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Methicillin Resistant *Staphylococcus aureus* (MRSA) in raw buffalo milk and its fate along the human gastrointestinal tract: an *in vitro* study

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“Io sono soltanto uno,

ma comunque sono uno.

Non posso fare tutto,

ma comunque posso fare qualcosa;

e il fatto di non poter fare tutto,

non mi fermerà dal fare quel poco

che posso fare.”

Edward Everett Hale

A questo dottorato di ricerca,

che mi ha mostrato quello che voglio e

non voglio diventare

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Abstract

Methicillin resistant *Staphylococcus aureus* (MRSA) has become one of the major public health challenges worldwide. MRSA strains are capable of causing from mild non-life-threatening to severe infections of the skin and soft tissues, and even death. Skin infections caused by MRSA include primary pyodermas such as folliculitis, furuncles, carbuncles, and impetigo. Infections involving the soft tissues include cellulitis and pyo-myositis, which are less common but can cause serious morbidity. The contamination of raw food, especially meat and milk, by MRSA is well documented as well as its transmission to humans *via* animal contact. In addition, humans can act as reservoirs of MRSA without showing any clinical signs, thus they can contaminate foods by handling. Its presence in several kinds of food has suggested the possibility of MRSA to act as a foodborne pathogen. Although the hypothesis is suggestive, there are insufficient evidence to consider the foodstuff a vehicle of MRSA infection. For instance, nothing is known about its ability to survive under human gastroenteric conditions. Despite of the potential hazard for human health there is a lack of date on prevalence of MRSA in some foods, such as buffalo milk and buffalo dairy products. The high consumptions of buffalo drinking milk and dairy products worldwide involve an elevated number of consumers of all ages, and this is crucial considering their potential role in the transmission of foodborne pathogens. To address these issues, the aims of the thesis are: i) to assess the occurrence of MRSA in new ecological niches such as buffalo dairy farms and buffalo tank milk from Italy in order to better understand the epidemiology of MRSA ii) to study the fate of MRSA strains isolated from foods and from humans along the human gastrointestinal tract and its inter-species interaction with the human gut microbiota.

Regarding the occurrence of MRSA in raw buffalo milk, seventy-five bulk tank milk (BTM) samples from farms and 24 nasal swabs from farm workers were collected, respectively. Three (4%) out of 75 BTM samples and 1 (4%) out of 24 nasal swabs were MRSA-positive. The milk isolates showed the following genotypes: ST1/t127/Va and ST72/t3092/V, while the human isolate was characterized as

ST1/t127/IVa. No ST398 were found. All the isolates were multidrug resistant but vancomycin susceptible, carrying the *icaA* gene, while they tested negative for *pvl* and *ses* genes. This study demonstrates for the first time in Europe that MRSA might be present in dairy buffalo farms and in raw buffalo milk. For what concern the second aim, a MRSA ST398/t011/V strain, previously isolated from raw cow milk, and a human origin MRSA strain were inoculated into two foods of animal origin respectively. The pH of the matrices was gradually decreased to 2.0 in 2 hours, during which time they were kept at 37°C and periodically homogenized. The same MRSA strains levels were inoculated within an intestinal in vitro simulator and it was periodically analyzed their fate along the whole transit. Mucin agar carriers replaced the intestinal mucus layer and a basic feed medium represented the intestinal lumen contents. A three-day in vitro study was performed using microbiota from the pooled faeces of healthy individuals that were stabilized simulating colon conditions. The MRSA population survived the decreasing gastric pH levels unharmed, but it was affected by the organic acids produced by the enteric microbiota along the transit into the simulator. It was, in fact, no longer viable after 24 h of incubation with luminal colon microbiota, whereas counts of 4 log cfu/g were still obtained in the mucin agar carriers after 72 h of incubation. Despite the ability of MRSA to overcome human stomach acidic conditions, these results confirm the hypothesis that competitive microbiota may control MRSA intestinal colonization.

Chapter 1. General Introduction

1.1 Zoonoses in the Food-Chain with Public Health Relevance

"Zoonoses are infections or diseases that can be transmitted directly or indirectly between animals and humans by eating contaminated foods or drinking water (foodborne/waterborne zoonotic diseases) or through contact with infected animals" (EFSA, 2014). A foodborne disease outbreak occurs when two or more people develop a similar illness after ingesting the same contaminated food or drink (WHO, 2008).

The contamination of food can occur at any point along the food chain: at farm level, at slaughter, during food processing or preparation (Tab 1). It can also occur at home if food is incorrectly handled or undercooked (EFSA, 2019).

Farms	**Slaughterhouses**	**Food industries**	
		During processing	**During Preparation**
Animal feed contaminated with bacteria that cause infections in animals	Meat contaminated by intestinal contents	Microorganisms present in other raw agricultural products (cross-contamination) or on food contact surfaces (poor sanification)	Improper use of utensils or kitchen surfaces, which can contribute to the spread of bacteria
Parasites that infect food-producing animals		Food handled by infected people (post-process contaminations)	
Milk contaminated through contact with faeces or environmental dust			
Animal skin and fur contaminated by faeces and environment			

Table 1 Main sources of food contamination. EFSA, 2019

Microorganisms in food might determine 2 types of foodborne diseases:

- Food Infections: occur after the ingestion of food contaminated by live bacteria or other microbes which grow and, once reached the intestine, multiply, causing local damage (enteroinvasive infection);
- Food Intoxications: are caused by eating food containing toxins pre-formed by bacteria which resulted from the bacterial growth in the food items before the ingestion. The intoxication happens in presence of the bacterial toxins even when the microorganism is no longer present.

Contaminated foods commonly cause gastroenteritis, which is the infection and inflammation of the digestive system (Lamps, 2007). For many people, symptoms (such as abdominal pain, diarrhoea and vomiting) settle within few days; however, some people, particularly the very young, the elderly, pregnant women, and people with underlying health problems or a weakened immune system (Y.O.P.I. categories), may experience more severe disease and complications, including death (WHO, 2019). Although, more than 250 agents, including germs (such as bacteria, viruses, and parasites) and chemicals (such as ciguatoxin) are known to cause foodborne illness (CDC, 2019), most foodborne diseases are caused by *Campylobacter, Salmonella, Listeria,* pathogenic *E. coli* and *Yersinia* (*Tab 1*) (EFSA, 2018).

Bacteria	*Campylobacter* spp., *Salmonella* spp., *Listeria monocytogenes*, pathogenic *Escherichia coli, Yersinia enterocolitica.*
Bacterial toxins	Toxins of *Staphylococcus aureus, Clostridium perfringens, Clostridium botulinum* and *Bacillus cereus*
Viruses	*Calicivirus* (including norovirus), *Rotavirus, Hepatitis A virus, Hepatitis E virus*
Parasites	*Trichinella* spp., *Toxoplasma gondii, Cryptosporidium* spp., *Giardia* spp.

Table 2. The most common causes of foodborne disease. EFSA, 2019.

Data collected during the 2017 from the European Food Safety Authority (EFSA) and the European Centre for Disease Prevention and Control (ECDC) including 37 European countries (28 Member States and nine non-member-states) showed that on a total amount of 5,079 foodborne (including waterborne) outbreaks, the bacterial agents are the most frequently (34.3 %) implicated causative agents (EFSA, 2018). The foodborne outbreaks by bacterial agents were predominantly associated with *Salmonella, Campylobacter* and enterohaemorrhagic *Escherichia coli*; while the foodborne outbreaks by bacterial toxins (16.1% of all outbreaks) were predominantly associated with *Clostridium perfringens*, *Bacillus cereus* and *Staphylococcus aureus* toxins (EFSA, 2018).

Although the statistically significant increasing trend of confirmed listeriosis cases in the Europe during the period 2013–2017, foodborne outbreaks by *Listeria monocytogenes* were reported only in 6 member states during the 2017 (EFSA, 2018), with a long-lasting outbreak of listeriosis linked to cold-smoked salmon and a large multi-country outbreak of invasive listeriosis (serogroup IVb) linked to frozen vegetables (EFSA, 2018). Other bacterial agents were less reported as causative agent for foodborne outbreaks by the investigated member states, during the 2017.

1.1.1 The Bacterial Pathogens

***Campylobacter* spp.** *Campylobacter* is the most common cause of gastroenteritis in many developed and developing countries (Nichols *et al.,* 2012; WHO, 2013).

While there are 17 species in the *Campylobacter* genus, human campylobacteriosis is primarily caused by *Campylobacter jejuni,* with 170 reported outbreaks in the Europe, while *C. coli* was reported in 14 outbreaks only (EFSA 2018). Diseases in humans might be from mild (gastroenteric symptoms) to severe (20-40% of cases of Guillian-Barrè syndrome) (WHO, 2011; Tam *et al.,* 2009; CDC, 2012). Although the main route of transmission from animals to humans is through undercooked meat and meat products, contaminated raw milk, shellfish, or contaminated water (WHO, 2011), the focus for intervention is the poultry meat food chain because of the importance of commercially-produced poultry as a source of infections (EFSA BIOHAZ 2011).

***Salmonella* spp.** Salmonellosis is one of the most common foodborne diseases in Europe (1,241 foodborne outbreaks affecting 9,600 people in 25 member states) (EFSA 2018).

It is caused by different serovars of the species *Salmonella enterica* (Rausch *et al.,* 2015). The top five most commonly reported serovars in human cases acquired in the EU during 2017 were, in decreasing order*: S*. Enteritidis, *S.* Typhimurium, monophasic *S*. Typhimurium, *S*. Infantis and *S.* Newport (EFSA, 2018).

S. Enteritidis is attaining major public health significance because it is dominantly infecting humans, transmitted by food (61.1% of all *Salmonella* foodborne outbreaks); while *S.* Typhimurium including monophasic variants was reported in 7.9 % of Salmonella foodborne outbreaks (EFSA, 2018).

Food could be contaminated by Salmonella serovars due to faecal contamination during the course of food processing or because of infected animals (ECDC, 2013).

The most common vehicles of transmission are meat, meat products (poultry, pork, cattle), eggs, egg products, fruits and ready-to-eat vegetables, and seafood.

Pathogenic *Escherichia coli*. The gastrointestinal tract of humans and other warm-blooded animals are the primary hosts of *Escherichia coli* as part of the normal microflora (Cheleste *et al.,* 2002; Bell, 2002). Although most of these microorganisms are non-pathogenic, some strains have evolved as pathogenic (Mainil, 2013; Bell, 2002).

Based on their virulence traits, pathogenic *E. coli* are categorized into at least six groups: enteropathogenic *E. coli* (EPEC), enteroaggregative *E. coli* (EAEC), enterotoxigenic *E. coli* (ETEC), enteroinvasive *E. coli* (EIEC), enterohaemorrhagic *E. coli* (EHEC), and diffusely adherent *E. coli* (DAEC) (Bell, 2002; Catalina Lopez-Saucedo *et al.,* 2003).

Shiga toxin-producing *E. coli* (STEC), also known as Verocytotoxin-producing *E. coli* (VTEC) are diverse EHEC pathotypes that have become of significant worldwide public health concern (Bell 2002).

During 2017 foodborne outbreaks associated with infection by pathogenic *E. coli* (n=37) including EAEC, ETEC, EIEC, EPEC, and STEC were reported (EFSA 2018), with the latter as the most reported causative agent with 50% of all outbreaks (including waterborne outbreaks) reported in Ireland (EFSA 2018). As in previous years, the most commonly reported STEC serogroup in confirmed cases of human STEC infections as well as the most frequently reported cause of haemolytic uremic syndrome cases in EU in 2017 was O157 (31.9%), followed by O26, O103 and O91 (EFSA 2018).

The most common vehicles of transmission involved in the foodborne outbreaks reported in 2017 were meat and meat products (especially bovine meat), milk, cheese and dairy products (other than cheeses), and vegetables (EFSA, 2018).

Listeria monocytogenes. The large majority of listeriosis cases (sporadic and outbreak-related) are caused by foodborne transmission, which accounts for 99% of human cases (Scallan *et al.,* 2011). Listeria infections were most commonly reported in the elderly population in the age group over 64 years and particularly in the age group over 84 years (EFSA, 2018). Investigation of several outbreaks has demonstrated that all epidemic listeriosis was caused by foodborne transmission of *Listeria monocytogenes*, which may be present in a wide range of retail foods (Schuchat *et al*., 1992; Pinner *et al.,* 1992; Allerberger *et al.,* 2015).

Outbreaks of listeriosis have been associated with the ingestion of raw milk, soft cheeses, and contaminated vegetables (Allerberger *et al.,* 2015). Also, evidence from EU-wide routine food safety investigations indicates that a substantial proportion of ready-to-eat products is contaminated by *L. monocytogenes* (EFSA, 2012).

In 2017, on 39 human cases of listeriosis, *L. monocytogenes* occurrence was highest in fish and fishery products (6%) followed by RTE salads (4.2%), RTE meat and meat products (1.8%), soft and semi-soft cheeses (0.9%), fruit and vegetables (0.6%) and hard cheeses (0.1%) (EFSA, 2018).

1.1.2 The Bacterial Toxins

Clostridium perfringens. First isolated and identified as a novel bacterium (the original name *Bacillus aerogenes capsulatus*) in 1891 by William H. Welch in infected blood vessels (Kiu and Hall, 2018), *Clostridium perfringens* has been associated with intestinal diseases in both animals and humans throughout the past century. *C. perfringens* strains are known to secrete more than 20 identified toxins or enzymes that could potentially be the principal virulence factors involved in pathophysiology (Revitt-Mills *et al.,* 2019). In fact, it has clinically been associated with various significant systemic and enteric diseases, in both humans and animals, including gas gangrene (Clostridial myonecrosis), food poisoning and non-foodborne diarrhoea, and enterocolitis (Sim *et al.,* 2017; Heida *et al.,* 2016). The microorganism is associated with diverse environments including soils, food, sewage, and as a member of the gastrointestinal (GI) tract microbial community of humans and animals (Kiu and Hall, 2018). The strains of *C. perfringens* are usually classified into 5 toxin types (A, B, C, D and E) according to the main toxins produced, and only the enterotoxigenic strains are responsible for food poisoning (Brunestad and Granum, 2002). The main foods incriminated in *C. perfringens* foodborne illness outbreaks are cooked dishes prepared in advance and large quantities (EFSA, 2005); in fact, raw materials are usually only slightly contaminated well below the threshold presenting a risk of poisoning (10^5/g). Cooking conditions and subsequent storage of prepared food are determinant factors in the change in the level of contamination (Brunestad and Granum, 2002). The most typical example is, during collective catering, the meat in gravy, cooked in large volumes and in advance, which has not been cooled rapidly enough between preparation and serving (EFSA, 2005).

Bacillus cereus. *Bacillus cereus* causes two different types of food poisoning: the diarrhoeal and the emetic types (Granum *et al.,* 1997). The diarrhoeal type is caused by complex enterotoxins produced during the vegetative growth of the microorganism in the small intestine (Beecher *et al.,* 1997), and

the emetic one is caused by the emetic toxins produced by growing cells in foods (Lund and Granum, 1997; Granum, 1994). Food usually implicated in both types of foodborne outbreaks has usually been heat treated and the surviving spore are responsible for the food poisoning (Kramer and Gilbert, 1989). The dominating type of disease caused by *B. cereus* differs from country to country. In Europe the diarrhoeal type is the most frequently reported (Kramer and Gilbert, 1989; EFSA, 2018).

Staphylococcus aureus **and staphylococcal food poisoning** The association between *Staphylococcus aureus* and food poisoning was firstly described by Vaughan in 1884 in Michigan (USA) (Vaughan, 1884; Hennekinne *et al.,*2012); thirty years later M.A. Barber confirmed the role of *S. aureus* as possible source of food intoxication by demonstrating that the consumption of milk from a cow suffering staphylococcal mastitis was able to cause food poisoning (Barber, 1914). Finally, Dack *et al.* (1930) demonstrated that a heat-resistant toxin and not the microorganism itself was responsible for the poisoning (Dack *et al.,*1930).

Since the growth of microorganism in the host is not required, this kind of food poisoning is considered an intoxication. Although *S. aureus* is not the only species within its genus to produce Staphylococcal Enterotoxin(s) (SE(s)), it is the main source of staphylococcal foodborne outbreaks (Seo and Bohach, 2013; Le Loir *et al.,* 2003; Hennekinne *et al.,* 2012). To date, 24 different SEs have been described, based on sequence homology (Hennekinne, 2018). Five of them have been fully characterized: SEA, SEB, SEC (presenting 5 variants - C1, C2, C3, SEC ovine and SEC bovine), SED, and SEE. These "classical" SEs are characterized by superantigenic and emetic activities (Hennekinne, 2018). Although heat-treatment (e.g., cooking) easily kill *S. aureus* bacteria, SEs are resistant to heat, freezing and irradiation (Hennekinne *et al*., 2012).

Foods most commonly involved in foodborne outbreaks are rich in proteins, and some examples are meat and meat products, poultry and egg products, milk and dairy products, ready-to-eat (RTE) products, salads, and bakery products, reflecting the different food habits among countries (Seo and Bohach, 2013; EFSA, 2015, 2016, 2017, 2018).

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

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Chapter 2. Methicillin resistant *Staphylococcus aureus* (MRSA)

2.1 Antimicrobial Resistance and Food Chain

Antimicrobial resistance (AMR) is the ability of a microorganism (like bacteria, viruses, and some parasites) to stop an antimicrobial (such as antibiotics, antivirals and antimalarials) from working against it, as a result, standard treatments become ineffective, infections persist and may spread to others (WHO, 2018). The contribution of food sources to the burden of AMR in humans is another important issue in the AMR global crisis, considering that foods contaminated by antimicrobial-resistant bacteria may contribute to the spread of these microorganisms within the human population as well as to a rapid transfer of resistance genes from foodborne commensals to human pathogens (Robinson *et al.,* 2016). Bacteria may be intrinsically resistant to a class of antimicrobial agents (natural resistance) or may acquire resistance by *de novo* mutation or *via* the acquisition of resistance genes from other organisms (acquired resistance); it depends on physiological or anatomical characteristics of the bacteria, the structure of the organism, the characteristic of antibiotic (Tenover, 2006). The natural AMR is a constitutive resistance: the microorganisms are deprived of the targets on which antibiotics act and it is usually a trait shared by all organisms within the same genus or species (Courvalin, 2008).

The acquired AMR can develop:

- Spontaneously: (chromosomal) from a random mutation of the genetic material of the microorganism that makes it resistant to a certain type of antimicrobial. In presence of that antibiotic, sensitive bacteria do not grow while the "mutants" grow. This kind of resistance can be transfer between bacteria by vertical transmission (Tenover, 2006).
- By acquisition of resistance genes directly from other microorganisms (extrachromosomal): the resistance genes are located in pieces of DNA that can be transferred between the various bacteria (horizontal gene transfer). Acquisition of new genetic material by antimicrobial-susceptible bacteria from resistant strains of bacteria may occur through conjugation,

transformation, or transduction, with transposons often facilitating the incorporation of the multiple resistance genes into the host's genome or plasmids. These mobile genetic elements (plasmids) often contain clusters of genes that may also harbour multiple resistance factors, conferring to the receiver micro-organism multiple resistance's characteristics (Tenover, 2006).

The modern era of antibiotics started with the discovery of penicillin by Sir Alexander Fleming in 1928 (Ventola, 2015). Since the introduction in 1937 of the first effective antimicrobials (the sulphonamides) the development of specific mechanisms of resistance has plagued their therapeutic use (**Fig 1**) (Davies and Davies, 2010).

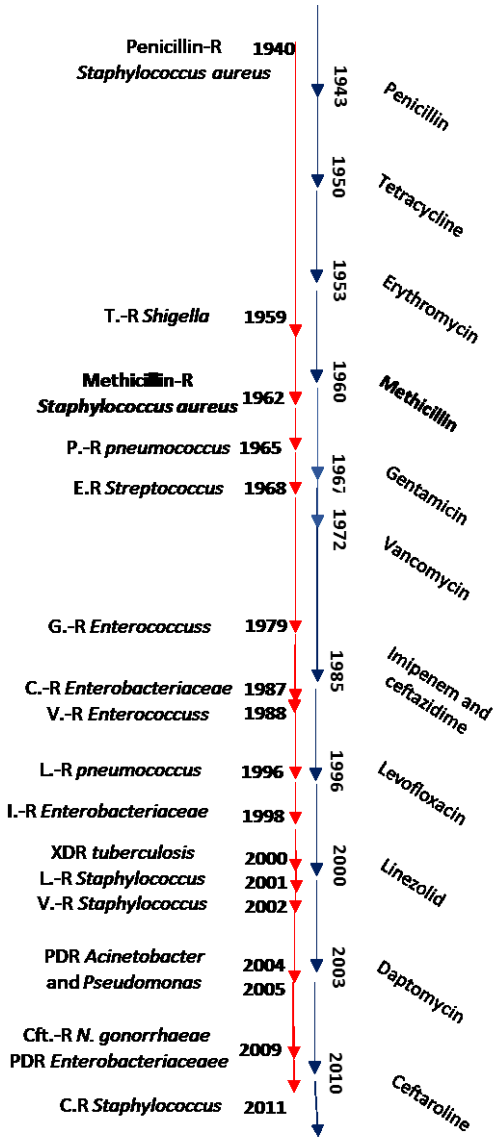

Figure 1. Antimicrobial resistance Timeline. Drawn by Elisa Spinelli, from Ventola et al. (2015).

That's the case of penicillin, which was successful in controlling bacterial infections during the World War II (Sengupta *et al.,*2013), but shortly thereafter, penicillin resistance became a substantial clinical problem (Spellberg and Gilbert, 2014). In response, new semi-synthetic penicillin resistant to beta-lactamases (such as methicillin, ampicillin, carbenicillin, amoxicillin) were discovered (Sengupta *et al.,*2013; Spellberg and Gilbert, 2014). However, in the same decade in United Kingdom (1962) and in the United States (1968) the first case of methicillin-resistant *Staphylococcus aureus* (MRSA) infection was identified (CDC, 2013). In 1972 vancomycin was introduced into clinical practice for the treatment of methicillin resistance in both *S. aureus* and coagulase-negative staphylococci

(Ventola, 2015), unfortunately few years later, some cases of vancomycin resistance were reported in coagulase-negative staphylococci (Sengupta *et al.,*2013). The introduction of a large number of new antibiotics to solve the resistance problem, restored confidence from the late 1960s through the early 1980s (Ventola, 2015). Unfortunately, resistance has eventually been seen to nearly all antibiotics that have been developed (CDC, 2013). As a result, over the past several decades, the over prescription of antibiotics in human medicine and the overuse or misuse of antimicrobials in human and veterinary medicines has leading to an increase in the number and types of antimicrobial resistant microorganisms (Mehndiratta, 2014) and bacterial infections have again become a public health threat (Ventola, 2015).

The cost of AMR is very high, with about 700 thousand of annual deaths related to antimicrobial resistant bacterial infections, of which 33 thousand only in the Europe, and a cost of hospitalization of 1.5 bn euros per year (Cassini *et al.,* 2019). Recent reports predict drug resistance will increase substantially, causing millions of extra deaths and costing trillions of dollars by mid-21st century, worldwide (Grace, 2015).

The prevalence of resistance varies between geographical regions and over time, and the massive increases in trade and human mobility brought about by globalization have enabled the rapid spread of infectious agents, including those that are drug resistant (Cassini *et al.,* 2019). Even if AMR is a biological phenomenon of the evolutionary adaptation in response to selective pressures in environments where antibiotics are heavily used (Toprak *et al.,* 2012; Hiltunen *et al.,* 2017), the misuse and overuse of antimicrobials will accelerate this process. This problem involves three levels (risk categories): the veterinary field, with loss of efficacy of antimicrobials for therapeutic use; the livestock sector, with loss of effectiveness of auxinic resulting in loss of productivity; public health, with loss of effectiveness of therapeutic treatments as a result of transmission of single or multiple resistance between animal and human microorganisms (Woolhouse *et al.,* 2015). AMR was firstly studied in human medicine when the severity and spread of hospital-acquired (nosocomial) infections, often supported by multi-resistant microorganisms, became apparent (Struelens, 1998). Clearly,

hospitals facilitate the spread of antimicrobial resistance for many reasons, including the presence of people with bacterial infections, the need to manage a large volume of contaminated materials, intrusive medical procedures, immunocompromised persons, the level of use of antibiotics and so on (Silbergeld, 2008). The combination of highly susceptible patients, intensive and prolonged antimicrobial use, and cross-infection has resulted in nosocomial infections with highly resistant bacterial pathogens such as multi resistant Gram-negative rods, most commonly *Klebsiella* species, *Enterobacter* species, and *Pseudomonas aeruginosa*; vancomycin resistant enterococci (VRE), and Gram positive cocci, such as hospital acquired methicillin resistant *Staphylococcus aureus* strains (HA-MRSA), which have become predominant over the past two decades (CDC, 2019). Among gram-positive pathogens, a global pandemic of resistant *S. aureus* and *Enterococcus* species currently poses the biggest threat (CDC, 2013; Rossolini *et al.*, 2014)

This trend is related to these pathogens' capacity for accumulating antibiotic resistance determinants (Gould, 2013). Some of these resistant strains have now spread outside the hospital causing infections in the community (WHO, 2001).

The use of antimicrobials outside the field of human medicine also has an impact on human health; thus, the risks of becoming infected by a resistant pathogen are higher in hospitals, but the source of resistance is greater outside the hospital, largely related to the size of the animal reservoir of resistance (Silbergeld, 2008).

The extensive literature on the prevalence of AMR in both commensal and pathogenic bacteria in food animal production, establishes a link between the use of antimicrobials in food-producing animals and aquatic species and the emergence of resistance among common pathogens.

Such resistance has an impact on animal health and on human health if these pathogens enter the food chain (Landers, 2012). Factors associated with the emergence of antimicrobial resistance in food-producing animals and the farming industry appear to be similar to those responsible for such resistance in humans (Mehndiratta, 2014). Oral medication of large groups of animals results in the exposure to frequently subtherapeutic concentrations of antimicrobials, and it's particularly likely to

favour emergence of and selection for AMR (Knobler, 2003). The frequent use of antimicrobials in commercial poultry production has raised concerns regarding the potential impact of antimicrobials on human health due to selection for resistant bacteria. Several studies have reported similarities between extraintestinal pathogenic *Escherichia coli* (ExPEC) strains isolated from birds and humans, indicating that these contaminant bacteria in poultry may be linked to human disease (Koga, 2015).

The potential threat to human health resulting from inappropriate antibiotic use in food animals is significant, as pathogenic-resistant organisms propagated in these livestock are poised to enter the food supply and could be widely disseminated in food products. Commensal bacteria found in livestock are frequently present in fresh meat products and may serve as reservoirs for resistant genes that could potentially be transferred to pathogenic organisms in humans (Landers, 2012).

Cross-contamination with AMR bacteria resulting from improper handling of food is a well-known phenomenon and has been widely studied (Kusumaningrum, 2003; Mylius, 2007).

2.2. *Staphylococcus aureus:* general description

Staphylococcus aureus belongs to genus *Staphylococcus*, family *Staphylococcaceae*, order Bacillales, class Bacilli, phylum Firmicutes (Schleifer and Bell, 2009; Foster and Geoghegan, 2015). Although fossil evidence suggests that Staphylococci have existed on earth for more than a billion years (Moellering, 2011), *S. aureus* was actually first described as bacterial pathogens in 1880 by the Scottish surgeon Alexander Ogston (Ogston, 1882).

Genus *Staphylococcus* comprises more than 50 species and subspecies that are divided into two groups, based on the ability to clot blood plasma by the action of the enzyme coagulase: coagulase-positive staphylococci (CoPS), and coagulase-negative staphylococci (CoNS). *S. aureus* belongs to CoPS and is the major pathogen within the genus (Harris *et al.,* 2002; Foster, 2009).

S. aureus is a Gram-positive, facultative anaerobic, non-motile, catalase and coagulase positive, oxidase-negative spherical microorganism that does not form spores. On microscopic examination, it

appears in pairs, short chains, or bunched in grape-like clusters of cells, as suggested by its name from Greek words *staphyle* (a bunch of grapes) and *coccus* (grain or berry) (Ogston, 1882).

S. aureus is a ubiquitous microorganism and it can be found in the air, soil, water, sand, dust, sewage, vegetal (Grace and Fetsch, 2018). In fact, although it is not a spore-forming microorganism, it is able to survive to different environmental conditions, such as dry conditions, high salt concentrations and it can grow at a wide range of temperatures (6-48 °C) (Jay *et al.,* 2005; Hennekinne *et al.,* 2012).

The main habitat for such microorganisms is the skin and upper respiratory tract of many warm-blooded animals (Grace and Fetsch, 2018). It is often present asymptomatically on parts of the human body such as skin, skin glands, and mucous membranes, including noses and gut of healthy individuals (Wertheim *et al.,* 2005). Studies have shown that about 20% of individuals are persistent nasal carriers and around 30% are intermittent carriers of *S. aureus* (Williams *et al.,* 1959; Wertheim *et al.,* 2005). This colonization therefore significantly increases the chances of infections by providing a reservoir of the pathogen; in fact, when it has the opportunity (*e.g.* in presence of damaged skin or mucosal membranes), *S. aureus* can cause a wide variety of infection, since many virulence factors enables it to overcome the host immunity defence and to invade and colonize tissues (Foster, 2009; Lakundi and Zang, 2018). Staphylococcal infections include infection of superficial soft tissue and skin, such as pimples, boils and abscesses, but also severe systemic infections, i.e. endocarditis, bacteremia, pneumonia and toxic shock syndrome (Otto, 2012). *S. aureus* strains may produce a large variety of toxins: cytolitic, including leukocidins, alpha-toxin and phenol-soluble modulins. Further important toxins are the staphylococcal superantigens, which include toxic shock syndrome toxin (TSST-1) and lead to exacerbate immune response by polyclonal T cell activation and massive cytokine release (Otto, 2013). Virulence factors are encoded in phages, plasmids, pathogenicity islands, and in the staphylococcal cassette chromosome.

The colonization and invasion of host tissues are mediated by the production of a variety of molecules, known as microbial surface component recognizing adhesive matrix molecules (MSCRAMMs) (Foster and Höök, 1998; Bien *et al.,* 2011). These surface associated adhesins include (**Fig. 2**):

- Fibronectin binding proteins (FnBPs): FnBA and FnBB are involved in the attachment of *S. aureus* to fibronectin and plasma clot (Ton-That *et al.,* 2001);
- Collagen binding proteins (Cna): Cna is responsible for adherence of *S. aureus* to collagenous tissues and cartilage (Ton-That *et al.,* 2001);
- Clumping factor proteins (Clf). ClfA and ClfB mediate clumping and adherence to fibrinogen, in presence of the fibronectin (TonThat *et al.,* 2001);
- Staphylococcal protein A (Spa): Spa is able to bind immunoglobulins (especially IgGs), inhibiting opsonization and phagocytosis (Ton-That *et al.,* 2001).

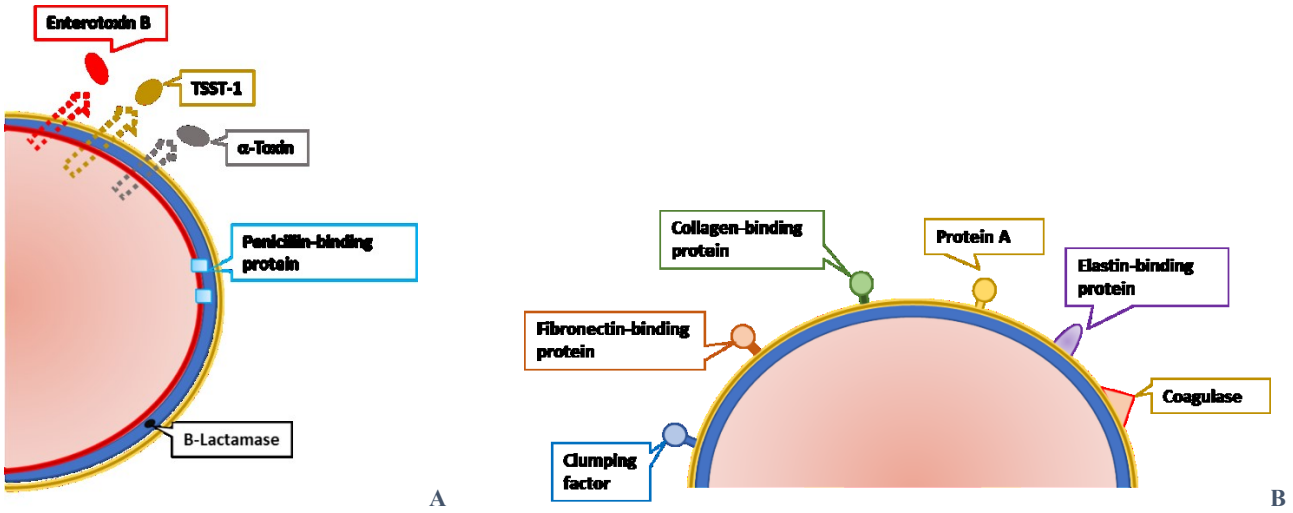

Figure 2. Pathogenic factors of *S. aureus* (A: cross-section of the cell envelope and secreted proteins; B: surface proteins) playing roles as virulence factors. Drawn by Elisa Spinelli, from From Vatansever *et al.* (2013).

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

The production of virulence factors is regulated by a series of genes coding for global regulator molecules, including:

- accessory gene regulator (*agr*). *agr* is a two-component system which has a crucial role in pathogenesis; in fact, it is involved in the bacterial cell to cell communication mechanism of

Quorum Sensing (QS), on virulence factors production and biofilm formation (Singh and Ray, 2014; Kavanaugh and Horswill, 2016).

- staphylococcal accessory regulator (*sar*A). *sar*A is involved in the activation of *agr* expression, in the synthesis of some MSCRAMMS and toxins, and in the inhibition of the expression of *spa* and proteases (Cheung *et al.,* 2008)
- sigma factor B (*Sig*B). *Sig*B is responsible for the transcription of genes that can confer resistance to heat, oxidative and antibiotic stresses (Bischoff *et al*., 2004; Hecker *et al.,* 2007).

S. aureus toxins play an important role in the pathogenicity of this microorganism, since they are able to damage biological membranes, leading to cell death (Otto, 2014). These include:

- haemolysins: *S. aureus* lysis of red blood cells is primarily mediated by the haemolysins known as alpha (α), beta (β) and delta (δ) toxins (Burnside *et al.,* 2010). The α-toxin, encoded by the *hla* gene, is important for *S. aureus* pneumonia, sepsis, septic arthritis, brain abscess and corneal infections (Bubeck *et al.,* 2017; Kielian *et al.,* 2001; Hume *et al.,* 2000; Nilsson *et al.,* 1999; Callegan *et al.,* 1994; Patel *et al.,* 1987). In addition to its pore forming ability, α-toxin induces the release of cytokines and chemokines such as IL-6, IL-1b, IL-1a, IL-8, TNF-a, KC andMIP-2 (Kielian *et al.,* 2001; Bhakdi *et al.,* 1989; Dragneva *et al.,* 2001; Onogawa, 2002; Bartlett *et al.,* 2008; Hruz *et al.,* 2009).
- leukocidins (*e.g.,* Panton-Valentine Leukocidin): Panton-Valentine Leukocidin (PVL) is a harmful cytotoxin which indirectly mediates tissue necrosis and sepsis by either the release of cytotoxic lysosomal granule contents from lysed polymorphonuclear leukocytes or by an inflammatory cascade set in motion by polymorphonuclear leukocytes lysis or apoptosis (Boyle-Vavra and Danum, 2007). In support of this, PVL-mediated lysis induces the release of reactive oxygen species and a variety of inflammatory mediators from granulocytes (Kaneko and Kamio, 2004).

Some of the staphylococcal toxins, such as the staphylococcal enterotoxins (SEs) and the Toxic Shock Syndrome Toxin 1 (TSST- 1), present also superantigenic activity, being able to stimulate the release of large amounts of cytokines (Dinges *et al.,* 2000).

2.2.1 Emergence and selection of methicillin resistance

AMR is a public health challenge considering that nearly as quickly as life-saving antibiotics are created, new antibiotic resistant bacteria appear (Ventola, 2015). The current situation is extremely serious and methicillin resistant *Staphylococcus aureus* (MRSA) is a particularly problematic nosocomial pathogen (Oniciuc *et al.,* 2017) considering its remarkable level of acquisition of resistance against multiple antibiotic classes (Lakundi and Zang, 2018). *S. aureus* has been considered a potential pathogen since its first detection in 1880, and before the introduction of penicillin the mortality rate of patients with infections caused by *S. aureus* was about 80% (Lowy, 2003).

As previously described in the AMR timeline, in 1942, within two years from the introduction of penicillin, the first penicillin-resistant *S. aureus* strain was detected (Rammelkamp, 1942) and now the high rate (over 90%) of drug-resistant in human *S. aureus* isolates confirms that penicillin almost lost therapeutical effect against staphylococcal infections (Peacock and Paterson, 2015). The same happened in 1960, when the first methicillin-resistant *S. aureus* (MRSA) was clinically identified soon after the development of the semisynthetic antibiotic methicillin (Jevons, 1961). Subsequently, MRSA, along with the resistance to other antibiotics, such as erythromycin, streptomycin, the tetracyclines (Finland, 1955; Brumfitt and Hamilton-Miller, 1989; Jessen *et al.,* 1969), also showed resistance to an entire class of penicillin-like antibiotics, including methicillin (Stefani *et al.,* 2015), and more recently to vancomycin and daptomycin (**Tab. 3**) (CDC, 2002; Stryjewski and Corey, 2014).

Antibiotics	Resistance genes	Gene products	Mechanisms of resistance
β-Lactams	*blaZ*	β-Lactamase	Enzymatic hydrolysis of β-lactam nucleus
	*mec*A	PBP2a	Reduced affinity for PBP
Glycopeptides	GISA: unknown	Altered peptidoglycan	Trapping of vancomycin in the cell wall
	VRSA: *van*A	D-ala-D-Lac	Synthesis of dipeptide with reduced affinity for vancomycin
Quinolones	*par*C	parC (or grlA) component of topoisomerase IV	Mutation in QRDR region reducing the affinity of
	*gyr*A or *gyr*B	gyrA or gyrB components of gyrase	enzyme-DNA complex for quinolones
Aminoglycosides *e.g.* gentamycin	Aminoglycosides modifying enzymes (*eg, aac, aph*)	Acetyltransferase, phosphotransferase	Acetylating and/or phosphorylating enzymes modify aminoglycosides
Trimethoprim-sulfamethoxazole (TMP-SMZ)	Sulfonamine: *sul*A	Dihydropteroate synthase	Overproduction of p-aminobenzoic acid by the enzyme
	TMP: dfrB	DHFR	Reduced affinity for DHFR
Tetracyclines	Tetracycline, doxycycline, minocycline: *tet*M	Ribosome protection protein	Binding to the ribosome and chasing the drug from its binding site
	Tetracycline: *tetK*	Efflux protein	Efflux pump
Erythromycin	*msr*A	Efflux protein	Efflux pump
	erm (A, C)	Ribosomal methylase (constitutive or inducible)	Alteration of 23S rRNA
Clindamycin	*erm* (A, C)	Ribosomal methylase (constitutive or inducible)	Alteration of 23S rRNA
Linezolid	*cfr*	Ribosomal methyltransferase	Methylation of 23S rRNA that interferes with ribosomal binding
Daptomycin	*mpr*F	Lysylphosphatidylglycerol synthetase (LPG)	Increasing: synthesis of total LPG translocation and positive net charges on cell membrane

Table 3. Representative mechanisms of Staphylococcus aureus resistance to antimicrobials (Zhu Li, 2018).

Beginning in the 1980s, this epidemic initially largely restricted to Europe has advanced so greatly to become a worldwide issue that is still ongoing (Lakundi and Zang, 2018). In fact, in 2016, the World Health Organization (WHO) has included in three priority risk lists (critical, high and medium) pathogens for which we need very soon new antibiotics, and MRSA was included in the priority list 2: "high risk" (**Tab. 4**) (WHO, 2016).

Priority 1: CRITICAL	Priority 2: HIGH	Priority 3: MEDIUM
Acinetobacter baumannii, carbapenem-resistant	*Enterococcus faecium, vancomycin-resistant*	*Streptococcus pneumoniae, penicillin-non-susceptible*
Pseudomonas aeruginosa, carbapenem-resistant	*Staphylococcus aureus, methicillin-resistant, vancomycin-intermediate and resistant*	*Haemophilus influenzae, ampicillin-resistant*
Enterobacteriaceae, carbapenem-resistant, ESBL-producing	*Helicobacter pylori, clarithromycin-resistant*	*Shigella spp., fluoroquinolone-resistant*
	Campylobacter spp., fluoroquinolone-resistant	
	Salmonellae, fluoroquinolone-resistant	
	Neisseria gonorrhoeae, cephalosporin-resistant, fluoroquinolone-resistant	

Table 4. WHO priority pathogens list for R&D of new antibiotics WHO, 2016.

Methicillin resistance is due to the carriage of mobile genetic elements, named the staphylococcal chromosomal cassettes (SCC*mec*) (Milheiriço, Oliveira and de Lencastre, 2007). SCC*mec* typically harbour *mec*A or *mec*C genes, encoding a penicillin binding protein 2a (PBP2a) (Paterson, Morgan, *et al.,* 2014; Petinaki and Spiliopoulou, 2012). MRSA is generated by the integration of a *mec*A-carrying SCC*mec* element into a methicillin-susceptible *S. aureus* (Enright *et al.,* 2002). A novel methicillin-resistance gene (named *mec*C) was first described in a *S. aureus* isolated from dairy cattle in England in 2011 (García-Alvarez *et al.,* 2011). This *mec*C gene is located in a novel SCC*mec* element, type XI-SCC*mec*, and shares only 70% nucleotide sequence identity with *mec*A (Oniciuc *et al.,* 2017). *mec*A gene encoded PBP2a, a modified form of PBP, regularly presents on staphylococcal

cells essential for cell wall peptidoglycan synthesis (Oniciuc *et al.,* 2017). β-lactams lead to the staphylococcal cells' lysis by binding to the PBPs and stopping the peptidoglycan synthesis (Matsuhashi, 1986; Ubukata, 1989). However, the varieties PBP2a have a low affinity for methicillin, oxacillin and virtually for all the β-lactams, such that peptidoglycan synthesis can continue in MRSA strains (Paterson, Morgan *et al.,* 2014) even in the presence of diverse β-lactam drugs (Oniuc *et al.,* 2017). There are three basic structural/genetic elements in SCC*mec* (IWG-SCC, 2009; Katayama and Hiramatsu, 2001):

- the *mec* gene complex: containing the *mec* gene (*mec*A, *mec*B, *mec*C, and/or *mec*D) and its regulatory elements that control its expression (*mec*R1, encoding a signal transducer protein, and *mec*I, encoding a repressor protein);
- the *ccr* gene complex: encoding the site-specific recombinases, *i.e.,* cassette chromosome recombinase (*ccr*) genes (*ccr*AB and/or *ccr*C);
- the joining regions (J regions): these nonessential cassette components may contain determinants for additional antimicrobial resistance.

Although all SCC*mec* share several common structural characteristics, the high diversity in the structural organization and the genetic content of these mobile elements has resulted in their classification into types and subtypes. Nowadays are known 13 types of SCC*mec* (**Fig. 4**) (Gostev and Sidorenko, 2012; Oniuc *et al.,* 2017; Lakhundi and Zhang, 2018). The most relevant from an epidemiological point of view are the first 5; of which the types I-IV-V confer only beta-lactams resistance; the types II and III confer multi-drug resistance. In fact, they typically harbor plasmidic sequences, transposons with other antimicrobial resistance genes or virulence factors (Oniuc *et al.,* 2017).

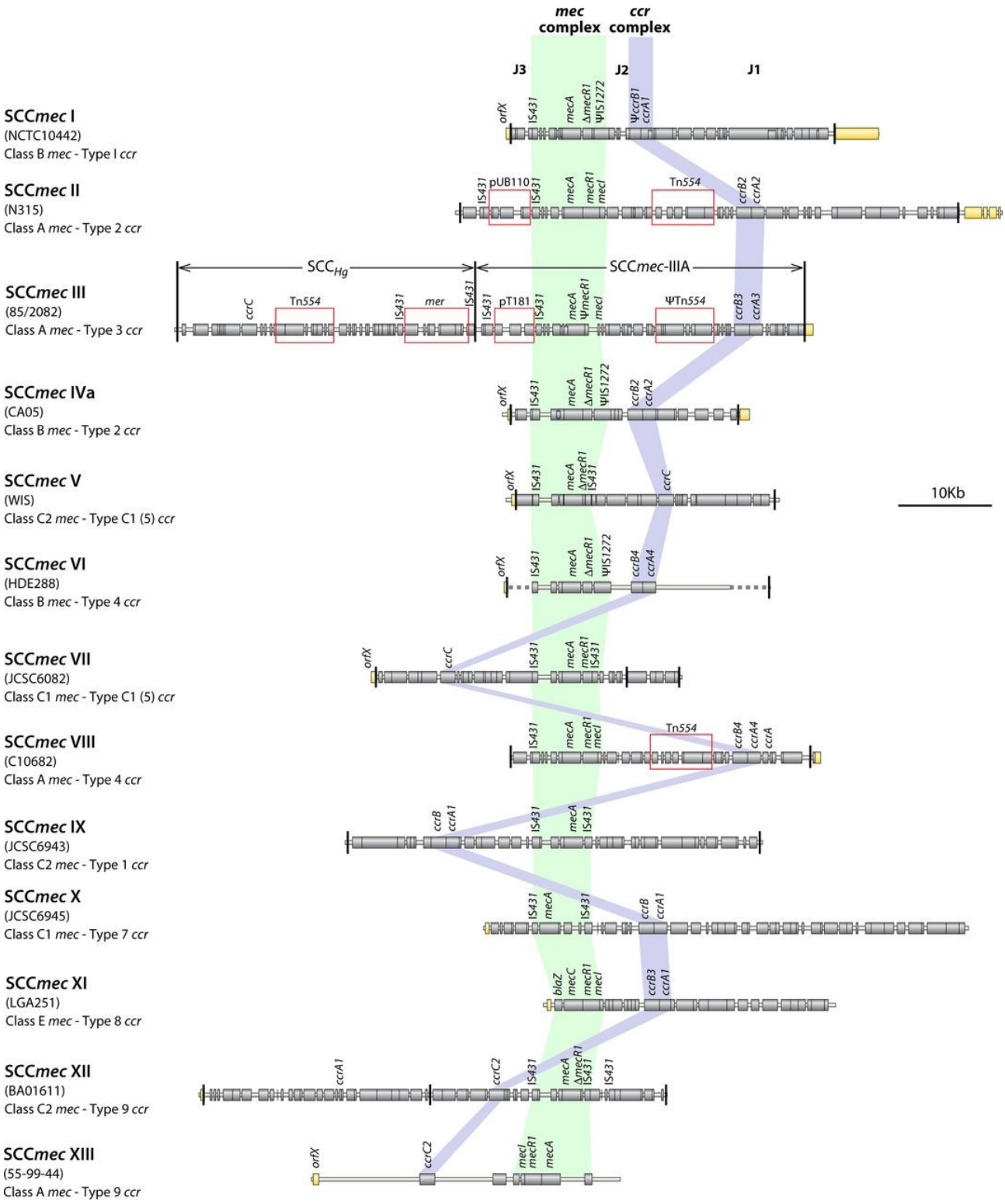

Figure 3. The Staphylococcal Cassette Chromosome mec (SCCmec) from *Lakhundi and Zhang, 2018*.

2.2.2. MRSA Typing methods

In the last few decades, several typing techniques have been developed for the characterization and discrimination of MRSA isolates based on their phenotypic and/or genotypic characteristics (Lakhundi and Zhang, 2018):

- Phage typing: is based on the different abilities of bacteria to be infected by different bacteriophages. Depending on whether *S. aureus* strains survive or not to the attack of a series of phages, it is given a number representing the phage type. A set of 23 internationally accepted phages is used for typing human strains of *S. aureus,* with a window of two local phages (Weller, 2000). However, a high proportion of MRSA isolates (20-30% of tested samples) remain non-typeable when this technique is used in an outbreak situation (Blair, 1966).
- Multilocus Enzyme Electrophoresis (MLEE): also called isoenzyme typing, allows the identification of genetically related types within a species, which can be associated with particular characteristics (Boerlin, 1997). Although MRSA isolates are generally typeable with good reproducibility (Tenover *et al.,* 1994; Mulligan and Arbeit, 1991), the results produced are difficult to compare between laboratories.

PCR-based typing systems:

- Amplified Fragment length Polymorphism (AFLP): based on the polymorphisms of amplified fragments of genomic DNA, it involves the digestion of genomic DNA with restriction enzymes, followed by ligation of double-stranded adaptors to the sticky ends of the restriction fragments (Vos *et al.,*1995). The genetic relatedness among studied bacterial isolates is determined via high-resolution banding patterns generated during AFLP analysis (Mortimer and Arnold, 2001).

- Random Amplification of polymorphic DNA (RAPD) and, its variant, Arbitrarily Primed PCR (AP-PCR): are based on the principle of rapid parallel amplification of random DNA segments under non-stringent conditions, producing a gel map unique to a particular bacterial strain (Williams *et al.,* 1990; Welsh and McClelland, 1990; Li *et al.,* 2009). The differences between the two techniques include the amplification. RAPD is less discriminatory, and the discriminatory power depends on the number and nucleotide sequences of primers. It has, however, been widely used for typing of isolates in outbreaks (Welsh and McClelland, 1990; Lanini *et al.,* 2011; Farber, 1996; Li *et al.,* 2009; Chang *et al.,* 2009; van Belkum *et al.,*1995; Saulnier *et al.,*1993; Struelens *et al.,* 1993).
- Repetitive Element Sequence-Based PCR (Rep-PCR): utilizes primers based on short sequences of repetitive elements dispersed throughout the prokaryote domain. This technique has a higher discriminatory power than many other typing techniques. As Rep-PCR targets specific sequences, allowing the use of stringent PCR conditions, the reproducibility of this technique is much better than that of RAPD (van der Zee *et al.,* 1999). The results obtained via the Rep-PCR technique have good correlation with PFGE, although the discriminatory power is slightly lower. It is, in fact, considered a typing method insufficiently discriminative for some organisms, including MRSA (Healy *et al.,* 2005; Fluit *et al.,*2010; Overdevest *et al.,* 2011; Babouee *et al.,*2011; Sabat *et al.,*2006; Wilson *et al.,*2009).
- *agr* typing: the accessory gene regulator *(agr*) is a crucial regulatory component in *S. aureus,* involved in the control of bacterial virulence factor expression. It is the variations among the hypervariable regions of the gene that divide *S. aureus* into at least 4 *agr* specificity groups (I to IV) (Novick, 2003).
- Pulsed-Field Gel Electrophoresis (PFGE): was once considered the gold standard for MRSA typing (Schwartz and Cantor, 1984). A number of studies have compared the usefulness of PFGE with that of other methods for MRSA isotyping (Struelens, 1996; Tenover *et al.,* 1994; Bannerman *et al.*, 1995; van Belkum *et al.,* 1995; Yoshida *et al.,* 1997; Chiou *et al.,* 2000;

Kumari *et al.,* 1997), and a number of restriction endonucleases have also been tested; SmaI was found to be the most useful for MRSA, allowing nearly all isolates to be typeable and results from standard strains to be reproducible after extensive subculturing (Tenover *et al.,* 1994; Bannerman *et al.*, 1995; Carles-Nurit *et al.,*1992; Ichiyama *et al.,* 1991; Prevost *et al.,* 1992; Blanc *et al.,* 2001; Tenover *et al.,*1997). The SmaI-based method has been proposed as the gold standard for MRSA typing (Struelens *et al.,*1992), even though not all pathogenic bacteria, such as MRSA strains of CC398, are typeable via PFGE (Rasschaert *et al.,* 2009). A molecular subtyping standard has been established by the Centers for Disease Control and Prevention (CDC) (Struelens *et al.,*1992), and all *S. aureus* PFGE profiles have been assembled in a national database for the investigation of MRSA outbreaks and global tracking of MRSA strain types (McDougal *et al.,* 2003; Swaminathan *et al.,* 2001).

- Multilocus Sequence Typing (MLST): is a modification of the MLEE phenotypic typing technique. MLST in conjunction with SCC*mec* typing, offers a universal nomenclature system for S. *aureus* strains. In 2000, Enright and colleagues (Enright *et al*., 2000) applied and validated the MLST scheme for *S. aureus* against PFGE. Out of 14 genes investigated, seven housekeeping genes were selected: *arc*C (encoding carbamate kinase), *aro*E (shikimate dehydrogenase), *glp*F (glycerol kinase), *gmk* (guanylate kinase), *pta* (phosphate acetyltransferase), *tpi* (triosephosphate isomerase), and *yqiL* (acetyl coenzyme A). MLST is defined by its allelic profile, which consists of a string of seven numbers assigned to each isolate (Aanensen and Spratt BG, 2005), all available on a web-based database. Feil and coworkers developed the BURST (Based Upon Related Sequence Type) algorithm for interpreting and analyzing the data, as well as developing evolutionary relationships among isolates (Feil *et al.,*2003; Feil *et al.,* 2004; Spratt *et al.,* 2004). Isolates sharing the exact same allelic profile belong to the same ST and, hence, the same genetic lineage. However, isolates with single-locus variants [SLVs] or double-locus variants [DLVs]) are considered to be genetically related and belonging to a cluster of related lineages, termed a clonal cluster (CC).

Based on this notion, for the first time there is an unambiguous, widespread, common, and universal language for MRSA (Enright *et al*., 2000; Lakundi and Zang, 2018).

- *spa* Typing: it is the first method based upon PCR amplification and the *spa* gene, developed specifically for the characterization of *S. aureus*, considering that the *spa* gene codes for protein A (conserved among *S. aureus* strains). *spa* type clusters specifically associated with MRSA lineages seem to be stable over time, making this method valuable for long-term global epidemiological studies (Faria *et al.,* 2008; Koreen *et al.,* 2004; Cookson *et al.,* 2007; Hallin *et al.,* 2007; Strommenger *et al.,* 2006). In addition, using the *StaphType* software, data are fully portable via the Ridom database, making it the most useful instrument and method of choice for characterizing *S. aureus* isolates at local, national, and international levels (Harmsen *et al.,* 2003; Hallin *et al.,* 2007; Deurenberg *et al.,* 2009; Friedrich *et al.,* 2008; Grundmann *et al.,* 2010; Kock *et al.,* 2009).
- SCC*mec* Typing: This technique is based on the assumption that two MRSA strains carrying different SCC*mec* elements are different, even if they belong to the same MLST type or pulsotype. Unfortunately, to date typing of SCC*mec* by PCR techniques has, because of its heterogeneity, been challenging. In fact, no single PCR method is available that can identify all SCC*mec* types and subtypes (Lakhundi and Zhang, 2018). Currently, SCC*mec* typing by multiplex PCR is limited to SCC*mec* types I to V. Other methods are therefore needed for typing the increasing number of SCC*mec* types, including types VI to XIII (Lakundi and Zang, 2018), and whole-genome sequencing has only partially solved this since no good bioinformatic tools have been available.
- DNA Microarray analysis: represent a tool well suited for bacterial typing (Sabat *et al.,* 2013). It is widely used for the analysis of genomic mutations and for the detection of extragenomic elements, including uncommon antibiotic resistance genes or patterns of virulence genes. Moreover, it has the potential to detect new epidemiological markers for clones (McCarthy *et al.,* 2012; McCarthy and Lindsay, 2012). Using the technique of microarray-based gene

content analysis, the identification of regulons of *S. aureus* pathogenesis, including *Agr* (Dunman *et al.,* 2001), *ArlRS* (Liang *et al.,* 2005), *SaeRS* (Liang *et al.,* 2005), *YhcSR/AirSR* (Yan *et al.*, 2012; Sun *et al.,* 2012), *Sar* (Dunman *et al.,* 2001), *SigB* (Bischoff *et al.,* 2004), *Rot* (Said-Salim *et al.,* 2003), and *Mgr* (Luong *et al.,* 2006), it is possible to better understand the molecular mechanisms of pathogenesis and to investigate *S. aureus* resistance and virulence capabilities. It is noteworthy that using whole-genome microarrays, 10 major *S. aureus* lineages responsible for causing the majority of human infections was revealed (Lindsay *et al.,* 2006). Because of the high cost of this technique, Alere Technologies has developed the Alere StaphType DNA, a new rapid and economic microarray assay for *S. aureus* (Monecke *et al.,* 2011), which automatically assigne the isolates to particular genetic lineages *via* software, based on their hybridization profiles.

- Whole-Genome Sequencing (WGS): is the ultimate identification of DNA diversity in any organism. Providing a cost-effective method of identifying genome-wide variations, this technique is currently considered an extremely powerful tool for epidemiological purposes (Mellmann *et al.,* 2011; Ben Zakour *et al.,* 2012; Chin *et al.,* 2011; Grad *et al.,* 2012), which allow to predict the number of open reading frames, through appropriate bioinformatics software. Several *S. aureus* genomes (including the genomes of methicillin-resistant strains) are now publically available, making the study of its biological systems possible. WGS can compare different genomes with single-nucleotide resolution, and this is very useful to characterize the transmission events and outbreaks, in an epidemiological perspective. Based on the literature, it seems that this technology will take over from routine investigation techniques currently used in clinical practice for the identification and characterization of bacterial isolates (Lakundi and Zang, 2018).

2.2.3. Epidemiology of MRSA in humans

The epidemiology of MRSA is quite complex, considering that it can be spread in several ways (Oniuc *et al.,* 2017) (Fig. 5).

For decades, the isolation of MRSA has been considered a good marker of the nosocomial (Hospital-Acquired -HA-) MRSA-related infections (Moellering, 2012). In the early 90s, the detection of various MRSA lineages in community setting (community-associated MRSA -CA-MRSA-), such as nursing homes and kindergartens or in patients without previous healthcare exposure, represented a dramatic change in the epidemiology of MRSA (Boyle-Vavra and Daum, 2007; Oniuc *et al.,* 2017). CA-MRSA strains were initially thought to be nosocomial strains that had spread from hospital to community settings; however, the current accepted model to explain the origin of CA-MRSA states that a small methicillin resistance cassette (*eg,* SCC*mec* IV) independently integrated into the genomes of many different methicillin-susceptible *S. aureus* (MSSA) ancestral clones circulating in different geographic regions (Boyle-Vavra and Daum, 2007).

Although its capacity to persist as a commensal, its ability to resist to multiple antimicrobial agents, and its multitude of virulence factors are important factors which contribute to the success of this pathogen (Fluit *et al*., 2001; Foster, 2005; Otto, 2010), one of the major MRSA infection-associated risk factors is the nasal colonization with *S. aureus* (Lakhundi and Zhang, 2018; 2, Davis *et al.,* 2004; Pujol *et al.,* 1996; von Eiff *et al.,* 2001; Huang and Platt, 2003).

Furthermore, it has also been considered a risk of MRSA acquisition (5% of all incident MRSA cases) the admission into a hospital room previously occupied by an MRSA-infected patient (Huang, 2006). Although according to CDC, a MRSA infection could be defined "community-acquired" if diagnosed in an out-patient, or within 48 hours of hospitalization when traditional risk factors for MRSA infection are lacking (CDC, 2000), to date, CA-MRSA and HA-MRSA are distinguished by means of genetic determinants (Boyle-Vavra and Danum *et al.,* 2007). They have a different accessory genome, which carries different SCC*mec* elements, and cause different clinical symptoms (Crombé *et al*., 2013; Enright *et al.,* 2002). HA-MRSA are mainly related to SCC*mec* types I, II and

III (Al-Zubeidi *et al.,* 2014) and the main HA-MRSA clonal complexes (CCs) isolated from hospitalized patients are CC5, CC8, CC22, CC30 and CC45 (Cortes *et al.,* 2015). On the other hand, SCC*mec* types IV and V are usually find in CA-MRSA and the main CA-MRSA strains belonged to the Sequence Type (ST) 1, 8 (USA 300), ST 80 (Europe), ST 30 and ST 93 (Australia) (Doulgeraki *et al.,* 2017).

HA-MRSA strains are often multidrug resistant, while CA-MRSA is usually limited to β-lactam resistance. This is consistent with the fact that, unlike the large SCC*mec* types (*i.e.,* SCC*mec* types I to III) present in HA-MRSA strains, which bear genes for multiple antibiotic resistance, SCC*mec* types IV and V carry only the *mec*A gene resistance to β-lactams (Ma *et al.,* 2002; Daskalaki *et al.,* 2007; Robinson and Enright, 2004; Herold *et al.,*1998). In fact, the majority of CA-MRSA isolates are therefore susceptible to fluoroquinolones, aminoglycosides, erythromycin, and clindamycin (Loughrey *et al.,* 2007).

However, although CA-MRSA are more often susceptible to non-β-lactam antibiotics, they are commonly associated with more serious and virulent skin and soft tissue infections such as severe and rapidly fatal necrotizing pneumonia and necrotizing fasciitis, than HA-MRSA (Loughrey *et al.,* 2007; Weigelt, 2008).

CA-MRSA strains test positive for haemolysins, leucocidin, and exfoliative toxins, whereas HA-MRSA usually does not contain these toxins. Among these virulence factors, CA-MRSA strains also encode β-lactamase and hyaluronidase (Weigelt, 2008) as well as the major virulence determinant associated with CA-MRSA disease: the Panton-Valentine Leucocidin (PVL) (Chambers and Deleo, 2009; Rossney *et al.,* 2007; O'Brien *et al.,* 2004; Kim *et al.,* 2007; Tristan *et al.,* 2007; Boyle-Vavra and Daum, 2007).

The prevalence of CA-MRSA varies worldwide, ranging from less than 1% in some countries to more than 50% in others, with the prevalence been higher in children than in adults (Kanerva *et al.,* 2009; Immergluck, 2007; Elston and Barlow, 2009; Olesevich and Kennedy, 2007). In the United States, Taiwan, Canada, and Australia, reported outbreaks have been more extensive, with infection

becoming endemic in certain populations in each of these countries (Nimmo and Coombs, 2008; David and Daum, 2010; DeLeo *et al.,* 2010; Otter and French, 2010). In other parts of the world, only small outbreaks or cases have been reported. In 2016, in the USA, the Centres for Diseases Control and Prevention (CDC) reported that, compared to 2013, the incidence of HA-MRSA decreased by 5.36%, while the incidence of CA-MRSA increased by 1.57% (Oniciuc *et al.,* 2017). In Europe, although with a stable incidence, MRSA remains a public health priority as its incidence is still above 25% in seven of 29 reporting countries, mainly in Southern and Eastern Europe (ECDC, 2015). There is fear, however, that if CA-MRSA becomes endemic in resource-poor nations, it would result in devastating consequences.

2.2.4. Epidemiology of MRSA in animals

In the last decades, an ever-changing epidemiology of MRSA has raised concerns about its presence in livestock, as livestock-associated MRSA (LA-MRSA). In veterinary medicine the abuse and misuse of antibiotics, especially in overcrowded intensive livestock has led to the selection of resistant bacteria adapted to animals which can spread to the environment and the related foodstuff.

The earliest published report of MRSA in farm animals described the detection of MRSA in dairy cows with mastitis in Belgium (Dreviese, 1975). LA-MRSA gained significant attention over a decade ago, with an alarming report about infections and high rates of MRSA colonization among Dutch pig farmers in 2005 (Voss *et al.,* 2005; Armand-Lefreve *et al.,* 2005; Huijsdens *et al.,* 2006). LA-MRSA isolates are genetically distinct from human isolates and, comprising mostly of MLST type ST398 (CC398), they represent the largest source of MRSA outside hospitals (Nicholson *et al.,* 2013). CC398 is reported from various parts of the world, where it is associated mainly with food animal species such as pigs and veal calves but has the capacity to colonize a wide range of hosts, including dogs, cats, sheep, cows, goat, poultry, rabbits, and horses (Weese, 2010; Graveland *et al.,* 2011; Dorado-Garcia *et al.,* 2013; Cuny *et al.,* 2010; van Duijkeren *et al.,* 2010). LA-MRSA strains are important from a monetary prospective as they cause infections in economically important

livestock animals worldwide, such as intramammary infections in dairy cows as well as in sheep and goat, skeletal infections in commercial broiler chickens, and mastitis and septicemia in rabbits (Bradley, 2002; Menzies and Ramanoon, 2001; McNamee and Smyth, 2000; Vancraeynest *et al.,* 2006). Many reports support the notions that persons in direct contact with livestock colonized by MRSA, such as farmers, personnel at slaughterhouses, transporters of livestock, and veterinarians, are at potential risk of becoming colonized with and suffering from infection caused by LA-MRSA. (Kluytmans *et al.,* 1995; Voss *et al.*, 2005; Garcia-Graells *et al.,* 2012; Wulf *et al.,* 2008; Huber *et al.,* 2010; Lakundi and Zang, 2018). MRSA CC398 is rapidly evolving considering that at the beginning it only had a few sequence types and *spa* types, and currently, CC398 harbors 43 sequence types (Stegger *et al.,* 2013). However, ST398 is still the most common sequence type colonizing pigs worldwide (Lakundi and Zang, 2018). In Europe, a comparative longitudinal study performed in the Netherlands, Denmark, and Belgium demonstrated that contact with pigs was the most important determinant for carriage of MRSA among household members of pig farmers (Garcia-Graells *et al.,* 2013). In southern Italy, the MRSA presence in pigs and workers at industrial abattoirs was also found to be as high as 37.6% (99 out of 215 pig nasal swabs) (Normanno *et al.,* 2015), and similarly in milk and dairy products a high MRSA rate was found among farmers, cattle, and bulk tank milk samples with 55% (344 out of 622) and 61% (283 out of 461) of bovine samples tested positive for MRSA, in comparison with 36% (40 out of 113) in human samples and 44% (21 out of 48) of bulk tank milk samples (Antoci *et al.,* 2013). Moreover, a study performed in the Netherlands, Europe's largest exporter of live pigs (Petinaki and Spiliopoulou, 2012), also concluded that working with sows and living with an MRSA-positive pig farmer increased MRSA carriage among household members (van Cleef *et al.,* 2015). Therefore, animal-to-human transmission could occur via three routes: direct contact, environmental contamination, or handling of an infected animal's products, and the direct contact is the most important way of transmission. The presence of human clones in pigs can result from human-to-pig contamination, but some strains, such as the t127/ST1 clone, may be animal-adapted (Pantosti, 2012). In fact, some human MRSA strains, such as ST5, ST8, ST22, ST30, and

ST45, have been found in pigs or pig farms in Europe, USA, and Africa (Crombé *et al*., 2013; Oniuc *et al.,* 2017). However, infected/colonized animals are not the only source of transmission. In fact, the first LA-MRSA ST398 outbreak in a Dutch hospital was reported in patients with no apparent contact with pigs or veal calves, suggesting possible human-to-human transmission (Wulf *et al.,* 2008).

2.2.5. Presence of MRSA in food and its role as foodborne pathogen

A multitude of studies support the notion that MRSA could be present in several kinds of food of animal origin and some of these isolates were identical to isolates from HA-MRSA and CA-MRSA human infections (Sergedilis *et al.,* 2017). MRSA has been found with different prevalence in meats such as pork (0.004 - 50%), poultry (0.7-43.8%), beef (1-15.2%), lamb (6.2%), rabbit (12.5%) and wild boar (25%), as well as in raw milk (1.7-17.6%), table eggs (11%) and fish (13.5%) (Tenhagen *et al.,* 2014; Feßler *et al.,* 2011; de Boer *et al.,* 2009; O'Brien *et al.,* 2012; Normanno *et al.*, 2007; Carfora *et al.,* 2016; Obaidat *et al.,* 2015). LA-ST398 MRSA strain is considered the major strain responsible for 85% of the contamination (de Boer *et al.,* 2009). In addition, according to Sergedilis and colleague (2017), even if underreported, the development of invasive disease following the ingestion of food contaminated with enterotixigenic MRSA is yet another risk (Sergedilis and Angelidis, 2017). Although the transmission of infection by food products appears to be very rare, and certainly much reduced from that following contact with live animals or humans (EFSA, 2009), there are descriptions of 2 outbreaks of foodborne disease due to MRSA. In 1994, the first foodborne outbreak at the University Hospital Rotterdam, where 5 out of 27 patients died, occurred; the outbreak was caused by a human strain isolated from a worker who contaminated the food during meal preparation (Kluytmans *et al.,* 1995). The second one, occured in 2001 and it was the first food poisoning outbreak caused by a staphylococcal enterotoxin C (SEC) producing MRSA isolated from roasted pork contaminated by food handlers (Jones *et al.,* 2002). Particularly notewhorty is a more recently case of MRSA colitis reported in a patient without any predisposing medical condition in the

United States (Kalakond *et al.,* 2015). On the other hand, it is important to stress that human MRSA strains, too, have been isolated from food, because of contamination during food handling (Weese, 2010); in fact, depending on the epidemiology of the geographical area considered, about 0.7 - 1.5% of human beings are colonized by MRSA (Gorwitz *et al.,* 2008; Wertheim, 2004; Munckhof *et al.,* 2009). Incorrect handling during animal slaughter can lead to MRSA contamination of carcasses (Argudín *et al.,* 2015), and consequently a risk for human consumption and dissemination in the community (Oniuc *et al.,* 2017). Therefore, based on the data reported from the aforementioned surveys, it is well known that MRSA could be present in food and there exists an interchange between human and animals' MRSA clones (Zarazaga, 2018). However, there is currently no evidence for increased risk of human colonisation or infection following contact or consumption of food contaminated by MRSA both in the community and in hospital (EFSA, 2009). The risk largerly depends on the hygienic measures taken, the populations of MRSA present in food and the ability of the strain itself to colonize the host (Sergedilis and Agelidis, 2017). Further studies are needed to clarify wether or not MRSA can act as a foodborne pathogen.

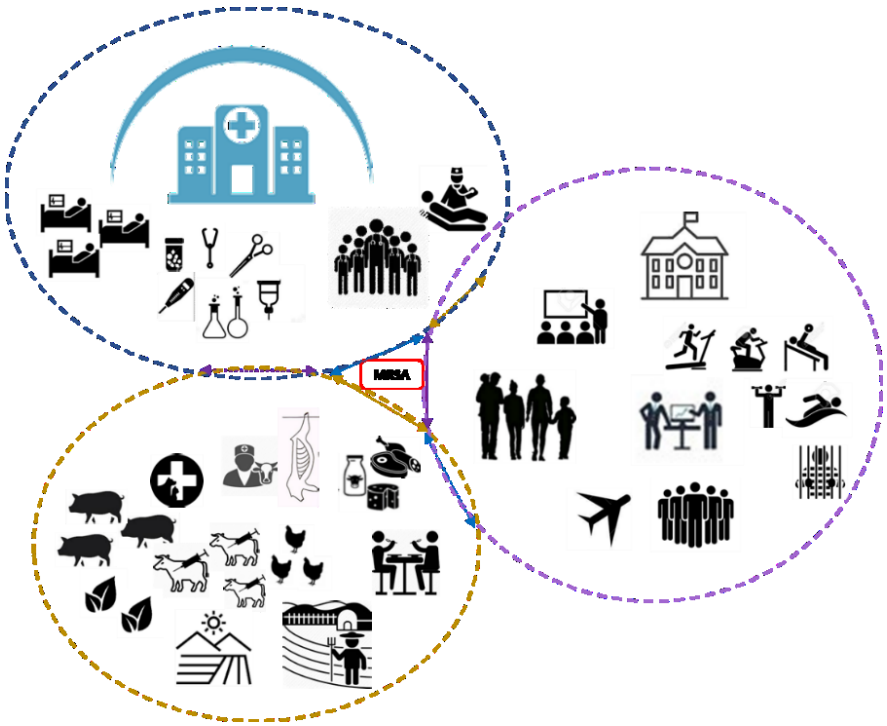

Figure 4. Potential routes of transmission of MRSA. Drawn by Elisa Spinelli, from Oniuc et al. (2017).

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Aims

The aims of the study are: i) to assess the occurrence of MRSA in buffalo buffalo tank milk from Italy, and to provide information about the antimicrobial resistance profile and molecular characteristics of the isolates ii) to study the fate of MRSA along the human gastrointestinal tract and its interaction with the gut microbiota iii) to evaluate the survival of MRSA in simulated human ascendant colon conditions and its interaction with gut microbiota into the mucus layer.

Part 1.

Occurrence and characteristics of Methicillin- resistant *Staphylococcus aureus* (MRSA) in buffalo bulk tank milk and the farm workers in Italy

Abstract

The aim of the present study was to assess the occurrence of MRSA on buffalo dairy farms and in buffalo tank milk from Italy, and to provide information about the antimicrobial resistance profile and molecular characteristics of the isolates. We collected 75 bulk tank milk (BTM) samples from 75 farms and 24 nasal swabs from 24 farm operators. Three (4%) of the 75 BTM samples and 1 (4%) of the 24 human nasal swabs tested positive for MRSA. The milk isolates belonged to the genotypes ST1/t127/Va and ST72/t3092/V, while the human isolate was characterized as ST1/t127/IVa. No ST398 was found. All isolates were multidrug resistant but vancomycin susceptible; they carried the *ica*A gene but tested negative for the *pvl* and *ses* genes. ST72 is a CA-MRSA commonly found in South Korea, and this is the first report of its detection in Europe. Although we found a low prevalence of MRSA on the farms we surveyed, this study clearly demonstrates, for the first time in Europe, that MRSA can be found on dairy buffalo farms and in raw buffalo milk. Therefore, the risk of human colonization/infection with MRSA linked to the handling of raw milk or consumption of contaminated dairy products should not be ruled out.

1. Introduction

Water buffaloes (*Bubalus bubalis*) are bred in all continents of the world. The global population of water buffalo is approximately 194 million head: 97% are reared in Asia; 2% are in Africa, particularly Egypt; 1% are in South America; and less than 1% are in Australia and Europe (FAOSTAT, 2014). The countries with the largest numbers of dairy buffalo are India, Pakistan, China, Egypt, and Nepal (FAOSTAT, 2014). Although milk production is dominated by dairy cows worldwide (FAO, 2017), water buffaloes are the greatest source of non-cattle milk (13.2%) (FAO, 2016). Buffalo milk has long been valued by its important chemical composition, determining nutritive properties and suitability in the manufacture of traditional as well as industrial dairy products (Ahmad, 2013). It has a different composition to that of cow's milk: it's richer in protein, fats (which

represents the major constituent) and, above all, in overall calcium (Tab 1) (CLAL, 2017; Barlowska *et al.,* 2011).

The high consumptions of buffalo drinking milk and dairy products worldwide involve an elevated number of consumers of all ages. And this is crucial considering the potential role of buffalo milk in the transmission of foodborne pathogens.

It is well known that *S. aureus*, including MRSA, is animportant cause of mastitis in dairy cows (Feßler *et al.,* 2010; Haran *et al.,* 2012; Vanderhaeghen *et al.,* 2010; Pu *et al,* 2014) and buffaloes (Khan and Muhammad, 2005; El-Ashker *et al.,* 2015); during subclinical mastitis, these organisms could be shed in milk without organoleptic alterations, allowing them to enter and spread through the food chain.

Buffalo milk has beneficial nutritional properties due to its chemical composition, and it is suitable for the manufacture of traditional and industrial dairy products (Ahmad, 2013). It is often processed into butter and cheese, but traditional products such as *dahi* (India, Pakistan) and *mozzarella* (Italy) are the most widely consumed buffalo dairy products (Alexandraki, 2016). Italy is the EU's leading buffalo milk producer, with 88% of total European buffalo milk production, and ranks seventh in the world (EFSA, 2015). Italian buffalo milk production amounts to 2,109,084 quintals, of which 1,799,606 are produced in the southern regions (ISTAT, 2018). Buffalo milk is mostly processed into *mozzarella* (Ercolini, 2012), a fresh and stringy-textured Italian cheese. Since buffalo milk has a higher fat to protein ratio (2:1 c.ca) than cow's milk, buffalo *mozzarella* is softer and tastier than other kinds (Bartocci, 2002) and is exported worldwide.

The appreciation of this buffalo mozzarella by consumers is demonstrated by the "Mozzarella di Bufala Campana" PDO recognition obtained in 1996. The Mozzarella di Bufala Campana PDO production data in Italy show a positive trend on 2017 with an increment of 6.1% from 2016. The database on its exports all over the world also shows an increment from 5.280 tons on 2006 to 14.190 tons on 2016 (Consorzio Tutela Mozzarella di Bufala Campana, 2018).

The high consumption levels of buffalo milk and its dairy products involve great numbers of consumers of all ages worldwide; this represents a food safety concern, since milk and dairy products can be important sources of foodborne pathogens because their high nutrient content can allow pathogens to multiply (Han *et al.*, 2007). Previous studies on the microbiological quality of buffalo milk have revealed the presence of coliform bacteria, pathogenic *Escherichia coli*, lactic acid bacteria, *Listeria* spp., *Staphylococcus aureus, Clostridium* spp. and some species of yeasts (Lorusso *et al*., 2009; Boycheva, *et al* 2002; Rahimi *et al.*, 2014; Corbo *et al.,* 2001; Oliveira *et al.,* 2011; Ercolini *et al.,* 2012). *S. aureus* may be present in bulk milk via direct excretion from the udders of dairy cattle with clinical and subclinical staphylococcal mastitis, or via fecal contamination (Callon *et al.*, 2008). Recently, the spread of methicillin-resistant *S. aureus* (MRSA) in food, especially meat and milk, has raised the question as to whether buffalo milk is actually a potential vehicle of transmission. Despite the potential hazard for human health linked to contact with animals and to the handling/consumption of raw milk (Khanna *et al.*, 2008; Weese, 2010; Parisi *et al.,* 2017), there is a lack of data on the prevalence of MRSA in buffalo milk and its dairy products. The aim of this study was to acquire better understanding of the epidemiology of MRSA, investigating the occurrence of MRSA in buffalo farms in southern Italy by examining the prevalence, molecular characteristics and antimicrobial resistance profile of MRSA isolated from raw buffalo milk and from farm workers.

2. Materials and Methods

2.1 Collection of bulk tank milk samples and nasal swabs

Between April 2017 and May 2018, 75 samples (100 mL) of bulk tank buffalo milk (BTM) were collected from 75 buffalo farms in southern Italy. Nasal swabs were collected (Laboindustria, Arzergrande-PD, Italy) on 24 of these farms from 24 farm operators working in contact with the animals (1 operator per farm) whose written consent had been obtained in advance. The samples were collected aseptically and immediately transported under refrigeration to the laboratory, where they were stored at -80°C before testing.

2.2 Isolation of MRSA

Samples were thawed and kept at room temperature for approximately one hour. Then 1 mL of milk was added to Mueller-Hinton broth (BiolifeItaliana, Milan, Italy) supplemented with 6.5% (w/v) NaCl (Sigma Aldrich, St Louis MO, USA). After incubation for 24 h at 37°C, 20 µl of each culture was spread onto a MRSA-SELECT® plate (Bio-Rad, Marnes la Coquette, France) and incubated at 37°C for 24-48-72h (Nahimana *et al.,* 2006). Suspected MRSA colonies (pink colonies) were subcultured on a Columbia Sheep Blood Agar plate (Oxoid, Basingstoke, Hampshire, UK) for purification and then screening for methicillin resistance and molecular characterization.

2.3 Molecular identification, confirmation of methicillin resistance and genetic characterization of MRSA

Genomic DNA was extracted from the presumptive MRSA isolates using a GenomicPrep® cell and tissue isolation kit (Amersham, Piscataway, NJ, USA) according to the manufacturer's instructions. DNA concentration was determined at a wavelength of 260 nm using a DU 640 spectrophotometer (Beckman, Fullerton, CA, USA) and adjusted with distilled water to 10 ng/µL.

Two separate PCR assays were performed to assess the species identification and to detect the *mec*A gene, using previously described primers *sau*1 and *sau*2, and *mec*A147-F and *mec*A147-R (Strommenger *et al.,* 2003; Zhang *et al.,* 2005), respectively. One isolate per sample, identified as MRSA, was further characterized as described below.

2.3.1 MLST analysis of MRSA

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

of identical loci out of seven loci for group definition and three minimum single locus variant contents for subgroup definition (Kwon *et al.,* 2005).

2.3.2 *spa*-typing

The x region of the *spa* gene was amplified by PCR using the primers spa-1113f (5′ TAA AGA CGA TCC TTC GGT GAG C 3′) and spa-1514r (5′ CAG CAG TAG TGC CGT TTG CTT 3′) (Strommenger *et al.,* 2006). DNA sequences were obtained using an ABI 3130 Genetic Analyzer (Applied Biosystems, Foster City, Calif.) with BigDye 3.1 Ready reaction mix (Applied Biosystems) according to the manufacturer's instructions. *Spa*-types were determined using BioNumerics 7.1 (Applied Maths, Belgium) software.

2.3.3 SCC-*mec* characterization

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

2.3.4 Detection of gene encoding PVL

All the MRSA strains were tested by PCR for *luk*S-*luk*F-PV, encoding Panton-Valentine leukocidin (PVL), as described elsewhere (Hesje *et al.,* 2011).

2.3.5 Detection of icaA gene encoding for polysaccharide intercellular adhesin (PIA)

All the MRSA strains were tested by PCR for the *ica*A gene (intercellular adhesion) as described elsewhere (Zmantar *et al.,* 2008).

2.3.6 Detection of staphylococcal enterotoxin encoding genes

MRSA isolates were tested by PCR for *sea* to *seg, seh, sei, sej, sen, seo and sem* encoding staphylococcal enterotoxins as described elsewhere (Boerema *et al.,* 2006).

2.4 Microbiological confirmation of methicillin resistance and detection of antimicrobial resistance pattern of MRSA

2.4.1 Oxacillin and cefoxitin disc diffusion test

Oxacillin and cefoxitin disc diffusion susceptibility tests were performed with 1 µg oxacillin and 30 µg cefoxitin discs (RoscoDiagnostica, Taastrup, Denmark), following CLSI (CLSI, 2012) recommendations. Mueller-Hinton agar plates (Biolife) were inoculated with a suspension (equivalent to a 0.5 McFarland standard) of each MRSA considered. Plates were incubated at 37°C and zone diameters were read after 18-24 h. The following breakpoints were considered: oxacillin: resistant ≤10 mm, intermediate 11-12 mm, susceptible ≥13 mm; cefoxitin: resistant ≤21 mm, susceptible ≥22 mm (Shariati *et al.,* 2010).

2.4.2 Agar screening method

The MRSA suspension (adjusted to match 0.5 McFarland turbidity standard) was inoculated on Oxacillin Salt Screen Agar® (Mueller-Hinton agar containing 4% NaCl and 6 µg oxacillin/ml-Biolife). Plates were incubated at 37°C for 24 h and any growth on the plate was considered as resistant to methicillin (Shariati *et al.,* 2010).

2.4.3 Oxacillin E-test

Mueller-Hinton agar plates supplemented with 2% NaCl (Biolife) were inoculated by streaking the standardized inoculums (equivalent to a 0.5 McFarland standard) with a sterile swab. Oxacillin E-test strips (bioMèrieux, Marcy l'Etoile, France) were placed on the plates, followed by incubation at 37°C for 18-24h. The minimum inhibitory concentration (MIC) for each isolate was read at the intersection point of the zone of growth inhibition with the graduated strip (resistant ≥ 4 µg/ml; susceptible: ≤ 2 µg/ml) (Shariati *et al.,* 2010).

2.4.4 Antimicrobic susceptibility testing of MRSA

MRSA isolates were tested for susceptibility to a panel of 21 antimicrobial agents using the disc agar diffusion method on Mueller-Hinton agar, following the guidelines of the CLSI (CLSI, 2012). The antibiotic discs (antibiotic concentration in µg) from Liofilchem (Liofilchem s.r.l, Roseto d. A., Italy) were as follows: amikacin (30), amoxicillin/clavulanic acid (30), ampicillin (10) cephalotin (30), cefotaxime (30), cefoxitin (30), clindamycin (2), choramphenicol (30), doxycycline (30), enrofloxacin (5), erythromycin (15), gentamicin (10), kanamicin (30), oxacillin (1), penicillin (10), streptomycin (10), sulfisoxazole (250), tetracycline (30), trimethoprim-sulfamethoxazole (25), tobramycin (10), and vancomycin (30). The results were recorded after 24 h incubation at 37°C and interpreted according to charts supplied with the discs.

3. Results

3.1 Prevalence and molecular characteristics of MRSA in buffalo tank milk and farm operators

MRSA was detected in 4 (5.3%) of the 75 investigated farms. Out of 75 bulk tank milk samples, 3 (4%) tested positive for MRSA; two strains were genotyped as ST1/t127/IVa and one strain as ST72/t3092/V. A MRSA strain ST1/t127/IVa was isolated from a farm operator of one (1.3%) of the investigated farms but not from the milk produced by the same farm. All isolates carried the *ica*A gene but were negative for the presence of the *luk*S-*luk*F and the *se*s genes (Table 2).

3.2 Antimicrobial resistance characteristics of the isolates

The results of antimicrobial susceptibility testing of MRSA isolates are shown in Table 3.

All isolates were confirmed as MRSA by the microbiological confirmation assays: the oxacillin disc diffusion test, the oxacillin agar screen test and oxacillin E-test. One strain isolated from buffalo milk resulted susceptible with the cefoxitin disk diffusion test (Table 3). All isolates were multidrug resistant, but susceptible to vancomycin, amikacin, cephalothin, clindamycin and chloramphenicol. One strain was susceptible to tetracycline (Table 3).

4. Discussion

This study assessed the prevalence of MRSA in buffalo bulk tank milk (BTM) produced in Italy, and also in farm operators, and reports the phenotypic and molecular characteristics of the isolates.

In our study, the overall prevalence of MRSA on the investigated farms was 5.4%, and 4% in the BTM. The rates of contamination found were higher than those reported by Pamuk, who investigated 120 raw buffalo milk samples sold in Turkey and found two *S. aureus* strains (1.66%) carrying the *mec*A gene (Pamuk *et al*., 2012). In another survey on the aetiology of subclinical mastitis in water buffaloes reared in South India, Preethirani and colleagues reported an overall prevalence of staphylococci of 18.1%; among these, 10.1% were identified as *S. aureus* and all the 14 isolates resulted resistant to cefoxitin. The authors concluded that a high proportion of the *S. aureus* strains isolated from domesticated water buffaloes of the investigated area was methicillin resistant (Preethirani *et al.,* 2015). More recently, Erdem Saka and Goknur Terzi Gulel reported a prevalence of 30% (99 isolated) of *S. aureus* from buffalo's milk product in Turkey, of which 9 (9%) were found to be *mec*A positive (Saka and Gulel, 2018).

Here, MRSA was detected in one farm operator at one of the sampled farms, but no MRSA was isolated from the BTM produced by the same farm. A possible explanation for this finding could be that the operator was actually an MRSA carrier but had not yet transferred the strain to the animals. On the other hand, farms were visited only once during the survey, and it is thus possible that this strain was simply missed by sampling due to the high dilution factor in large volumes of BTM. Further understanding is required in order to provide a better explanation of this finding.

The MRSA strain isolated from the operator was genotyped as ST1/t127/SCC*mec*IVa; in our survey, this genotype was also detected in two out of three strains isolated from buffalo BTM, and all these isolates were *ica*A positive, *luk*S-*luk*F and *ses* negative. MRSA ST1/t127 is a well-known genotype primarily associated with human infections worldwide (Lin *et al.,* 2011; Köck *et al.*, 2011), and was also isolated from Italian pigs by Battisti; in addition, MRSA t127 is a *spa*-type identified as a cause of mastitis in Italian dairy cows (Battisti *et al.,* 2010; Benedetti *et al.,* 2010). On the other hand, ST1

(t286/SCC*mec*IVa/*pvl*$^-$) is a genotype isolated from mastitic milk in Korea, where the same genotype is also considered a Community-Acquired (CA)-MRSA prevalent in humans (Nam *et al.,* 2011). In a recent survey on the occurrence of MRSA in bovine BTM produced in southern Italy, the authors found great genetic diversity among the isolates, identifying both Livestock-Associated (LA)-MRSA strains and typical human strains, which suggests an exchange of strains between humans and animal and *vice versa* (Parisi *et al.,* 2016). In the present study, although the number of samples was smaller than in this latter work, we found only strains known to be human-associated. Surprisingly, no ST398 strains were isolated, and this finding indicates the need for further research to establish whether MRSA ST398 is able to colonize water buffalo. In fact, to the best of our knowledge, although MSSA 398 was found in buffaloes (El-Ashker *et al.,* 2015) this genotype of MRSA has never been isolated from this species.

Our detection of an ST72/t3092/SCC*mec*V strain was unexpected. ST72 is the most common Community-Acquired (CA) MRSA genotype in South Korea, and ST72/SCC*mec*IV can cause bacteremia, invasive infections and death (Lee *et al.,* 2010; Park *et al.,* 2015). ST72/t3092/SCC*mec*VIII has also been isolated in hospitals in South Africa, where it is considered a "sporadic" clinical genotype (Jansen van Rensburg *et al.,* 2011). In addition, ST72 SCC*mec*IV has been isolated in a Brazilian hospital (Schuenck *et al.,* 2009). Regarding food-related MRSA ST72, only two studies from Korea report its presence in the meat and milk production chain; the only genotype associated with milk, farmers and the farm environment was ST72/t324/SCC*mec* IV, whereas several genotypes were detected in domestic and imported meats (Lim *et al.,* 2013; Kim *et al.,* 2015). The identification of ST72 in buffalo milk products in Italy might be explained by the global trade in livestock animals or by the on-farm presence of extra-European operators carrying the strain; further on-farm research is required in order to clarify our finding.

The antimicrobial susceptibility tests have shown that human and buffalo BTM strains are resistant to a wide range of antimicrobials belonging to different classes, but it must be emphasized that all isolates in our study were susceptible to vancomycin. At present, multidrug resistant MRSA strains

of both animal and human origin are quite frequently found worldwide (Weese and van Duikeren, 2010; Parisi *et al.,* 2017). Interestingly, we found one buffalo MRSA isolate (the ST72 strain) resistant to tobramycin. This is an uncommon finding in MRSA animal isolates, since tobramycin is an antimicrobial rarely used in veterinary medicine, and its use in Italy is only allowed for pet animals (Italian Ministry of Health, 2019). This finding could be explained by the widespread diffusion of antimicrobial genes in the environment and in the bacterial population living on farms; in fact, antimicrobial-resistant bacteria can also be found in farmed animals where antimicrobials are never used (Gebreyes *et al.,* 2006; Mollenkopf *et al.,* 2014). On the other hand, the same strain was susceptible to tetracycline, although animal-associated MRSA is frequently resistant to tetracycline due to large-scale use of this antimicrobial in animal farming (Wendlandt *et al*., 2013). The susceptibility of the ST72 isolates to tetracycline reinforces the hypothesis that this isolate is a human-derived strain.

One buffalo milk strain was *mec*A positive/susceptible to the cefoxitin test (Table 2); given that *mec*A gene detection is considered the most reliable method for identification of MRSA. This finding confirms that phenotypic methods may not identify MRSA, which could raise concerns about the choice of an antimicrobial molecule for the treatment of mastitis; in fact, an antimicrobial treatment based on the results reached by the phenotypic methods could be incorrect as the molecule used might not affect the microorganism tested.

On the other hand, phenotypic oxacillin-susceptible *mecA* positive *S. aureus* (OS-MRSA) is known and has been isolated from cows with mastitis (Pu *et al.,* 2014). Further insights are required in order to clarify our finding.

With regard to the foodborne risk of staphylococcal food poisoning (SFP) linked to the presence of MRSA in raw buffalo milk, the results of our survey suggest that this risk is quite limited, considering that no isolates were enterotoxigenic. These findings contrast with those of other surveys on the occurrence of MRSA in cow's milk, where enterotoxigenic strains have been isolated (Haran *et al.,* 2012; Normanno *et al.,* 2007; Wang *et al.,* 2014). It is actually difficult to compare our findings with

other studies on the prevalence and characteristics of MRSA in buffalo milk, due to the scarcity of research regarding these issues.

Nonetheless, in a previous study, El-Ashker and colleagues reported the isolation of two MRSA strains from mastitic buffaloes belonged to the CC5/V and CC22/IV, carrying the genes encoded for SEA and SED and the *ecg* cluster, respectively (El-Ashker *et al*., 2015).

It is well known that food-related MRSA, particularly those isolated from milk, may be able to synthesize a biofilm, allowing their persistence and spread in dairy plants (Vergara *et al*., 2017). Biofilm formation by *S. aureus* is mediated by the intercellular adhesion operon (*ica*) (Gad *et al.,* 2009) and our milk isolates carry the *ica*A gene, which raises concerns about the potential hazard due to the presence of MRSA in dairy plants.

In conclusion, our findings suggest that contact with buffalo on the farm and the handling of raw milk during cheese production pose a potential threat to human health, as does the consumption of buffalo dairy products, since most are traditionally made using raw milk. In addition, raw buffalo milk could be considered a vehicle for the spread of antimicrobial resistant bacteria and their genes along the food chain. Our detection of MRSA in buffalo BTM samples confirms the potential zoonotic risk associated with direct contact with farm animals or the handling and consumption of raw buffalo milk and/or its dairy products, indicating the need to consider careful assessment of the related foodborne and occupational risks.

Tables

Species	Protein %	Fat %	Lactose %
Buffalo (*Bubalus bubalis*)	4.38 (min 3.44; max 6.29)	7.73 (min 4.90; max 13.39)	4.79 (min 2.95; max 6.10)
Cattle (*Bos taurus*)	3.42 (min 2.54; max 4.19)	4.09 (min 3.23; 5.34)	4.82 (min 4.40; max 5.33)
Sheep (*Ovis aries*)	5.73 (min 3.35; max 6.60)	6.99 (min 4.10; max 9.30)	4.75 (min 3.70; max 5.21)
Goat (*Capra hircus*)	3.26 (min 2.38; max 4.43)	4.07 (min 3.06; max 6.02)	4.51 (min 4.08; max 5.09)

Table 1. Chemical composition of milk from various animal species. From Barlowska *et al*., 2011

Isolates	Origin	*sau*	*mec*A	ST	*spa*-type	SCC*mec*	*ica*A	*luk*	*se*
1_{1439}	Buffalo milk	+	+	1	t127	IVa	+	-	-
2_{1440}	Worker	+	+	1	t127	IVa	+	-	-
3_{1442}	Buffalo milk	+	+	72	t3092	V	+	-	-
4_{1443}	Buffalo milk	+	+	1	t127	IVa	+	-	-

Table 2. Genotypic characteristics of MRSA isolates

Isolates	Disc diffusion test		Oxacillin agar screen test	Oxacillin E-test	Antimicrobial resistance profile
	Oxacillin	Cefoxitin			
1_{1439}	R	R	R	R	AUG-AMP-CTX-FOX-DXT-E-K-OX-P-S-ST-TE-SXT
2_{1440}	R	R	R	R	AUG-AMP-CTX-FOX-DXT-E-K-OX-P-S-ST-TE-SXT
3_{1442}	R	R	R	R	AMP-CTX-FOX-ENR-E-CN-K-OX-P-ST-SXT-TOB
4_{1443}	R	S	R	R	AMP-CXT-E-K-OX-P-S-ST-TE

Table 3. Phenotypic characteristics and antimicrobial resistance profile of MRSA isolate

Abbreviations.
R: resistant (disc diffusion test - oxacillin: 10 mm; cefoxitin: 21 mm).
AUG, amoxicillin/clavulanic acid; AMP, ampicillin; CTX, cefotaxime; FOX, cefoxitin; DXT, doxycycline; ENR, enrofloxacin; E, erythromycin; K, kanamicin; OX, oxacillin; P, penicillin; S, streptomycin; ST, sulfisoxazole; TE, tetracycline; SXT, trimethoprim-sulfamethoxazole; TOB, tobramycin.
Isolate numbers are in accordance with those in Table 1

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Part 2.

Could MRSA survive in the acidic conditions of the human stomach?

Abstract

It is well known that Methicillin resistant *Staphylococcus aureus* (MRSA) can enter the food chain and its presence in several kinds of food has suggested that it could act as a foodborne pathogen; although this hypothesis is interesting, there are still some unclear features for the hypothesis to be define. For instance, nothing is known about the ability of MRSA included in food, to overcome the acid-dependent bactericidal barrier of the human stomach. In this *in vitro* study we investigated the survival of MRSA inoculated in two foods of animal origin exposed to the simulated acidic conditions of the human stomach. To address this issue, a known amount (10^7 cfu mL^{-1}) of animal origin MRSA ST398/t011/V and of human origin MRSA ST1/t127/IVa strains were inoculated into *ricotta* cheese and hamburger samples. The pH of the matrices was gradually decreased from 6.0 down to 2.0 during a period of about 2 h, under conditions simulating gastric mechanical digestion and MRSA were recovered by MRSA-SELECT® (BioRad). Although both strains showed a certain acidic resistance, they showed different responses at the lower pH during the experiment: ST398 survived unharmed during the course of the experiments to the last stage at pH 2 where counts of 6.4 cfu/g for the hamburger and 7.5 log cfu/g for *ricotta* cheese assays were obtained at the end of the digestion. In contrast, the ST1 population was affected by the lower acidic levels with a different acidic resistance between the two matrices, being no longer detectable at pH 3 for the hamburger and at pH 2 for the *ricotta* cheese assays. This is the first study that has investigated the ability of MRSA to overcome the acidic conditions of the human stomach and add a new evidence that might contribute to expanding knowledge about the significance of MRSA in the food safety debate.

1. Introduction

Methicillin resistant *Staphylococcus aureus* (MRSA) is a widely known human and animal antimicrobial resistant bacteria responsible for mild to severe pathologies (Doulgeraki *et al.,* 2017). MRSA was considered almost exclusively a nosocomial pathogen for decades (Doyle *et al.,* 2012); later, a great number of studies reported the detection of MRSA in the community, from food producing animals and from humans in close contact with them, such as farmers, veterinarians, slaughterhouse workers (DeLeo *et al.*, 2010; Vanderhaeghen *et al.*, 2010; Khanna *et al.*, 2008; Weese *et al.,* 2010), suggesting its zoonotic role (EFSA, 2009). Moreover, both animal and human MRSA strains have been found in several foods of animal origin such as pork (de Boer *et al.,* 2009; O'Brien *et al.,* 2012), poultry (Feβler *et al.,* 2011; Hanson *et al.,* 2011), beef (Tenhagen *et al*., 2014) and horse meat (Parisi *et al.,* 2017), as well as in raw milk and dairy products (Normanno *et al*., 2007; Parisi *et al.,* 2016) as a consequence of animal contamination during slaughter and milking and human contamination during food handling. In addition, two food-related outbreaks (Kluytmans *et al.*, 1995; Jones *et al.,* 2002) and some cases of enterocolitis due to MRSA (Pressly *et al.,* 2016; Bergevin *et al.,* 2017) have been reported. The presence of MRSA at different prevalence in foodstuffs, has suggested the possibility of it being a foodborne pathogen; however, according to the European Food Safety Authority (EFSA), eating and handling food contaminated by MRSA is still considered a potential vehicle of transmission (EFSA, 2009). Although a study has supported the hypothesis of the zoonotic foodborne role of MRSA in such human infections (Larsen *et al.,* 2016), to date, there are too many missing pieces to complete the whole picture in order to define MRSA as foodborne pathogen. In fact, the first requirement for the enteric colonization during an active infection is the ability of the bacteria to circumvent the acidic environment of the stomach and pass into the intestinal tract (Smith, 2003; Gahan and Hill, 2005).

It is widely recognized that a normal gastric acidity by killing ingested pathogens (Donskey, 2004) may provide an important host defense against these microorganisms (Rao *et al.,* 2006), as previously

demonstrated for several gram-negative bacilli (Giannella *et al.,* 1972) and vegetative cells of *Clostridium difficile* (Wilson *et al.,*1985), as well as for nosocomial pathogens, such as *C. difficile, Candida albicans, methicillin-resistant Staphylococcus aureus* (MRSA), vancomycin-resistant *Enterococcus* spp. (VRE), and extended-spectrum-β-lactamase-producing *enterobacteriaceae* (Ben-Ami *et al.,*2006; Dial *et al.,* 2004; Dial *et al.,* 2005; McNeil *et al.,* 2006; Puzniak *et al.,* 2004).

A number of studies have examined the adaptation responses of *S. aureus* exposed to HCl stress in fermented foods (Bore *et al.,* 2007; Rode *et al.,* 2010), however, relatively little information is available regarding the ability of acidic conditions of the human stomach to kill ingested MRSA. The aim of this study was to evaluate the survival of two MRSA strains included in two foods of animal origin exposed to the human stomach environment by miming the gastric acid conditions and its mechanical digestion.

2. Material and Methods

2.1 Preparation of Methicillin resistant *S. aureus* (MRSA) *inoculum.* A MRSA ST398/t011/V strain, previously isolated from raw cow's milk, and a MRSA ST1/t127/IVa strain, previously isolated from human nasal swabs (Parisi *et al*., 2016), were individually suspended in 5 mL of BHI broth and incubated at 37°C for 24 h. The MRSA culture of the strains was prepared by resuspending for each one 20 µL of the pre-inoculum in 5 mL of fresh BHI broth incubated at 37°C for 24h. The MRSA concentration of each inoculum, subsequently used in the simulated gastric acidity experiment, was 5 mL x 10^7 cfu mL^{-1} according to the McFarland standard.

2.2 Preparation of food matrices. The fed medium was prepared as previously described by Barroso and colleagues (Barroso *et al.*, 2015). The medium contained arabinogalactan (1 g/L), pectin from apple (2 g/L), xylan (1 g/L), potato starch (3 g/L), glucose (0.4 g/L), yeast extract (3 g/L), peptone (1 g/L), mucin (4 g/L) and L-cysteine (0.5 g/L). Once the powders were dissolved, the mixture was then autoclaved, and the pH value was adjusted until it reached the value of 6.5.

Two foods of animal origin, *ricotta* cheese and hamburger, were used in the experiments. To exclude bias in the results, each food was tested prior to the experiment for the presence of *S. aureus* and MRSA using protocols described elsewhere (Parisi *et al.* 2016).

For the preparation of the experimental suspension (ES) used in this study, fifty grams of each matrix were individually added to 50 mL of fed medium at 37°C and homogenized for 10 min at 230 RPM using a stomacher at room temperature. Finally, 5 mL of each inoculum was added (5% of the volume) to each of the two prepared ES.

2.3 Gastric acidity experiment. The simulated gastric acidic experiment was performed as described by Haffner *et al*. (Haffner *et al.,* 2017) with slight modifications (*Fig 1*). Briefly, the pH of the samples was gradually decreased during a period of about 2 hours and periodically homogenized by using a stomacher miming the mechanical digestion of the stomach (Haffner *et al.,* 2017; Maisanaba *et al.,* 2018). In detail: after the MRSA inoculation into each prepared ES, and its first homogenization (230 RPM for 10 min) in a stomacher bag, the pH of the *ricotta* cheese and the hamburger (stabilized at pH 6.0 starting from pH 6.5 and pH 5.8, respectively) was decreased in steps from 6.0 (T0) to 2.0 (T4) by adding a specific amount of 1 M HCl (from 500 µL to 19000 µL). Each sample was shaken for 2 min at room temperature and incubated at 37°C for 15 min at each point of the experiment, except for the last one, when they were incubated for 30 min at 37°C, as suggested by Haffner *et al.* (Haffner *et al.,* 2017). For each food experiment a 50 mL amount of fed medium without food matrices inoculated with each MRSA strain was used as a control. Each control had the same initial pH as its matrix, and they were both processed under the abovementioned conditions.

Each experiment was carried out twice.

2.4 MRSA count. Appropriate serial dilutions of each sample at each sampling time, from T0 (inoculum time) to T4, were seeded onto plates of MRSA-SELECT® (BioRad) and incubated at 37°C for 24h.

2.5 Total Bacteria Count (TBC). A total bacterial colony count (TBC) was carried out on both non-inoculated *ricotta* cheese and hamburger at three different points during the experiment: at T_0 (pH 6), at T_2 (pH 4), at T_4 (pH 2) by using the Plate Count Agar (Microbiol) according to the protocol reported in the ISO 4833-1:2013 (ISO 4833-1:2013).

2.6 Statistical analysis. To compare the behavior of MRSA strains in both the matrices and the controls, the differences in MRSA average counts between the food matrices and in comparison, with their controls, were statistically tested with a Student-t-test ($0.05 < P < 0.10$).

To evaluate the variation in acidic resistance of each MRSA strain during the course of the experiment at each stage in the decrease of the pH values (from T_0 to T_4) in both the matrices and the controls the coefficient of variation (CV%) was used.

3. Results

3.1 MRSA count. The MRSA count of both strains showed a decrease during the course of the experiments, as reported in Table 1 and in Figures 1, 2, 3. The two MRSA strains showed a different variation in acidic resistance at given pH. In detail, in the controls and in both the matrices, the variation in acidic resistance of ST398 corresponded to a coefficient of variation under the factor 10, while for ST1 it was over the factor 10^2.

The survival threshold of both strains was higher in the *ricotta* cheese than into the hamburger (*Fig 2, 3, 4)*. A count of log 7.5-7.6 cfu/g of ST398 was still obtained at T_4 (pH 2) in the controls and in the *ricotta* cheese, respectively. The most significant decrease for ST398 was recorded at T_3 (pH 3) in the *ricotta* cheese experiment and also in the hamburger experiment, in which we recorded one log cfu/g less than its concentration at its initial pH (T_0; pH 6.0) (*Tab 1*).

Similarly, ST1 strain in the *ricotta* cheese was detectable with slight decreases during the course of the experiment until T_3 (pH 3), when a count of 7.6 cfu/g was still obtained. After that, it was no

longer detectable (T_4; pH 2.0). In contrast, in the hamburgers there was found to be a significant decrease, with a count of log 6.5 cfu/g, at T_2 (pH 4), after which it was no longer detectable (*Tab* 1). A statistically significant difference between the behavior of the ST398 in the *ricotta* cheese and in the hamburgers, as well as in the food matrices compared to their relative controls, was found. In contrast, the ST1 population, showed no statistically significant difference, keeping the same acidic resistance during the course of the experiments both when it was included in a solid matrix (*ricotta* cheese or hamburger) and when it was in the fed medium.

3.2 Total Bacteria Count. Total bacterial count showed a decrease during the course of the experiment. In detail, at T_0 the total bacterial counts, in the *ricotta* cheese and the hamburger experiment, ranged between log 7.4 and 7.0 cfu/g, respectively. At T_2 (pH 4) the total bacterial count, in both matrices, kept the same values with a light increment for the *ricotta* cheese (log 7.6 cfu/g) and a slight decrease for the hamburger (log 6.9 cfu/g). At T_4 (pH 2), total bacterial counts were no longer detectable in either of the matrices.

4. Discussion

S. aureus and its methicillin-resistant variant (MRSA) are microorganisms which have a great impact on both human and veterinary medicine (WHO, 2014); its marked adaptability and its coevolution with its host(s) enable it to be successful as an opportunistic pathogen and to be resistant to changing environments (Clement *et al.,* 1998). *S. aureus*, is also a major foodborne pathogen, representing the leading source of foodborne intoxication (Le Loir *et al.,* 2003; Fetsch and Johler, 2018). In addition, MRSA has been identified as an important cause of enterocolitis especially in hospitalized patients and in those who have a decreased gastric acidic production (Pressly *et al.,* 2016). The detection of MRSA in a variety of foods of animal origin, as a consequence of animal and/or human contamination (Normanno *et al.,* 2007), launched a scientific debate on its role in causing infections *via* food

consumption, but the survival of MRSA in the acidic conditions of the human stomach has not yet been investigated.

Assuming that the gastric bactericidal barrier is primarily acid dependent (Drasar, 1969; Hornick, 1971; Peterson, 1999) because the low pH is able to control bacterial population in gastric environment (Smith, 2003), we investigated the ability of MRSA to overcome the human gastric barrier by miming the acidic conditions of the stomach.

Considering that MRSA has been detected in meats and cheeses, in our experiments we hypothesized a contamination of hamburger and *ricotta* cheese samples with animal and human MRSA strains and to evaluate their fate under the acidic environment of the human stomach; we have chosen a ST398 strain because of its zoonotic ability (Van den Eede *et al*., 2013) and a ST1 strain, known as a human pathogen (Monaco *et al.,* 2013).

A long-term mechanical homogenization of each matrix was performed in order to recreate the conditions in which food arrives (as *bolus*) in the proximal part of the stomach after the oral chewing and the transit through the esophagus (Kong and Singh, 2008). According to Haffner *et al.* (Haffner *et al.,* 2017) and on the basis of the human gastric digestive phases, the MRSA population was thus exposed to a decreasing pH for different incubation times right down to the last step (T_4; pH=2), when the time of exposure to the lower acidic environment was doubled (from 15 to 30 minutes). In fact, solid foods initially remain in the proximal part of the stomach while liquids are passing into the duodenum (Pal *et al.,* 2007; in a second phase, food particles are mixed, pumped out of the *atrium* and moved from the fundus to the duodenum by propelling actions (Kong and Singh, 2008). Although the remarkable decrease of the TBC, which confirmed the efficacy of our procedure, MRSA ST398 survived unharmed during the course of the entire experiment with a slight decrease from the higher permissive pH value (pH=6.0) to the final stage at pH 2. In contrast MRSA ST1 showed a dramatic reduction during the course of the experiments.

Although not strictly comparable, these results confirmed what previously reported on the resistance of *S. aureus* under acid conditions. For example, Chan and colleagues, reported that *S. aureus* is

rapidly killed by acid (pH 2) but it is able to resist and adapt to acidic stress if it is first exposed to a higher, non-lethal pH (Chan *et al.* 1998; Smith, 2003); moreover, Rao and colleagues observed a significant *in vitro* killing of MRSA exposed to the lower gastric acid (pH 1 and 2) (Rao *et al.,* 2006). On the other hand, a study of acidic stress induced by both non-permeant inorganic acid (HCl) and weak-permeant organic acid showed that *S. aureus* was affected the most by the organic acid, at the same pH (Lund *et al.* 2000). Non-permeant acids do not affect the pH of the cytoplasm as much as weak permeable acids, and microorganisms are generally more sensitive to the internal pH modification than to a change in external pH (Beales, 2004), confirming the role of the low-pH-control in reducing or inhibiting the growth of certain bacteria in food (Rode *et al.* 2010).

The ST1 strain showed a different acidic resistance under the lower pH in both the matrices and their relative controls. This behaviour could be explained by the different composition of the food matrices, considering that food nutrients, especially the fat content, play a protective role for microorganisms against the acidic stress (Drouault, *et al.* 1999). However, although the hamburger used in this study was composed by a fat content (16%) higher that the *ricotta* cheese (11.6%), ST1 population showed less acidic resistance in the hamburger experiment, where it was not detectable at the T_3 (pH 3) than in the *ricotta* cheese experiment, where it was significantly affected by the low pH at T_4 (pH 2) (*Tab.* 1). Further studies are needed to explain this behaviour.

Further studies need to be carried out in order to explain the behavioural difference between *S. aureus* and MRSA strains, and between the different MRSA strains under acidic conditions, as well as to assess the role of the *mec*A and other antimicrobial-resistance genes in this finding.

To the best of our knowledge, this is the first study which investigates the behaviour of MRSA strains in the acidic conditions of the human stomach. Although we detected differences between the acidic resistance of the two MRSA strains used in our experiments, our results demonstrate that certain strains of MRSA have a strong (prob)ability of surviving under acidic stress conditions. As consequence, they could pass the gastric barrier and reach the bowel where they could cause an active infection (Watanabe *et al.,* 2001; Pressly *et al.,* 2016; Bergevin *et al.,* 2017). In conclusion, our

results add new knowledge about the fate of MRSA in the acidic condition miming the human stomach; these findings may contribute to better define its role in the food safety debate.

Tables and Figures

		Control				*Ricotta* cheese				Hamburger			
		ST 398		ST 1		ST 398		ST 1		ST 398		ST 1	
T	pH	Log	R	Log	R	Log	R	Log	R	Log	R	Log	R
T_0	6	7.7	0.1	7.4	0.4	7.9	0.4	7.9	0.3	7.5	0.4	7.5	0.3
T_1	5	7.5	0.8	7.4	0.1	7.7	0.5	7.5	0.3	7.2	0.9	6.3	0.3
T_2	4	7.6	0.2	6.1	0.3	7.8	0.3	7.4	0.2	7.2	0.4	6.7	0.4
T_3	3	7.6	0.1	0.0	0.0	7.8	0.2	7.6	0.1	6.9	0.2	0.0	0.0
T_4	2	7.5	0.3	0.0	0.0	7.6	0.2	0.0	0.0	6.5	0.0	0.0	0.0

Table 1. MRSA ST398 and ST1 counts at each given pH level in the *ricotta* cheese, hamburger and control assays.

R= range (of variation) between the two repetitions of each assay.

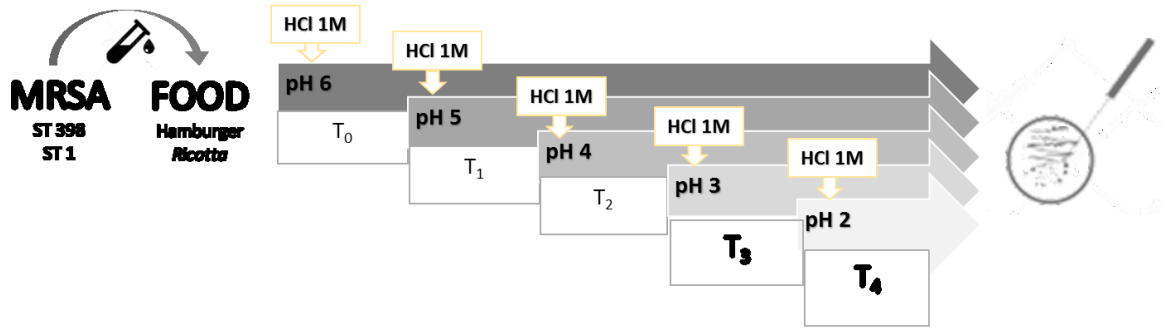

Figura 1. Experimental design. *Drawn by Dr.* Elisa Spinelli

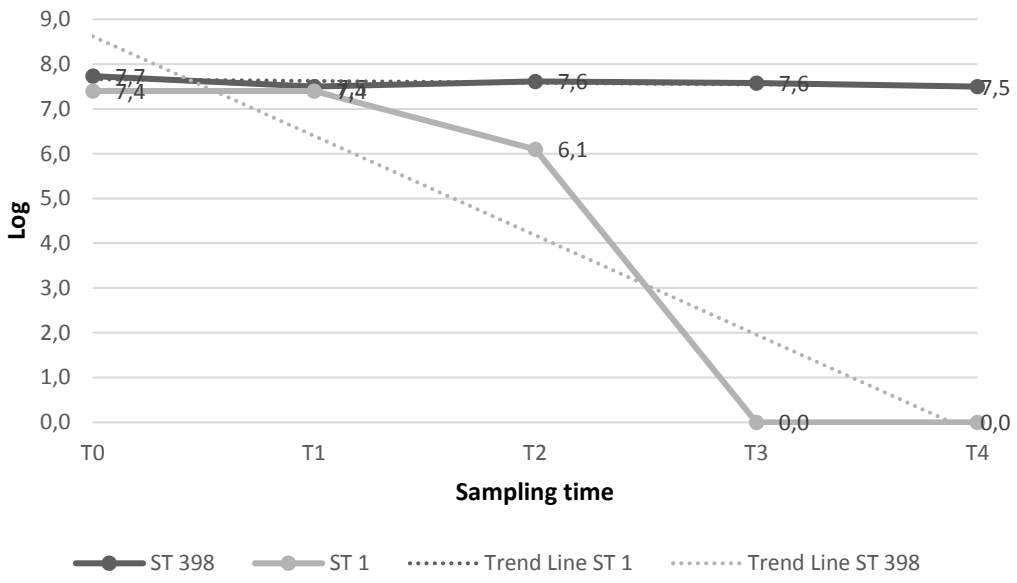

Figure 2 MRSA ST 398 and ST1 counts under acidic stress conditions: control.

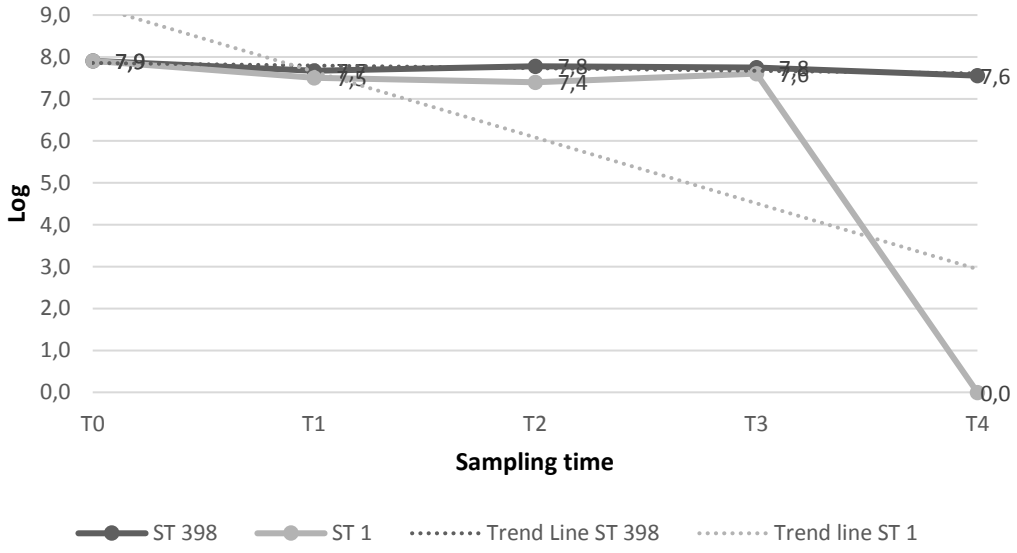

Figure 3 MRSA ST 398 and ST1 counts under acidic stress conditions: ricotta cheese.

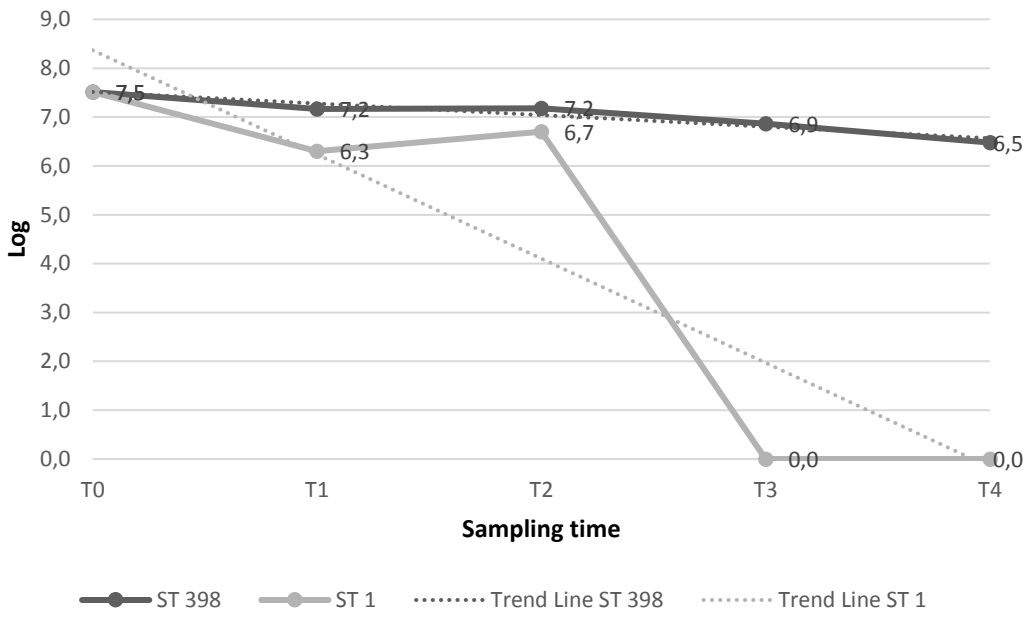

Figure 4 MRSA ST 398 and ST1 counts under acidic stress conditions: hamburger.

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Part 3.

Fate of livestock associated Methicillin-resistant *Staphylococcus aureus* (MRSA) during the transit along the human intestinal tract and its interaction with gut microbiota: an *in vitro* study

Abstract

Intestinal colonization by methicillin-resistant *Staphylococcus aureus* (MRSA) has been associated with an increased risk of staphylococcal infection. In fact, intestinal mucus layer may provide a niche for many nosocomial pathogens, including *S. aureus* which can occasionally cause a staphylococcal enterocolitis. Recent exciting researches support the notion that a healthy intestinal microbiota composition can promote resistance to invading pathogenic bacterial species.

The aim of this study was to track the intestinal survival of MRSA along the transit in human intestinal tract by using an *in vitro* dynamic simulator. To address this issue, a known amount (10^7 cfu mL^{-1}) of an animal origin MRSA ST398/t011/V strain, previously isolated from raw cow milk, was inoculated within the dynamic multistage BFBL gut model and it was periodically analyzed its fate along the whole transit. A three-day *in vitro* study was performed using microbiota from the pooled faeces of healthy individuals that were stabilized simulating ascendant, transverse and descendent colon conditions. Each day we checked the viability of MRSA in the SI and in each colon reactor. The results were confirmed by quantitative PCR. The same MRSA strains levels were inoculated within a vessel simulating the human ascendant colon conditions, where mucin agar carriers replaced the intestinal mucus layer and a basic feed medium represented the intestinal lumen contents. A three-day in vitro study was performed using microbiota from the pooled faeces of healthy individuals that were stabilized simulating ascendant colon conditions. Each day we checked the viability of MRSA both into the mucin agar carriers and in the feed medium.

Besides monitorization of MRSA ST398/t011/V strain, products of fermentative metabolism in the colon reactors were also quantified: SCFA (acetic, propionic, butyric and formic acids) as well as lactic acid (fermentative metabolism) and ammonium (derived from proteolytic metabolism). The MRSA population was affected by the organic acids produced by the enteric microbiota along the transit into the simulator as well as during the incubation with luminal microbiota of the ascendent

colon, where it was not viable after 24 h. However, counts of 4 log cfu/g were still obtained in the mucin agar carriers after 72 h of incubation. The results support the hypothesis that a competitive microbiota may control MRSA intestinal colonization empathizing the important role of specific groups, such as *Bifidobacterium* and *Akkermansia,* which can inhibit the adhesion of/displace MRSA from the intestinal mucus layer.

1. Introduction

The abuse/misuse of antimicrobials in food producing animals has led to the selection of antimicrobial-resistant zoonotic foodborne bacterial pathogens which can be transmitted to humans *via* contaminated food (White *et al*., 2002).

The contamination of several raw food, especially meat and milk, by MRSA is well documented (de Boer *et al.,* 2009; O'Brien *et al.,* 2012; Feβler *et al.,* 2011; Hanson *et al.,* 2011; Tenhagen *et al.,* 2014; Normanno *et al.,* 2007; Parisi *et al.,* 2016), as well as, the transmission of zoonotic MRSA to humans *via* animal contact (DeLeo *et al.*, 2010; Vanderhaeghen *et al.*, 2010; Khanna *et al.*, 2008; Weese *et al.,* 2010). In addition, asymptomatic MRSA food handlers are an additional source of food contamination, sometimes causing food poisoning outbreaks (Weese *et al.,* 2010; Kluytmans *et al.,* 1995; Jones *et al.,* 2002).

The colonization of human intestinal tract by MRSA has been described (Singh *et al.,* 2003; Gries *et al.,* 2005; Rosenthal *et al.,* 2007; Acton *et al.,* 2009; Dulon *et al.*, 2014). However, there is insufficient evidence of an increased risk of human colonization or infections associated with contact or consumption of food contaminated by MRSA; it is, in fact, still unclear whether these microorganisms can act as foodborne pathogens (EFSA, 2009).

The intestinal carriage of *S. aureus* has been associated with negative health effects, such as antibiotic associated diarrhoea (Ackermann *et al.,* 2005; Boyce and Havill, 2005), the inflammatory bowel

disease (Lu *et al.,* 2003) and an increased risk of staphylococcal infections (Gries *et al*., 2005); but the clinical implications linked to the intestinal colonization of MRSA are still largely unknown.

Despite the protective role of the commensal intestinal microbiota from ingested antibiotic-resistant microorganisms (Buffie and Pamer, 2013), several food-ingested bacteria are able to temporarily integrate into the gut microbiota, to affect the biological intestinal *equilibrium* and to cause intestinal and/or extraintestinal diseases (Derrien *et al.,* 2015).

The aim of this study was to determine the fate of MRSA along the human intestinal tract and its interaction with the gut microbiota, by using the dynamic multistage BFBL gut model. The results could be useful to better understand the ability of MRSA to cause intestinal and/or extraintestinal human diseases *via* food consumption.

2. Material and methods

2.1. Growth conditions of MRSA *inoculum*

A MRSA ST398/t011/V strain, previously isolated from raw cow milk (Parisi *et al*., 2016) was suspended in 5 mL of BHI broth and incubated at 37°C for 24h. The MRSA culture of the strain was prepared by resuspending 20 µL of the pre-inoculum in 5 mL of fresh BHI broth incubated at 37°C for 24 h. The MRSA concentration of the *inoculum*, subsequently used in the BFBL gut model, was 5 mL x 10^7 UFC g^{-1} according to the McFarland standard.

2.2. Preparation of the nutritive medium and mucin agar carriers

The three colon reactors were filled and pre-conditioned with nutritive medium in a volume of 250, 400 and 300 mL, respectively. The nutritive medium was prepared as previously described by Barroso *et al.* (2015). In detail, the medium contained arabinogalactan (1 g/L), pectin from apple (2 g/L), xylan (1 g/L), potato starch (3 g/L), glucose (0.4 g/L), yeast extract (3 g/L), peptone (1 g/L), mucin (4 g/L) and L-cysteine (0.5 g/L). In order to give to the simulator an overall residence time of 76 h,

the nutritive medium of each colonic reactor was adapted as the conditions standardized for the SHIME model (De Boever, Deplancke and Verstraete, 2000; Barroso *et al.*, 2015).

In the current experiment, the AC bioreactor also contained 12 mucin carriers together with the luminal phase stabilized at the ascendant colon conditions. According to Liu and colleagues (2018) the mucosal carriers provide a mucin surface for bacterial to grow. In this way, the mucosal surface of the intestines can be simulated. Mucin-Agar containing carriers were prepared by dipping plastic, hollowed carriers (DI, 5 mm) into a mucin-agar solution, as previously described by Liu and colleagues with slight modifications (Liu *et al.*, 2018). Briefly, the Mucin-Agar solution was prepared by boiling 1.5% bacterial agar in autoclaved MilliQ water three times, to dissolve 5% type II porcine mucin (Sigma-Aldrich). The filled carriers were solidified under laminar flow in a biosafety cabinet at room temperature and stored at -3 °C until use.

2.3. Dynamic simulation of intestinal conditions by using the BFBL gut model

The BFBL gut model is constituted by four compartments (glass reactor vessels), simulating *in* vitro the small intestine (SI) and the microbial conditions of the ascending (AC), transverse (TC) and descending colon (DC) tracts, which are interconnected by pipes and peristaltic valve pumps (Watson-Marlow 120 U/DV) that transfer the content between the successive units (*Fig 1*).

The four above mentioned reactors (SI, AC, TC and DC) are continuously stirred by magnetic stirrers (150 rpm). Each vessel contains different point of access for the transit of intestinal content, a continuous flushing of nitrogen, sampling points, and pH and temperature probes. The temperature was kept at 37 °C. The pH in the colonic units was controlled by addition of 0.5 M NaOH and 0.5 M HCl to keep values of 5.6 ± 0.2 in the AC, 6.3 ± 0.2 in the TC and 6.8 ± 0.2 in the DC. All the vessels were maintained under anaerobic conditions by continuously flushing N2. The main software (master) controls the addition of the pancreatic juice to the SI unit and the transit times for intestinal content transfer to the SI, AC, TC and DC units.

A three-day-long experiment was carried out using the abovementioned MRSA strain and the microbiota from the pooled faeces of healthy individuals that were stabilized simulating ascendant, transverse and descendent colon conditions, as previously reported by Barroso *et al.,* 2015.

At the beginning of the experiment, each colon reactor was all simultaneously inoculated by 20 mL of a fresh 20% (w/v) faecal slurry from a healthy volunteer who had no received any antibiotic treatment in the previous 3 months of the experiment, prepared in anaerobic conditions with sodium phosphate buffer (0.1 M, pH 7.0), containing 1 g/L sodium thioglycolate as reducing agent, as described by De Boever *et al.* (2000). The bacterial species included in the used gut microbiota and their own load are listed in Table (1). The colonic microbiota was allowed to stabilize, reaching the steady state after 2 weeks (stabilization period), as described by Barroso *et al.* (2015).

After the *inoculum* (5 mL x 10^7 UFC g^{-1}) of MRSA into the small intestine (SI), the digestion was performed during 2 h at 37 °C and the content of the SI vessel was automatically transferred to the following colon compartment (AC) at a flow rate of 5 mL/min, which simultaneously activated the transit of colonic content between the AC, TC and DC compartments at the same flow rate.

At the time of sampling for each colon region vessels, a microbiological plate count analysis and a quantitative PCR (qPCR) was performed. The experiment was carried out twice.

2.4. The metabolic context of the colonic reactors

Short Chain Fatty-Acids (SCFA) of each colon compartments were determined following the method described by Sanz *et al.* (2005). Data acquisition and processing was carried out using a ChromNAV Data System software (Jasco). Calibration curves of acetic, propionic, butyric and lactic acid were built up in the range concentration of 1e100 mM. Ammonia was determined from the supernatant fraction of samples (13,000 x g, 15 min, 4 °C) using an enzymatic kit for ammonia determination (R-Biopharm).

2.5. The BFBL gut model: MRSA count and molecular confirmation of MRSA

Appropriate serial dilutions of each sample, from T0 (*inoculum* time) to T4 (48 h) were seeded onto plates containing a selective agar media MRSA-SELECT® (BioRad) and incubated at 37°C for 24-48-72h. A quantitative PCR (qPCR) targeting *sau* and *mec*A genes was performed to confirm the results.

2.6 MRSA interspecies interactions into the mucus layer of ascendant colon

The study was performed at ascendant colon environment: body-like temperature (37°C), anaerobiosis (N2), pH 5.7, constant slow shaking (40 RPM). Mucin agar carriers stand for the intestinal mucus layer and a basic feed medium represented the intestinal lumen contents. A three-day-long in vitro study was performed by using microbiota from pooled faeces of healthy individuals that were stabilized simulating ascendant colon conditions and a MRSA strain of animal origin (ST398-t011-SCCmecV; 107 UFC/mL). Each day we checked the viability of MRSA both into the mucin agar carriers (the mucosal samples) and in the feed medium (the luminal samples) by using MRSA-SELECT® plates (BioRad). The results were confirmed by quantitative PCR.

3. Results

3.1. The microbiological and metabolic context of the colonic reactors

Tables 1 and 2 shows the results of the SCFA and ammonia contents, and the microbiological context in each reactor (AC, TC, DC), respectively, during the course of the experiment.

3.2. MRSA count and molecular confirmation of MRSA

Into the BFBL gut model: The MRSA ST398/t011/V strain count showed a decrease during the course of the experiments, as reported in Table 3.

In detail, the first day of the experiment at T0 (*inoculum* time, before the transit) MRSA was present into the SI vessel (Log 7.7 cfu/mL), but it was not yet transferred into the colon reactors. After 2 h of

incubation into the SI, during the course of the transfer (T1) a MRSA count of Log 6,1 and Log 5.4 cfu/mL was found in the AC, TC and DC respectively. After that, in the AC reactor MRSA count decreased as a function of time, being no longer detectable until the end of the experiment. On the other hand, MRSA was still found after 5 h (T2) in TC (Log 4.2 cfu/mL) and DC (3 cfu/mL) reactors. Finally, at 24 h (T3) a MRSA count of Log 2 and 3 cfu/mL was still obtained in TC and DC reactors, respectively, being no longer detectable in both the reactors at 48 h (T4).

Into the ascendant colon: MRSA population decreased as a function of time during the incubation with luminal colon microbiota where it was not viable after 24 h. Counts of 4 log cfu/g were still obtained in the mucin agar carriers after 72 h of incubation. On the other hand, counts *of Bifidobacterium* and *Akkermansia* increased in the mucin agar carriers as a function of time.

4. Discussion

It is generally recognized that MRSA, especially the livestock associated MRSA clone CC398, could be present in several food products, suggesting food as a pathway for MRSA dissemination (Oniciuc *et al.,* 2017). After the consumption, the food-ingested bacteria that overcome the acid bactericidal barrier of the stomach, enter the small intestine. Here, more permissive pH values (>6), unable the recovery and even the growth of the survived cells which might continue in the colon (Derrien *et al.,* 2015). MRSA has been shown to be resistant to a high pH in gastric juice (Milton-Thompson *et al.,* 1982; Ruddell and Losowsky, 1980), and when it moves downward and proliferates in the lower digestive tract, MRSA enteritis can easily occur (Takesue *et al.,*1993). Moreover, people are the natural reservoir of MRSA, but its growth and reproduction are limited by other intestinal flora (Takesue *et al.,*1993; Wei *et al.,* 2015). It is, in fact, generally recognized that one of the major functions of a healthy intestinal microbial community is to protect against colonization by pathogens and to restrain their growth (Kamada *et al.,* 2013). However, disturbances of the microbiota (dysbiosis) caused by antibiotics can lead to an increase in antibiotic resistant organisms such as

vancomycin-resistant *enterococci* and MRSA (Jernberg *et al.,* 2010; Wei *et al.,* 2015). In our study, MRSA ST398/t011/V was detected, with the highest value (Log 7.7 cfu/mL), into the SI vessel at T0. During the course of the transfer (T1), a MRSA count of Log 6.1 and Log 5.4 cfu/mL was found in the AC, TC and DC respectively. Thereafter, MRSA count decreased as a function of time, until being no longer detectable in the lumen of the AC reactor 5 h post-administration (T2). On the contrary, it is noteworthy that count of 4 log cfu/g was still obtained in the mucin agar carriers after 72 h of incubation in the AC, suggesting that the mucosal carriers might have provided a mucin surface for bacterial to grow (Liu *et al.,* 2018). Moreover, despite the loss of bacterial viability during the transit along the colon reactors, MRSA was found in the TC and DC vessels at T2 and a small number of the bacterium was still detected after 24 h in the two reactors. However, since the strain was not recovered after 48h *post*-administration, it can be assumed that although MRSA ST398/t011/V survived the passage through the intestinal tract, it was not able to colonize the reactors. In agreement with the results previously found by Ceuppens and colleagues (2012), our findings suggest that MRSA was able only to maintain itself temporarily in competition with the intestinal bacteria, and its permanence in each vessel, followed by a progressive decrease along the transit, could be explained by the possible effect of the washing out of the machine. Many *in vitro* and *in vivo* studies support the notion that, ranging from direct inhibition to indirect pathways through the stimulation of immune system, certain commensal bacteria can confer resistance against pathogens, including *S. aureus* (Pascual *et al.,* 1999; Van Coillie *et al*., 2007; Lawley and Walker, 2013; Zipper *et al.,* 2016; Ubeda *et al.,* 2017). For example, it is known that the production of short chain fatty acids (SCFA) may suppress the growth of the pathogenic *E. coli* strain O157:H7 (Shin *et al.,*2002) as well as butyrate and propionate are able to downregulate the expression gene responsible for invasion of intestinal epithelial cells in *S.* Typhimurium (Lawhon *et al.,* 2002; Gantois *et al.,* 2006). Based on the aforementioned studies, we expected the inhibition of MRSA due to the metabolic environment, especially the pool of acetate, of the reactors.

It is, in fact, known that SCFA-dependent toxicity involves the entry of nonionized acids into the bacterial cytoplasm (Sun *et al.,* 2013). This mechanism depends on the luminal external pH as well as the cytoplasmatic pH of bacterial cells (Lambert and Stratford, 1999; Repaske and Adler, 1981; Russell, 1992; Salmond, Kroll, and Booth, 1984). When external pH is low, despite the intrinsic impermeability of the bacterial membrane (Raven and Beardall, 1981), a higher transmembrane pH gradient with acid influx may occur, resulting in a more susceptibility to SCFA toxicity (Diez-Gonzalez and Russell, 1997; Russell, 1991; Sun *et al.,* 2013). Further studies are needed for understand the beahvoiur of human MRSA strains.

Tables And Figures

Phylum	Bacterial group	Compartment	Media
Firmicutes	*Blautia coccoides*	AC	8,12 ± 0,43
		TC	7,96 ± 0,35
		DC	7,73 ± 0,17
	Roseburia	AC	nd
		TC	4,84 ± 0,58
		DC	4,52 ± 0,65
	Faecalibacterium	AC	nd
		TC	8,12 ± 0,31
		DC	7,93 ± 0,47
	Enterococcus	AC	5,53 ± 0,20
		TC	5,56 ± 0,46
		DC	5,75 ± 0,61
	Ruminococcus	AC	nd
		TC	5,36 ± 0,43
		DC	5,21 ± 0,43
	Lactobacillus	AC	5,54 ± 0,90
		TC	5,46 ± 0,61
		DC	5,52 ± 0,69
	Clostridium leptum	AC	nd
		TC	7,10 ± 0,30
		DC	7,01 ± 0,37
Proteobacteria	*Enterobacteriaceae*	AC	8,04 ± 0,49
		TC	7,82 ± 0,53
		DC	7,79 ± 1,00
Actinobacteria	*Bifidobacterium*	AC	7,33 ± 0,59
		TC	7,13 ± 0,52
		DC	7,16 ± 0,64
Bacteroidetes	*Bacteroides*	AC	8,04 ± 0,95
		TC	8,24 ± 0,29
		DC	7,56 ± 0,73
	Prevotella	AC	3,89 ± 0,68
		TC	3,91 ± 0,87
		DC	3,84 ± 0,77
Others*	*Akkermansia*	AC	4,74 ± 0,33
		TC	7,86 ± 0,45
		DC	8,06± 0,30

*Others: *Verrucomicrobia.* Typically, less abundant members of the gut microbiota

Tab 1. Microbiological context of the colonic reactors

Compound	Compartment	Media
Total SCFA	AC	38,97 ± 15,62
	TC	63,06 ± 9,10
	DC	68,92 ± 8,53
Acetic acid	AC	11,01 ± 6,79
	TC	33,66 ± 0,98
	DC	43,42 ± 4,76
Propionic acid	AC	4,82 ± 1,63
	TC	8,01 ± 1,05
	DC	10,53 ± 1,56
Butyric acid	AC	8,15 ± 1,54
	TC	10,45 ± 1,46
	DC	8,84 ± 1,60
Lactic acid	AC	1,19 ± 0,55
	TC	0,27 ± 0,25
	DC	0,65 ± 0,11
Ammonium	AC	6,50 ± 1,24
	TC	11,82 ± 0,72
	DC	13,58 ± 1,04

Tab 2. Metabolic context of colonic reactors

T	h	Media			qPCR *mec*A		
		AC	**TC**	**DC**	**qAC**	**qTC**	**qDC**
T0	**0**	0	0	0	0	0	0
T1	**2**	6,9	6,1	5,4	6,9	6	3,8
T2	**5**	0	4,2	3	6,8	5,8	4,8
T3	**24**	0	2	3	5,4	5,9	5,8
T4	**48**	0	0	0	5,2	5,9	5,9

Tab 3 MRSA count and molecular confirmation by targeting *mec*A gene

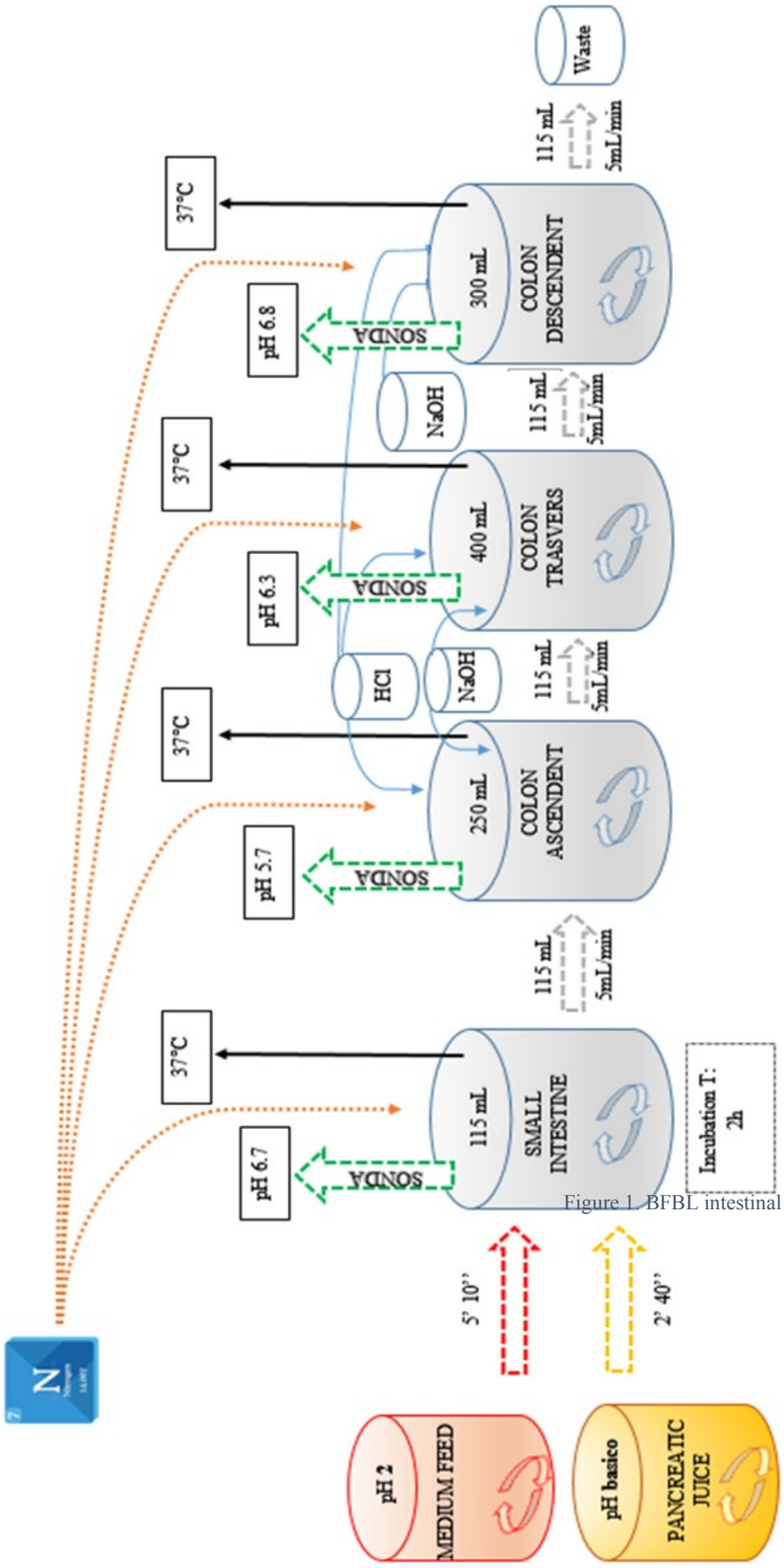

Figure 1. BFBL intestinal simulator (CIAL-CSIS). Drawn by E

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Concluding Remarks

Being an extraordinarily adaptable pathogen with the proven ability to develop resistance, MRSA, a virulent and difficult-to-treat "superbug," is now considered one of the top-priority antimicrobials-resistant pathogens.

Our detection of MRSA in buffalo BTM samples confirms the potential zoonotic risk associated with direct contact with farm animals or the handling and consumption of raw buffalo milk, indicating the need to consider careful assessment of the related foodborne and occupational risks. In addition, raw buffalo milk could be considered a vehicle for the spread of antimicrobial resistant bacteria and their genes along the food chain.

Ingested pathogens, including MRSA, transmitted from contaminated foods, enter the body *via* the gastrointestinal (GI) tract. The chance and severity of illness may be different among people eating the same contaminated food, depending on an overwhelming dose of pathogens and/or the virulence of the pathogen, and the health status of the host. The first requirement for the enteric colonization during an active infection is the ability of the bacteria to circumvent the acidic environment of the stomach and pass into the intestinal tract, where the intestinal homeostasis is particularly relevant in the host defence. Our *in vitro* study on the fate of MRSA during the human gastrointestinal transit, confirmed the notion that competitive gut microbiota in a healthy adult may control food MRSA intestinal colonization. In fact, despite the ability of MRSA to overcome human stomach acidic conditions, the LA-MRSA strain used in this study was affected by the organic acids produced by the enteric microbiota along the transit into the human intestinal simulator. Further studies are needed to analyse the behavior of human MRSA strains.

Publications

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Poster Presentation

Spinelli E., Normanno G. Model for the survival of Methicillin-Resistant *Staphylococcus aureus* (MRSA) in aged cheese. Risk Assessment Research Assembly (RARA) EFSA, 7 February 2018, Utrecht, Netherlands.

Spinelli E., Caruso M., Santagada G., Parisi A., Barlaam A., Normanno G. Methicillin resistant *Staphylococcus aureus* (MRSA) in raw buffalo milk from Italy: preliminary results. V International Conference on Antimicrobial Research (ICAR), 24-25 May 2018, Torremolinos, Spain.

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Oral Presentation

Model for the survival of Methicillin-Resistant Staphylococcus aureus (MRSA) in aged cheese. Risk Assessment Research Assembly (RARA), 7 February 2017, Utrecht, Netherlands.

Methicillin-resistant *Staphylococcus aureus* (MRSA) in food of animal origin: professional and foodborne risk. New infectious and parasitic risks in the food chain, 22 November 2018, University of Foggia, Puglia, Italy.

Methicillin resistant *Staphylococcus aureus* (MRSA) in raw buffalo milk and its fate along the human gastrointestinal tract. Higher Education for Sustainable Food Production 1st Joint Meeting of Agriculture-Oriented PhD Programs at UniCT, UniFG, and UniUD, 17-21 June 2019, University of Catania, Sicily, Italy.

Methicillin Resistant *Staphylococcus aureus* (MRSA) ST 398 and gut microbiota: interspecies interactions into the mucus layer of ascendant colon. 14th International Conference on Microbial Interactions & Microbial Ecology. 19-20 August, Vienna, 2019 Austria.