodríguez-Lázaro, D.- Comparative genomic analysis for understanding evolution of MRSA Strains - **First prize** - Scientific Conference of Doctoral Schools from Dunarea de Jos University of Galati, 5th edition, 08-09/06/2017, Dunarea de Jos University of Galati, Romania

Oniciuc, E.A., Nicolau, A.I.- Protein based matrices evidenced in biofilm structure of *Staphylococcus aureus* isolated from food sources - **First prize** - Scientific Conference of Doctoral Schools from Dunarea de Jos University of Galati, 4th edition, 2-3/06/2016, Dunarea de Jos University of Galati, Romania

Oniciuc, E.A., Ariza-Miguel, J., Bolocan, A.S., Diez-Valcarce, M., Rovira, J., Hernández, M., Fernández-Natal, I., Nicolau, A.I., Rodríguez-Lázaro, D.- Incidence of *Staphylococcus aureus* in food products illegally sold in a Romanian market and characterization of the isolated strains - **Second prize** - Scientific Conference of Doctoral Schools from Dunarea de Jos University of Galati, 3rd edition, 4-5/06/2015, Dunarea de Jos University of Galati, Romania

Memberships

2015-Present- Romanian Society of Biochemistry and Molecular Biology

2014-Present- Romanian Society of Microbiology

2014-Present- Scientific Council of Doctoral School of Engineering (Dunarea de Jos University of Galati, Galati, Romania)

Other activities carried out during doctoral studies

Articles

Bleoanca, I., Saje, K., Mihalcea, L., **Oniciuc, E.A.**, Smole-Mozina, S., Nicolau, A.I., Borda, D. (2016). Contribution of high pressure and thyme extract to control *Listeria monocytogenes* in fresh cheese- A hurdle approach. Innovative Food Science and Emerging Technologies, 38:7-14, doi.org/10.1016/j.ifset.2016.09.002. Impact factor: 2.997

Bolocan, A.S., Nicolau, A.I., Alvarez-Ordonez, A., Borda, D., **Oniciuc, E.A.,** Stessl, B., Gurgu, L., Wagner, M., Jordan, K. (2016). Dinamics of *Listeria monocytogenes* colonisation in a newly-opened meat processing facility. Meat Science, 113:26-34, doi:10.1016/j.meatsci.2015.10.016. Impact factor: 2.615

Bolocan, A.S., **Oniciuc, E.A.**, Alvarez-Ordonez, A., Wagner, M., Rychli, K., Jordan, K., Nicolau, A.I. (2015). Putative cross-contamination routes of *Listeria monocytogenes* in a meat processing facility in Romania. Journal of Food Protection, 78 (9):1624-1769, https://doi.org/10.4315/0362-028X.JFP-14-539. Impact factor: 1.974

Book chapter

Bolocan, A.S., Ciolacu, L., **Oniciuc, E-A.**, Draper, L., Nicolau, A.I., Wagner, M., Hill, C.- Book chapter- Utilization of Bacteriophages Targeting *Listeria monocytogenes* in Dairy and Food Industry. Apple Academic Press, Inc., *accepted for publishing*

International conferences

Bolocan, A.S., **Oniciuc, E.A.**, Alvarez-Ordóñez, A., Wagner, M., Rychli, K., Jordan, K., Borda, D., Nicolau, A.I.- What could explain contamination in a Romanian food processing environment?, **PROMISE Conference**- Sensible data: A challenge for Risk Communication?, 18-19/12/2014, Vienna, Austria

Nicolau, A.I., Bolocan, A.S., Rychli, K., **Oniciuc, E.A.**, Alvarez-Ordóñez, A., W Jordan, K., Wagner, M.- To be persistent or not to be persistent? That is the question for *Listeria monocytogenes* strains isolated from a meat processing environment. **General Assembly of the Hungarian Society for Microbiology**, 15-17/10/2014, Lake-Balaton, Hungary

Bolocan, A.S., Rychli, K., **Oniciuc, E.A.**, Wagner, M., Nicolau, A.I.- Genetic characterization of some *Listeria monocytogenes* strains isolated from a meat processing facility in relation to their tolerance to disinfectants. **Food Micro Conference**, 1-4/09/2014, Nantes, France

National conferences

Saje, K., **Oniciuc, E.A.**, Mihalcea, L., Coman, G., Bleoancă, I., Smole-Mozina, S., Nicolau, A.I., Borda, D.- Synergistic effect of high pressure processing and thyme extract on *Listeria monocytogenes* in fresh cheese. **7th International Symposium EuroAliment**, 24-26/09/2015, Galati, Romania

Projects

Present- SafeConsumE project- Safer food through changed consumer behavior: Effective tools and products, communication strategies, education and a food safety policy reducing health burden from foodborne illnesses (no. 727580/2017) (Dunarea de Jos University of Galati, Galati, Romania)

Present- SafeFood project- Development of a novel industrial process for safe, sustainable and higher quality foods, using biotechnology and cybernetic approach (ERA-IB-16-014) (Dunarea de Jos University of Galati, Galati, Romania)

2016- COST FA1408- A European Network for Foodborne Parasites (Euro-FBP)

Internships

01/03/2016-15/04/2016- Doctoral internship- Screening and detection of *Toxoplasma gondii* in pig samples from slaughterhouses (COST FA1408- Universidad de Burgos, Spain)

Honours and awards

Bolocan, A.S., Crăiță, A., Stângă, C.T., **Oniciuc, E.A**. -"Magic Bite product"- **Bronz medal** - UGAL INVENT, 8-10/10/2014, Dunarea de Jos University of Galati, Romania

Trends in Food Science & Technology 61 (2017) 49–59

Contents lists available at ScienceDirect

Trends in Food Science & Technology

journal homepage: http://www.journals.elsevier.com/trends-in-food-science-and-technology

Review

Presence of methicillin-resistant *Staphylococcus aureus* in the food chain

Elena-Alexandra Oniciuc [a, d], Anca Ioana Nicolau [a], Marta Hernández [b, c], David Rodríguez-Lázaro [d, *]

[a] Dunarea de Jos University of Galati, Faculty of Food Science and Engineering, Domneasca Street 47, 800008, Galati, Romania
[b] Laboratory of Molecular Biology and Microbiology, Instituto Tecnológico Agrario de Castilla y León, Ctra. de Burgos, km 118, 47071, Valladolid, Spain
[c] Departamento de Ingeniería Agrícola y Forestal, Tecnología de los Alimentos, E.T.S. Ingenierías Agrarias, Universidad de Valladolid, 34004, Palencia, Spain
[d] Microbiology Division, Department of Biotechnology and Food Science, Faculty of Science, University of Burgos, Plaza Misael Bañuelos s/n, 09001, Burgos, Spain

ARTICLE INFO

Article history:
Received 19 October 2016
Received in revised form
7 December 2016
Accepted 8 December 2016
Available online 5 January 2017

Keywords:
Staphylococcus aureus
Antibiotics
β-lactam
Transmission
Safety
Surveillance

ABSTRACT

Background: Antimicrobial resistance is one of the major challenges in medicine and **methicillin-resistant *Staphylococcus aureus* (MRSA)** is a particularly problematic nosocomial pathogen. Many recent studies document successful MRSA lineages in farm animals and derived foodstuffs, highlighting the need for adequate control and prevention programmes to avoid food transmission.
Scope and approach: We review the presence of MRSA along the food chain, and the potential of food-producing animals and associated foodstuffs for the transmission of MRSA. The massive use of **antibiotics** in feed to promote growth, and the inappropriate use of antimicrobial agents in veterinary and human medicine are considered to be major contributors to the emergence of resistance. Developments in the epidemiology of MRSA, in hospital (human) settings and primary food production, and MRSA spread along the food chain are described here. Information from EU surveillance programmes is also taken into consideration.
Keys findings and conclusions: The emergence of MRSA has implications for **food safety** and surveillance programmes are required for rapid MRSA detection and control.

1. Introduction

Methicillin-resistant *Staphylococcus aureus* (MRSA) is becoming clinically problematic worldwide. The appearance of antimicrobial-resistant bacteria as a result of inappropriate use of antibiotics either as antimicrobial therapy or for growth promotion is affecting the food chain sectors, with implications for food-producing animals and associated foodstuff. Here, we review recent evidence of the increasing prevalence of MRSA, with particular attention to the food chain and the genetic background.

2. Methicillin-resistant *Staphylococcus aureus* (MRSA)

Resistance to penicillinase-stable penicillins, also called "methicillin resistance" or "oxacillin resistance", in *S. aureus* is manifested as resistance to all β-lactam antimicrobial agents including cephalosporins and carbapenems and potential susceptibility to the newest class of MRSA-active cephalosporins (e.g. ceftaroline).

The appearance of methicillin-resistant *Staphylococcus aureus* (MRSA) dates back to 1961 in the United Kingdom (Petinaki & Spiliopoulou, 2012), one year after the first introduction of methicillin in clinical practice to treat infections with penicillin-resistant *S. aureus* (Jevons, 1961). MRSA became a serious threat in the early 90s in the USA and UK (Kluytmans, 2010). Resistance is conferred by a mobile genetic element, named the staphylococcal chromosomal cassette (SCC*mec*) (Milheiriço, Oliveira, & de Lencastre, 2007), carrying *mec*A or *mec*C genes, encoding a penicillin-binding protein 2a (PBP2a) (Paterson, Morgan, et al., 2014;; Petinaki & Spiliopoulou, 2012). Beta-lactam drugs bind to PBPs, essential for cell wall peptidoglycan synthesis, leading to bacterial cell lysis. However, PBP2a has low affinity for β-lactam agents, such that peptidoglycan synthesis can continue in MRSA strains (Paterson, Morgan, et al., 2014) even in the presence of diverse β-

* Corresponding author.
E-mail addresses: rodlazda@gmail.com, drlazaro@ubu.es (D. Rodríguez-Lázaro).

http://dx.doi.org/10.1016/j.tifs.2016.12.002
0924-2244/

International Journal of Food Microbiology 209 (2015) 34–38

Contents lists available at ScienceDirect

International Journal of Food Microbiology

journal homepage: www.elsevier.com/locate/ijfoodmicro

Foods from black market at EU border as a neglected route of potential methicillin-resistant *Staphylococcus aureus* transmission

Elena-Alexandra Oniciuc [a,1], Jaime Ariza-Miguel [b,1], Andrei-Sorin Bolocan [a], Marta Diez-Valcarce [c], Jordi Rovira [c], Marta Hernández [b], Isabel Fernández-Natal [d,e], Anca Ioana Nicolau [a], David Rodríguez-Lázaro [b,f,*]

[a] Faculty of Food Science and Engineering, Dunarea de Jos University of Galati, Romania
[b] Instituto Tecnológico Agrario de Castilla y León, Valladolid, Spain
[c] Department of Biotechnology and Food Science, Faculty of Sciences, University of Burgos, Burgos, Spain
[d] Department of Clinical Microbiology, Complejo Asistencial Universitario de León, León, Spain
[e] Institute of Biomedicine (IBIOMED), University of León, León, Spain
[f] Microbiology Section, Faculty of Sciences, University of Burgos, Burgos, Spain

ARTICLE INFO

Available online 22 November 2014

Keywords:
Methicillin-resistant *Staphylococcus aureus*
LA-MRSA
Food
Black market
Border

ABSTRACT

The illegal entrance of foods to EU through black markets at the EU borders can constitute a neglected route of dissemination of foodborne pathogens, and in particular of methicillin-resistant *Staphylococcus aureus* (MRSA). In this study, we have assessed the presence of MRSA in foods sold in a black market at an EU border (the south-east part of Romania, on the border with Republic of Moldavia). We performed a search for MRSA among 200 food samples collected from 2012 to 2013. All *S. aureus* were studied by pulsed-field gel electrophoresis (PFGE) and antimicrobial susceptibility testing. MRSA isolates were further characterized by multilocus sequence typing (MLST) and SCC*mec* typing, and tested for the presence of Panton–Valentine leukocidin (PVL) virulence factors. Overall, 32 *S. aureus* isolates were recovered from 16 food samples (8%). One isolate detected in a pork lard sample was MRSA (0.5%). PFGE with the restriction enzyme *Sma*I revealed 12 genotypes among the 32 *S. aureus* isolates. The MRSA isolate belonged to sequence type 398, harbored SCC*mec* type V, tested negative for the presence of the PVL genes and was resistant to ciprofloxacin, tetracycline and cefazolin, besides all β-lactams. Among 31 methicillin-sensitive *S. aureus* (MSSA), 29% were resistant to penicillin, 9.7% to tetracycline and 3.2% to ciprofloxacin. In conclusion, in this study we report the presence of livestock-associated MRSA in foods sold in a black market at an EU border: ST398-MRSA-V. These results confirm the potential role of food in the dissemination of MRSA lineages among population, and the potential role of illegally introduced food to EU in the prevalence and evolution of MRSA clones in the community.

1. Introduction

It is estimated that *Staphylococcus aureus* produced an average of 241,148 episodes of domestically acquired foodborne illnesses caused in United States (Scallan et al., 2011), representing an important cause of food poisoning. In addition, this foodborne pathogen cause nosocomial invasive infections ranging from mild skin and soft tissue infections to life-threatening diseases such as septicaemia, endocarditis and necrotizing pneumonia (Lowy, 1998). It has been estimated that around 30% of healthy human individuals are colonized by this opportunistic pathogen (Graveland et al., 2011). *S. aureus* frequently harbor antibiotic resistance determinants which complicate treatment and significantly increase the associated costs. Currently, methicillin-resistant *S. aureus* (MRSA) is distributed worldwide and constitutes a major concern in human health because of its complex epidemiology and its ability to acquire novel antibiotic resistance mechanisms.

MRSA was first described in 1960, within a year after the inclusion of methicillin in the clinical practice to treat infections caused by the emergence of penicillin-resistant *S. aureus* (Jevons, 1961). Its presence was restricted to the clinical environment initially, but at the end of the past decade first cases of MRSA infections in the community were reported affecting people who exhibited no typical risk factors of hospital acquisition (Otter and French, 2010). Until the beginning of this century, MRSA had been rarely reported in livestock. It was first described in 1975, and after that, only sporadic cases were reported in the following 25 years. From 2005 onwards, MRSA belonging to sequence type (ST)

Abbreviations: MRSA, methicillin-resistant *Staphylococcus aureus*; SCC*mec*, staphylococcal cassette chromosome *mec*; PLV, Panton–Valentine leukocidin; CA-MRSA, community-associated MRSA; LA-MRSA, livestock-associated MRSA; PFGE, pulsed-field gel electrophoresis; MLST, multilocus sequence typing; ST, sequence type

* Corresponding author at: Instituto Tecnológico Agrario de Castilla y León (ITACyL), Consejería de Agricultura y Ganadería, Junta de Castilla y León, Carretera de Burgos km. 119, Valladolid, Spain. Tel.: +34 983 415233; fax: +34 983 410462.
E-mail address: rodlazda@gmail.com (D. Rodríguez-Lázaro).

[1] These authors contributed equally to the work.

http://dx.doi.org/10.1016/j.ijfoodmicro.2014.11.015
0168-1605/

FOODBORNE PATHOGENS AND DISEASE
Volume 13, Number 3, 2016

DOI: 10.1089/fpd.2015.2057

Tracking Foodborne Pathogenic Bacteria in Raw and Ready-to-Eat Food Illegally Sold at the Eastern EU Border

Luminita Ciolacu,[1,2] Beatrix Stessl,[2] Andrei Sorin Bolocan,[1] Elena Alexandra Oniciuc,[1] Martin Wagner,[2] Kathrin Rychli,[2] and Anca Ioana Nicolau[1]

Abstract

Food illegally brought into the European Union, mainly in the personal luggage of travelers, represents a potential threat to consumers' health. The aim of this study was to investigate the presence of five pathogens in food brought into the European Union by Moldavian citizens as personal goods and illegally sold in Romania in the vicinity of the border. The occurrence of *Staphylococcus aureus* and *Listeria monocytogenes* was 7.5% and 8%, while *Campylobacter* spp., *Escherichia coli* O157:H7, and *Salmonella* spp. were absent in all samples. *L. monocytogenes* sequence type 2, 9, 121, and 155, highly prevalent among foodstuffs worldwide, was also present among isolates from ready-to-eat food illegally sold in Romania, even at the same date of sampling, indicating cross-contamination during food handling. *S. aureus spa* types t449, t304, and t524 were most often isolated from raw-milk cheeses contaminated with 10^3–10^5 colony-forming units per gram, evidencing a contamination at herd level or unhygienic conditions during processing. *S. aureus* t011 and t3625, both included in the livestock-associated CC398, were isolated from pork lard and poultry meat. This study shows that cross-border trade from nonmember states represents a neglected route of transmission of foodborne pathogens into the European Union that could lead to sporadic or family-associated cases of disease.

Introduction

Illegally imported food, also known as contraband or smuggled food, is a worldwide problem. In the European Union (EU), the high number of foreigners to illegally import food is proved by the amounts of confiscated items in different points of entering (ports, airports, terrestrial borders) with the largest amounts in Spain, the United Kingdom, and Germany (EC, 2011). Food is mainly carried in the personal luggage of travelers and is primarily designated for personal consumption, and secondly for illegal sale (Noordhuizen *et al*., 2013; Beutlich *et al*., 2015; de Melo *et al*., 2015; Rodriguez-Lazaro *et al*., 2015; Schoder *et al*., 2015).

As neither raw material origin and quality, nor technological process and hygienic conditions during food processing are known, smuggled food poses a potential health risk. Furthermore, the conditions during transportation and sale might violate the safety rules since refrigeration and adequate packaging are missing. Usually little information is available regarding associated risks and prevalence of pathogens in these foods. A better situation exists in the United States, where some of such foods are microbiologically examined. For example, it is known that cheese smuggled into the country by Mexican citizens harbors *Salmonella* (13%), *Listeria* spp. (4%), and *Mycobacterium* spp. (Kinde *et al*., 2007).

To fill this gap, one objective of the EU research project ''Protection of consumers by microbial risk mitigation through combating segregation of expertise'' (PROMISE) was to assess five significant foodborne pathogens for being introduced into EU *via* uncontrolled imports (www.promise-net.eu). Thus, the prevalence of *Salmonella* spp., *Campylobacter* spp., *Escherichia coli* O157:H7, *Listeria monocytogenes*, and *Staphylococcus aureus* was investigated by all project partners in raw and ready-to-eat (RTE) food collected either from ports and airports or from terrestrial EU borders. At the Eastern EU border, the Romanian Law 10/2010 ratifies the agreement on cross-border traffic between Romania and the Republic of Moldova. Among other goods, foods that are officially declared for personal use are legally brought into European Union, but illegally sold in local Romanian markets organized to sell fresh fruits and vegetables. There are nine crossing points between Romania and the Republic of Moldova that are

[1]Faculty of Food Science and Engineering, Dunarea de Jos University of Galati, Galati, Romania.
[2]Institute for Milk Hygiene, University of Veterinary Medicine Vienna, Vienna, Austria.

frontiers in Microbiology

PERSPECTIVE
published: 30 March 2016
doi: 10.3389/fmicb.2016.00390

Compositional Analysis of Biofilms Formed by *Staphylococcus aureus* Isolated from Food Sources

Elena-Alexandra Oniciuc[1,2], *Nuno Cerca*[2] *and Anca I. Nicolau*[1]*

[1] Faculty of Food Science and Engineering, Dunarea de Jos University of Galati, Galati, Romania, [2] Centre of Biological Engineering, Universidade do Minho, Braga, Portugal

Sixteen *Staphylococcus aureus* isolates originating from foods (eight from dairy products, five from fish and fish products and three from meat and meat products) were evaluated regarding their biofilms formation ability. Six strains (E2, E6, E8, E10, E16, and E23) distinguished as strong biofilm formers, either in standard Tryptic Soy Broth or in Tryptic Soy Broth supplemented with 0.4% glucose or with 4% NaCl. The composition of the biofilms formed by these *S. aureus* strains on polystyrene surfaces was first inferred using enzymatic and chemical treatments. Later on, biofilms were characterized by confocal laser scanning microscope (CLSM). Our experiments proved that protein-based matrices are of prime importance for the structure of biofilms formed by *S. aureus* strains isolated from food sources. These biofilm matrix compositions are similar to those put into evidence for coagulase negative staphylococci. This is a new finding having in view that scientific literature mentions exopolysaccharide abundance in biofilms produced by clinical isolates and food processing environment isolates of *S. aureus*.

Keywords: *Staphylococcus aureus*, biofilm, food, CLSM, exopolysaccharide, protein

OPEN ACCESS

Edited by:
Romain Briandet, French National Institute for Agricultural Research, France

Reviewed by:
Giovanni Di Bonaventura, "G. D'Annunzio" University of Chieti-Pescara, Italy
Arnaud Bridier, French Agency for Food, Environmental and Occupational Health & Safety, France

***Correspondence:**
Anca I. Nicolau
anca.nicolau@ugal.ro

Specialty section:
This article was submitted to Food Microbiology, a section of the journal Frontiers in Microbiology

Received: *05 January 2016*
Accepted: *11 March 2016*
Published: *30 March 2016*

Citation:
Oniciuc E-A, Cerca N and Nicolau AI (2016) Compositional Analysis of Biofilms Formed by Staphylococcus aureus Isolated from Food Sources. Front. Microbiol. 7:390. doi: 10.3389/fmicb.2016.00390

INTRODUCTION

Few studies have been reported so far regarding the biofilm formation by *Staphylococcus aureus* isolated from foods (Di Ciccio et al., 2015) and the impact of the environmental factors encountered in food processing plants on the adherence and biofilm formation (Vázquez-Sánchez et al., 2013; Santos et al., 2014).

In food industry it is important to know the conditions under which *S. aureus* is able to survive, adhere to surfaces and form biofilms (Futagawa-Saito et al., 2006), leading to contamination of food products. In planktonic form, *S. aureus* does not appear resistant to disinfectants, compared to other bacteria, but it may be among the most resistant ones when is attached to a surface (Fratamico et al., 2009). *S. aureus* can produce a multilayered biofilm embedded within a glycocalix with heterogeneous protein expression throughout, forming at least two types of biofilms: *ica*-dependent, mediated by polysaccharide intercellular adhesin (PIA)/poly-N-acetyl-1,6-β-glucosamine (PNAG), and *ica*-independent, mediated by proteins (Beloin and Ghico, 2005). Biofilm-associated protein (Bap), which shows global organizational similarities to surface proteins of Gram-negative (*Pseudomonas aeruginosa* and *Salmonella enterica* serovar Typhi) and Gram-positive (*Enterococcus faecalis*) bacteria (Cucarella et al., 2001), was the first protein that has been found to be involved in biofilm formation by staphylococcal strains isolated from mammary glands in ruminants suffering from mastitis (Speziale et al., 2014). Meanwhile, Foulston et al. (2014) discovered

International Journal of Food Microbiology 209 (2015) 26–28

Contents lists available at ScienceDirect

International Journal of Food Microbiology

journal homepage: www.elsevier.com/locate/ijfoodmicro

Evaluation of two commercially available chromogenic media for confirmation of methicillin-resistant *Staphylococcus aureus* from human, animal, and food samples

Jaime Ariza-Miguel [a], Elena-Alexandra Oniciuc [b], Iván Sanz [c], Isabel Fernández-Natal [d,e], Marta Hernández [a], David Rodríguez-Lázaro [a,f,*]

[a] Instituto Tecnológico Agrario de Castilla y León, Valladolid, Spain
[b] Faculty of Food Science and Engineering, Dunarea de Jos University of Galati, Romania
[c] Valladolid National Influenza Center, Valladolid, Spain
[d] Complejo Asistencial Universitario de León, León, Spain
[e] Institute of Biomedicine (IBIOMED), University of León, Leon, Spain
[f] Microbiology Section, Department of Biotechnology and Food science, Faculty of Science, University of Burgos, Burgos, Spain

ARTICLE INFO

Article history:
Received 30 March 2015
Received in revised form 26 April 2015
Accepted 10 May 2015
Available online 12 May 2015

Keywords:
Methicillin-resistant *Staphylococcus aureus*
Chromogenic media
Screening

ABSTRACT

We compared the diagnostic performance of two chromogenic media, Brilliance MRSA 2 agar (Thermo Fisher Scientific) and ChromID MRSA agar (bioMérieux), for MRSA confirmation of 239 *Staphylococcus aureus* isolates from clinical, animal and food samples. Statistically significant differences were not observed between MRSA confirmation by *mec*A/*mec*C PCR, and by culture in both chromogenic media. However, a statistically significant difference was observed between the results obtained by both chromogenic media ($p = 0.003$). Segregated analysis of the results depending on the origin of the isolates (clinical, animal, and food) revealed a significant lower performance in the MRSA confirmation of food-derived isolates by using Brilliance MRSA 2 agar in comparison to PCR confirmation ($p = 0.003$) or ChromID MRSA agar ($p < 0.001$). Both chromogenic media provided a good diagnostic performance for detection of MRSA isolates of human and animal origin. In conclusion, the use of chromogenic agar plates for MRSA confirmation of *S. aureus* isolates can provide a good diagnostic performance (sensitivity >92% and specificity >89%) regardless of the type of chromogenic media used or the origin of the *S. aureus* isolates. However, our results revealed a lower diagnostic performance for MRSA confirmation of *S. aureus* isolates from food samples by using Brilliance MRSA 2 agar.

1. Introduction

Dissemination of methicillin-resistant *Staphylococcus aureus* (MRSA) from the health care system to community and animal settings in the last two decades has elicited a great concern (Graveland et al., 2011; Okuma et al., 2002). Furthermore, the emergence of MRSA in food-producing animals and derived food products has raised the question on the potential role of food as a route for transmission of successful livestock and community associated MRSA lineages (Oniciuc et al., 2015–in this issue; Rodríguez-Lázaro et al., 2015–in this issue). Therefore, monitoring the presence and genetic features of MRSA in all environments and potential reservoirs is required to better understand the dissemination, genetic evolution and evolutionary success of epidemic MRSA lineages.

Several commercially available chromogenic media have been developed to facilitate the screening of MRSA, and some studies have assessed their diagnostic performance (McElhinney et al., 2013; Veenemans et al., 2013; Verkade et al., 2011). However, they have been mainly focused in human clinical samples, and there is a knowledge gap regarding MRSA from animal and food samples. Therefore, in this study we evaluated the performance of Brilliance MRSA 2 Agar (Thermo Fisher Scientific, Waltham, MA, USA) and ChromID MRSA Agar (bioMérieux, France) as rapid MRSA confirmation screening assays for *S. aureus* isolates from a wide range of origins: clinical, animal and food samples. We assessed, by using the McNemar's test for paired samples, if there are statistically significant differences among a reference method, the molecular detection of resistance genes *mec*A and *mec*C, and MRSA confirmation by using both chromogenic media. Furthermore, we have compared the diagnostic performance between the chromogenic media.

2. Material and methods

2.1. Bacterial isolates

A collection of 239 *S. aureus* isolates, comprising 154 methicillin-sensitive *S. aureus* isolates (MSSA), 83 MRSA isolates harbouring *mec*A, and two MRSA isolates harbouring *mec*C, were selected to perform the

* Corresponding author at: Instituto Tecnológico Agrario de Castilla y León (ITACyL), Consejería de Agricultura y Ganadería, Junta de Castilla y León, Carretera de Burgos km. 119, Valladolid, Spain.
E-mail address: rodlazda@gmail.com (D. Rodríguez-Lázaro).

http://dx.doi.org/10.1016/j.ijfoodmicro.2015.05.004
0168-1605/

europass Curriculum vitae

PERSONAL INFORMATION

Elena-Alexandra ONICIUC

193 TECUCI Street, Bl. N7, ap. 38, 800295 Galati (Romania)

+40 744998122 +34 691324210

Elena.Oniciuc@ugal.ro; Oniciuc_Elena@yahoo.com

Skype Oniciuc.Elena

Sex Female | Date of birth 18/10/1989 | Nationality Romanian

WORK EXPERIENCE

01/08/2014–Present — Laboratory for Physico-chemical and Microbiological Analysis of Food
„Dunarea de Jos" University, Faculty „Food Science and Engineering", Galati (Romania)

01/03/2013–01/09/2013 — Laboratory for Physico-chemical Analysis of Meat Products
SC.SALTEMPO.SRL, Galati (Romania)

EDUCATION AND TRAINING

01/10/2014–Present — PhD student
„Dunarea de Jos" University of Galati, Romania and University of Burgos, Spain

Topic of the thesis: Characterization of methicillin resistant *Staphylococcus aureus* strains isolated from foods

2012–2014 — Engineer- Control, Expertise and Food Safety
„Dunarea de Jos" University, Faculty „Food Science and Engineering", Galati (Romania)

Topic of the thesis: The incidence of *Staphylococcus aureus* in food imported from Republic of Moldavia and illegally sold in Romania. Characterization of isolates

2012–2013 — Graduate Pedagogical Training Module- Level II
„Dunarea de Jos" University, Department for Teacher Training, Galati (Romania)

2008–2012 — Bachelor in Engineering and Management in catering and tourism
„Dunarea de Jos" University, Faculty „Food Science and Engineering", Galati (Romania)

Topic of the thesis: Designing a catering unit for high school with a capacity of 200 seats

2008–2012 — Graduate Pedagogical Training Module- Level I
„Dunarea de Jos" University, Department for Teacher Training, Galati (Romania)

2004–2008 — Food analysis technician
College of Food Industry „Elena Doamna", Galati (Romania)

PERSONAL SKILLS

Mother tongue(s) Romanian

Other language(s)

	UNDERSTANDING		SPEAKING		WRITING
	Listening	Reading	Spoken interaction	Spoken production	
English	B2	B2	B2	B2	B2
Spanish	B1	B1	B1	B1	B1
French	A1	A1	A1	A1	A1

Levels: A1 and A2: Basic user - B1 and B2: Independent user - C1 and C2: Proficient user
Common European Framework of Reference for Languages

Communication skills

- Good communication skills;
- Team spirit;

Organisational / managerial skills

- Passionate, optimistic;
- Adaptation to work in a team;
- Dynamic person, motivated, responsible;

Digital competence

SELF-ASSESSMENT				
Information processing	Communication	Content creation	Safety	Problem solving
Proficient user	Proficient user	Independent user	Independent user	Independent user

Digital competences - Self-assessment grid

Good command of MS Office (Word, Excel, PowerPoint, Publisher); Autocad; Origin; Bionumerics

Driving licence

B

ADDITIONAL INFORMATION

Publications

Oniciuc, E.A., Nicolau, A.I., Hernández, M., Rodríguez-Lázaro, D.(2017). Presence of methicillin-resistant *Staphylococcus aureus* in the food chain. Trends in Food Science and Technology, 61:49-59, http://dx.doi.org/10.1016/j.tifs.2016.12.002

Bleoanca, I., Saje, K., Mihalcea, L., **Oniciuc, E.A**., Smole-Mozina, S., Nicolau, A.I., Borda, D. (2016). Contribution of high pressure and thyme extract to control *Listeria monocytogenes* in fresh cheese- A hurdle approach. Innovative Food Science and Emerging Technologies, 38:7-14, http://dx.doi.org/10.1016/j.ifset.2016.09.002

Oniciuc, E-A., Cerca, N., Nicolau, A.I. (2016). Compositional analysis of biofilms formed by *Staphylococcus aureus* isolated from food sources. Frontiers in Microbiology, 7:1-4, http://dx.doi.org/10.3389/fmicb.2016.00390

Ciolacu, L., Stessl, B., Bolocan, A.S., **Oniciuc, E.A**., Wagner, M., Rychli, B., Nicolau, A.I. (2016). Tracking foodborne pathogenic bacteria in raw and ready-to-eat food illegally sold at the Eastern EU border. Foodborne Pathogens and Disease,13(3):148-155, http://dx.doi.org/10.1089/fpd.2015.2057

Bolocan, A.S., Nicolau, A.I., Alvarez-Ordonez, A., Borda, D., **Oniciuc, E.A**., Stessl, B., Gurgu, L., Wagner, M., Jordan, K. (2016). Dynamics of *Listeria monocytogenes* colonisation in a newly-opened meat processing facility. Meat Science, 113:26-34, http://dx.doi.org/10.1016/j.meatsci.2015.10.016

Bolocan, A.S., **Oniciuc, E.A**., Alvarez-Ordonez, A., Wagner, M., Rychli, K., Jordan, K., Nicolau, A.I. (2015). Putative cross-contamination routes of *Listeria monocytogenes* in a meat processing facility in Romania. Journal of Food Protection, 78 (9):1624-1769, https://doi.org/10.4315/0362-028X.JFP-14-539

Ariza-Miguel, J., **Oniciuc, E.A.,** Sanz, I., Fernández-Natal, I., Hernández, M., Rodríguez-Lázaro, D. (2015). Evaluation of two commercially available chromogenic media for confirmation of methicillin-resistant *Staphylococcus aureus* from human, animal, and food samples. International Journal of

Food Microbiology, 209: 26-28, http://dx.doi.org/10.1016/j.ijfoodmicro.2015.05.004

Oniciuc, E.A., Ariza-Miguel, J., Bolocan, A.S., Diez-Valcarce, M., Rovira, J., Hernández, M., Fernández-Natal, I., Nicolau, A.I., Rodríguez-Lázaro, D. (2015). Foods from black market at EU border as a neglected route of potential Methicillin-Resistant *Staphylococcus aureus* transmission, International Journal of Food Microbiology, 209: 34-38, http://dx.doi.org/10.1016/j.ijfoodmicro.2014.11.015

Projects

Present- PhD student in SafeConsumE project- Safer food through changed consumer behavior: Effective tools and products, communication strategies, education and a food safety policy reducing health burden from foodborne illnesses (no. 727580/2017) („Dunarea de Jos" University of Galati, Galati, Romania)

Present- PhD student in SafeFood project- Development of a novel industrial process for safe, sustainable and higher quality foods, using biotechnology and cybernetic approach (ERA-IB-16-014) („Dunarea de Jos" University of Galati, Galati, Romania)

01/06/2014-31/12/2014- PhD student in the FP7th PROMISE project no 265877- Protection of consumers through mitigation of segregation of expertise („Dunarea de Jos" University of Galati, Galati, Romania)

2012-2013- Member in POSDRU project no 86/1.2 /S/52 422- Management correlating education system with labor market („Dunarea de Jos" University of Galati, Galati, Romania)

Internships

16/01/2017- 15/04/2017- Erasmus internship- Whole genome sequencing analysis of an OS-MRSA strain associated with food (ITACyL and Universidad de Burgos, Spain)

01/03/2016- 15/04/2016-Doctoral internship- Screening and detection of *Toxoplasma gondii* in pig samples from slaughterhouses (COST FA1408- Universidad de Burgos, Spain)

01/12/2015- 28/02/2016- Doctoral internship- Characterization of the methicillin resistant *Staphylococcus aureus* strains from different neglected routes to Europe (FEMS grant- ITACyL and Universidad de Burgos, Spain)

01/10/2015- 30/11/2015- Doctoral internship- Characterization of the methicillin resistant *Staphylococcus aureus* strains from different neglected routes to Europe (international airports and open market close to EU borders) (COST FA1202 grant- ITACyL and Universidad de Burgos, Spain)

10/03/2015-30/04/2015- Doctoral internship- Characterization of *Staphylococcus aureus* biofilms by CLSM (COST FA1202 grant- Center of Biological Engineering, University of Minho, Braga, Portugal)

06/10/2014-12/12/2014- Doctoral internship- Characterization of *Staphylococcus aureus* isolates by PFGE (PROMISE grant- Instituto Tecnológico Agrario de Castilla y León, Valladolid, Spain)

17/02/2014-18/05/2014- Erasmus internship- Characterization of *Staphylococcus aureus* strains isolated from foods originating from a black market in Romania (Universidad de Burgos, Burgos, Spain)

Workshops

09/09/2016- One day Summer School- *In vitro/ In silico* approaches for food science (EFSA, Parma, Italy)

18/12/2014-19/12/2014- Workshop on sensible data produced by the PROMISE consortium with involved research partners and Food Safety Agencies (PROMISE consortium and AGES, Wien, Austria)

15/09/2014-20/09/2014- Hands-on course in advanced techniques to study food-biofilms (COST FA1202- Center of Biological Engineering, University of Minho, Braga, Portugal)

14/07/2014-16/07/2014- PROMISE Summer School- "Protocols on host-pathogen interactions" (Veterinary Research Institution, Brno, Czech Republic)

01/07/2014-04/07/2014- Cell Culture Course- *Listeria monocytogenes* infection assay (Faculty of Food Science and Engineering, "Dunarea de Jos" University of Galati, Romania, course held by Dr. rer. nat. Kathrin Rychli from University of Veterinary Medicine of Vienna)

07/10/2013-08/10/2013- Erasmus intensive course- Physical state effects on food stability (Faculty of Food Science and Engineering, "Dunarea de Jos" University of Galati, Romania, course held by prof. Kirsi Jouppila from University of Helsinki)

14/04/2013-27/04/2013- Erasmus intensive programme “Functional Ingredients for Tailored Food" (Cork University, Cork, Ireland)

Conferences

Oniciuc, E.A., Bucur, F.I., Rodríguez Lázaro, D., Barbu, V., Hernandez, M., Nicolau, A.I.- Correlation between biofilm formation and composition and molecular aspects of methicillin-resistant *Staphylococcus aureus*. FEMS Congress, 09-13 July 2017, Valencia, Spain

Rodríguez Lázaro, D., Ariza-Miguel, J., **Oniciuc, E.A**., Nicolau, A.I., Rovira, J., Wagner, M., Fernández Natal, I., Hernandez, M. -"Evidence on an emerging risk associated with the methicillin-resistant *Staphylococcus aureus* (MRSA) in the food chain". Food Micro Conference, 19-22 July 2016, Dublin, Ireland

Fernández Natal, I., Ariza Miguel, J., **Oniciuc, E.A**., Nicolau, A.I., Rovira, J., Wagner, M., Hernandez, M., Rodríguez Lázaro, D. -"Descripcion del primer caso de la variante OXA sensible de una cepa de MRSA aislada en alimentos". XX Congreso Nacional de la Sociedad Española de Enfermedades Infecciosas y Microbiología Clínica, 26-28 May 2016, Barcelona, Spain

Saje, K., **Oniciuc, E.A**., Mihalcea, L., Coman, G., Bleoancă, I., Smole-Mozina, S., Nicolau, A.I., Borda, D.- Synergistic effect of high pressure processing and thyme extract on *Listeria monocytogenes* in fresh cheese. 7th International Symposium EuroAliment, 24-26/09/2015, Galati, Romania

Oniciuc, E.A., Bolocan, A.S., Fotin, A.A., Dajbog, A., Nicolau, A.- "Comparative assessment of disk diffusion and micro dilution methods using CLSI guidelines for antimicrobial susceptibility testing of *Staphylococcus aureus* isolated from foods". Food Safety Congress, 7-8 May 2015, Istanbul, Turkey

Bolocan, A.S**., Oniciuc, E.A.**, Alvarez-Ordóñez, A., Wagner, M., Rychli, K., Jordan, K., Borda, D., Nicolau, A.I.- "What could explain contamination in a Romanian food processing environment?**"**, Conference- Sensible data: A challenge for Risk Communication?, 18-19 December 2014, Wien, Austria

Nicolau, A.I., Bolocan, A.S**.,** Rychli, K., **Oniciuc, E.A.**, Alvarez-Ordóñez, A., W Jordan, K., Wagner, M.- "To be persistent or not to be persistent? That is the question for *Listeria monocytogenes* strains isolated from a meat processing environment". General Assembly of the Hungarian Society for Microbiology, 15-17 October 2014, Lake-Balaton, Hungary

Bolocan, A.S., Rychli, K., **Oniciuc, E.A.**, Wagner, M., Nicolau, A.I.- "Genetic characterization of some *Listeria monocytogenes* strains isolated from a meat processing facility in relation to their tolerance to disinfectants". Food Micro Conference, 1-4 September 2014, Nantes, France

Bolocan, A.S.,**Oniciuc, E.A.**, Dalmasso, M., Alvarez-Ordonez, A., Jordan, K., Nicolau, A.- "The necessity of changes in the legislation for *Listeria monocytogenes* control in the meat industry". Safefood Annual Conference and 1st Promise Stakeholder Meeting on Listeria, 20 November 2013, Dublin, Ireland

Bolocan, A.S., **Oniciuc, E.A.**, Barbu, V., Nicolau, A.- "Validation of a PCR method for detection of *Listeria monocytogenes* in meat industry". The 6th International Symposium, 3-5 October 2013, "Dunarea de Jos" University of Galati, Romania

Oniciuc, E.A., Moscu, T., Bolocan, A.S., Udrea, I., Stoica, M., Bleoancă, I., Borda, D.- "Utilization of the revolutionary treatment HPLT for preservation of the amazing whey based beverage: WoWEnergy". The 6th International Symposium, 3-5 October 2013, "Dunarea de Jos" University of Galati, Romania

Bolocan, A.S., **Oniciuc, E.A.**, Nicolau, A.-"How fast *Listeria monocytogenes* installs in a meat processing environment". The 42nd Annual Food Research Conference, 27-28 June 2013, Dublin, Ireland

Oniciuc, E.A., Gîtin, L., Ciortan, S., Maftei (Aron), N., Nicolau, A.- 'Intense light pulses effect on fungal burden of mustard and black pepper ". International Conference "Biotechnologies, Present and Perspectives", 19-20 November 2010, Faculty of Food Engineering "Stefan cel Mare" Suceava, Romania

Scientific Session for Students

Fotin, A.A., **Oniciuc, E.A.,** Nicolau, A.- "Antibiotic susceptibility of *Staphylococcus aureus* strains isolated from animal origin foods sold in an open market". Student Scientific Session, Faculty "Food Science and Engineering", 22 May 2015, Galati, Romania

Oniciuc, E.A. -"Intense light pulses effect on fungal burden of mustard berries". International

Conference "Student in Bucovina", Faculty of Food Engineering "Stefan cel Mare", 8-10 July 2010, Suceava, Romania

Oniciuc, E.A.-"What do we know about ionizing radiation?". Student Scientific Session, Faculty "Food Science and Engineering", 14-15 May 2010, Galati, Romania

Oniciuc, E.A.- "Effect of light pulses on the *Penicillium* molds". Student Scientific Session, Faculty "Food Science and Engineering", 14-15 May 2010, Galati, Romania

Oniciuc, E.A. –Degree of participation– in the event "Chemex Cluj-Napoca", 3rd Edition, "Babes Bolyai" University, 25- 28 March 2010, Cluj-Napoca, Romania

Oniciuc, E.A.-"Vegetable concentrates". International Conference "Students for Students", 6th edition, "Babes Bolyai" University, 10-12 April 2009, Cluj-Napoca, Romania

Honours and awards

Oniciuc, E.A., Martín Quijada, N., Nicolau, A.I., Hernández, M., Rodríguez-Lázaro, D.- "Comparative genomic analysis for understanding evolution of MRSA Strains"- First prize- Scientific Conference of Doctoral Schools from "Dunarea de Jos" University of Galati, 5th edition, 08-09 June 2017, "Dunărea de Jos" University of Galati, Romania

Oniciuc, E.A., Nicolau, A.I.- "Protein based matrices evidenced in biofilm structure of *Staphylococcus aureus* isolated from food sources"- First prize- Scientific Conference of Doctoral Schools from "Dunarea de Jos" University of Galati, 4th edition, 2-3 June 2016, "Dunărea de Jos" University of Galati, Romania

Oniciuc, E.A., Ariza-Miguel, J., Bolocan, A.S., Diez-Valcarce, M., Rovira, J., Hernández, M., Fernández-Natal, I., Nicolau, A.I., Rodríguez-Lázaro, D.- "Incidence of *Staphylococcus aureus* in food products illegally sold in a Romanian market and characterization of the isolated strains"- Second prize- Scientific Conference of Doctoral Schools from "Dunărea de Jos" University of Galati, 3rd edition, 4-5 June 2015, "Dunărea de Jos" University of Galati, Romania

Bolocan, A.S., Crăiță, A., Stângă, C.T., **Oniciuc, E.A.** -"Magic Bite product"-Bronz medal- UGAL INVENT, 8-10 october 2014, "Dunărea de Jos" University of Galati, Romania

Stângă, C.T., Crăiță, A., Bolocan, A.S., **Oniciuc, E.A.**- "Magic Bite cupcakes having a complex structure that capitalize two waste products often met in food industry: eggshell and whey" –First prize– Ecotrophelia local competition, 9 may 2014, Faculty "Food Science and Engineering", Galati, Romania

Moscu, T., **Oniciuc, E.A.**, Bolocan, A.S., Udrea, I.- "WoWEnergy product, a whey based beverage with fruit juice preserved by HPLT"-Second prize- Ecotrophelia national competition, 8-9 july 2013, „Stefan cel Mare" University of Suceava, Romania

Oniciuc, E.A., Moisa, D., Danilov, C.- "Studies on the action of light pulses on a grain of mustard on an installation prototype consisting of a vibratory sieve" –First Prize– Student Scientific Session, 14-15 May 2010, Faculty "Science and Food Engineering", Galati, Romania

Memberships

2015-Present- Member of Romanian Society of Biochemistry and Molecular Biology

2014-Present- Member of Romanian Society of Microbiology

2014-Present- Member of the Scientific Council of Doctoral School of Engineering („Dunarea de Jos" University, Galati, Romania)

2012-2014- Member of Scientific Research Committee of the University „Dunarea de Jos" Senate („Dunarea de Jos" University, Galati, Romania)

2011–2012- Member of HACCP system implementation project in the School Inspectorate of Galati (School Inspectorate, Galati, Romania)

Others

23/05/2013-24/05/2013- Secretary Section 1- Food Technology and applied Biotechnology-Student Scientific Session (Faculty of Food Science and Engineering, "Dunarea de Jos" University of Galati, Romania)