„DUNĂREA DE JOS”
UNIVERSITY OF GALAȚI

UNIVERSITY OF BURGOS

Doctoral School of Engineering

Advances in Food Science and Biotechnology

PHD THESIS

SUMMARY

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

Characterization of Methicillin-Resistant Staphylococcus aureus Strains Isolated from Foods

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PhD. Eng. Elena-Alexandra ONICIUC

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Serie I4: Industrial Engineering **No.** 47

Galați 2017

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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

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.

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.

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.

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.

CHAPTER 1

Staphylococcus aureus and Its Main Characteristics

S. aureus is one of the most known and studied of its genus. It is an opportunistic bacterium 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 1.1) is characterized by the production of specific enzymes (coagulase, catalase, thermonuclease, hyaluronidase) and exotoxins. *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).

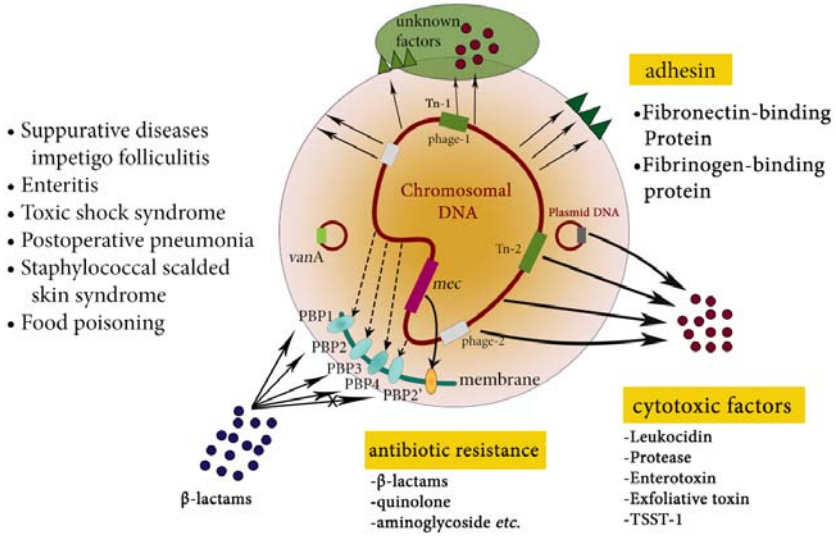


Figure 1.1. Virulence factors in *Staphylococcus aureus*

Moreover, cell wall adhesion (CWA) components (adhesins, protein A, teichoic acid, peptidoglycan) of *S. aureus* are also involved in virulence (Gordon and Lowy, 2008).

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). 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 1.2).

MRSA has been isolated from food products, implicating food as a pathway for MRSA dissemination.

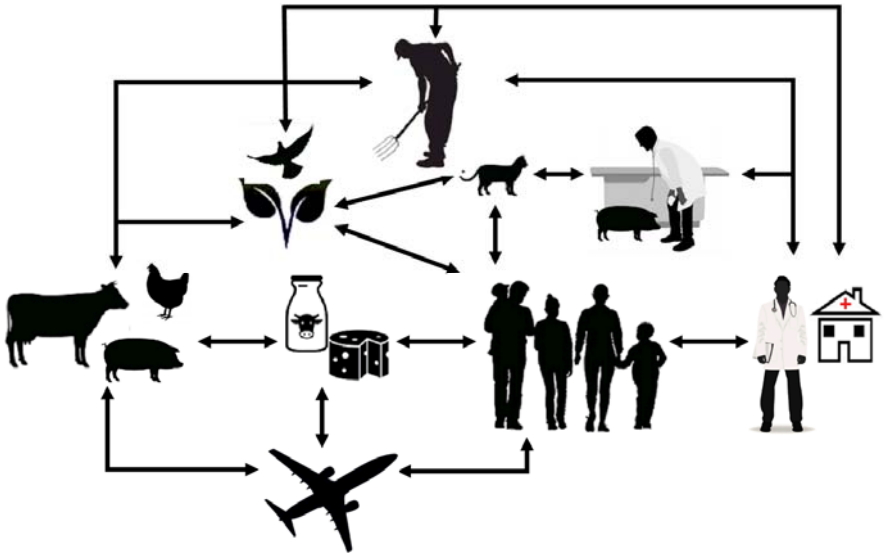


Figure 1.2. Potential routes of transmission of MRSA

CHAPTER 2

Procedures Used for Detection and Identification of *Staphylococcus aureus*

2.1. Conventional Detection and Identification Methods

ISO 6888 describes two horizontal methods (part 1 and part 2) (Figure 2.1) 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).

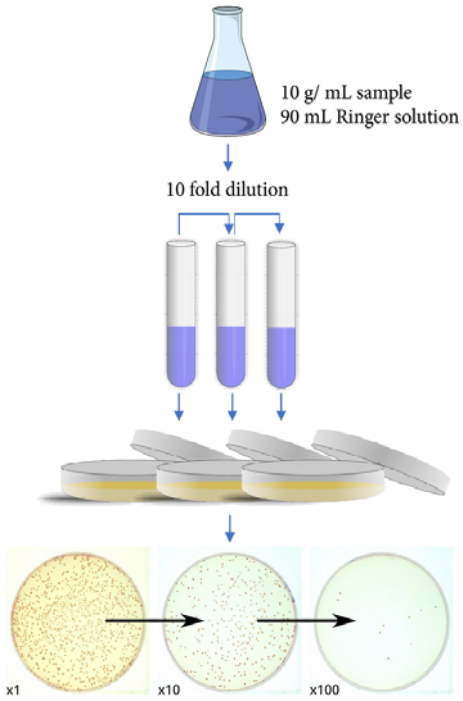


Figure 2.1. Horizontal method for the enumeration of coagulase-positive staphylococci (*S. aureus* and other species)

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.

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).

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.

Multilocus sequence typing. 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/).

***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/).

SCCmec 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).

Whole Genome Sequencing. 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.

CHAPTER 3

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).

CHAPTER 4

Methods

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.

Global results regarding identification, isolation and characterization of *S. aureus*, particularly MRSA strains were obtained by following phenotypical and genotypical approaches.

CHAPTER 5

Detection and Identification of *Staphylococcus aureus* in Food Isolated from Black Market

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.

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.

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 5.1).

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.

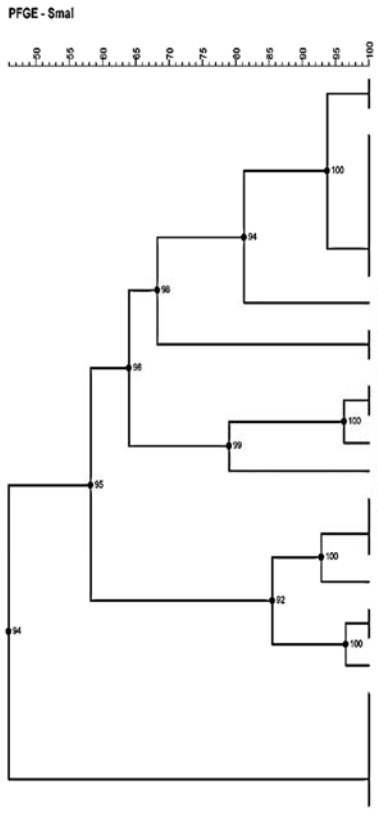
A further particular livestock-associated problem is the increasing number of MRSA (Haran *et al.*, 2012). Apart from dairy herds as a reservoir for MRSA, raw meat could also harbor higher loads of *S. aureus* (15–65%), among them 1–11% MRSA (Bhargava *et al.*, 2011; Jackson *et al.*, 2013).

Table 5.1. *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 5.1). 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.



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 5.1. 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 5.2). 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 5.2**. 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 5.3). 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 5.3).

Table 5.3. *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 5.3). 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

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).

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. 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. 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 6.1). 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 6.1. *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 6.1-6.2).

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 6.1 in comparison with those formed by the reference strain.

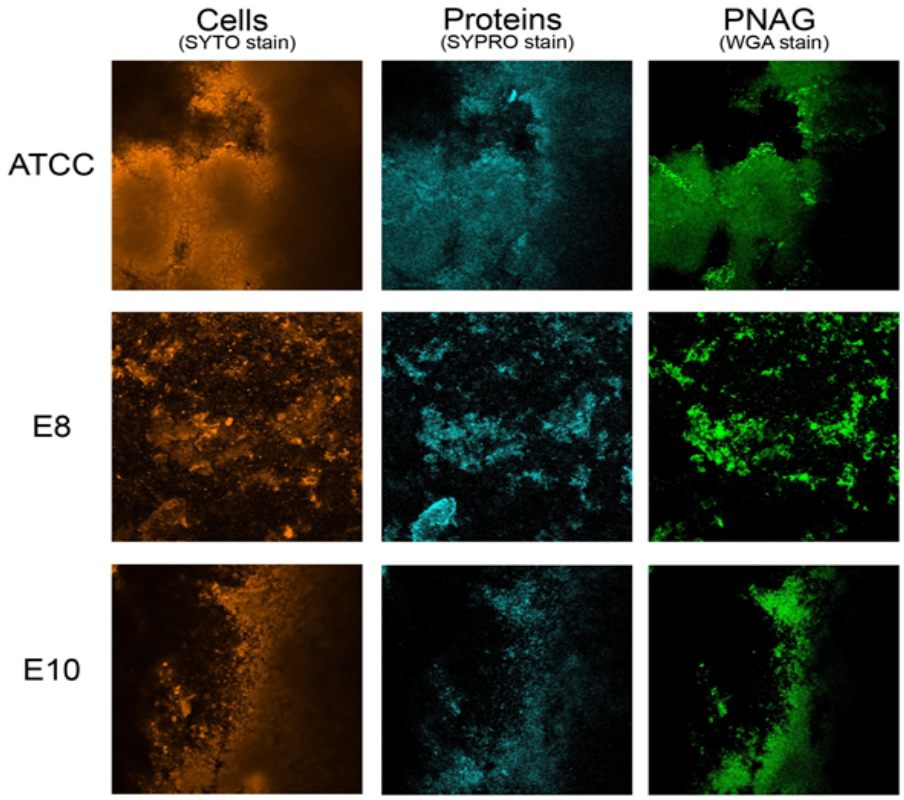


Figure 6.1. Biofilm matrix structure obtained from confocal microscopy observations of *S. aureus* ATCC® 25923, E8 isolated from poultry, and E10 isolated from artificial red caviar. One z-stack is represented for each biofilm

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.

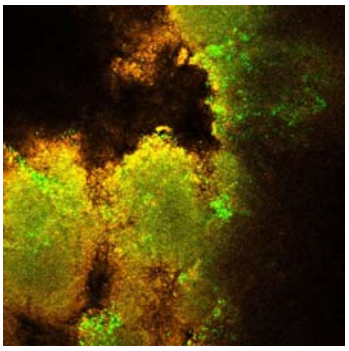


Figure 6.2. Biofilm structure obtained from confocal microscopy observation of *S. aureus* ATCC® 25923, exposed to three types of dyes: SYTO dye for cells, (WGA)-TRITC conjugate for polysaccharides visualization, and SYPRO Ruby for extracellular proteins. One z-stack is represented

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.

CHAPTER 7

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

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.

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 7.1-7.2), 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 7.3).

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.

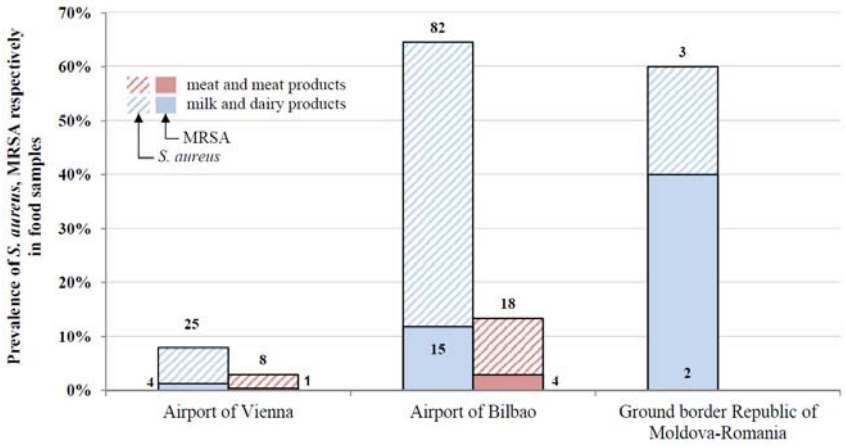


Figure 7.2. Prevalence (%) of *S. aureus* in food samples analyzed. The solid columns represent the number of MRSA positive samples, while the columns with stripes represent the number of *S. aureus* positive isolates

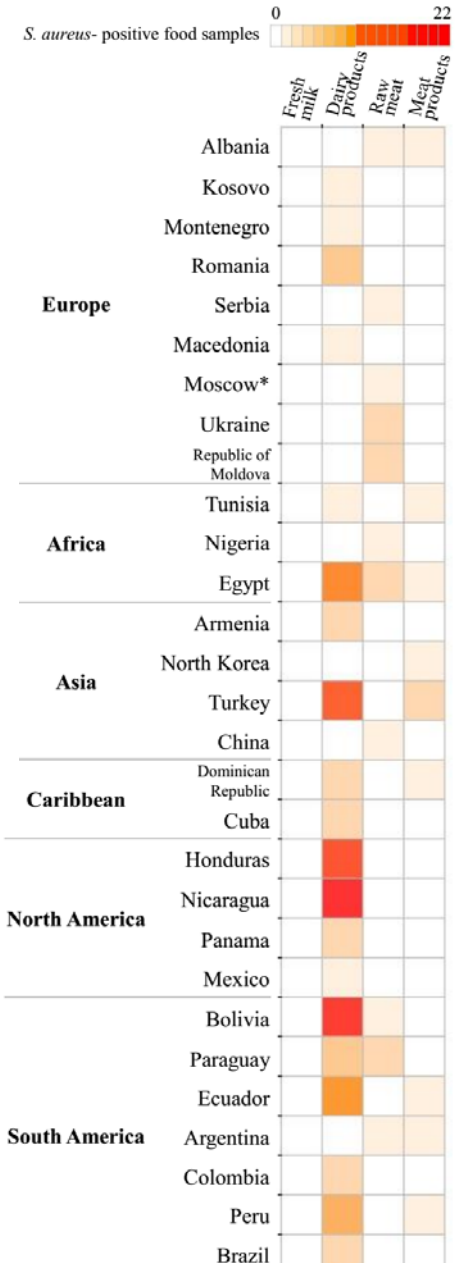


Figure 7.1. Heat map regarding the origin and distribution of *S. aureus*-positive food samples

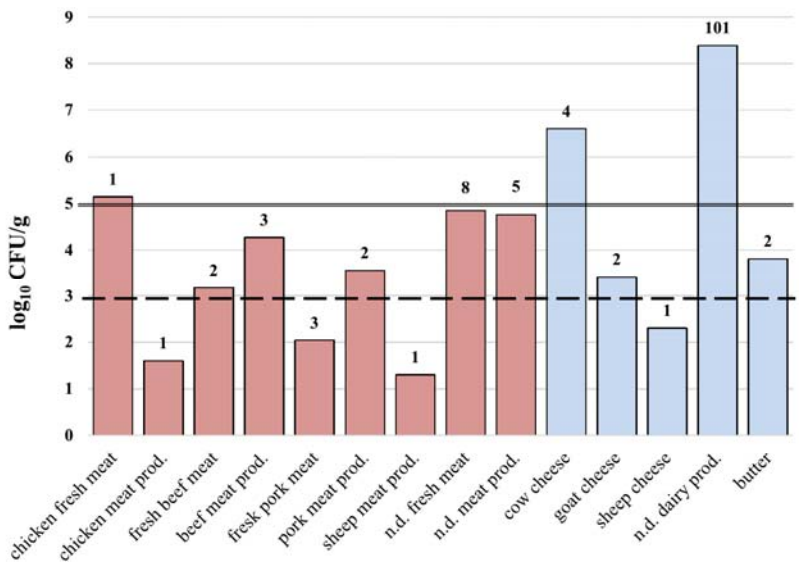


Figure 7.3. *Staphylococcus aureus* counts (log_{10}CFU/g) per food category, type and origin. The number of food samples analyzed per food type are displayed above each column. Lines passing through the columns (- -) and (−) represent the maximum (M) value in the microbiological criteria for raw milk intended for processing and in powdered milk, and M value for cheeses made from raw milk respectively, according to EC 2073/2005. ICMSF recommends 10^3 CFU/g for meat and poultry cooked products as M value (ICMSF, 2011)

Antibiotic profile of the MRSA isolates

Antibiotic susceptibility testing reported 14 resistance profiles (Table 7.1). From all the MRSA strains studied, 16 of them were multiresistant. Moreover, MRSA isolates were sensitive to all non β-lactam antibiotics tested.

Table 7.1. *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 7.2).

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 7.2**. 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)	-

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).

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 7.4). 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).

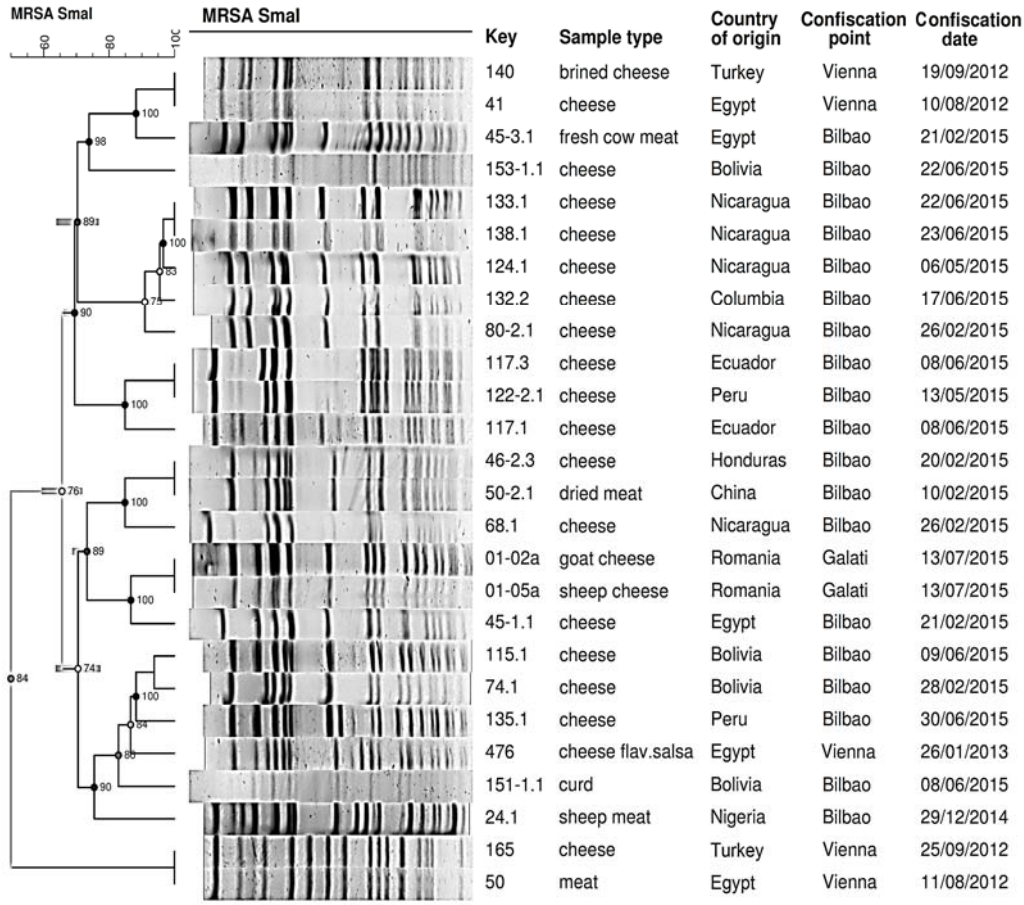


Figure 7.4. Genetic relationship among 26 methicillin-resistant *Staphylococcus aureus* (MRSA) isolates obtained by comparison of pulsed-field gel electrophoresis profiles, using the restriction enzyme *Sma*I. MRSA isolates were observed among a total of 136 food samples positive to *S. aureus*, confiscated 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 7.5) 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.

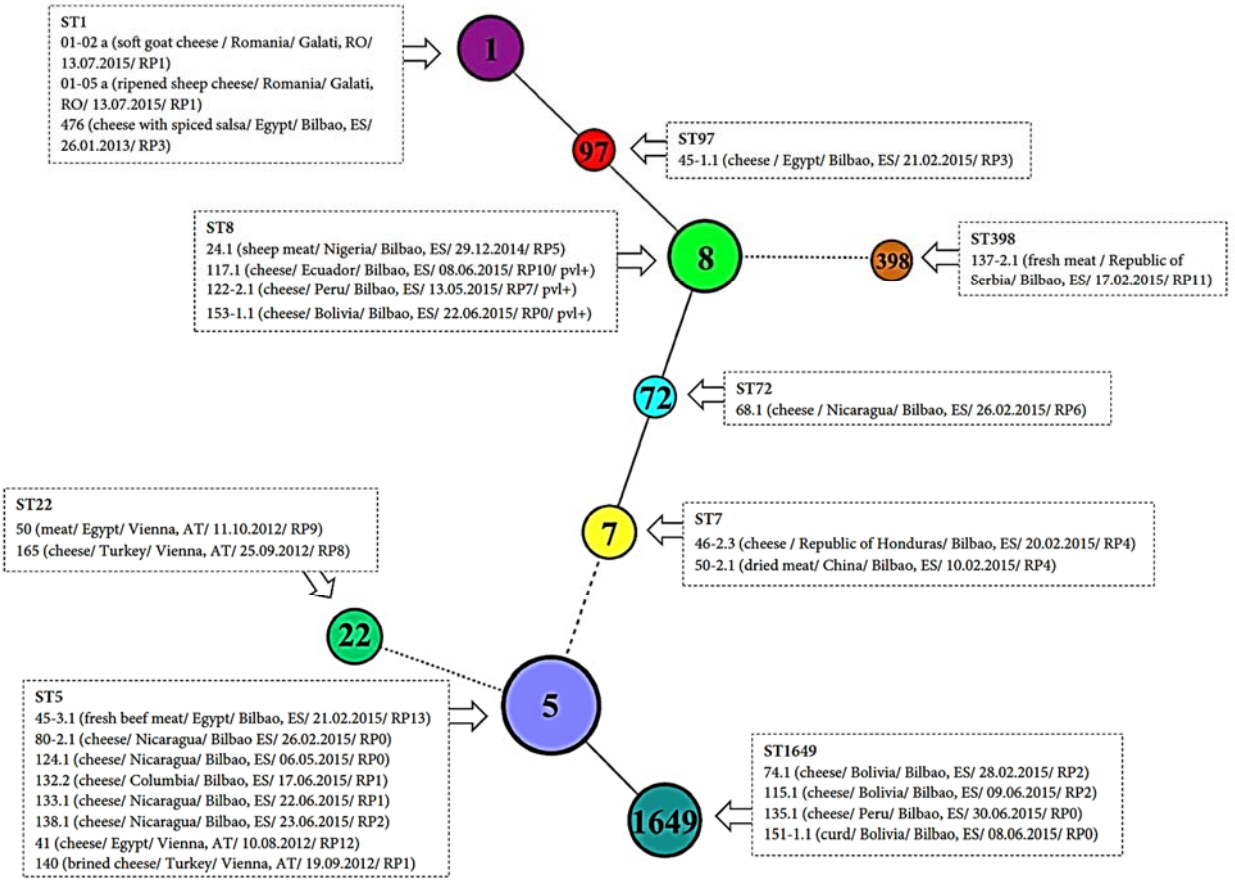


Figure 7.5. Multi-Locus Sequence Typing of 26 MRSA strains isolated from illegally introduced food into the EU. The STs were clustered according to the seven housekeeping genes using a minimum spanning tree (MST). A different randomized color was attributed to each ST. MRSA strains belonging to the same 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 7.1). 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.

Another issue highlighted in our study is the great amount of food samples contaminated with *S. aureus* (Figure 7.2), in which *S. aureus* exceeded the EU microbiological criteria (Figure 7.3) (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 7.3). 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.

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 7.1), 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.

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 7.5). 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

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 aims 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.

Results

Biofilm forming ability of MRSA isolates

Isolates were categorized based on their ability to produce biofilms (Figure 8.1). 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 8.1 describes the summarized results of MRSA isolates from different food sources based of their ability to produce biofilms.

Table 8.1. *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 8.2), 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 8.2. *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	cheese	Nicaragua	IV	5	+	
80-2.2						
80-2.3						
138.1	cheese	Nicaragua	IV	5	+	
138.2						
476	cheese in spicy sauce	Egypt	V	1	+	
124.1	cheese	Nicaragua	IV	5	+	
68.1	cheese	Nicaragua	V	72	+	
68.2						
68.3						
133-1.1	cheese	Nicaragua	IV	5	+	
133-1.2						
133-1.3						
115-1.1	cheese	Bolivia	IV	1649	+	
115-1.2						
115-1.3						

01-05a	maturated sheep cheese	Romania	IV	1	+	
01-05b						
01-02a	soft goat cheese	Romania	IV	1	+	
135.1	cheese	Peru	IV	1649	+	
135.2						
135.3						
45-3.1	fresh beef meat	Egypt	V	5	+	
117.1*	cheese	Ecuador	IV	8	+	+
117.2*						
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	cheese	Bolivia	IV	1649		+
74.2						
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 8.2). Based on these findings, we further characterize MRSA isolates after 48 h of incubation.

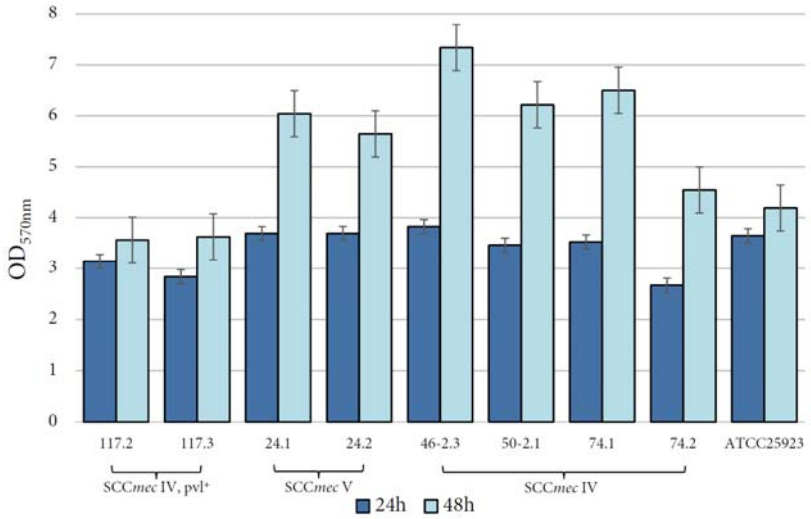


Figure 8.2. Quantification of 24- and 48 h MRSA biofilm biomasses using TSBG. Bars indicate the average of the OD value ± standard deviation (SD) from three independent experiments. Negative samples have been extracted from the shown values

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 8.3).

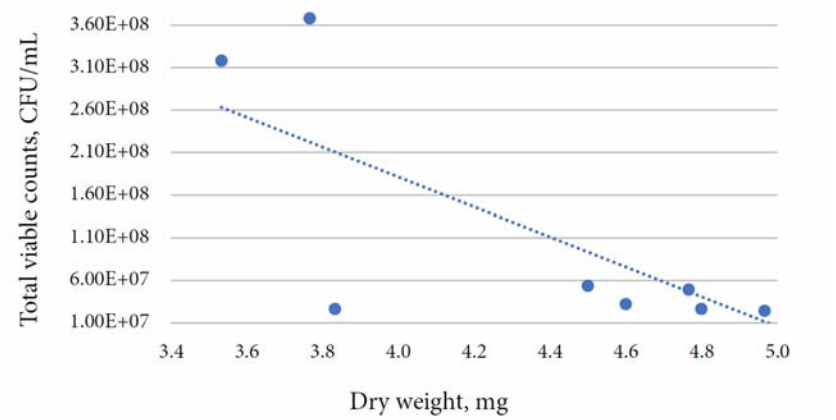


Figure 8.3. Biomass and viable cell quantification of 48 h MRSA biofilms. Values indicate the average ± standard deviation from 3 independent experiments

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.

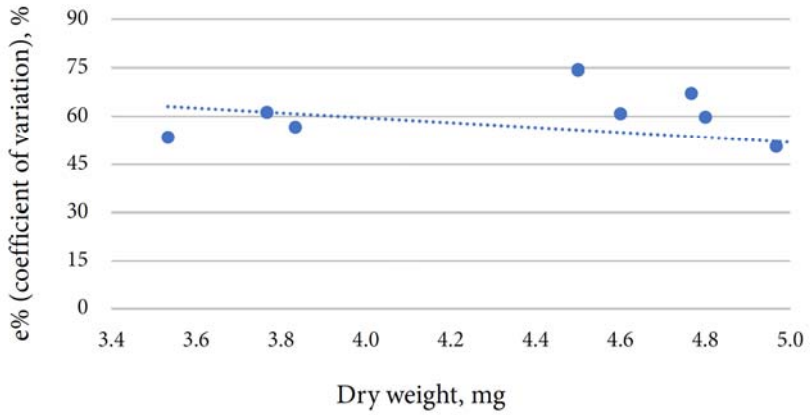


Figure 8.4. Coefficient of variation as a function of biofilm biomass in MRSA isolates

In Figure 8.4 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 8.5. CLSM images differentiate bacterial cells (green) from proteins (red) and PNAG (blue) within the biofilm matrix.

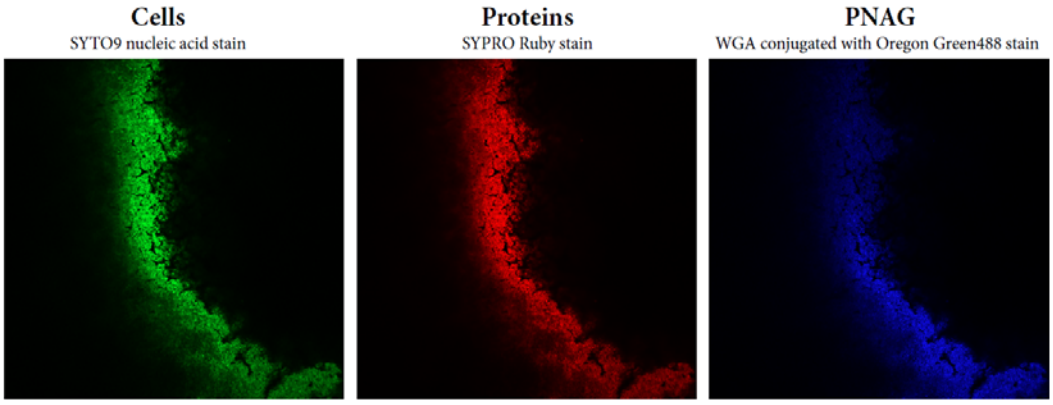


Figure 8.5. Biofilm imaging obtained from confocal observations of MRSA isolate 117.2, with different biofilm components differentiated (cells- left; proteins- center; polysaccharides- right). One z-stack is represented

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 8.6). 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.

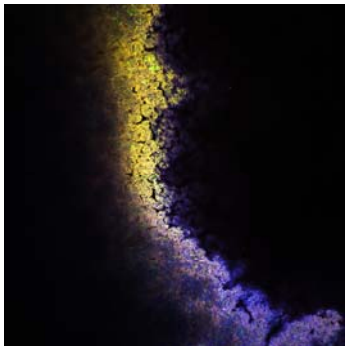


Figure 8.6. Biofilm imaging obtained from 63X confocal observations of MRSA isolate 117.2 stained with all three types of dyes

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).

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).

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.

CHAPTER 9

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

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. 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 *mec* and *bla* regulatory systems 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.

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 9.1).

Table 9.1. *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
*luk*S-PV *& luk*F	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 9.1.

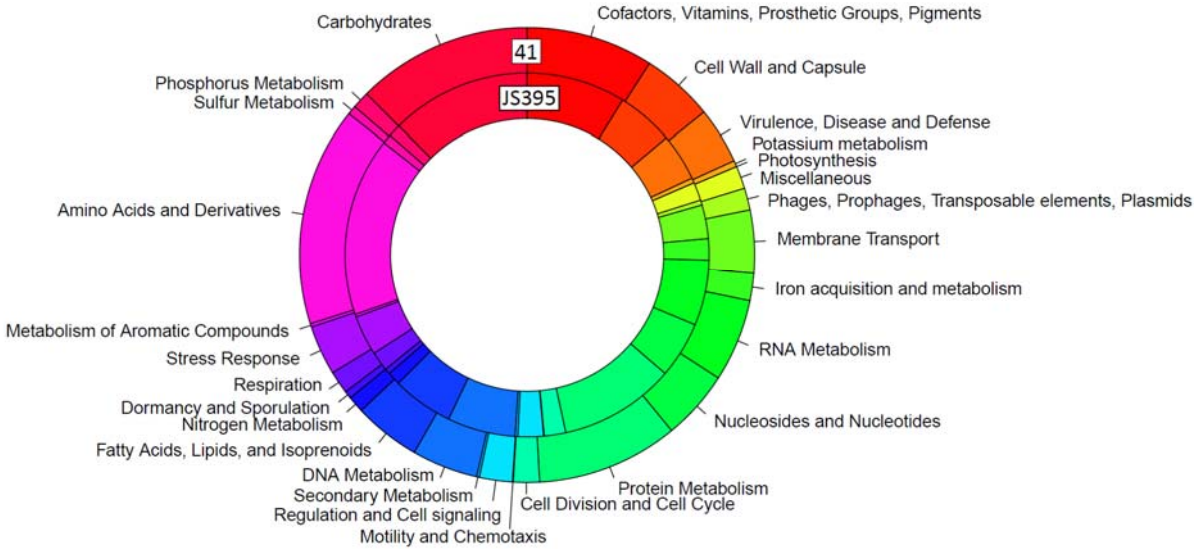


Figure 9.1. Comparison between various subsystems present in the OS-MRSA 41 and JS295 genomes

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.

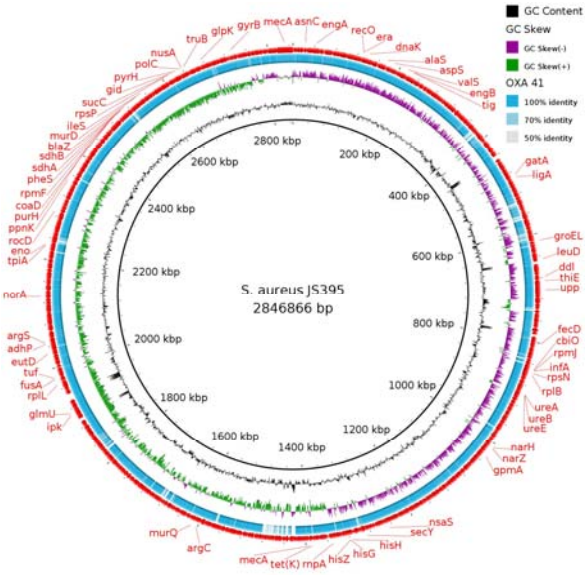


Figure 9.2. Chromosome of OS-MRSA 41

The OS-MRSA 41 genome consists of a circular 2,819,217 bp chromosome size, with a GC content of 32.8% (Figure 9.2). 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 9.3). 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.

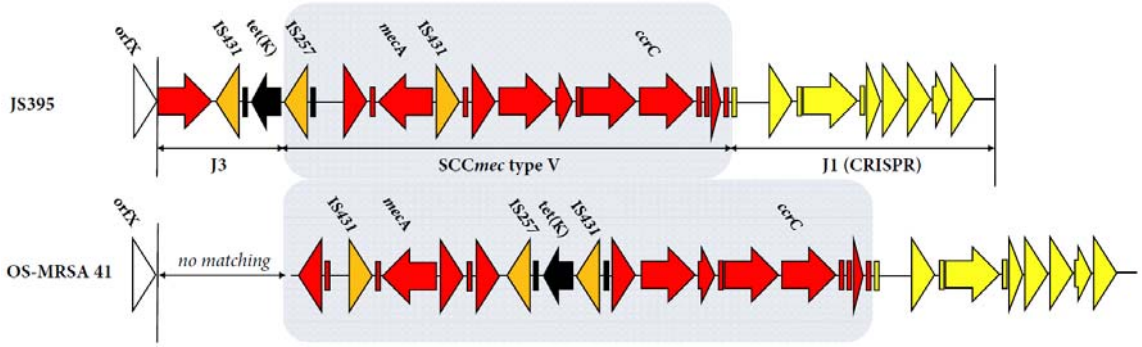


Figure 9.3. Comparative structure analysis of the SCC*mec* element in the *S. aureus* JS395 (DDBJ/ENA/GenBank accession number CP012756) and OS-MRSA 41 strain

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 9.2). 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 9.2 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.

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.

CHAPTER 10

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

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 10.1) 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.

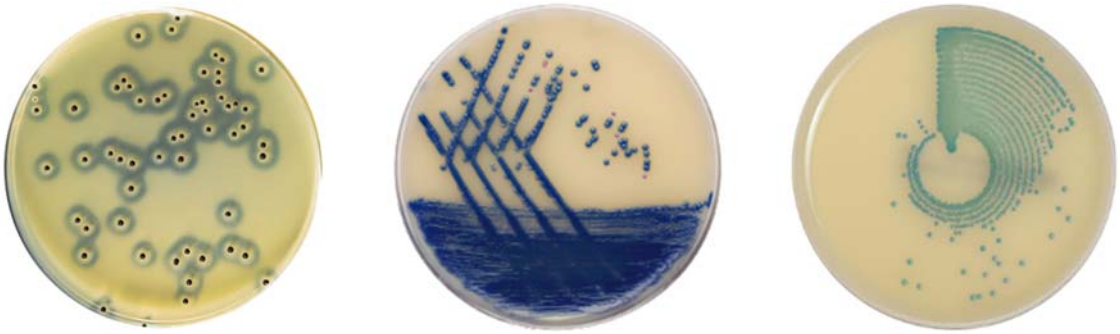


Figure 10.1. Evaluation of chromogenic media for MRSA detection: Baird Parker media (left side), Brilliance MRSA 2 Agar (center) and ChromID MRSA Agar (right side) (www.oxoid.com; www.biomerieux.com)

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 10.1): 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 10.1). Remarkably, both chromogenic media were capable of detecting *mec*C-positive MRSA.

Table 10.1. *Comparative diagnostic performance of Brilliance MRSA 2 Agar and Chrom ID MRSA Agar for detection of animal, human and food isolates of MRSA*

Performance*	Clinical isolates		Animal isolates (n=48)		Food isolates (n=121)	
	Brilliance	*Chrom ID*	*Brilliance*	*Chrom ID*	*Brilliance*	*Chrom ID*
Positive	67	68	4	4	12	12
False negative	1	0	0	0	1	1
Negative	2	2	44	44	96	108
False positive	0	0	0	0	12	0
Sensitivity	98.5	100	100	100	92.3	92.3
Specificity	100	100	100	100	88.9	100
PPV	100	100	100	100	50.0	100
NPV	66.7	100	100	100	99.0	99.1

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

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.

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.

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.

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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