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PROTEOMICS OF DIFFERENT CLINICAL ISOLATES OF STAPHYLOCOCCUS AUREUS

(VET05)

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I dedicate this work to all the people who never stop believing in me and who along with god, have been my ‘footprints in the sand’

My mother

My wife

My bother Mohamed

My brother Hussein

My beloved baby, Ahmed

And lastly to my Father spirit, who taught me to get up after a fall and start again.

They are the true roses of my life………..

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Summary

S. *aureus* causes severe infection even if it is mainly a harmless bacterium. It exists in a oxacillin-resistant (meticillin-resistant S. aureus, MRSA) form that represents the most important cause of antibiotic-resistant healthcare-associated infections worldwide. Most MRSA strains contain Panton-Valentine leukocidin (PVL) genes, that encode for a citotoxin that is one of the essential components of the virulence mechanisms of S. *aureus*. Furthermore, the presence of PVL is also associated with increased virulence of certain strains (isolates) of *S. aureus*. Although clinicians are currently concerned primarily with MRSA infections, methicillin-susceptible S. *aureus* (MSSA) infections can be present with similar epidemiologic and clinical characteristics when are PVL positive. In this study, 2-DE coupled with mass spectrometry has been used to compare protein profiles of (PVL+) and (PVL-) MRSA and MSSA S. *aureus* in order to highlight protein differences related to PVL presence or absence. Bacterial samples were isolated from human infections and classified on the basis of PCR analysis and antibiotic susceptibility test. Quantitative two dimensional electrophoresis was performed on MSSA and MRSA samples further classified in PVL+ and PVL-. Experiments were done in triplicate using custom IPG strips pH 4-5.5 to optimize spot resolution and to visualize different isoforms on gels. Image and statistical analysis were performed with Progenesis SameSpots software (Nonlinear Dynamics), proteins differentially expressed (ANOVA $p<0.05$) were identified by MALDI-TOF-TOF for identification. 2-DE image analysis revealed the differential expression of several proteins involved in iron metabolism and oxidative stress.

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Abbreviations

1D	One-dimensional
2D	Two-dimension
2D-PAGE	Two dimensional polyacrylamide gel electrophoresis
ADHs	Alcohol dehydrogenases
AdoMet	S-adenosylmethionine
ADP	Adenosine-5'-diphosphate
ahp	alkyl hydroperoxide reductase
ATP	Adenosine-5'-triphosphate
CaCl2	Calcium Chloride
CA-MRSA	Community acquired MRSA
CETP	Cholesterol ester transfer protein
CHAPS	3-[(3-cholamidopropyl) dimethylammonio]-1 propanesulfonate
CoNS	Coagulase-Negative Staphylococci
DL	Detection Limits
DNA	Deoxyribonucleic Acid
DTT	Dithiothreitol
EF-G	Elongation Factor G
emPAI	Exponentially Modified Protein Abundance Index
ESI	Electrospray Ionization
EU countries	European Countries
FA	Formic Acid
Fur	Ferric Uptake Regulator
GDP	Guanosine-5'-diphosphate
GMP	Guanosine 50-monophosphate
GTP	Guanosine-5'-triphosphate
GyrB	Gyrase B subunit
H_2O_2	Hydrogen Peroxide
HA-MRSA	Hospital Acquired MRSA
HAP	Hospital-Acquired Pneumonia
HCl	Hydrochloric Acid
HEPES	N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid
HGPRT1	Hypoxanthine–guanine phosphoribosyltransferase
HprT	Hypoxanthine phosphoribosyltransferase
icaR	intercellular adhesion locus repressor
IEF	Iso-electric focusing
IPG	immobilized pH gradient
LC	Liquid chromatography
m/z	mass-to-charge ratio
MALDI-TOF-MS	Matrix-assisted laser desorption/ionization- time of flight -mass spectrometer
mM	Milli molar

mRNA	Messenger Ribonucleic acid
MRSA	Methicillin Resistant St. sureus
MS	Mass spectrometry
MSSA	Methicillin- Susceptible St. aureus
NaCl	Sodium Chloride
NAD (P)	Nicotinamide adenine dinucleotide (Phosphate)
NADH	Nicotinamide adenine dinucleotide dehydrogenase
nl	Nano-liter
nLC	Nanoscale liquid chromatography
O^{-}_{2}	Superoxide radical
O^{2-}	superoxide anion
$OH^{\bullet}$	hydroxyl radical
ON	Over night
oQ	Epoxyqueunosine
PAGE	Poly acrylamide gel electrophoresis
PBP	Penicillin-Binding Protein
PCR	Polymerase Chain Reaction
PDH	pyruvate dehydrogenase
PDHC	pyruvate dehydrogenase complex
PGDS	prostaglandin-D synthase
PGF2α	prostaglandin F2α
PLTP	phospholipid transfer protein
PMF	Peptide mass fingerprinting
PMNs	Poly morpho-nuclear cells
PMSF	Phenylmethanesulfonyl fluoride
PPi	inorganic pyrophosphate
PRPP	phosphoribosyl pyrophosphate
PRR	pattern recognition receptors
PRTase	phosphoribosyltransferase
tpx	probable thiol peroxidase
PVL	Panton Valentine Leukocidine
PVL^{-}	Panton Valentine Leukocidine gene negative
PVL^{+}	Panton Valentine Leukocidine gene positive
Q	queuosine
ROS	Reactive oxygen species
Rpm	Revolutions per minute
RT	Room temperature
S. aureus	Staphylococcus aureus
S. epidermidis	Staphylococcus epidermidis
S. saprophyticus	Staphylococcus saprophyticus
SCCmec	Staphylococcal Cassette Chromosome mec
ScdA	Iron-sulfur cluster repair protein
SDS	Sodium dodecyl sulphate
Se	Sensitivity

SOD	Superoxide dismutase
Sp	Specifity
Spa	staphylococcal protein A
Srr	Staphylococcal respiratory response
SSTIs	Skin and Soft Tissue Infections
TBS	Tris-buffered saline
TCA	Tricarboxylic acid
TFA	Tri fluoroacetic acid
Tg	Toxoplasma gondii
TgEFG	Toxoplasma gondii elongation factor G
TLR	Toll-like receptors
TNF-α	tumor necrosis factor-α
TPBC	triphenylbismuthdichloride
TPx	Thiol Peroxidase
tRNA	Transfer Ribonucleic acid
Trx	Thioredoxin
TSST	Toxic Shock Syndrome Toxin
U/l	international units
UV	Ultra violet
V/V	Volume/volume
w/w	Weight/weight
µl	Micro-liter

1. Introduction

Staphylococcus aureus is both a colonizer of 20–30% of the human population and one of the most important pathogenic microorganism, being responsible for a wide range of infections both in the hospital and in the community(1). More than fifty years ago *S. aureus* isolates acquired resistance to methicillin, a semisynthetic derivate of penicillin, and, since then meticillin-resistant Staphylococcus aureus (MRSA) has become the most important cause of infections in patients in hospitals settings. At the end of 1990s a new lineage of MRSA, designated (CA)-MRSA, emerged in the community causing infections in healthy people, without known risk factors(2, 3). The most common clinical presentations associated to CA-MRSA are skin and soft-tissue infections (SSTIs) or deep-seated infections such as necrotizing pneumonia(2-4). One of the main features of CA-MRSA is the presence of the Panton–Valentine leukocidin (PVL), a phage-encoded pore-forming protein that lyses neutrophils and causes tissue necrosis(5). Panton–Valentine leukocidin was initially considered as the factor responsible for the peculiar CA-MRSA virulence. Nevertheless, the pathogenic role of PVL is still controversial(5). Studies in rodent animal models could not associate PVL production to virulence, while studies in rabbits demonstrated that PVL producing *S. aureus* caused tissue damage and necrosis (6). More recently it has been hypothesized that other factors such as phenol-soluble modulin-alpha peptides (PSMa) could contribute to the toxic effects produced in infections sustained by CA-MRSA since PSMa cause lysis of neutrophils from different animal species and humans (7). Hence CA-MRSA virulence could be attributable to the association of multiple virulence factors.

1.1 General aspects

Staphylococci were firstly named in 1882 by Ogston as he named the clustered micrococci "staphylococci," from the Greek staphyle, meaning bunch of grapes(8**.** Later in 1884 Anton J. Rosenbach isolated two strains of staphylococci, which he named for the pigmented appearance of their colonies: *S. aureus*, from the Latin aurum for gold, and Staphylococcus albus (now called epidermidis), from the Latin albus for white**.**

Until the early 1970s, the genus Staphylococcus consisted of three species: coagulase-positive species S.aureus, coagulase-negative species S. epidermidis, and S. saprophyticus, but a deeper look into the chemotaxonomic and genotypic properties of staphylococci led to the description of many new staphylococcal species. Currently, 36 species and several subspecies are recognized in the genus Staphylococcus (9)**.** Staphylococci are Gram positive cocci (0.5−1.5 µm), occur singly, in pairs, tetrads, short chains, and irregular grape-like clusters, nonmotile, nonsporeforming, and usually are unencapsulated or have limited capsule formation**,** widespread in nature and occupy a variety of niches, inhabiting the skin, skin glands, and mucous membranes of humans, other mammals, and birds**.**

S. aureus is a facultatively anaerobic, Gram-positive, catalase-positive, golden-yellow colonies, often with hemolysis**.** It is a major species of primates, though specific ecovars or biotypes can be found occasionally living on different domestic animals or birds**.** *S. aureus* is found infrequently on nonprimate wild animals, as well as, has the ability to survive for up to 12 days on abiotic surfaces (10) which may serve as a reservoir for contamination in people who come into contact with these surfaces (11)**.** In humans, *S. aureus* has a niche preference for the anterior nares, especially in the adult, asymptomatically colonizes the anterior nares of approximately a third of the population (12)**,** where it can exist as a resident or as a transient member of the normal flora.

1.2 S. aureus antibiotic resistance, the MSSA and MRSA phenotypes

Antimicrobial resistance can occur because an organism is intrinsically resistant to one or more antimicrobials, or can be acquired by spontaneous mutation or acquisition of resistance genes from another organism via conjugation (sexual transfer of DNA), transduction (bacteriophage transfer), or transformation (acquisition and incorporation of DNA released into the bacteria environment by lysis of other bacteria) (13)**.** In *S. aureus*, both intrinsic and acquired resistances exist to the β-lactam class of antimicrobials. Penicillin and other β-lactam antimicrobials act by binding to a transpeptidase involved in cell wall peptidoglycan synthesis disrupting bacterial cell walls. Staphylococci can become resistant to β-lactam antimicrobials through the production of a β-lactamase enzyme that destroys the antimicrobial β-lactam ring. β-lactamase inhibitors such as clavulanate and sulbactam are often combined with β-lactam antimicrobials to overcome this type of resistance, but resistance to methicillin is not mediated through production of β-lactamase(14). Instead, MRSA have acquired a mobile genetic element known as staphylococcal cassette chromosome mec (SCCmec). This SCC carries a gene known as mecA, which encodes for an altered penicillin-binding protein (PBP2a or PBP20). The PBP2a has a lower affinity for b-lactam antimicrobials than the normal PBP such that these antimicrobials are ineffective (15). The SCC containing the mecA gene can spread horizontally between staphylococcal populations (16, 17)**.** The SCC contains additional insertional sequences that allow incorporation of additional antimicrobial resistance markers (18). These insertional sequences explain why many methicillin resistant staphylococci are resistant to non β-lactam antimicrobials, which act through mechanisms other than interference with bacterial cell wall synthesis (eg, macrolides, fluoroquinolones).

Staphylococci possessing the mecA gene are not necessarily more virulent than those without the gene. Both methicillin-susceptible *S. aureus* (MSSA) and MRSA can cause serious infection and may possess virulence factors such as superantigenic toxins, leukocidin, and fibronectins (14, 19)**.** However, the major reason for concern over infection with methicillin-resistant staphylococci as opposed to infection with methicillin-susceptible strains is the difficulty encountered in effective use of antimicrobial drugs to combat infection.

As in humans, the methicillin-resistant staphylococci causing infection are not necessarily more virulent than their methicillin susceptible counterparts but they are more difficult to treat.

1.3 Overview of epidemiological aspects of S. aureus infection

Previously, the majority of attention was concerned with methicillin resistant *S. aureus* (MRSA) as it was rationalized that the resistant isolates are the most predominant isotypes and those strains were more associated with sever clinical manifestations. Inversely, recent epidemiological studies on *S. aureus* demonstrated that the most predominant isolate in some localities was confirmed to be methicillin sensitive *S. aureus* (MSSA) with the outcome of same degree of severity in clinical manifestations (20-22). In Taiwan, there was no difference in the mortality rate for bacteremia due to CA-MRSA strains and that of bacteremia resulting from MSSA (23, 24), in a study, also demonstrated that MRSA and MSSA cause similar infections in a comparable group of patients, with similarities in disease spectrum, clinical outcomes, demographic and epidemiological features. MSSA was associated with bone and joint infection, but not MRSA (25). In a previous report from Westmead Hospital, Sydney, the authors reported 20 cases of MRSA osteomyelitis or septic arthritis in their tertiary neonatal unit over six years (26)**,** they do not comment on MSSA osteomyelitis over the same period. However, (27) reported that 16 of 30 babies with neonatal osteomyelitis had MSSA infection, and only seven had MRSA, while (28) found that MSSA was significantly more likely to cause bone and joint infection, which occurred in only one of seven babies with MRSA sepsis. In the United States, several researchers have estimated that 25 to 30 % of people are colonized with methicillin-sensitive *S. aureus* (MSSA) and 1 to 2 % harbor MRSA (18, 29)**.** Teterycz (30) assessed total joint arthroplasty infections and other orthopedic implant infections due to MSSA, MRSA, and coagulase-negative staphylococci (CoNS) in Switzerland.

There were 44 infections due to MRSA, 58 due to MSSA, and 61 due to CoNS. On the other hand, in vitro studies failed to confirm the low pathogenicity of MSSA, as MRSA and MSSA strains have been shown to possess similar virulence factors, adherence properties, and bacteriological pathogenicity (31, 32). Duckworth and Jordens even found significantly less attachment of MRSA to cells and fibronectin compared with methicillin-sensitive strains (33). In 1992 Coia and his collegues recorded that the proportion of haemolysin-producing isolates did not differ amongst the isolates of MSSA and MRSA (34).

1.4 Mechanisms of virulence: Panton Valentine Leukocidine (PVL)

Pathogenicity of *S. aureus* is attributed to the expression of an arsenal of virulence factors (35). Among the different virulence factors employed by *S. aureus*, there are hemolysins and leukocidins (14). Panton-Valentine Leukocidin (PVL) is a staphylococcal synergohymenotropic bi-component exotoxin encoded by two genes lukFPV and lukS-PV PVL genes, of pore-forming toxin family. Its genes, lukS-PV and lukF-PV, are encoded on prophages and can be found in diverse genetic lineages of *S. aureus*. Strains which are positive for this leukocidin are usually associated with community-acquired infections which generally affect previously healthy children and young adults. It was first described in 1932 by Panton and Valentine, and is therefore known as Panton-Valentine leukocidin, or PVL(5, 36).

PVL is thought to be a key factor in the pathogenesis of necrotizing pneumonia. It forms pores in the cell and mitochondrial membrane of neutrophils, monocytes, and macrophages, and recently been associated with necrotizing pneumonia and thus provokes cell lysis and apoptosis with subsequent liberation of inflammatory mediators (37-40).

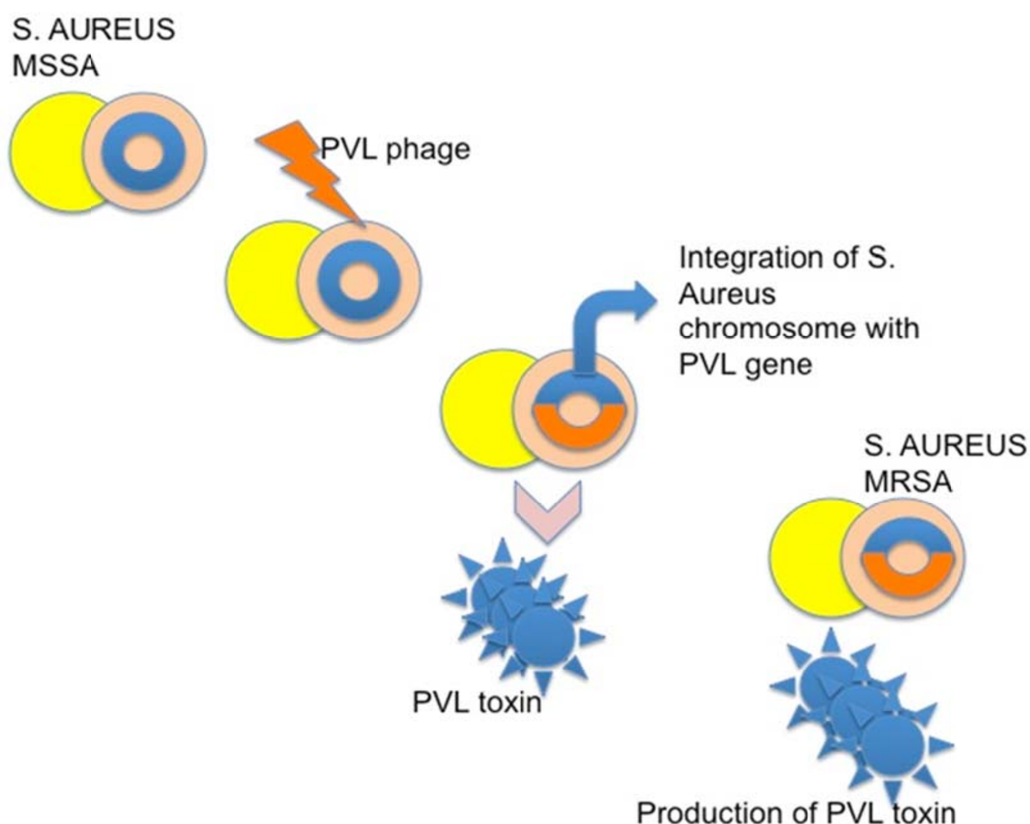


Figure 1. Model for emergence of PVL producing CA-MRSA (36)

Experimental and clinical evidence shows that PVL-producing strains are associated with severe, necrotizing skin infections and pneumonia (4, 37, 41) and this made some authors contest the pathogenic potential of PVL and suggest the presence of PVL genes to be a marker of other virulence determinants (42, 43). For example, PVL has been linked to skin and soft tissue infections (SSTIs)(44), necrotizing pneumonia(37, 40, 45), and bone and joint infections in humans (46) (41) (37). Rabbit and human leukocytes are highly sensitive to PVL-mediated leukocytosis (47), and animal studies have shown that PVL causes more severe disease in dermo-necrosis (44), osteomyelitis (48), and necrotizing pneumonia. Recently the issue of the emergence of novel, community-acquired methicillin-resistant *S. aureus* (MRSA) strains being positive for PVL has been emphasized. However, PVL is also common in methicillin-susceptible *S. aureus* (MSSA) and can be detected in as much as 30% of abscess isolates. Osteomyelitis caused by PVL^+ *S. aureus* strains were associated with more severe local disease and a greater systemic inflammatory response compared with osteomyelitis caused by PVL^- *S. aureus* in both methicillin-susceptible and methicillin-resistant S aureus (46, 49). Intradermal injection of PVL in rabbits produces severe necrotizing skin lesions, consistent with the association between PVL-positive staphylococcal strains and the formation of furuncles, cutaneous abscesses, and severe necrotic skin lesions (44). Interestingly, the strains of *S. aureus* responsible for widespread epidemics in newborn nurseries during the 1950s and 1960s were recently shown to produce PVL (50). The importance of PVL as a virulence factor has recently been demonstrated in a mouse model of pneumonia (4); however, it was foundamental for virulence in mouse models of sepsis and skin infection (45). Recent clinical experience strongly suggests that PVL-positive strains of *S. aureus* exhibit enhanced virulence (37, 51, 52), although it is unclear whether this phenotype is attributable to PVL itself, to linked and yet unidentified virulence determinants, or to altered regulation of toxin expression.

However, not all studies have reached this conclusion and controversy has emerged on the pathogenic role of PVL. For instance, CA-MRSA strains lacking PVL were as virulent in mouse sepsis and abscess models as those with the toxin (45), α-haemolysin and not PVL was responsible for mortality in a murine pneumonia model using USA300 and USA400 CA-MRSA strains (40), a PVL-negative *S. aureus* strain (USA500) produced skin lesions similar to the PVL-positive strain (53), in SSTIs suffering rabbit caused CA-MRSA USA300 strain, authors did not identify any significant role of PVL in propagation of the disease (7). In patients with hospital-acquired pneumonia (HAP) caused by MRSA, the presence of PVL was not associated with either higher risk for clinical failure or mortality (39). Similar findings were reported from a multicentre observational study of patients with HAP and ventilator associated pneumonia caused by MRSA (54). Moreover, a large, multinational study found that PVL was not associated with better or worse outcome in complicated SSTIs (55).

The diversity of the animal models used in the different studies may explain these discordant results. Additional evidence has called into question the comparability of murine models of PVL-associated disease and human infections. In a study, neutrophils from mice, humans, and rabbits were used to test the cytotoxic effect of PVL and to elucidate differences among species. Murine neutrophils were insensitive to the effects of PVL, suggesting that models using mice do not correctly replicate PVL-bearing *S. aureus* disease in humans. Rabbit compared to murine neutrophils were much more susceptible to PVL, indicating a closer approximation to human disease (47, 56).

It was noted in a recent review that most of the evidence from rabbit models of infection to date suggests that PVL contributes to the virulence of CA-MRSA, but it is not the only factor contributing to the CA-MRSA epidemic (57).

Watkins et al. 2012 concluded that the role of PVL in human MRSA infections remains controversial (58), but it appears that PVL is not likely to be a useful single target for vaccine development and is not likely the main factor determining the severity of CA-MRSA infections. Hence, additional or alternative virulence factors are likely to play an important role in the pathogenesis of both HA-MRSA and CA-MRSA infections.

1.5 S. aureus infection in animals

S. aureus is not only a human pathogen but it causes an array of infections in economically important livestock animals. History of MRSA infection in animals started in 1972, where methicillin resistant *S. aureus* (MRSA) isolate was found in milk from Belgian cows with mastitis, however the MRSA status of the dairymen was not investigated and since this date cows with mastitis seem the most likely to harbour MRSA. MRSA has been reported in many different species, including pets, farm animals and wild animals.

Infection in animals is manifested by skin and soft tissue infections (especially post-surgical) in companion animals (58, 59), in horses there are many clinical forms reported as, Skin and soft tissue pathologies**,** bacteraemia and septic arthritis(59-62), osteomyelitis**,** implant-related infections and metritis, omphalitis (59, 60), and catheter-related infections and pneumonia(60)**.** In cattle *S. aureus* causes mastitis in milking herds, and occasionally purulent dermatitis in their milkers**.** In pigs, *S. aureus* causes exudative epidermitis**.** *S. aureus* is also a major cause of lameness in commercial broiler chickens (63) Furthermore, in continental Europe where rabbit farming is an expanding industry, *S. aureus* epidemics causing skin abscesses, mastitis and septicaemia are common(64).

1.6 S. aureus infection in humans

S. aureus, since its early discovery as an opportunistic pathogen, continues to be a major cause of mortality and is responsible for a variety of infections(12). In the late 1950s and early 1960s, *S. aureus* caused considerable morbidity and mortality as a nosocomial pathogen. Among the major human infections caused by this species are furuncles, carbuncles, impetigo, toxic epidermal necrolysis (scalded skin syndrome), pneumonia, osteomyelitis, acute endocarditis, myocarditis, pericarditis, enterocolitis, mastitis, cystitis, prostatitis, cervicitis, cerebritis, meningitis, bacteremia, toxic shock syndrome, and abscesses of the muscle, skin, urogenital tract, central nervous system, and various intraabdominal organs. Serious invasive disease arises when *S. aureus* enters the blood stream, resulting in a number of life-threatening conditions, such as sepsis, endocarditis, osteomyelitis or meningitis. The risk of intravascular and systemic infection by *S. aureus* arises when the epithelial barrier is disrupted by surgery, intravascular catheters, implants (e.g. orthopaedic devices and artificial heart valves), mucosal damage or trauma. Accordingly, postoperative wound infections are frequently caused by staphylococcal strains carried by the patient(65, 66).Another important clinical problem is that staphylococci form thick multilayered biofilms not only on indwelling catheters and other implanted devices, but also on chronic wounds such as pressure sores, diabetic foot ulcers and venous stasis ulcers. Bacteria within such biofilms are protected against insults from the host's immune system, antibiotics and disinfectants(9, 57) and they interfere severely with wound re-epithelialization (67). *S. aureus* represents a serious public health burden worldwide, and particularly within health-care settings. Nevertheless, *S. aureus* is primarily a commensal(12). Notably, this organism can also be detected in other moist regions of the human body, such as the axilla, perineum, vagina, and rectum, thereby forming a major reservoir for infections.

1.7 S. aureus, a potential zoonoses?

Close human contact with animals provides more opportunity for transmission between the species. Once acquired, further horizontal transmission of the pathogen between animals or humans and their families can occur. Transmission of MRSA was reported between Hungarian cows with sub-clinical mastitis and an agricultural worker who was throat swab positive(68). Human skin scales with MRSA are easily shed from leg ulcers, eczematous skin and pressure areas during the activities

of daily living. Undetected colonized animals provide a reservoir for continuing relapsing infection in humans(69-72). In 2004, a Dutch pig farmer's wife developed MRSA mastitis and pleural effusion. Although successfully treated with teicoplanin, eradication therapy failed, and subsequent screening found her husband and daughter to be MRSA carriers. Six months later, with the baby and parents still colonized, wider sampling revealed that 3 co-workers and 10 pigs from the closest holding were carriers of the PFGE non-typeable MRSA, all of which were identical (73). Agricultural workers and their families involved with pig, and (to a far lesser extent) cattle, farming have a high likelihood of MRSA colonization, with up to 23% Dutch pig farmers being nasal carriers of MRSA (74)

Also, Pet animals as cats and dogs represent potential sources of infection due to their close contact with humans. The number of cats and dogs has substantially increased in modern society, with an estimated population of above 70 million in the EU countries. Close physical contact by touching, petting and licking occurs at high frequency on the basis of the current perception of household pets as actual family members**.** Price and colleagues reported that livestock-associated MRSA CC398 originated as MSSA in humans, which attenuated its zoonotic potential and exemplified a bidirectional zoonotic exchange(75).

Infection in animals is done by horizontal spread of the pathogen between animals on farms and in veterinary establishments(62).

S. aureus colonization of food has long been associated with a form of gastroenteritis that is manifested clinically as emesis with or without diarrhea, called staphylococcal food poisoning and results from ingestion of one or more preformed staphylococcus entertoxins on food that has been contaminated with *S. aureus*.

2. Aim of this study

The new therapeutic strategies depend on deep understand of both cell physiology and adaptation mechanisms raised by invading pathogens during infection and allowing those virulence armies to act efficiently and in a harmony manner offering security and safety of the pathogen from any attack either from host immune system or static bactericidal and antimicrobials. Simultaneously with the availability of genomic sequences, the power of 2-D electrophoresis to separate complex mixtures of proteins, recent advances in protein analytical methods including mass spectrometry, and developments in computational methods, it is now possible to separate, identify and list the proteins expressed in a cell under a given set of conditions**.**

Aim of this study is to identify the difference in proteomic profile of genetically and phenotypically different isolates of *S. aureus*. This has been done in agreement with the new policy of the Ministry of Health to counteract *S. aureus* raising incidence in Italy.

Samples were obtained from human patients suffering from skin and pulmonary affections with a history of close contacts with livestock or hosting pet animals. The patients were confirmed to be infected with *S. aureus* as the pathogen was isolated from the lesions. 20 isolates from cases similar in clinical manifestations were chosen and used for this study. All the isolates were collected and classified according to microbial sensitivity and molecular biology into 4 groups, each one is composed of 5 isolates; 1st group was MRSA positive for PVL gene, 2nd group was MRSA negative for PVL gene, 3rd group was MSSA positive for PVL gene, and 4th group was MSSA negative for PVL gene.

All the obtained groups were analyzed through 2DE and Mass Spectrometry in order to detect the differentially expressed proteins and to highlight the different pathways involved in the differernt virulence mechanisms.

3. Materials and methods

3.1 Bacterial isolates

A total of 20 characterized *S. aureus* isolates, 10 MSSA and 10 MRSA, were examined. *S. aureus* characterization included the detection of nuc, mecA and lukS/F-PVgenes to confirm *S. aureus* species, methicillin resistance and the presence of PVL toxin. The determination of sequence type (ST), was performed by multi-locus sequence typing (MLST) to identify clonal complexes (CCs)(76). The isolates were selected to include 10 pairs of strains, 5 pairs of MSSA and 5 pairs of MRSA. The isolates of each pair belonged to the same CC but were divergent for the presence of PVL. Isolates from frozen stock were cultured on Muller Hinton agar plates (Becton Dickinson Diagnostic Systems, Sparks, MD, USA) at 37°C overnight. One loopful of confluent culture was inoculated into 100 ml of Brain Heart Infusion (Thermo Fisher Scientific, Milan, Italy) and incubated overnight at 37°C with light shaking. Bacterial cells were pelleted from the culture medium by centrifugation at 3000 g at 4°C for 15 min. The bacterial pellets were kept refrigerated at 4°C until used.

3.2 2-DE

Protein extraction was performed as previously described (77, 78) , adapting protocol to *S. aureus* samples. Bacteria were harvested at 9000 g, at 4°C, for 10 minutes and quickly washed five times with cold PBS. Cellular pellets for 2-DE analysis were suspended in lysis buffer (7 M urea, 2M Tiourea, 4% CHAPS, 1% DTT, 2% ampholine 3.5-10) containing protease inhibitors cocktail (GE Healthcare) and nuclease mix (GE Healthcare) to remove nucleic acids according to the manufacturer's instructions and disrupted by sonication 5 times for 4 min at maximum power on ice. Cell debris were removed by centrifugation at 9000 g, at 4°C, for 60 min. To minimize contamination by lipids, phospholipids and other cell constituents, supernatant was cleaned by precipitation (ref) using a solution consisting of tri-n-butyl phosphate: acetone: methanol (1:12:1), cooled on ice. 1.4 mL of this solution were added to each sample to reach a final acetone concentration of 80% (v/v) and incubated at 4 °C for 90 min. The precipitate was isolated by centrifugation at 9000g, for 20 minutes at 4 °C. After washing with the same precipitant buffer, it was centrifuged again and then air dried. Cellular pellets were re-suspended in 7 M urea, 2M Tiourea, 4% CHAPS, 1% DTT, and 2% Ampholine pH 3,5-10. Protein concentration in all samples was determined using 2D Quant Kit (GE Healthcare).

For 2-DE three experimental replicates were performed for each sample. Homemade immobilized pH gradient (IPG) strips (7 cm) with a ultranarrow linear pH range of 4-5.5 were rehydrated overnight in a buffer containing 7M urea, 2% CHAPS, 0.4%DTT, 0.5% Ampholine pH 3,5-10. 100 µg of protein sample were loaded on each IPG strip using cup loading at cathodic side. Isoelectric focusing was performed using EttanIPGphor III IEF system (GE Healthcare) at 20°C with a current of 120 µA/strip. For IEF was used the following protocol: 30 V (4 h), 50 V (3 h), 100 V (3 h), 500 V (3 h), 1000 V (3 h), 3000 V (3 h),4000 V (3 h), 6000 V (3 h) and 8000 V (8 h). After the first dimension, IPG strips were equilibrated twice with a solution containing 6 M urea, 2% SDS, 50 mM Tris-HCl pH 8.8 and 30% glycerol, for 15 min, under gentle stirring. For the first equilibration step was used 1% DTT and for the second 2.5% iodoacetamide. The second dimension was performed using homemade 12% acrylamide gradient vertical SDS-PAGE slab gels on Protean tetra cell (BioRad). IPG strips were put on top of the SDS gels which were poured up to 1 cm from the top of the plates and then sealed with 1.5 ml of a solution containing 0.5% low-melting-point agarose diluted in hot SDS running buffer (25 mM Tris-HCl pH 8.3, 192 mM glycine, 0.1% SDS). Molecular weight protein markers (Precision Plus, Biorad) were applied on one end of the IPG strips. In the second dimension, gels were run at 15 mA/gel, for 20 min. and then at 40 mA/gel,

until the bromophenol blue front-line came out of the gel. After runs, gels were stained with colloidal Coomassie and digitalized with PharosFX Plus Laser Imaging System (BioRad).

3.3 Image analysis.

Gel images were imported both into Progenesis SameSpots (v 4.5); Nonlinear Dynamics, Newcastle, UK). All imported images were processed to check image quality (saturation, dimension, background). The aligned images were then automatically analyzed using the 2D analysis module for spot detection, background subtraction, normalization, and spot matching, and all spots were manually reviewed and validated to ensure proper detection and matching.

3.4 Statistical analysis

Statistical analysis was performed by the Progenesis Stats module on the log-normalized volumes for all spots. Mann-Whitney test and one-way ANOVA were used to confirm the p value between different groups, p-values under 0.05 were considered statistically significant. FDR (false discovery rate) and power analysis were also calculated, q values <0.05 and power values >0.8 respectively were considered to be significant.

3.5 Mass spectrometry

Single spots were excised from Coomassie-stained 2DE gels and gradually dehydrated with 50 mM ammonium bicarbonate, a mixture of 50 mM ammonium bicarbonate and acetonitrile (1:1), and 100% acetonitrile. Proteins were reduced using 10 mM DTT and alkylated with 55 mM iodoacetamide. Gel plugs were alternatively hydrated with 50 mM ammonium bicarbonate and dehydrated in acetonitrile and then left to completely dry. Proteins were digested with 0.1 µg of porcine trypsin (Promega, Madison, WI) at 37 °C overnight. Reactions were stopped with 1% TFA. Peptides were desalted and concentrated by ZipTip C18 (Millipore) and eluted with a solution of 0.5 mg/ml α-ciano-4-hydroxycinnamic acid dissolved in 50% acetonitrile, 0.05% TFA on a Ground Steel plate (Bruker-Daltonics) previously spotted with a thin layer of 10 mg/ml α-ciano-4-hydroxycinnamic acid. Mass spectra were acquired with an Ultraflex III MALDI-TOF/TOF spectrometer (Bruker-Daltonics). External calibration was performed using the Peptide calibration standard (m/z: 1046.5418, 1296.6848, 1347.7354, 1619.8223, 2093.0862, 2465.1983, 3147.4710; Bruker-Daltonics). FlexAnalysis 3.0 software (Bruker-Daltonics) was used for the selection of the monoisotopic peptide masses. Internal calibration was performed on autolysis peaks from porcine trypsin (m/z: 842.509 and 2211.104).

After MS spectra acquisition, the instrument was switched in LIFT mode and precursor ions were manually selected for the subsequent fragmentation. MS/MS spectra were acquired with 4–8 × 103 laser shots adjusting the laser power and the number of shots per shot trains in order to obtain at least 104 ion counts for the prominent fragmentation peak. The precursor mass window was automatically set after the precursor ion selection. Spectra baseline subtraction, smoothing (Savitsky–Golay) and centroiding were operated by FlexAnalysis 3.0 software (Bruker-Daltonics).

4. Results

Proteome profile of the compared microbial categories demonstrated a significant difference in protein expression level between MSSA/MRSA groups. In figure 2 is shown the reference map of *S. aureus* with identified proteins. The proteome clusterization of MSSA/MRSA/PVL/PVL- are strongly according with the previous genetic classification. Furthermore these results are strongly supported by the evidence that all isolates were microbially cultivated under the same conditions and same nutrients to avoid protein expression differences due to modified extra environment.

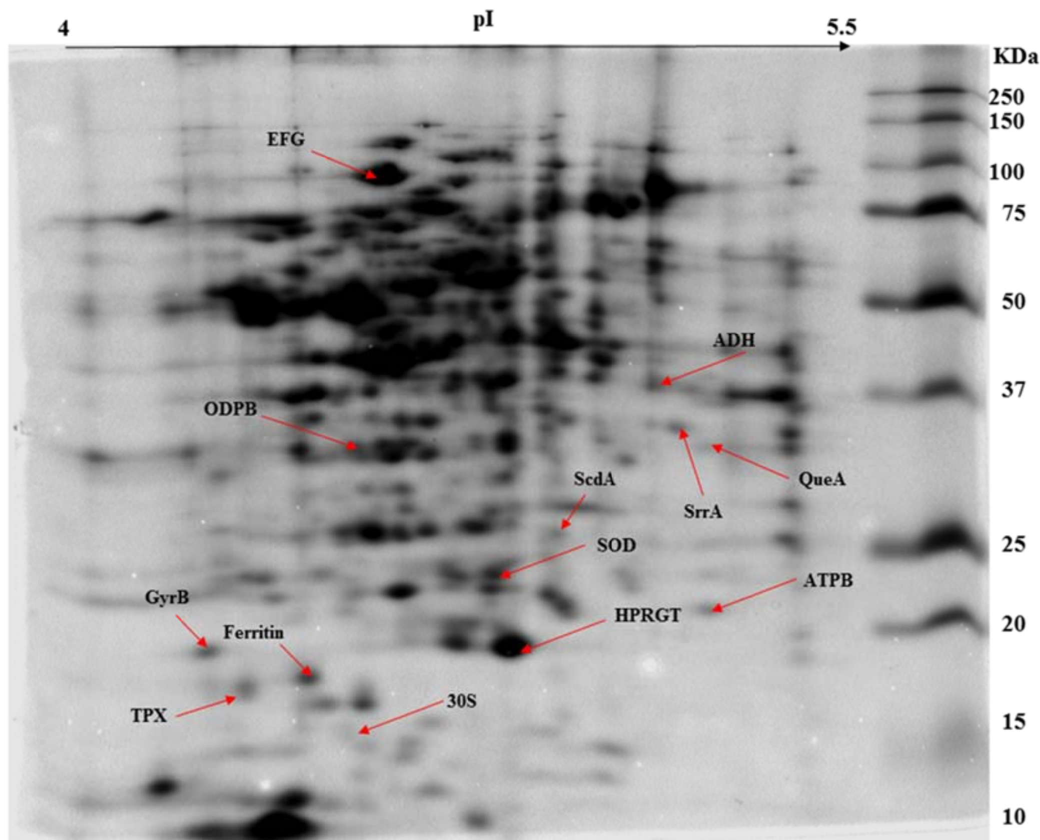


Figure 2. Representative ultra-narrow 2-DE map of *S. aureus* MSSA/MRSA clinical isolates. Isoelectric point and molecular weight are reported. Legend of differentially expressed proteins; srrA: Transcriptional regulatory protein srrA ; ODPB: Pyruvate dehydrogenase E1 component subunit beta; scdA: Iron-sulfer cluster repair protein ScdA; queA: S adenosylmethionine: tRNA ribosyltransferase-isomerase; EFG: Elongation factor G; ADH : Alcohol dehydrogenase ; SOD : Superoxide dismutase [Mn/Fe] 1; gyrB: DNA gyrase subunit B;TPX thiol peroxidase; 30S : 30S ribosomal subunit s6; ATPB : ATP synthase subunit B

Among the huge number of well separated and significantly detected protein spots by the software image analysis, it was demonstrated that there are 7 protein differentially expressed between MRSA and MSSA PVL^{+} (fig. 3, table 1).

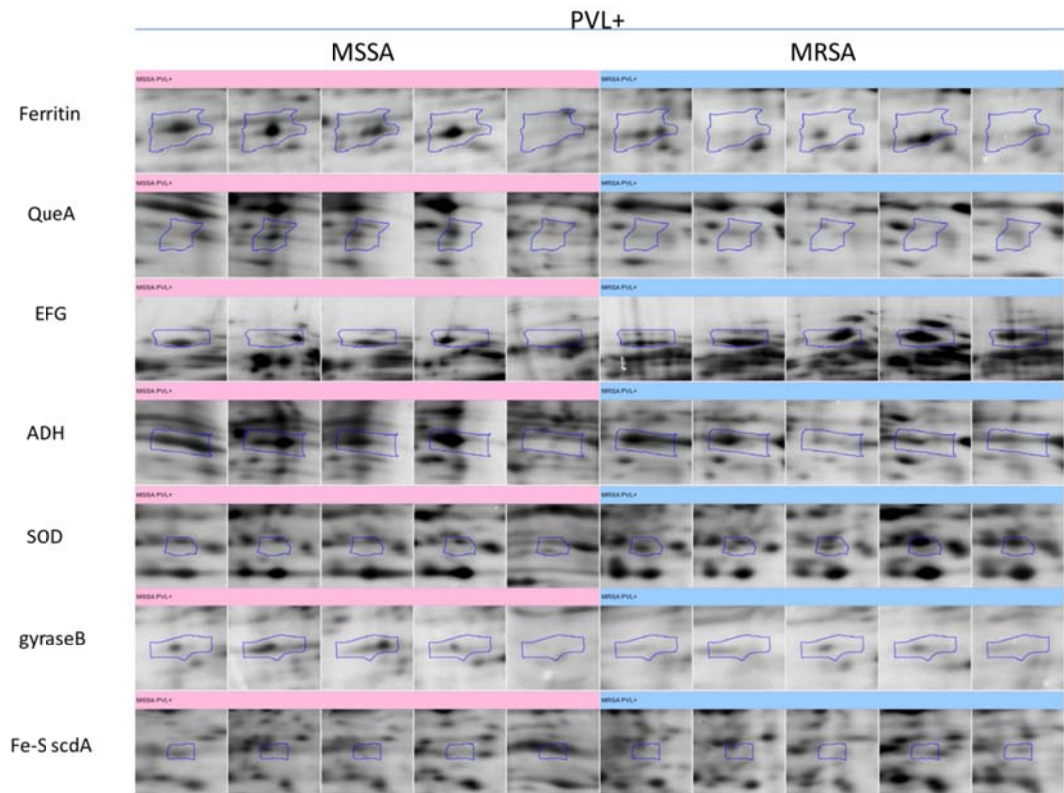


Figure 3. Differentially expressed proteins in *S. aureus* mssa pvl+ vs mrsa pvl+ strain. Legend; Fe-S scdA: Iron-sulfer cluster repair protein ScdA; queA: S adenosylmethionine: tRNA ribosyltransferase-isomerase; EFG: Elongation factor G; ADH : Alcohol dehydrogenase ; SOD : Superoxide dismutase [Mn/Fe] 1; gyrase B: DNA gyrase subunit B.

spot number	Identified protein	Accession	MRSA PVL$^+$	MSSA PVL$^+$
306	Ferritin	FTN_STAA3	4,742e+007	7,274e+007
201	S adenosylmethionine: tRNA ribosyltransferase-isomerase	QUEA_STAA3	2,027e+007	3,695e+007
415	Elongation factor G	EFG_STAA1	5,256e+007	3,775e+007
417	Alcohol dehydrogenase	ADH_STAA3	5,054e+007	9,275e+007
420	Superoxide dismutase [Mn/Fe] 1	SODM1_STAA3	2,443e+007	1,578e+007
390	DNA gyrase subunit B	GYRB_STAA3	2,130e+007	3,944e+007
422	Iron-sulfur cluster repair protein ScdA	SCDA_STAA3	9,433e+006	1,325e+007

Table 1. Mass spectrometry results and differential protein expression in PVL+ *S. aureus*.

About the Panton-Valentine leukocidin gene lacking isolates, confronting MRSA and MSSA PVL^{-} proteome profile, the image analysis software reported 8 protein spots differentially expressed between MRSA and MSSA isolates with p value ≤ 0.05 (Fig. 4, Table 2).

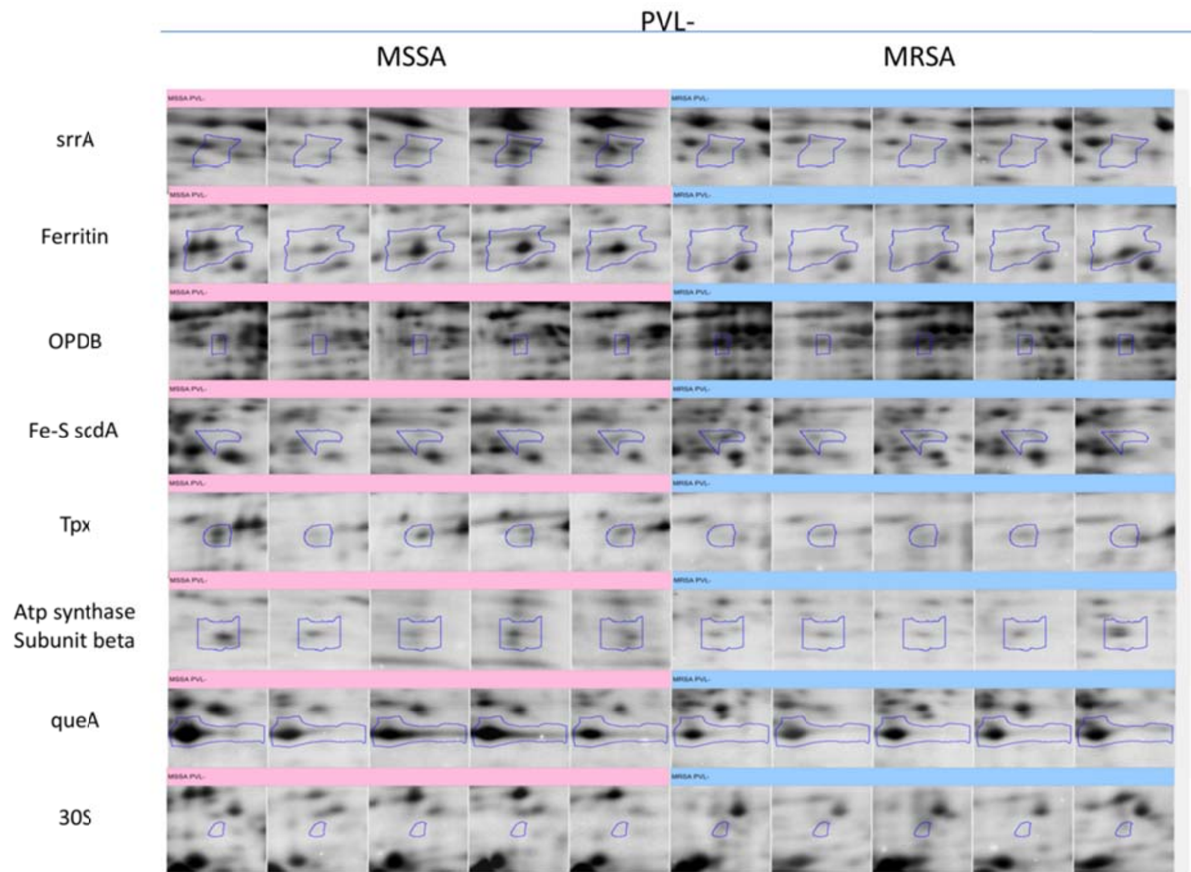


Figure 4. Differentially expressed proteins in *S. aureus* mssa pvl- vs mrsa pvl-strain. Legend; srrA: Transcriptional regulatory protein srrA ; ODPB: Pyruvate dehydrogenase E1 component subunit beta; Fe-S scdA: Iron-sulfer cluster repair protein ScdA; queA: S adenosylmethionine: tRNA ribosyltransferase-isomerase; EFG: Elongation factor G; SOD : Superoxide dismutase [Mn/Fe] 1; Tpx : thiol peroxidase; 30S : 30S ribosomal subunit s6.

	Spot number	Identified protein	accession	MRSA PVL-	MSSA PVL-
1	201	Transcriptional regulatory protein srrA	SRRA_STAA8	1,766e+007	3,455e+007
2	306	Ferritin	FTN_STAA3	3,797e+007	6,509e+007
3	210	Pyruvate dehydrogenase E1 component subunit beta	OPDB_STAAC	1,175e+007	8,600e+006
4	398	Iron-sulfer cluster repair protein ScdA	SCDA_STAA3	1,391e+007	7,534e+006
5	310	thiol peroxidase	TPX_STAAC	8,821e+006	1,443e+007
6	342	30S ribosomal protein S6	RS6_STAA1	2,938e+006	1,212e+006
7	408	ATP synthase subunit beta	ATPB_STAA3	1,704e+007	2,812e+007
8	416	Hypoxanthine-guanine phosphoribosyltransferase	HPRT_STAAC	7,550e+007	1,093e+008

Table 2. Mass spectrometry results and differential protein expression in PVL- *S. aureus*.

The statistical analysis shown in figure 5 resumes the data about different protein expression of all the experimental groups previously described.

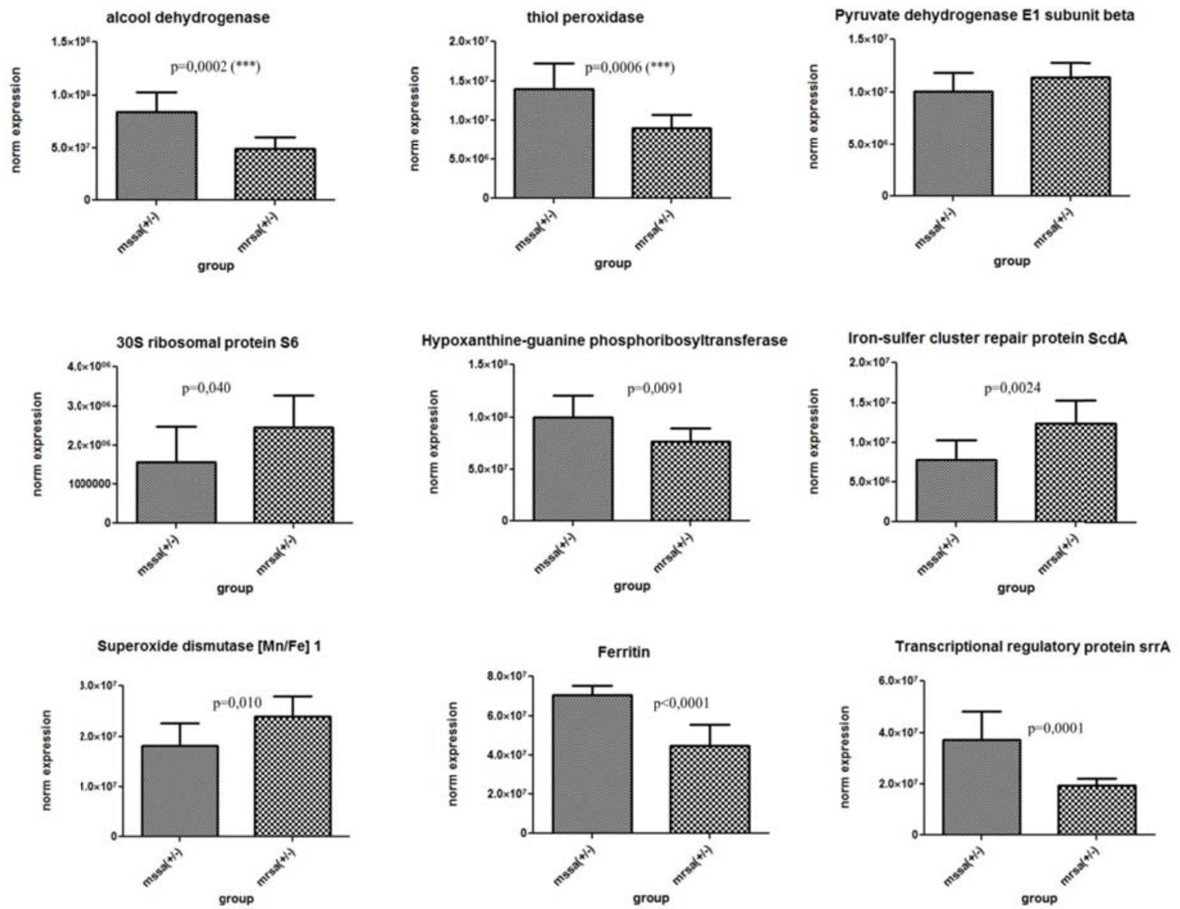


Figure 5. Statistycal analysis of global protein expression between the clinical isolates MSSA/MRSA. $p \leq 0.05$ has been considered statistically significant.

In the discussion setion it has been choosen to discuss all the proteins that, according to the Progenesis SameSpots image analysis software, showed a difference of 1,5 fold change or a p-value ≤ 0.05.

5. Discussion

5.1 MSSA PVL+ vs MRSA PVL+.

5.1.1 Superoxide dismutase [Mn/Fe] 1(Upregulated in MRSA PVL+)

Phagocytic leukocytes have a considerable arsenal of killing mechanisms at their disposal for the destruction of invading microorganisms through the action of superoxide ions, hypochlorite, hydrogen peroxide, hydrolytic enzymes, and other soluble defence factors such as lactoferrin and defensins(79). After ingestion of a microorganism by a leukocyte, oxygen consumption is found to increase, along with an increase in the consumption of glucose(80, 81).

Reactive oxygen species (ROS) such as the superoxide anion (O^-_2), hydrogen peroxide (H_2O_2) and hydroxyl radical (NOH) are also natural products of metabolism formed when oxygen becomes partially reduced(82). The known sources of metabolically generated superoxide are primarily respiratory enzymes whose flavin moieties chemically reduce molecular oxygen on the cytosolic face of the cytoplasmic membrane(83). Molecular oxygen has two unpaired electrons in its outer orbitals. Each of these outer orbitals can accommodate an additional electron. The addition of one electron produces the superoxide anion (O^-_2); this anion can function either as an oxidant, in which case it gains an electron and produces hydrogen peroxide, or as a reductant, in which case it loses its electron and is oxidized to oxygen(84-89). Lipid hydroperoxides can be generated from the attack of ROS to the bacterial membrane. Organic hydroperoxides can also be formed during metabolism of certain drugs or during oxidation of n-alkanes. These peroxides can then react with metals or with metalloproteins leading to the production of secondary free radicals which may be related to the fact that organic peroxides possess bactericidal activity(90). A novel study indicated that exposure to different classes of bactericidal drugs led to the formation of hydroxyl radicals in both Gram-positive (*S. aureus*) and Gram-negative (Escherichia coli) bacteria, and proposed that this reactive oxygen species (ROS) induced damage to various bacterial structures, leading to cell death(91).

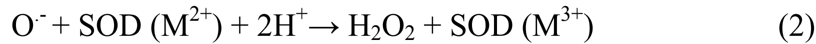
Superoxide fluxes have been shown to kill cells(92), inactivate viruses, induce lipid peroxidation(93, 94) and damage DNA(95) and membranes(93, 94, 96).

All aerobic and facultative anaerobes must be able to defend themselves against superoxide (O^-_2), the most potent reactive oxygen intermediate, generated in the cytosol in the course of oxidative metabolism. Antioxidant enzymes such as superoxide dismutases (SODs), catalases, and peroxidases are primary defense mechanisms utilized by bacteria for preventing oxidative damage(97). A key strategy by which bacteria avoid killing by O^-_2 involves a group of enzymes called superoxide dismutases (SODs). These enzymes are vital metalloenzymes catalyze the conversion of superoxide to hydrogen peroxide (H_2O_2), which can then be detoxified by other enzymes. These enzymes, SODs, are usually co-factored with metal ions, such as Mn^{2+}, Fe^{2+}, Cu^{2+}, or Zn^{2+}.Three types of SOD have been reported in bacteria (SodA, SodB and SodC), classified according to their corresponding metal co-factor [Mn2+, Fe3+ or Cu2+/Zn2+, respectively.

Searching in the biophysiology of those identified proteins revealed that, superoxide dismutase [Mn/Fe] 1 is known to possess defensive functions for even the most rudimentary of prokaryotes while also being implicated in the degeneration of the most complex organ in nature, the human brain. Besides the enzyme's relatively well described defensive functions within bacteria, it is thought to play a significant role in the pathogenicity of certain infections in animal hosts. The primary function of SOD is that of protecting the cell from endogenously generated ROS, that is the normal respiratory events of aerobes and facultative organisms account for most of the oxidative stress with which these cells are burdened.

Disproportion occurs in two sequential steps (see equations 1 and 2)

$$O^- + SOD\ (M^{3+}) \rightarrow O_2 + SOD\ (M^{2+}) \qquad (1)$$



$$O^{-} + SOD\ (M^{2+}) + 2H^{+} \rightarrow H_2O_2 + SOD\ (M^{3+}) \quad (2)$$

Where SOD signifies the enzyme and M indicates the bound iron or manganese ion, which cycles between its +3 and +2 oxidation states.

SODs of each class have been implicated as being contributors to virulence in multiple pathogens including Salmonella enteric serovar Typhimurium(98, 99). Mycobacterium tuberculosis(100-103), *S. aureus*(104)**,** Streptococcus agalactiae(105), Francisella tularensis(106), Neisseria meningitidis(107), Brucella abortus(108),E. coli(109), and Enterococcus faecalis(110). A common element in the infectious course of many of these pathogens is that they are capable of surviving interactions with ROS-generating phagocytic cells such as macrophages(84).

The absence of SOD activity is known to produce a variety of oxygen-dependent phenotypic alterations. These alterations were manifested in E. coli mutant strains, and including severe defects in amino acid biosynthesis(111), structural instability of the cell envelope(112), and a high rate of spontaneous mutagenesis(113), Bizzini demonstrate that the E. faecalis superoxide dismutase is the key to its non-susceptibility towards the bactericidal effect of penicillin and vancomycin, and that cell death pathways in E. faecalis are induced by the superoxide anion. Indeed, protection against oxygen-dependent DNA damage is an important property of SOD(114, 115).

Mn-SOD proved to be virulence factor responsible for resistance and tolerance against oxidative stress in fungi, as Schizosaccharomyces cerevisiae(116, 117). Candida albicans, Schizosaccharomyces pombe(118, 119) and human fungal pathogens(120, 121) by knocking out the SOD gene as the mutant isolates rendered less virulent and more sensitive to oxidative stress.

Mn-SOD has proven to make significant contributions to both stress tolerance and virulence of Beauveria bassiana, an insect fungus, and can be exploited for improving fungal biocontrol agents less tolerant to oxidative and UV-B stresses(122).

Esteve-Gassent and coworkers indicate that SodA plays an important role in combating oxidative stress and is essential for the colonization and dissemination of Borrelia burgdorferi(123). The importance of O_2^- as a toxic species can also be appreciated by the use of bacteria deficient in SOD activity. O_2^- was shown to be quite toxic to Streptococcus sanguis, an organism deficient in catalase and heme synthesis, and this toxicity is apparently independent of formation of HO•. A study carried out on Nocardia asteroids revealed that, there was a relationship among the production of a surface-associated and secreted SOD, high levels of catalase, phase of growth, virulence, and resistance of Nocardia asteroides to the microbicidal activities of human PMNs. Thus, the less virulent strain, N. asteroides 10905, was more susceptible to the oxidative killing mechanisms of PMNs than the more virulent N. asteroides GUH-2. Furthermore, exogenously added SOD and catalase purified from strain GUH-2 completely protect strain 10905 from the lethal effects of PMNs, and specific antibody active against the surface associated SOD enhances the susceptibility of N. asteroids GUH-2 to the lethal effects of the PMNs(124). The same result was obtained from another study done on *S. aureus*, showing the relatively higher SOD activities in virulent *S. aureus* strains as compared with the much lower activities in nonvirulent strains with a marked correlation of SOD activities with virulence among the *S. aureus* strains tested(125).

Cybulski and colleagues concluded that SOD molecules within the spore afford Bacillus anthracis protection against oxidative stress and enhance the pathogenicity of Bacillus anthracis in the lung. The sensitivity of spores to macrophages in the absence of SODs suggests the potential therapeutic utility of small molecular inhibitors that would be capable of blocking the activity of spore-associated SOD molecules(126).

Karavolos accentuated and confirmed the significance of SOD activity for the survival of staphylococci either within the host or in the external environment(104).

The upregulation of this protein in MRSA PVL+ demonstrates that this isolate could be less sensitive to ROS. Reactive oxidants cause damage to the essential biomaterials of cells; for instance, by reacting with intracellular iron, hydrogen peroxide can form the hydroxyl radical through the Fenton reaction, which injures various cellular molecules including lipids, proteins, and DNA(82). Superoxide is also capable of promoting oxidative damage by increasing the intracellular

concentration of free iron(112). All organisms have developed various means of protecting themselves against ROS, represented by specific defenses and global responses that enable cells to survive periods of oxidative stress, which are regulated and respond to the environment-associated oxidative threat(97). The survival of the bacterium depends on the evolution of a series of defenses, which include 1) detoxifying enzymes and free radical-scavenging substrates, 2) DNA and protein repair systems, and 3) competition for substrates favoring bacterial survival. In many cases, these defenses may be coordinately regulated. In the presence of high oxygen concentrations, bacteria employ multiple strategies to protect against reactive oxygen derivatives that are generated by the partial reduction of oxygen. They regulate the expression of genes involved in aerobic metabolism and in the enzymatic defense against reactive oxygen species(127). Secondly, bacteria may use non-coupled respiration and a mild uncoupling mechanism to accelerate the respiratory consumption of oxygen, and thereby lower the concentration of oxygen(128-130). Thirdly, motile bacteria take advantage of directed motility to escape from microenvironments where the oxygen concentration is too high for their metabolic lifestyle. The higher expression of this protein in MRSA PVL+ could be linked to a more efficient adaptation of this *S. aureus* isolate to invade host organisms. The evolutionary process could have brought this isolate to synthesize high amounts of SOD to counteract the host defense system.

5.1.2 Elongation factor G (Upregulated in MRSA PVL+)

Regulation of translation is a widespread mechanism for the control of gene expression. In many organisms, elongation factors play a role in the regulation of translation(131-133) **.** The factor EF-G catalyzes the translocation of the tRNA and mRNA down the ribosome at the end of each round of polypeptide elongation. Translation elongation factor EF-G uses GTP to catalyze translocation of peptidyl-tRNA from the ribosomal A/P site to the P/P site (134) After GTP hydrolysis and translocation, EF-G·GDP leaves the ribosome and is regenerated by the spontaneous exchange of GDP for GTP off the ribosome(134, 135). EF-G·GDP also plays a role with RRF (ribosome recycling factor) in splitting the ribosome into its two subunits after translation termination(136, 137). EF-G mutations result in inappropriate global transcriptional patterns, attributed to perturbations to ppGpp levels, and associate with a variety of phenotypes, including reduced translation rate, increased cell size at division, cell filamentation, cell clumping, reduced susceptibility to bacteriophage infection and reduced virulence(138). Where, ppGpp acts as a nutritional stress signal which binds to the β-subunit of RNA polymerase(139, 140) and reduces its affinity for promoters of stable RNA(141, 142) by inhibiting formation of a ternary transcription initiation complex(143, 144).

In Toxoplasma gondii (Tg), a mutant (49E10) containing an insertion within the 3′UTR of TgEFG showed a dramatic reduction in its lethality in an acute infection mouse model. Decrease in TgEFG expression in the 49E10 mutant causes a severe virulence defect. These results suggest that regulation of translation in the apicoplast, a plastid-like organelle essential for proper growth of the Parasite, is essential during an animal infection, and TgEFG is absolutely required for survival in an animal infection(145).

Fusidic acid is a steroid antibiotic by binding to a complex of the ribosome and elongation factor G (EF-G)(146) stabilizes EF-G·GDP on the ribosome after translocation of peptidyl-tRNA, blocking further protein synthesis(147). Resistance to fusidic acid in Salmonella enterica serovar Typhimurium is caused by mutations in fusA encoding EF-G (148).

Macvanin and coworkers concluded that, while fusidic acid directly inhibits protein synthesis and thus inhibits the growth of sensitive Salmonella typhimurium cells, cells that acquire resistance through mutations of EF-G suffer from perturbations in ppGpp levels, potentially resulting in loss of virulence because of inappropriate transcription patterns(149, 150). Similar results were obtained on demonstrating physiological role of EF-G also in salmonella, authors concluded that Fusr

mutants are sensitive to oxidative stress and significantly impaired in growth or survival. They explained the reduced in vivo fitness of the Fusr mutants to be resulted from their failure to respond appropriately to stress conditions(149, 150).
Protein synthesis in prokaryotic and eukaryotic organisms is suppressed by oxidizing reagents(151). Elongation factor G was identified as one of proteins that were abundantly carbonylated in cells of Escherichia coli that had been exposed to H_2O_2(152, 153) and in mutant E. coli cells that lacked a superoxide dismutase(154). Biochemical studies using a translation system in vitro from Synechocystis revealed that EF-G is a primary target of inactivation by ROS, and that inactivation of EF-G by H_2O_2 is caused by the oxidation of two specific cysteine residues with the resultant formation of an intramolecular disulfide bond(155, 156). Treatment of EF-G in E.coli with H_2O_2 resulted in the oxidation of two specific cysteine residues and subsequent formation of an intramolecular disulfide bond. This oxidation was responsible for the inactivation of EF-G in translation. Treatment of EF-G with H_2O_2 resulted in a decrease in the GTPase activity of EF-G via interference with an appropriate conformational change in EF-G that is induced after EF-G binds to the ribosome(157).
Interestinguily, obtained results showed an increased expression of this protein in MRSA PVL+. This experimental evidence could be due to several factors. The first one, as previously described, could be the depletion of this protein due to oxidative stress (see above). Indeed the lack of SOD in MSSA PVL+ could enhance oxidative stress and deplete the amounts of EF-G. The second one is the involvement of EF-G in the antibiotic resistance. It has been demonstrated that EF-G is a protein that, if blocked, could result in deficient mechanism *S. aureus* adaptation against xenobiotics and environmental changes. The overexpression of this protein in MRSA PVL+ highlighted in our results can further explain the importance of this protein in the development of antibiotic resistance(158).

5.1.3 Ferritin (Downregulated in MRSA PVL+)

Ferritins are ancient proteins that store iron away from the delicate machinery of the cell to be released again in a controlled fashion at the time of need. Ferritins are the major iron storage proteins found in eukaryotes (H chain) and gram-negative prokaryotes(159). The iron storage mechanism involves binding of ferrous iron to ferritin protein followed by migration to the ferroxidase catalytic site where ferrous iron [Fe (II)] is oxidized to the ferric [Fe (III)] state. Oxygen and hydrogen peroxide represent the major cellular oxidants for this oxidation reaction(160) Subsequently Fe (III) is transferred and sequestered as ferric mineral in the storage cavity of ferritin making it available for the cell at the time of iron deprivation(161).
The iron acquisition and iron storage pathways of Mycobacterium tuberculosis (Mtb) have been linked to its growth and disease-causing ability(162-164) as well as are important for the survival and hematogenous spread of the pathogen(165).
Iron is an essential micronutrient, which is required for many key metabolic processes in both bacterial and mammalian cells. Iron is needed for important cellular functions, such as the transport and storage of oxygen, and as a catalyst in electron transport processes. It is a very versatile biocatalyst. A variety of enzymes that require iron for activity have been characterized; these include ribotide reductase, nitrogenase, peroxidase, catalase, cytochromes, and succinic dehydrogenase. These enzymes serve important cellular roles, such as the reduction of ribonucleotides and dinitrogen and the activation and decomposition of peroxides. While, iron deprivation in an organism causes cessation of growth leading to its death, an increase in iron results in the production of reactive oxygen species (ROS) that damage the basic components of the cell such as nucleic acids and lipid membranes(166).
Iron exerts an inhibitory effect towards interferon-γ (IFN-γ) activity, which negatively affects antimicrobial effector pathways of macrophages including the expression of inducible nitric oxide synthase (iNOS) and of tumour necrosis factor-α (TNF-α)(167-170). Despites its biological

importance, high levels of free intracellular ferric iron are toxic to cells, so the amount of free iron in the cytoplasm must be strictly regulated. In particular, iron is notorious in its ability to catalyze the formation of hydroxyl radicals that can cause cellular death(171).

Because of iron's insolubility and iron availability is further limited by the induction of hypoferremia during infection, which has long been recognized as a component of the acute-phase response to infection. This acute-phase response, that can be induced by microbes and certain microbial products such as endotoxins, is mediated through the increased release of various cytokines, particularly interleukin-1 and tumor necrosis factor alpha (cachectin)(172, 173), microorganisms have evolved a number of mechanisms for the acquisition of adequate iron from the environment(174, 175); these mechanisms are closely linked to bacterial virulence. The competitive growth and survival of bacteria in the host require a number of adaptive responses on the part of the bacteria; the acquisition of iron is one of the most important of these adaptive responses for bacterial pathogenesis.

In addition to its physiological functions in bacteria, iron levels regulate different virulence determinants such as toxins, enzymes and adhesins. Expression of iron acquisition systems by pathogens has been linked to their pathogenicity and is essential for microbial growth and proliferation(176, 177).

Several iron-regulated virulence factors have been identified in a number of pathogenic bacteria that include iron-repressible outer-membrane proteins in Escherichia coli, Enterobacter cloacae, Erwinia spp., Klebsiella mobilis, Neisseria gonorrhoeae, Pseudomonas spp., Salmonella typhimurium, Shigella fleexneri, Vibrio spp. and Yersinia spp.(178, 179). The haemolysin of Vibrio cholerae, diphtheria toxin of Coryne bacterium diphtheriae, Shiga toxin of Shigella dysenteriae and Shiga-like toxin of E. coli are also iron-regulated toxins(180).

As iron uptake must be tightly regulated to avoid the toxic effects of iron over accumulation(181) bacteria have global regulators such as Fur usually mediate this iron regulation. An additional mechanism for overcoming intracellular iron toxicity is to remove free iron from the cytoplasm by compartmentalizing the iron into specialized intracellular iron storage proteins. This results in a reserve of nontoxic iron that can be used as a nutrient source during conditions of iron starvation.

In Vibrio parahaemolyticus, Escherichia coli, Porphyromonas gingivalis, and Campylobacter jejuni, ferritins are required for maximal growth under iron-restricted conditions(27, 182-185) but the C. jejuni ferritin also has an important role in protection against iron-mediated oxidative stress that is not observed in E. coli or P. gingivalis. In contrast, the primary role of the Helicobacter pylori ferritin appears to be in protecting this gastric pathogen against metal toxicity(186). Besides its function in dispatching metabolic iron, ferritin also has a protective effect against iron-catalysed oxidative damage including lipid peroxidation(187-189)**,** DNA modifications, and alterations in proteins. The oxidative damage is thought to be caused by O2 radicals (hydroxy-OH, alkoxy-OR or organic peroxyl RO2) which appear in the presence of iron ions and O2 through cyclical oxidoreduction, called the Fenton reaction(190). This fact was reviewed by Theil who reported that DNA in bacteria is protected from hydrogen peroxide damage by dps (DNA protection in starvation) proteins, which are mini-ferritins with the characteristic quaternary and secondary structure constructed with 12 rather than 24 polypeptide subunits(191). Author mentioned that Dps represents the use of ferritin by the pathogen to defeat the release of toxic oxygen species produced by the host, while the host uses ferritin to decrease pathogen access to iron. The amino acid sequence of dps proteins is very different from the other ferritins, but the subunit folding and protein cage around a solvent or mineral-filled core are conserved(191).

One particularly deleterious reaction that free iron can promote is reaction with reactive oxygen species (ROS) to form highly reactive hydroxyl radicals via Fenton chemistry.

Wai et al studied the biological function of ferritin in Campylobacter Jejuni by constructing a ferritin-deficient mutant isolate. They found that growth of ferritin-deficient strain SNA1 was clearly inhibited under iron deprivation, the ferritin-deficient mutant was more sensitive to killing by H2O2 and paraquat than the isogenic parent strain. These findings demonstrate that ferritin in C.

jejuni makes a significant contribution to both iron storage and protection from intracellular iron overload, and resulting iron mediated oxidative stress(184).
Obtained data demonstrate that the expression of ferritin in MSSA is increased of two folds in comparison to MRSA and support the suggestion that MSSA could be more virulent than MRSA.
This concept could be particularly true, specially according with the role of ferritin as a key protein essential for iron storage in body fluids where iron level is insufficient for microbial growth requirements.

5.1.4 Iron-sulfur cluster repair protein (ScdA) (Downregulated in MRSA PVL+)
Iron-sulfur (Fe-S) clusters are important prosthetic groups with unusual chemical properties that enable the proteins that contain them (Fe-S proteins) to function in pathways ranging from metabolism to DNA repair. They are evolutionarily ancient and are present in essentially all organisms, including Archaea, bacteria, plants and animals. Fe-S clusters are crucial for facilitating enzyme activities in all kingdoms of life because they can bind electronrich enzymatic substrates, accept or donate single electrons and stabilize specific protein conformations that are important to the activities of numerous proteins.
As it is well established that, in case of infection neutrophils and macrophages of the mammalian immune system produce reactive oxygen and reactive nitrogen species, that have important roles in killing pathogenic bacteria by damaging such cellular components as DNA, lipids, and proteins. Particularly vulnerable to inactivation are iron-sulfur (Fe-S) proteins, which were among the first catalysts used by nature (192). They participate in numerous cellular processes, including crucial biological events like DNA synthesis and processing of dioxygen, oxygen/nitrogen sensing, control of labile iron pool and DNA damage recognition and repair**.** They are source of free iron (193). Finally, Fe-S clusters are used as “molecular switches” for gene regulation at both the transcriptional and translational levels due to their sensitivity to cellular redox conditions (194). In virtually all organisms where they fulfill crucial redox, catalytic, and regulatory functions (194-197).
Iron-sulfur clusters are found in a variety of metalloproteins, such as the ferredoxins, as well as NADH dehydrogenase, hydrogenases, Coenzyme Q-cytochrome c reductase, Succinate-coenzyme Q reductase and nitrogenase**.** Iron-sulfur clusters are best known for their role in the oxidation-reduction reactions of mitochondrial electron transport. They have many other functions including catalysis as illustrated by aconitase, generation of radicals as illustrated by SAM-dependent (S-adenosylmethionine) enzymes, and as sulfur donors in the biosynthesis of lipoic acid and biotin**.**
About Aconitase (ACO), it belongs to the lyases class of enzymes. It contains a [4Fe-4S] cluster and catalyses the conversion of citrate to isocitrate (198). Isocitrate is the substrate for NADP-dependent isocitrate dehydrogenase, an important producer of NADPH in the cytosol. Glutathione reductase uses NADPH to regenerate reduced gluthatione, which may be oxidized by glutathione peroxidase to remove H_2O_2, an oxidative stress inducer (199). In this respect, production of isocitrate by ACO may be considered as a contribution to the cellular defense against oxidative stress.
S. aureus synthesizes a single aconitase, a dehydratase of the tricarboxylic acid cycle, that contains a $[4Fe\text{-}4S]^{+2/+1}$ cluster that is susceptible to damage by NO and oxidants such as hydrogen peroxide. In the absence of scdA, the activity of aconitase was found to be 33% lower than in the *S. aureus* parent strain. Also, authors recorded that *S. aureus* scdA mutant strains was more sensitive to oxidative conditions than its parent. Hence, ScdA constitutes an efficient protection system against hydrogen peroxide, and essential for the repair of an $[4Fe\text{-}4S]^{+2/+1}$ protein whose cluster is damaged by oxidative or nitrosative compounds as fumarase.
As recorded that as oxidative stress may result in DNA damage, oxidatively damaged single DNA base is preferentially repaired by BER, the repair system that directly removes a damaged base with or without nicking the adjacent sugar backbone**.** The key enzymes in this process are DNA

glycosylases, enzymes that cut the N-glycosidic bond between the damaged base and sugar, thus initiating the first step of repair. Most glycosylases cut out the damaged base from the DNA leaving an abasic site or a single-strand break. And many of DNA glycosylase containing Iron sulfer clusters (200-204). Lukianova and David 2005 (205) reviewed that several possible roles for the iron–sulfur cluster in DNA repair enzymes, they recorded that, the cluster plays a unique structural role by recognizing, positioning and distorting duplex DNA for the base removal activity.

The role of iron sulfer clusters in combating the released host immune defenses was demonstrated in many issues. It was found that, isc (iron sulfur cluster) and suf (sulfer formation) are repressed under anaerobic conditions but highly induced in response to ROS, such as H_2O_2. In E. coli, exposure to hydrogen peroxide (H_2O_2) leads to a strong increase in suf transcription (206, 207). H_2O_2 is known to directly oxidize exposed [4Fe-4S] clusters in dehydratase enzymes (208). H_2O_2 also can oxidize protein cysteinyl residues to form sulfenic and sulfinic acid. Oxidation by H_2O_2 would perturb sulfur transfer during Fe-S cluster assembly, since those steps consist of donation of persulfide sulfur by protein cysteinyls. Given the consequences of H_2O_2 exposure, it seems likely that suf is activated to help meet an increased demand for de novo Fe-S assembly (209).

According with our findings this protein is downregulated in MRSA PVL+ and has a higher expression level in MSSA PVL+. This result is in accordance with the results obtained for other proteins especially if considering the overview that in MSSA there is an enhanced iron metabolism linked and necessary to counteract the higher oxidative stress due to the lower SOD activity.

5.1.5 Alcohol dehydrogenase (Downregulated in MRSA PVL+)

Alcohol dehydrogenases (ADHs) display a wide range of substrate specificities and fulfill several key physiological functions.

In many bacteria, alcohol dehydrogenase plays an important part in fermentation: pyruvate resulting from glycolysis is converted to acetaldehyde and carbon dioxide, and the acetaldehyde is then reduced to ethanol by an alcohol dehydrogenase called ADH1. The purpose of this latter step is the regeneration of NAD+, so that the energy-generating glycolysis can continue.

Bacteria are sensitive to environmental changes as oxygen concentration and then respond by switching their regulatory mechanisms to ensure that the most energetically favorable process is active under a given environmental condition. Fuchs et al.(210) carried out a study for demonstrating the gene expressed in *S. aureus* grown under anaerobic condition to estimate the regulators of microbial survival, obtained results indicate that under fermentative conditions *S. aureus* may undergo mixed-acid (lactate, formate, and acetate) and butanediol fermentation. Therefore, pyruvate could be reduced either to lactate by the activity of lactate dehydrogenase or metabolized to acetoin and 2,3-butanediol by the activity of acetolactate synthase (BudB), α-acetolactate decarboxylase (BudA1), and acetoin reductase (SACOL0111). Each conversion described above contributes to oxidation of NADH, which is a requisite under fermentation conditions. Finally, pyruvate could be converted to acetyl-CoA or acetyl-phosphate, which is further metabolized to acetate or ethanol. While the former reaction is accompanied by the synthesis of ATP, the latter involves recycling of NADH.

While in abscesses bacteria live under very anaerobic conditions, the cardiac endothelial tissues might be characterized by high oxygen concentrations. The ability of *S. aureus* to adapt to extreme changes in external oxygen concentrations implies the existence of one or more systems that regulate anaerobic gene expression. These findings indicate the physiological impact of fermentation enzymes in the virulence and pathogenesis of *S. aureus* and the influence of oxygen availability on physiology and virulence factor production might thus be of some importance for understanding the pathogenicity of *S. aureus* in different tissues.

Despites the role of alcohol dehydrogenase as one of the fermentation enzymes utilized for survival and multiplication of *S. aureus* under restricted conditions, it might play a significant role in antibiotic resistance. As regarding to Mycobacterium bovis, mycolic acids and free lipids are

thought to contribute to the unusually low permeability of the mycobacterial cell wall which is believed to play a role in the resistance of mycobacteria to therapeutic agents. Wilkin et al. 1999 (211) in a study demonstrated that the M. bovis BCG ADH might be involved in the biosynthesis of the free lipids required for the formation of the mycobacteria cell envelope. Their results implicate the M. bovis BCG ADH in the mycobacterial defence system, producing a more lipidic and resistant mycobacterial cell envelope. Another study suggested a potential role of one or several ADHs in the virulence of M. tuberculosis (212).
Alcohol dehydrogenase is downregulated in MRSA PVL+ in comparison to MSSA PVL+. Higer levels of this protein in MSSA PVL+ could correlate with enhanced resistance of *S. aureus* to several disinfectant as reported by other studies (213). As demonstrated by Korem and colleagues the higher reducing power generated by the higher activity of the alcohol dehydrogenase is responsible of a higher virulence and of a higher resistance to the host defence mechanisms and alcohol-based disinfectant methods (214). This finding too is in accordance with the previously described results and with the suggestion of a higher virulence activity of the MSSA isolates.

5.1.6 DNA gyrase subunit B (Downregulated in MRSA PVL+)

DNA gyrase, bacterial topoisomerase II, catalyzes ATP dependent DNA supercoiling and is ssential for bacterial growth, being involved in DNA replication, transcription, and recombination (215-218).
They are essential to the cell, which makes them attractive targets for developing more effective antimicrobials to fight the ever increasing threat of resistance to existing antibiotics in pathogenic bacteria, particularly methicillin-resistant *S. aureus* (MRSA) (219). DNA gyrase introduces negative supercoils into DNA, which is essential for DNA replication, elongation and transcription. The activity of this enzyme is energetically driven by the hydrolysis of ATP, catalysed by the GyrB subunit (220-222).
Two classes of drug act by targeting DNA gyrase. Antibacterial 4-quinolones, such as ciprofloxacin, interrupt DNA breakage and resealing by the A subunits, whereas the coumarin drugs novobiocin and coumermycin interfere with ATP utilization by the B proteins (217, 218, 223, 224).
Also The aminocoumarin antibiotics, natural products of various Streptomyces strains are powerful inhibitors of gyrase, binding to this target with higher affinity than modern fluoroquinolones (219). Aminocoumarins bind to the GyrB subunit of gyrase or the ParE subunit of topo IV, competing with the binding of ATP (219, 225).
Gyrase inhibitors have been widely used, particularly against *S. aureus* (226). Both ciprofloxacin and novobiocin are active against *S. aureus*, including methicillin-resistant strains (226-229), although resistance to fluoroquinolones has rapidly emerged (230, 231)
Obtained results show how this protein is downregulated in MRSA PVL+ and has a higher expression in MSSA. The reason of this differential expression is still unknown and it has to be interpreted.

5.1.7 S-adenosylmethionine: tRNA ribosyltransferase-isomerase (QueA) (Upregulated in MSSA PVL+)

The enzyme S-adenosylmethionine:tRNA ribosyltransferase-isomerase catalyzes the penultimate step in the biosynthesis of the hypermodified tRNA nucleoside queuosine (Q), an unprecedented ribosyl transfer from the cofactor S-adenosylmethionine (AdoMet) to a modified-tRNA precursor to generate epoxyqueuosine (oQ).
Queuosine and its derivatives occur exclusively at position 34 (the wobble position) in the anticodons of tRNAs coding for the amino acids asparagine, aspartic acid, histidine, and tyrosine**.** A definitive picture of the biochemical function or functions of queuosine has yet to emerge, but it has been correlated with eukaryotic cell development and proliferation (232), neoplastic transformation

(232-235), tyrosine biosynthesis in animals, translational frame shifts essential to retroviral protein biosynthesis (236), and the ability of pathogenic bacteria to invade and proliferate in human tissue (237). The overexpression of this protein in MSSA is in accordance with the overview of this work that is oriented to describe MSSA isolates more virulent than MRSA. Especially in the light of the putative involvement of this protein in the bacterial survive (238).

5.2 MSSA PVL- vs MRSA PVL-.

5.2.1 30S ribosomal protein S6 (Upregulated in MRSA PVL-)

Ribosomes are essential, highly abundant components of all cells. The rate of ribosome biosynthesis is directly correlated to the rate of bacterial cell proliferation. Ribosomes are the ribonucleoprotein organelles on which the mRNA-directed synthesis of proteins takes place. They consist of a large and a small subunit which assemble during the initial stages of protein synthesis.

30S ribosomal protein S6 has been identified as a cold shock protein in E. coli and B. subtilis (239), and induced by heat shock in Myxococcus Xanthus, suggesting that 30S ribosomal protein S6 may play a unique role in sensing temperature differences to control ribosome function. It was more expressed in response to osmotic shock salt stress in Listeria monocytogenes (240). It is one of the ribosomal units responsible for formation of a ribosomal initiation complex (241).

5.2.2 Hypoxanthine-guanine phosphoribosyltransferase (Downregulated in MRSA PVL-)

Many bacteria are able to produce nucleotides by de novo synthesis pathways. In the purine nucleotide synthesis pathway, inosine monophosphate (IMP), a molecule produced from reactions originating in sugar and amino acid metabolism, is converted into either ATP or GTP. The external environment of bacteria often contains nucleosides (e.g. guanosine, xanthosine) and nucleobases (e.g. guanine, xanthine) that have been excreted by living cells or arise from degradation of dead cells. Intracellular breakdown of nucleic acids may also be a source of nucleosides and nucleobases. To prevent the loss of valuable precursors, bacteria have evolved salvage pathways that enable recovery of purine and pyrimidine bases and nucleosides, which can then be recycled into nucleotides or used as a source of energy, carbon or nitrogen**.** HprT catalyses the addition of a ribose-phosphate moiety to either guanine or hypoxanthine, using phosphoribosyl pyrophosphate (PRPP). Thus, guanine or hypoxanthine is converted into the nucleotides GMP or IMP, respectively.

Enzymes of the purine salvage pathway, the purine phosphoribosyltransferase (PRTase) family, are found in most microbes and mammals; their reaction involves the ribophosphorylation in one step of purine nucleobases (hypoxanthine, guanine, adenine, or xanthine) and their analogues to their respective nucleoside 50-monophosphate and pyrophosphate (242). Hypoxanthine–guanine phosphoribosyltransferase (HGPRT1) is a purine salvage enzyme that catalyzes the Mg2+-dependent reversible transfer of the 50-phosphoribosyl group from a-D-ribofuranose 10-pyrophosphate 50-phosphate (also known as 50-phosphoribosil-a-10-pyrophosphate; PRPP) to the N9 position of 6-oxopurines (guanine, hypoxanthine) to form the corresponding ribonucleotides GMP (guanosine 50-monophosphate) and IMP (inosine 50-monophosphate), with the release of PPi (inorganic pyrophosphate) (243). PRTases are of significant interest in both human genetic diseases and parasite treatments, such as, respectively, Lesch–Nyhan syndrome, and Chagas' disease (244).

During stationary phase and long-term starvation, nutrient scavenging and salvaging systems are induced. SPW 20 has an insertion in a gene homologous to hprT from B. subtilis (55% over 90 aa). Hypoxanthineguanine phosphoribosyltransferase is a key enzyme in the salvaging system for purine nucleotides. This salvage pathway comprises a collection of enzymes capable of converting nucleobases and nucleosides to nucleotides (245, 246). This enables organisms to use exogenous

bases liberated from lysed cells or recycle those produced by the breakdown of unstable RNA. The purine salvage pathway is important during starvation, both as a source of energy and for the maintenance of purine pools. Hypoxanthine is the predominant intracellular nucleobase during stationary phase in E. coli (247), and in *S. aureus* (248).
The hprT gene is clearly not essential for the growth of *S. aureus*, although it has a minor role in the ability to grow in high-salt and survive in starvation conditions due to likely defects in nucleotide recycling. The expression of hprT–lacZ in rich medium is maximal in post-exponential phase, which matches the role of hprT in recycling as the culture becomes nutrient-limited. In the presence of highsalt concentrations, the defect in nucleotide recycling results in a decreased growth rate which implies an important role for nucleotide recycling under these stressful environmental conditions (249).
As enzymes of the purine phosphoribosyltransferase (PRTase) family are components of purine salvage pathway and have been proposed as drug targets for the development of chemotherapeutic agents against infective and parasitic diseases as Mycobacterium tuberculosis (250). Antagonists of hypoxanthine-guanine phosphoribosyltransferase coul be useful to counteract resistance in MRSA.

5.2.3 Pyruvate dehydrogenase E1 component subunit beta (Upregulated in MRSA PVL-)
The tricarboxylic acid (TCA) cycle is an essential source of energy and biosynthetic intermediates for many organisms. Pathogenic organisms can be divided into three categories based on the TCA cycle. Those in the first group do not possess a TCA cycle and have become dependent upon the host to provide amino acids or intermediates for biosynthesis (e.g., Borrelia burgdorferi and Streptococcus pyogenes). Those in the second group have an incomplete TCA cycle and are auxotrophic for some amino acids (e.g., Yersinia pestis and Haemophilus influenzae). Lastly, the third group is characterized as having a complete TCA cycle (e.g., Pseudomonas aeruginosa and *S. aureus*) but, depending upon other metabolic limitations, can be auxotrophic for certain amino acids. The relative independence of the latter two groups of pathogens on the host for amino acids suggests that the TCA cycle may perform important functions in these organisms during pathogenesis. This supposition is supported by extensive experimental data demonstrating that TCA cycle function is involved in virulence, survival, and persistence (251-256).
The organism produces many extracellular virulence factors and cell wall-associated adherence proteins that are important for colonization, tissue invasion, evasion of host defenses, and nutrient acquisition. The expression of many virulence factors is negatively regulated by glucose and is maximal during the postexponential phase of growth (257). *S. aureus* uses the pentose phosphate and glycolytic pathways to catabolize glucose to pyruvate. The catabolic fate of pyruvate is determined by the growth conditions. Under anaerobic growth, pyruvate is reduced to lactic acid (258), whereas during aerobic growth, pyruvate undergoes oxidative decarboxylation to produce acetyl-coenzyme A (259). Acetylcoenzyme A is converted into acetylphosphate, which is then used for substrate-level phosphorylation to generate ATP and acetate.
The pyruvate dehydrogenase (PDH) complex, a member of the 2-oxo acid dehydrogenase family, catalyzes the irreversible oxidative decarboxylation of pyruvate to acetyl-coenzyme A, linking glycolysis with the TCA cycle (260). The complex is composed of multiple copies of three different enzymes: pyruvate decarboxylase (E1), dihydrolipoamide dehydrogenase (E2), and lipoamide dehydrogenase (E3). E1 and E2 are unique to the complex, whereas E3 is shared with other 2-oxo acid dehydrogenase complexes (261, 262). The PDH complexes of gram positive bacteria consist of a core of 60 E2 subunits with icosahedral symmetry and 30 E1 and 6 E3 subunits associated. E1 of gram-negative bacteria is a homodimer, while this component from gram-positive bacteria is a heterotetramer ($E1\alpha_2E1\beta_2$).
Birkenstock et al. 2012 characterized the antibacterial properties of triphenylbismuthdichloride (TPBC), which has recently been successfully used against device-associated infections. They demonstrate that TPBC has potent antimicrobial activity against many bacterial pathogens. Using

an exometabolome profiling approach, a unique TPBC-mediated change in the metabolites of *S. aureus* was identified, indicating that TPBC blocks bacterial pyruvate catabolism. Enzymatic studies showed that TPBC is a highly efficient, uncompetitive inhibitor of the bacterial pyruvate dehydrogenase complex. TPBC was shown to block the bacterial pyruvate dehydrogenase complex (PDHC), thereby abrogating central metabolic activities. Their study indicates that inhibition of the bacterial pyruvate dehydrogenase complex may represent a promising strategy for combating multidrug-resistant bacteria. The higher amount of this protein in MRSA PVL- could be linked to the mechanisms involved in the higher bacterial resistance of this isolate to the action of antibiotics (citare articolo coli multidrug resistance).

5.2.4 Probable thiol peroxidase (Downregulated in MRSA PVL-)

Most aerobes have multiple, often overlapping pathways for detoxifying reactive oxygen species (171). Of these defense mechanisms, a new type of peroxidase, named thiol peroxidase (TPx) has been known to eliminate H2O2 and alkyl hydroperoxides with use of a thiol-reducing equivalent. Thiol peroxidases (Tpx), also known as peroxiredoxins, are present in organisms from all kingdoms (263). As peroxidases, they act to remove peroxides and provide defence against oxidative damage. Tpx contains thiol-dependent peroxidase activity and is capable of reducing either organic hydroperoxides or H_2O_2 by using reduced thioredoxin (Trx) as an electron donor. The resultant oxidized thioredoxin is cycled back to the reduced form by the thioredoxin reductase reaction, which utilizes NADPH as a reductant (264). In addition to protecting against reactive oxygen species, some peroxiredoxins can reduce and thereby detoxify reactive nitrogen and sulfur (118) species.

H2O2 is both a toxic compound that can cause oxidative stress and a second messenger that is required for cell proliferation (265). Its signaling function is thought to result from direct oxidation of various cell signaling and regulatory components, and its toxicity from stochastic oxidative damage to proteins, lipids, and nucleic acids (266, 267). Several classes of enzymes, such as catalases and peroxidases, have evolved that specifically act on H2O2 or other hydroperoxides as substrates. Prominent among them are thiol-dependent peroxidases, which belong to peroxiredoxin (Prx) and glutathione peroxidase (Gpx) protein families. Thiol peroxidase genes are present in all previously characterized organisms, suggesting that these enzymes serve important functions conserved throughout evolution. Prx and Gpx have been implicated in cell signaling due to their ability to reduce intracellular levels of hydroperoxides and to serve as floodgates of H2O2 signaling (263, 268).

The TPx family, also referred to as TSA/AhpC family, is a large family of newly coming peroxidases that have been discovered from prokaryotes to eukaryotes (264).

The role of thiol peroxidase as a virulence factor in many pathogens responsible for confronting the oxidative stress raised either as a fate of microbial metabolism or as a mechanism of immune defense in mammalian host. It acts as scavenger of H_2O_2. In Cryptococcus neoformans, a fungal pathogen causing meningitis in immunocompromised patients, The thiol peroxidase mutant isolates was sensitive to NO and antifungal drugs known previously to be inefficient in treatment and the mutants were significantly less virulent. In Pseudomonas aeruginosa, tpx mutant reveals differential patterns of H_2O_2 sensitivity with different H_2O_2 concentrations. By the same way, the same result was obtained in E.coli (264), Streptococcus pneumonia. Tpx plays a significant role in peroxide and superoxide resistance in Helicobacter pylori. Over expression of peroxiredoxin in Leishmania chagasi parasites enhances its survival within U937 macrophage cells.

La Carbona et al. 2007 (269) showed that thiol peroxidase (Tpx) mutant is significantly more sensitive against macrophage killing than the alkyl hydroperoxide reductase (ahp) and also NADH peroxidase (npr) mutants, identifying the thiol peroxidase as the most important protective enzymatic activity among the three peroxidases of E. faecalis inside murine macrophages. Authors concluded that Tpx enzyme is clearly the most important activity to protect Enterococcus faecalis

cells against the complex antibacterial weapons synthesized during the oxidative burst inside mouse peritoneal macrophages. The attenuated virulence of the Δtpx mutant demonstrated further the in vivo functional importance of the thiol peroxidase and, in consequence, may identify it as a potential drug target.
Tpx was described as a culture filtrate protein in search for vaccine candidates in Mycobacterium tuberculosis, and proteomic studies identified it in cell wall fractions (270-272). It is characterized as one of the antigens strongly recognized in animals infected with this organism and induces a strong proliferative response in humans and mice (273).
Obtained resuts showed how this protein is downregulated in MRSA PVL- and has a higher expression in MSSA PVL-. This result too is in accordance with other obtained result that demonstrate how the MSSA isolates could need to developed a more efficient way to counteract the host defence system.

5.2.5 Transcriptional regulatory protein srrA (Downregulated in MRSA PVL-)

During in vivo and in vitro growth, bacteria encounter oxygen in several forms, from the beneficial triplet oxygen (O2) and water (H2O) to the more harmful oxidants superoxide ($O2^-$), hydrogen peroxide (H2O2), and hydroxyl radicals ($OH^.$). The beneficial forms are necessary for numerous biochemical reactions (e.g., the hydration of cis-aconitate to form isocitrate and the oxidation of reduced flavin adenine dinucleotide). However, in the example of reduced flavin adenine dinucleotide, the addition of one electron to O2 generates $O2^-$, a ROS that can damage iron-sulfur clusters, DNA, and sulfhydryl groups. Dismutation of the superoxide anion radical generates the more-stable H2O2. If oxidative stress has induced the release of iron from iron-sulfur clusters, this creates an intracellular environment permissive to Fenton chemistry. Fenton chemistry generates the highly reactive $OH^.$ from ferrous iron and H2O2. Pathogenic bacteria must respond not only to endogenous metabolism-generated ROS but also to host-generated exogenous ROS. These observations highlight the bacterial need for oxygen-responsive regulators. Besides, differences in virulence factor regulation contribute to the variable pathogenic potential of the organism in humans or other animals. *S. aureus* encodes many global regulators of virulence, including a quorum-sensing system, the Sar family of virulence regulators, two-component systems, and transcriptional regulators. The staphylococcal respiratory response (SrrAB) two-component system regulates energy metabolism as well as the genes tst (toxic shock syndrome toxin 1 [TSST-1]), spa (staphylococcal protein A), and icaR (intercellular adhesion locus repressor) in response to oxygen (274-278). SrrB is a membrane-associated protein, and SrrA is a cytoplasmic DNA binding protein whose DNA binding activity is influenced by phosphorylation (274, 277).
Two-component regulatory systems have attracted interest as possible targets for antimicrobial chemotherapy (279, 280). Indeed, compounds that inhibit signal transduction through these systems have been shown to be effective even against multi-drug-resistant pathogens, including methicillin-resistant *S. aureus* and vancomycin-resistant Enterococcus faecium (281).
Yarwood et al. 2001 (275) investigated effects of srrAB expression on expression of RNAIII (the effector molecule of the agr locus) and on production of TSST-1 (an exotoxin) and protein A (a surfaceassociated virulence factor). Authors concluded that the putative two-component system encoded by srrAB, SrrA-SrrB, acts in the global regulation of staphylococcal virulence factors, and may repress virulence factors under low-oxygen conditions. Furthermore, srrAB may provide a mechanistic link between respiratory metabolism, environmental signals, and regulation of virulence factors in *S. aureus*. They suggest that manipulation of SrrA-SrrB in *S. aureus* may suppress production of exotoxins. This suggestion is in accordance with results obtained by Schlievert and his collegues as they showed that the presence of glycerol monolaurate can significantly reduce the amount of toxin produced in culture or when applied to tampons while having only weak antimicrobial action (257). A possible mechanism of glycerol monolaurate, which acts at the lipid-water interface, is to interfere with the activity of the membrane-bound sensor

proteins. Inhibition of toxin production would reduce systemic disorders in patients while helping to prevent further spread of the organism. Treatment combining drugs that interfere with staphylococcal environmental sensing mechanisms with standard antibiotics may provide an effective two-pronged approach by quickly eliminating toxin production and clearing the organism from the patient.

In another study, truncations in srrA affected growth and virulence factor regulation. N2 demonstrated decreased growth and increased hemolysin and TSST-1 activity with oxygen limitation, while N19 showed decreased growth and increased hemolysin activity with oxygen limitation. Where, N2 and N19 were two strains with N-terminal truncations in SrrA (282)

It has been demonstrated that SrrAB can indirecly contribute to the protection of *S. aureus* against non-oxidative defence mechanisms (274). Its upregulation in MSSA PVL- could be explained as an adaptive phenomenon to overcome the lower resistance of this isolate to antibiotics.

5.2.6 ATP synthase subunit beta (Downregulated in MRSA PVL-)

ATP synthase (F0F1) is a multisubunit, membrane-associated protein complex, present in all living organisms and is located in the membranes of mitochondria, bacteria, and chloroplast thylakoids as well as on the surfaces of various cell types, including endothelial cells, keratinocytes, and adipocytes (283-285). It catalyzes the phosphorylation of ADP to ATP at the expense of a proton motive force generated by an electron transport chain in energy-transducing membranes (286). In some organisms, as bacteria growing under anaerobic conditions, it also works in the reverse direction by hydrolyzing ATP and generating an electrochemical proton gradient across a membrane to support locomotion or nutrient uptake. ATP synthase is an exceptionally complicated protein complex. ATP synthases from different sources have a common structure; the enzyme consists of two sectors, a soluble globular, membrane-peripheral F1 catalytic sector, carries the catalytic domains of the enzyme and is composed of five different subunits designated α, β, γ, δ, and ε and a membrane-integral F0 proton-translocating sector. F1 is bound to the membrane by interaction with F0 (287-289). The ATP synthase complex plays a central role in energy transduction in living cells. According to Mitchell's theory of chemiosmosis it uses the proton gradient of bacterial, mitochondrial, and chloroplast membranes to promote the final step in oxidative phosphorylation (or photophosphorylation) of ADP.

It has recently been demonstrated that blocking the ATP synthase enzyme by diarylquinoline TMC207, a new clinical diarylquinoline drug candidate, is a highly efficient strategy for the treatment of drug resistant Mycobacterium tuberculosis (290). In Leishmania amazonensis avirulent promastigotes were less efficient than the virulent promastigotes in hydrolyzing ATP (291).

In eukaryotes and prokaryotes, the degradation of most cell proteins requires metabolic energy (96). This feature of intracellular proteolysis applies not only for cytosolic proteins in bacterial and animal cells. Mitochondria (292) and chloroplasts also contain systems for complete degradation of abnormal proteins, and this process also requires ATP. An ATP requirement for proteolysis is surprising on thermodynamic grounds, since hydrolysis of peptide bonds should be a spontaneous, exergonic process, and protein breakdown by traditional proteases does not require energy-rich cofactors (96). Initial speculations concerning the energy requirement for proteolysis in eukaryotic cells led to the suggestion that ATP might be necessary for the function of lysosomes in which protein degradation was assumed to occur. However, bacteria lack such organelles, but do show a similar ATP dependence for proteolysis as animal cells (292). Therefore, this requirement must represent a more fundamental property of the degradative process.

As mentioned, Bacteria can produce ATP by phosphorylation of fermentable carbon sources or by oxidative phosphorylation using the respiratory chain and ATP synthase. ATP represents an efficient source of energy required for many cellular biological processes and functions.

Higgins and his colleagues described a class of closely related ATP-binding proteins, from several bacterial species, which are associated with a variety of cellular functions including membrane

transport, cell division, nodulation in Rhizobium and haemolysin export. These proteins comprise a family of structurally and functionally related subunits, which share a common evolutionary origin, bind ATP and probably serve to couple ATP hydrolysis to each of these biological processes. This finding suggests a specific role for ATP in cell division, nodulation during nitrogen fixation and protein export (293).
Transition metals (Cu+, Zn2+, Fe2+, Co2+, Ni2+, Mn2+) are essential micronutrients participate in biochemical pathways ranging from cellular respiration to gene expression. Consequently, these are key structural and catalytic components of a large fraction of bacterial proteomes (294-296). Conversely, the capability of metals to participate in Fenton reactions and directly interact with DNA and proteins renders them as potent cytotoxics if present at high levels or free in the cellular milieus (296-298). Transition metal transport P1B-ATPases couple the energy provided by ATP hydrolysis to the efflux of cytoplasmic substrates. P1B-ATPases drive cytoplasmic metal efflux, P1B-ATPases translocate metal outside of the cell, it would be expected that Cu+-ATPases play a role in virulence. many pathogenic bacteria have multiple isoforms of Cu+-ATPases encoded in their genome. they contribute to maintain cytoplasmic metal levels. However, their participation in the assembly of periplasmic and secreted metalloproteins has been shown. Both general functions appear essential for bacterial infection of host organisms (299-304).
Cellular ATP is the signal upregulating mgtC transcription when Salmonella experiences mild acidification (Lee and Groisman 2012). mgtC is the gene required for survival inside macrophages and for growth in low Mg2+ media in several phylogenetically unrelated intracellular pathogens. Expression of mgtC must be tightly regulated for a normal course of infection. Because inactivation of the mgtC gene attenuates Salmonella virulence in mice (305), whereas preventing transcription of AmgR, a PhoP-dependent anti-sense RNA that promotes the preferential degradation of the mgtC portion of the polycistronic mgtCBR message, renders Salmonella hypervirulent (306).
It has been demonstrated that the biofilm formation could be enhaced in case of decreasement of the overall cellular metabolism (307).
The obtained result about the MRSA PVL- that shows a decrement of this protein coul be explained through the theory that this isolate coud have a lower metabolism that could bring to a higher biofilm and quorum sensing conformation that causes a higher resistance to antibiotics (308).

6. Conclusion

S. aureus must continuously deal with stress situations in vivo. Such stress conditions may include changes in environmental temperature, pH, humidity, oxygen tension and the presence fo ROS and other stress agents.
To preserve this balance state, biological systems and living organisms have developed highly complex nonenzymatic and enzymatic protection, repair and detoxification mechanisms (171).
Reactive oxidants cause damage to the essential biomaterials of cells; for instance, by reacting with intracellular iron, hydrogen peroxide can form the hydroxyl radical through the Fenton reaction, which injures various cellular molecules including lipids, proteins, and DNA (82). Superoxide is also capable of promoting oxidative damage by increasing the intracellular concentration of free iron (97).
All organisms have developed various means of protecting themselves against ROS, represented by specific defenses and global responses that enable cells to survive periods of oxidative stress, which are regulated and respond to the environment-associated oxidative threat (111, 206). The survival of the bacterium depends on the evolution of a series of defenses, which include 1) detoxifying enzymes and free radical-scavenging substrates, 2) DNA and protein repair systems, and 3) competition for substrates favoring bacterial survival. In many cases, these defenses may be coordinately regulated. In the presence of high oxygen concentrations, bacteria employ multiple strategies to protect against reactive oxygen derivatives that are generated by the partial reduction of oxygen. They regulate the expression of genes involved in aerobic metabolism and in the enzymatic defense against reactive oxygen species (154). Secondly, bacteria may use non-coupled respiration and a mild uncoupling mechanism to accelerate the respiratory consumption of oxygen, and thereby lower the concentration of oxygen. Thirdly, motile bacteria take advantage of directed motility to escape from microenvironments where the oxygen concentration is too high for their metabolic lifestyle.
Previous studies indicate that oxygen plays an important role in virulence gene regulation and in the bacteria's ability to persist and grow in ecological niches similar to those of the host environment (248, 275, 277). And since *S. aureus* has the ability to invade different tissues, where oxygen tension varies in between different sites in the host, as in abscesses, completely anaerobic conditions have often been found. *S. aureus* was found to have the ability to grow under low-oxygen conditions by fermentation or nitrate respiration (259), that confirmed by synthesis of fermentative enzymes like lactate dehydrogenase, alcohol dehydrogenase, and pyruvate formate lyase (276).
In this study it has been observed that SOD is more expressed in MRSA than in MSSA. SOD is an antioxidant metalloenzyme, shares with other enzymes as primary defense mechanisms utilized by bacteria for preventing oxidative damage and protect against oxygen-dependent DNA damage, co-factored with metal ions, such as Mn^{2+}, Fe^{2+}, Cu^{2+}, or Zn^{2+}. The MSSA isolate overcomes the SOD lack with the production if higher amounts of thiol peroxidase (ptx). This feedback mechanism could also be linked to the expression of ferritin and iron sulfer cluster as explained in the discussion section.
This findings coul suggest that MSSA and MRSA have different mechanisms to countereact oxidative stress results from ROS. One of the biggest differences, as resumed in figure x is that the MRSA show a higher expression of SOD. This has not been observed in MRSA that seem to have a different pathway to fight oxidateive stress, more based on the thiol peroxidase-dependent pathway.
The antioxidant enzyme of MSSA isolate is the protein probable thiol peroxidase (ptx) inspite of superoxide dismutase in MRSA. This enzyme is one of the detoxifying and free radical-scavenging enzyme that is required to balance oxidative stress.
Iron plays a crucial role in infectious diseases due to its significant influence on the proliferation of immune cells, the effectiveness of macrophage immune functions (169), the binding affinity of antibodies (309) as well as on the multiplication of most pathogens (310-312).

Touati et al. (111) demonstrated that loss of iron regulation led to oxidative stress and consequent deleterious effects, provided by a study of E. coli fur mutant isolate. The mutant was viable but had impaired growth, and there was evidence for oxidative DNA damage. As in SOD deficient mutants, there was an oxygen-dependent increase in spontaneous mutagenesis in the fur mutant. Authors found that those deleterious effects could be suppressed by scavenging iron with ferrozine, a ferrous iron chelator, by blocking ferric iron import into a tonB mutant, or by increasing the iron storage capacity of the cell by overproducing the ferritin H-like protein. They concluded that, the deregulation of the control of iron acquisition in conditions of iron sufficiency leads to oxidative stress in an aerobic environment. Touati reviewed the role of iron in oxidative stressed in bacteria and demonstrated that that the lack of iron availability in the presence of oxygen is a major problem. Strict and elaborate iron regulation enables cells to acquire the iron necessary for survival and maintain low levels of "free" iron, which could cause oxidative stress and damage (111). This mechanism too could be explained as a compensative mechanism to supply the lack of reducing power due to the lower levels of SOD in MSSA.
Ferritin is mentioned in the previous group with its essential role for microbial sustain and resistance against oxidative stress either results from microbial metabolism or attack of immune cells during infection. Also, hypoxanthine-guanine phosphoribosyltransferase (HprT) which is a purine salvage enzyme enables recovery of purine and pyrimidine bases and nucleosides, which can then be recycled into nucleotides or used as a source of energy, carbon or nitrogen, enables organisms to use exogenous bases liberated from lysed cells or recycle those produced by the breakdown of unstable RNA. Finally, ATP synthase protein catalyzes the phosphorylation of ADP to ATP and under anaerobic conditions works in the reverse direction by hydrolyzing ATP and generating an electrochemical proton gradient across a membrane to support locomotion or nutrient uptake, that it plays a central role in energy transduction in living cells (see the above mentioned pathophysiology of identified proteins). This can explain the prevalence of MSSA through utilizing its own tools to adapt and counteract the raised immune system action and stress conditions. This results in an increased virulence potential of this isolate in comparison to MRSA.
The explained mechanism is resumed in figure 6 and highlights the importance of the iron metabolism represented by ferritin and iron-sulfur cluster protein for bacterial virulence.
This scheme also efforts the theory that MSSA isolates of S.aureus are more virulent than MRSA because of the higher expression of proteins such as ferritin, ATP synthase, QueA and alcohol dehydrogenase.

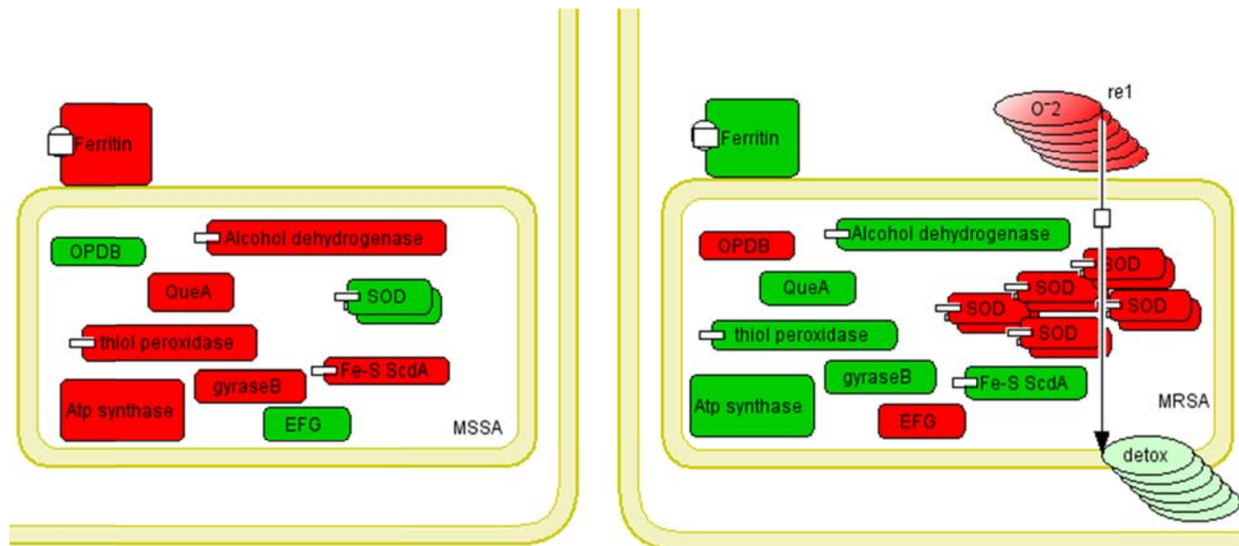


Figure 6. General scheme of differential expressed proteins in *S. aureus* mssa vs mrsa strain. Red squares represents overexpressed proteins , green squares underexpressed proteins. Legend; srrA: Transcriptional regulatory protein srrA ; ODPB: Pyruvate dehydrogenase E1 component subunit beta; Fe-S scdA: Iron-sulfer cluster repair protein ScdA; queA: S adenosylmethionine: tRNA ribosyltransferase-isomerase; EFG: Elongation factor G; ADH : Alcohol dehydrogenase ; SOD : Superoxide dismutase [Mn/Fe] 1; gyrase B: DNA gyrase subunit B

Furthermore it has been found that MSSA has a high level of expression of ADH that has not been previously reported. This finding could be a valuable effort to hypothesize the increased virulence of MSSA in response to the use of alcohol-based disinfectants.

7. Concluding remarks

Proteomics opened new insights for the identification of the proteins as regulators and protagonists of adaptative microbial tolerance against stress condition.
This study helps to elucidate the synergism of this microbial pathogen and highlights the putative targets useful to block the adaptative mechanism of this pathogen.
The described findings obtainded through this proteomic approach highlight new insights on the mechanisms involved in microbial tolerance against stress conditions.
About Panton Valentine Leukocidine gene it has been demonstrated that it is not absolutely determinant for virulence in clinically isolated *S. aureus*.
Resuming the most important points of the present study it has to be mentioned that the present classification of pathogenecity of *S. aureus* according to methicillin resistance should be readjusted. The described findings demonstrated that the MSSA isolate should be considered more virulent than the MRSA.

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9. Appendix

Because Staphylococcus aureus is one of the major causes of mastitis in farm animals, during my Ph.D. training, thanks to a collaboration provided by EU COST project-farm animal proteomics (FA1002 action), I had the possibility to apply proteomics to the study of bovine mastitis. So I report below the published paper on the study where I have been involved as coauthor.

Proteomics of inflammatory and oxidative stress response in cows with subclinical and clinical mastitis.
Turk R, Piras C, Kovačić M, Samardžija M, Ahmed H, De Canio M, Urbani A, Meštrić ZF, Soggiu A, Bonizzi L, Roncada P.
J Proteomics. 2012 Jul 19;75 (14):4412-28. doi: 10.1016/j.jprot.2012.05.021.

Inflammatory and oxidative stress response in cows with subclinical and clinical mastitis

Introduction

Bovine mastitis is a major disease that causes economic losses to dairy industry going beyond decreased milk production to reproductive disorders in dairy cows [1, 2]. Mastitis is still a main topic in veterinary research due to financial losses and food safety concerns over antimicrobial use, but also because there are only a limited number of efficacious mastitis treatment options. In addition to detection of clinical mastitis which is relatively easy, early diagnosis of subclinical mastitis, where an increased somatic cells count (SCC) is the only finding, is also very important not only for the benefit for milk production but also for reproductive efficiency. Subclinical mastitis is difficult to detect due to the absence of any visible clinical signs [3]. Clear understanding of the pathogenesis of mastitis is crucial for development of adequate tools for mastitis diagnostics [4].

The pathogenesis of mastitis includes an inflammatory reaction resulting in response to many factors including intra-mammary infections by microorganisms. Once the mammary gland is affected by pathogens, the innate immune system responds by leukocytes infiltration and production of cytokines at the site of infection [5]. Proinflammatory cytokines stimulate the synthesis of acute phase proteins (APPs) predominantly in liver but also in other tissues including mammary gland [6, 7]. Serum amyloid A (SAA) is one of the major APP in ruminants which concentration rises dramatically during acute phase response[8, 9].

During inflammatory conditions, phagocytes produce reactive oxygen species (ROS) that are needed for killing bacteria [10]. However, raised amount of ROS can overcome the antioxidant system [11-15] and compromise immune function of cows [16]. Directly, ROS can oxidize macromolecules such as lipids, proteins and DNA and cause oxidative cell injury. Indirectly, ROS can damage cellular components and membranes and thus modify metabolic pathways [17]. Oxidative stress can enhance the adhesion of activated neutrophils to mammary endothelial cells and intensify inflammation[18]. Several studies have shown that supplementing dairy cows with adequate level of antioxidants increased immune function of cows, i.e. phagocytosis, bacterial killing and oxidative metabolism of neutrophils [19-21]. Additionally, antioxidants can decrease platelet activating factor (PAF) synthesis [22] which is an inflammatory mediator promoting chemotaxis of leukocytes, their adhesion to the endothelium and granule secretion, platelet activation, vascular permeability and oxygen radical production from leukocytes [23-25]. The limitation of PAF bioactivity and its catabolism is regulated by the Platelet-activating factor acetylhydrolase (PAF-AH). Pathogenic changes in blood and milk constituents including cytokines and ROS can affect not only mammary gland but also other organs and systems such as reproductive system.

Paraoxonase-1 (PON1) and PAF-AH are both antioxidative/anti-inflammatory enzymes which hydrolyze lipid hydroperoxides and oxidatively fragmented phospholipids produced during oxidative stress [26-28]. Oxidatively fragmented phospholipids have a similar structure to PAF and provoke the same acute phase responses [29].

The first aim of this work was to evaluate the concentration of Serum amyloid A (SAA) and the activity of PON1 and PAF-AH in serum of cows with subclinical and clinical mastitis in order to evaluate systemic inflammatory and oxidative stress response. Evaluation of inflammatory and oxidative stress response is necessary for a better comprehension of proteomic data and to evaluate other parameters helpful for subclinical mastitis diagnoses.

The interest in research for biomarkers discovery for the diagnoses of bovine mastitis stems largely from the need to better characterize mechanisms of the disease, to identify reliable biomarkers for use as measures of early detection and drug efficacy, and to uncover potentially novel targets for the development of alternative therapeutics.

Most of proteomic studies on mastitis have been performed on milk and somatic cells [30-32]. Differential expression analysis performed from Baeker *et al.* [32, 33] of the whey from both mastitic and non-mastitic milk revealed a marked increase in the expression of a series of four

proteins during infection. Peptide mass fingerprinting (PMF) analysis revealed all four spots as Prostaglandin-D synthase (PGDS).

Although proteomic profile of bovine milk whey proteins have been well characterized still poor information have been provided on serum and plasma proteomics of bovine mastitis.

Previously it has been demonstrated how acute phase proteins (APPs) are subject to modifications, such as glycosylation, in altered physiological states [34-36]. Despite APPs are thought to lack specificity as candidate biomarkers of disease because of their high expression during the innate immune response, there are several evidences suggesting that the modification of APPs could be disease-specific. Thus, characterization of the post-translational modifications in APPs during the inflammatory response in different diseases could aid in the evaluation of the specificity of APPs as biomarkers.

Because APPs have demonstrated changes as great as 25% in serum concentrations during altered physiological states, APPs have been considered usable as potential biomarkers for diagnosing animal disease, monitoring health status, and evaluating responses to primary and adjunctive veterinary therapies [37]. In particular, APPs have been investigated as potential biomarkers for inflammatory diseases in food animals, because usually the etiological agents are bacteria, and the use of antibiotics for treatment and prevention is common. Antibiotic use in food animals, however, causes concern for food safety, and could increase growth of antibiotic resistant bacteria [38]. Additionally, antibiotics are not the appropriate treatment for diseases in food animals with affiliated inflammatory responses. Differences in APP expression during disease across species have been reported, and evidence exists that circulating concentrations of APPs in blood and other biological fluids are directly proportional to disease severity[34, 37, 39]. Moreover, recent data based on the proteomic analyses of APPs during disease suggests that modification of glycoproteins could be disease specific [8, 34-36]. Interest in APPs as potential veterinary biomarkers has involved the application of proteomic strategies for the evaluation of APPs and measures of the host response in complex biological samples [40-43].

In this manuscript we specifically aim to extend the current knowledge on molecular circulating biomarkers of mastitis including both subclinical and clinical animal group. Whole serum proteome was extensively evaluated in all three clinical groups in order to possibly find differential protein expression useful to help in early diagnoses of this pathology. The study design was tailored in order to possibly associate major molecular effects on serum proteome with the different clinical groups. It has been evaluated how serum sample conservation is important to

avoid the formation of *ex-vivo* artefacts due to an inappropriate storage [44]. In this case it has been used a linear MALDI-TOF-MS approach to evaluate the quality of serum samples.

Shotgun MS analysis was used to investigate the most abundant serum proteins among the three different groups and to evaluate if there are some differences in the acute phase proteins concentration. Moreover in order to investigate the differential protein expression and to find some putative biomarkers it was used 2D electrophoresis coupled with MS analysis.

The overall collected evidences showed data strong association between oxidative stress response, lipid metabolism and the differential protein expression.

Materials and methods

Animals and serum samples

The study was conducted on a total of 80 Holstein-Frisian dairy cows located on farms in the region of Eastern Croatia. Cows were fed with voluminous hay made of silage of whole plan (corn and clover). Additives contained glycerol (energy source) and bypass fat. Parameters for diet were established according to the following normative: Intake of dry matter kg/day, intake of proteins g/day, intake of net lactation energy/day and intake of crude fiber g/day.

All cows were checked by physical examination and somatic cells count (SCC) and California mastitis test in milk samples were performed. According to the results, cows were divided into 3 groups: Group I (control, n=30) consisted of healthy cows with SCC below 200,000 cells/ml with negative California mastitis test and without any clinical sign of mastitis. Group II (subclinical mastitis, n=30) comprised cows without clinical signs of mastitis but with SCC above 200,000 cells/ml and positive California mastitis test. Group III (clinical mastitis, n=20) consisted of cows with clinical signs of mastitis which include changes in milk appearance (flakes and clots in milk), different stages of udder inflammation (hyperemia, edema, pain, udder enlargement and elevated udder temperature) and disturbance of general health (depression, relaxed cold ears,

dehydration, elevated body temperature, increased heart and respiratory rate, decreased ruminal contraction and decreased appetite).

Blood samples were taken from *v. coccygea* and centrifuged at 1500 rpm for 15 min after clotting for two hours at room temperature. Serum samples were stored at -70°C until analysis.

Analysis procedures

Biochemical analysis

PON1 activity was assayed by the slightly modified method of hydrolysis of paraoxon previously described by Mackness *et al*. [45, 46] and Schiavon *et al*. [47]. Briefly, 10 µl serum was added to 350 µl 0.1 M Tris-HCl buffer, pH 8.0 containing 2.0 mM paraoxon (O,O-diethyl-O-p-nitrophenylphosphate, Sigma Chemical Co, London, UK) as substrate, 2.0 mM $CaCl_2$ and 1 mM NaCl. The formation of p-nitrophenol was monitored at 405 nm and 37°C on Olympus AU 600. PON1 activity was expressed in international units (U/L) as the amount of substrate hydrolyzed per minute and per litre of serum (µmolmin^{-1}/L). The ratio of PON1 activity and HDL-cholesterol concentration was also presented (U/mmol or µmolmin^{-1}/mmol).

PAF-AH activity was determined by the spectrophotometric assay described by Kosaka *et al*. [48]. Briefly, 2 µl serum was added to 240 µl of 200 mmol/L HEPES (N-2-hydroxyethylpiperazine–N`-2-ethanesulfonic acid) buffer (Reagent 1), pH 7.6 and pre-incubated at 37°C for 5 min. The reaction was started by adding 80 µl of 20 mmol/L citric acid monohydrate buffer, pH 4.5 containing 90 mmol/L 1-myristoyl-2- (4-nitrophenylsuccinyl) phosphatidylcholine (Reagent 2), (Azwell Inc, Osaka, Japan). The liberation of p-nitrophenol was monitored at 405 nm and 505 nm at 1 and 3 min after the addition of Reagent 2 using the automatic biochemical analyzer Olympus AU 600 (Olympus Optical Co., LTDL, Tokyo, Japan).

SAA concentration was assayed using the multispesies Tridelta Phase™ range ELISA SAA kit (Tridelta Development Ltd., Ireland).

Triglyceride, total cholesterol, HDL-C and calcium concentrations were measured by standard commercial kits (Olympus Diagnostica GmbH, Hamburg, Germany and ThermoTrace, Victoria, Australia) using automatic analyzer Olympus AU 600.

Proteomic analysis

All proteomic analysis was performed on thirty serum samples, 10 control serum, 10 subclinical mastitis and 10 clinical mastitis.

MALDI-TOF MS Analysis

MALDI-TOF MS Analysis was performed on a total of thirty serum samples (10 control serum, 10 subclinical mastitis, 10 clinical mastitis).

Five volumes of 0.1 % acetic acid were added to serum samples and stored at -80 °C until analysis. 20 µl of each sample, acidified with 0.1 % TFA, were desalted and concentrated by ZipTip C_4 (Millipore, Billerica, MA) according to to Della Donna et al. (53). Desalted samples were co-crystallized with 2 µl of a saturated solution of sinapinic acid dissolved in 25 % acetonitrile, 0.05 % trifluoroacetic acid (TFA) on a Ground Steel plate (Bruker-Daltonics, Bremen, Germany) pre-spotted with a thin layer of a saturated solution of sinapinic acid dissolved in 100 % ethanol. Mass spectra were acquired with an Ultraflex III MALDI-TOF/TOF spectrometer (Bruker-Daltonics) equipped with SmartBeam laser, working in positive linear mode. The acquisition range was set to 1–20 kDa, laser power and total number of shots were adjusted to obtain 10^3 ion counts *per* shot train and $2.5\text{-}3.5 \times 10^4$ total ion counts for the base peak. External calibration was performed using a mix of Peptide calibration standard II (*m/z*: 757.3992, 1046.5418, 1296.6848, 1347.7354, 1619.8223, 1758.9326, 2093.0862, 2465.1983, 3147.4710; Bruker-Daltonics) and Protein calibration standard II (*m/z*: 5734.51, 6180.99, 8476.65, 8565.76, 12360.97, 16952.30; Bruker-Daltonics). Processing and interpretation of data were performed by ClinProTools 2.1 software (Bruker-Daltonics). Data processing included baseline subtraction, obtained by Top Hat Baseline algorithm (minimal baseline width = 10 %), smoothing by Savitsky Golay algorithm (width = 10, cycle = 1), noise spectra exclusion (threshold = 2) and recalibration using prominent internal signal

peaks (maximal peak shift = 1000ppm; match to calibrant peaks = 30). Spectra were divided in three classes according to sample source and Genetic Algorithm was run for generation of a model of classification (Maximal number of peaks in model = 5; Maximal number of generations = 50).

2D electrophoresis

Serum protein amount was determined using Bio-Rad Protein Assay according to the manufacturer instructions.

Once determined the protein concentration of each sample it was diluted up to desidered concentration in a buffer containing 8 M urea, 4 % CHAPS, 1 % DTT and 2 % Ampholine pH 3,5-10.

Three experimental replicates were performed for each serum sample collected from a single animal. Euroclone immobilized pH gradient (IPG) strips (7 cm) with a linear pH range of 4.0-8.0 were rehydrated overnight in a buffer containing 8 M urea, 4 % CHAPS, 1 % DTT, 10 mM TRIS and 2 % Ampholine pH 3,5-10. 120 µg of protein sample were loaded on each IPG strip using cup loading at cathodic side. Isoelectric focusing was performed using EttanIPGphor III IEF system (GE Healthcare) at 20 °C with a current of 120 µA/strip. For IEF was used the following protocol: 30 V (4 h), 50 V (3 h), 100 V (3 h), 500 V (3 h), 1000 V (3 h), 3000 V (3 h), 4000 V (3 h), 6000 V (3 h) and 8000 V (8 h). After the first dimension, IPG strips were equilibrated twice with a solution containing 6 M urea, 2 % SDS, 50 mM Tris-HCl pH 8.8 and 30 % glycerol, for 15 min, under gentle stirring. For the first equilibration step was used 1 % DTT and for the second 2.5 % iodoacetamide. The second dimension was performed using 12 % polyacrylamide SDS-PAGE slab gels). IPG strips were put on top of the SDS gels which were poured up to 1 cm from the top of the plates and then sealed with 1.5 ml of a solution containing 0.5 % low-melting-point agarose diluted in hot SDS running buffer (25 mM Tris-HCl pH 8.3, 192 mM glycine, 0.1 % SDS). Molecular weight protein markers (Precision Plus- BioRad) were applied on one end of the IPG strips. In the second dimension, gels were run at 7.5 mA/gel, for 20 min. and then at 20 mA/gel, until the bromophenol blue front-line came out of the gel. After runs, gels were stained with colloidal Coomassie and digitalized with laser scanner Pharos FX (Biorad)[49].

Protein identification from two-dimensional electrophoresis gel

Single spots were excised from coomassie stained bidimensional gels and gradually dehydrated with 50 mM ammonium bicarbonate, a mixture of 50 mM ammonium bicarbonate and acetonitrile (1:1), and 100 % acetonitrile. Proteins were reduced using 10 mM DTT and alkylated with 55 mM iodoacetamide. Gel plugs were alternatively hydrated with 50 mM ammonium bicarbonate and dehydrated in acetonitrile and then left to completely dry. Proteins were digested with 0.1 g of porcine trypsin (Promega, Madison, WI) at 37 °C overnight. Reactions were stopped with 1 % TFA. Peptides were desalted and concentrated by ZipTip C_{18} (Millipore) and eluted with a solution of 0.5 mg/ml -ciano-4-hydroxycinnamic acid dissolved in 50 % acetonitrile, 0.05 % TFA on a Ground Steel plate (Bruker-Daltonics) previously spotted with a thin layer of 10 mg/ml -ciano-4-hydroxycinnamic acid. Mass spectra were acquired with an Ultraflex III MALDI-TOF/TOF spectrometer (Bruker-Daltonics). External calibration was performed using the Peptide calibration standard (*m/z*: 1046.5418, 1296.6848, 1347.7354, 1619.8223, 2093.0862, 2465.1983, 3147.4710; Bruker-Daltonics). FlexAnalysis 3.0 software (Bruker-Daltonics) was used for the selection of the monoisotopic peptide masses. Internal calibration was performed on autolysis peaks from porcine trypsin (*m/z*: 842.509 and 2211.104).

After MS spectra acquisition, the instrument was switched in LIFT mode and precursor ions were manually selected for the subsequent fragmentation. MS/MS spectra were acquired with 4-8 x 10^3 laser shots adjusting the laser power and the number of shots *per* shot train in order to obtain at least 10^4 ion counts for the prominent fragmentation peak. The precursor mass window was automatically set after the precursor ion selection. Spectra baseline subtraction, smoothing (Savitsky Golay) and centroiding were operated by FlexAnalysis 3.0 software (Bruker-Daltonics

Not identified samples were analyzed by nLC-MS/MS. The digested products were extracted from gel plugs twice with a solution of 0.1 % TFA and 50 % acetonitrile (1:1), and then with 100 % acetonitrile, collecting supernatants in autosampler vials. The extracted mixtures were dried by Speed-Vac until the final volume of 20 µl. 2 µl of each sample were separated by a Proxeon Easy-nLC II (Thermo Scientific, Waltham, MA) chromatographic system equipped with an EASY-Column C18, 5 µm, 100 µm x 20 mm precolumn (Thermo Scientific) and an EASY-Column C18, 3 µm, 75 µm x 100 mm nanoscale LC column (Thermo Scientific). Mobile phase A was water with 0.1 % formic acid and mobile phase B was 0.1 % formic acid in acetonitrile. Peptides were separated with a reverse phase gradient of 5-35 % mobile phase B over 60 min at flow rate of 300 nl/min, followed by a plateau of 35 % mobile phase B and a rinse with 100 % mobile phase B for 15 min. The chromatographic system was interfaced to an amaZon ion trap

(Bruker-Daltonics) by means of a nano-Flow ESI sprayer. Ion trap operated in data-dependent scan mode (AutoMSn with n = 2) and enhanced resolution (maximum speed = 8100 *m/z* s^{-1}). The mass-acquisition mode involved scans in the range from 300 to 1500 *m/z* followed by three tandem scans in the ion trap. The three most intense peaks (over an intensity threshold of 25000 a.u.) from each scans were selected in the ion trap for further fragmentation and tandem mass spectrometry. Proteomic data were analyzed by Compass DataAnalysis 4.0 software (Bruker-Daltonics) for mass spectra deconvolution.

Monodimensional immunoblotting

One-dimensional (1D) electrophoresis (reducing conditions, 8% polyacrylamide gels, Mini-PROTEAN Tetra Cell, BioRad) and immunoblotting were performed as described previously [50], except that Tris-buffered saline, pH 7.4 (TBS), containing 5% non-fat dry milk was used for all washing steps and for dilution of second antibodies. Primary antibody (Anti-Vitronectin Monoclonal Antibody, A27, Thermo) was used ON at a concentration of 1:1000 and secondary antibody (anti mouse IgG, Sigma-Aldrich) was used at a concentration of 1:20000 for 1h.

Shotgun analysis by nLC-MS/MS

A total of 100 µg of serum samples were precipitated with a mixture of ethanol, acetone and methanol (2:1:1) and dissolved in 100 mM Tris/HCl pH 7.9 containing 6 M urea. Equal amounts of proteins from each sample were mixed in order to create three different serum pools representative of the bovine classes in examination. Reduction and alkylation of proteins were obtained by adding 100 mM DTT (1 h at 37 °C) and 200 mM iodoacetamide (1 h at R.T.). Protein samples, at final concentration of 2 µg/µl, were digested with 1:20 (w/w) sequence grade porcine trypsin (Promega) at 37 °C overnight. Reactions were stopped adding 1 µl of 10 % (v/v) TFA. Digestion mixture was diluted 1:54 with 0.1 % (v/v) formic acid and 0.25 µg of each sample were loaded onto Proxeon Easy-nLC II (Thermo Scientific) chromatographic system for peptide separation. Each pool was run three times to minimize experimental variability. Peptides were trapped on a EASY-Column C18, 5 µm, 100 µm x 20 mm precolumn (Thermo Scientific) and separated using a Acclaim PepMap100 C18, 5 µm, 75 µm x 25 mm (Dionex, Thermo Scientific) nanoscale LC column. Mobile phase A was water with 0.1 % formic acid and mobile phase B was 0.1 % formic acid in acetonitrile. Peptides were separated with a gradient of 5-35 % mobile phase B over 140 min at flow rate of 300

nl/min, followed by a rinse with 90 % mobile phase B for 10 min. The chromatographic system was coupled online with a MaXis 4G ultra-high resolution time of flight mass spectrometer (Bruker-Daltonics), equipped with a ESI nano Sprayer ion source and a calibrant reservoir providing a signal of *m/z* = 1221.99 for internal calibration. Spectra acquisition in the mass range 50-2200 *m/z*, was performed by using an Auto (MS/MS) mode method optimized for complex samples, with number of precursor ions set to 5, spectra rate of 2.0 Hz, using Argon in collision gas cell (Bruker-Daltonics). Spectra were processed by using Compass DataAnalysis 4.0 software, setting the intensity threshold to 1000 and number of maximum compounds to 3000.

In order to extend the peptide list of analyzed samples, a second data set was collected coupling the chromatographic system to an amaZon ion trap (Bruker-Daltonics). The ion trap operated in data-dependent scan mode (AutoMSn with n = 2) and enhanced resolution (maximum speed = 8100 $m/z\ s^{-1}$). The mass range from 300 to 1500 *m/z* was scanned for peptide signals survey and three most intense peaks (over the threshold of 25000 a.u.) from each scans were automatically selected and further analyzed by tandem mass spectrometry. Acquired mass spectra were processed by Compass DataAnalysis 4.0 software (Bruker-Daltonics) allowing a maximum of 5000 compounds over the intensity threshold of 1000 u.a.

Database search

The identification of peptides and proteins from mass spectrometry data was achieved by MASCOT algorithm (www.matrixscience.com) interrogating the UniProtKB/Swiss-Prot database restricted to the "*Other_Mammalia*" taxonomy. All database searches were conducted with carbamidomethylation of cysteines as fixed modification and oxidation of methionines as variable modification, allowing one missing cleavage on tryptic peptides. In MALDI-TOF experiments peptide mass lists were matched against version of SwissProt 2011_11 (533,028 sequences entries). The identifications by Peptide Mass Fingerprinting (PMF) were obtained allowing 50 ppm as maximal error tolerance while identifications by peptide sequencing were achieved setting maximal error tolerances to 75 ppm and 0.5 Da for parent and fragment ions, respectively. nLC-MS/MS data searches were run against version of SwissProt 2011_08 (531,473 sequences entries). For ion trap data, maximal error tolerances of 0.3 Da and 0.6 Da for parent and fragment ions were set while data from maXis 4G mass spectrometer were searched allowing 5 ppm as maximal error on parent ions and 0.05 Da on fragment ions.

The identification of peptides and the recognition of corresponding proteins in shotgun experiments were achieved by ProteinScape 2.1 Bioinformatics Platform (Bruker-Daltonics). nLC-MS/MS data acquired both by maXis 4G mass spectrometer and by amaZon ion trap were combined by Protein extractor method. The protein hits were accepted if meet the following criteria: i) to belong to *"Bos taurus"* taxonomy; ii) to own at least one peptide with a Mascot score above 29 (corresponding to the threshold of identity or extensive homology); iii) to be detected in at least two of three technical replicates. emPAI values for each protein were obtained according to definition of Ishihama [51].

Statistical analysis

Kolmogorov-Smirnov test and Leven´s test were used for testing data for normality and equal variance. Differences between investigated groups were tested by the non-parametric analysis using Mann-Whitney rank sum-test. SigmaStat 3.0 (SPSS Inc., Chicago, Illinois, USA) was applied for statistical analysis. Statistical significance between median values was based on values $P<0.05$.

Results

Biochemical Characterization

Subclinical and clinical mastitis result in significant changes in the activities of anti-inflamatory/antioxidative enzymes and the acute phase protein serum amyloid A. The mean values of PON1 and PAF-AH activities and SAA concentration are shown in Table 1. PON1 activity is significantly lower ($p<0.05$) in subclinical and clinical mastitis compared to control cows. PAF-AH activity is significantly lower ($p<0.05$) only in clinical mastitis but not in cows with subclinical

mastitis. SAA concentration is statistically higher ($p<0.05$) in clinical mastitis and there are no significant difference between subclinical mastitis and healthy cows.

Table 2 presents lipid status and calcium concentration in all three investigated groups. Triglyceride concentration is significantly higher ($p<0.05$) in serum of cows with clinical mastitis, while total cholesterol, HDL-C and calcium concentrations are significantly lower ($p<0.05$) in serum of cows with clinical mastitis. There are no significant differences in concentrations in lipids and calcium between healthy cows and those with subclinical mastitis.

Proteomics Investigation

Describe what kind of selection criteria has been adopted to choose the samples for proteomic analysis (Romana).

Linear MALDI-TOF-MS analysis was performed in order to preliminary evaluate the sample collection quality and to possibly examine the presence of distinctive low molecular mass proteins and peptides fragments in serum samples. In fact, massive protein degradation and oxidation products have been observed whenever sample pre-analytical phase has not been properly followed[52].

Single mass spectra show very similar peaks distributions in the range 3,000-20,000 m/z (Fig. 1B). Principal peak differences were observed in 8,200-9,000 m/z region whereas slight variability was observed in the remaining areas of mass spectra. This region is known to contain mass signals relative to apolipoproteins isoforms. In the light of our previous studies, mass signals 8,219.5 m/z and 8,553.5 m/z were assigned to Apolipoprotein C-III and Apolipoprotein A-II (for more details, see reference [53]). No signal indicating partial degradation of serum samples was detected. Average mass spectra obtained from the three classes of sera (clinical mastitis, subclinical mastitis and control) were highly superimposable (Fig. 1A). Genetic Algorithm of ClinProtools software was run to construct a model for the class distribution. The algorithm failed to indicate discriminating peaks for this classification, even if recognition capability of generated model was relatively high (79.17%). This result was not unexpected indicating a substantial homogeneity of serum mass spectra in low molecular weight range.

In order to achieve information on the most abundant serum proteins a nLC-MS/MS analysis was performed on tryptic peptides obtained by digestion of sera pools of the three bovine groups in examination. A semi-quantitative analysis was achieved by reverse phase C18 separation

coupled to ultra high resolution Q-TOF analyzer. The collected experimental data were used to obtain the emPAI differential protein composition indices for all three groups as shown in Table 3. Such an experiment allowed us to provide a relative abundance index for 34 non-redundant proteins, in fact post-translational modifications were either not included in the database search nor detectable given the peptide based profiling strategy. In fact, this experimental design tends to level out the potential molecular differences associated with a single peptide since the overall probabilistic emPAI scores are based on the reconstruction on a defined RNA transcript. Two proteins (Haptoglobin and Apolipoprotein A-II) were exclusively detected in serum of cattle affected by clinical mastitis whereas three proteins (Serpin A3-4, Inter-alpha-trypsin inhibitor heavy chain H1 and Protein AMBP) were shared in clinical and subclinical mastitis cattle. Moreover, by comparing average emPAI index, we observed a significant increase of serum levels of 11 proteins in the clinical mastitis group compared with the control group, 3 of them (Alpha-1B-glycoprotein, Alpha-1-acid glycoprotein and Vitamin D-binding protein) were also increased in subclinical mastitis respect to the control group (Table 3).

A more subtle characterization of serum protein profile was pursued by a 2DE profiling on intact proteins. This analysis highlighted 10 differentially expressed proteins among all three groups. Three proteins were found to be differentially represented between control and subclinical group and seven proteins were found to be differentially represented between subclinical and clinical group. In the subclinical group in comparison to the control group Serpin A3-1 and a Vitronectin-like protein were found to be over-represented while Complement factor H was found to be under-represented (Fig. 2, Table 4). Vitronectin expression was evaluated with 1D immunoblotting confirming results obtained with 2D electrophoresis (Fig. 3).

Seven proteins, respectively Inter-alpha-trypsin inhibitor heavy chain H4, Serpin A3-1, C4b-binding protein alpha chain (Fig. 4) and three different isoforms of Haptoglobin were found to be upregulated in the clinical group in comparison to the subclinical group, and only an isoform of Apolipoprotein A-I was found to be downregulated (Fig. 5). All these data have been resumed in table 4.

In figure 6 is represented the whole bovine serum map with the identified proteins included in this study. The number assigned to each spot corresponds to the number of the protein described in table 4.

As shown in Table 3 and 4 four proteins were found both with 2DE and nLC-MS/MS shotgun experiments (Serpin A3-1, Inter-alpha-trypsin inhibitor heavy chain H4, Haptoglobin, Apolipoprotein A-I).

Discussion

The present study describes systemic acute phase response in cows with subclinical and clinical mastitis. Serum PON1, PAF-AH and SAA demonstrate different responses in subclinical and clinical mastitis. PON1 activity showed a significant decrease in subclinical and clinical mastitis compared to control while PAF-AH activity showed a significant decrease only in clinical mastitis. SAA is strongly up-regulated only in clinical mastitis, during which its concentration is 2.5 fold higher than in healthy cows.

An increase in SAA concentration has been found previously in serum and milk of cows with clinical mastitis [54]. In the present study SAA was not increased in subclinical mastitis indicating probably SAA expression only in the mammary gland [6]. It has been suggested that acute phase response (APR) is associated with a loss of PON1 and PAF-AH from HDL during the SAA enrichment of HDL [55]. In the present study, only PON1 was lower in both subclinical and clinical mastitis compared to healthy cows indicating that PON1 activity is a more sensitive marker then SAA. As PON1 is an antioxidant enzyme, low activity indicate an involvement of oxidative stress in the pathogenesis of mastitis. Low antioxidant status and enhanced oxidative stress in mastitis of cows has been found previously [46, 47]. Several studies have shown that supplementing dairy cows with adequate level of antioxidants increased immune function of cows, i.e. phagocytosis, bacterial killing and oxidative metabolism of neutrophils [19-21] supporting the hypothesis that low level of antioxidants is related to acute phase response. Lower PON1 and PAF-AH activities were found in our previous studies in cows during periparturient period [56, 57] and in cows with liver hypertrophy indicating oxidative stress and systemic acute phase response.

Our results showed that inflammation interferes with lipid metabolism affecting concentrations of total cholesterol, HDL-C and triglycerides (TG) in cows with clinical mastitis.

HDL-C and total cholesterol were lower in clinical mastitis while TG concentration was higher in cows with clinical mastitis. The reasons for lipid alteration probably lie in the remodeling of lipoprotein particles during the interaction of APPs with plasma lipoproteins [58, 59]. Hypertriglyceridemia during APR could contribute to triglyceride enrichment of HDL that could also contribute to release of PON1 and PAF-AH from HDL[60]. A significant reduction in PON1 and PAF-AH might be a factors contributing to inflammation in the pathogenesis of subclinical and clinical mastitis.

Results demonstrate that SAA concentration in serum is a reliable marker for the diagnosis of clinical mastitis sharply increasing in cows with clinical form of disease but cannot distinguish subclinical mastitis from healthy animals. On the other hand, in spite of the moderate extent of its extent, the reduction of PON1 activity in subclinical mastitis is significant and validates PON1 as a potential marker for diagnosis of the subclinical form of the disease.

A semi-quantitative proteomic analysis was performed to investigate the levels of serum proteins in clinical and subclinical mastitis cattle in order to obtain a general picture of response to this disease. We detect a total of 34 serum proteins, 12 of them were found positively modulated in clinical and/or subclinical mastitis groups relatively to the control group, whereas 5 proteins were found only in serum of cattle affected by mastitis both clinical and/or subclinical. Shotgun proteomic analysis identified Haptoglobin (see forward in Discussion) and Apolipoprotein A-II, exclusively in clinical mastitis group suggesting an enrichment of these proteins in serum of cattle affected by this pathologic condition. Apolipoprotein A-II is the second component of HDL. It was reported that the increment of Apolipoprotein A-II in HDL leads to impairment of lipoprotein antioxidant properties, causing the displacement PON1 and PAF-AH [61]. Hence, results obtained by shotgun analysis, in agreement with biochemical characterization of serum samples, suggest a deep change in HDL proteome. Studies on the modifications of the HDL proteome in human rheumatoid arthritis [62] and coronary artery disease [63], disorders displaying abnormalities in lipid metabolism similar to those occurring during infection and inflammation, indicate that the HDL-associated proteins are mainly involved in lipid metabolism, acute phase response, complement activation, protease inhibition. Our findings well agree with this key of interpretation. Ten modulated proteins are APPs whose serum levels are known to increase during early phase of infections. This group includes: Haptoglobin, Kininogen, Hemopexin, Vitamin D-binding protein, Transthyretin, Alpha-1-acid glycoprotein, Complement C3, two isoforms of Inter-alpha-trypsin heavy chain H and Protein AMBP (REF). Two proteins, Complement C3 and Complement factor B are directly involved in the activation of complement cascade, which represents the “front line” of

the response of innate immune system in the infection caused by different species of pathogens. The great part of modulated proteins regulate the biological process related to protease inhibition. Eight overexpressed proteins belong to this category: Haptoglobin, Kininogen, three isoforms of Serpin A3, two isoforms of Inter-alpha-trypsin heavy chain H and Protein AMBP. It is known that bacterial pathogens produce an array of proteases acting as toxin factors, which are known to aid in colonization, evasion of host defense, facilitation of dissemination and host tissue damage during infection. The observed increased levels of serine and cysteine protease inhibitors may be explained considering the attempt of the animals to contrast a bacterial infection (REF). Finally, shotgun proteomics analysis highlights increased serum level of Alpha-1B-glycoprotein, Alpha-1-acid glycoprotein and Vitamin D-binding protein in subclinical mastitis respect to the control group. These proteins are the most promising candidates for the diagnosis of the onset of the disease. In this perspective it is relevant to considerer that these putative biomarkers are known to be increased in a way independent by the bacterial pathogen species [64].

On the other hand proteomic analysis through 2D electrophoresis highlighted ten differentially expressed spots among the three groups.

Two differentially expressed proteins were found between control group and subclinical group. Serpin A3-1 (Antichymotrypsin) was found to be upregulated in subclinical group in comparison to control group (Fig. 2). Serpin A3-1 is a potent inhibitor of the serine proteases Elastase and Trypsin. Moderately inhibits the serine proteases Plasmin and Chymotrypsin, and the thiol protease Proenkephalin-processing enzyme [65, 66]. It is an acute phase protein and its expression is usually increased during inflammation.

Complement factor H is an essential regulatory protein that plays a critical role in the homeostasis of the complement system in plasma and in the protection of bystander host cells and tissues from damage by complement activation. Factor H regulates complement both in fluid-phase and on cellular surfaces. However, while factor H binds and inactivates promptly C3b in fluid-phase, the inactivation of surface-bound C3b by factor H is dependent on the chemical composition of the surface to which C3b is bound [67]. Its downregulation as shown in Figure 2 indicates the higher activation of C3b and of all complement pathways to counteract the colonization of mammary glands that causes mastitis. Evaluating the concentration of complement factor H could help to the early diagnose of mastitis avoiding its worsening to clinical levels when it would be disadvantageous any kind of therapeutic solution.

Another characteristic protein upregulated during subclinical mastitis was found to be a Vitronectin-like protein (bovine protein S). Vitronectin is a multifunctional protein that plays an important role in complement-dependent cell lysis [68], in the coagulation system [69], and in cellular adhesion [70]. The bovine S protein exists in plasma with molecular weight of 76,000 and 65,000 Da [71] and its biological properties were indicated by the ability to spread cultured endothelial cells, as described for human S protein [72]. There are experimental evidences that demonstrate the role of Vitronectin in the process of adherence of specific pathogen to bovine epithelial cells [72]. As shown in Figure 2, our results highlighted an over-representation of this Vitronectin-like protein in the subclinical group in comparison to control group. Since this protein is linked to the phagocytosis process of pathogens monitoring its concentration could be helpful for the early diagnoses of subclinical mastitis.

As described in results and as shown in Figure 3 and in Table 2, seven proteins were found to be upregulated in clinical group in comparison to subclinical group. The first one was Inter-alpha-trypsin inhibitor heavy chain H4, it is an acute phase protein and its concentration was found to follow an increasing trend starting from control to clinical group, but the bigger difference was found between the subclinical and the clinical group. It is a 120 kDa protein and its concentration during clinical mastitis has already being studied [32, 73], but it has never been studied during subclinical mastitis, its increasing trend in concentration between control and clinical mastitis suggests that it can be used as marker for monitoring the inflammation status.

In clinical group versus subclinical group has been successfully identified through mass spectrometry analysis the Serpin A3-1 (antichymotrypsin). This protein was already over-represented in control group versus subclinical group but in this case this is a different isoform that is visualized only in clinical group. It is characterized, as shown in Figure 3, by a slightly different isoelectric point that could suggest that it is differenced by some kind of different post-translational modification. Serpin A3-1 overexpression during the CM status has been detected both with 2D electrophoresis and shotgun analysis by nLC-MS/MS.

C4b binding protein alpha chain controls the classical pathway of complement activation. It binds as a cofactor to C3b/C4b inactivator (C3bINA), which then hydrolyzes the complement fragment C4b. It also accelerates the degradation of the C4bC2a complex (C3 convertase) by dissociating the complement fragment C2a. Alpha chain binds C4b. It interacts also with anticoagulant protein S and with serum amyloid P component. The final action of C4b binding protein alpha chain results in a strong inhibition of the complement cascade. As shown in Figure 3 it is overexpressed in clinical group in comparison to subclinical group. Its overexpression could be

due to a mechanism involved in the control of the high activation of complement system in order to regulate the excess of inflammation generated by the bacterial colonization of mammary gland.

Three isoforms of Haptoglobin have been found to be overexpressed only in clinical mastitis group, they were not present in the gels obtained from control and subclinical group (Fig. 4). This data have been confirmed by shotgun MS analysis that, as shown in Figure 4 and in Table 3, shows a relative emPAI index of 0.32 only in the clinical mastitis group. Increased Haptoglobin expression has already been documented in bovine *E. coli*-induced mastitis [74], our work highlighted three different Haptoglobin isoforms that could be potentially addressed as specific molecular features for mastitis diagnoses. Haptoglobin (Hp) is an acidic glycoprotein present in most body fluids of humans and other mammals. Its function is still not fully understood but it seems to play an important role in the suppression of inflammatory response. Moreover there is considerable evidence that Hp could work as an extracellular chaperon protein that could be found in high level in body fluids [75]. Its anti-inflammatory task, its function as chaperone protein makes of Hp an important acute phase protein necessary to avoid oxidative stress-dependent tissue damage. As previously described oxidative stress could be a key component of mastitis pathogenesis and the presence of high levels of Hp could be due to a protection mechanism against oxidative stress. A recent paper of Yunee [76] and colleagues showed how different strain of *Staphylococcus aureus*-dependent mastitis could have a different response in the synthesis of IL-8, IFN- , TGF- 1 but there were not found any statistically significant differences in the overexpression of Haptoglobin.

Apolipoprotein A-I (Apo A1) is produced by both the liver and the small intestine. In cattle ApoA1 is a major apolipoprotein in the HDL fraction [77, 78]. Though apolipoproteins are known to be involved in lipid transport and are major components of high density lipoproteins, other roles for the apolipoproteins during disease and inflammation have been proposed [79]. The specific role of the apolipoproteins during inflammation related to mastitis has not yet been determined, but implications are that the apolipoproteins could inhibit neutrophil activation, as well as the release of inflammatory cytokines [30, 80, 81]. Obtained results showed an upregulation of total concentration of Apo A1 with a concomitant downregulation of only an isoform of Apo A1. At the same time shotgun proteomics experiments showed an increased total concentration of Apo A1. Apo A1 anti-inflammatory function could be important to control the inflammatory process due to high inflammatory response against bacterial activity. Discuss lipoproteins, cholesterol and triglyceride link. Discuss link between apolipoprotein A-I and haptoglobins.

Conclusion

Bovine mastitis is characterized by a strong release of white blood cells into the mammary gland due to bacterial invasion. Milk-secreting tissue and various ducts throughout the mammary gland are damaged due to bacterial toxins. Immune response is necessary and causes a strong increment of proinflammatory cytokines and oxidative stress that brings to higher production of acute phase reactants. As described in results and discussion we founded three differentially expressed proteins in subclinical mastitis versus control. As previously described Serpin A3-1 and Vitronectin were found to be upregulated and Complement factor H was found to be downregulated in subclinical mastitis. All these three proteins could be further explored as possible molecular biomarkers. In particular the specific Vitronectin-like protein can be considered the most appropriate candidate. Its production is linked to the process of bacterial opsonization that involves both Vitronectin and complement cascade. For this reason it is a serum marker that, if overexpressed, indicates the presence of a strong bacterial infection. Moreover its overexpression during subclinical mastitis could help in the early diagnoses of this pathology.

During the clinical mastitis in comparison to subclinical mastitis it was found the overexpression of three different isoforms of Haptoglobin and with shotgun proteomics it was found also the overexpression of Haptoglobin and Apoliporotein A-I. The possibility of the interaction of these two proteins was already described [82]. In particular the interaction and the lower renal excretion of these two proteins could be due to the formation of a complex between apoA1 and Haptoglobin due to oxidative stress that is enhanced during inflammation. This is really interesting if considering that during both subclinical and clinical mastitis is present a downregulation of PON1 that is necessary to hydrolyze lipid hydroperoxides and oxidatively fragmented phospholipids produced during oxidative stress. Moreover the upregulation of C4b binding protein alpha chain could suggest the development during clinical mastitis of a mechanism to control the complement pathway in order to slow down the inflammation process.

The proposed molecular features for the stratification of subclinical mastitis and control group and of the ones that differentiate subclinical and clinical mastitis could be useful for the early detection of mastitis during the subclinical phase.

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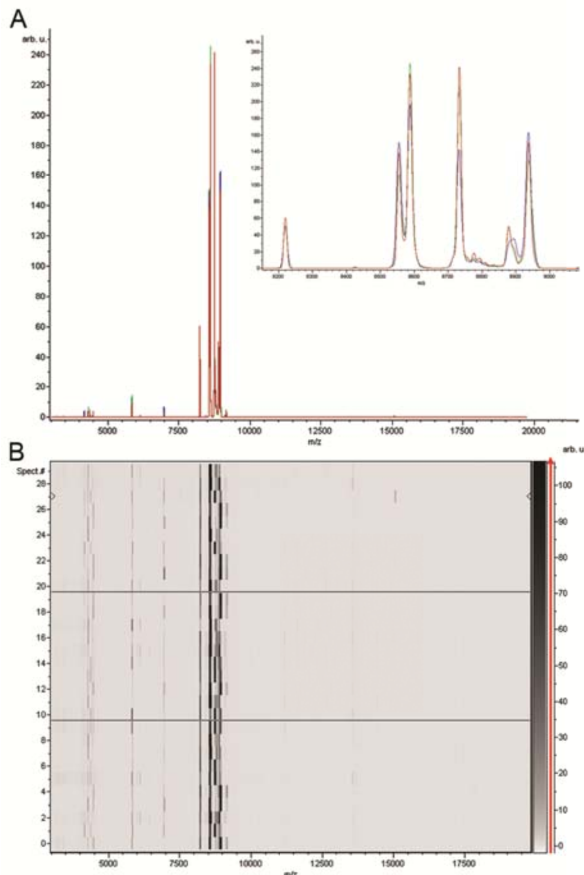
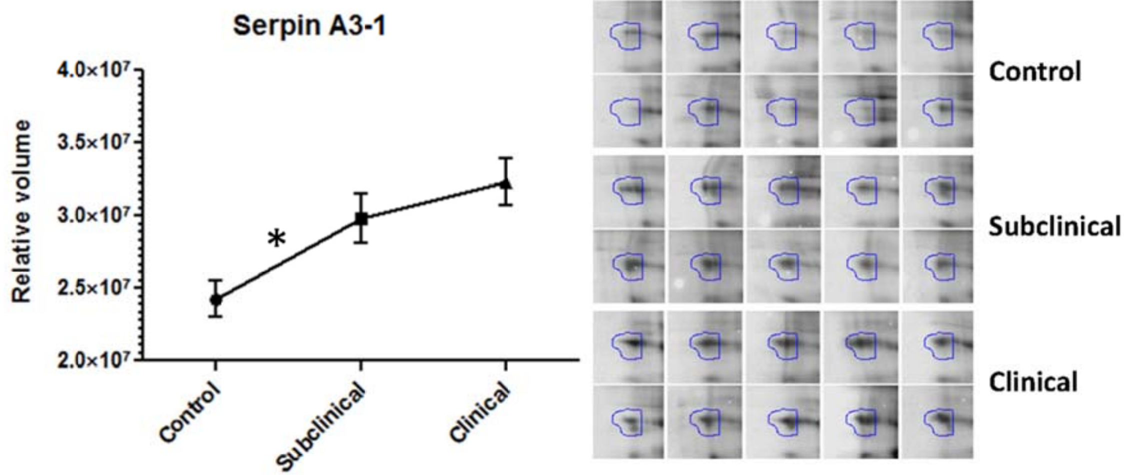
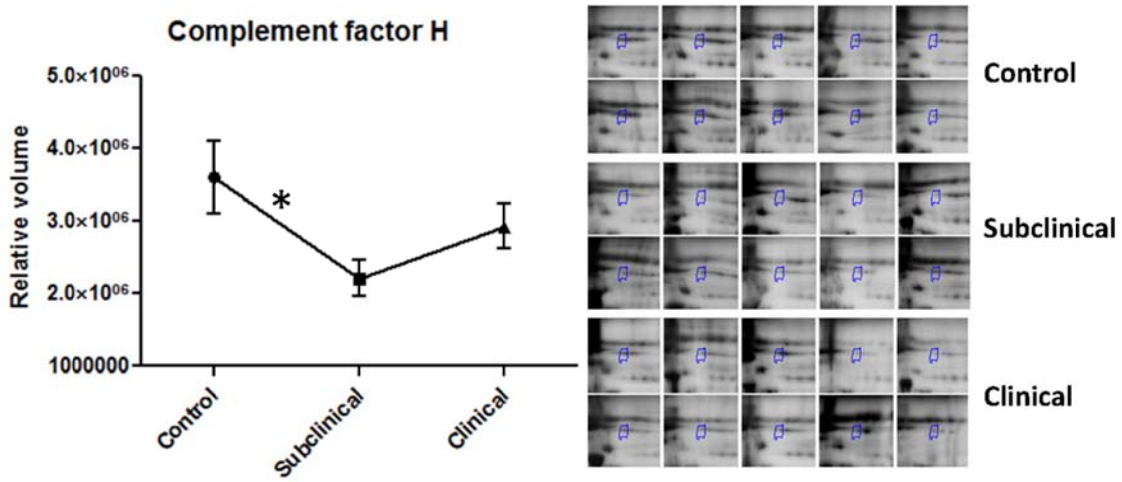


Figure 1: Serum PON1, PAF-AH and SAA in control cows and cows with subclinical and clinical mastitis. a,b Values with different superscript letters are significantly different.


Serpin A3-1
Relative volume
4.0×10⁷
3.5×10⁷
3.0×10⁷
2.5×10⁷
2.0×10⁷
*
Control
Subclinical
Clinical
Control
Subclinical
Clinical


Complement factor H
Relative volume
5.0×10⁰⁶
4.0×10⁰⁶
3.0×10⁰⁶
2.0×10⁰⁶
1000000
*
Control
Subclinical
Clinical
Control
Subclinical
Clinical

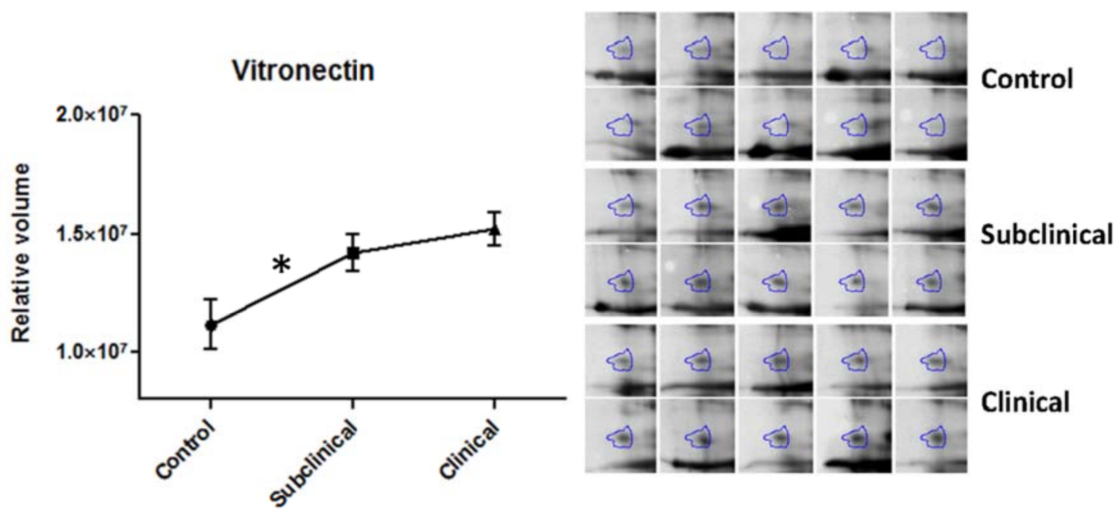
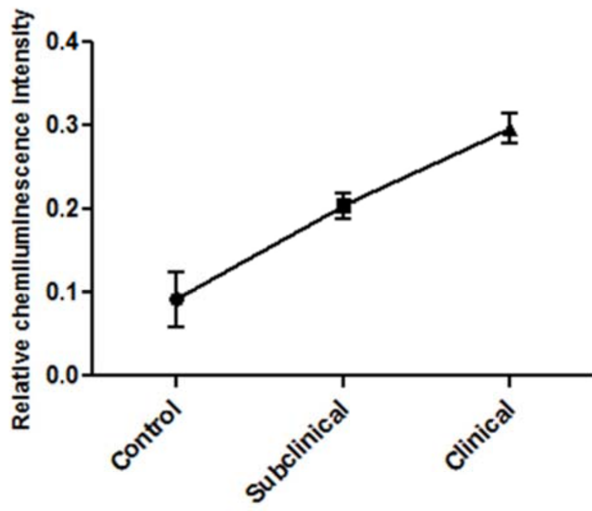
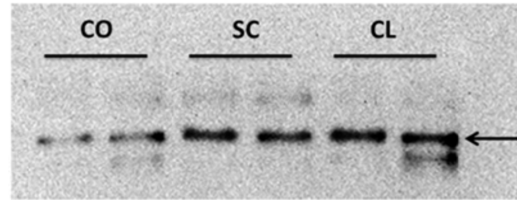
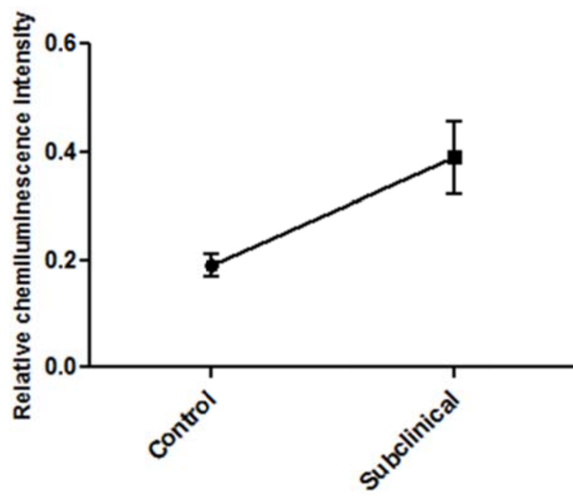
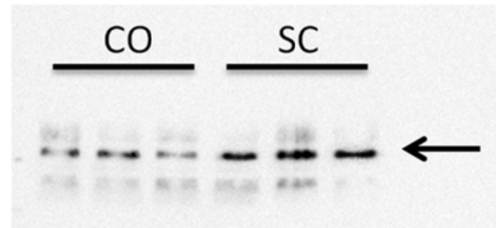

Vitronectin
Relative volume
2.0×10⁷
1.5×10⁷
1.0×10⁷
*
Control
Subclinical
Clinical
Control
Subclinical
Clinical

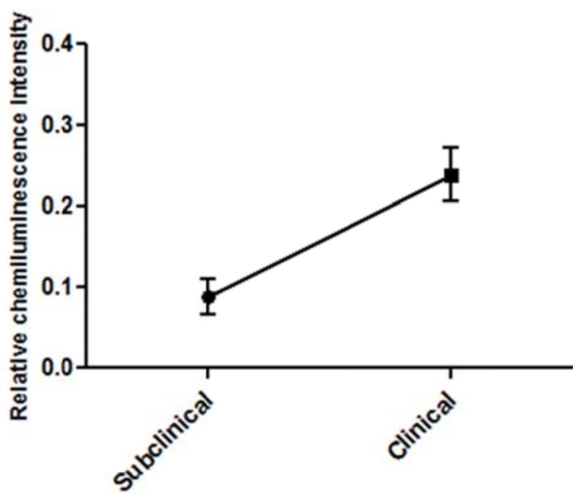
Figure 2: Superimposed average mass spectra of bovine serum samples obtained by MALDI-TOF analysis (A). The average spectrum of sera from cattle affected by clinical mastitis is indicated by a blue line, subclinical mastitis and control average mass spectra are shown by green and red lines, respectively. The region from 8,200 to 9,000 m/z including the most prominent peaks is shown in the enlarged view. Gel view of single mass spectra (B). Spectra are grouped in three classes according to the origin of serum samples. Mass spectra of sera from cattle affected by clinical mastitis are shown at the top of the panel, spectra from subclinical mastitis and control sera are shown in the middle and at the bottom of the panel, respectively.


Relative chemiluminescence Intensity
0.4
0.3
0.2
0.1
0.0
Control
Subclinical
Clinical


CO
SC
CL


Relative chemiluminescence Intensity
0.6
0.4
0.2
0.0
Control
Subclinical


CO
SC


Relative chemiluminescence Intensity
0.4
0.3
0.2
0.1
0.0
Subclinical
Clinical

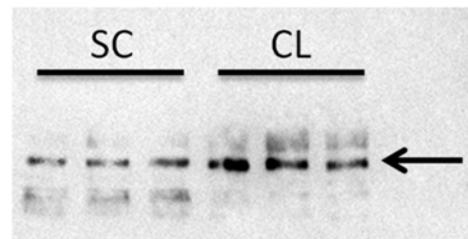
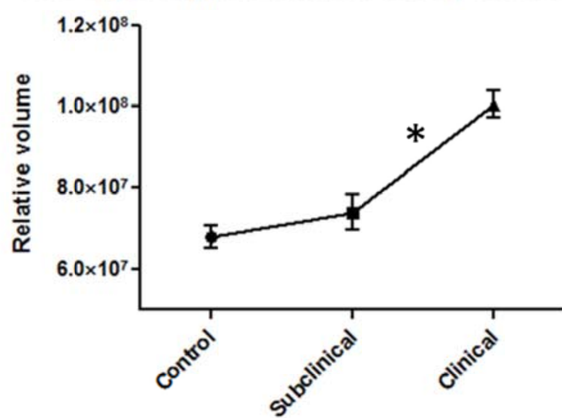
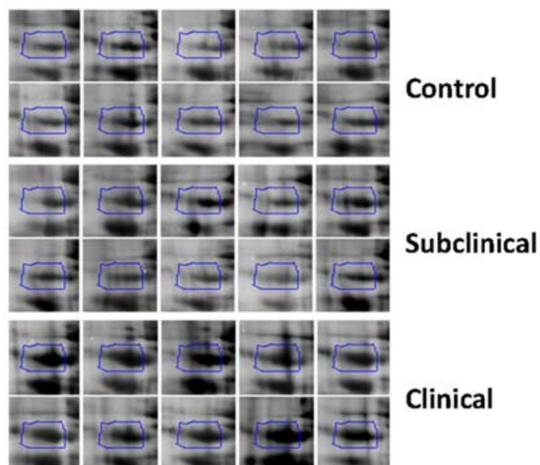
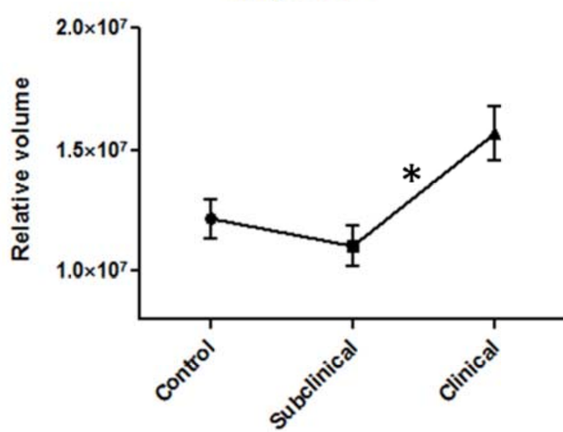
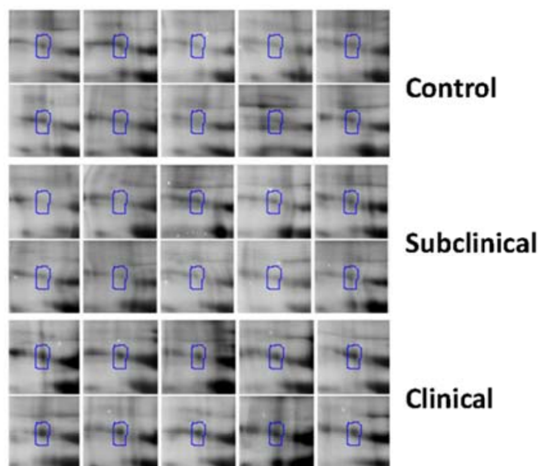

SC
CL

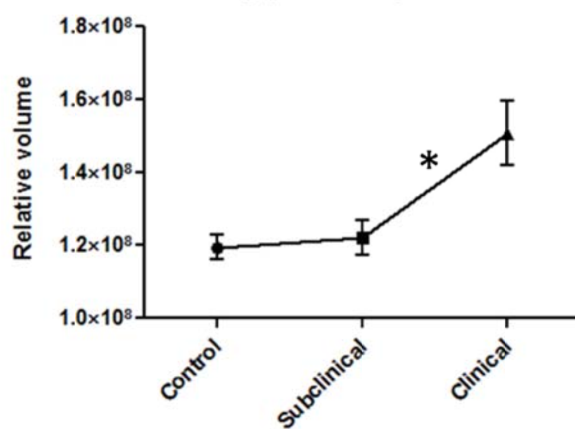
Figure 3: Differentially expressed proteins in subclinical group versus control group, respectively spots n° 1,2,3 ($p = \leq 0,05$)


Inter-alpha-trypsin inhibitor heavy chain H4
Relative volume
1.2×10⁸
1.0×10⁸
8.0×10⁷
6.0×10⁷
*
Control
Subclinical
Clinical


Control
Subclinical
Clinical


Serpin A3-1
Relative volume
2.0×10⁷
1.5×10⁷
1.0×10⁷
*
Control
Subclinical
Clinical


Control
Subclinical
Clinical


C4b-binding protein alpha chain
Relative volume
1.8×10⁸
1.6×10⁸
1.4×10⁸
1.2×10⁸
1.0×10⁸
*
Control
Subclinical
Clinical

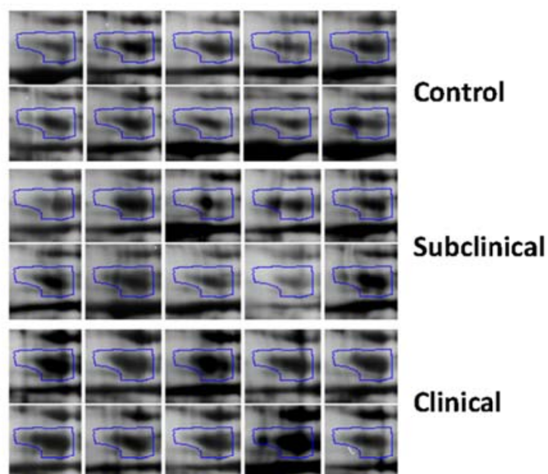

Control
Subclinical
Clinical

Figure 4: Differentially expressed proteins in clinical group versus subclinical group, respectively spots n° 4,5,6 (p=≤0,05)

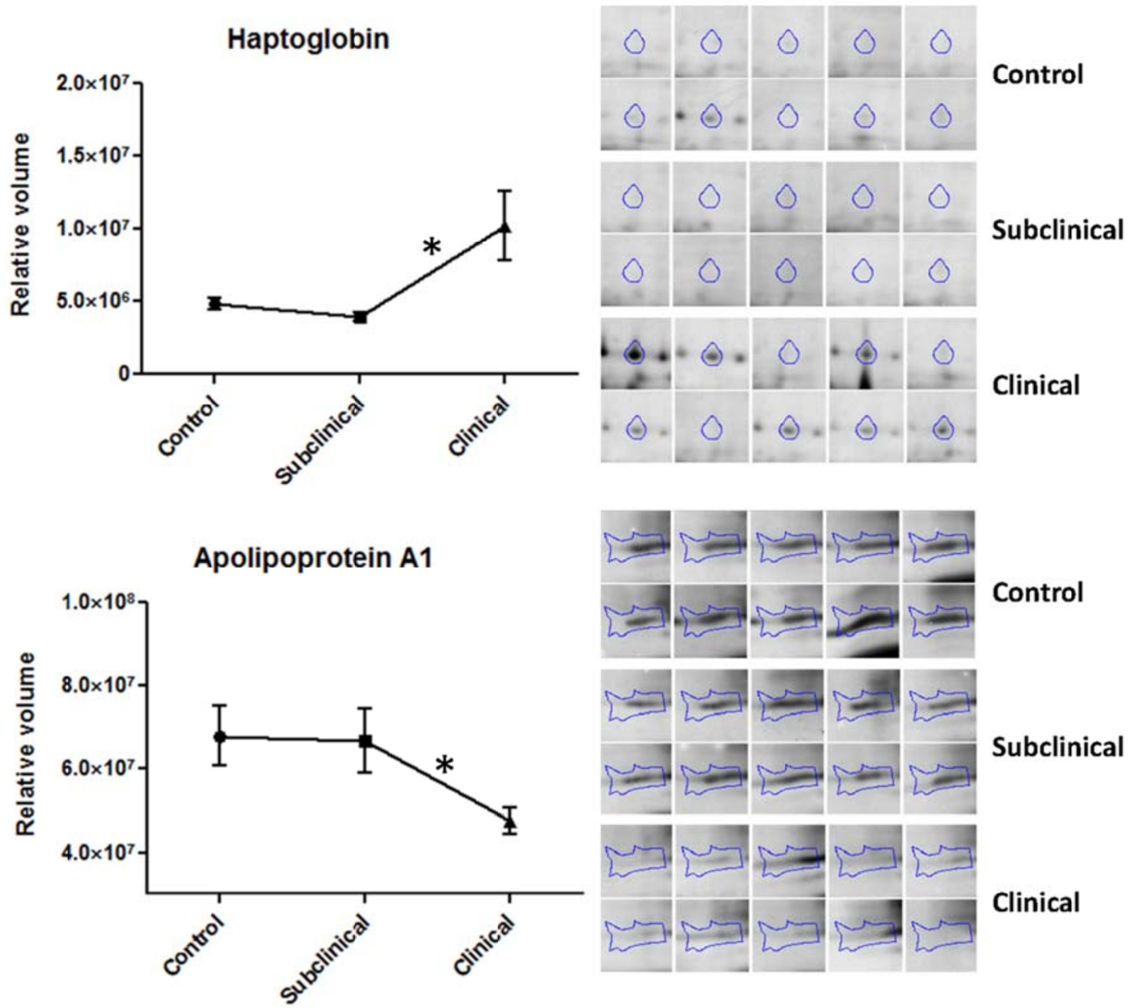


Figure 5: Differentially expressed proteins in clinical group versus subclinical group, respectively spots n° 7 and 10 (p=≤0,05)

Table 2. Shotgun Analysis

hit	[ǂ]Accession	Description	Mascot Score	MW [kDa]	pI	Sequence Cover. [%]	CTR [#]mean	CTR [§]SD	SM [#]mean	SM [§]SD	CM [#]mean	CM [§]SD	ratio SM/CTR	ratio CM/SM	ratio CM/CTR
1	P02769	Serum albumin precursor	2667	69.2	5.8	73.0	7.73	0.51	7.59	0.44	7.89	0.69	1.0	1.0	1.0
2	Q7SIH1	Alpha-2-macroglobulin	1891	167.5	5.7	37.0	1.10	0.03	1.11	0.20	1.16	0.08	1.0	1.0	1.0
3	Q29443	Serotransferrin precursor	1689	77.7	6.9	50.9	2.28	0.09	2.49	0.24	2.33	0.09	1.1	0.9	1.0
4	Q2UVX4	Complement C3	1681	187.1	6.4	30.7	0.61	0.05	0.59	0.05	0.69	0.02	1.0	1.2	1.1*
5	P15497	Apolipoprotein A-I	1154	30.3	5.6	63.0	17.02	1.44	17.85	1.44	18.68	-	1.0	1.0	1.1
6	Q3SZV7	Hemopexin	418	52.2	8.9	32.0	0.27	0.10	0.41	0.07	0.89	-	1.5	2.2*	3.3*
7	Q9TTE1	Serpin A3-1	301	46.2	5.6	18.7	0.47	0.10	0.31	0.09	1.00	-	0.7	3.2*	2.1*
8	Q2KJF1	Alpha-1B-glycoprotein	297	53.5	5.2	16.1	0.44	-	0.83	-	0.83	-	1.9*	1.0	1.9*
9	Q3SZR3	Alpha-1-acid glycoprotein	269	23.2	5.5	35.1	1.15	-	2.16	-	1.98	0.32	1.9*	0.9	1.7*
10	Q3T052	Inter-alpha-trypsin inhibitor heavy chain H4	261	101.4	6.2	8.4	0.04	-	0.05	0.02	0.12	-	1.3	2.3*	3.1*
11	Q3MHN5	Vitamin D-binding protein	260	53.3	5.2	23.8	0.33	-	0.43	-	0.54	-	1.3*	1.2*	1.6*
12	P01044	Kininogen-1	259	68.8	6.1	13.0	0.22	0.04	0.19	-	0.34	-	0.9	1.8*	1.6*
13	P34955	Alpha-1-antiproteinase (Serpin A1)	202	46.1	6.1	18.8	0.31	0.12	0.27	0.06	0.34	0.07	0.9	1.3	1.1
14	P19035	Apolipoprotein C-III	175	10.7	4.9	35.4	1.43	0.43	1.43	0.43	0.93	-	1.0	0.6	0.6
15	P02070	Hemoglobin subunit beta	171	15.9	7.9	31.0	0.93	-	0.28	0.11	0.64	-	0.3	2.3*	0.7
16	A2I7N3	Serpin A3-7	154	46.9	5.9	10.3	0.12	-	0.12	-	0.25	-	1.0	2.1*	2.1*
17	P81187	Complement factor B	132	85.3	8.8	6.0	0.05	-	-	-	0.15	-	-	-	3.1*
18	Q32PJ2	Apolipoprotein A-IV precursor	128	43.0	5.2	21.8	0.33	-	0.35	0.23	0.21	-	1.0	0.6	0.6
19	P12763	Alpha-2-HS-glycoprotein	126	38.4	5.2	12.0	0.23	0.11	0.17	-	0.17	-	0.7	1.0	0.7
20	O46375	Transthyretin	112	15.7	5.9	40.8	0.39	-	0.39	-	1.68	-	1.0	4.3*	4.3*
21	Q2KIT0	Protein HP-20 homolog	94	20.6	9.9	18.3	0.28	0.15	0.39	-	0.39	-	1.4	1.0	1.4
22	Q2KIX7	Protein HP-25 homolog 1	68	22.5	7.8	17.9	0.48	-	0.48	0.26	0.33	-	1.0	0.7	0.7
23	P33433	Histidine-rich glycoprotein (Fragments)	65	44.4	7.1	4.0	0.12	-	0.12	-	0.12	-	1.0	1.0	1.0
24	P01966	Hemoglobin subunit alpha	63	15.0	9.5	10.6	0.23	-	-	-	0.23	-	-	-	1.0
25	Q2KIU3	Protein HP-25 homolog 2	55	22.9	5.0	13.0	0.26	-	0.26	-	-	-	1.0	-	-
26	P17690	Beta-2-glycoprotein 1	40	38.2	9.7	7.2	0.12	-	0.12	-	-	-	1.0	-	-
27	Q4JIJ2	Methionine synthase reductase	40	77.1	6.0	4.2	0.07	-	0.07	-	0.07	-	1.0	1.0	1.0
28	P06868	Plasminogen	36	91.2	8.8	2.3	0.04	-	0.04	-	0.04	-	1.0	1.0	1.0
29	Q08E43	Ankyrin repeat and SOCS box protein 8	35	31.7	5.6	4.9	0.10	-	0.10	-	0.10	-	1.0	1.0	1.0
30	A2I7N0	Serpin A3-4	152	46.3	5.9	17.3	-	-	0.12	0.05	0.51	-	-	4.4*	-
31	Q0VCM5	Inter-alpha-trypsin inhibitor heavy chain H1	60	101.2	7.1	3.6	-	-	0.04	-	0.04	-	-	1.0	-
32	P00978	Protein AMBP	48	39.2	9.0	3.4	-	-	0.10	-	0.10	-	-	1.0	-
33	Q2TBU0	Haptoglobin	239	44.8	8.8	20.4	-	-	-	-	0.33	-	-	-	-
34	P81644	Apolipoprotein A-II	56	11.2	9.1	16.0	-	-	-	-	0.58	-	-	-	-

[ǂ]Accession number according to UniProtKB/Swiss-Prot database. [#]emPAI average value of three experimental replicates of serum pool from control (CTR) subclinical mastitis (SM) and clinical mastitis (CM) groups. [§]Standard deviation was not assessed if the same number of peptide was detected in technical replicates. *Fold change significance (*p* <0.05) assessed by Mann-Whitney test.