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PhD Thesis

"Development and validation of BIOVITAE® lamps use in the milking parlor to control buffalo mastitis in relation to animal welfare and antibiotic resistance issue"

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Al mio papà

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List of abbreviations

AK	Amikacin
AMC	Amoxicillin–clavulanate
AMR	Antimicrobial resistance
AW	Animal welfare
CD	Clindamycin
CFU/mL	Colony forming unit/milliliter
CIP	Ciprofloxacin
CLSI	Clinical Laboratory Standards Institute
CM	Clinical mastitis
CMT	California Mastitis Test
CN	Gentamicin
CW	Continuous wave
DTT	Dithiothreitol
E	Erythromycin
EC	Electrical conductivity
EM	Electromagnetic
EAEC	Enteroaggregative
EHEC	Enterohemorrhagic
EIEC	Enteroinvasive
EPEC	Enteropathogenic
ETEC	Enterotoxigenic
EUCAST	European Committee on Antimicrobial Susceptibility Testing
FOX	Cefoxitin
GSH	Glutathione
H	Healthy
H_2O_2	Hydrogen peroxide
IMI	Imipenem
IMI	Intramammary infection
K	Kanamycin
LED	Light-emitting diode
MBC	Mozzarella di Bufala Campana
MDR	Multridrug resistant
MRP	Meropenem
NAS	Non-*aureus* staphylococci
$O2^{-}$	Superoxide anion radical
$^1O_2$	Singlet oxygen
•OH	Hydroxyl groups
OD	Optical density
OX	Oxacillin
P	Penicillin

PDI	Photodynamic inactivation
PDO	Protected Designation of Origin
PS	Photosensitizer
ROS	Reactive oxygen species
S	Streptomycin
SCC	Somatic cells count
SCM	Subclinical mastitis
STEC	Shiga toxin-producing *E. coli*
SXT	Sulfamethoxazole–trimethoprimprim
T	Oxytetracycline
TE	Tetracycline
UV	Ultraviolet light
VIS	Visible spectrum

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Abstract

The Mediterranean buffalo (*Bubalis bubalis*) is the second most important livestock species after the bovine (*Bos taurus*) (IDF, 2007). This large bovid is widely distributed, especially in Southern Italy, and it is an important economic resource for milk production to produce Mozzarella di Bufala Campana (MBC), a traditional cheese of the Campania Region which is renowned worldwide.
Breeding lactating buffaloes can result in the occurrence of mastitis cases. Mastitis is an inflammatory condition of the udder that can be caused by physical and biological hazards, genetic factors, and inadequate environmental and management conditions. In the dairy industry, mastitis is almost always caused by pathogenic and environmental bacteria which, under certain favorable conditions, can enter and multiply in the mammary gland, altering its health and function. The presence of mastitis cases in herds is a critical problem for producers, as the inflammation has consequences on animal health, the quality and quantity of produced milk and, on farmers' income (Halasa et al., 2007). Antibiotic therapy plays an important role in the mastitis treatment. Its success depends on both early detection and proper diagnosis, including identification of the pathogen involved in the inflammation, which will determine the choice of appropriate antibiotic therapy (Erskine et al., 2003). The phenomenon of antimicrobial resistance (AMR), becoming widespread in recent years, is a worrying problem, clinically and economically, and requires innovative strategies for antibiotic treatment.
Light-based antimicrobial approaches are becoming a growing translational part of antimicrobial treatments in the current age of resistance. The purpose of this PhD project was to evaluate the antimicrobial action of light device with Biovitae® technology in a milking parlor of the chosen buffalo farm. The experimental plan included an *in vitro* and *in vivo* study, in which LED devices, supplied by the Italian company Nextsense, were tested. Through a special combination of frequencies in the visible spectrum (VIS), LED devices create a system of multispectral interfering waves that result in an antimicrobial effect.

In Chapter 1, the *in vitro* use of Master light strip and the light bulb was described. We tested these lights on three bacterial strains previously isolated from the buffalo farm: *Escherichia coli* (*E. coli*) and *Staphylococcus microti* (*S. microti*) strains, isolated from milk samples, and *Staphylococcus aureus* (*S. aureus*) strain isolated from worker's hand swab. The effect of light exposure resulted in a statistically significant ($P \leq 0.05$) reduction in bacterial growth for *E. coli* and *S. aureus* strains after 4 hours of exposure to both the Master light strip and the bulb. The bacterial reduction for the *S. microti* strain was not statistically significant after 4 hours of exposure to both light devices. Given the link between the excitation of endogenous porphyrins and reactive oxygen species (ROS) production, Dithiothreitol (DTT) and Glutathione (GSH) reagents were tested to regulate intracellular redox reactions

and to defend the bacterial cell viability from ROS production. DTT or GSH presence in bacterial cultures showed a protective effect expressed by the reduction of photodynamic microbicidal activity induced by Biovitae® light.

In vivo study, reported in Chapter 2, described the monitoring of light effect performing ten samplings in milking parlor. Differences in the bacteria load and number of isolated bacterial genus and species were detected, comparing the results before and after the lights installation. The study included the collection of different samples (milk samples, milking parlor surfaces and workers' hands and nostrils swabs), bacteriological examination and proteomic identification of isolated bacteria. In addition, somatic cell count (SCC) and composition analysis were performed for milk samples to detect intramammary infections, mastitis cases, and milk quality changes. No case of clinical mastitis was detected based on SCC analysis and bacteriological examination. Whereases, 22 out of 200 (11%) milk samples were associated with subclinical mastitis (SCM) cases and 124 out of 200 (62%) milk samples with intramammary infection (IMI).
The obtained results from the use of antimicrobial device showed that the light progressively reduced the presence of Gram-negative bacteria, whereas the Gram-positive bacterial load fluctuated during all ten sampling. In addition, colony forming unit (CFU) and the number of bacterial genus and species isolated increased significantly when action of Biovitae® light was stopped, demonstrating the antimicrobial role of light in *vivo*.

Bacterial identification by MALDI-TOF-MS led to the detection in milk samples and milking parlor swabs of a little-known staphylococcus species within non-staphylococcus *aureus* (NAS) group, identified as *Staphylococcus microti* (Chapter 3). The identification of 53 *S. microti* strains was further confirmed by whole-genome 16S rRNA sequencing. Their phenotypic resistance profiles were evaluated by a disk diffusion method, and the tetracycline resistance of strains (100%) was also performed by genotypic characterization. Genotypic analysis, evaluated for the *tet*M and *tet*K genes by multiplex PCR, detected the presence of the *tet*M gene in all isolated *S. microti* strains. In addition, SCC was performed and the association of this value with the presence of *S. microti* strains showed that 37.9% of the strains isolated from milk were associated with IMI cases and 18.2% with SCM cases.

From all collected samples, the predominant Gram-negative bacteria was E. coli, which is generally found in manure, soil, and the farm environment. It often indicates improperly performed hygienic and management conditions. In Chapter 4, we described the isolation of *E. coli* strains (n.88) and antimicrobial susceptibility profiles which showed a high level of resistance to penicillin (100%) and clindamycin (98.9%). In addition, the strains showed against tetracycline a percentage of 29.5% resistance and a higher of intermediate susceptibility (65.9%)

representative of an emerging resistance, probably related to its frequent use in mastitis cases. The genotypic characterization of the most fGram-negative bacteria's most frequently detected genes med on phenotypically tetracycline resistant *E coli*. By multiplex PCR, we detected the presence of the *tet*A, *tet*B, *tet*C, *tet*D e *tet*G genes, alone or in combination. For the *E. coli* exhibiting intermediate susceptibility to tetracycline no tested genes showed positivity suggesting the need of further studies. In addition, the association for the milk samples of SCC value with *E. coli* detection showed that 33.9% of samples were categorized as IMI cases and 27.3% as SCM cases.

In parallel with the rise of AMR, innovative approaches are being developed. One possible addition to the current range of treatments is the use of light with antimicrobial activity, able to eliminate a range of common pathogens. The results herein obtained from the antimicrobial photodynamic action produced by the Biovitae® LED devices are encouraging both *in vitro* and *in vivo*, when installed in milking parlor. We can conclude that the light alone has the advantages of being easy to install, useful for lighting and lower electricity consumption, and associated with correct routine cleaning practices, could be a valid and innovative approach to controlling bacteria capable of causing mastitis.

Riassunto

Il bufalo mediterraneo (*Bubalis bubalis*) è la seconda specie zootecnica più importante dopo la bovina (*Bos taurus*) (IDF, 2007). Questo bovino di grandi dimensioni è ampiamente diffuso soprattutto nell'Italia meridionale e rappresenta un'importante risorsa economica per la produzione di latte e, in particolare, per la produzione di Mozzarella di Bufala Campana (MBC), un formaggio tradizionalmente prodotto nella Regione Campania e rinomato in tutto il mondo. L'allevamento di bufale in lattazione può comportare l'insorgenza di casi di mastite. La mastite è una condizione infiammatoria della mammella che può essere causate da pericoli fisici e biologici, da fattori genetici e da non adeguate condizioni ambientali e gestionali. Nel settore lattiero-caseario, la mastite è quasi sempre causata da batteri patogeni ed ambientali i quali, sfruttando determinate condizioni favorevoli, riescono ad entrare e a moltiplicarsi nella ghiandola mammaria alterandone lo stato di salute e la funzionalità. La presenza di casi di mastite negli allevamenti è un tema critico per i produttori, poiché l'infiammazione comporta principalmente conseguenze sulla salute degli animali, sulla produzione qualitativa e quantitativa del latte e, infine, sul reddito degli allevatori (Halasa et al., 2007).

La terapia antibiotica gioca un ruolo importante nel trattamento delle mastiti. Il suo successo dipende sia da un rilevamento precoce che da una corretta diagnosi che comprende l'identificazione dell'agente patogeno coinvolto nell'infiammazione ed in base al quale verrà scelta la terapia antibiotica appropriata (Erskine et al., 2003). Il fenomeno dell'antimicrobico resistenza (AMR) diffusasi negli ultimi anni è una questione preoccupante, sia da un punto di vista clinico che economico, e che necessita di strategie innovative al trattamento antibiotico. Gli approcci antimicrobici basati sulla luce stanno diventando una parte traslazionale crescente dei trattamenti antimicrobici nell'attuale era della resistenza. Questo progetto di dottorato ha valutato l'azione antimicrobica dei dispositivi luminosi con tecnologia Biovitae® con un piano sperimentale *in vivo*, con installazione di essi in sala di mungitura di una azienda bufalina, e uno *in vitro*, utilizzando ceppi batterici isolati dalla stessa azienda. I dispositivi LED, forniti dall'azienda italiana Nextense, presentano una speciale combinazione di frequenze nello spettro visibile (VIS) capace di creare un sistema di onde multispettrali che producono un effetto antimicrobico.

Nel Capitolo 1, è stato descritto l'uso *in vitro* della Master light strip e della lampadina. Abbiamo testato queste luci su tre ceppi batterici precedentemente isolati dall'azienda bufalina: un ceppo di *Escherichia coli* (*E. coli*) e uno di *Staphylococcus microti* (*S. microti*), isolati dai campioni di latte, e un ceppo di *Staphylococcus aureus* (*S. aureus*) isolato da un tampone effettuato sulle mani di un addetto alla mungitura. L'effetto conseguente all'esposizione luminosa ha

determinato una riduzione della crescita batterica, statisticamente significativa (P ≤0,05) per i ceppi di *E. coli* e di *S. aureus*, dopo 4 ore di esposizione sia all'azione della Master light strip che della lampadina. La riduzione batterica del ceppo di *S. microti* non è risultata statisticamente significativa dopo le 4 ore di esposizione ad entrambi i dispositivi luminosi.
Dato il legame tra l'eccitazione delle porfirine endogene e la produzione di specie reattive dell'ossigeno (ROS), i reagenti Ditiotreitolo (DTT) e Glutatione (GSH) sono stati testati per regolare le reazioni redox intracellulari e difendere la vitalità delle cellule batteriche dalla produzione dei ROS. La loro presenza in colture batteriche ha mostrato un significativo effetto protettivo riducendo l'attività microbicida indotta dalla luce Biovitae®.

Lo studio *in vivo*, riportato nel Capitolo 2, ha descritto il monitoraggio batterico relativo all'effetto della luce mediante l'esecuzione di dieci campionamenti nella sala di mungitura. Confrontando i risultati prima e dopo l'installazione delle luci, sono state rilevate differenze nella carica batterica e nel numero di generi e specie batteriche isolate. Lo studio ha previsto la raccolta di diversi campioni (campioni di latte, tamponi delle superfici della sala di mungitura e tamponi delle mani e delle narici dei lavoratori), l'esame batteriologico e l'identificazione proteomica dei batteri isolati.
Inoltre, la conta delle cellule somatiche (SCC) e l'analisi della composizione sono state effettuate per i campioni di latte al fine di individuare la presenza di infezioni intramammarie, casi di mastite e alterazioni della qualità del latte. Basandoci sull'analisi SCC e sull'esame batteriologico, non sono stati rilevati casi di mastite clinica. Invece, 22 dei 200 campioni di latte (11%) sono stati associati con casi di mastite subclinica (SCM) e 124 campioni di latte (62%) con casi di infezione intramammaria (IMI). I risultati ottenuti in seguito all'uso dei dispositivi antimicrobici ha mostrato che la luce ha progressivamente ridotto la presenza di batteri Gram-negativi, mentre la carica batterica dei Gram-positivi ha avuto un andamento altalenante durante tutti i dieci campionamenti. Inoltre, la conta delle unità formanti colonia (CFU) e il numero di generi e di specie batteriche isolate sono aumentati in modo significativo quando l'azione della luce è stata interrotta, dimostrando il ruolo antimicrobico delle luci *in vivo.*

L'identificazione batterica mediante MALDI-TOF-MS di campioni di latte e tamponi della sala mungitura ha portato alla rilevazione di una specie di stafilococco poco conosciuta, all'interno del gruppo dei ceppi stafilococchi non-*aureus* (NAS), identificata come *Staphylococcus microti* (*S. microti*) (Capitolo 3). L'identificazione di 53 ceppi isolati di *S. microti* è stata ulteriormente confermata dal sequenziamento dell'intero genoma 16S rRNA. I loro profili di resistenza fenotipica sono stati valutati con un metodo di diffusione su disco e la caratterizzazione genotipica dei ceppi resistenti alla tetraciclina (100%), eseguita

mediante PCR multiplex, ha rilevato la presenza del gene *tet*M in tutti i ceppi di *S. microti* isolati. Inoltre, SCC è stata effettuata e l'associazione di questo valore con la presenza dei ceppi di *S. microti* ha mostrato che il 37.9% dei ceppi isolati dal latte erano associati a casi di IMI e il 18.2% a casi di SCM.

Da tutti i campioni raccolti (campioni di latte, tamponi delle superficie e tamponi dei lavoratori), il batterio Gram-negativo predominante era *Escherichia coli* (*E. coli*) che, solitamente presente nel letame, nel suolo e nell'ambiente aziendale, è indicative di condizioni igieniche e gestionali non correttamente eseguite. Nel Capitolo 4, abbiamo descritto l'isolamento di 88 ceppi di *E. coli* ed I profili di suscettibilità antimicrobica, i quali mostravano un alto livelli di resistenza alla penicillina (100%) ed alla clindamicina (98,9%). Inoltre, i ceppi esibivano verso la tetraciclina una percentuale di resistenza del 29.5% ed un valore più alto di suscettibilità intermedia (65.9%), rappresentativo di una resistenza emergente e probabilmente collegata al suo frequente uso nei casi di mastite. La caratterizzazione genotipica dei geni maggiormente rilevati nei batteri Gram-negativi è stata effettuata sui ceppi di *E. coli* resistenti. Mediante una PCR multipla, abbiamo rilevato la presenza dei geni *tet*A, *tet*B, *tet*C, *tet*D e *tet*G, da soli o in combinazione. Nessuno dei geni testati ha mostrato positività per i ceppi di *E. coli* che esibivano una sensibilità intermedia alla tetraciclina, suggerendo la necessità di ulteriori studi. Inoltre, l'associazione tra il valore di SCC dei campioni di latte e il rilevamento di *E. coli* ha evidenziato che il 33,9% dei campioni è stato classificato come casi di IMI ed il 27.3% come casi di SCM.

Parallelamente all'aumento dell'AMR, si stanno sviluppando approcci innovativi. Una possibile integrazione all'attuale gamma di trattamenti è l'uso della luce con attività antimicrobica, in grado di eliminare parte dei comuni agenti patogeni. I risultati qui ottenuti dall'azione fotodinamica antimicrobica prodotta dai dispositivi Biovitae® LED sono incoraggianti sia *in vitro* che *in vivo*, quando installati nella sala di mungitura. Possiamo concludere che la luce da sola ha i vantaggi di essere facile da installare, utile per l'illuminazione e consumare meno elettricità e che, associata a corrette pratiche di pulizia ordinaria, potrebbe essere un approccio valido e innovativo per il controllo dei batteri capaci di provocare mastiti.

1. Introduction

1.1 Mastitis classification and causes

Animal welfare (AW) is based on the "Five Freedoms" that must be observed in management practices within intensive livestock production systems (Farm Animal Welfare Council, 2009). The five freedoms for animal welfare include: 1) freedom from thirst, hunger, and malnutrition, 2) freedom from discomfort, 3) freedom from pain, injury, and disease, 4) freedom to express normal behavior and 5) freedom from fear and distress (Fig. 1.1)
If raised animals live in inadequate conditions and their health status is not optimal, their welfare will be affected, as will their production. Animal welfare is a broad concept that also includes animal health, food safety, and public health (De Passille and Rushen, 2005).

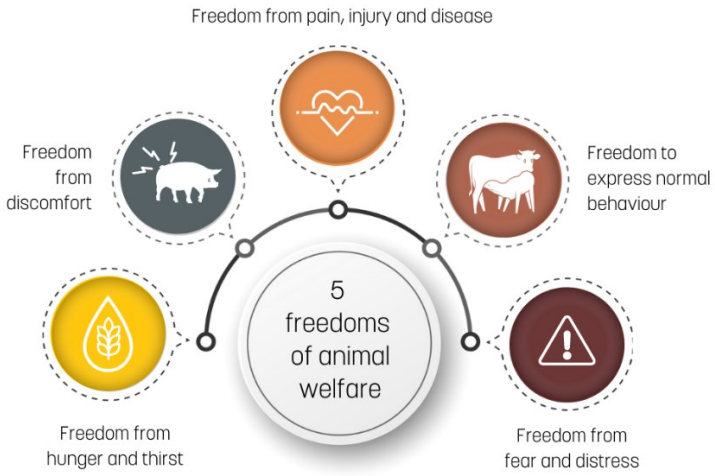

Figure 1.1 - Representation of "Five Freedoms" regulated by the European Union for animals' welfare and protection (*https://www.euractiv.com/section/agriculture-food/infographic/animal-welfare-in-the-eu/).*

Mastitis represents one of the main problems that alter the welfare and health status of the herd. Mastitis is an inflammation of the mammary parenchyma and involves physical and chemical changes in mammary gland tissue and glandular secretions. Physical trauma, infection by microorganisms (bacteria, viruses, algae, fungi), poor nutrition, or ineffective management conditions in animal husbandry can cause an inflammatory response in mammary tissue (Cheng and Han, 2020; Libera et al., 2021; Ajose et al., 2022). The occurrence of mastitis may be due to different factors: genotype, environmental conditions, diet, and the addition of dietary supplements (Abebe et al., 2010). In the dairy industry, mastitis is almost always caused by

bacteria (Nagasawa et al., 2019) that enter and multiply in the udder, leading to compositional changes related to the production of numerous mediators of inflammation (Wellnitz and Bruckmaier, 2012) and the change in permeability of blood capillaries.

Infections due to bacteria are classified as contagious and environmental. Bacteria that cause contagious infections can spread during milking time by transferring from one infected animal to another healthy animal through the milking cluster. Dirty hands and inadequately cleaned milking equipment and clothes can also spread contamination. Environmental mastitis pathogens are mainly found in feces, soil, water, and bedding (Garcia, 2004) and in areas such as stalls and corridors where animals spend most of their time and serve as primary sources of infection. Another risk for infection is dirt on the lower legs and feet, which can come in contact with the udder and teats when the animal is lying down.

Mastitis cases can manifest as clinical mastitis (CM) or subclinical mastitis (SCM), depending on host-pathogen interactions (Hoque et al., 2020). Subclinical mastitis is often undiagnosed due to limited clinical signs (FAO, 2014) because the mammary gland and milk appear normal, and it is frequently related to an intramammary bacterial infection (IMI) in quarters (Alekish, 2015). Milk from SCM-infected animals is characterized by increased numbers of somatic cells (SCC), such as leukocytes, neutrophils, macrophages, lymphocytes, and epithelial cells, which alter the composition and coagulative properties of the milk. Screening tests, such as bacteriological examination, California Mastitis Test (CMT), and electrical conductivity (EC), are essential to detect subclinical mastitis. Clinical mastitis is detected by the presence of visible abnormalities, like red, sore, and swollen breasts, and also by the presence of fever. Other indicators are inappetence and dehydration, reduced milk production, and watery milk with clots and flakes (Khan and Khan, 2006).

When the signs detected are severe and occur suddenly, they indicate acute mastitis cases (Rienesl et al., 2022) and can be further divided into hyperacute, acute, and subacute depending on the degree of inflammation detected in the animal. Chronic mastitis indicates cases of prolonged persistence of inflammation or a relapsing condition (Blowey and Edmondson, 2010), and the inflammatory process may last for several months, with clinical flare-ups occurring at irregular intervals (Cheng and Han, 2020). SCM cases are more prevalent in dairy farms worldwide among the various types of udder inflammation (Abebe et al., 2016)

In addition, mastitis cases are responsible for significant economic losses (Hoque et al., 2015; Ruegg, 2017), and economic damage is related to direct and indirect losses. The costs of treatments, veterinary services, and costs associated with repeated mastitis cases and milk discarded because it cannot be marketed are direct economic losses, while indirect losses are related to decreased milk quality and quantity, increased early culling, and reduced animal welfare and health (Petrovski et al., 2006; Ajose et al., 2022). Especially cases of clinical mastitis, as well as

reducing animal welfare, can have a negative impact on the reproductive system of dairy animals (Santos et al., 2004). Economic losses due to subclinical mastitis would be more significant than in clinical mastitis cases (Romero et al., 2018) and due to the persistence of pathogens within the herd (Bradley, 2022). In fact, bacteria can often persist within the herd, even because infected animals become asymptomatic and carriers of pathogens.
Maintaining hygienic practices during milking and milking tools are essential to reducing mastitis infections. Poor hygienic conditions in the milking environment can contaminate milk, making it a reservoir of pathogens that may spoil the milk produced and adversely affect consumers' health.
In confirmed mastitis cases, the primary strategy is antibiotic therapy. The overuse and misuse of antibiotics may also have implications for human health because of the increase and rapid spread of antibiotic-resistant strains and the entry of resistant bacteria into the food chain. More attention should be directed toward forms of mastitis prevention rather than acting when inflammation is already present in the breast.

1.2 Occurrence of mastitis cases in buffalo dairy animals

The water buffalo (Bubalus bubalis) is an animal species, the second most important dairy species after the bovine species (*Bos taurus*) (IDF, 2007), raised primarily for milk production, especially in Asia and Europe, but also for meat production or as a dual-purpose animal (Borghese A., 2005).
Italian Mediterranean buffalo was introduced from India to Europe during the 8th-century Arab occupation of Sicily and southern Italy. Currently, the water buffalo is mainly bred in central and southern Italy, especially in the Campania region, and is principally selected for milk production.
The buffalo udder is medium-sized, has four quarters and cylindrical teats, and a buffalo's average daily milk production depends on factors such as genetics and feeding system. During lactation, milk production can range from 3-4 kg per day for poorly fed animals to 15 kg per day in intensive production systems (Borghese and Moioli, 2016).
The composition of buffalo milk is different from cow's milk, being higher in protein, fat, and calcium, and lacking β- this reason, buffalo milk is a valuable product whose market price is almost double that of cow's milk. The explanation is due both to its characteristics and to the traditional production in the Campania region of typically Italian cheese, *Mozzarella di Bufala Campana* (MBC), which presents PDO (Protected Designation of Origin) status and is made with only milk from Italian Mediterranean buffalo (*Bubalus bubalis*) (EU Regulation Nº. 1107/1996 of June 12, 1996).

Even buffaloes, unlike previously believed, can be affected by the same diseases and parasites as cattle, as well as cases of mastitis. Mastitis cases, however, occur mainly based on three components: exposure to microbes, animal defense mechanisms, and environmental and management factors (Suriyasathaporn et al., 2000) (Fig. 1.2)

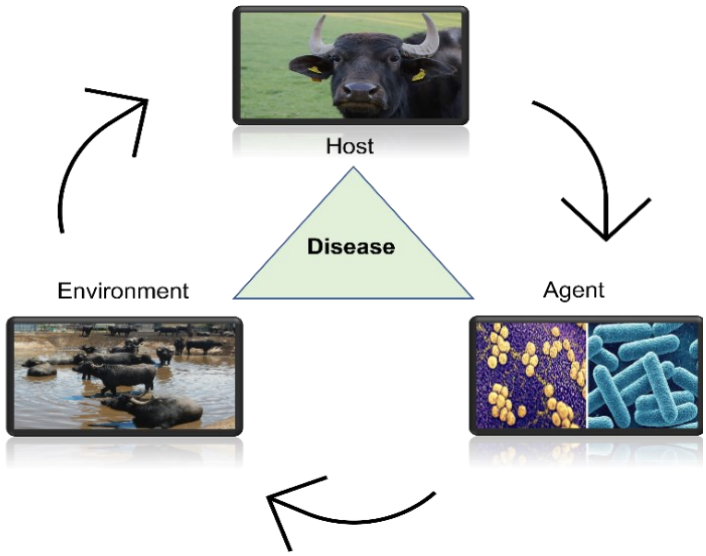

Figure 1.2 - Factors contributing to the occurrence of mastitis cases.

Although buffaloes are traditionally considered less susceptible to mastitis than cattle (Guccione and Ciaramella, 2017), and this may have underestimated its presence in herds, similar mastitis frequencies have been reported for both species (Guccione et al., 2017). Udder and teat characteristics are also different from bovine. Dairy buffalo have a more pendulous udder, and the teats are longer, which may contribute to a higher risk of mastitis during lactation. However, one condition that may prevent the invasion of microorganisms is that buffaloes have a long, narrow teat canal and the teat sphincter has a smoother muscle fiber. In addition, microorganisms can multiply rapidly in raw buffalo milk because of the high nutrient content.

1.2.1 Risk factors of mastitis

Several risk factors can play a role in the incidence of mastitis. First, the presence of contagious and environmental bacteria, which can take advantage of circumstances or poor cleanliness to start processes of infection and inflammation. Genetic factors and production indices may also influence the animal's susceptibility or defense against mastitis. Indeed, whether purebred or crossbred,

high-yielding dairy cows appear to be genetically more vulnerable to mastitis than medium-yielding breeds (Shaheen et al., 2016).

Susceptibility of dairy animals to mastitis is mainly linked to the conditions of the intrinsic immune system of the mammary gland (Burton and Erskine, 2003), and a higher incidence of mastitis can be detected during the peripartum period, during parturition and the first month of lactation (De Visscher et al., 2016; Fadlelmula et al., 2009) due to immunosuppression, associated with increased oxidative stress and poor antioxidant defense (Sharma et al., 2011). These distinctions are probably due to differences in the management of heifers compared to older animals and physiological differences, including the fact that heifers are starting lactation for the first time and are still growing (De Vliegher et al., 2012). In addition, during lactation, animals consume more energy and nutrients for colostrum and milk synthesis and may have a negative energy balance (Kibebew, 2017). In fact, if the nutrition provided does not follow the dietary requirements of the animal's physiological state, there may be nutritional deficiencies, which can bring immunosuppression and predispose to udder inflammation. Macro minerals (calcium, phosphorus, sodium, chlorine, sulfur, and magnesium) and micronutrients (iron, copper, manganese, zinc, cobalt, chromium, iodine, molybdenum, and selenium) participate in the formation of the body's structural components and the proper functioning of enzymes, hormones, vitamins, and cells, and their deficiency can affect breast health status (Libera et al., 2021). Also, a reduction of amino acids (lysine, L-histidine) and vitamins (A, C, E, β-carotene, lycopene) can increase the susceptibility of the udder (Shaheen et al., 2016; Matsui T., 2012).

The management practices and the cleanliness conditions of the farm can influence the presence of mastitis-causing bacteria and increased animal exposure. Especially on farms with high animal density, poor cleanliness of environments, damp bedding, poor ventilation, and inadequate climate (Abebe et al., 2016; Shaheen et al., 2016; Zeinhom et al., 2016). Cleaning practices are essential not only for lactating animals but also for dry cows and pregnant heifers because bacteria can enter the partially or totally open teat.

Mistakes related to milking practice can also increase the incidence of mastitis, like problems due to overmilking or defects in milking equipment. The effects of vacuum level, vacuum stability, and milking duration should be checked periodically to reduce the risk of new mammary infections and inflammation (Ruegg, 2017).

It is important to perform udder stimulation before milking and before machine attachment to promote the release of oxytocin in the bloodstream and allow optimal milk ejection, limiting teat and mammary gland stress (Costa et al., 2020). Older animals are more susceptible to infection, most likely because of the wider or more open teat canal due to frequent milking (Kibebew, 2017). The mammary epithelium

of older cows also shows increased permeability, mainly due to irreversible damage caused by previous inflammation (Król et al., 2013).
If sanitary standards for milking parlor maintenance and hygiene are lacking, microorganisms can easily penetrate and colonize the teat, causing an infectious process of varying severity, from subclinical to clinical status (Qaisar et al., 2017). Management practices highly influence environmental mastitis (Garcia, 2004), and cleaning must increase with the rising number of animals per area.
Farm cleaning combines chemical and physical processes because udder washing removes material on the udder, reducing the bacteria concentration but not entirely removing them from the skin. The cleanliness of udders and milking equipment is a key part of the milking routine, especially since the lack of cleanliness could affect the level of bacterial contamination of bulk milk in the tank (Bava et al., 2010; Oliver et al., 2005) and the presence of pathogens could negatively affect the health and nutritional status of consumers (Amenu et al., 2019).
Better knowledge of safety regulations and adherence to milk quality standards are essential to reduce the chances of disease transmission from milk (Dongol et al., 2017; Kumar et al., 2017).

1.2.2 Use of antibiotics as treatment for mastitis

On dairy farms worldwide, mastitis cases are common and severe because of the consequences on animal health, milk production, and farm profits. When cases of udder inflammation occur, depending on the severity of the case, animals may heal spontaneously or require treatment, such as the use of antibiotics, which are the main treatment of choice (Pol and Ruegg, 2007). The healing process and restoration of the affected quarter occur concurrently with treatment.
Antibiotic therapy aims to eliminate the microorganisms responsible for inflammation, reduce their spread, prevent the worsening of existing cases, and avoid further infections, especially during the dry period. The treatment also prevents mortality and improves animal and herd welfare by restoring the expected quality and quantity of milk produced.
Clinical signs and animals' medical history support the choice of antimicrobial treatment (Griffioen et al., 2016) but cannot provide information on etiology (Ruegg, 2018). Bacteriological examination and antimicrobial resistance profile are extremely important to support mastitis treatment decisions. Mastitis cases in which antimicrobial treatment may not be successful must be differentiated from those susceptible to therapy to choose and use antimicrobials prudently (Griffioen et al., 2021).
Since the discovery of the first antibiotic to the present, numerous antimicrobial compounds have been introduced, but at the same time, resistance mechanisms toward these compounds have occurred (Clatworthy et al., 2007), resulting in

prolonged illness or even death (Fig. 1.3). Unfortunately, currently the rate of antimicrobial resistance acquisition is inversely proportional to the scarcity of antibiotic discoveries (Skwor et al., 2016).

"Antimicrobial resistance (AMR) is the inability or reduced ability of an antimicrobial agent to inhibit the growth of a bacterium, which, in the case of a pathogenic organism, can lead to therapy failure" (EMA and EFSA, 2017).

A bacterial strain can acquire resistance by mutation, by uptake of exogenous genes via horizontal transfer from other bacterial strains, or by activation/triggering of a genetic cascade, thereby inducing the expression of resistance mechanisms. Antimicrobial resistance results from the continuous positive selection of resistant bacterial clones, whether pathogenic, commensal, or environmental bacteria, changing the population structure of microbial communities (EMA and EFSA, 2017). Multi-resistant bacteria have been shown to survive in environments such as sludge and wastewater treatment systems (Miller et al., 2016), allowing transmission and exchange of infectious bacteria between human and animal populations.

Recent studies have focused attention on a serious and emerging issue: cross-resistance between antibiotics and biocides in bacteria. Mechanisms of resistance also exist for biocides, chemicals used to disinfect environments, surfaces, and tools, which have been identified as an established cause of cross-resistance to antibiotics (Grimaldi et al., 2017). Incorrect concentrations of biocides (sub-inhibitory concentrations), inexact exposure times, inadequate temperatures of the water used, or the presence of residues on the surfaces to be disinfected, result in selective pressure that can lead to the development and spread of genetic mutations that confer resistance to both the biocides themselves and the antibiotics. Biocides act on multiple sites within the microorganism, and resistance may be due to reduced envelope permeability or active efflux pump systems, as for antibiotics. It has also been indicated that antibiotic resistance does not necessarily encode biocide resistance, but biocide resistance is more likely to lead the antibiotic resistance (Vali et al., 2008). Therefore, it is essential to perform adequate disinfection to reduce the bacterial load and avoid the emergence of cross-resistance to antibiotics.

Inappropriate use of antimicrobials, biocides and improper hygiene practices on farms or in the food chain facilitate the transmission of resistant microorganisms. Antibiotic resistance has spread rapidly in both animal and human populations (Birkegård et al., 2018) and it is a concern for consumers, who are increasingly seeking sustainable, ethical, and antimicrobial-free animal foods, changing market demand. All of these indications are factors that change the routines and procedures used in animal production (Boogaard et al., 2011).

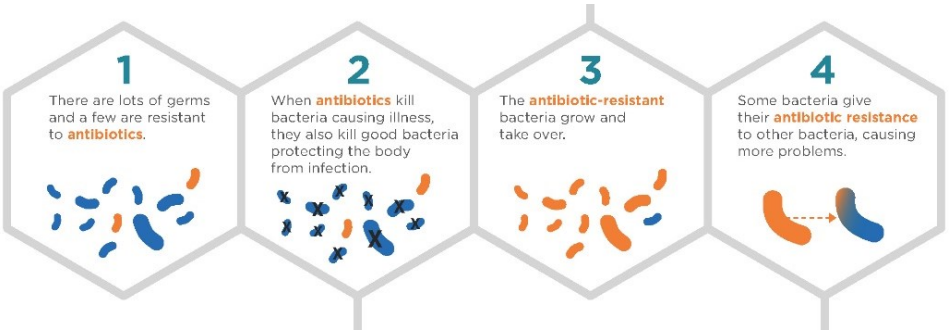

Figure 1.3 – How antibiotic resistance happens (*www.cdc.gov/antibiotic-use*).

1.3 Previous strategies used for bacterial reduction (UV and PDI)

Several strategies have been adopted in the dairy and livestock industry to reduce and eliminate microorganisms that can harm animals, raw materials, and various production processes.

Although effective, bacteria reduction strategies used in the past are not always applicable *in vivo* because they can damage eukaryotic cells and kill bacteria indiscriminately.

Ultraviolet (UV) lights are nonchemical disinfection technologies. UV irradiation is a physical method in which energy is the germicidal agent and includes wavelengths ranging from 100 to 400 nm on the electromagnetic spectrum: UV-A (315-400 nm), UV-B (280-315 nm), UV-C (200-280 nm) and vacuum UV (100-200 nm) (Fig 1.4).

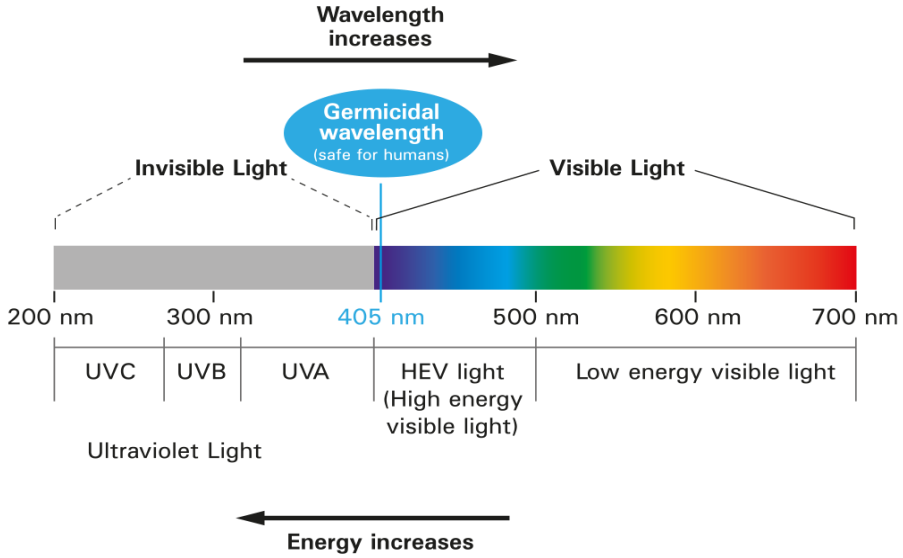

Figure 1.4 – The electromagnetic spectrum, including the wavelengths emitted by UV and visible lights (https://eaelighting.com/en-en/what-is-uv-disinfection).

Among the indicated wavelengths, UV-C light appears to have the most effective germicidal effect on bacteria, viruses, protozoa, fungi, and algae (Bintsis et al., 2000), leading to the inhibition of microbial growth or inactivation of the cell. Absorption of UV-C irradiation causes the formation of DNA photoproducts (cyclobutane pyrimidine dimers and the pyrimidine 6-4 pyrimidone photoproducts) which hinder transcription and replication, leading to mutagenesis and cell death (Gayan et al., 2014). Depending on the microorganism and UV doses, metabolism may attempt to repair DNA damage through photo-reactivation or dimming; however, at high UV doses, repair may not be possible due to increased damage.

In the dairy industry, UV light has been used to disinfect various production areas. UV technology enables the disinfection of the air in production areas by inhibiting the presence of pathogenic microorganisms and preventing their spread using low-pressure mercury vapor lamps (Koca et al., 2018). UV-C lights are also used to disinfect and eliminate various types of microorganisms in water used during dairy industry processing: drinking water, wastewater, process water, and brine. (Koca et al., 2018).

Bacterial inactivation carried out by ultraviolet light treatment is an approved and used alternative method on fruit juices (FDA, 2000) and has also been proposed as a possible alternative in milk processing (Matak et al., 2005). In fact, it can also be used as an alternative to heat treatment of raw milk for sale or calves. Milk can be easily contaminated during handling or storage from various sources, which may facilitate the growth of many pathogenic microorganisms due to its rich composition. Treatment with UV lights reduces the presence of microorganisms in milk, although these treatments can reduce the content of vitamins (A, B2, C, E) (Guneser and Karagul Yuceer, 2012). Inactivation by UV light depends on the bacterium types and their numbers, the dose of UV light, the concentration of solids, and the volume and transparency of the liquid medium (Guerrero and Barbosa-Canovas, 2004).

Unfortunately, extensive studies on the use of UV light, and particularly UVC light, have shown harmful effects on ocular components (Young, 2006) and human skin with an increased risk of cancer-related to constant exposure to low-intensity UV light or with the presence of injury from accidental exposure to high-intensity UV light (Hadi et al., 2021). For these reasons, lamps that adopt UV technology are shielded or closed in places that limit their exposure and require protective devices. Furthermore, it has been shown that the absorption of UV rays does not cause the destruction of a microorganism but causes its inactivation, damaging the nucleic acids and making it unable to perform its pathogenic function of replicating within a host. Bacteria have mechanisms that, during the cell replication process, can repair or bypass thymine dimers within DNA, and some viruses can also reactivate by exploiting specific host cell enzymes.

A promising technology is photodynamic inactivation (PDI). PDI involves the use of a photosensitizer (PS), either endogenous or exogenous, which is excited and

activated by light in the visible spectrum (VIS) and emitted through laser or LEDs, leading to the production of reactive oxygen species (ROS) and consequently the inactivation of microbial cells (Kovacikova et al., 2010). The inactivation of microbes is caused by ROS, which has the potential to deconstruct proteins, lipids, and nucleic acids within microbial cells and lead to cell death (Luby et al., 2019).
However, oxidative damage may not be the only cause of cell death. A transcriptomic study by Yang et al. (2017) examined a mechanism of toxicity related to the upregulation of phage proteins after irradiation. Phage maturation was inhibited, which would prevent cell death, suggesting that this mechanism would be phage-dependent, with important implications for antimicrobial selectivity.
PDT is entirely non-invasive, quick to use, and requires only a light source to inactivate many microorganisms, including bacteria, fungi, and viruses.
Its multi-target mechanism provides for rapid eradication of the microorganism, and it is unlikely to develop a resistance mechanism in microorganisms (Brovko L., 2010; Seidi Damyeh et al., 2020). Bacteria may respond differently to treatment, which depends on the photosensitizer and is much more effective if it penetrates inside the bacteria instead of acting only on the bacterial surface (Hamblin et al., 2002).
Light energy emanating from PDT is considered safe for humans, using recommended irradiation levels, because mammalian cells have better resistance, probably related to using more complex mechanisms to counteract oxidative damage, than bacterial cells (Maclean et al., 2014).
Photodynamic therapy (PDT), using PDI, was initially applied to neoplasms and skin infections (Lipson et Baldes, 1960) and subsequently to microorganisms (Fekrazada et al., 2016; Sellera et al., 2016) with *in vitro* experiments to counteract the multi-resistance profile, the ability to form biofilms, the bacterial spores, and the virulent bacterial determinants (St. Denis et al., 2011). PDT has advantages over traditional therapeutic alternatives, allowing minimal invasiveness and reducing systemic toxicity and minimal functional disturbances (Kaczorowska et al., 2021).
The effect of PDT was also evaluated in the treatment of subclinical bovine mastitis to develop an *in vivo* therapeutic protocol. During the study, a photosensitizer and an LED device were used in contact with the teat inducing a significant reduction in the total number of bacteria (Moreira et al., 2018).
PDT is an advantageous therapeutic strategy that could also be a promising support in open environments with a greater chance of inflammation caused by bacteria developing.

1.4 Innovative strategies: Biovitae® lamps

Master light strip and light bulb are light devices produced by the Italian company Nextense and present the Biovitae® technology. They consist of innovative white LEDs that emit a special multi-spectral system of electromagnetic (EM) waves interfering in the visible spectrum (VIS). The energy radiated by the lamps uses a photodynamic inactivation (PDI) mechanism in which the reduction of microorganism growth is produced by emitted light in combination with atmospheric oxygen and bacterial porphyrins, which are used as endogenous photosensitizers. The energy emitted by the combination of LEDs and the different wavelengths of VIS is not harmful to eukaryotic cells and is an innovative strategy to reduce pathogenic bacteria and counter antimicrobial resistance.
Porphyrins are photosensitizing chromophores present in bacteria and exhibit different profiles and absorbance bands: Soret-band (380-500nm) and Q-band (500-750nm), in the blue and red spectral region, respectively (Bernardini et al., 2021), with the strongest absorbers and most efficient bacterial inactivation in the visible blue-violet spectrum range (400-420nm) (Haridas and Atreya, 2022). Porphyrins are a group of macrocyclic heterocyclic organic compounds consisting of 4 pyrrolic rings, formed by 4 carbon atoms and 1 nitrogen atom, and are intermediate species in heme biosynthesis (Choby and Skaar, 2016) (Fig. 1.5). The two most important porphyrins are protoporphyrin (IX) and coproporphyrin (I and III), which absorb blue light in the range of 405-420 nm and 390 nm, respectively (Hessling et al., 2017). Different bacteria synthesize porphyrin molecules internally, and porphyrin types and levels can differ among bacterial species.

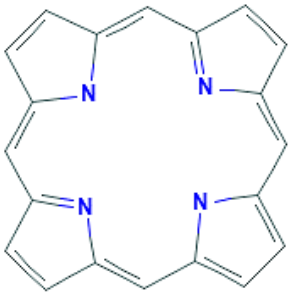

Figure 1.5 – Chemical structure of a porphyrin (https://pubchem.ncbi.nlm.nih.gov).

The presence and action of these molecules have been studied in various bacterial strains, both Gram-negative and Gram-positive (Wang et al., 2016; Dai et al., 2013), but bacteria such as the genera *Streptococcus* and *Enterococcus* do not synthesize endogenous porphyrins and are not sensitive to blue light antimicrobial treatments (Hadi et al., 2021). When porphyrins are hit by light at an appropriate wavelength, they switch to an excited state that can undergo molecular collisions with oxygen to form reactive oxygen species (ROS) and free radicals as singlet oxygen ($^1O_2$), superoxide anion radical ($O2^-$), hydrogen peroxide (H_2O_2) and, in the presence of

free transition metals, hydroxyl groups (•OH). In healthy and untreated bacterial cells, reactive species production is a natural side effect of aerobic respiration (Ong et al., 2017), but their overproduction leads to a toxic environment and the alteration of redox balances between free radical production and antioxidant defenses (Lobo et al., 2010). Cell damage occurs through lipid peroxidation, protein, and nucleic acid oxidation, and enzyme inhibition, ultimately leading to cell death by activating a programmed cell death mechanism (Ramakrishnan et al., 2016). ROS and radicals attack the intracellular structure and destroy it from the inside (Hessling et al., 2020) through a direct (type I) and indirect (type II) process: In the type I process, the excited photosensitizer releases excess energy, in the form of an electron, to other biomolecules, forming free radicals; in the type II process, the photosensitizer reacts with molecular oxygen to form singlet oxygen (Chukuka et al., 2021).
The action of the Master light strip was also evaluated against infected cells with SARS-CoV-2 (severe acute respiratory syndrome coronavirus 2) in order to test the lamp's ability to reduce the transmission of infection through aerosols and contact. Using Biovitae® technology, a significant decrease in cell viability was obtained for the first-time using LED irradiation with multiple wavelengths of VIS (De Santis et al., 2021). Initial concentrations of 8×10^1 PFU/ml, 8×10^2 PFU/ml, 7 x 10^3 PFU/ml, and 3×10^4 PFU/ml were reduced by 2 log (99%) and viral inactivation of about 96% was achieved using a concentration of 1.7×10^5 PFU/ml after 60 min of exposure.

1.4.1 Biovitae® Master light strip

Master light strip device has 13 LEDs with Biovitae® technology and 37 white LEDs, and a particular combination of multispectral interfering waves covers the visible spectrum with wavelengths at 400–420 nm, 400–450 nm, 400–700 nm at intensities of 3.51 mW/cm^2, 5.85 mW/cm^2, 12.53 mW/cm^2, respectively (Fig. 1.6 and 1.7). The Biovitae® strip was installed in a hood 30 cm above the working surface, operating on Petri dishes without lids in continuous wave (CW) throughout the experimental period. To prevent the heating of samples during exposure, the light strip has a heat sink for thermal management.

Figure 1.6 - Master light strip Biovitae®.

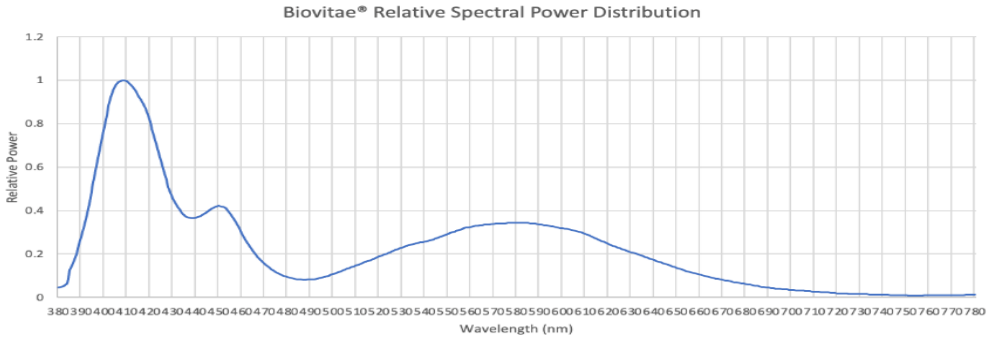

Figure 1.7 - Radiometric spectral distribution of Biovitae® Master light strip.

1.4.2 Biovitae® Light bulb

Light bulb has the same technology of Master light strip, presenting LEDs with Biovitae® technology and 21 white LEDs (Fig. 1.8). The device has a combination of frequencies covering the visible spectrum with wavelengths at 400–420 nm, 400–450 nm, 400–700 nm, at intensities of 0.83 mW cm^2, 1.13 mW cm^2, 2.89 mW cm^2, respectively (Fig. 1.9). Light bulb was installed in a closed box at 15 cm above the plates, operating in continuous wave (CW) throughout the experimental period. Petri dishes without lids were kept in the box and directed toward the light.

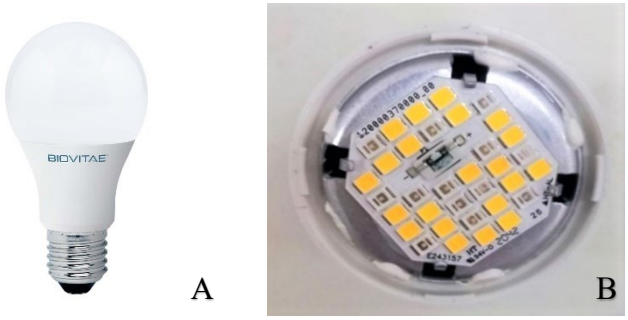

Figure 1.8 - Biovitae® Light Bulb (A), light bulb without cap (B).

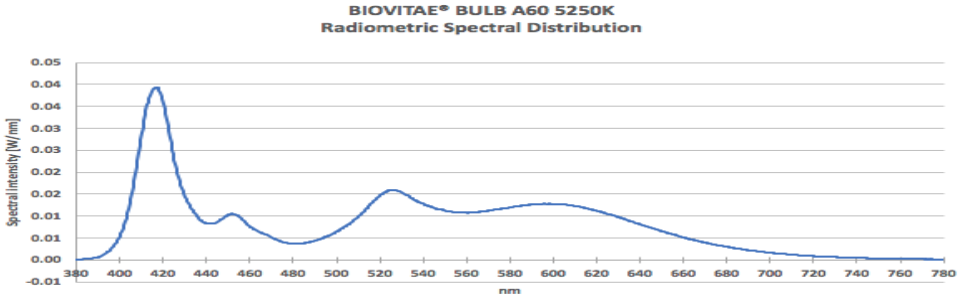

Figure 1.9 - Radiometric spectral distribution of the Biovitae® Light bulb.

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1. AIM:

The attention paid to mastitis cases on dairy farms is mainly related to their impact on animal and consumer welfare, as well as the economic losses they cause.
Antibiotics have been and are the main treatment against inflammatory processes caused by bacteria, although their extensive and inappropriate use has gradually led to the development of widespread antimicrobial resistance.
The search for innovative and effective solutions, as alternatives to antibiotic treatment, has led to the development of a system with antimicrobial capabilities that enables bacterial reduction without harming humans or animals.
In this study, we investigated the action of lamps with Biovitae® technology in an open environment, such as a milking parlor, where bacteria can easily circulate through the passage of animals and humans. A smallholder family-owned raising Italian Mediterranean buffaloes in the Campania region with about 100 lactating animals maintained in free stalls was chosen. Master light strips were installed at the ceiling of the milking parlor and were turned on 1 hour before the start of milking,were kept on for the next 2 hours (average milking time) and left on for the next 2 hours, to reduce the presence of bacteria causing udder inflammation, control mastitis cases, and improve animal welfare.
The experimental plan included an initial part of *in vitro* experiments in which both white LED devices supplied by Nextense, Master light strip and light bulb, were tested. The light action was used on different Petri dishes containing three bacterial strains isolated from the selected buffalo farm. *Escherichia coli*, Gram-negative strain, *Staphylococcus aureus,* and *Staphylococcus microti*, Gram-positive strains, were isolated from the environment, a milker, and a milk sample, respectively. In addition, the light strip was also tested on the same three strains using 96-well plates and maintaining the same exposure conditions. Given the link between the excitation of endogenous porphyrins and ROS production, Dithiothreitol (DTT) and Glutathione (GSH) reagents were tested to regulate intracellular redox reactions and defense of bacterial cell viability from ROS **(Chapter 1).**
In vivo experimental part included collecting milk samples from lactating buffaloes to assess the bacteriological quality and degree of contamination. In addition, milking parlor surfaces swabs were carried out to detect the isolation of the bacterial strains most prevalent in the environment before and after milking time. Workers' hands and nostrils swabs were performed because they are in direct contact with animals and milking parlor surfaces and are more exposed to bacteria circulating on the farm that can be transferred to humans during daily activities. The sample collection, before and after installing Master light strips at the ceiling of the milking parlor, was carried out. In addition to bacteriological examination and identification, for milk samples, somatic cell count and fat, protein, and lactose

content analysis of milk samples were also carried out to detect intramammary infection or mastitis cases that may alter the count and composition **(Chapter 2).** Bacterial identification by MALDI-TOF-MS led to the detection in milk samples and milking parlor swabs of a little-known staphylococcus species, identified as *Staphylococcus microti*. Phenotypic and genotypic studies related to this bacterial species and their correlation with IMI, SCM, CM and health status are presented in **Chapter 3**.

Escherichia coli strains were isolated from milk samples and surface and worker's swabs and were among the predominant bacteria detected in the collected samples. Analyses of the characteristics, antimicrobial resistance profiles and associations among the *E. coli* strains and milk samples to define animal health status are shown in **Chapter 4.**

2. Chapter 1 - *In vitro* studies: Biovitae® lights on Gram-negative and Gram-positive strains

3.1 Introduction

Since their discovery about 100 years ago, antibiotics have become the main drugs used to prevent and treat infections in humans and animals (WHO, 2022). Unfortunately, bacteria can develop antibiotic resistance strategies that limit available treatment options. For several years, health organizations such as the World Health Organization (WHO) have urged the development of new and alternative antimicrobial approaches (Boucher et al., 2009).

Photodynamic inactivation (PDI) is a useful and alternative approach to the use of conventional treatment with antibiotics. PDI is based on photophysical and photochemical reactions, which require the presence of light using wavelengths emitted in visible light spectrum (VIS), oxygen, and a photosensitizer (PS) (Penha et al., 2017), which can absorb photons in photochemical reactions and transfer energy to specific molecules (Jiang et al., 2013). Light using wavelengths between 400 and 420 nm, peaking at 405 nm, has been shown to have an important antimicrobial effect (Endarko et al., 2012, Maclean et al., 2008) and broad-spectrum action against both Gram-positive and Gram-negative bacteria (Maclean et al., 2009) including multi-resistant bacterial species. During light exposure, photosensitizers such as endogenous porphyrin molecules present in bacterial cells are excited and induce the reactive oxygen species (ROS) formation. ROS are molecules formed by the incomplete reduction of molecular oxygen (O_2) as an inevitable consequence of mitochondrial metabolism (Forkink et al., 2010) and are molecules with a highly positive redox potential (pE) and depending on how positive the potential becomes, the species' affinity for electrons and its strength as an oxidizing agent increase. ROS act as essential signaling molecules for cell growth and proliferation, but their overproduction, due to changes in intracellular redox balance, can cause oxidative damage to cells (Kwon et al., 2019) damaging membrane lipids, enzymes, proteins, or DNA, consequently inducing bacterial death (Ghate et al., 2013). When ROS and free radicals concentrations increase significantly, the cell's defense mechanisms (such as catalase, alkyl hydroperoxide reductase, thioredoxin, superoxide dismutase and DNA repair enzymes) are overwhelmed (Vatansever et al., 2013). The photodynamic mechanism used by antimicrobial lights leads to the ROS overproduction, resulting in oxidative stress and bacterial cell damage (Latifi et al., 2009). To determine if cellular damage is due to the action of free radicals, reducing agents, such as Dithiothreitol and Glutathione, have been tested for their ability to reduce the presence and damage caused by highly reactive oxygen species.

1,4-Dithiothreitol (DTT), also known as Cleland's Reagent ($C_4H_{10}O_2S_2$), is a chelator that protects free sulfhydryl groups from oxidation or reduces disulfide bonds to free sulfhydryl groups in proteins and enzymes (Fig. 3.1 A). DTT has a molar mass of 154.253 g/mol, contains two thiol groups and two hydroxyl groups, and is soluble in water, allowing it to permeate the cell membrane easily. Due to its low redox potential (-0.332 V at pH 7), DTT can protect sulfhydryl groups from oxidation or reduce disulfide bonds to free sulfhydryl groups in proteins and enzymes. This mechanism is important because disulfide bond formation is reversible and involves many biological processes, such as the activation and inactivation of proteins and the regulation of gene expression. DTT has little tendency to be oxidized directly by air, so it has the advantage of being a protective reagent compared with other thiol compounds.

Reduced glutathione (GSH) is another antioxidant molecule ($C_{10}H_{17}N_3O_6S$), is soluble in water, has a molar mass of 307.33 g/mol, and is the most abundant form in the cytosol, nucleus, and mitochondria (Forkink et al., 2010). Glutathione is a ubiquitous thiol-containing tripeptide composed of L-cysteine, L-glutamic acid, and glycine (Fig. 3.1 B), which can inactivate radicals and reactive oxidants and participates in thiol protection and redox regulation of cellular thiol proteins under conditions of oxidative stress (Kwon et al., 2019).

In this chapter, *in vitro* experiments are exposed performed to test the action of Biovitae® lights. The light device position is unchanged in all experiments, whereas the variable condition is the used plates in the tests. In addition, different concentrations of DTT and GSH were tested on *E. coli*, *S. aureus*, and *S. microti* strains. The molecules were added to bacterial cultures using 96-well plates, and the bacterial growth of the three strains was monitored for the plates exposed to Biovitae® light and control plates, to evaluate the protective role played by the molecules against the action of ROS.

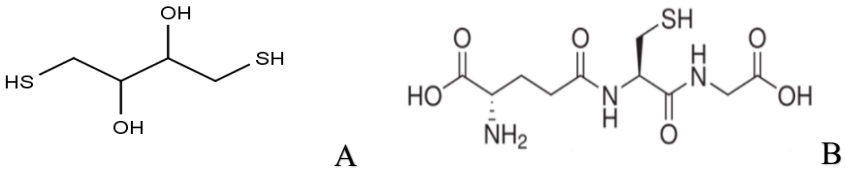

Figure 3.1 - Structure of a Dithiothreitol molecule (A); Structure of a Glutathione molecule (B).

3.2 Materials and methods

3.2.1 Bacterial and culture conditions

Three strains*, Escherichia coli*, *Staphylococcus aureus and Staphylococcus microti* were selected for this study. Bacteria strains were of animal origin and were

previously isolated from the Italian Mediterranean buffalo herd in the Campania region chosen for the study. Bacteria were identified before by proteomic analysis using matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF-MS) (Bruker Daltonik, Germany) and then were stored at −80 ◦C in Microbank™ vials (Pro-lab Diagnostics, Richmond Hill, ON, Canada) in Microbiology Laboratory of the Department of Veterinary Medicine and Animal Production of the University of Naples Federico II (Naples, Italy). The bacterial strains were transferred from the bacterial stock in appropriate growth media, McConkey agar for *E. coli* and Columbia CNA agar for *S. aureus* and *S. microti*, respectively. The plates were successively placed in an incubator for 24 hours at 37°C.

3.2.2 Experimental procedure using Master light strip, light bulb, and Petri dishes

To carry out the test, a loop of fresh overnight cultures of bacterial strains was transferred into 3 mL of brain heart infusion broth (BHI broth), a non-selective enrichment medium for aerobic bacteria, and then incubated aerobically at 37°C. To obtain the desired initial population of bacteria for experimental use, a suspension of 0,5 McFarland turbidity (concentration,1~2 $x10^8$ colony-forming units, CFU/mL) was serially diluted in peptone water until a final concentration of 2×10^3 CFU/mL. At each dilution step, the suspension was mixed using a vortexer (Thermo Fisher Scientific, UK) to ensure that the microorganism was evenly dispersed throughout the suspension. Plates were enumerated, and 10 µl of suspension, in quintuplicate, was distributed for each plate of MacConkey agar for *E. coli*, and Columbia CNA agar, for *S. aureus* and *S. microti*. The drops inoculated on the plate were left to dry and then exposed to the antimicrobial light without the lid. The exposure times were 2, 3, and 4 hours for Master light strip, whereas 2 and 4 hours for Light Bulb. Control plates were not exposed to light. After each indicated time, the plates were incubated at 37°C for 24 hours, and then the colony forming units (CFU/mL) were performed.
The bacterial reduction value was calculated using the following formula: $(N_f - N_i) \times 100/ N_f$, where N_f is the number of colonies grown on the LED-irradiated plates, while N_i is the number of colonies grown on unirradiated plates. Six tests were carried out both for Master light strip and light bulb.

3.2.3 Experimental procedure using Master light strip and 96-well plates

To perform the test, a loop of fresh overnight cultures of bacterial strains was transferred into 3 mL of LB medium and then incubated aerobically at 37°C for 24h. 200 µl of bacterial suspension, with an initial concentration of ~ 1-2 $x10^8$ and a mean optical density (OD595nm) of A 0.140 for *E. coli*, A 0.150 for *S. aureus*, and A 0.150 for *S. microti*. The absorbance of the empty culture medium was

subtracted from the initial value of samples containing bacteria. Tests (n=24) were performed using 96-well plates, and OD value was measured by Victor spectrophotometer (PerkinElmer). The initial bacterial suspension was diluted twice, using 1:2 and 1:4 dilutions. Plates with bacteria were exposed to the antimicrobial light strip for six hours, and bacterial growth was monitored by measuring OD at time zero, at 2, 4, 6, 21, and up to 24 hours for exposed and unexposed control plates.

3.2.4 Experiments in the presence of DTT and GSH as reducing agents

To establish the link between the redox changes induced by ROS overproduction and reduced bacterial growth, the reducing agents dithiothreitol and glutathione were tested in association with the photodynamic action produced by the Master light strip. The same initial concentrations and dilutions of the bacterial suspensions used in the previous procedure were used, in triplicate, in combination with different molar concentrations of the two protective agents. Test (n=6) were performed using 96-well plates with DTT molar concentrations of 0,05 mM, 0,1 mM, 0,5 mM, and 1 mM, and GSH molar concentrations of 0,03 mM, 0,3 mM e 3 mM. Plates containing bacteria and reducing agents were exposed to the light strip action for 6 hours. Exposed and unexposed plates were placed in the incubator at 37°C for 24 hours. Bacterial growth was monitored by measuring OD at time zero, at 2, 4, 6, 21, and up to 24 hours for exposed and unexposed control plates.

3.2.5 Antimicrobial susceptibility test

After the experimental procedure using the Master light strip on Petri plates, colonies from the control plate (unexposed to light) and colonies that survived (grown after 4 hours of exposure to the light strip) were used to perform the antimicrobial susceptibility test using the Kirby-Bauer disc diffusion method on Muller Hinton agar plates (Liofilchem, Teramo, Italy).
The following panel of antibiotics, which belonged to 8 different classes, were tested for *E. coli*, *S. aureus,* and *S. microti* strains: amikacin (AK, 30 µg), amoxicillin–clavulanate (AMC, 20/10 µg), ciprofloxacin (CIP, 5 µg), erythromycin (E, 15 µg), gentamicin (CN, 10 µg), oxytetracycline (T, 30 µg), streptomycin (S, 10 µg), sulfamethoxazole-trimethoprim (SXT, 25 µg), tetracycline (TE, 30 µg).
Gram-negative bacteria were also tested for imipenem (IMI, 10 µg) and meropenem (MRP, 10 µg). Gram-positive bacteria were also tested for penicillin (P, 10 IU), clindamycin (CD, 2 µg), oxacillin (OX, 1 µg), and cefoxitin (FOX, 30 µg). All antibiotic discs were from Oxoid (Milan, Italy). After a 24-hour incubation at 37°C, the zone of inhibition of each antibiotic disc was reported in millimeters (mm) following the interpretation by Clinical Laboratory Standards Institute (CLSI) (2018) and European Committee on Antimicrobial Susceptibility Testing

(EUCAST) (2015). Three replicate agar plates were used for each bacterium.

3.2.6 Statistical Analysis

The significant differences of the obtained data were calculated using Student t-test and Mann-Whitney U-test, depending on the data, using SigmaPlot software (version 11.0, Systat Software, Inc. Germany). The arithmetic mean and standard deviation were calculated for each test group.

3.3 Results

3.3.1 Master light strip effect on Petri dishes

CFU/mL counts were performed 18 hours after the exposure of bacterial suspension to Biovitae® Master light strip and a combination of multispectral interfering waves in VIS at intensities of 3.51 mW/cm^2, 5.85 mW/cm^2, 12.53 mW/cm^2, respectively. The results showed that *E. coli* reduction after light exposure was 65.4% after 2 hours, 88.6% after 3 hours and 98.3% after 4 hours. *S. aureus*, exposed to the light for 2, 3, and 4 hours, showed a colony reduction of 18%, 20.2%, and 26.4%, and *S. microti* reduction, after exposure for 2, 3, and 4 hours was 8.6%, 10.3%, and 15%, individually (Fig. 3.2). Significant differences were calculated at the 95% confidence interval using Mann-Whitney U-test, indicating a statistically significant reduction (P <0.05) for *E. coli* strain after 2, 3 and 4 hours of exposure, whereas for *S. aureus* strain after 4 hours of exposure. *S. microti* reduction was not statistically significant.

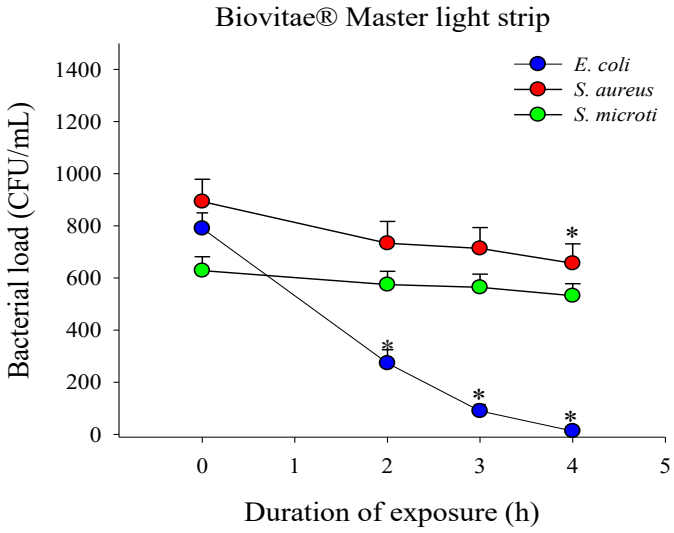

Figure 3.2 - *E. coli*, *S. aureus,* and *S. microti* growth reduction using Master light strip after 2, 3, and 4 hours of light exposure. Data show the mean counts (n = 30) ± SD. Statistical analysis showed significant difference (*, P <0.05), using Mann-Whitney U-test.

3.3.2 Light bulb effect on Petri dishes

CFU/mL counts were performed 18 hours after the light exposure to the Biovitae® Light bulb and a combination of multispectral interfering waves in VIS at intensities of 0.83 mW cm^2, 1.13 mW cm^2, 2.89 mW cm^2, respectively. The results, presented in Fig. 3.3, show that *E. coli* reduction was 19% after 2 hours of light exposure and 29.6% after 4 hours of light exposure. *S. aureus*, exposed for 2 and 4 hours, demonstrated a colony reduction of 12.3% and 20.8%, and *S. microti* reduction, after light exposure for 2 and 4 hours there was 11.2% and 24.4%, respectively. Significant differences were calculated at the 95% confidence interval using Mann-Whitney U-test, indicating a statistically significant reduction (P <0.05) for *E. coli* and *S. aureus* strains after 2 and 4 hours of exposure, whereas *S. microti* reduction was not statistically significant.

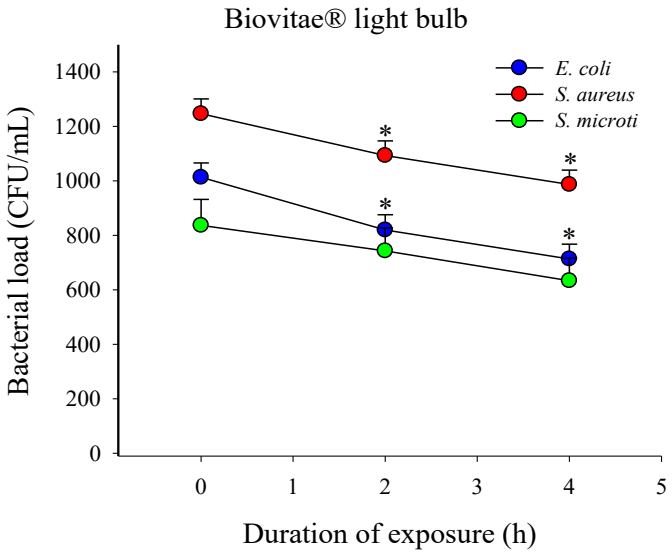

Figure 3.3 - *E. coli*, *S. aureus,* and *S. microti* growth reduction using Light bulb after 2 and 4 hours of light exposure. Data show the mean counts (n = 30) ± SD. Statistical analysis showed significant difference (*, P <0.05), using Mann-Whitney U-test.

3.3.3 Master light strip effect on 96-well plates

Antimicrobial light effect on the strains also showed, even in this experimental condition, a reductive effect on *E. coli*, *S. aureus*, and *S. microti* growth. During Biovitae® light exposure, bacterial growth was slightly and steadily increased (Fig. 3.4, 3.5, 3.6). However, 18 hours after exposure, there was a significant decrease in bacterial growth compared to control plates not exposed to the light action. Student t-test indicated a significant bacterial reduction of Gram-negative bacteria for the initial bacterial suspension (P<0.05) and for the two dilutions (P<0.001). Gram-

positive bacterial reduction was significant (P<0.001) for both the initial bacterial suspensions and the two dilutions.

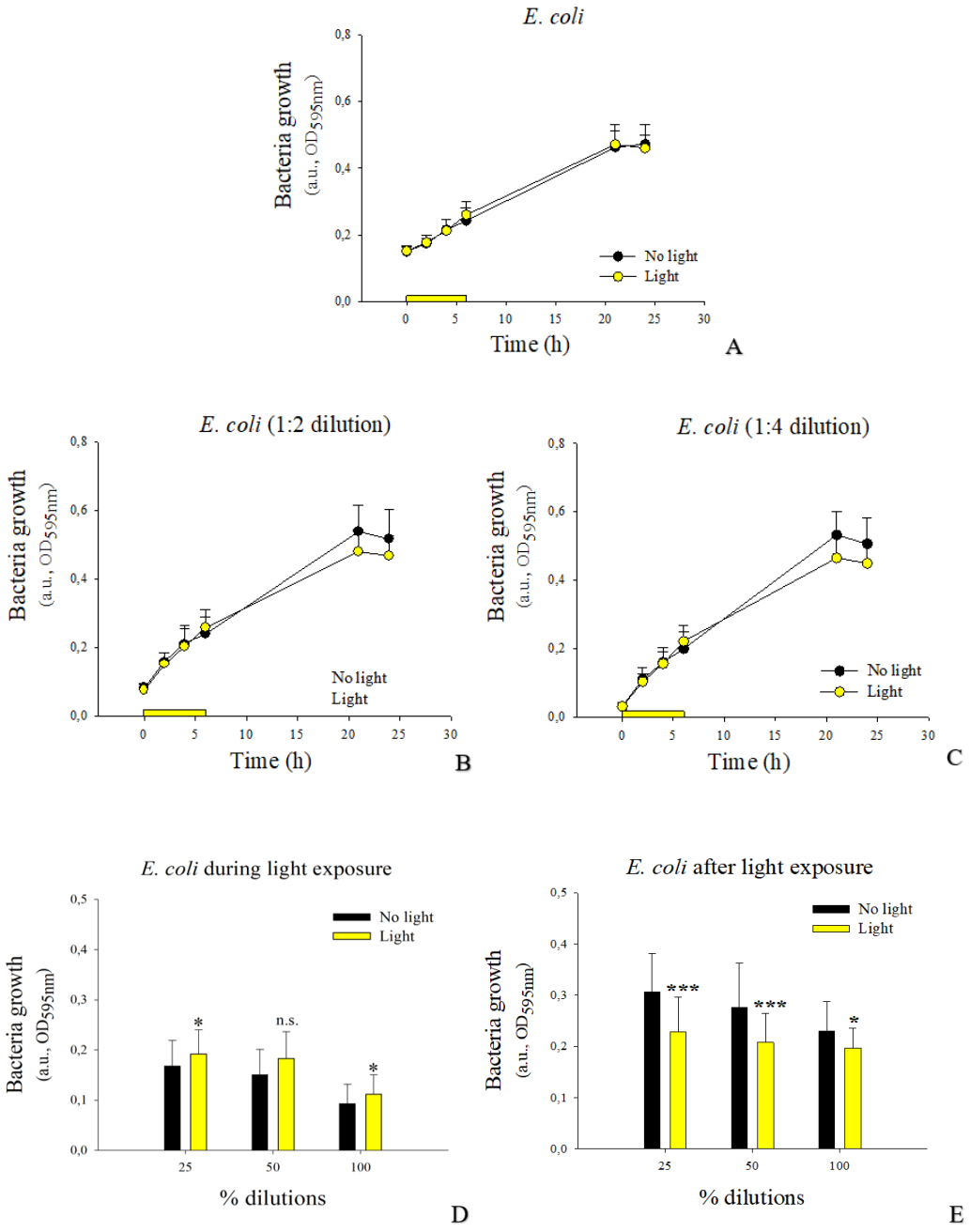

Figure 3.4 – Bacterial growth trend of light-exposed and unexposed *Escherichia coli* strain (letters A, B, and C). Effect on bacterial growth during the six hours of light action (letter D) and 18 hours after the end of exposure (letter E). (* P<0.05, ** P<0.005, *** P<0.001, n.s. = not significant).

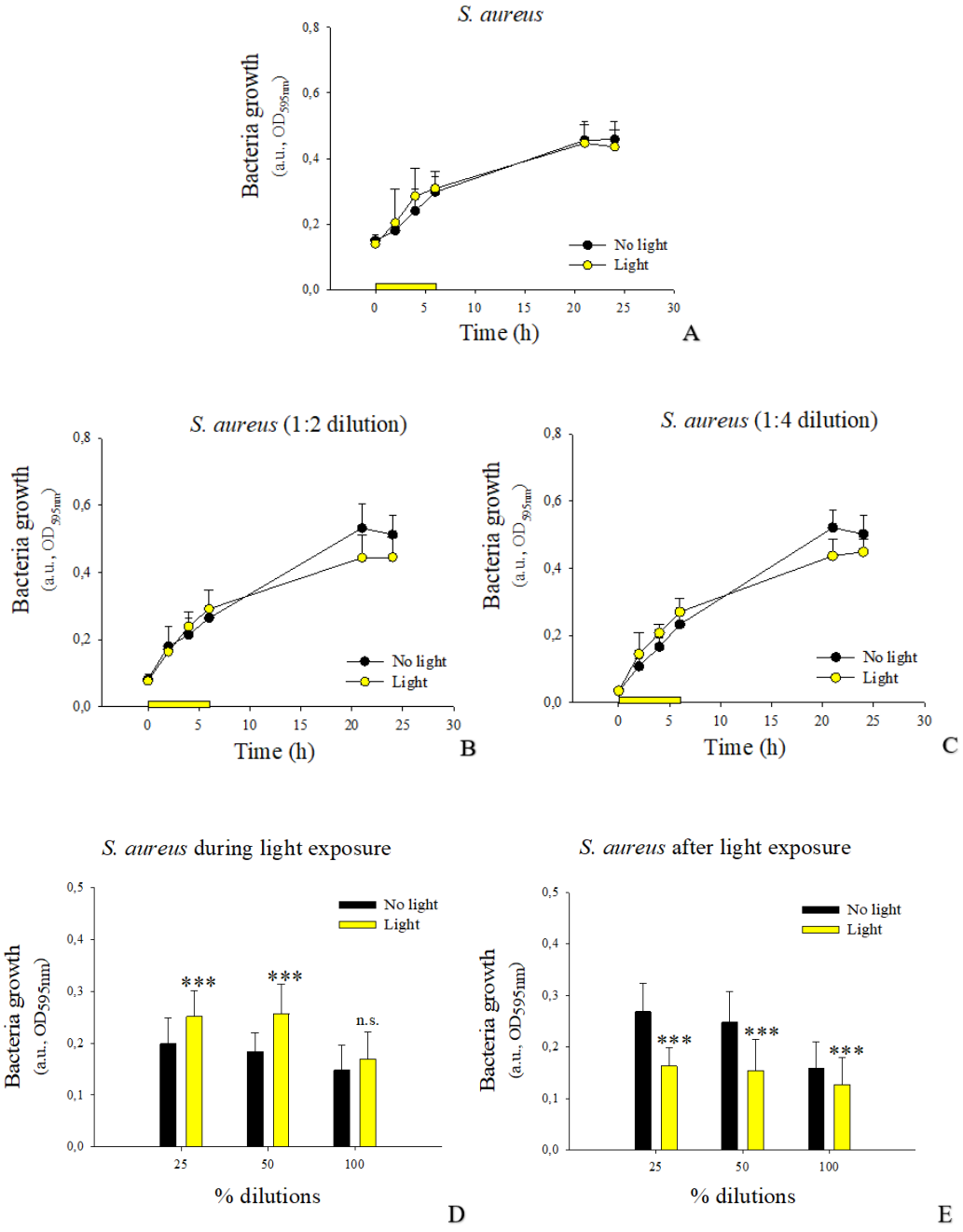

Figure 3.5 - Bacterial growth trend of light-exposed and unexposed *Staphylococcus aureus* strain (letters A, B, and C). Effect on bacterial growth during the six hours of light action (letter D) and 18 hours after the end of exposure (letter E). (* P<0.05, ** P<0.005, *** P<0.001, n.s. = not significant).

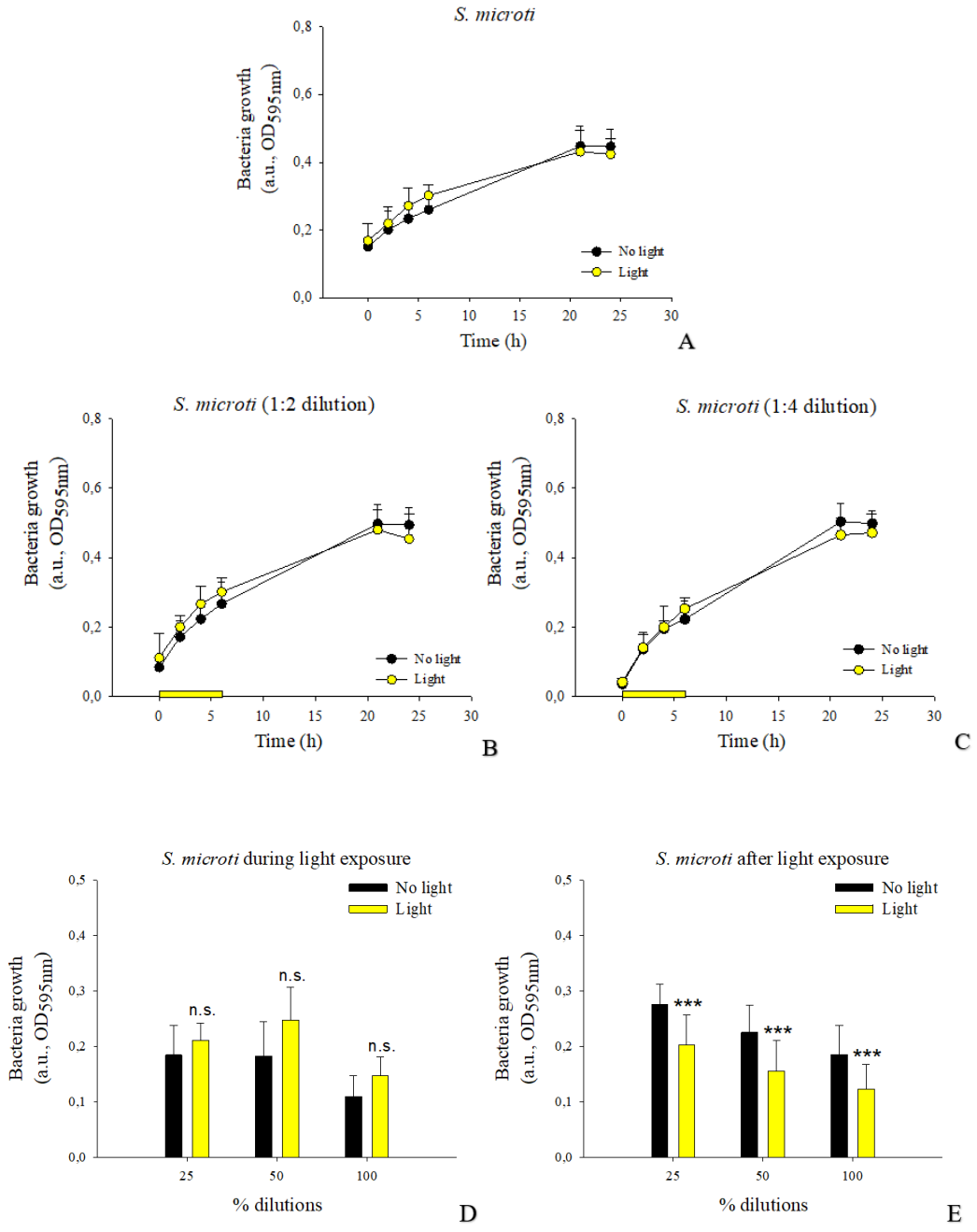

Figure 3.6 - Bacterial growth trend of light-exposed and unexposed *Staphylococcus microti* strain (letters A, B, and C). Effect on bacterial growth during the six hours of light action (letter D) and 18 hours after the end of exposure (letter E). P<0.05, ** P<0.005, *** P<0.001, n.s. = not significant).

3.3.4 Dithiothreitol and Glutathione effects

The addition of DTT and GSH to the bacterial suspensions showed a redox-sensitive mechanism of action, reducing the photodynamic microbicidal effect of Biovitae® light. The activity of the two reagents was monitored for both light-exposed and control plates from the initial time until 24 hours. During 6-hours exposure, DTT effect showed a growth reduction, which was indirectly proportional to the increase in concentration. In fact, the mechanism that induced a slight and steady growth increase during light exposure seems to be scaled down by the reagent action (Fig. 3.7, 3.8, 3.9, letters A, C, E). Moreover, bacterial growth of all three bacteria strains was generally reduced even without light, mainly when DTT concentrations were 0.5 mM and 1 mM.

The results also show that, 18 hours after exposure, bacterial growth of the three strains in the presence of DTT increased proportionally to the used concentration, indicating a protective role against ROS (Fig. 3.7, 3.8, 3.9, letters B, D, F). In contrast, at 24 hours, bacterial growth of unexposed strains was found to have decreased in some cases when the concentration of DTT increased.

DTT acted differently according to the used concentrations and the strains exposed to antimicrobial light. The protective effect on bacterial growth, detected at 24 h and using the highest concentration of DTT (1 mM), was significant for the *E. coli* strain, both for the initial bacterial suspension ($P<0.005$) and the 1:4 dilution ($P<0.001$); the effect was also significant for the initial bacterial suspension ($P<0.05$) and the two dilutions ($P<0.001$) of the *S. aureus* strain; no significant protective effect was observed for the *S. microti* strain exposed to the light action.

Results obtained from GSH use were similar to those obtained using DTT in the presence and absence of light. GSH action showed a stronger redox-sensitive effect for both exposed and unexposed plates, probably due to the higher concentrations used than DTT (Fig 3.10, 3.11, 3.12, letters A, C, E). Even after 24 hours, the trend obtained from the GSH effect is comparable to that obtained for the three strains in the presence of DTT (Fig 3.10, 3.11, 3.12, letters B, D, F).

GSH also acted differently depending on the used concentrations and the strains exposed to the antimicrobial light. The protective effect of GSH, detected at 24 h and using the highest concentration (3 mM), was significant ($P<0.001$) for the *E. coli* strain, for both the initial bacterial suspension and the 1:4 dilution; the effect was significant ($P<0.001$) also for the initial bacterial suspension and the two dilutions of the *S. aureus* strain; the GSH effect was statistically significant ($P<0.001$) for the initial bacterial suspension and the 1:4 dilution of the *S. microti* strain.

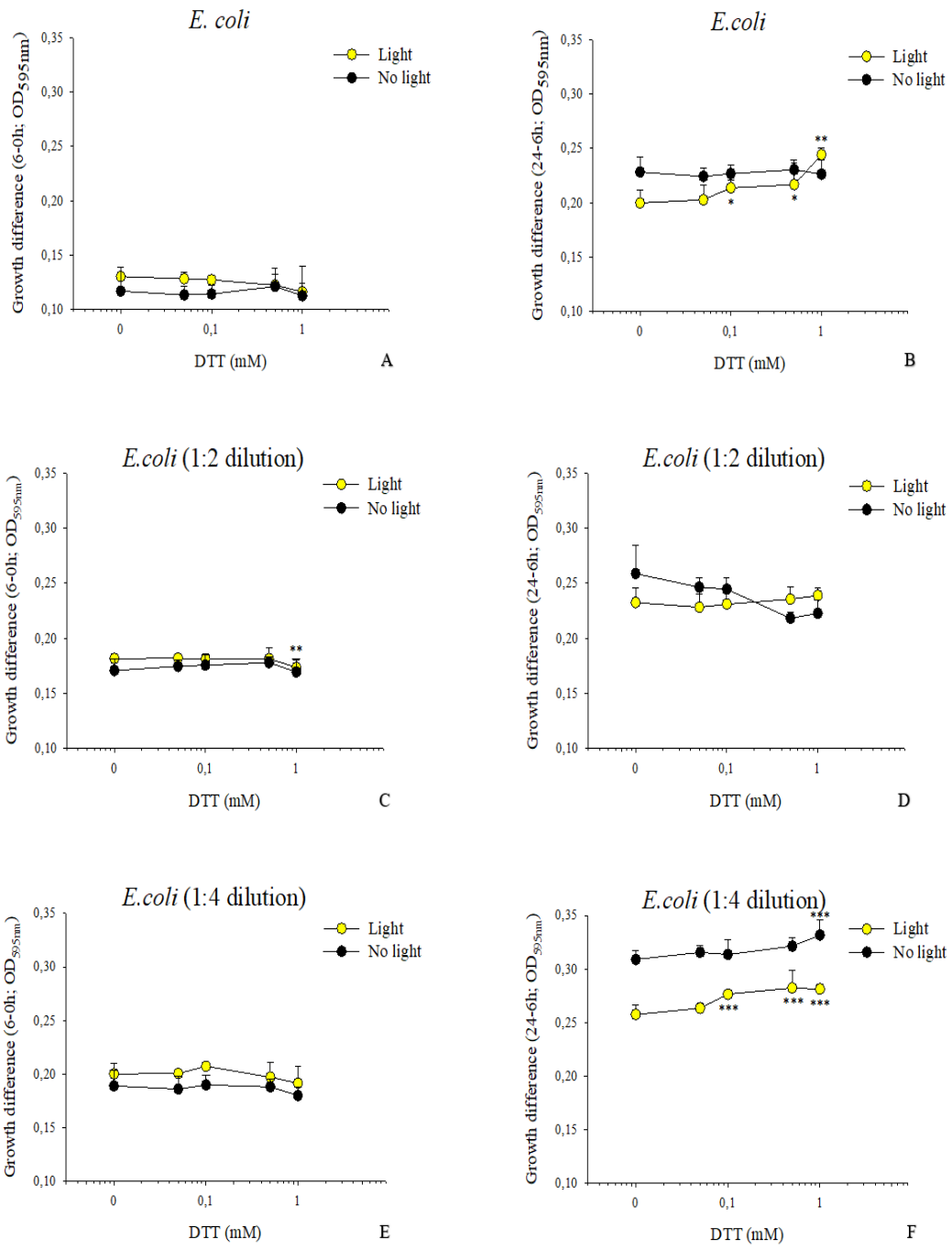

Figure 3.7 – Effect of different DTT concentrations (0.05 mM, 0.1 mM, 0.5 mM, and 1 mM) on *Escherichia coli* bacterial growth in the presence and absence of the light action. Growth trend during six hours of light exposure (letters A, C, E) and at 24 hours (letters B, D, F). (* P<0.05, ** P<0.005, *** P<0.001).

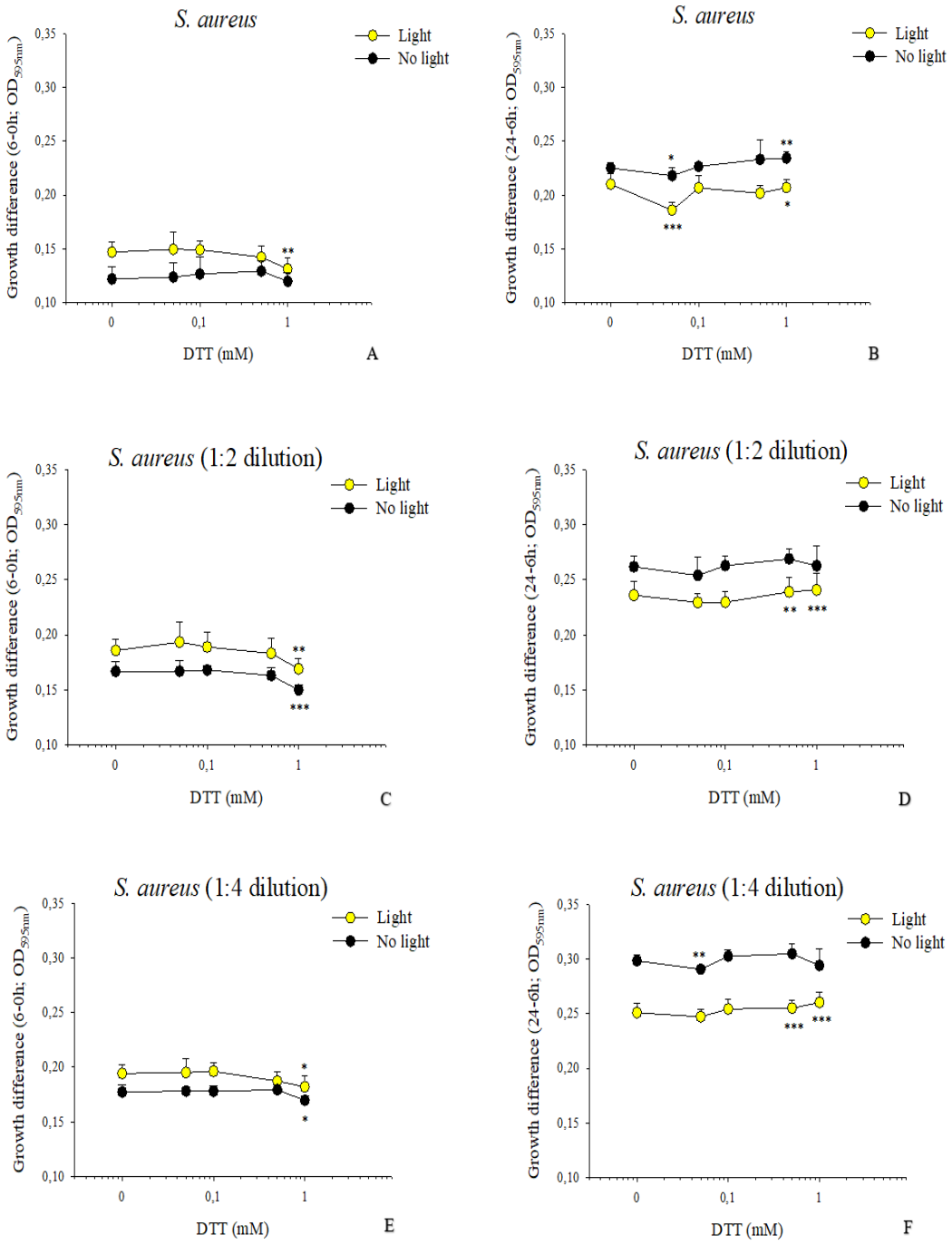

Figure 3.8 - Effect of different DTT concentrations (0.05 mM, 0.1 mM, 0.5 mM, and 1 mM) on *Staphylococcus aureus* bacterial growth in the presence and absence of the light action. Growth trend during six hours of light exposure (letters A, C, E) and at 24 hours (letters B, D, F). (* P<0.05, ** P<0.005, *** P<0.001).

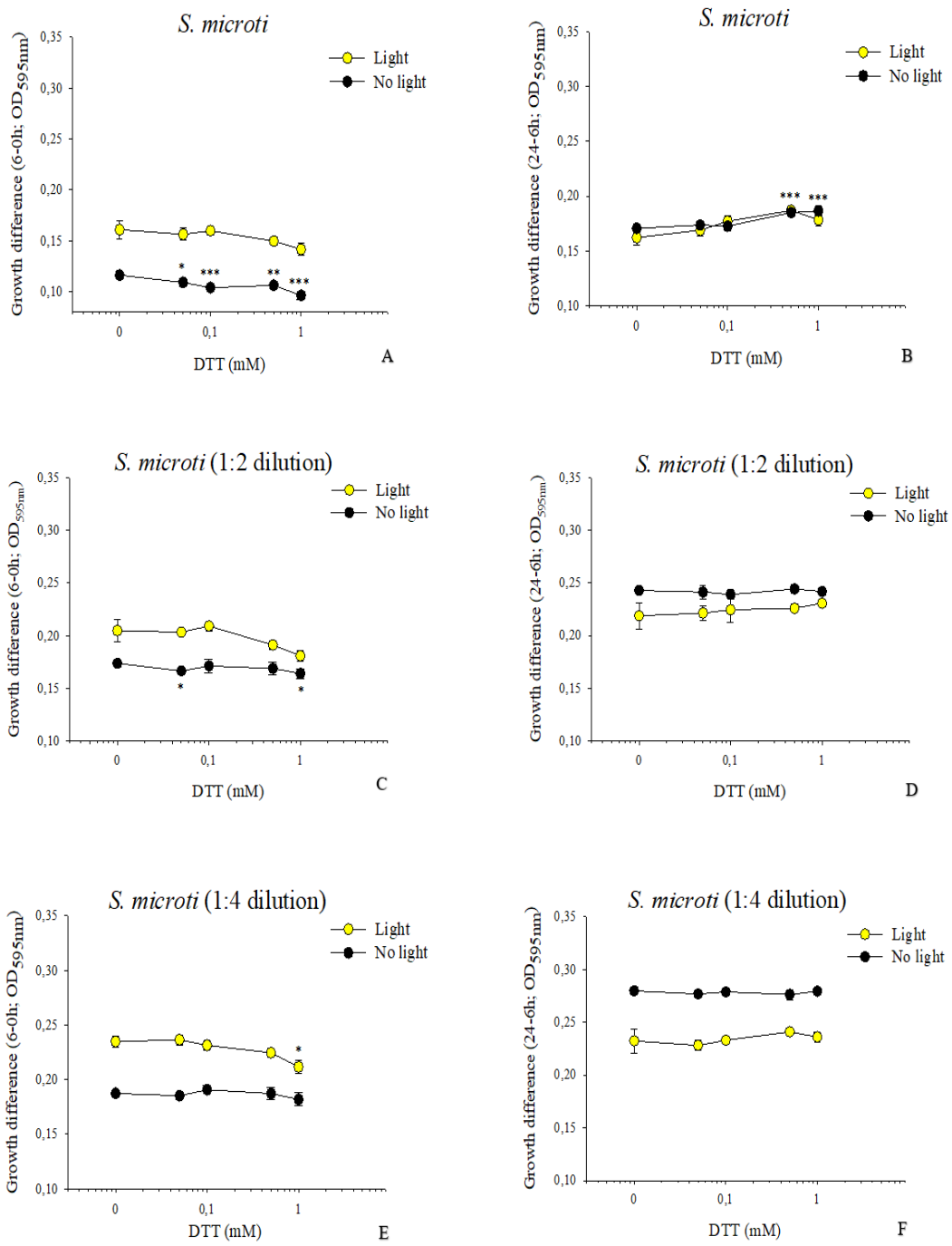

Figure 3.9 - Effect of different DTT concentrations (0.05 mM, 0.1 mM, 0.5 mM, and 1 mM) on *Staphylococcus microti* bacterial growth in the presence and absence of the light action. Growth trend during six hours of light exposure (letters A, C, E) and at 24 hours (letters B, D, F). (* P<0.05, ** P<0.005, *** P<0.001).

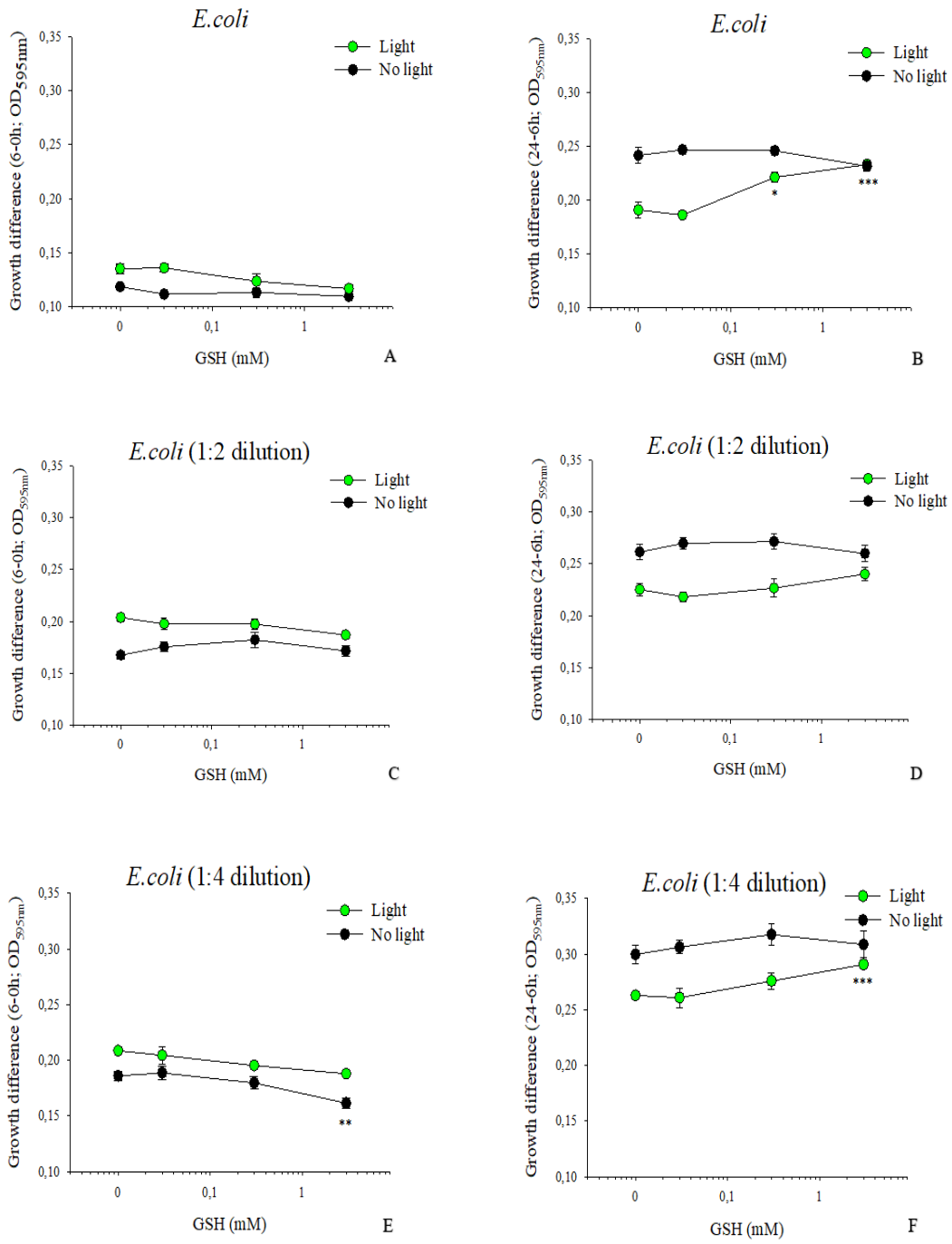

Figure 3.10 - Effect of different concentrations of GSH (0.03 mM, 0.3 mM, and 3 mM) on *Escherichia coli* bacterial growth in the presence and absence of the light action. Growth trend during six hours of light exposure (letters A, C, E) and at 24 hours (letters B, D, F). (* P<0.05, ** P<0.005, *** P<0.001).

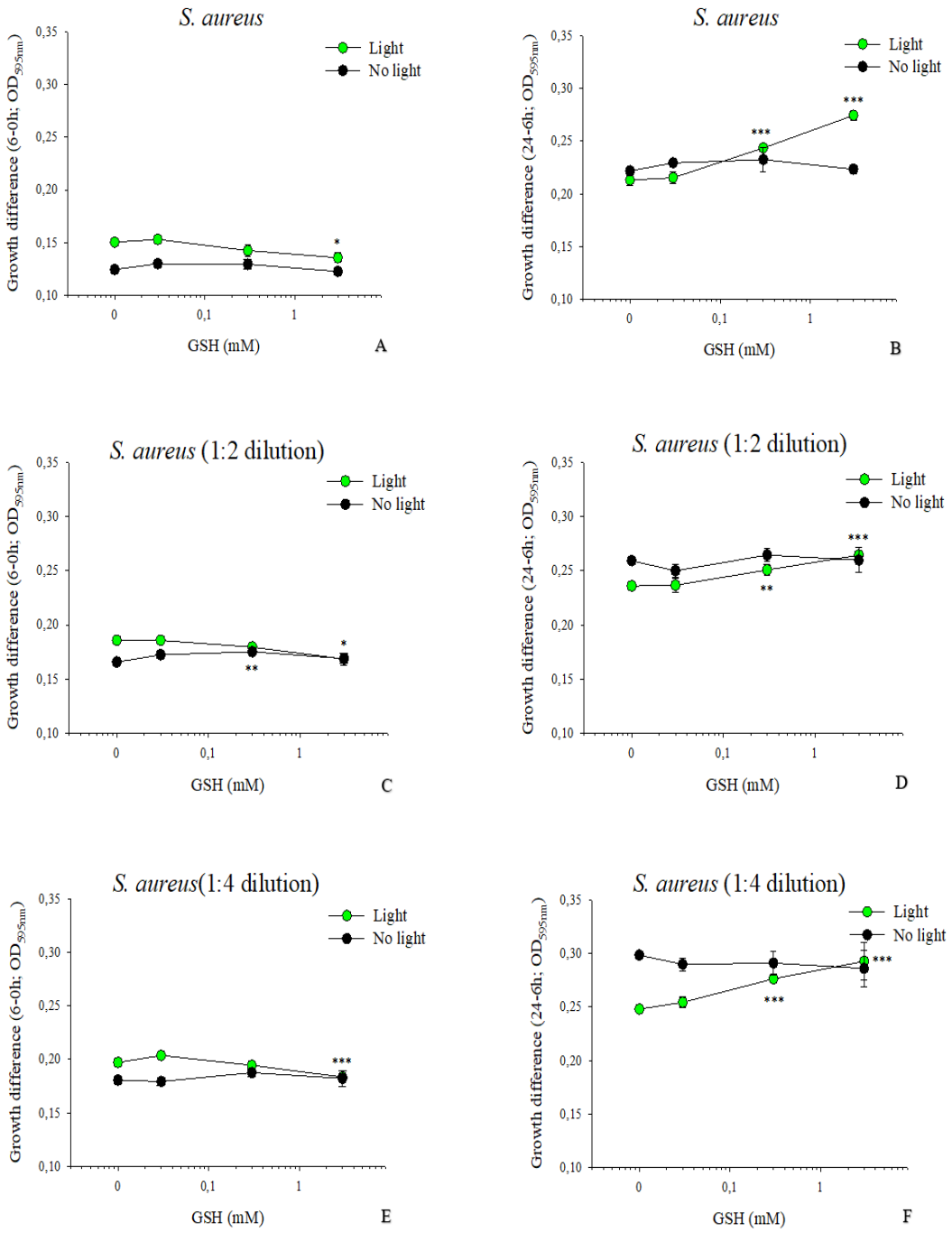

Figure 3.11 - Effect of different concentrations of GSH (0.03 mM, 0.3 mM, and 3 mM) on *Staphylococcus aureus* bacterial growth in the presence and absence of the light action. Growth trend during six hours of light exposure (letters A, C, E) and at 24 hours (letters B, D, F). (* P<0.05, ** P<0.005, *** P<0.001).

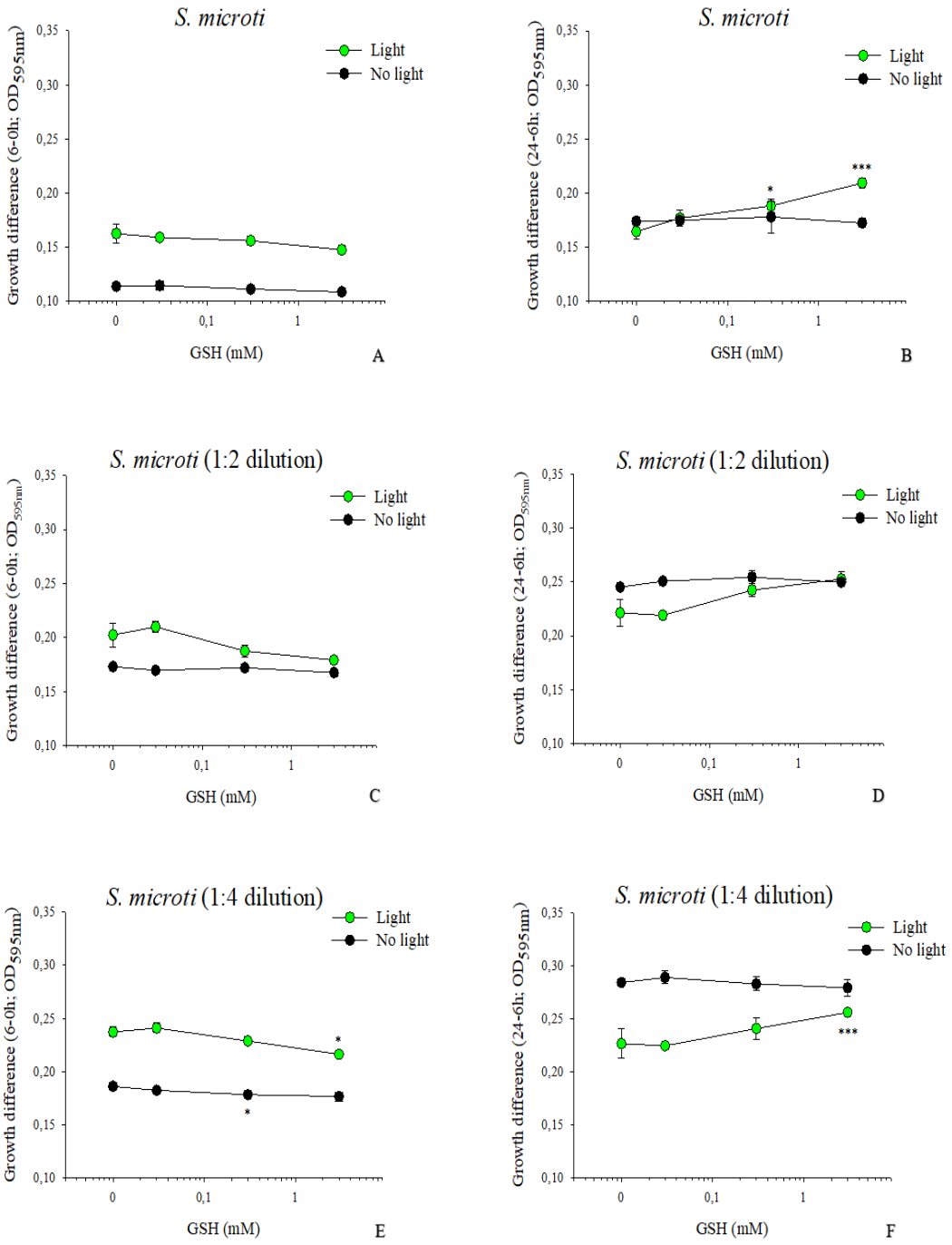

Figure 3.12 - Effect of different concentrations of GSH (0.03 mM, 0.3 mM, and 3 mM) on *Staphylococcus microti* bacterial growth in the presence and absence of the light action. Growth trend during six hours of light exposure (letters A, C, E) and at 24 hours (letters B, D, F). (* P<0.05, ** P<0.005, *** P<0.001).

3.3.5 Antimicrobial susceptibility profile

The diameter of the zone of inhibition for each of the three strains taken from exposed and unexposed plates was measured. The results of the antibiotic sensitivity tests are shown in Tables 3.1, 3.2, and 3.3 and indicate that the diameter obtained for the light-exposed strains was identical to that of the unexposed strains.

Table. 3.1 Panel of antibiotics tested on *Escherichia coli* strain exposed and unexposed to Biovitae® LEDs.

Antibiotic	µg/mL	Inhibition zone diameter range		After exposure		Non-exposure	
		R ≤	S ≥	Diameter (mm)	Interpretation	Diameter (mm)	Interpretation
Amikacin	30 µg	14	17	20	S	20	S
Amoxicillin–clavulanate	20/10 µg	13	18	15	I	15	I
Ciprofloxacin	5 µg	15	21	32	S	32	S
Erythromycin	15 µg	13	23	12	R	12	R
Imipenem	10 µg	13	16	12	R	12	R
Gentamicin	10 µg	12	15	18	S	18	S
Meropenem	10 µg	13	16	15	I	15	I
Oxytetracycline	30 µg	14	19	17	I	17	I
Streptomycin	10 µg	11	15	17	S	17	S
Sulfamethoxazole-trimethoprim	25 µg	10	16	25	S	25	S
Tetracycline	30 µg	14	19	18	I	18	I

Table. 3.2 Panel of antibiotics tested on *Staphylococcus aureus* strain exposed and unexposed to Biovitae® LEDs.

Antibiotic	µg/mL	Inhibition zone diameter range		Under exposure		Non-exposure	
		R ≤	S ≥	Diameter (mm)	Interpretation	Diameter (mm)	Interpretation
Amikacin	30 µg	14	17	18	S	18	S
Amoxicillin–clavulanate	20/10 µg	19	20	32	S	32	S
Cefoxitin	30 µg	14	18	10	R	10	R
Ciprofloxacin	5 µg	14	21	0	R	0	R
Clindamycin	2 µg	15	19	16	I	16	I
Erythromycin	15 µg	13	23	15	I	15	I
Gentamicin	10 µg	12	15	15	S	15	S
Oxacillin	1 µg	10	17	17	S	17	S
Oxytetracycline	30 µg	14	19	18	I	18	I
Penicillin	10 IU	-	29	40	S	40	S
Streptomycin	10 µg	10	15	11	I	11	I
Sulfamethoxazole-trimethoprim	25 µg	10	16	21	S	21	S
Tetracycline	30 µg	14	19	18	I	18	I

Table. 3.3 Panel of antibiotics tested on *Staphylococcus microti* strain exposed and unexposed to Biovitae® LEDs.

Antibiotic	µg/mL	Inhibition zone diameter range		Under exposure		Non-exposure	
		R ≤	S ≥	Diameter (mm)	Interpretation	Diameter (mm)	Interpretation
Amikacin	30 µg	14	17	12	R	12	R
Amoxicillin–clavulanate	20/10 µg	19	20	26	S	26	S
Cefoxitin	30 µg	14	18	19	S	19	S
Ciprofloxacin	5 µg	14	21	18	I	18	I
Clindamycin	2 µg	15	19	20	S	20	S
Erythromycin	15 µg	13	23	23	S	23	S
Gentamicin	10 µg	12	15	15	S	15	S
Oxacillin	1 µg	10	17	15	I	15	I
Oxytetracycline	30 µg	14	19	14	R	14	R
Penicillin	10 IU	-	29	18	R	18	R
Streptomycin	10 µg	10	15	13	I	13	I
Sulfamethoxazole-trimethoprim	25 µg	10	16	21	S	21	S
Tetracycline	30 µg	14	19	-	R	-	R

3.4 Discussions and conclusions

Scientific studies on visible light exposure indicate that, at certain wavelengths, it causes photodynamic inactivation (PDI) of bacterial species through an oxygen-dependent process and photostimulation of endogenous intracellular porphyrins (Maclean et al., 2008). Different porphyrins are present in bacterial cells, and bacterial susceptibility is related to the composition of porphyrins rather than the levels of individual porphyrins (Ghate et al., 2019).

In this study, we tested the photodynamic inactivation of Biovitae® LEDs, produced by the company Nextense, on three animal strains previously isolated from buffalo farms. *E. coli, S. aureus* and *S. microti* strains were exposed to the two antimicrobial light devices at different intensity values and under different experimental conditions.

Bacterial cultures grown on solid agar media and exposed to the Master light strip showed a significant ($P < 0.05$) reduction after 4 hours of exposure of 98.3% for *E. coli* strain and of 26.4% for *S. aureus* strain. Whereas the light strip action showed a reduction value of 15% for *S. microti* strain.

The light bulb produced a significant ($P < 0.05$) reduction of 29.6% in *E. coli* growth and 20.8% for *S. aureus* strain at the end of the 4-hour exposure. Whereas the light bulb action showed a reduction value of 24.4% for *S. microti* strain after 4 hours of light exposure.

Nitzan et al. (2004) studied the types and amounts of porphyrins produced after photodynamic treatment. They hypothesized that the different responses to photoinactivation of tested staphylococcal strains (*Staphylococcus aureus* and *Staphylococcus epidermidis*), other Gram-positive bacteria (*Streptococcus faecalis* and *Bacillus cereus*), and Gram-negative bacteria (*Escherichia coli*, *Acinetobacter*

baumannii, and *Aeromonas hydrophila*) might depend on the diversity and amount of produced porphyrins within the cell of each bacterial species.
The more prominent reduction in bacterial growth in *E. coli* might depend on the faster metabolism induced by a higher replication rate, leading to a higher production of reactive oxygen species (ROS) by Gram-negative strains than Gram-positive strains.
The results of the experiments using the 96-well plates and the Master light strip showed a very important mechanism that occurs during the exposure time. During Biovitae® light exposure, bacterial growth was slightly and steadily increased. However, 18 hours after exposure, there was a significant decrease in bacterial growth compared to control plates not exposed to the action of light. This mechanism could indicate that initially the bacteria take advantage of the energy received to replicate but that, subsequently, the overproduction of ROS would create considerable oxidative damage that the bacteria are unable to handle.
The results of the experiments conducted with the 96-well plates showed significant bacterial reduction for both *E. coli* strains ($P<0.05$) and *S. aureus* and *S. microti* strains ($P<0.001$) after 6 hours of exposure. The results of these experiments conducted with the 96-well plates showed slightly greater bacterial reduction for the *S. aureus* and *S. microti* strains than *E. coli* strain. The difference from previous experiments may depend on the exposure of liquid medium to light. The bactericidal efficacy of light in the liquid medium may vary among the different bacterial species and strains tested, leading to an increase or decrease in light efficacy (Hadi et al., 2020). The attenuation of irradiation, the result of absorption and scattering of light as it passes through a bacterial sample, could affect the result during the inactivation process. Maclean et al. (2009) exposed Gram-negative and Gram-positive bacteria suspensions to the inactivating action of LEDs. They reported that bacterial growth was reduced for both groups, but Gram-negative species required longer exposure times than Gram-positive species. A 300-minute exposure was required to achieve a 3.1 log10 reduction for *Escherichia coli*, whereas the highest levels of inactivation (5 log10) were recorded for *Staphylococcus strains* after exposure between 60 and 90.
Photodynamic action can bring both sub-lethal and lethal damage to bacterial colonies (McKenzie et al., 2016) due to the reaction of ROS and free radicals, consequently inducing damage to the cell itself, such as loss of cellular components, complete cell lysis, and cell death (McKenzie et al., 2016). Dithiothreitol (DTT) and Glutathione (GSH) molecules are reducing agents that protect bacterial cells from oxidative damage. In our experiments, adding these two molecules to bacterial suspensions highlighted this aspect, confirming that the cause of inactivation is due to the overproduction of ROS and the oxidative damage they are capable of causing. Indeed, both DTT and GSH limited the reduction of bacterial growth by counteracting the mechanism of action caused by porphyrins excitation and ROS

production. In contrast, bacterial growth of unexposed strains was sometimes reduced as the concentration of reducing agents increased.

A further study included the antimicrobial susceptibility of the three bacterial strains to examine any differences in the diameter of the zone of inhibition for each of the 16 tested antibiotics. Colonies from unexposed control plates and plates exposed to light for 4 hours showed no change in susceptibility to the used antibiotics. The antimicrobial susceptibility of the three strains was also tested to examine any differences in the diameter of the zone of inhibition for each of the 16 antibiotics tested. Analyzed colonies from unexposed control plates and plates exposed to light for 4 hours showed no change in susceptibility to the antibiotics used. Although previously reported that the photosensitization of bacteria does not depend on their susceptibility or resistance to antibiotics (Malik et al., 1992), subsequent studies report different results regarding antimicrobial susceptibility testing after antimicrobial light treatment. Rapacka-Zdonczyk et al. (2019) reported that *S. aureus* strains (methicillin-sensitive and methicillin-resistant) treated with blue light (411 nm; 150 J/cm^2 per cycle; 15 cycles) had, in contrast to untreated control strains, increased susceptibility to some antibiotics such as gentamicin and doxycycline, but not to other antibiotics such as vancomycin, ciprofloxacin, chloramphenicol, and rifampin. In contrast, a study by Tomb et al. (2017) tested in vitro *S. aureus* strains (methicillin-sensitive and methicillin-resistant) by performing 15 cycles of sublethal blue light exposures (405 nm; 108 J/ cm^2 per cycle). They then performed an antimicrobial susceptibility test but reported no change in the susceptibility of the strains to the panel of 10 tested antibiotics.

The results showed that Biovitae® LEDs with a clear antimicrobial action could be an optimal tool for containing pathogens, regardless of their antibiotic resistance.

The photodynamic mechanism using wavelengths in the VIS and excitation of endogenous porphyrins has several advantages, unlike previous strategies used for bacterial reduction. PDI does not produce toxic chemicals, requires only an energy-producing light source, and the possibility of causing microbial resistance is low due to its multi-target nature (Costa et al., 2011). It is hypothesized that tolerance to this procedure is unlikely because of the nonselective nature of the mechanism of action (Dai et al., 2013) and also because porphyrins possess different ways of targeting microbial pathogens (Almeida et al., 2011). Indeed, unlike antibiotics, this treatment targets various structures and components of bacterial cells, which reduces the possibility of developing resistance against such approaches (Maisch, 2015). In addition, habituation or incubation of bacterial cells with sublethal doses of PDI does not lead to the development of resistance to photo-treatment (Wozniak et al., 2018).

Photodynamic action does not harm cultured human cells (Hamblin, 2016) and the health status of the healthy population (Proykova et al., 2018), and the light devices could be used to reduce the microbial load present in environments without achieving levels of complete environmental sterility. This process would promote

mechanisms of competitive antagonism among microorganisms and maintain the immune system's resilience, preserving a continuous interaction between living things and microorganisms (De Angelis et al., 2021).
Results obtained in vitro show LEDs action and growth reduction for all three bacteria tested, even under different experimental conditions. Biovitae® devices could find application in vivo, in open environments easily contaminated by the presence of people or animals, playing an important role in the overall control of both Gram-negative and Gram-positive bacterial infections.

3.5 References

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4. Chapter 2 *In vivo* studies: use of Biovitae® lamps in a buffalo milking parlor

4.1 Introduction

Milking is an essential practice among those carried out on a dairy farm, and the milking parlor is a key area because milking takes place for a few hours a day, about twice a day, and throughout the year. In a milking parlor, it is necessary to create a suitable and hospitable environment for the animals, to carry out precise and fast interventions to limit the waiting time of the animals, to perform milking and cleaning routines, to allow the collection of quality milk from a hygienic and sanitary point of view without altering its chemical and physical characteristics, and to reduce the incidence of risks for both the milker and the animal.
Proper environmental management, cleanliness of the animals, and milking parlor, including the equipment used for milking, are crucial factors in having efficient milking and reducing the risk of intramammary infections and mastitis.
The milking time is an ideal opportunity for bacteria to enter the teat canal if environmental or pathogenic bacteria are present on the udder or teat. Animals can contaminate the milking unit and the bacteria can spread to other lactating animals during milking. Preventive measures to limit contamination mainly include proper hygiene practices, which should be carried out before milking, and can reduce the number of bacteria on the teat skin (Baumberg et al., 2016), but also the washing and disinfection of milkers' hands, cleaning of tools, and sterilization of containers.
Studies on light systems with antimicrobial effects have been conducted mainly in healthcare settings, such as hospitals and clinics (Rutala et al., 2018, Bache et al., 2012, Maclean et al., 2010), to reduce microbial growth on surfaces after disinfection and microbial load due to recontamination. Luminous devices are advantageous for several reasons: used continuously, they are not dangerous in the presence of humans and animals because the wavelengths of light do not belong to the UV region and do not create cases of photokeratitis or skin damage; they are durable devices and can illuminate consuming less energy than standard fluorescent lighting (Ghate et al., 2013; Yeh et al., 2015); they are more environmentally friendly and require less maintenance; unlike UV lights, they do not damage surfaces or materials such as plastics and polymers during repeated exposures (Yin et al., 2013).
The objective of installing Biovitae® LED lamps in the buffalo milking parlor is to help to inactivate and reduce the bacteria presence and load that can cause mastitis cases, reducing the excessive and incorrect use of antibiotics and biocides. The use of white-light LEDs would also be helpful to illuminate the milking parlor, facilitating the work of milkers during preparation, udder cleaning, and visual examination of the first jets of milk removed before the attachment of the mechanical milking group.

The study aimed to approach the knowledge of bacteria circulating in the buffalo milking parlor and among lactating animals. Composite milk samples were collected from the buffaloes to understand the health status of the udder and obtain information about the microorganisms in the milk. SCC made it possible to identify milk samples from animals with cases of infection or inflammation of the mammary gland, whereas analysis of milk composition allowed the detection of the percentages of fat, protein, and lactose present in samples from healthy or diseased animals. Swabs were taken from the surfaces of the milking parlor and the milkers' hands and nostrils to obtain more information about the environment and people working in close contact with the animals. Sampling was carried out during the first two samplings before and in the eight samplings after installing the Master light strips at the ceiling of the buffalo milking parlor.

4.2 Materials and methods

4.2.1 Ethical statement

This study was approved by the Institutional Animal Ethics Committee of the University of Naples Federico II (Italy) PG/2020/0092228 of 11/06/2020. All the dairy buffaloes sampled in this study were from a single herd and were subjected to routine milk sampling for diagnostic purposes. Milk and milking parlor surface samples were collected with the owner’s permission, and written informed consent was required.

4.2.2 Lighting design and installation of Biovitae® lamps in a buffalo milking parlor

The operation of a milking parlor is regulated by ad hoc laws necessary to create safe conditions for operators and animals. Situations such as traumatic contact with the animal, slips or falls, and contact with biological materials can occur, which must be mitigated by proper lighting. The lighting design was carried out to verify that the lighting complied with EN-12464-1 regulation. The norms are summarized in Table 4.1, and the data are expressed in lux. Lux is the unit of illuminance measurement accepted by the International System. The milking parlor, which had dimensions of 30 m^2 and a height of 4 m, was illuminated with 6 Biovitae® Master light 120 cm lighting devices manufactured by the company Nextsense.

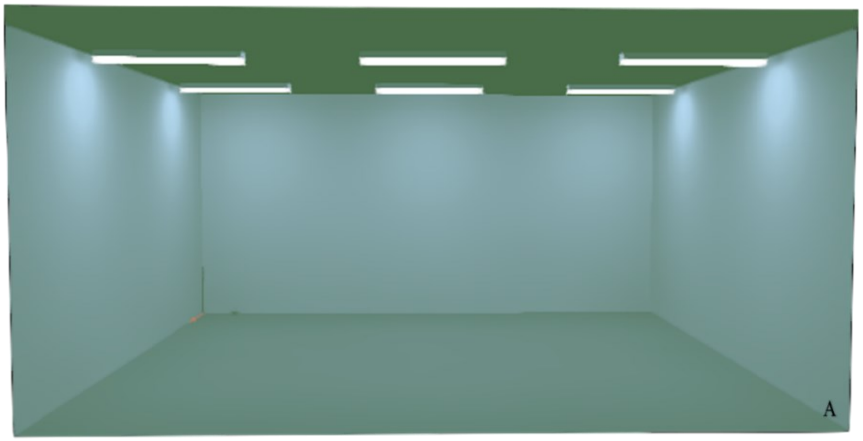
Table 4.1 - Norms regulating lighting design.

Type of room	Maintained average illuminance (lx)	Glare	Heat yield
Sick animal housing and calving rooms.	200	25	80
Loading and handling of stock, processing with machinery and tools.	200	25	80
Feed preparation, milking, tool washing	200	25	80

Table 4.2 - Lighting data of lamps installed in the buffalo milking parlor.

Input Voltage	230 Vac – 50/60 Hz
Input Wattage	43 W
CCT	6425 °K
CRI	83
PPFD @ 100 cm	23.05 μmol m-2 s-1
PPFD @ 400 cm	1.48 μmol m-2 s-1
400-420 @ 400cm	0.0364 W m-2
400-450 @ 400cm	0.0908 W m-2
400-700 @ 400cm	0.3344 W m-2
380-780 @ 400cm	0.3434 W m-2

Fig. 4.1 - Biovitae® Master light.



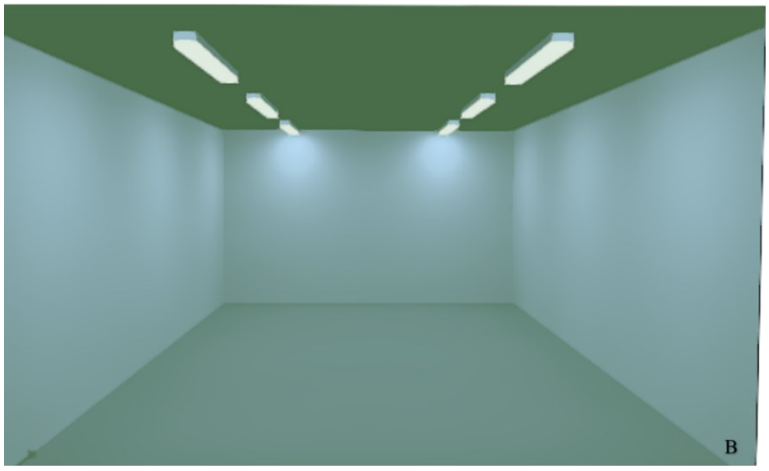
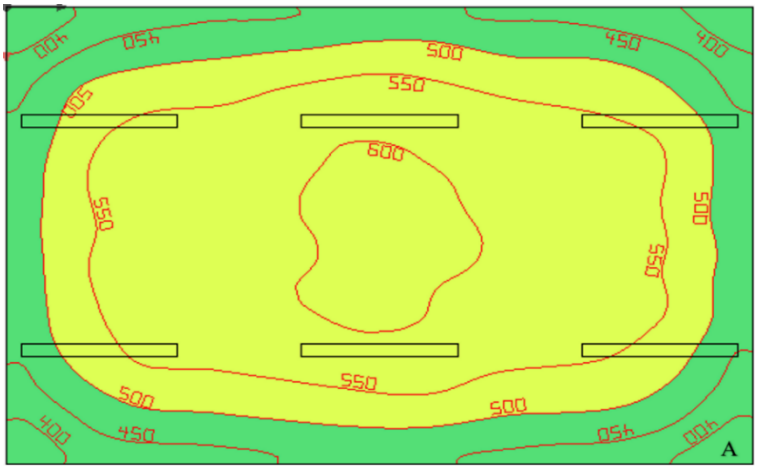


Fig. 4.2 (A-B) - Milking parlor typological image.



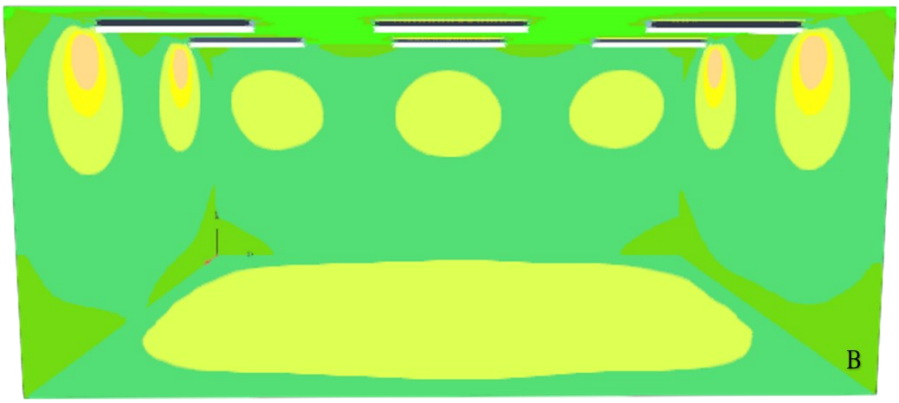


Fig. 4.3 (A-B) - Surface lux of milking parlor. The yellow zone represents 500 lux, the blue zone 450 lux while the green zone 400 lux.

4.2.3 Sample collection

The chosen buffalo herd had about 100 lactating animals, 3-9 years old, which were milked twice a day in a tandem milking parlor. On ten occasions, 200 composite milk samples were collected aseptically over a sampling period from June 2020 to November 2021. Two samplings were performed before the installation of the Biovitae® lamps at the ceiling of the milking parlor, and the other eight samplings after their installation.

For the microbiological safety part, an on/off protocol of the light sources with Biovitae® technology was applied. The protocol included in the two daily milking phases, the lamps were turned on 1 hour before the start of milking operations, were kept on for about the next 2 hours (average milking time of all lactating animals) and left on for the next 2 hours. The Biovitae® light was turned off during the period when the eighth and ninth samplings were done, and a standard LED device was used to illuminate the milking parlor. During this period, two samplings were conducted to study any changes in the microbial population after prolonged exposure to the antimicrobial light and then without exposure. Subsequently, light activity was restored, and a final (tenth) sampling was performed after one month to assess any changes that had occurred since the restart of antimicrobial activity.

For each sampling, 20 buffaloes in different lactation stages were haphazardly selected. The 200 milk samples were collected from 120 different animals; among them, 68 animals were sampled once, and 52 animals two or more times during the study. During the period, the breeder did not observe clinical signs of mastitis in any of the sampled buffaloes, and no antibiotic therapy was administered.

Two composite milk samples were collected from each buffalo during evening milking, after routine pre-milking cleaning and discharging the first stream of foremilk to minimize chances of sample contamination from bacteria in the teat end. The samples were placed in two sterile tubes (Thermo Fisher Scientific, Str. Rivoltana, Km 4 - 20090 Rodano, Milan, Italy): a total of 50 mL was collected for somatic cell count (SCC) and 15 mL for bacterial culture. The milk samples were kept at 4 °C and transported to the laboratory within 24 h.

In addition, 104 swabs were collected from the milking parlor surfaces (milking boxes, teatcups of the milking unit and room walls), sampling ca. 10cm^2 areas before and after the milking time of sampled animals, and the two workers' hands and nostrils. Each swab was placed in Stuart W/O CH transport medium (Aptaca Spa, Asti, Italy) and transferred within 24 h to the laboratory for bacteriological examination.

The cleanliness of the animals and the milking parlor was assessed for each occasion before sampling to get an indication of cleanliness before milking. Precisely, the body parts of animals (mainly the upper rear limb, the lower rear limb, the udder side, the ventral abdomen, the hind udder, and the tail head) were evaluated following a scheme with a 4-point scale: 1 = clean, 2 = some spot of dirt,

3 = dirty, or 4 = very dirty with encrusted dirt (Ruud et al., 2010), and the animals' score was calculated using the mean of the scores of the different animals.
The milking parlor cleanliness was scored on each occasion with a 3-point scale: 1 = clean, 2 = slightly dirty, and 3 = dirty.

4.2.4 Bacteriological examination and identification

All bacteriological examinations were performed at the Microbiology Laboratory of the Department of Veterinary Medicine and Animal Production of the University of Naples "Federico II". According to the standards of the National Mastitis Council (NMC, 2017), ten microliters of all milk samples were spread on each of the following plates: Mac Conkey Agar, 5% Columbia Sheep Blood agar, Mannitol Salt Agar, and Saboraud Dextrose Agar (Oxoid, Milan, Italy). Following an internal protocol, 50 µL of each milk sample were diluted in 1 mL of buffered peptone water, and 200 µL of inoculum were spread on the plates to perform the CFU/mL. The same plates were also used for the collected swabs from the surfaces and the workers. All plates inoculated with the samples were then aerobically incubated at 37°C for 24–48 hours in aerobic conditions. The isolation of 1 to 3 different colony types and ≥500 CFU/mL for each colony type was considered as a positive sample, whereas milk samples for which more than 3 colony types and/or <500 CFU/mL colonies of any bacterial colony was considered as a contaminated sample. Single colonies were subcultured on their respective culture media to obtain pure cultures using the same incubation conditions.
After 24 hours, the colonies of a fresh overnight culture were subsequently identified by matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF MS) (Bruker Daltonics Inc., Germany). A small amount of biological material from a single colony was spread using a toothpick on a spot on the MALDI target plate. When the biological material was dry, it was coated with 1 µL of matrix solution (a-cyano-4-hydroxycinnamic acid diluted in 50% acetonitrile and 2.5% trifluoroacetic acid) and allowed to dry at room temperature. The target plate was then loaded onto a Biotyper Bruker MALDI-TOF and identified by comparing the protein detection pattern in the mass spectrum with the reference pattern in the database. Score values below 1.7 indicated a non-reliable identification, between 1.7 and 1.99 a probable genus identification, and equal or above 2.0 a certain genus identification and probable or highly probable species identification. A bacterial test standard (BTS) (Bruker Daltonics, Germany) was used as a calibrator for quality control.

4.2.5 Antimicrobial Susceptibility Testing

The 14 *Streptococcus agalactiae* strains isolated from milk samples were assessed for *in vitro* antimicrobial susceptibility testing based on the Kirby–Bauer method and the inoculated Muller–Hinton agar plates were incubated at 37 °C for 24 h in an aerobic atmosphere. The following panel of antibiotics were tested: amoxicillin–clavulanate (AMC, disk content: 20/10 µg), amikacin (AK, disk content: 30 µg), cefoxitin (FOX, disk content: 30 µg), clindamycin (CD, disk content: 2 µg), ciprofloxacin (CIP, disk content: 5 µg), erythromycin (E, disk content: 15 µg), gentamicin (CN, disk content: 10 µg), kanamycin (K, disk content: 30 µg), oxacillin (OX, disk content: 1 µg), oxytetracycline (T, disk content: 30 µg), penicillin (P, disk content: 10 IU), streptomycin (S, disk content: 10 µg), sulfamethoxazole–trimethoprim (SXT, disk content: 25 µg) and tetracycline (TE, disk content: 30 µg). The chosen antibiotics belonged to eight classes: Aminoglycosides, Cephalosporins, Lincosamides, Macrolides, Penicillins, Quinolones, Sulfonamides, and Tetracyclines. The isolates were classified as susceptible (S), intermediate (I) or resistant (R) according to the Clinical and Laboratory Standards Institute (CLSI, 2015) and to the European Committee on Antimicrobial Susceptibility Testing (EUCAST, 2018) guidelines.

4.2.6 Somatic cell count (SCC) and milk component analysis

The SCC was performed using a NucleoCounter® SCC-100™ (ChemoMetec Inc. 8950 Villa La Jolla Drive – Suite A127 La Jolla, CA, USA) at the Istituto Zooprofilattico Sperimentale del Mezzogiorno (Portici - Naples, Italy), with a measurement range of 1×10^4 to 200×10^4 SCC/mL. The milk composition was analyzed using a Milkoscan FT120 (Foss Electric A/S, Denmark), based on Fourier transform infrared (FTIR) spectra, to measure the fat, protein, and lactose content.
Bacteriologically negative milk samples with $<200\times10^3$ cells/mL were considered healthy (H). Differently, bacteriologically positive milk samples were categorized in three groups considered diseased: samples with IMI presenting SCC $<200\times10^3$ cells/mL; samples with SCM presenting SCC values $>200\times10^3$ cells/mL in the absence of clinical signs; samples with CM presenting SCC values $>200\times10^3$ cells/mL in the presence of clinical signs.
A possible correlation between increased SCC and change in milk composition was investigated.

4.2.7 Statistical analysis

Collected data were evaluated graphically using Microsoft Office Excel (version 2211, Microsoft Corporation, USA) and statistically using SigmaPlot software (version 11.0, Systat Software, Inc. Germany). The obtained data from experiments were compared statistically with Student t-test and two-tailed Fisher's exact test.

4.3 Results

4.3.1 Identification of bacterial strains isolated from milk samples

The 200 analyzed milk samples were from a total of 120 animals haphazardly chosen during the ten samplings: 68 animals were sampled once (56.7%), 33 animals twice (27.5%), 11 animals three times (9.20%), 7 animals four times (5.80%) and 1 animal five times (0.80%).

Microbiological analysis of collected milk samples showed bacterial counts of Gram-negative ranging from 2 x 10^2 to 2.4 x 10^3 CFU/mL. Analysis of Gram-positive bacteria showed a bacterial count ranging between 6 x 10^2 and 4.1 x 10^3 CFU/mL and revealed a more fluctuating trend than Gram-negative bacteria during all sampling (Fig. 4.4). In addition, Gram-negative and Gram-positive bacterial load was evaluated for the two samplings in which the action of Biovitae® light was stopped (eighth and ninth) by comparing them with the samplings performed before and after the interruption. Fisher's two-tailed exact test showed a statistically significant value ($P< 0.05$) for bacterial load grown in the absence of light.

The number of bacterial genera identified is mainly related to the cleanliness conditions of the animals and the period when sampling was conducted. The number of bacterial genera detected is almost always higher in autumn and winter seasons, with wet and rainy periods, and in cases where cleanliness is not optimal (Table 4.4).

Bacteriological examination was positive for 144 out of 200 samples (72%), and none were identified as contaminated samples because for all positive samples 1-3 different colony types and ≥500 CFU/mL for each colony type were isolated. 32.3% (82/254) of identified bacterial species were Gram-negative, and 67.7% (172/254) were Gram-positive. The most common isolated bacterial species and genus from milk samples were non-*aureus* staphylococci (NAS; *S. cohnii, S. chromogenes, S. epidermidis, S. microti, S. saprophyticus, S. sciuri, S. simulans, S. xylosus*) (25.6%, 65/254), *Escherichia coli* (18.9%, 48/254), *Aerococcus viridans* (16.5%, 42/254), *Streptococcus* spp (8.3%, 21/254), *Bacillus* spp (5.9%, 15/254) (Fig. 4.2). The classification of isolated strains in contagious and environmental bacteria is shown in Table 4.5.

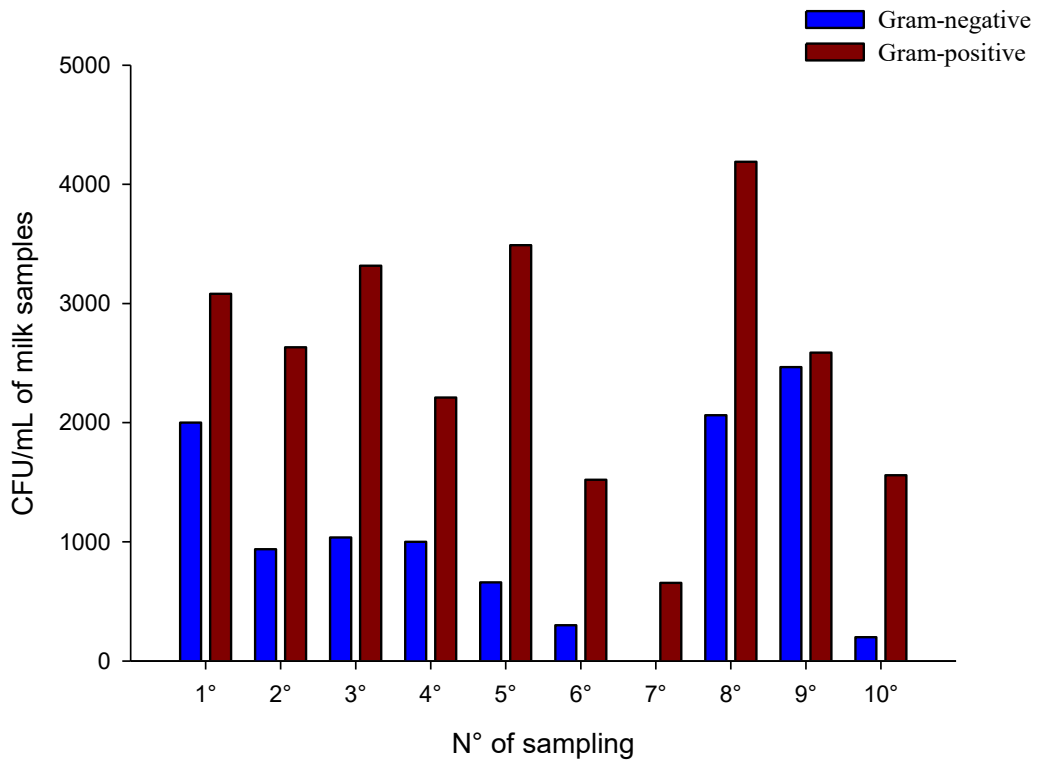


Figure 4.4 - Mean CFU/mL of Gram-negative and Gram-positive bacteria isolated from milk samples collected during the ten samplings (the first two before and the other eight after the installation of the Biovitae® Master light strip.

Table 4.3 - The number of Gram-negative and Gram-positive bacteria and Mycetes genera identified from the samples in relation to the sampling month, and animal and milking parlor cleanliness conditions. The value of the animal cleanliness rating was expressed as an average and evaluated using a a 4-point scale: 1 = clean, 2 = some spot of dirt, 3 = dirty, or 4 = very dirty with encrusted dirt. The milking parlor cleanliness was scored with a 3-point scale: 1 = clean, 2 = slightly dirty, and 3 = dirty.

N° sampling/ month	**Gram-negative**	**Gram-positive**	**Mycetes**	**Gram-negative**	**Gram-positive**	**Mycetes**	**Gram-negative**	**Gram-positive**	**Mycetes**	**Animal cleanliness score**	**Milking parlor cleanliness score**
	Milk samples	Milk samples	Milk samples	Surfaces swabs	Surfaces swabs	Surfaces swabs	Workers' swabs	Workers' swabs	Workers' swabs	(score 1 to 4)	(score 1 to 3)
1°- June		3		5	3	1	2	3		1	1
2°- July	7	3		6	1	1	3	2	1	2	1
3°- Nov	2	5		1	1		1	3		3	2
4°- Nov	1	8		3	1		1	3		3	1
5°- Dec	1	6		1	4		1	6		3	2
6°- Dec	1	2		0	4		0	3		3	1
7°- Jan	2	5		1	5		0	2		2	2
8°- May	2	7		6	8		3	3		1	2
9°- Oct	2	6		3	8	1	2	2		2	2
10°-Nov	1	2		3	2		1	2		2	1

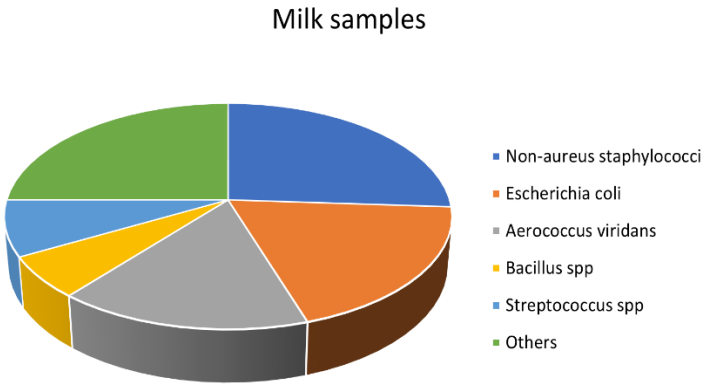


Figure 4.5- Prevalence of identified bacteria from milk samples.

Table 4.4 - Groups of contagious and environmental bacteria isolated from buffalo milk samples.

Contagious	**n°**	**%**
Streptococcus agalactiae	14	5,50%
Staphylococcus aureus	0	
Corynebacterium bovis	0	
Environmental	**n°**	**%**
Non-*aureus Staphylococci*	65	25,60%
Escherichia coli	48	18,90%
Streptococcus uberis	6	2,40%
Enterococcus spp	3	1,20%
Pseudomonas spp	2	0,80%
Streptococcus dysgalactiae	1	0,40%
Klebsiella spp	1	0,40%

4.3.2 Identification of bacterial strains isolated from swabs

Microbiological analysis of collected milking parlor surface swabs showed Gram-negative bacterial counts ranging from 3 to 9 x 10 CFU/10 cm^2 before milking. Before milking, Gram-positives bacterial counts ranged from 2 to 1 x 10^2 CFU/10cm^2. After milking, Gram-negative bacteria counts ranged from 4 to 1 x 10^2 CFU/10cm^2 and Gram-positive bacterial counts ranged from 2 to 1 x 10^2 CFU/10cm^2. Figures 4.6 and 4.7 show the clear increase in CFU/cm2 of Gram-negative and Gram-positive bacteria on the sampled surfaces after milking operations. In addition, microbiological analysis of samples collected when the milking parlor was not subjected to bactericidal light activity (eighth and ninth sampling) showed increased bacterial load and isolated bacterial genera and species. In particular, there was an increase in Gram-negative bacteria, which had been considerably reduced in the previous sampling during the light exposure.

Bacteriological analysis was positive for 76 out of 104 milking parlor surface swabs (73%). 47.9% (66/138) of the identified bacterial species were Gram-negative strains, 48.5% (67/138) were Gram-positive strains, and 3.6% (5/138) were Mycetes. The most common isolated bacterial species and genus from samples were: *Escherichia coli* (22.5%, 31/138), *Aerococcus viridans* (12.3%, 17/138), *Bacillus* spp (10.1%, 14/138), *Acinetobacter* spp (8.7%, 12/138), *Rothia* spp (8%, 11/138) and non-*aureus* staphylococci (5.8%, 8/138).

Bacillus spp (17%, 9/53), *Escherichia coli, Aerococcus viridans*, and non-*aureus* staphylococci (*Staphylococcus microti*, *Staphylococcus sciuri* and *Staphylococcus chromogenes*) (11.3%, 6/53), *Rothia* spp and *Acinetobacter* spp (9.4%, 5/85) were the bacteria most identified from 53 isolated strains of surface swabs collected before milking (Fig. 4.8).

Escherichia coli (29.4%, 25/85), *Aerococcus viridans* (12.9%, 11/85), *Acinetobacter* spp (9.2%, 7/85), *Rothia* spp (7%, 6/85), *Aeromonas* spp (5.9%, 5/85) and *Bacillus licheniformis* (5.9%, 5/85) on the other hand, were the bacteria most identified from 85 isolated bacteria of surface swabs collected after milking (Fig. 4.9).

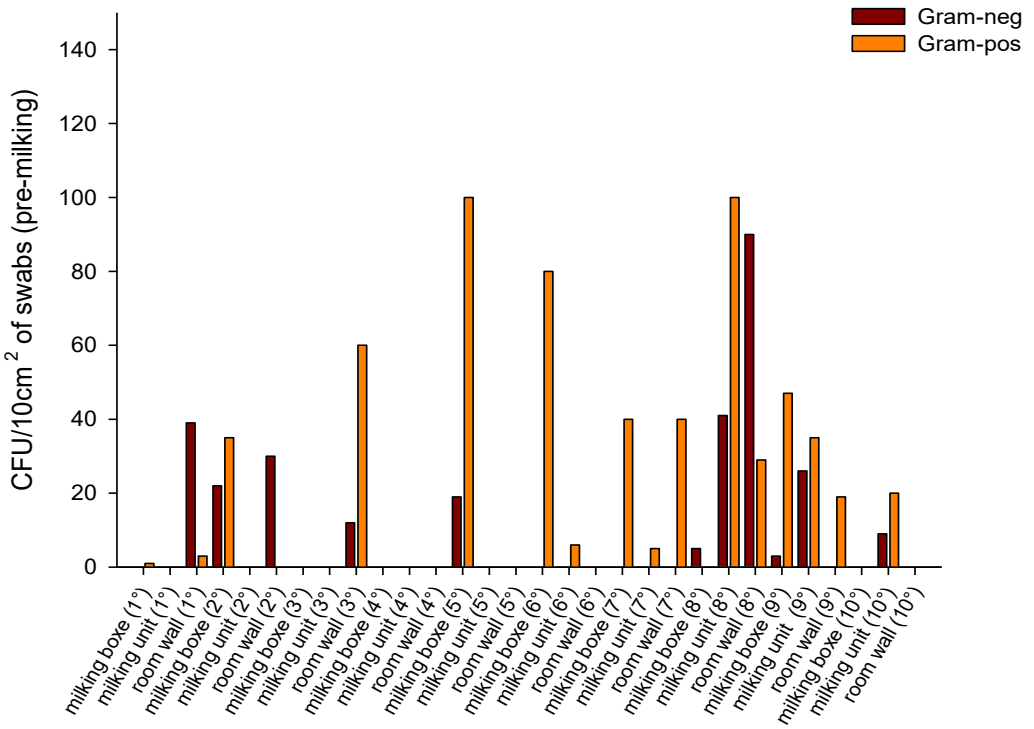


Figure 4.6- CFU/cm10^2 of Gram-negative and Gram-positive bacteria isolated from surface swabs (milking boxes, milking units and room walls) collected before the milking.

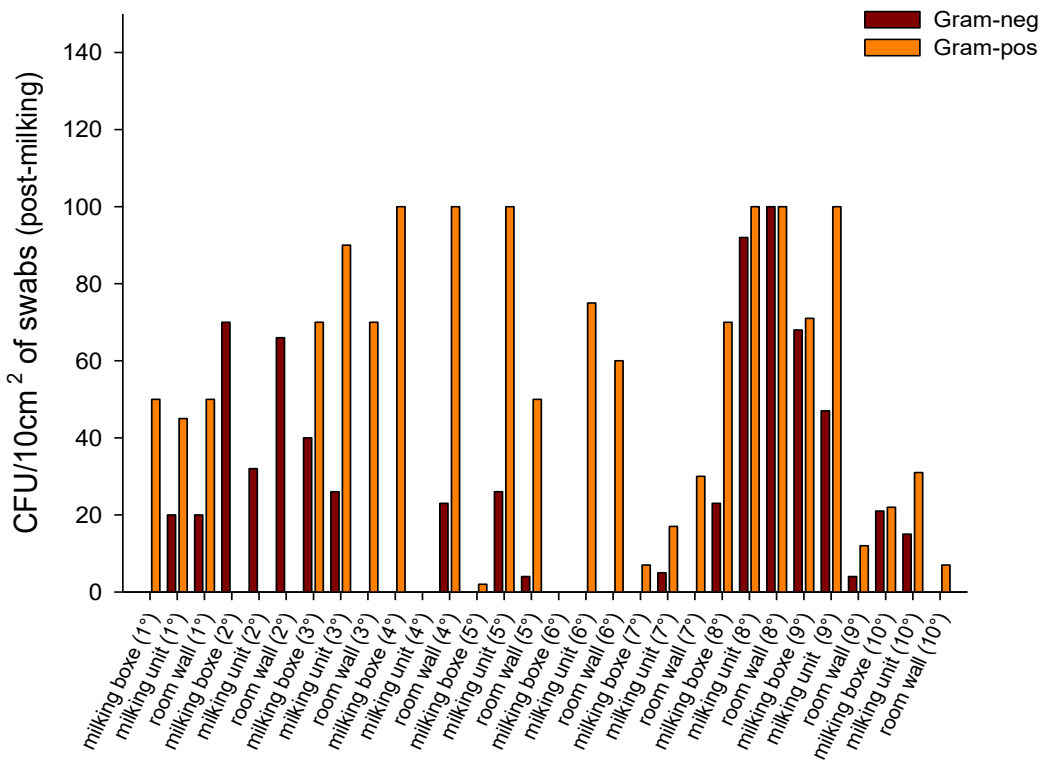


Figure 4.7 - CFU/cm10^2 of Gram-negative and Gram-positive bacteria isolated from surface swabs (milking boxes, milking units and room walls) collected after the milking.

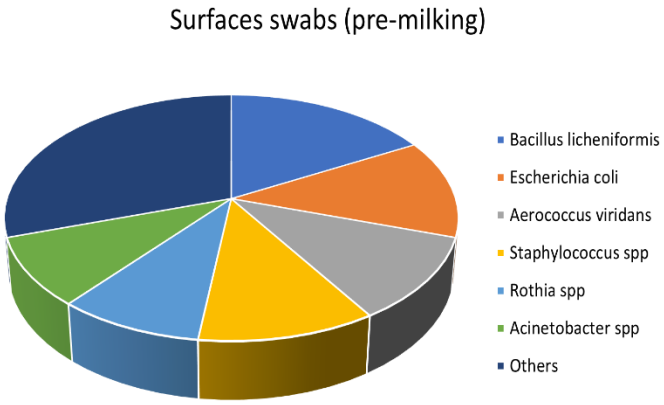


Figure 4.8- Prevalence of identified bacteria from surface swabs (pre-milking).

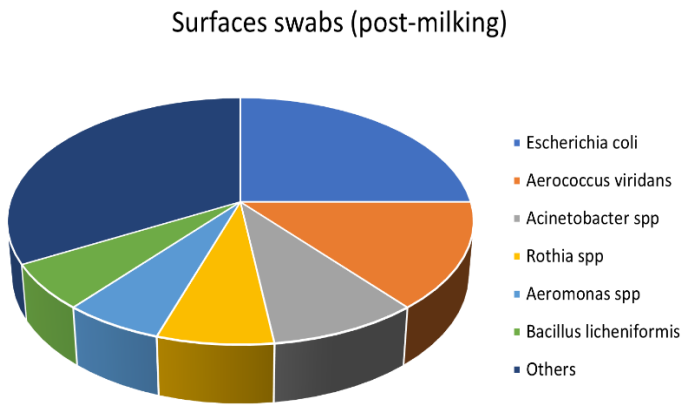


Figure 4.9- Prevalence of identified bacteria from surface swabs (post-milking).

Bacteriological examination was positive for 55 out of 60 swabs of workers' hands and nostrils (91,6%).

The identified bacterial species from worker's hands were Gram-negative strains (29.2%, 19/65), Gram-positive strains (67,7%, 44/65) and Mycetes (3.1% (2/65). The most common bacteria isolated were *Rothia amarae* (37%, 24/65), *Escherichia coli* (13.8%, 9/65), non-*aureus* staphylococci (10.8%, 7/65), *Bacillus licheniformis* (7.7%, 5/65) and *Aerococcus viridans* (6.1%, 4/65) (Fig. 4.10).

Bacteriological examination of workers' nostril swabs showed that all the identified bacterial species were Gram-positive strains. Isolated bacteria strains were *Staphylococcus epidermidis* (83.3%, 15/18), *Staphylococcus aureus* (11.1%, 2/18) and *Bacillus licheniformis* (5.6%, 1/18) (Fig. 4.11).

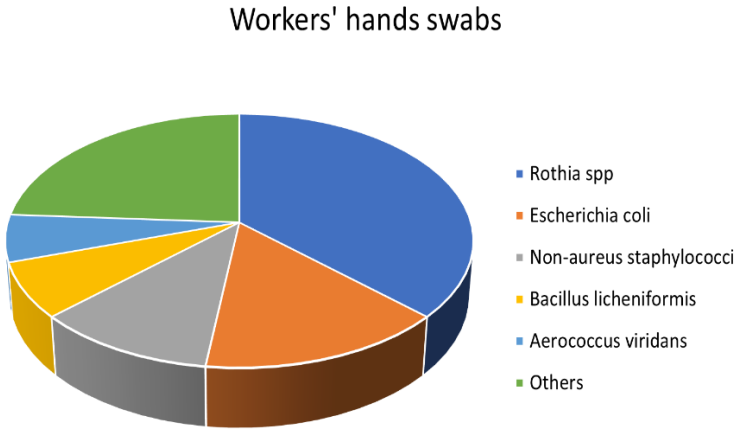


Figure 4.10- Prevalence of identified bacteria from workers' hands swabs.

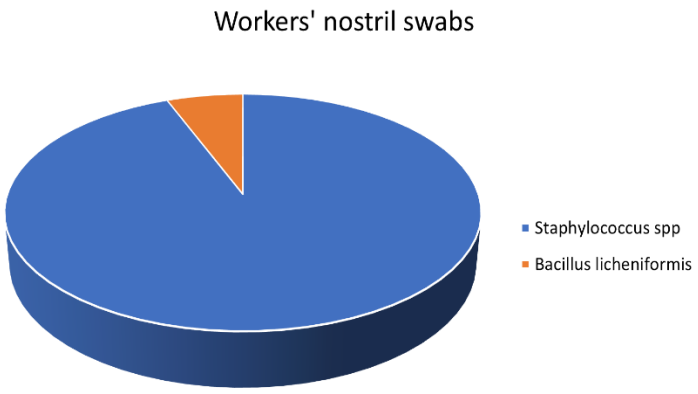


Figure 4.11- Prevalence of identified bacteria from workers' nostrils swabs.

4.3.3 Phenotypic profiles of antimicrobial resistance

The antimicrobial susceptibility testing of *Streptococcus agalactiae* (*S. agalactiae*) strains results highlighted a complete resistance to both cefoxitin and oxacillin (100%) for all 14 *S. agalactiae* isolated strains, and a high resistance value toward gentamicin (92.8%, 13/14), amikacin (85.7%, 12/14), streptomycin (78.6%, 11/14) and kanamycin (71.4%, 10/14). All strains were sensitive to penicillin (100%). High levels of antimicrobial susceptibility were found in many antibiotics tested: ciprofloxacin, clindamycin and amoxicillin-clavulanate (71.4%, 10/14), erythromycin, tetracycline and oxytetracycline (64.3%, 9/14), and sulfamethoxazole-trimethoprim (35.7%, 5/14) (Fig. 4.12).

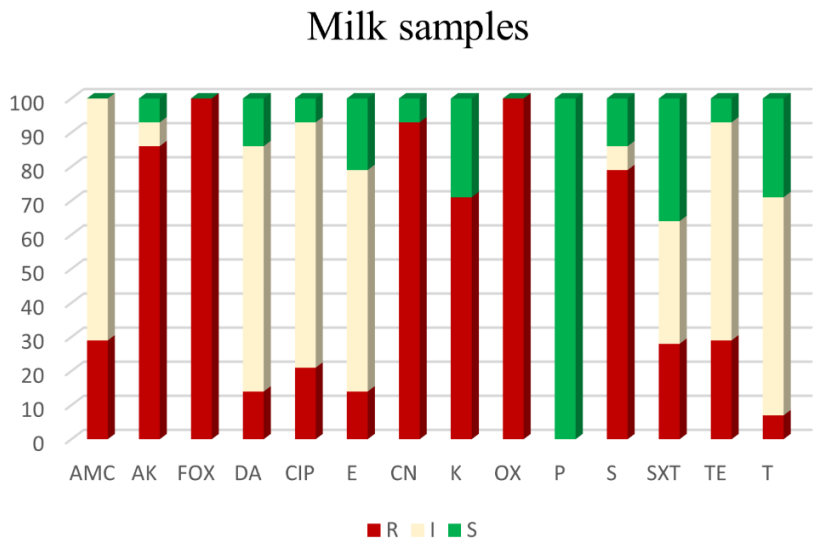


Figure 4.12 - Antibiotic resistance profiles of 14 *Streptococcus agalactiae* isolates. Tested antibiotics: amoxicillin–clavulanate (AMC), amikacin (AK), cefoxitin (FOX), clindamycin (CD), ciprofloxacin (CIP), erythromycin (E), gentamicin (CN), kanamycin (K), oxacillin (OX), penicillin (P), sulfamethoxazole–trimethoprim (SXT), tetracycline (TE), oxytetracycline (T) and streptomycin (S).

4.3.4 Relation between SCC values and SCM/IMI cases

SCC results in relation to the bacteriological analysis of milk samples showed that 54 of the 200 samples (27%) were defined as 'healthy', having an SCC < 200×10^3 cells/mL and no bacterial growth; 124 samples (62%) were classified as IMI, having an SCC < 200×10^3 cells/mL and bacterial growth; whereas 22 samples (11%) presented SCC values > 200×10^3 cells/mL and bacterial growth compatible with the definition of SCM.

Furthermore, the two-tailed Fisher's exact test evidenced a value statistically significant with $p < 0.05$ between the two dependent variables: bacterial growth (presence or absence) and values of SCC ($\leq200\times10^3$ cells/mL or $>200\times10^3$ cells/mL) in milk samples, as reported in Table 4.5.

Aeroccus viridans (21%), non-*aureus* staphylococci (18.4%), *Streptococcus agalactiae* and *Escherichia coli* (15, 8%), *Aeromonas hydrophila* and *Rothia* spp (8%), *Citrobacter freundii*, *Acinetobacter johnsonii*, *Pseudomonas aeruginosa, Corynebacterium xerosis* and *Lactococcus lactis* (2.6%) were the bacteria strains identified and associated with the 22 SCM cases (Table 4.6). Because the animals were chosen randomly, it was possible to monitor 13 of them that were sampled more than once. Specifically, animal "8922" was sampled 4 times: *Escherichia* coli strains were identified in 3 of 4 milk samples and on 2 occasions in samples with high SCC. Whereas milk collected from animal "7985" had elevated SCC in 3 out of 4 samples, always associated with the presence of *Streptococcus agalactiae.*

Antimicrobial susceptibility tests were performed for *Escherichia coli* strains (reported in Chapter 4) and *Streptococcus agalactiae* strains associated with SCM cases. The results showed that the phenotypic resistance profiles were different for both 2 strains of *E. coli* and 3 strains of *S. agalactiae*, suggesting that the lineage was not the same and different strains caused the mammary inflammations.

Table 4.5 - Bacterial culture results and somatic cell counts (SCCs) in buffalo milk samples (***** H (healthy), SCM (subclinical mastitis) and IMI (intramammary infection).

Bacterial Culture	SCC Values (cell/mL)	Status *	No. of Samples/ 200 Samples	%	Fisher's Two-Tailed
No bacterial growth	SCC ≤ 200.000	H	54	27.0%	
	SCC > 200.000	SCM	0	0%	$p < 0.05$
Bacterial growth	SCC ≤ 200.000	IMI	124	62%	
	SCC > 200.000	SCM	22	11%	$p < 0.05$

Table 4.6 - Bacterial species and MALDI-TOF scores in 22 buffalo milk samples showing high somatic cells counts (SCC) compatible with the definition of subclinical mastitis (SCM).

Sample	SCC/mL	Identified Bacterial Strains (Colony Forming Unit, CFU)	MALDI-TOF Score
1	2×10^5	*Aeromonas hydrophila* (3000 CFU/mL)	2.11
2	2.9×10^5	*Acinetobacter johnsonii* (5000 CFU/mL)	2.26
		Staphylococcus simulans (1000 CFU/mL)	2.20
3	2.5×10^5	*Staphylococcus microti* (10000 CFU/mL)	2.07
		Citrobacter freundii (10000 CFU/mL)	1.96
4	2.8×10^5	*Escherichia coli* (5000 CFU/mL)	2.09
		Staphylococcus simulans (1000 CFU/mL)	2.20
		Staphylococcus sciuri (500 CFU/mL)	2.09
5	2×10^6	*Streptococcus agalactiae* (6000 CFU/mL)	2.10
		Lactococcus lactis (1100 CFU/mL)	2.17
		Aeromonas hydrophila (5000 CFU/mL)	1.88
6	2.7×10^5	*Aeromonas hydrophila* (3000 CFU/mL)	2.12
7	5.3×10^5	*Streptococcus agalactiae* (10000 CFU/mL)	2.27
		Corynebacterium xerosis (1000 CFU/mL)	2.04
8	3.6×10^5	*Staphylococcus microti* (3000 CFU/mL)	2.04
		Aerococcus viridans (2000 CFU/mL)	1.79
		Rothia endophytica (200 CFU/mL)	1.91
9	2.9×10^5	*Aerococcus viridans* (2000 CFU/mL)	1.81
10	2.7×10^5	*Staphylococcus microti* (4000 CFU/mL)	2.04
11	2.3×10^5	*Escherichia coli* (4000 CFU/mL)	2.16
12	2.9×10^5	*Pseudomonas aeruginosa* (4000 CFU/mL)	2.34
13	2.2×10^5	*Streptococcus agalactiae* (3000 CFU/mL)	2.43
14	2.5×10^5	*Rothia amarae* (6000 CFU/mL)	2.05
15	5.1×10^5	*Escherichia coli* (2800 CFU/mL)	2.22
		Rothia amarae (500 CFU/mL)	1.94
		Aerococcus viridans (700 CFU/mL)	1.82
16	2.9×10^5	*Escherichia coli* (400 CFU/mL)	2.14
		Streptococcus agalactiae (5400 CFU/mL)	2.29
		Aerococcus viridans (200 CFU/mL)	1.99
17	1.5×10^6	*Escherichia coli* (2000 CFU/mL)	2.17
		Aerococcus viridans (1100 CFU/mL)	2.14
18	8.3×10^5	*Streptococcus agalactiae* (5000 CFU/mL)	2.19
19	2.1×10^5	*Staphylococcus microti* (200 CFU/mL)	2.06
		Aerococcus viridans (100 CFU/mL)	1.89
		Escherichia coli (500 CFU/mL)	2.31
20	4.5×10^5	*Aerococcus viridans* (400 CFU/mL)	1.90
21	8.8×10^5	*Streptococcus agalactiae* (4700 CFU/mL)	2.04
22	2.3×10^5	*Aerococcus viridans* (4800 CFU/mL)	1.99

4.3.5 Analysis of the major constituents of milk samples

Data on the nutritional composition of almost all milk samples showed optimal values: an average fat content of 7.0 g/100 g, an average protein content of 4.2 g/100 g and an average lactose content of 4.1 g/100 g. Only one milk sample, exhibiting a positive bacteriological examination with the growth of *Staphylococcus microti* and *Citrobacter freundii* and an SCC > 200×10^3 cells/mL, showed a slight decrease in the value of the tested milk constituents (i.e., fat, protein and lactose).

4.4 Discussions and conclusions

Previous *in vivo* studies on light systems with antimicrobial effects have been conducted mainly in healthcare settings, such as hospitals and clinics (Rutala et al., 2018, Bache et al., 2012, Maclean et al., 2010), to reduce the microbial growth on surfaces after disinfection and the microbial load due to recontamination.
Challenges to counter bacterial infections are present not only in the healthcare environment but also in the dairy industry because bacteria cause udder infections in dairy animals, altering their health status and the quality and quantity of produced milk.
In this study, the action of antimicrobial lights, after an *in vitro* evaluation, was tested *in vivo* to act on microorganisms in the milking parlor environment of a buffalo farm. The Master light strips effect installed at the ceiling of the milking parlor was monitored by performing ten samplings. Analysis of the collected samples (milk samples, surface swabs and workers' hands and nostrils swabs) provided indications about the bacterial species and genera and their load in the environment. The study also considered the effect that turning off the antimicrobial light and using a typical lighting source (eighth and ninth sampling) had on the microorganisms.
Milk is a rich and nutritious product with its own endogenous microbiota, but it can be contaminated by external and different bacteria that result from penetration of the teat canal, udder skin, milking tools or tanks used for storage and preservation (Addis et al., 2016). The microbial load of milk from healthy animals would be slightly above the value of 10^3 CFU/mL at the time of release (Kurweil and Busse, 1973), might contain more than 10^5 CFU/mL in the case of animals with inadequately cleaned udders and teats before milking (Bramley and McKinnon, 1990), and increase significantly in cases of mastitis. CFU/mL counts of collected milk samples ranged from 2 x 10^2 to 2.4 x 10^3 CFU/mL for Gram-negative bacteria and from 6 x 10^2 to 4.1 x 10^3 CFU/mL for Gram-positive bacteria. The values obtained were below the total bacterial count limits (at 30°C) set by European guidelines (Regulation (EC) Nº. 853/2004), which indicate a range of 5 x 10^5 to 1.5 x 10^6 CFU/mL for raw milk from species other than bovine (European Parliament, 2004).
An interesting study by Vacheyrou et al. (2011) analyzed the presence and the transfer of bacteria from the environment to raw milk, finding that most of the environmental bacteria isolated from milk samples were also present in the barn and in the milking parlor. The most bacteria isolated from our milk samples were environmental and opportunistic bacteria: non-*aureus* staphylococci (25.6%), *Escherichia coli* (18.9%) and *Aerococcus viridans* (16.5%). Only 5.5 % of the isolates were contagious bacteria, represented by *Streptococcus agalactiae* strains. This finding can be related to the cleanliness conditions of the milking parlor and animals observed during the sampling occasions. In fact, the number of isolated

bacterial genera and bacterial load was usually directly related to environmental and animal cleanliness conditions and sampling periods. Teat and udder cleaning and sanitization practices before milking reduce the possibility that environmental bacteria can be isolated from the milk, altering its quality and sanitation, and entering the udder can cause mastitis (Berge and Baars, 2020, Baumberg et al., 2016).

The Mediterranean buffalo (*Bubalus bubalis*) has generally been considered less susceptible to mastitis than cows. The reason is related to the morphological difference of the teat canal and sphincter, which would reduce the possibility of the pathogenic bacterium invading the udder (Fagiolo and Lai, 2007). However, mastitis is also common in the buffalo dairy industry and has a negative impact on the buffaloes' health and the industry's economy.

Somatic cell count (SCC) of raw milk samples is an essential method to monitor animal health and detect the presence of clinical or subclinical mastitis cases. SCC analysis of the 200 milk samples associated with bacteriological examination indicated the absence of clinical mastitis (no samples showing visible clinical signs, SCC values > 200×10^3 cells/mL and bacterial growth) and the presence of subclinical mastitis in 11% of the cases (SCC values > 200×10^3 cells/mL and bacterial growth). Our SCM rate agrees with the values between 5% and 20% reported by Joshi and Gokhale (2006), whereas Srinivasan et al. (2013) and Hoque et al. (2022) reported a higher prevalence of mastitis cases of 26.2% and 37.6%, respectively.

Mastitis cases were mainly caused by the presence of *Aerococcus viridans* strains (21%), non-*aureus* staphylococci (18.4%), *Streptococcus agalactiae* and *Escherichia coli* (15, 8%).

S. agalactiae is a common cause of mastitis in dairy farms, with a prevalence of 20-40% reported by Li et al. (2012). *S. agalactiae* was the only infectious pathogen isolated from milk samples and in 6 cases its presence was associated with SCM cases. Our isolation rates are close to 11.3% and 10.3% values reported by Gianneechini et al. (2002) and Amin et al. (2011), respectively.

β-lactams are among the most frequently used antimicrobial classes for streptococcal mastitis treatment (Denamiel et al., 2005), resulting in high levels of penicillin resistance (Guo et al., 2018). *S. agalactiae* strains isolated from our samples showed higher levels of resistance against oxacillin (100%), while they showed complete susceptibility to penicillin (100%) and a high intermediate susceptibility value to amoxicillin-clavulanate (71.4%).

The etiology of *Escherichia coli*, non-*aureus* staphylococci and *Streptococcus agalactiae* strains in mastitis cases are well observed, while the etiology of *Aerococcus viridans* in mastitis cases needs to be better understood. *Aerococcus viridans* has been associated with cases of bovine mastitis (Spaková et al., 2012), and in the study of Arruda et al. (2013) it was the second most commonly isolated

species after non-*aureus* staphylococci, reporting a result very close to the percentage of our isolates from milk samples.

Bacteriological analysis of surface swabs showed that the most isolated bacteria were *Escherichia coli*, *Aerococcus viridans*, *Bacillus* spp, *Acinetobacter* spp, *Rothia* spp and non-*aureus* staphylococci. The presence of these bacteria was found mainly in the teatcups of the milking machine and detected predominantly in the samples collected after milking, being numerically four times higher than the bacteria isolated from the samples collected before milking.

In addition, the most relevant result is that the microbial load and the number of bacterial genera and species detected were strongly increased in the two samplings when the Biovitae® light was turned off and the standard lighting was used, and then reduced again in the last sampling when the light action was restarted. As reported by Maclean et al. (2010), when the use of the antimicrobial light is terminated, recontamination of the hospital room in which it was tested can be observed, reporting similar levels of contamination as before treatment.

Isolated bacteria from milking parlor surface swabs are generally present in the farm environment and are transported in the milking parlor by the animals and milkers, also contaminating the milking boxes and milking machine. It is necessary to perform proper pre-milking hygiene practices that can reduce the number of bacteria on the teat skin, as well as to perform post-milking practices, and take care of milking machine hygiene.

A similar issue must be made for operators. Because workers are in contact with animals and milking parlor surfaces, they are exposed to bacteria circulating on the farm and potentially transferable to humans during daily activities. Bacteriological examination of worker's hand swabs showed that the most common bacteria isolated were *Rothia amarae* (37%), *Escherichia coli* (13.8%), non-*aureus* staphylococci (10.8%), *Bacillus licheniformis* (7.7%) and *Aerococcus viridans* (6.1%). The presence of these bacteria could be linked to contamination due to improper use of personal protective equipment or inadequate cleaning of hands before milking operations.

In conclusion, the use of Biovitae® lamps installed in the milking parlor appears to have a satisfactory effect on microbial load, although it is influenced by several critical points such as the hygienic conditions of the milking parlor, animal cleaning practices, attention to milking routines, and the integrity of the milking machine. Cleaning is essential because it reduces organic dirt that might otherwise reduce the photodynamic inactivation effect (Tuladhar et al., 2012).

The action of Biovitae® lamps could be a useful tool to reduce the possibility of infections caused by microorganisms, preserve animal health, reduce the use of antibiotics and biocides, ensure quality milk, and also the safety of operators.

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5. Chapter 3 - Isolation of *Staphylococcus microti* from collected samples

5.1 Introduction

The Mediterranean buffalo (*Bubalis bubalis*) is a large bovid widely distributed throughout Southern Italy. Buffalo milk has a high economic and social value in the Campania Region, as it is the raw material used to produce Mozzarella di Bufala Campana Protected Denomination of Origin (PDO), a worldwide renowned cheese. Mastitis is one of the most economically important diseases affecting the dairy industry worldwide, and it is almost always caused by bacteria (Nagasawa et al., 2019). Thus, the identification of bacterial pathogens associated with clinical and subclinical mastitis is essential to understand the etiology of this disease, reduce animal culling and develop suitable therapy and preventative measures. According to the National Mastitis Council, the diagnosis of mastitis should be based on an interpretation of the somatic cell count (SCC) and bacteriological milk culture results (Middleton et al., 2017).

The milk somatic cell count (SCC) is adopted as an indicator of udder health in single milk samples and is also important for monitoring farm hygiene in bulk milk. Clinically, cow's milk samples with an SCC < 200×10^3 cells/mL and negative to bacteriological culture are indicative of a healthy status. An SCC < 200×10^3 cells/mL and a positive bacteriological culture are indicative of an intramammary infection (IMI), and an SCC > 200×10^3 cells/mL and a positive bacteriological culture are indicative of a subclinical mastitis (SCM) or clinical mastitis, according to the absence or presence of clinical signs, respectively (Puggioni et al., 2020).

Staphylococci, especially the coagulase-positive *Staphylococcus aureus*, are among the pathogens associated with mastitis in farmed ruminants. The causative role of non-*aureus* staphylococci (NAS) has grown over the years, and today they are considered as etiological agents of mastitis in cows, goats, and sheep (Lange et al., 2015) and, in addition, they are more frequently isolated from milk (Moroni et al., 2006).

More than 45 recognized NAS species have been described so far, and *S. microti* is a new species firstly described in 2010 (Nováková et al., 2010). Precisely, the first two strains of *S. microti* were isolated between 1999 and 2002 from the liver and kidneys of common voles (*Microtus arvalis* Pallas) in the Czech Republic (Nováková et al., 2010). Another strain was recovered from the skin of a small mammal (belonging to rodents or insectivores) in northeastern Poland (Hauschild et al., 2010). Phylogenetic studies based on the sequence analysis of the 16S rRNA gene and several housekeeping genes revealed that *S. microti* is closely related to *S. rostri* and *S. muscae* (Riesen et al., 2010). Subsequently, eleven *S. microti* strains were identified in milk samples collected from dairy cows affected with clinical and subclinical mastitis characterized by a high self-curing rate (Król et al., 2016). The complete genomic sequence of the *S. microti* DSM 22,147 strain, isolated from the

viscera of common voles, has been reported by Hu et al. (2018), which described a genome without plasmids and with a GC content of 38% in a 2,381,859 bp long sequence.
Data on the presence and causal role of *S. microti* in cattle are scarce, but recently the species was found in a buffalo herd and was associated with cases of subclinical mastitis and alterations in milk protein composition (Addis et al., 2022).
In this study, we investigated the occurrence, supplemented by two identification analyses of *S. microti* in buffalo milk and milking parlor surface samples of a buffalo farm in the Campania Region (Salerno, Italy). The strains' *in vitro* susceptibility to commonly used antimicrobials was investigated, and a genotypic tetracycline resistance analysis was also performed. In addition, *S. microti*'s association with different clinical statuses of udders was evaluated.

5.2 Materials and methods

5.2.1 Bacterial isolation, dentification and somatic cell count

As reported in Chapter 2 "*In vivo* studies," after sample collection, bacteriological examinations of milk samples (200) and swabs collected from the surfaces of the milking parlor (104) and worker (60) were carried out at the Microbiology Laboratory of the Department of Veterinary Medicine and Animal Production of the University of Naples "Federico II". Diluted milk samples were streaked on Mac Conkey Agar, 5% Columbia Sheep Blood agar, Mannitol Salt Agar, and Saboraud Dextrose Agar (Oxoid, Milan, Italy). The same plates were used for the swabs, and all agar plates were incubated for 24 h at 37 °C in aerobic conditions. The phenotypically equal colonies from the milk samples were counted to perform an enumeration of the CFUs. To obtain pure cultures, single colonies were subcultured on Columbia Sheep Blood agar and, after incubation time, were identified by MALDI TOF MS (Bruker Daltonics Inc., Germany). Score values below 1.7 indicated a nonreliable identification, between 1.7 and 1.99 a probable genus identification and equal or above 2.0 a certain genus identification and probable or highly probable species identification. A bacterial test standard (BTS) (Bruker Daltonics, Germany) was used as a calibrator for quality control.
SCC of milk samples performed using a NucleoCounter® SCC-100™ (ChemoMetec Inc. 8950 Villa La Jolla Drive – Suite A127 La Jolla, CA, USA) and their composition in fat, protein and lactose, analyzed using a Milkoscan FT120 (Foss Electric A/S, Denmark) were determined at the Istituto Zooprofilattico Sperimentale del Mezzogiorno (Portici - Naples, Italy). All bacteriologically positive milk samples were considered 'diseased' and were divided into three groups: samples with IMI (presenting SCC $< 200\times10^3$ cells/mL); samples with SCM (presenting SCC values $> 200\times10^3$ cells/mL and the absence of clinical signs) and samples with CM (presenting SCC values >

200×10^3 cells/mL in the presence of clinical signs). Whereas bacteriologically negative milk samples with $<200\times10^3$ cells/mL were considered 'healthy' (H).

5.2.2 DNA extraction and 16S rRNA gene sequencing

The 16S ribosomal gene was amplified from each DNA and then entirely sequenced. The genomic DNA was extracted from the overnight solid cultures of each identified *S. microti* strain using the commercial Isolate II Genomic DNA kit (Bioline, London, UK) according to the manufacturer's instructions. The quantity and quality of the DNA were assessed by spectrophotometric reading of the A260/A280 ratio (Eppendorf BioPhotometer 6131). The DNA samples were stored at −20 °C until use. Table 5.1 shows all of the primers used in this study. We designed the primers Smi16-11F, Smi16-750F, Smi16Fdown, Smi16-337R, Smi16Rup, Smi16-1072R and Smi16-1527R based on the preliminary sequencing data and on the reference sequence MF678892.1 found in GenBank. The other primers were retrieved from the cited literature. We first tested the presence of *S. microti* in our samples by amplifying the genomic DNA with the primers Smi16F and Smi16R (20 ng each DNA, 2 mM each dNTP and 0.3 υM each primer, 5U Takara PrimeSTAR GXL DNA polymerase, Takara Bio, Inc., USA). Amplification protocol: initial denaturation at 98 °C 3 min, 35 cycles at 98 °C 10 s, 60 °C 15 s, 68 °C 1 min, obtaining a fragment of 370 bp, which sequence matched the *S. microti* and *S. rostri* 16S rRNA genes. We attempted to identify the whole 16S gene using 2 generic bacterial primers, B27F and B1492R (20 ng each DNA, 2 mM each dNTP and 0.7 μM each primer, 5U Takara PrimeSTAR GXL DNA polymerase, Takara Bio, Inc., USA). Amplification protocol: initial denaturation at 98 °C 3 min, 35 cycles at 98 °C 10 s, 57 °C 15 s, 68 °C 1.5 min and a final step at 68 °C 5 min. The amplified product of approximately 1400 bp was then sequenced from its terminal regions using internal primers: Smi16F, Smi16Fdown, Smi16Rup and Smi16R. The sequence obtained showed that we amplified a single fragment that matched exactly the *S. microti* 16S rRNA gene. Based on our sequencing data and the reference sequence in GenBank, MF678892.1, we designed the primers Smi16-11F and Smi16-1527R, which encompassed almost the whole rDNA 16S sequence, except for the first 10 bp. With these two primers, we were able to amplify the whole 16S gene from each DNA sample (20 ng each DNA, 2 mM each dNTP and 0.3 uM each primer, 5U Takara PrimeSTAR GXL DNA polymerase, Takara Bio, Inc., USA). Amplification protocol: initial denaturation at 98 °C 3 min, 35 cycles at 98 °C 10 s, 60 °C 15 s, 68 °C 1.5 min and a final step at 68 °C 5 min, obtaining a 1516 bp product in each case. Each PCR product was sequenced with all of the primers listed in Table 5.1 using the Brilliant Dye Terminator kit v3.1 (Nimagen, The Netherlands) and run on the 3700 xl DNA analyzer (Thermo Scientific, USA). The obtained sequences were assembled with SeqMan II sequence analysis software

(DNASTAR Inc., Madison, WI, USA) independently for each of the 1511 bp amplicons.

Table 5.1 - Primers used for the PCR sequencing of the 16S ribosomal gene.

Primer	Sequence	Reference
Smi16-11F	GGCGGCGTGCCTAATACATG	This study
Smi16-750F	GTGGGGATCAAACAGGAT	This study
Smi16F	CCTCTTCGGAGGACAAAGTGA	Bannoehr et al., 2007
Smi16Fdown	GAATACGTTCCCGGGTCTTG	This study
Smi16-337R	CTGCTGCCTCCCGTAGG	This study
Smi16Rup	ATCCTGTTTGATCCCCAC	This study
Smi16-1027R	TCACTTTGTCCTCCGAAGAGG	This study
Smi16R	GACCCGGGAACGTATTCACC	Bannoehr et al., 2007
Smi16-1527R	TAGAAAGGAGGTGATCCAGC	This study
B27F	AGAGTTTGATCMTGGCTCAG	Hongoh et al., 2003
B1492R	TACCTTGTTACGACTT	McAllister et al., 2011

5.2.3 Phenotypic antibiotic resistance profiles

All of the *S. microti* isolates were assessed for *in vitro* antimicrobial susceptibility based on the Kirby–Bauer method and inoculated Muller–Hinton agar plates were incubated at 37 °C for 24 h in an aerobic atmosphere. The following panel of antibiotics were tested: amoxicillin–clavulanate (AMC, disk content: 20/10 µg), amikacin (AK, disk content: 30 µg), cefoxitin (FOX, disk content: 30 µg), clindamycin (CD, disk content: 2 µg), ciprofloxacin (CIP, disk content: 5 µg), erythromycin (E, disk content: 15 µg), gentamicin (CN, disk content: 10 µg), kanamycin (K, disk content: 30 µg), oxacillin (OX, disk con-tent: 1 µg), oxytetracycline (T, disk content: 30 µg), penicillin (P, disk content: 10 IU), streptomycin (S, disk content: 10 µg), sulfamethoxazole–trimethoprim (SXT, disk con-tent: 25 µg) and tetracycline (TE, disk content: 30 µg). The chosen antibiotics belonged to eight classes (Table 5.2). The isolates were classified as susceptible (S), intermediate (I) or resistant (R) according to the Clinical and Laboratory Standards Institute (2015) and to the European Committee on Antimicrobial Susceptibility Testing (2018) guidelines. In according to Magiorakos (2012), *S. microti* isolates non-susceptible to at least one antibiotic in more than two families were considered multidrug-resistant strains.

Table 5.2 – Antibiotic classes tested to define the antimicrobial susceptibility profiles of the isolates.

Antibiotics	Disk Content	Antibiotic Class	Reference for Breakpoints
Amoxicillin–clavulanate (AMC)	20/10 µg	Penicillins	(CLSI, 2015)
Penicillin (P)	10 IU		
Oxacillin (OX)	1 µg		
Amikacin (AK)	30 µg	Aminoglycosides	(CLSI, 2015)
Kanamycin (K)	30 µg		
Gentamicin (CN)	10 µg		
Streptomycin (S)	10 µg		
Cefoxitin (FOX)	30 µg	Cephalosporins	(EUCAST, 2018)
Ciprofloxacin (CIP)	5 µg	Quinolones	(CLSI, 2015)
Clindamycin (DA)	2 µg	Lincosamides	(CLSI, 2015)
Erythromycin (E)	15 µg	Macrolides	(CLSI, 2015)
Tetracycline (TE)	30 µg	Tetracyclines	(CLSI, 2015)
Oxytetracycline (T)	30 µg		
Sulfamethoxazole–trimethoprim (SXT)	25 µg	Sulfonamides	(EUCAST, 2018)

5.2.4 Genotypic profiles of resistance to Tetracycline

Resistance to tetracyclines was further investigated in *S. microti*-isolated strains by performing multiplex PCR for the *tet*M and *tet*K genes. It has been reported that tetracycline resistance is mediated above all by these two genes in NAS isolated from bovine subclinical or clinical mastitis cases (Schwarz and Chaslus-Dancla, 2001; Qu et al., 2019). The *Tet*M gene encodes for ribosomal protection proteins, which reduce the affinity of tetracycline for the ribosome, and the *tet*K gene encodes for efflux proteins, which prevent tetracycline from accumulating inside the cell. Multiplex PCR was performed for all 54 *S. microti* isolates, as already described by Ullah et al. (2012). The list of primers and the multiplex PCR conditions used for amplification of tetracycline resistance genes are described in Table 5.3. For all of the multiplex PCR reactions, there was always a positive (DNA from a clinical *S. aureus* strain positive to *tet*M and *tet*K genes) and a negative (without bacterial DNA) control. Multiplex PCR was performed using Biorad T100TM Thermo cycler (BioRad, Hercules, CA).

Table 5.3 - Primer sequences, amplicon size and amplicon program of *tet*M and *tet*K genes.

Gene	Primer Sequences (5'-3' Sense and Antisense)	Amplicon Size (bp)	Amplifications Program
*tet*M	F: AGTTTTAGCTCATGTTGATG R: TCCGACTATTTAGACGACGG	1862	94 °C 15 s; 94 °C 1 min, 52 °C 1 min, 72 °C 90 s, for 30 cycles; 72 °C 5 min
*tet*K	F: GTAGCGACAATAGGTAATAGT R: GTAGTGACAATAAACCTCCTA	360	

5.3 Results

5.3.1 Occurrence of *S. microti* strains and SCC in milk samples

White and weakly hemolytic, catalase-positive, coagulase-negative and oxidase-negative colonies were identified as *S. microti* by MALDI-TOF analysis, with a log(score) ≥ 2.0 in all identifications, indicating a reliable identification at the species level.

The *S. microti* isolation was revealed in 51/200 (25.5%) milk samples. Only 23 animals were sampled two times with different sampling intervals, and precisely 10 showed positivity to *S. microti* in both samplings, with a maximum interval of 6 months, and 13 animals resulted positive to *S. microti* in one of the two samplings. Furthermore, 18 animals positive to *S. microti* were sampled only once.

The evaluation of the SCC for each milk sample revealed the presence of *S. microti* in four milk samples (4/22, 18.2%) with an SCC value > 200×10^3 cells/mL, and 47 strains were isolated from the milk samples (47/124, 37.9%) with an SCC value < 200×10^3 cells/mL, compatible with SCM and IMI, respectively. *S. microti* were isolated alone or in combination with other bacteria, as described for the *S. microti*-associated SCM cases (Table 5.4).

In addition, the antimicrobial resistance profile exhibited by 4 strains of *S. microti*, associated with the presence of SCM cases and isolated in different samplings, showed a matching profile for two strains, leading us to speculate that the lineage was the same.

As demonstrated in Table 5.4, the MALDI-TOF identification of *S. microti* always presented an optimal score > 2.0, only 11/38 (28.9%) identifications of other bacterial species showed a slightly <2.0 score. 47 *S. microti*-associated IMI cases exhibited a high level of genus and species identification are shown in Table 5.5. Moreover, 3/104 (2.9%) *S. microti* were isolated from surface swabs in two different samplings, as well as precisely two strains from a teatcups of the milking unit and one from a milking box, which presented a good score of identification (>2.0), as reported in Table 5.6.

Table 5.4 - Bacterial species and MALDI-TOF scores in 22 buffalo milk samples showing high somatic cells counts (SCC) compatible with the definition of subclinical mastitis (SCM).

Sample	SCC/mL	Identified Bacterial Strains (Colony Forming Unit, CFU)	MALDI-TOF Score
1	2×10^5	*Aeromonas hydrophila* (3000 CFU/mL)	2.11
2	2.9×10^5	*Acinetobacter johnsonii* (5000 CFU/mL)	2.26
		Staphylococcus simulans (1000 CFU/mL)	2.20
3	2.5×10^5	*Staphylococcus microti* (10000 CFU/mL)	2.07
		Citrobacter freundii (10000 CFU/mL)	1.96
4	2.8×10^5	*Escherichia coli* (5000 CFU/mL)	2.09
		Staphylococcus simulans (1000 CFU/mL)	2.20
		Staphylococcus sciuri (500 CFU/mL)	2.09
5	2×10^6	*Streptococcus agalactiae* (6000 CFU/mL)	2.10
		Lactococcus lactis (1100 CFU/mL)	2.17
		Aeromonas hydrophila (5000 CFU/mL)	1.88
6	2.7×10^5	*Aeromonas hydrophila* (3000 CFU/mL)	2.12
7	5.3×10^5	*Streptococcus agalactiae* (10000 CFU/mL)	2.27
		Corynebacterium xerosis (1000 CFU/mL)	2.04
8	3.6×10^5	*Staphylococcus microti* (3000 CFU/mL)	2.04
		Aerococcus viridans (2000 CFU/mL)	1.79
		Rothia endophytica (200 CFU/mL)	1.91
9	2.9×10^5	*Aerococcus viridans* (2000 CFU/mL)	1.81
10	2.7×10^5	*Staphylococcus microti* (4000 CFU/mL)	2.04
11	2.3×10^5	*Escherichia coli* (4000 CFU/mL)	2.16
12	2.9×10^5	*Pseudomonas aeruginosa* (4000 CFU/mL)	2.34
13	2.2×10^5	*Streptococcus agalactiae* (3000 CFU/mL)	2.43
14	2.5×10^5	*Rothia amarae* (6000 CFU/mL)	2.05
15	5.1×10^5	*Escherichia coli* (2800 CFU/mL)	2.22
		Rothia amarae (500 CFU/mL)	1.94
		Aerococcus viridans (700 CFU/mL)	1.82
16	2.9×10^5	*Escherichia coli* (400 CFU/mL)	2.14
		Streptococcus agalactiae (5400 CFU/mL)	2.29
		Aerococcus viridans (200 CFU/mL)	1.99
17	1.5×10^6	*Escherichia coli* (2000 CFU/mL)	2.17
		Aerococcus viridans (1100 CFU/mL)	2.14
18	8.3×10^5	*Streptococcus agalactiae* (5000 CFU/mL)	2.19
19	2.1×10^5	*Staphylococcus microti* (200 CFU/mL)	2.06
		Aerococcus viridans (100 CFU/mL)	1.89
		Escherichia coli (500 CFU/mL)	2.31
20	4.5×10^5	*Aerococcus viridans* (400 CFU/mL)	1.90
21	8.8×10^5	*Streptococcus agalactiae* (4700 CFU/mL)	2.04
22	2.3×10^5	*Aerococcus viridans* (4800 CFU/mL)	1.99

Table 5.5 – Bacterial species identification by MALDI-TOF of 42 milk samples positive to S. *microti* growth with or without other bacterial species. All milk samples presented somatic cell count (SCC) <200 x 10^3 cells/mL indicative of intramammary infection (IMI).

Sample	SCC/ml	Identified bacterial strains (colony forming unit, CFU)	MALDI-TOF score
1	1.7 x 10^5	*Staphylococcus microti* (3000 CFU/mL)	2.03
2	3.5 x 10^4	*Staphylococcus microti* (6000 CFU/mL)	2.02
3	4.2 x 10^4	*Staphylococcus microti* (4000 CFU/mL)	2.07
4	1.6 x 10^4	*Staphylococcus microti* (600 CFU/mL)	2.14
5	6.2 x 10^4	*Staphylococcus microti* (2000 CFU/mL) *Escherichia coli* (500 CFU/mL)	2.08 2.23
6	8.7 x 10^4	*Staphylococcus microti* (800 CFU/mL) *Klebsiella oxytoca* (500 CFU/mL) *Acinetobacter proteolyticus* (500 CFU/mL)	2.01 2.15 2.16
7	2.9 x 10^4	*Staphylococcus microti* (2000 CFU/mL) *Citrobacter freundii* (1500 CFU/mL)	2.05 2.22
8	7.3 x 10^4	*Staphylococcus microti* (600 CFU/mL) *Raoultella ornithinolyca* (1000 CFU/mL)	2.06 2.27
9	9.2 x 10^4	*Staphylococcus microti* (3100 CFU/mL) *Escherichia coli* (500 CFU/mL) *Staphylococcus saprophyticus* (1400 CFU/mL)	2.02 2.47 1.96
10	3.7 x 10^4	*Staphylococcus microti* (500 CFU/mL) *Aeromonas hydrophila* (2000 CFU/mL)	2.09 2.28
11	4.8 x 10^4	*Staphylococcus microti* (1100 CFU/mL) *Staphylococcus xylosus* (500 CFU/mL) *Aeromonas media* (700 CFU/mL)	2.07 2.23 1.70
12	1.1 x 10^4	*Staphylococcus microti* (2000 CFU/mL) *Escherichia coli* (500 CFU/mL) *Macrococcus caseolyticus* (500 CFU/mL)	2.08 2.27 2.06
13	1 x 10	*Staphylococcus microti* (700 CFU/mL)	2.06
14	8 x x 10^4	*Staphylococcus microti* (1500 CFU/mL) *Aeromonas hydrophila* (1000 CFU/mL)	2.04 2.12
15	2.5 x 10^4	*Staphylococcus microti* (1500 CFU/mL)	2.04
16	2.8 x 10^4	*Staphylococcus microti* (1500 CFU/mL) *Aeromonas hydrophila* (500 CFU/mL)	2.01 2.11
17	1.1 x 10^4	*Staphylococcus microti* (500 CFU/mL)	2.01
18	2.8 x 10^4	*Staphylococcus microti* (1200 CFU/mL) *Escherichia coli* (1000 CFU/mL) *Enterococcus aquimarinus* (800 CFU/mL)	2.12 2.26 1.76
	5.7 x 10^4	*Staphylococcus microti* (600 CFU/mL)	2.02
19	2.9 x 10^4	*Staphylococcus microti* (800 CFU/mL) *Aerococcus viridans* (200 CFU/mL) *Enterococcus aquimarinus* (1500 CFU/mL)	2.08 1.80 1.84
20	1.5 x 10^5	*Staphylococcus microti* (2000 CFU/mL)	2.10
21	5.8 x 10^4	*Staphylococcus microti* (2000 CFU/mL)	2.01
22	6.4 x 10^4	*Staphylococcus microti* (1000 CFU/mL)	2.01

23	1.5 x 10^5	*Staphylococcus microti* (900 CFU/mL) *Aerococcus viridans* (600 CFU/mL) *Lactococcus lactis* (100 CFU/mL)	2.10 1.96 2.00
24	3.1 x 10^4	*Staphylococcus microti* (1000 CFU/mL) *Escherichia coli* (200 CFU/mL) *Streptococcus uberis* (700 CFU/mL)	2.12 2.29 2.11
25	1 x 10^4	*Staphylococcus microti* (600 CFU/mL)	2.04
26	1.1 x 10^5	*Staphylococcus microti* (500 CFU/mL) *Escherichia coli* (2500 CFU/mL) *Aerococcus viridans* (100 CFU/mL)	2.09 2.13 1.80
27	4.8 x 10^4	*Staphylococcus microti* (500 CFU/mL) *Staphylococcus epidermidis* (500 CFU/mL)	2.07 2.19
28	5.1 x 10^4	*Staphylococcus microti* (600 CFU/mL)	2.01
29	1.4 x 10^5	*Staphylococcus microti* (800 CFU/mL) *Microbacterium oxydans* (400 CFU/mL)	2.08 2.03
30	2.6 x 10^4	*Staphylococcus microti* (1000 CFU/mL) *Staphylococcus sciuri* (1200 CFU/mL) *Aerococcus viridans* (800 CFU/mL)	2.01 2.12 1.99
31	7.2 x 10^4	*Staphylococcus microti* (1600 CFU/mL) *Bacillus licheniformis* (1100 CFU/mL) *Macrococcus caseolyticus* (1100 CFU/mL)	2.06 1.99 2.01
32	1.1 x 10^5	*Staphylococcus microti* (800 CFU/mL) *Escherichia coli* (1400 CFU/mL) *Acinetobacter towneri* (300 CFU/mL)	2.06 2.24 1.99
33	5.1 x 10^4	*Staphylococcus microti* (1100 CFU/mL) *Staphylococcus chromogenes* (600 CFU/mL) *Lactococcus lactis* (1300 CFU/mL)	2.01 2.11 1.87
34	1.5 x 10^4	*Staphylococcus microti* (1000 CFU/mL) *Staphylococcus chromogenes* (800 CFU/mL) *Aerococcus viridans* (1000 CFU/mL)	2.08 2.10 1.99
35	8.1 x 10^4	*Staphylococcus microti* (500 CFU/mL) *Bacillus licheniformis* (800 CFU/mL) *Aerococcus viridans* (2900 CFU/mL)	2.02 2.01 1.98
36	2.2 x 10^4	*Staphylococcus microti* (1600 CFU/mL) *Aerococcus viridans* (1800 CFU/mL)	2.08 2.11
37	3.9 x 10^4	*Staphylococcus microti* (2000 CFU/mL) *Bacillus licheniformis* (1200 CFU/mL) *Aerococcus viridans* (1200 CFU/mL)	2.08 1.99 2.12
38	9.6 x 10^4	*Staphylococcus microti* (2500 CFU/mL) *Bacillus licheniformis* (200 CFU/mL)	2.12 2.01
39	2.8 x 10^4	*Staphylococcus microti* (1200 CFU/mL) *Escherichia coli* (500 CFU/mL) *Bacillus licheniformis* (200 CFU/mL)	2.12 2.12 2.01
40	1.5 x 10^4	*Staphylococcus microti (*1200 CFU/mL) *Streptococcus uberis (*600 CFU/mL) *Acinetobacter indicus (*200 CFU/mL)	2.08 2.23 1.77
41	6.6 x 10^4	*Staphylococcus microti (*2400 CFU/mL) *Acinetobacter indicus (*500 CFU/mL) *Bacillus licheniformis (*1700 CFU/mL)	2.02 1.77 1.88
42	1.1 x 10^5	*Staphylococcus microti (*700 CFU/mL) *Aerococcus viridans (*4300 CFU/mL) *Escherichia coli (*800 CFU/mL)	2.09 2.03 2.24

43	1.6 x 10^5	*Staphylococcus microti* (800 CFU/mL) *Escherichia coli* (7000 CFU/mL)	1.90 2.19
44	8.4 x 10^4	*Staphylococcus microti* (1000 CFU/mL) *Escherichia coli* (2000 CFU/mL)	2.20 2.25
45	1.6 x 10^5	*Staphylococcus microti* (600 CFU/mL) *Aerococcus viridans* (1100 CFU/mL)	1.95 1.98
46	3.3 x 10^4	*Staphylococcus microti* (200 CFU/mL) *Escherichia coli* (300 CFU/mL) *Aerococcus viridans* (900 CFU/mL)	2.09 2.24 1.88
47	1.2 x 10^5	*Staphylococcus microti (*30000 CFU/mL) *Aerococcus viridans* (600 CFU/mL)	2.05 2.09

Table 5.6 – *S. microti* and other bacterial species isolated from 3 milking surface samples.

Sample	Identified bacterial strains (number of colonies forming units, CFU)	MALDI-TOF score
1	*Staphylococcus microti* (3400 CFU/mL) *Bacillus licheniformis (*3000 CFU/mL)	2.07 1.88
2	*Staphylococcus microti* (4800 CFU/mL) *Escherichia coli* (1600 CFU/mL) *Acinetobacter indicus* (4200 CFU/mL)	2.14 2.23 1.83
3	*Staphylococcus microti* (2000 CFU/mL) *Acinetobacter towneri* (600 CFU/mL) *Corynetobacter xerosis* (1000 CFU/mL)	2.15 1.99 1.78

5.3.2 16S rRNA gene sequencing

The 16S rRNA genes were amplified and sequenced from genomic DNA purified from the bacterial isolates with the primers listed in Table 5.1. The sequences were analyzed with the BLAST algorithm (National Center for Biotechnology Information, NCBI, USA) against the Nucleotide Collection database, retrieving the *S. microti* 16S gene (accession MF678892.1, 99.28% identity).

5.3.3 Phenotypic resistance profiles and genotypic characterization of tetracycline resistance

The antimicrobial susceptibility testing results highlighted a complete resistance to both tetracycline and oxytetracycline (100%) for all 54 *S. microti* isolated strains, while the majority of the strains (52/54, 96.5%) were susceptible to amoxicillin–clavulanate, gentamicin and sulfamethoxazole–trimethoprim, followed by amikacin (90.7%, 49/54), kanamycin (87%, 47/54), streptomycin (77.8%, 42/54) and ciprofloxacin (76%, 41/54). Referring to betalactams antibiotics, high levels of susceptibility were observed for oxacillin and cefoxitin, being 94.4% (51/54) and 81.5% (44/54), respectively; but nearly half of the isolated strains were resistant to

penicillin (51.9%, 28/54). Clindamycin and erythromycin resulted to be the antibiotics with the highest number of strains showing intermediate susceptibility (66.7%, 36/54 and 50%, 27/54, respectively). Figure 5.1 shows the antimicrobial susceptibility pattern of all bacterial isolates obtained in this study, classifying them as sensitive (S), intermediate (I) or resistance (R). In addition, an important finding concerns the prevalence of *S. microti* strains with a multidrug-resistance (MDR) profile, as described in Table 5.7. In fact, 20.4% (11/54) of the total isolates showed resistance to at least three classes of antibiotics, and all were isolated from the milk samples. In addition, the genotypic characterization of tetracycline resistance revealed the presence of the *tet*M gene in all collected *S. microti* strains. No *tet*K gene was found.

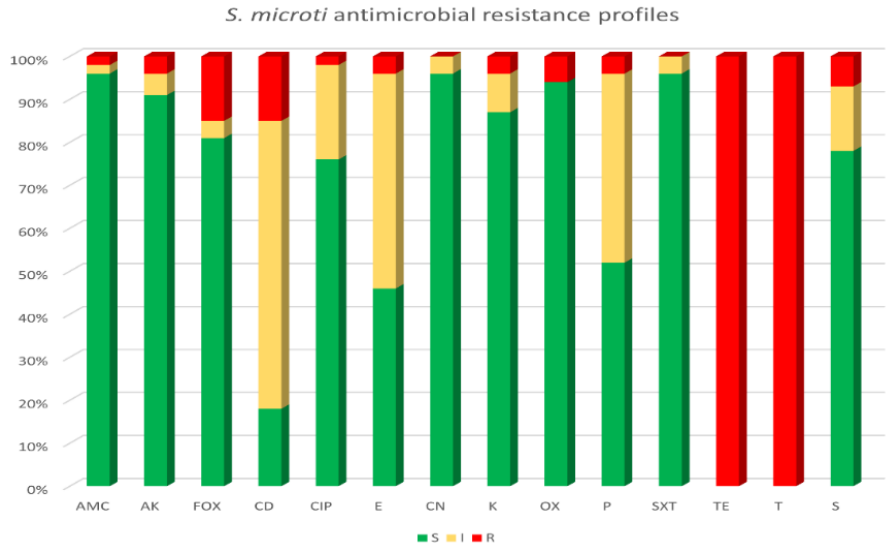

Figure 5.1 - Antibiotic resistance profiles of 54 S. microti isolates. Tested antibiotics: amoxicillin–clavulanate (AMC), amikacin (AK), cefoxitin (FOX), clindamycin (CD), ciprofloxacin (CIP), erythromycin (E), gentamicin (CN), kanamycin (K), oxacillin (OX), penicillin (P), sulfamethoxazole–trimethoprim (SXT), tetracycline (TE), oxytetracycline (T) and streptomycin (S).

Table 5.7 - Antibiotic resistance profiles of 11 multidrug-resistant *S. microti* strains.

	Penicillins			Aminoglycosides			Cephalosporins		Quinolones	Lincosamides	Macrolides	Tetracyclines		Sulfonamides
S. microti strains	AMC	P	OX	AK	K	CN	S	FOX	CIP	DA	E	TE	T	SXT
7				R							R	R	R	
11							R	R		R		R	R	
12								R		R		R	R	
13	R	R						R				R	R	
14								R		R		R	R	
15								R		R		R	R	
16				R						R		R	R	
17									R	R		R	R	
19			R		R							R	R	
20		R						R				R	R	
22			R				R				R	R	R	

5.4 Discussions and conclusions

Bacteria of the genus *Staphylococcus* have been considered the main etiological agents associated with cases of SCM (Dhakal et al., 2008; Vásquez-Garcia et al., 2017) and CM (Pizauro et al., 2014) in buffaloes. However, non-*aureus* staphylococci (NAS) species are currently the most prevalent pathogens causing SCM, IMI and, rarely, CM in dairy buffaloes (Condas et al., 2017; Thorberg et al., 2009). The predominance of NAS in milk samples is remarkable (Supré et al., 2011; Hosseinzadeh and Saei, 2014), and their ability to cause inflammatory processes should not be unerestimated. Together with microbial analysis, the somatic cell counts (SCCs) contained in milk can provide an accurate evaluation of the milk quality.

Based on this principle, in this study, we considered milk samples with an SCC < 200×10^3 cells/mL and negative to bacteriological culture as a healthy status of the buffaloes, and milk samples with an SCC < 200×10^3 cells/mL and a positive bacteriological culture indicative of IMI, and an SCC > 200×10^3 cells/mL with a positive bacteriological culture as SCM cases, as already reported by Puggioni et al. (2020). Moreover, animals with clinical signs, SCC values > 200×10^3 and positive for bacteriological examination were defined as CM cases (Puggioni et al., 2020). Our study performed in a buffalo farm determined a statistically significant higher level of IMI (62%) than SCM (11%) cases, with no cases of CM.

The SCC level changed over time through the course of the cases, and the lack of this information represents a limitation of this study; in fact, only 23 animals were sampled twice, and relevant changes were not observed in the SCC values between the two sampling periods.

The bacterial identification performed in this study, among the isolated NAS, highlighted a new species known as *S. microti*, a bacterial species that is still not well known on buffalo farms. In fact, the identification of *S. microti* has been only once reported as the predominant species in the milk of water buffaloes with SCM (Addis et al., 2022). Previously, a first case of IMI in cattle was reported in Poland (Król et al., 2016) and more recently in a bovine herd in Germany, where *S. microti* was detected as the causative pathogen of IMI in combination with other NAS (Hamel et al., 2020). To the best of our knowledge, there are no published studies investigating the presence of *S. microti* at the same time in milk and milking parlors of buffalo farming. Thus, for the first time, we isolated 54 strains of *S. microti* over seven samplings, with an average of eight strains per sampling, and no prevalence of isolation between the summer and autumn periods was observed. Moreover, we obtained in 10/41 animals the isolation of *S. microti* in two different samplings. Precisely, 51 strains were isolated from milk samples and 3 from the milking parlor surfaces, highlighting the predominant presence of this strain in the milk samples.

It is known that sequencing and taxonomic resolution based on 16S rRNA gene alone are limited (Rajendhran and Gunasekaran, 2020), and a cost-effective

alternative are taxonomic analyses using MALDI-TOF. Ribosomal RNA and ribosomal proteins can be used for phylogenetic analysis, being universally conserved in both eukaryotic and prokaryotic cells (Yutin et al., 2012). The identification of *S. microti*, here obtained by MALDI-TOF analysis, was perfectly confirmed by the classification method based on the complete sequence analysis of the 16S rRNA gene. However, MALDI-TOF analysis is a time-saving method for the routine identification of bacteria and can achieve 96% accuracy (Cameron et al., 2017).

In addition, *S. microti* isolates were evaluated for an antibiotic susceptibility profile and all bacteria isolated both from the SCM and IMI cases were found to be 100% resistant to tetracycline and oxytetracycline. The susceptibility to other antibiotics agreed with a previous study that reported a high susceptibility rate to a panel of antibiotics, not including tetracyclines, tested for *S. microti* strains isolated from bovine milk samples (Król et al., 2016). In our study, 20.4% (11/54) of the *S. microti* isolates, showing resistance to three or more classes of antibiotics, were defined as MDR. Further molecular investigations are needed for four strains, which resulted to be resistant to cefoxitin but susceptible to penicillins, while two strains were oxacillin-resistant but susceptible to penicillin and cefoxitin. The MDR profile was not detected in the *S. microti* isolates from the milking parlor surfaces, which exhibited resistance to the tetracycline class and one of them also resistance to lincosamides. Currently, to our knowledge, there are no other studies on the genotypic characterization of tetracycline resistance in *S. microti* strains from buffalo. In our study, all 54 isolates showed the presence of the *tet*M gene, justifiable by their isolation performed at the same farm. Previously, the detection of both the *tet*K and *tet*M genes in NAS strains has been reported (Ruegg et al., 2015; Mbindyo et al., 2021), as well as in *S. aureus* isolated from cows with mastitis (Guzmán-Rodríguez et al., 2020).

The hygienic status of animals is very important to ensure healthy and quality milk, and both microorganisms and SCC should be minimized in raw milk (Erdem et al., 2019). The monitoring of IMI, SCM, CM and cleaning milking parlor routines represent an important aspect to profitable livestock economics worldwide.

From this study, we can conclude that the pathological and epidemiological roles of *S. microti* need further investigation as well as its association with IMI or SCM. Furthermore, new information on the presence of this bacterium in Italian buffalo-breeding herds, especially in the Campania Region where buffalo herds represent 80% of the national buffalo assets and is an important zootechnical and economic reality, is very important. In the context of the surveillance of NAS strains and of studies on antibiotic resistance profiles, including resistance gene identification, future work should also be focused on the detection and monitoring of MDR *S. microti* in wider areas of the Campania Region.

5.5 References

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6. Chapter 4 - Isolation of *Escherichia coli* from milk samples and swabs

6.1 Introduction

Coliform bacteria are microorganisms typically found in the natural flora of the human and animal intestinal tract. *Escherichia coli* (*E. coli*) is a coliform bacterium, that belongs to the family Enterobacteriaceae and is categorized as a Gram-negative opportunistic environmental bacterium. *E. coli* can be found in manure, environment, soil, and contaminated water (Disassa et al., 2017) and is usually a harmless commensal. In dairy farms, *E. coli* appears to be one of most frequently isolated bacterium in bovine milk samples and often associated with intramammary infection (Olde Riekerink et al., 2008). Furthermore, it is responsible for subclinical (Ahmed et al., 2018) and clinical mastitis, which can be mild, severe, or fatal (Shpigel et al., 2008). In fact, it is able to pass from the environment to the udder through the teats canal, proliferating and generating an inflammatory reaction depending on the animal's health status, predisposition, genetic background and lactation stage (Burvenich et al., 2003).

E. coli strains can be commensal but also enteric and extraintestinal pathogenic bacteria (Kaper et al., 2004) and can cause many animal diseases. Enterotoxigenic (ETEC), enterohemorrhagic (EHEC), enteroinvasive (EIEC), enteropathogenic (EPEC), and enteroaggregative (EAEC) are enteric pathogenic strains divided in pathotypes, depending on the virulence factors and mechanisms used to cause disease. Enterohemorrhagic *Escherichia coli* serotype O157:H7 produces potent cytotoxins called Shiga (Shiga toxin-producing *E. coli,* STEC) and is transmitted by the fecal-oral route through consumption of contaminated food or water and by direct contact with infected animals and people.

The major carriers of *E. coli* O157:H7 are ruminants, and the main vehicles are raw milk and fresh meat (Gugsa et al., 2022). Adult buffaloes can be a reservoir of *E. coli* O157:H7 and calves of both ETEC and STEC strains (Galiero et al., 2005). These infectious strains are one of the most common causes of neonatal calf diarrhea (Nguyen et al. 2011), causing significant economic losses due to low animal growth rates, high morbidity and mortality rates, and high treatment costs (Ok et al., 2009). *E. coli* O157:H7 is also recognized as an important cause of human foodborne illness, causing hemorrhagic colitis and hemolytic-uremic syndrome.

E. coli strains are frequently isolated from milk and, as well as other coliform and pathogenic bacteria, are indicators of contamination from udders, equipment, or water (Chye et al., 2004). Their presence could be caused by inadequate hygienic techniques (Chatterjee et al., 2006), poor milk handling, and contaminated water and workers' hands. Foodborne microorganisms reduce milk quality and affect food safety by significantly impacting public health and the economic sector.

In addition, the presence of multidrug-resistant (MDR) pathogenic bacteria is

steadily increasing and becoming a challenge in terms of treatment and prevention of disease spread through zoonotic transmission. Studies concerning *E. coli* strains have shown an increasing number of resistance genes in isolates, and many of them have been acquired by horizontal gene transfer. This worrisome finding is due to the ability of *E. coli* strains to act as both donor and recipient of resistance genes, acquiring resistance genes from other bacteria and transferring its genes to other bacteria (Poirel et al., 2018).
During the *in vivo* study, 88 *E. coli* strains were isolated from milk samples, milking parlor surfaces and workers' hands. In this chapter, the strains' susceptibility to commonly used antimicrobials and partial genotypic tetracycline resistance analysis were investigated. In addition, the detection of *E. coli* O157:H7 was performed and *E. coli* association with different clinical statuses of udders was evaluated.

6.2 Materials and methods

6.2.1 Bacterial isolation, dentification and somatic cell count

As reported in Chapter 2 "*In vivo* studies," after sample collection, bacteriological examinations of milk samples (200) and swabs collected from the surfaces of the milking parlor (104) and worker (60) were carried out at the Microbiology Laboratory of the Department of Veterinary Medicine and Animal Production of the University of Naples "Federico II". Diluted milk samples were streaked on Mac Conkey Agar, 5% Columbia Sheep Blood agar, Mannitol Salt Agar, and Saboraud Dextrose Agar (Oxoid, Milan, Italy). The same plates were used for the swabs, and all agar plates were incubated for 24 h at 37 °C in aerobic conditions. To obtain pure cultures, single pink-colored colonies, considered presumptive of *E. coli* strains, were subcultured on Mac Conkey Agar and, after incubation time, were identified by MALDI-TOF-MS (Bruker Daltonics Inc., Germany). Score values below 1.7 indicated a nonreliable identification, between 1.7 and 1.99 a probable genus identification and equal or above 2.0 a certain genus identification and probable or highly probable species identification. A bacterial test standard (BTS) (Bruker Daltonics, Germany) was used as a calibrator for quality control.
SCC of milk samples performed using a NucleoCounter® SCC-100™ (ChemoMetec Inc. 8950 Villa La Jolla Drive – Suite A127 La Jolla, CA, USA) and their composition in fat, protein and lactose, analyzed using a Milkoscan FT120 (Foss Electric A/S, Denmark) were determined at the Istituto Zooprofilattico Sperimentale del Mezzogiorno (Portici - Naples, Italy). All bacteriologically positive milk samples were considered "diseased" and were divided into three groups: samples with IMI (presenting SCC $< 200\times10^3$ cells/mL); samples with SCM (presenting SCC values $> 200\times10^3$ cells/mL and

the absence of clinical signs) and samples with CM (presenting SCC values >200×10^3 cells/mL in the presence of clinical signs). Whereas bacteriologically negative milk samples with <200×10^3 cells/mL were considered 'healthy' (H).

6.2.2 Detection of *E. coli* O157:H7 by latex agglutination test

PROLEX^{TM} *E. coli* O157:H7 latex test reagent kit (PRO-LAB Diagnostic) was used to detect *E. coli* serogroup O157. The test was carried out in accordance with manufacturer's instructions and, precisely, *E. coli* colonies from an overnight culture were removed from the agar surface using a sterile loop and were suspended in a test tube with normal saline solution to achieve turbidity corresponding to a 3-5 McFarland Standard. A drop of Latex Reagent, test latex, consisting of latex particles sensitized with specific rabbit antibody reactive with the *E. coli* 0157 antigen, was placed in a test circle of the provided cards and then mixed with a drop of the bacterial suspension. The cards were gently rocked, and agglutination was examined for up to two minutes.

6.2.3 Phenotypic antibiotic resistance profiles

The *E. coli* isolates were assessed for *in vitro* antimicrobial susceptibility by Kirby–Bauer method and inoculated Muller–Hinton agar plates were incubated at 37 °C for 24 h in an aerobic atmosphere. The following panel of antibiotics was tested: amoxicillin–clavulanate (AMC, disk content: 20/10 µg), amikacin (AK, disk content: 30 µg), clindamycin (CD, disk content: 2 µg), ciprofloxacin (CIP, disk content: 5 µg), erythromycin (E, disk content: 15 µg), gentamicin (CN, disk content: 10 µg), kanamycin (K, disk content: 30 µg), imipenem (IMI, disk content: 10 µg), meropenem (MRP, disk content: 10 µg), oxytetracycline (T, disk content: 30 µg), penicillin (P, disk content: 10 IU), streptomycin (S, disk content: 10 µg), sulfamethoxazole–trimethoprim (SXT, disk con-tent: 25 µg) and tetracycline (TE, disk content: 30 µg). The chosen antibiotics belonged to eight classes: Aminoglycosides, Carbapenemes, Lincosamides, Macrolides, Penicillins, Quinolones, Sulfonamides and Tetracyclines. The isolates were classified as susceptible (S), intermediate (I) or resistant (R) according to the Clinical and Laboratory Standards Institute (2015) and to the European Committee on Antimicrobial Susceptibility Testing (2018) guidelines. In according to Magiorakos (2012), *E. coli* isolates non-susceptible to at least one antibiotic in more than two families were considered multidrug-resistant strains.

6.2.4 Genotypic characterization of tetracycline resistance

A preliminary study of genotypic characterization of tetracycline resistance was further performed to identify specific genes of resistance to tetracycline that resulted to be the molecule more frequently used in the buffalo farm chosen for this study.

Genomic DNA extraction of *Escherichia coli* strains was carried out by the commercial Isolate II Genomic DNA kit (Bioline, London, UK) according to the manufacturer's instructions. The quantity and quality of the DNA were assessed by the spectrophotometric reading of the A260/A280 ratio (Eppendorf BioPhotometer 6131). The DNA samples were stored at −20 °C until use.

All 26 tetracycline-resistant strains, isolated from milk samples (18), worker (2) and surface swabs (6), were assessed for carriage of tetracycline-resistance genes encoding the efflux pump mechanism [*Tet*(A), *Tet*(B), *Tet*(C), *Tet*(D), *Tet*(E), *Tet*(G), *Tet*(K)] by PCR. The used primers are shown in Table 6.1. For–PCR reactions, there was always a negative (without bacterial DNA) control. PCR was performed using Biorad T100TM Thermo cycler (BioRad, Hercules, CA).

Table 6.1. Primers used for detection of *tet* genes in *Escherichia coli* strains.

Resistance mechanism	Resistant gene	DNA Primers sequences (5′ to 3′ sense and antisense)	Size of product (bp)	References
Efflux pump	*Tet* (A)	F: GGTTCACTCGAACGACGTCA (20)	577	Torkan et al., 2016
		R: CTGTCCGACAAGTTGCATGA (20)		
Efflux pump	*Tet* (B)	F: CCTCAGCTTCTCAACGCGTG (20)	634	Torkan et al., 2016
		R: GCACCTTGCTCATGACTCTT (20)		
Efflux pump	*Tet* (C)	F: CTTGAGAGCCTTCAACCCAG (20)	418	Ng et al., 2001
		R: ATGGTGGTCATCTACCTGCC (20)		
Efflux pump	*Tet* (E)	F: AAACCACATCCTCCATACGC (20)	278	Ng et al., 2001
		R: AAATAGGCCACAACCGTCAG (20)		
Efflux pump	*Tet* (D)	F: TGTGCTGTGGATGTTGTATCTC (22)	844	Khan et al., 2019
		R: CAGTGCCGTGCCAATCAG (18)		
Efflux pump	*Tet* (G)	F: GCGCTNTATGCGTTGATGCA (20)	803	Khan et al., 2019
		R: ATGCCAACACCCCCGGCG (18)		
Efflux pump	*Tet* (K)	F: GTAGCGACAATAGGTAATAGT (21)	360	Ullah et al., 2012:
		R: GTAGTGACAATAAACCTCCTA (21)		
		R: TCCCACTGTTCCATATCGTCA (21)		

6.3. Results

6.3.1 Identification of *E. coli* strains

Bacteriological examination of the 200 milk samples showed that 31.5% of the isolates were Gram-negative bacteria (79/251) and among 60.7% were *E. coli* strains (48/79). Analysis of the 104 swabs collected from the surfaces before and after milking showed that Gram-negative bacteria were 50.7% of the total isolates

(72/142). From the collected swabs before milking, 10% of the isolated bacteria were *E. coli* strains (6/58): 3 from the teatcups of the milking unit, 2 from the milking parlor wall and 1 from the milking box. 84 bacterial species were isolated from swabs taken after milking, and 29.8% were *E. coli* strains (25/84): 13 strains from the teatcups of the milking unit, 4 strains from the milking parlor wall and 8 strains from the milking box.
Bacteriological examination of the 60 workers' swabs indicated that Gram-negative bacteria were 31% of the total isolates (21/70) and 12.9% were *E. coli* strains (9/70) isolated from the workers' hand swabs. No *E. coli* strains were isolated from the workers' nostrils swabs.

6.3.2 Occurrence of *E. coli* strains and SCC in milk samples

The results of the bacteriological analysis in relation to the SCC displayed that 54 of the 200 milk samples (27%) were defined as 'healthy', having an SCC < 200×10^3 cells/mL and no bacterial growth; 124 samples (62%) were classified as IMI, having an SCC < 200×10^3 cells/mL and bacterial growth; whereas 22 samples (11%) presented SCC values > 200×10^3 cells/mL and bacterial growth compatible with the definition of SCM. No animals with clinical signs of mastitis were observed during the study period.
Evaluation of bacteriological and SCC analysis of all milk samples showed the presence of *E. coli* strains in 6 milk samples (6/22, 27.3%) associated with SCM cases and in 42 milk samples compatible with IMI cases (42/124, 33.9%). The 200 milk samples were haphazardly collected from 120 different animals in different stages of lactation, and among them, 52 animals were sampled two or more times. In one case, animal "8922" was sampled 4 times and *E. coli* strains were identified in 3 milk samples alone or in combination with other bacteria (*Staphylococcus microti* and *Aerococcus viridans*). In addition, on 2 occasions *E. coli* was associated with high SCC ($2.3\text{x}10^5$ and $2.1\text{x}10^5$ cells/mL) and in one with low SCC ($1{,}2\text{x}10^5$ cell/mL). The other 4 samples, associated with SCM cases and *E. coli* growth, had the following SCC values and bacterial growth: 1) $1{,}5\text{x}10^6$ cell/mL and growth of *Aerococcus viridans* strains; 2) $2{,}8\text{x}10^5$ cell/mL and growth of *Staphylococcus simulans* and *Staphylococcus sciuri* strains; 3) $5{,}1\text{x}10^5$ cell/mL and growth of *Aerococcus viridans* and *Rothia amarae*; 4) $2{,}9\text{x}10^5$ cell/mL and growth of *Streptococcus agalactiae* and *Staphylococcus lugdunensis*. In addition, a different antimicrobial resistance profile exhibited by 6 *E. coli* isolates associated with SCM cases, at different time points, leads us to hypothesize that the lineage was not the same.
Table 6.2 displays all 42 *E. coli*-associated IMI cases that exhibited a high level of genus and species identification.

Table 6.2 – Bacterial species identification by MALDI-TOF of 42 milk samples positive for *E. coli* growth with or without other bacterial species. All milk samples presented somatic cell count (SCC) <200 x 10^3 cells/mL, indicative of intramammary infection.

Sample number	SCC/ml	Identified bacterial strains (colony forming unit, CFU)	MALDI-TOF score
1	1.3 x 10^5	*Escherichia coli* (3000 CFU/mL)	2.20
2	6.2 x 10^4	*Escherichia coli* (500 CFU/mL) *Staphylococcus microti* (2000 CFU/mL)	2.23 2.08
3	2.4 x 10^4	*Escherichia coli* (3000 CFU/mL) *Acinetobacter ursingii* *Staphylococcus saprophyticus*	2.07 2.15 1.71
4	7.3 x 10^4	*Escherichia coli* (3000 CFU/mL)	2.26
5	1.2 x 10^5	*Escherichia coli* (3000 CFU/mL) *Bacillus licheniformis*	2.12 1.81
6	1.1 x 10^4	*Escherichia coli* (3000 CFU/mL)	2.29
7	9.2 x 10^4	*Escherichia coli* (500 CFU/mL) *Staphylococcus microti* (3100 CFU/mL) *Staphylococcus saprophyticus* (1400 CFU/mL)	2.47 2.02 1.96
8	1.1 x 10^4	*Escherichia coli* (500 CFU/mL) *Staphylococcus microti* (2000 CFU/mL) *Macrococcus caseolyticus* (500 CFU/mL)	2.27 2.08 2.06
9	2 x 10^4	*Escherichia coli* (200 CFU/mL) *Lactococcus lactis* (6000 CFU/mL) *Aeromonas hyprophyla* (400 CFU/mL)	2.17 2.19 2.11
10	1.2 x 10^4	*Escherichia coli* (2000 CFU/mL) *Aerococcus viridans* (1000 CFU/mL) *Aeromonas hyprophyla* (300 CFU/mL)	2.36 1.89 1.72
11	10	*Escherichia coli* (300 CFU/mL) *Aerococcus viridans* (300 CFU/mL) *Aeromonas hyprophyla* (1000 CFU/mL)	2.07 2.23 1.70
12	2.8 x 10^4	*Escherichia coli* (1000 CFU/mL) *Staphylococcus microti* (1200 CFU/mL) *Enterococcus aquimarinus* (800 CFU/mL)	2.26 2.12 1.76
13	2 x 10^4	*Escherichia coli* (300 CFU/mL)	2.28
14	1 x 10^4	*Escherichia coli* (400 CFU/mL) *Streptococcus agalactiae* (800 CFU/mL)	2.06 2.02
15	1.2 x x 10^5	*Escherichia coli* (300 CFU/mL) *Aerococcus viridans* (400 CFU/mL)	2.04 2.12
16	2.9 x 10^4	*Escherichia coli* (3000 CFU/mL)	2.11
17	3.1 x 10^4	*Escherichia coli* (200 CFU/mL) *Staphylococcus microti* (1000 CFU/mL) *Streptococcus uberis* (700 CFU/mL)	2.29 2.12 2.11
18	1.6 x 10^4	*Escherichia coli* (300 CFU/mL)	2.13
19	1.1 x 10^4	*Escherichia coli* (300 CFU/mL)	2.14
20	1.1 x 10^5	*Escherichia coli* (2500 CFU/mL) *Staphylococcus microti* (500 CFU/mL) *Aerococcus viridans* (100 CFU/mL)	2.13 2.09 1.80

21	8.3×10^4	*Escherichia coli* (300 CFU/mL)	2.29
22	1.4×10^5	*Escherichia coli* (2000 CFU/mL)	2.39
23	1.4×10^5	*Escherichia coli* (10000 CFU/mL)	2.33
24	2.2×10^4	*Escherichia coli* (3000 CFU/mL)	2.27
25	1.5×10^4	*Escherichia coli* (3000 CFU/mL)	2.15
26	1.6×10^4	*Escherichia coli* (4000 CFU/mL)	2.27
27	1.3×10^4	*Escherichia coli* (600 CFU/mL)	2.39
28	3.8×10^4	*Escherichia coli* (200 CFU/mL)	2.28
29	1.1×10^5	*Escherichia coli* (1400 CFU/mL) *Staphylococcus microti* (800 CFU/mL) *Acinetobacter towneri* (300 CFU/mL)	2.24 2.06 1.99
30	6×10^4	*Escherichia coli* (300 CFU/mL) *Macrococcus caseolyticus* (10000 CFU/mL) *Bacillus licheniformis* (200 CFU/mL)	2.01 1.87 2.02
31	2.8×10^4	*Escherichia coli* (500 CFU/mL) *Staphylococcus microti* (1200 CFU/mL) *Bacillus licheniformis* (200 CFU/mL)	2.12 2.12 2.01
32	1.4×10^5	*Escherichia coli* (600 CFU/mL) *Aerococcus viridans* (5000 CFU/mL) *Streptococcus uberis* (9000 CFU/mL)	2.12 1.97 2.03
33	1.1×10^5	*Escherichia coli (800 CFU/mL)* *Staphylococcus microti (700 CFU/mL)* *Aerococcus viridans (4300 CFU/mL)*	2.23 2.09 2.03
34	1.6×10^5	*Escherichia coli (7000 CFU/mL)* *Staphylococcus microti (800 CFU/mL)*	2.19 2.00
35	8.4×10^4	*Escherichia coli (2000 CFU/mL)* *Staphylococcus microti (1000 CFU/mL)*	2.25 2.20
36	6×10^4	*Escherichia coli (200 CFU/mL)*	2.29
37	4.9×10^4	*Escherichia coli (1100 CFU/mL)* *Aerococcus viridans* (3300 CFU/mL)	2.26 1.81
38	8×10^4	*Escherichia coli (3000 CFU/mL)* *Acinetobacter tandoii* (3000 CFU/mL)	2.22 1.70
39	3.3×10^4	*Escherichia coli (300 CFU/mL)* *Staphylococcus microti (200 CFU/mL)* *Aerococcus viridans (900 CFU/mL)*	2.24 2.09 1.88
40	4.3×10^4	*Escherichia coli (400 CFU/mL)* *Acinetobacter johnsonii* (6000 CFU/mL)	2.02 2.00
41	1.6×10^5	*Escherichia coli* (200 CFU/mL) *Aerococcus viridans* (600 CFU/mL)	2.11 1.96
42	1.4×10^5	*Escherichia coli* (500 CFU/mL) *Aerococcus viridans* (300 CFU/mL)	2.08 2.23

6.3.3 *E. coli* O157:H7 latex agglutination test

After the waiting time, the latex agglutination reagent suspension remained smooth and white. Latex agglutination tests had a negative reaction for all tested *Escherichia coli* strains, indicating an absence of *E. coli* O157:H7 strains harmful to animal and human health.

6.3.4 Phenotypic resistance profiles and genotypic characterization of tetracycline resistance

Antimicrobial susceptibility testing of 48 *E. coli* strains isolated from milk samples showed complete resistance to penicillin and clindamycin (100%) and a high resistance value toward erythromycin (87.5%, 42/48). All strains were sensitive to ciprofloxacin (100%), the majority of them were sensitive to gentamicin and sulfamethoxazole-trimethoprim (89.6%, 43/48), followed by amikacin (85.4%, 41/48), kanamycin (75%, 36/48), and streptomycin (54.2%, 26/48). Regarding carbapenems antibiotics, more than half of the strains were sensitive to imipenem and meropenem (66.7%, 32/48 and 54.2%, 26/48). Tetracycline and oxytetracycline were the antibiotics with the highest number of strains with intermediate susceptibility (62.5%, 30/48), followed by amoxicillin-clavulanate (43.7%, 21/48).
Antimicrobial susceptibility profile of 31 *E. coli* strains isolated from surface swabs showed complete resistance to penicillin (100%), followed by clindamycin (96.8%, 30/31) and erythromycin (80.6%, 25/31). All strains were sensitive to ciprofloxacin and sulfamethoxazole-trimethoprim (100%). Regarding aminoglycoside antibiotics, high levels of susceptibility were observed for amikacin (87.1%, 27/31), gentamicin (80.6%, 25/31), kanamycin (67.7%, 21/31), streptomycin (45.2%, 14/31), followed by carbapenem antibiotics meropenem (67.7%, 21/31) and imipenem (54.8%, 17/31). Oxytetracycline and tetracycline were the antibiotics with the highest number of strains with intermediate susceptibility being (83.9%, 26/31) and (67.7%, 21/31), respectively, followed by amoxicillin-clavulanate (48.4%, 15/31).
Antimicrobial susceptibility testing of 9 *E. coli* strains isolated from workers' hand swabs showed complete resistance to penicillin and clindamycin (100%) and high resistance value to erythromycin (88.9%, 8/9) and amoxicillin-clavulanate and meropenem (55.5%, 5/9). All 9 strains displayed complete susceptibility to ciprofloxacin (100%), while most were susceptible to gentamicin (88.9%, 8/9), sulfamethoxazole-trimethoprim and imipenem (77.8%, 7/9), amikacin (66.7%, 6/9) and streptomycin (44.4%, 4/9). The isolates showed complete intermediate susceptibility to oxytetracycline (100%), followed by tetracycline (77.8%, 7/9) and kanamycin (62.5%, 5/8).

Figures 6.1, 6.2 and 6.3 show the antimicrobial susceptibility pattern of all bacterial isolated from milk samples and swabs, classifying them as susceptible (S), intermediate (I) or resistant (R).

In addition, a worrying finding is the high prevalence of *E. coli* strains with a multidrug-resistance (MDR) profile. 95.4 % of the total isolates (84/88) showed resistance to at least three classes of antibiotics. Precisely, 47 strains were from milk samples, 29 from milking parlor surface swabs and 8 from workers' hand swabs. The 84 MDR *E. coli* strains show 34 different phenotypes (Table 6.3).

Workers are in daily contact with animals and milking parlor surfaces and are exposed to an increased risk of contracting zoonoses caused by MDR *E. coli* strains. Antibiotic resistance profiles of MDR strains were cross-checked and four patterns, belonging to 5 different *E. coli* strains isolated from workers' hands, matched with the profiles of 14 strains isolated from milking parlor surfaces and 20 strains isolated from milk samples. The resistance profile most found exhibited resistance to P, DA, E (22.7%, 20/84 strains). Precisely, with this profile there were 8 strains isolated from milk, 1 from the hands of operators and 11 from surface swabs.

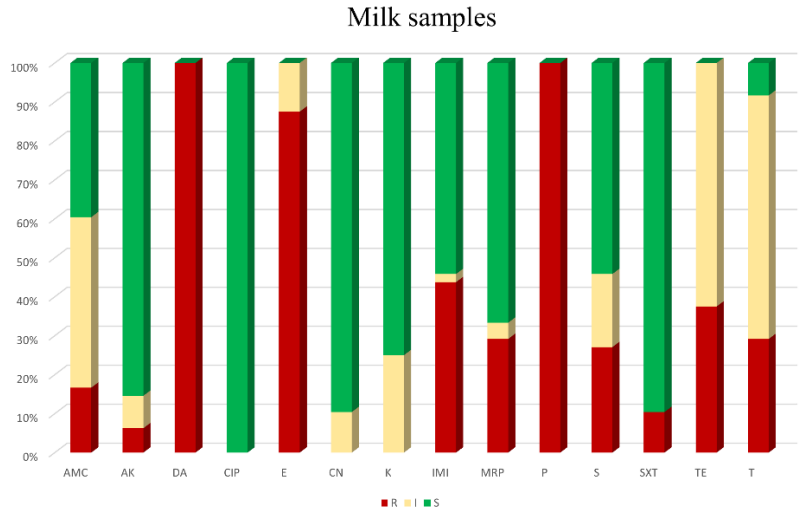

Figure 6.1 - Antibiotic resistance profiles of 48 *E. coli* strains isolated from milk samples. Tested antibiotics: amoxicillin–clavulanate (AMC), amikacin (AK), clindamycin (CD), ciprofloxacin (CIP), erythromycin (E), imipenem (IMI), gentamicin (CN), kanamycin (K), meropenem (MRP), penicillin (P), sulfamethoxazole–trimethoprimprim (SXT), tetracycline (TE), oxytetracycline (T) and streptomycin (S).

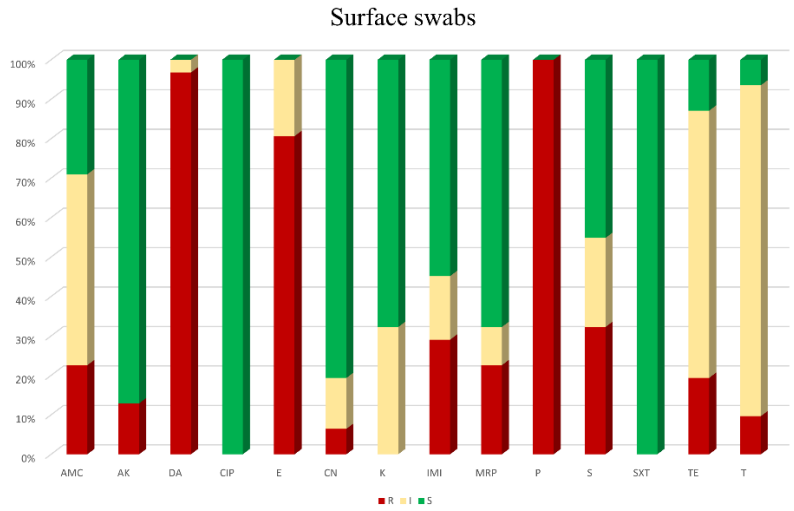

Figure 6.2 - Antibiotic resistance profiles of 31 *E. coli* strains isolated from milking parlor surface swabs. Tested antibiotics: amoxicillin–clavulanate (AMC), amikacin (AK), clindamycin (CD), ciprofloxacin (CIP), erythromycin (E), imipenem (IMI), gentamicin (CN), kanamycin (K), meropenem (MRP), penicillin (P), sulfamethoxazole–trimethoprimprim (SXT), tetracycline (TE), oxytetracycline (T) and streptomycin (S).

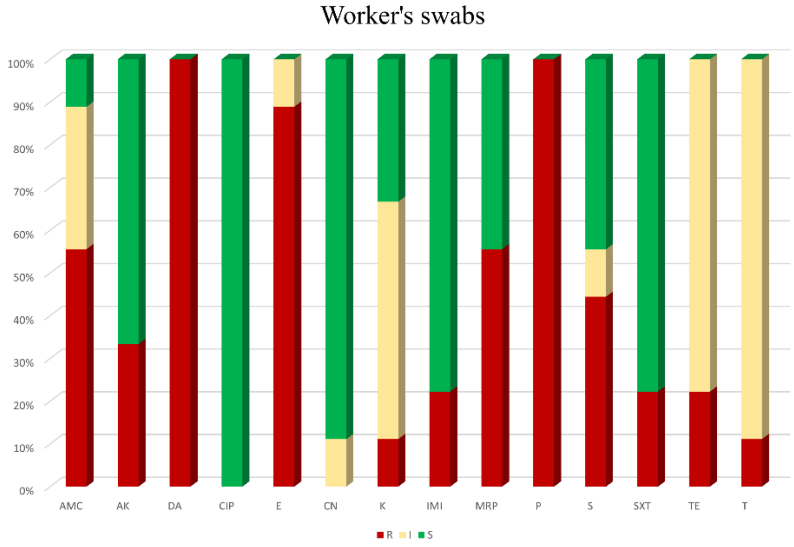

Figure 6.3 - Antibiotic resistance profiles of 9 *E. coli* strains isolated from workers' hand swabs. Tested antibiotics: amoxicillin–clavulanate (AMC), amikacin (AK), clindamycin (CD), ciprofloxacin (CIP), erythromycin (E), imipenem (IMI), gentamicin (CN), kanamycin (K), meropenem (MRP), penicillin (P), sulfamethoxazole–trimethoprimprim (SXT), tetracycline (TE), oxytetracycline (T) and streptomycin (S).

Table 6.3 - Antibiotic resistance pattern of 84 multidrug-resistant *E. coli* strains.

Antibiotic resistant pattern	N.	%
P, DA, E (*)	19	22.6
P, DA, E, IMI (*)	14	16.7
P, DA, IMI	4	4.8
P, DA, E, IMI, MRP, S	4	4.8
P, DA, E, TE, T	3	3.6
P, DA, AMC, E, MRP, TE, T	3	3.6
P, DA, AMC, E, MRP, S (*)	3	3.6
P, DA, AMC, AK, E, MRP, S, SXT, TE, T (*)	3	3.6
P, DA, E, IMI, TE, T	2	2.3
P, DA, E, MRP	2	2.3
P, DA, E, S	2	2.3
P, DA, E, IMI, S	2	2.3
P, DA, AMC, AK, E, CN, IMI, S, TE	2	2.3
P, DA, AMC, AK, E, TE, T	1	1.2
P, DA, AMC, AK, E, K, MRP, S, TE	1	1.2
P, DA, AMC, AK, E, MRP, S	1	1.2
P, DA, AMC, AK, E, MRP, S, SXT, TE	1	1.2
P, DA, AMC, E	1	1.2
P, DA, AMC, E, TE, T	1	1.2
P, DA, AMC, E, MRP	1	1.2
P, DA, AMC, E, MRP, SXT	1	1.2
P, DA, AMC, E, MRP, S, SXT, TE, T	1	1.2
P, DA, AK, E, S, TE	1	1.2
P, DA, MRP, S, SXT, TE, T	1	1.2
P, DA, E, MRP, S, SXT, TE, T	1	1.2
P, DA, IMI, MRP, S, TE, T	1	1.2
P, DA, E, IMI, MRP, S, T	1	1.2
P, DA, IMI, T	1	1.2
P, DA, E, IMI, TE	1	1.2
P, DA, E, S, TE	1	1.2
P, DA, TE	1	1.2
P, DA, E, TE	1	1.2
P, MRP, S	1	1.2
P, DA	1	1.2

* = Pattern of isolated strains from workers' hands corresponding with some of the isolated strains' profiles from milk and surface samples.

The results of the preliminary study on genotypic characterization of tetracycline resistance of the 26 *E. coli* strains isolated from the samples showed that 22 strains of them had at least one of the tested genes (Table 6.4).
The association of *Tet*(A) and *Tet*(G) genes was the most relevant and was found 5 times in isolates from milk samples and once in isolates from milking parlor surface swabs. The *Tet*(G) gene alone was found 6 times: 4 times in milk samples, 1 from

surface swabs and 1 from swabs of operators' hands. The other genes detected twice in the milk samples were: *Tet*(B+G), *Tet*(C), *Tet*(D+G) and *Tet*(D). *Tet*(D) and *tet*(E) genes were identified from the surfaces and workers' swabs, respectively. No *tet*K gene was detected.

Table 6.4 - Tet genes detected from *Escherichia coli* samples.

Milk samples		Surface swabs		Workers' swabs	
Tet(A+G)	5	*Tet*(A+G)	1	*Tet*(E)	1
Tet(G)	4	*Tet*(D)	1	*Tet*(G)	1
Tet(C)	2	*Tet*(G)	1		
Tet(D+G)	2				
Tet(D)	2				
Tet(B+G)	2				

6.4 Discussions and conclusions

The quality of milk is determined by aspects relating to its composition and hygiene. Pathogenic and environmental bacteria that enter the udder or accumulate in the milk due to poor hygiene or inadequate milking techniques result in milk contamination and altered hygiene status. In fact, bacteria on the skin of the teats act as a reservoir of microbes that can contaminate milk during milking (Vacheyrou et al., 2011) as well as tools. Bacteria in milk are able to grow and multiply using milk as an excellent breeding medium due to its nutritional composition and high-water activity.

E. coli strains are able to pass into the mammary gland through the teat canal and are among the most frequently isolated coliforms in intramammary infections and mastitis cases (Fahim et al., 2019). The bacteriological examination of milk samples collected during the ten samplings showed that 48 out of 200 milk samples (24%) were contaminated with *E. coli* strains. Our results are nearly similar to those reported by Silva et al. (2001) and Awadallah et al. (2016), who isolated *E. coli* strains in 22.1% and 22.4% of the milk samples collected, respectively.

Younis et al. (2021) found less contamination by *E. coli* strains in raw milk by isolating them in 10% of samples and Kumar and Prasad (2010) in 8.14%. More contaminated samples were shown by Mansour et al. (2013) and Fahim et al. (2019) who isolated *E. coli* strains in 50% and 47% of the sampled animals, respectively.

Analysis of 104 milking parlor surface swabs showed that 6 swabs collected before milking were positive for the *E. coli* growth (5.8%): 3 from the teatcups of the milking unit, 2 from the milking parlor wall and 1 from the milking box. Whereas *E. coli* isolates were detected from 25 swabs taken after milking (24%): 13 from the

teatcups of the milking unit, 4 from the milking parlor wall and 8 from the milking box.

E. coli isolation, generally found in manure, the environment and soil, provides indications of the cleanliness and hygienic conditions of the milking parlor and used milking tools. *E. coli* strains were isolated before the milking time and their presence quadrupled after milking. In the milking unit, 3 strains were isolated before milking and 13 strains were isolated at the end, clearly indicating that the cleaning routines of the milking parlor and animals were not properly performed.

Because workers are in contact with animals and milking parlor surfaces, they are more exposed to bacteria on the farm and potentially transferable to humans during daily activities. Bacteriological analysis of swabs obtained from workers allowed to study of cultured strains. *E. coli* strains were isolated in 15% of the swabs (9/60) and always from operator hands.

When the udder has a mastitis case, both the microbial load and the number of somatic cells increase significantly. In our study, no animals had clinical signs, positive bacteriological examination and an SCC value > $200x10^3$ cell/mL related to clinical mastitis cases. Among the milk samples obtained from animals without clinical signs, 22 out of 200 milk samples (11%) were bacteriologically positive and had SCC values above the indicated threshold (>$200x10^3$cells/mL) compatible with the definition of SCM, while 124 out of 200 milk samples (62%) had SCC values <$200x10^3$ cells/mL and bacterial growth compatible with the definition of IMI.

Our result is very similar to that found by Abdul wahid (2018) who reported 27.7% of *E. coli* strains isolated from buffaloes with SCM.

Lower values have been reported from previous studies indicating that *E. coli* was the most frequently isolated bacterial pathogen (19%, 14% and 13%) from animals with mastitis (Ali et al., 2021; Gao et al., 2017; Bhat et al., 2017).

Antibiotics are key to limiting disease cases and improving animal productivity, animal welfare and food safety of products (Nobrega et al., 2017). However, their use over time has led to an increase in antimicrobial resistance, making it essential to evaluate the phenotypic resistance profile of strains isolated from animals with mastitis cases to develop appropriate and treatment strategy.

In the current study, the highest resistance of bacterial isolates was observed against penicillin (100%) and clindamycin (98.9%, 87/88), in accordance with previous studies on *E. coli* isolated from milk samples which showed to be completely resistant to penicillin (Younis et al., 2021; Yu et al., 2019) and with significant levels of resistance to clindamycin (Younis et al., 2021; Tadesse et al., 2018).

The energy-dependent efflux pump system is the common mechanism in Gram-negative bacteria and is encoded by the *tet*A, *tet*B, *tet*C, *tet*D and *tet*G genes (Skočková et al., 2012).

Genotypic study of tetracycline resistance of phenotypically tetracyclin-resistant *E. coli* strains revealed that the main resistance genes were tet(A+G) and tet(G) both

isolated 6 times. In other studies, however, the genes that mainly conferred tetracycline resistance were the tet(A) and tet(B) genes (Younis et al., 2021; Jajarmi et al., 2017).

Bacteria resistance to antimicrobials affects animal health and is a growing concern in veterinary medicine.

Overuse of antimicrobials in animals led to selective pressure, intensifying favorable mutations in bacteria and developing antimicrobial resistance that can spread to humans through the food chain (Pokharel et al., 2020).

Multidrug resistance (MDR, resistance to at least three classes of antibiotics) of *E. coli* strains is also particularly alarming (Klemm et al., 2018). *E. coli* strains isolated from our samples showed a worrisome result regarding multidrug resistance. In fact, 95.4% of strains showed an MDR profile. Precisely, 47 strains were isolated from milk samples, 29 from milking parlor surface swabs and 8 from workers' hand swabs.

In addition, antibiotic resistance profiles of MDR strains were cross-checked. Four antibiotic patterns, belonging to 5 different *E. coli* strains isolated from workers' hands, matched the profiles of 14 strains isolated from milking parlor surfaces and 20 strains isolated from milk samples.

These data indicate that these bacteria spread easily in the environment, threatening the health of animals and humans.

Routine milking practices, improved milking parlor hygiene conditions and the use of protective devices for workers are highly recommended because they would help to reduce the pathogenic and environmental bacterial load on udder skin and parlor surfaces, prevent the entry of mastitis-causing bacteria and reduce the possibility of transmission to workers.

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7. Conclusions

One of the current challenges in animal breeding is to reduce the incidence of mastitis, which is a disease that alters animals' welfare and health status. Udder inflammation is due to many factors, but it is mainly caused by bacteria (Nagasawa et al., 2019). In general, antimicrobial treatment is essential to restore the udder health of large and small ruminants and avoid significant economic losses. On the contrary, the emergence and spread of antimicrobial resistance (AMR) is an urgent matter of public attention, and consequently, antimicrobial use in production livestock is a critically discussed topic.

In this scenario, the present PhD project involved the setting up of an innovative tool (Biovitae® lights) that fits fully in the "Health" thematic area of the national plan for national research and into the health, nutrition and quality of life area of the National Smart Specialization Strategy (SNSI) approved by the European Commission.

In particular, the research followed the main steps regarding:

- The choice of the buffalo farm in whose milking parlor the lighting devices would be installed by Nextesense s.r.l.
- Isolation and identification of etiologic agents from milk samples associated with buffalo mastitis, from milking parlor surface swabs, and from workers' hand and nostril swabs collected before, during, and after the installation of the lights
- Evaluation of the antimicrobial susceptibility profiles of the bacterial strains isolated from samples
- Evaluation of the efficacy of light devices *in vitro* and *in vivo*

In Chapter 1, *in vitro* action of Biovitae® lights was described. Three bacterial strains isolated from the chosen buffalo farm were used for these experiments. Precisely, the experiments carried out with *E. coli* strain and *S. microti* strain isolated from milk samples, and *S. aureus* strain isolated from workers' nostril swabs, showed that *in vitro* exposure of strains to two different lights (Master light strip and light bulb) had an antibacterial effect, statistically significant ($P \leq 0,05$) on *E. coli* and *S. aureus* strains after 4 hours exposure. The bacterial reduction for the *S. microti* strain was not statistically significant after 4 hours of exposure to both light devices. Furthermore, two reducing agents, DTT (Dithiothreitol) and GSH (Glutathione), have been tested separately for their ability to reduce the presence of and damage caused by highly reactive species. Their association to bacterial cultures showed a redox-sensitive mechanism of action that reduced the photodynamic microbicidal effect of Biovitae® light. This data led us to hypothesize that the light action is related to the overproduction of ROS, resulting in oxidative stress and bacterial cell damage.

In Chapter 2, experiments conducted *in vivo* on a buffalo farm with lactating animals have been described. The study involved the monitoring of the light efficacy in the milking parlor, and for this purpose milk samples, milking parlor surface swabs (milking boxes, teatcups of the milking unit and room walls) and workers' hand and nostril swabs were collected ten times. Samples taken before and after the Master light strip installation showed the trend of bacterial isolates during the study period. The results show that the light action, progressively reduced the presence of Gram-negative bacteria, while the Gram-positive bacterial load fluctuated during all sampling. Importantly, when the Biovitae® light action was stopped, and the two samplings were performed, the CFU and the number of isolated bacterial species significantly increased, demonstrating the antimicrobial role of lights *in vivo*.

The little-known strain of *Staphylococcus* genus, among non-aureus staphylococci (NAS), *Staphylococcus microti,* was often detected in the collected samples. For this reason, extensive investigations, described in Chapter 3, were carried out on this bacterial strain. The identification of n. 54 strains of *S. microti*, isolated from buffalo milk and milking parlor surface samples, were performed by MALDI-TOF MS and whole 16S rRNA gene sequencing. Their phenotypic resistance profiles were evaluated by a disk diffusion method, and the tetracycline resistance of the strains (100%) was studied by genotypic characterization. Genotypic analysis, performed for the *tet*M and *tet*K genes by multiplex PCR, detected the presence of the *tet*M gene in all isolated *S. microti* strains. In addition, SCC was performed and the association of this value with the presence of S. microti strains showed that 37.9% of the strains isolated from milk were associated with IMI cases and 18.2% with SCM cases. The presence of this bacterium in the milk of buffaloes in the absence of evident mastitis clinical signs underlines the need for further studies, and its finding on milking parlor surfaces suggests that the environmental quality of the milking parlor plays an important role in influencing both *S. microti*'s spread and the microbial communities of the milk. This study, subject of the publication in Animals (Ambrosio et al., 2023) highlights that *S. microti* may be commonly isolated from dairy buffalo milk and milking parlor equipment, whereas its association with SCM or IMI requires further study.

Another mammary pathogenic bacterium is *Escherichia* (*E. coli)*, one of dairy cattle's main etiologic agents of acute clinical mastitis. Chapter 4 concerns the study of 88 *E. coli* strains isolated from all collected samples in this study. The choice to deepen the study on *E. coli* strains, generally found in manure, the environments, and soil, was linked to the isolation of numerous strains with a high microbial load indicative of substandard hygienic and management conditions of the milking parlor and the used milking tools. Strains isolated from milk, surfaces, and workers' hands were tested for antimicrobial susceptibility, showing a high level of

resistance to penicillin (100%) and clindamycin (98.9%). Resistance (29.5%) profile to tetracycline, frequently used in antibiotic therapy in mastitis cases, encoded by several genes identified by genotypic characterization. Genotypic study revealed the presence of *tet* genes in 22 analyzed strains of 26 *E. coli* strains resistant to tetracycline. The mainly detected genes were *tet*A+G and *tet*G isolates 6 times, respectively, followed by *tet*D genes found 3 times, *tet*B+G, *Tet*C, *Tet*D+G twice, and *Tet*E gene once. In addition, a large proportion of *E. coli* strains exhibiting intermediate susceptibility to tetracycline (65.9%) and negative to genotypic investigations may be a signal of emerging resistance that requires monitoring studies and further genetic investigations. The extensive use of tetracycline in the treatment of human and animal infections has led over time to a wide range of resistant strains in Gram-positive and Gram-negative bacteria. These strains can spread easily and act as reservoirs of resistance genes, transferring them to pathogenic bacteria and causing increasing problems in the treatment of infectious diseases (Michalova et al., 2004). The genotypic characterization of *S. microti* and *E. coli* strains, phenotypically tetracycline-resistant, involved the detection of several genes encoding resistance. In Gram-positive bacteria, resistance to tetracycline is typically conferred by genes with a ribosomal protection mechanism, such as the *tet*M gene that is commonly isolated in NAS (Bryan et al., 2004, Schwarz and Chaslus-Dancla, 2001, Duran et al., 2012). Genes encoding for the efflux pump mechanism (*tet*A, *tet*B, *tet*C, *tet*D and *tet*G genes) are most frequently detected in Gram-negative bacteria (Jahantigh et al., 2020).

In conclusion, the results obtained from the antimicrobial photodynamic action produced by the LED devices are encouraging both *in vitro*, in controlled experimental conditions, and *in vivo*, in an environment such as the milking parlor on which several factors have an influence in increasing the present microbial flora. Biovitae® lamps exhibited an *in vitro* antimicrobial potential against *Escherichia coli*, *Staphylococcus microti* and *Staphylococcus aureus*. The result can be defined satisfactory, although the milking parlor hygienic conditions, animal cleaning practices, attention to milking routines, and milking machine integrity are always the main critical points. However, the use of Biovitae® lamps in milking parlor could be a valid support to further improve the cleanliness of environment and represent an innovative approach to control bacteria capable of causing mastitis.

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