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Full Length Research Paper

Effectiveness of temocillin on extended-spectrum beta-lactamase producing *Escherichia coli* isolates from patients attending the Niamey General Reference Hospital

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Extended-spectrum beta-lactamase (ESBL)-producing *Escherichia coli* urinary tract infections represent a major global health problem, constituting a public health emergency with high patient morbidity and mortality. Temocillin serves as an alternative antibiotic for treating these infections and conserving carbapenems. A total of 209 isolates of uropathogenic *Escherichia coli* were collected in the Microbiology Laboratory of Niamey General Reference Hospital. The isolates underwent analysis using standard bacteriological methods. Identification and antibiotic susceptibility testing of *Escherichia coli* isolates were determined using the VITEK-2 system with the GN and AST-N372 cards. Of these isolates, 104 were subjected to ESBL testing. ESBL testing was performed with antibiotic discs of amoxicillin/clavulanic acid (20/10 µg), ceftazidime (30 µg), aztreonam (30 µg), and cefotaxime (30 µg) on Mueller Hinton agar, following the CA-SFM recommendations for 2019. Isolates that did not exhibit an ESBL phenotype were further tested on Mueller Hinton agar with cloxacillin for ESBL associated with AmpC cephalosporinase. Data were recorded and analyzed using EPI INFO software version 7.2.2.6. Microsoft Word and Excel software were utilized for word processing and figure creation. Out of the 209 Escherichia coli isolates, only 104 exhibited ESBL phenotypes, distributed as follows: 91/104 (43.54%) on Mueller Hinton agar and 13/104 (6.22%) on Mueller Hinton agar with cloxacillin, while the remaining 105 isolates did not produce ESBL (50.24%). The sensitivity of ESBL Escherichia coli isolates to temocillin was 62.5%. Temocillin demonstrated good activity against ESBL *Escherichia coli* isolates. However, it is recommended that empirical treatment with temocillin be included in the guidelines, making it an alternative antibiotic for conserving carbapenems in the treatment of urinary tract infections. Additionally, it can also be used against isolates producing AmpC enzymes.

Key words: Extended Spectrum Beta-lactam (ESBL), *Escherichia coli*, Temocillin, effectiveness, Niger.

INTRODUCTION

Extended-spectrum beta-lactamase (ESBL)-producing *Escherichia coli* (*E. coli*) infections are a major global health problem, constitute a public health emergency and result in high morbidity and mortality in certain patients (Akpaka et al., 2021). Resistance to beta-lactams through the production of extended-spectrum beta-lactamases was first reported in the early 1980s in Europe, then in the United States, shortly after the introduction of third-generation cephalosporins into clinical practice. (Cantón et al., 2008a). The frequency of *E. coli* ESBL is fairly high in both hospital and community settings, and their incidence varies from one country to another (Cantón et al., 2008b; Seydou, 2021; Silago, 2021). Rising levels of *E. coli* ESBL have reduced treatment options to a limited number of antibiotics (Pitout, 2010). Extended-spectrum beta-lactamases are a group of enzymes that can hydrolyze a variety of beta-lactams, including fourth-generation cephalosporins, and compromise the efficacy of all beta-lactams except cephamycins and carbapenems (Saravanan et al., 2018). Beta-lactamases are encoded by genes that are mainly mediated by plasmids (Jena et al., 2017a). ESBL genes are classified into three main types. These are the temoneira (TEM) and sulfhydryl variable (SHV) enzymes, the Munich cefotaximase (CTX-M) (Manoharan et al., 2011). TEM (183), SHV (134) and CTX-M (103) variants are derived from point mutations in their parent genes (Nandagopal et al., 2015). Temocillin is a beta-lactamase-resistant penicillin. It is the 6-alpha-methoxy derivative of ticarcillin, synthesized in the early 1980s (Habayeb et al., 2015; Kuch et al., 2020; Soubirou et al., 2015). This antibiotic is used as an alternative treatment for urinary tract infections caused by ESBL-producing *Enterobacteriaceae* (Alexandre et al., 2018; Lacroix et al., 2021), but also AmpC-type enzymes encoded by plasmids (Kresken et al., 2021). Temocillin is stable against most beta-lactamases and AmpC enzymes, with the exception of metallo-carbapenemases (class B metalloenzymes) and OXA enzymes (Sacco et al., 2019). Temocillin is approved at doses of 4 to 6 g/day (in 2 to 3 divided doses or as a continuous infusion) (Heard et al., 2021). First-line temocillin treatment before carbapenems prevents the development of carbapenem resistance (Lupia et al., 2022). The aim of our study was to provide data on the activity of temocillin against *E. coli* ESBL isolates involved in urinary tract infections.

MATERIALS AND METHODS

Type, period and site of study

This was a prospective, descriptive study conducted on urine samples from patients received at the Microbiology Laboratory of the Niamey General Reference Hospital. Urine samples were received from January to December, 2021.

Bacteriological analysis

Macroscopic examination

The appearance of the patients' mid-jet urine in the containers was assessed macroscopically with the naked eye in a clear space to look for any changes in the urine.

Microscopic examination

Quantitative white and red cell cytology: White blood cells and red blood cells were counted in a KAVA SLIDE cell. To quantify the cells, the mid-jet urine was homogenized in the collection vessels, then the homogenized urine was introduced into the cell wells and deposited for 5 min to immobilize the cells inside the wells, after which the cells were counted under the microscope at objective X40 following the reading method in Table 1.

Qualitative cytology: To separate the supernatant from the urine pellet, patients' urine was placed in hemolysis tubes and centrifuged at 3,000 rpm for 5 min. Figurative elements in patients' urine pellets were assessed crosswise, following microscopic observation of the urine pellet between slides at X 40. These included renal epithelial cells (round), vesical cells (racket-shaped), squamous or endothelial cells (plated), hyaline, fatty, granular, hematic, leukocytic or epithelial cylinders, phosphate, oxalate or urate crystals of drug origin.

Inoculation of urine samples

Fresh patient urine was systematically inoculated onto CLED and Uriselect4 culture media using a 10 µL calibrated plastic loop by the streak method, and then incubated in an oven at 37°C for 24 h.

Gram staining of bacteria

Gram staining was performed on all bacterial growths obtained after 24 h incubation in an oven. Smears of the bacterial strains were mixed with sterile water in 10 ml ampoules, dried at room temperature, then passed over a flame to fix them and placed on the staining rac for Gram staining, following the manufacturer's recommendations. Gram staining is used to differentiate Gram-positive bacteria (stained purple) from Gram-negative bacteria (stained pink) under an X100 immersion oil microscope. The latter are the focus of our study.

Enterobacteriaceae identification method on vitek-2

Following isolation of Gram-negative bacilli, enterobacterial isolates were identified using the vitek-2 instrument. First, isolates were purified on agar media and used for identification. To do this, we aspirated 3 ml volumes of saline solution and introduced them into each of the numbered hemolysis tubes placed in a cassette. The

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Table 1. Urine cell counting method.

Variable	Few cells	Numerous cells	Large number of cells
Counting on	9 large squares	1 single large tile	1 single small tile
Number of cells counted	n	n	n
Cellular concentration	n cells / mL	n × 10 cells / mL	n × 100 cells / mL

hemolysis tubes were inoculated with a few colonies of the purified isolates, the hemolysis tubes were shaken and mixed until we had a bacterial suspension, then this suspension was measured with a densitometer to look for an optical density of 0.5 Mac.Farland. Once the 0.5 Mac Farland bacterial suspensions had been made, the GN vitek cards were inserted into the hemolysis tubes, lab ID numbers were assigned to each of the hemolysis tubes containing the bacterial suspensions of the isolates to be identified, and patient files were created on the vitek-2 for the patients from whom the strains had been isolated, with the same lab ID numbers assigned to the hemolysis tubes. After completing this step, the cassettes were inserted into the first chamber of the instrument to introduce the bacterial suspension into the vitek-2 cards, then transferred to the second chamber for loading and incubation in the instrument. The wells of the ID cards were read after every 15 min to check whether or not there was any biochemical reaction between the isolates and the substrates contained in the wells and then the software integrated into the instrument generated a code through the positivity or negativity of the biochemical reactions in the wells and finally the software compared the code generated with predefined codes that corresponded to bacterial species integrated into the CA-SFM/ EUCAST 2019 V2 database software.

Method for performing antibiograms of *E. coli* isolates on vitek-2

Once the *Enterobacteriaceae* had been identified, antibiograms of *E. coli* species were performed on the vitek-2 instrument. Firstly, isolates were purified on agar media and used to test the activities of temocillin and other antibiotics on the *E. coli* species identified. To do this, we aspirated 3 ml volumes of saline solution and introduced them into hemolysis tubes numbered and labelled ID and AST for each *E. coli* isolate and placed in a cassette. The first hemolysis tubes of each isolate were inoculated with a few colonies of the purified isolates, the hemolysis tubes were shaken and mixed until a bacterial suspension was obtained, then this suspension was measured with a densitometer to look for an optical density of 0.5 Mac.Farland. After making bacterial suspensions of 0.5 Mac. Farland, 145 µl of each bacterial suspension was transferred into the second hemolysis tubes of each AST-labeled isolate. The tubes were then shaken and vortexed, the first hemolysis tubes used to make the bacterial suspensions were removed, and the AST-N372 cards were introduced into the AST-labeled hemolysis tubes, lab ID numbers were assigned to each of the AST hemolysis tubes containing 3 ml saline solution + 145 µl parent bacterial suspension of the isolates, and patient files were created on vitek-2 for the patients from whom the strains had been isolated, with the same lab ID numbers assigned to the hemolysis tubes. After completing this step, the cassettes were inserted into the first chamber of the instrument to introduce the new bacterial suspension into the AST-N372 cards, then transferred to the second chamber for loading and incubation in the instrument. The wells of the AST-N372 cards were read after every 15 min to check whether or not there was bacterial growth despite the presence of different concentrations of antibiotic containing in the wells of the AST card and then the instrument measures the turbidity of the bacterial suspension every 15 min and compares the result to the turbidity of a control well containing no antibiotic and then the data is transmitted to the software integrated into the instrument to categorize the antibiotics either Sensitive, Intermediate or Resistant according to MIC with CA-SFM/ EUCAST (2019).

A total of 209 isolates of uropathogenic *E. coli* were collected at the Microbiology Laboratory of the Niamey General Reference Hospital. Isolates were analyzed using standard bacteriological methods. Identification and antibiotic susceptibility of *E. coli* isolates were determined on the vitek-2 system using the GN and AST-N372 card (Torres-Sangiao et al., 2022).

ESBL phenotype determination

To investigate the ESBL phenotypes of *E. coli* isolates showing resistance to third-generation cephalosporins at antibiotic susceptibility testing, we prepared bacterial suspensions with each isolate and measured the optical densities of the suspensions to bring them to 0.5 Mac. Farland with a densitometer; after 15 min, these suspensions were inoculated onto Muller Hinton culture media, and after 15 min, the following antibiotic discs were applied to the agar media: amoxicillin/clavulanic acid (30 µg), ceftazidime (30 µg), ceftriaxone (30µg), aztreonam (30 µg) and cefotaxime (30 µg), as shown in Figure 1. The distance between two antibiotic discs was 3 cm. Media plates were incubated at 37°C in the oven for 24 h according to the recommendations of CA-SFM, 2019 (CA-SFM/EUCAST, 2019) . ESBL phenotypes were read macroscopically by observing one or more synergistic images that appeared as champagne corks between the antibiotic discs. *E. coli* isolates that did not express ESBL phenotypes were subjected to a complementary test on Mueller Hinton medium supplemented with cloxacillin, to look for ESBL production associated with an AmpC cephalosporinase, following the same steps as above. (Figure 1) (Tiemtoré et al., 2019).

Antibiotic susceptibility testing of *E. coli* ESBL

The minimum inhibitory concentrations (MICs) of temocillin and 7 other antibiotics were studied on Vitek-2: amoxicillin/clavulanic acid, piperacillin/tazobactam, ceftazidime, ceftriaxone, cefixime, ertapenem and imipenem.

Data analysis

Data were recorded using EPI INFO software version 7.2.2.6. Statistical analyses were carried out using EPI INFO. A p value of < 0.05 was considered statistically significant. Word and Excel software were used for text processing and figure preparation.

Ethics

Written informed consent was obtained from all patients prior to the

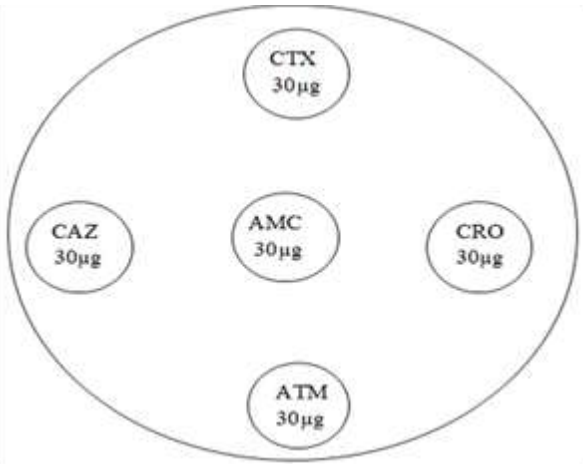

Figure 1. Disposition of antibiotic discs for synergy testing.
Source: Tiemtoré et al. (2019).

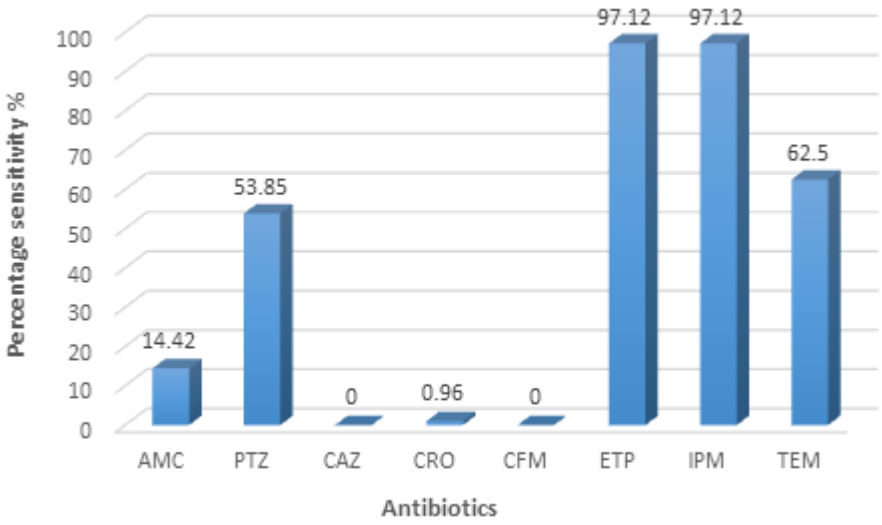

Figure 2. Susceptibility (%) of *E. coli* ESBL to various antibiotics.AMC = Amoxicillin/clavulanic acid, PTZ = Piperacillin/tazobactam, CAZ = Ceftazidime, CRO = Ceftriaxone, CFM = Cefixime, ETP = Ertapenem, IPM = Imipenem, TEM = Temocillin.

study. To protect patient anonymity, numbers were assigned to all patient samples. Written authorization was obtained from the authorities of the General Reference Hospital (Reference Code: 000744/DG/HGR/DA/S/SER) for the conduct of this study.

RESULTS

During the study period, two hundred and nine (209) *E. coli* were isolated. Of the 209 *E. coli* isolates, only 104 were tested for ESBL, 91 isolates produced ESBL on Muller Hinton medium without additives with a prevalence of 43.54%, 13 isolates expressed ESBL on Muller Hinton/cloxacillin medium with a prevalence of 6.22%, and the remaining 105 isolates were ESBL-negative (50.24%) (Table 2). The sensitivity of *E. coli* ESBL isolates to temocillin was 62.5%. Susceptibility to carbapenems was 97.12%, followed by piperacillin/tazobactam 53.85%, amoxicillin/clavulanic acid 14.42% and 3rd-generation cephalosporin 0.96% (Figure 2).

DISCUSSION

The number of infections due to ESBL *E. coli* is

Table 2. Prevalence of ESBL phenotype in *E. coli* isolates.

Variable	ESBL positive		ESBL negative
	MH without additive	MH + cloxacillin	
Number of isolates	91	13	105
Prevalence (%)	43.54	6.22	50.24

increasing, especially in African countries (Manyahi et al., 2014). In our study, 104 out of 209 *E. coli* isolates expressed ESBL, that is a prevalence of 49.72%. This result was superior to that reported by Alio et al. (2017) in a phenotypic detection study of extended-spectrum beta-lactamase in multidrug-resistant *E. coli* from clinical isolates in Niger. This shows that there is a worldwide increase in *E. coli* BLSE (Bevan et al., 2017). The spread of *E. coli* ESBL isolates reduces treatment preferences (Jena et al., 2017b). The sensitivity of *E. coli* ESBL isolates to temocillin was 62.5%. Urinary elimination of temocillin enhances its activity on ESBL *E. coli* isolates, according to a study by Vallee et al. (2017). Temocillin maintains a favorable profile on intestinal microbiota, with a low rate of *Clostridium difficile* infection (Lupia et al., 2022). Authors in various countries around the world, such as Belgium in 2006, have reported a prevalence of sensitivity of 92% (Rodriguez-Villalobos et al., 2006), prevalence in England was 86% in 2011 (Balakrishnan et al., 2011) and in France the prevalence was 71.3% in 2019 (Duployez et al., 2019). These rates showed that sensitivity to temocillin had decreased in different parts of the world. Susceptibility to carbapenems was 97.12%. This explains why carbapenems have been considered antibiotics of last resort against ESBL-producing *Enterobacteriaceae* (Lepeule et al., 2014). The prevalence of susceptibility to 3rd-generation cephalosporins was 0.96%. Extended-spectrum beta-lactamases have been recognized for their ability to hydrolyze 3rd and 4th generation cephalosporins (Rupp and Fey, 2003).

Conclusion

Temocillin has shown good activity against *E. coli* ESBL isolates. However, empirical treatment with temocillin should be included in the guidelines. It is therefore an alternative carbapenem-sparing antibiotic for the treatment of urinary tract infections caused by *E. coli* ESBL isolates, and can also be used against isolates producing AmpC enzymes.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Evaluation of the impact of the efflux pump modulation process on the anti-adhesion and antibiofilm activities of analogues of natural marine compounds in a marine bacterium

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One strategy for enhancing the effect of natural marine antifouling compounds has been to combine them with coumarin-type molecules (VL2, VL17 and VL19). These molecules are known to have an inhibitory effect on the efflux pumps (EP) of a large number of Gram-negative bacteria. To this end, they have been used as adjuvants to natural marine compounds (OB1, AS194 and AS162) to enhance their antibiofilm activity. On the other hand, the combination of synthetic analogues with coumarins had a synergistic effect on adhesion and biofilm formation in the bacterial strain studied, *Pseudoalteromonas ulvae*. In this strain, coumarins increased the effect of AS194 on adhesion and biofilm. This increase was more marked with the coumarins VL17 and VL19. These results were confirmed by EC50 calculations. With regard to adhesion, the EC50 of OB1 alone showed a reduction in combination, from 31.7 to 65.3%. As for AS194 and AS162, reductions ranged respectively from 16.9 to 43.6% and from 11.6 to 30.2%. With regard to biofilm, these two compounds in combination showed a significant decrease in their baseline EC50 in P. ulvae TC14. This EC50 decrease was marked by reduction rates ranging from 69.2 to 75.3% for OB1 and from 65.4 to 77% for AS194. In both cases (adhesion or biofilm), the effect variation in AS162 remained relatively small. Similar results were observed with two other marine strains, namely Pseudoalteromonas lypolitica TC8 and Paracoccus spp. 4M6. This study shows that inhibition of efflux pumps by coumarins enhances the anti-biofilm effect of natural marine compounds.

Key words: Biofouling, biofilm, coumarin, efflux pumps.

INTRODUCTION

One of the bacterial defense mechanisms is the active export of antimicrobial substances outside the cell. This mechanism leads to the rejection of these molecules into the external environment, ensuring a low level of intracellular concentration, below the threshold of efficacy. The term "efflux pumps" has been proposed to

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describe these mechanisms (Boulant et al., 2020). Efflux pumps play a major role in the development of multidrug resistance by actively exporting a wide variety of harmful compounds out of the bacterium (Lamers et al., 2013). PEs take the form of transporters capable of expelling different classes of antibiotics out of the bacterial cell, contributing significantly to therapeutic failure in the treatment of infectious diseases. In the medical field, one of the major contributors to multidrug resistance (MDR) and pathogenicity in Gram-negative bacteria is the overexpression of efflux pumps (EPs) belonging to the Resistance-Nodulation-Division (RND) family. These transporters are capable of expelling different classes of antibiotics from the bacterial cell, contributing significantly to therapeutic failure in the treatment of infectious diseases. In this context, EPs are interesting targets for the discovery of new antimicrobials. To combat this resistance mechanism, efflux pump inhibitors (EPIs) are being developed as adjuvants to antibiotics, with the aim of restoring or enhancing their activity. The mechanism of efflux pump inhibition has long been implicated in the enhancement of antibiotic activity. In this study, the modulation of PEs was demonstrated, along with its impact on adhesion and biofilm formation in marine bacteria. Speaking of biofilm inhibition, PEs have been presented by several authors as interesting targets. In the course of his research, Koch (2019), demonstrated a link between biofilm production and PEs in *Yersinia pestis* through a mutant encoding the TolC gene located in the outer membrane. According to the work of Cattoir (2004) and Boulant et al. (2020), one of the factors limiting the reduction of the harmful effect of antimicrobial substances is the defense mechanism based on efflux pumps. Clearly, the rejection of a proportion of compound by bacterial cells via EPs could limit their effect. Then, modulation of EPs has most often been an effective method of enhancing a compound's antimicrobial activity. This modulation can be achieved through genetic mutation in EPs (Luchao et al., 2020 ; Aires, 2011). In this study, efflux pump inhibitors were used to potentiate the antibiofilm effect of marine compound. This alternative approach is increasingly used in the medical field to make antibiotics more effective. This study aims to use a rarely employed method on marine biofilm. Three natural marine compounds with antibiofilm character, namely OB1, AS194 and AS162, were studied. These compounds have each been described to have a strong individual effect on adhesion and biofilm formation in the marine bacterium *Pseudoalteromonas ulvae* TC14 (Gozoua et al., 2019). Anti-adhesion and antibiofilm effects of these compound can be enhanced by combining them with the three coumarin derivatives VL2, VL17 and VL19. Potentiation of the anti-adhesion and antibiofilm effects was assessed by screening the compounds on adhesion and biofilm, and calculating the median concentration (EC50) on biofilm formation. These experiments were repeated with two other marine bacteria (*Pseudoalteromonas lypolitica* TC8 and *Paracoccus spp.* 4M6) to assess reproducibility.

Strategies for combating bacterial resistance

The fight against bacterial resistance relies on anti-resistant molecules. Anti-resistant molecules were associated as adjuvants to antibiotics (Gill et al., 2015). An adjuvant generally has no intrinsic antibacterial activity, but it enables an antibiotic to have a better action on its target when combined with it. Thus, a combinatorial approach is deemed opportune in order to potentiate the effects of antimicrobials (Sundaramoorthy et al., 2020; Jayan and Gupta 2023).

There are several classes of adjuvant compounds. These include efflux pump inhibitors and outer membrane permeabilizers (Urakawa et al., 2010; Abuzaid et al., 2012; Dias et al., 2022).

Efflux pump inhibition strategy

Overexpression of the efflux pump is an important mechanism of bacterial resistance, leading to the expulsion of antibiotics from bacterial cells. Pump inhibition is therefore a strategy that could restore the potency of current antibacterial compounds against resistant bacteria, and perhaps lead to the development of new compounds. RND efflux pumps are involved in the intrinsic resistance of many Gram-negative bacteria and, when expressed, lead to multiple drug resistance phenotypes in Enterobacteriaceae and *Pseudomonas aeruginosa*. EPs are potential sites for restoring antibiotic sensitivity (Wang et al., 2022). Possible inhibitors include phenylalanine-arginine ß-naphthylamide (PAßN), a dual permeabilizing and efflux pump inhibitor. This compound inhibits the efflux action of many RND family pumps and is capable of reducing intrinsic and mutational resistance to several antimicrobial compounds (Lamers et al., 2013; Luchao 2020). Another widely researched target is the NorA efflux pump of *Staphylococcus aureus*, which confers resistance to several antimicrobial agents, including fluoroquinolones (Kumar and Schweizer, 2005), giving rise to a multidrug resistance phenotype. Numerous compounds from different sources and classes have been tested for their ability to deactivate the NorA pump and restore antibiotic activity against resistant *S. aureus*. Research has also been carried out to develop fluoroquinolones to prevent efflux via the NorA pumps in order to improve their antimicrobial efficacy (Ince et al., 2002). Another possibility for reducing the deleterious effects of efflux pumps involves the use of antisense peptide nucleic acids, also known as ANPs. ANPs are synthetic nucleic acid homologues in which the phosphate polynucleotide backbone is replaced by a flexible pseudopeptide polymer. PNAs act as antisense

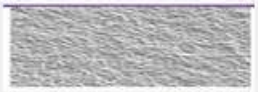
strains	TC14
Taxonomy	*Pseudoalteromonas ulvae*
Gram Type	Bacille Gram-
Morphology	Small purple colony (1,5 mm X 1.5mm)
Sampling site	Toulon military port
Surface used	fluorosilicone ELP08/55554/13
Image on VNSS Agar	
SEM Image	
References	(Briand-jaisson et al., 2014)

Figure 1. Description of the *Pseudoalteromonas ulvae* TC14 strain.

mediators, binding with high specificity to complementary DNA and RNA sequences and inhibiting gene expression and translation (Paulasova and Pellestor, 2004; Wang et al, 2022). A PNA compound was used to sensitize Campylobacter jejuni by decreasing expression of the efflux pump CmeABC, which generally confers resistance to several antimicrobials, including ciprofloxacin and erythromycin (Zhang et al., 2009).

Efflux pumps: Definitions and classification

Efflux pumps are either class-specific or responsible for Multidrug Resistance (MDR). They comprise several classes based on the form of energy utilization provided (Tahmina et al., 2017). Thus, proton dissipation is specific to the MFS, RND, and SMR families. The MATE family uses the sodium ion (Na+) as an energy source, while the ABC family draws its energy source from ATP hydrolysis (Vincent, 2004). In Gram-negative bacteria, efflux systems are often ternary protein complexes with a transmembrane pump, a periplasmic junction protein and an outer membrane porin. The most frequently encountered pumps are of the RND type, such as AcrB in *Escherichia coli* or MexB in *P. aeruginosa*. In Gram-positive bacteria, efflux systems consist solely of the pump. The most extensively studied are MFS pumps such as NorA or QacA in *S. aureus* and PmrA in *Streptococcus pneumoniae*. A few transporters have also been described in *mycobacteria* (Cattoir, 2004). The main efflux pump families are shown. A classification based on a soft spherical mask of the upper part of a pump is also possible (Shi et al., 2019).

MATERIALS AND METHODS

Description of the *Pseudoalteromonas ulvae* TC14 strain

The biological material used in this study consists of bacteria of marine origin, isolated from the Toulon roadstead, belonging to the collection (TC for Toulon Collection) of the MAPIEM laboratory. The main strain studied is *Pseudoalteromonas ulvae* TC14. It is described in Figure 1.

Preparation of *P. ulvae* TC14 strain

P. ulvae TC14 was first cultured in liquid VNSS at 20°C with 120 rpm agitation until the beginning of stationary phase. The cultures were then centrifuged at 6000 rpm for 10 min. The VNSS medium was then removed and replaced by ASW, to obtain a final bacterial concentration equal to 0.8 (OD600nm = 0.8). ASW keeps bacteria alive while limiting their growth, thus promoting their adhesion. Two other marine strains, *Pseudoalteromonas lypolitica* TC8 and *Paracoccus spp*. 4M6, were also tested in this study. They were used as control strains.

Presentation and structures of the coumarins tested in this study

The coumarin derivatives used in this study come from the Faculty of Pharmacy at the AIX University of Marseille, France. They are three molecules coded by the letters V and L, namely VL2, VL17, VL19. These molecules are previously known to be efflux pump inhibitors. The different structures are shown in Figure 2.

Preparation of coumarin-type molecules and natural marine compounds

Preparation of test molecules and compounds

Coumarins and compounds synthesized in the laboratory and other

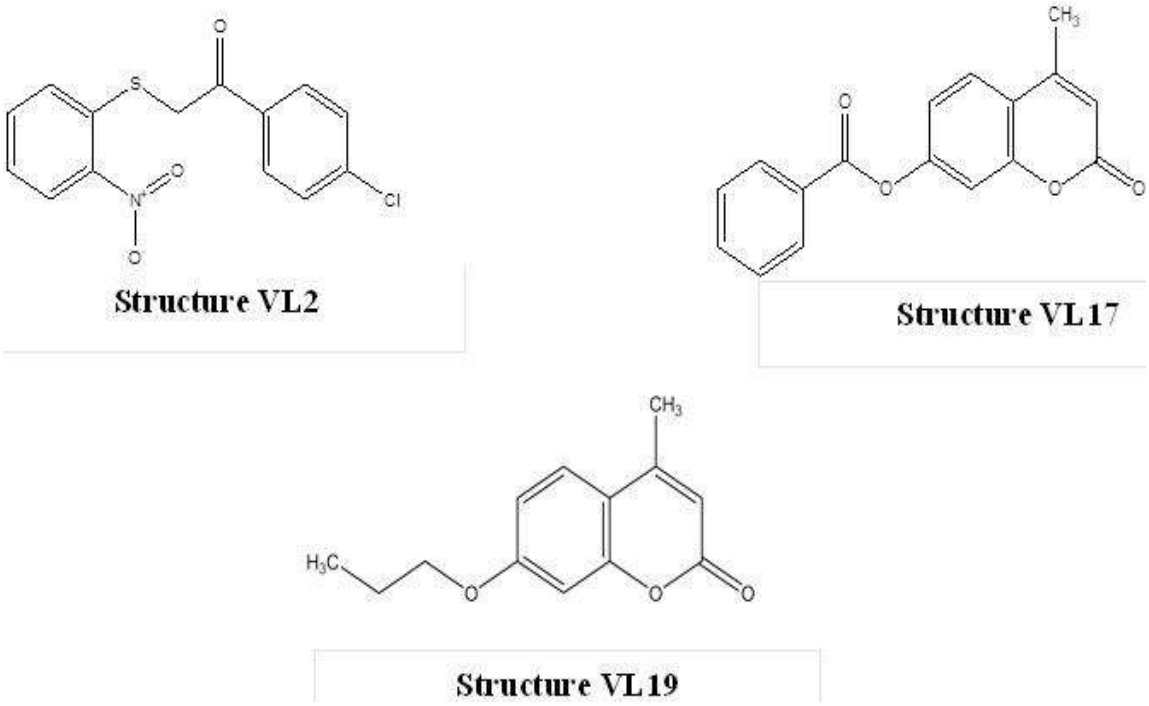


Figure 2. Schematic representations of Coumarin-type molecules.

molecules were first solubilized in DMSO to a stock concentration of 10 mM, then diluted in ASW to concentrations of 10, 50, 100, 150 and 200 µM.

Adhesion and biofilm principle and protocol

The marine bacterium *P. ulvae* TC14 has the ability to attach to natural or synthetic surfaces. These bacteria have been described to have a good adhesion capacity on polystyrene (Brian-Jaisson et al., 2014). The protocol used to perform the adhesion test was adapted from the anti-adhesion test protocol developed by Camps et al. (2011) and taken up by Aye et al. (2015). This protocol was repeated in the course of this study with the difference that the molecules tested in this case are coumarins, and the compounds synthesized in the laboratory. In some works, this technique is used to define cell migration, invasion, and adhesion strategies. It has been applied by Pijuan et al. (2019) on cancer cells. In our case, experiments were carried out in black 96-well polystyrene microplates for the adhesion test and in transparent polystyrene microplates for the biofilm test. It should be noted that during the adhesion and biofilm tests, 50 µM coumarins were added to the concentration range of marine compounds. Results were processed in TECAN, Infinit M 200 pro.

EC50 determination

EC50 is defined as the concentration capable of eliminating 50% of the effect. It correspond to the concentration of substance that elicits a response halfway between the baseline and the maximum response (Fechner et al., 2012).

EC50 value also makes it possible to avaluate an anti-biofilm compound's activity (Malouch et al., 2023). In this study, the EC50 of compounds and different combinations were determined using GraphPadPrism 5 software.

RESULTS AND DISCUSSION

Evaluation of the impact of efflux pump modulation on the anti-adhesion effect of compounds using a combinatorial approach

Adhesion tests, with combinations of antifouling compounds and coumarins, were carried out according to the adhesion protocol described by Camps et al. (2011) and taken up by Spriano et al. (2017). Coumarins previously tested alone showed no significant effect on adhesion and biofilm formation (results not reported in this study). Coumarin derivatives alone showed no antibiofilm effect here even though in the work of He et al. (2022), the coumarin derivatives tested showed anti-biofilm effects for the dispersion of advanced, pre-formed biofilms. However, the aim of this study goes beyond this aspect. Coumarin derivatives described as molecules that inhibit efflux pumps were instead tested in combination with antibiofilm compounds (OB1, AS194, AS162) with the aim of enhancing their effects, which will involve the notion of potentiation. These combination tests carried out on the adhesion of the *P. ulvae* strain showed a reinforcement of the effect of the marine compounds by the coumarins.

Although the synergistic effect was less pronounced for OB1 from 10 µM (Figure 3), for AS194 the synergistic effect was more pronounced from 25 µM. This result seems logical, since AS194 has a higher individual effect than OB1. On the other hand, a synergistic effect was only observed for VL17 and VL19 (Figure 3B, C, E and

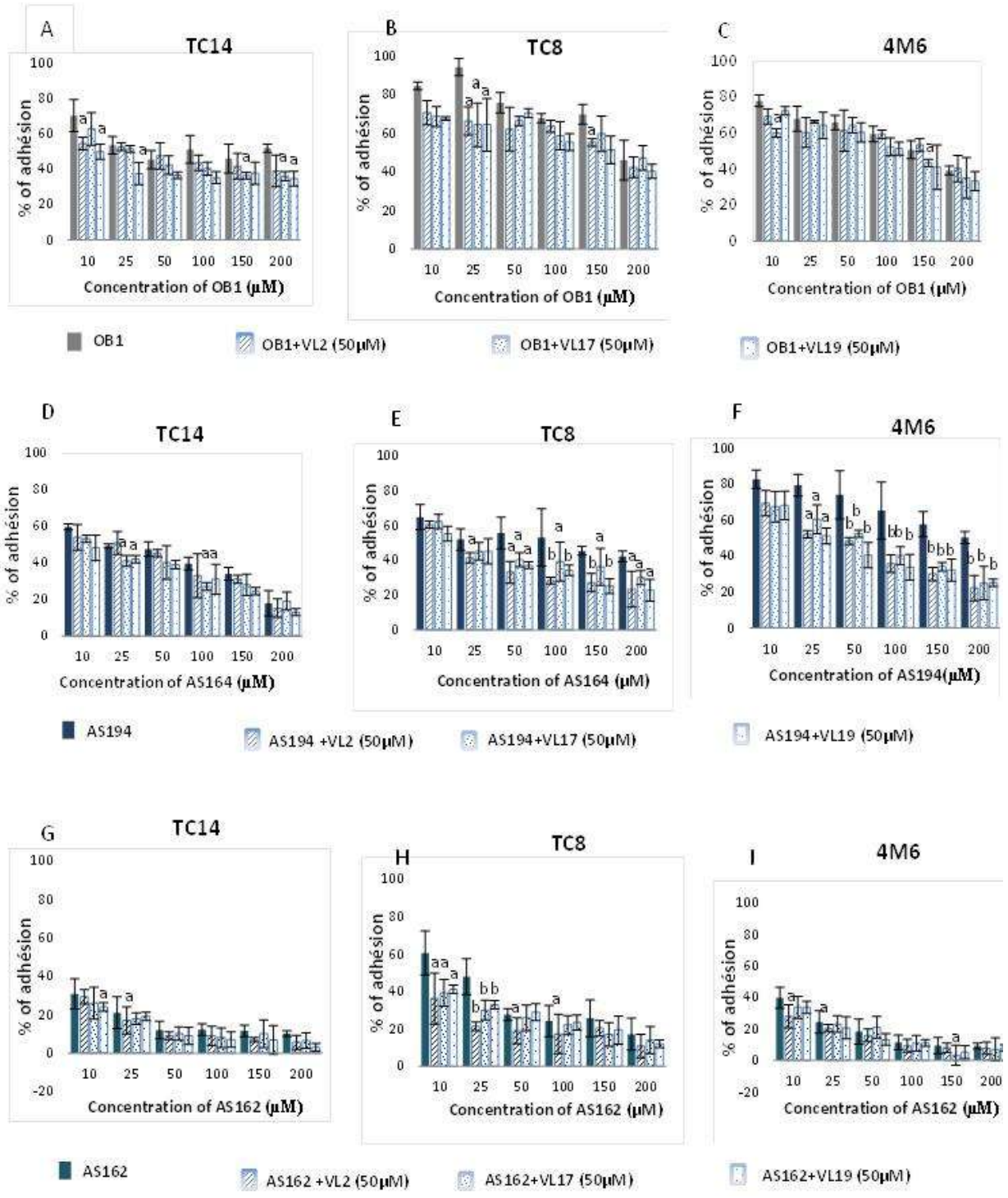


Figure 3. Effect of combining analogues and coumarins on adhesion in TC14, TC8 and 4M6. Trials were performed three times in replicates in black 96-well microplates. Assays marked with letters are significantly different (P<0.05) from the control (bacterial cultures without coumarins).

F). One of the three coumarin derivatives (VL2), in combination with the three antifouling compounds, showed no significant effect on *P. ulvae* adhesion. It has to be said that the latter was less active on the efflux pumps of this bacterium. This result was reproducible on one of the test bacteria used in this study. This was *P. lypolitica* TC8 (Figure 3H). In *Paracoccus sp*ecies 4M6, on the other hand, only slight variations in the effect of

AS162 were observed. This indicates a low level of coumarin activity in this bacterium. It should nevertheless be noted that the results observed in this chapter suggest that inhibition of efflux pumps is a pathway for potentiating the anti-adhesion effect in *P. ulvae* TC14.

Evaluation of the impact of efflux pump modulation on the biofilm effect of compounds using a combinatorial approach

Combination tests on biofilm were carried out according to the principle of Li et al. (2012) and repeat by Hawas et al. (2022). The results presented in Figure 4 showed a very marked synergistic effect of the combinations on biofilm formation in *P. ulvae* TC14 (Figures 4A, D and G) and *P. lipolytica* TC8 (Figures 4B, E and H). These results are similar to those obtained previously after the adhesion test. The adhesion stage precedes biofilm formation. Thus, a molecule with an anti-adhesive effect will have an impact on the next stage, which is biofilm formation (Rubio, 2002). In *Paracoccus spp*. 4M6 (Figures 4C, F, I), the synergistic effect of the combinations was less marked on biofilm formation, although there was a significant effect with OB1 at high concentrations (Figure 4C). In contrast, the combination of AS162 and coumarins (Figure 4I) showed a marked synergistic effect at low AS162 concentrations (10 and 25 µM).

Above 50 µM AS162, there was no synergistic effect on biofilm formation in 4M6. This shows that from 50 µM AS162 upwards, the effect of the combined molecule reaches a stationary phase. The potentiation of the bacteria's antibiofilm effect seems to be more real with relatively low concentrations of antibiofilm compounds. Above 50 µM, there seems to be a form of antagonism between the two types of compound. This is explained by the results obtained in Figure 4F, which reveal a relapse in the antibiofilm effect of compound AS194 on 4M6 when 200 µM of antibiofilm compound is reached. These results show that the quantities of antibiofilm products must be carefully adjusted to avoid antagonism.

This was explained in the work of Côté et al. (2016), who demonstrated cases of antagonism between two antibiofilm effects in which one of the effects attenuated the other in the event of concentration variation. In our case, this method of potentiating the antibiofilm effect may appear complex, as it would require rigorous monitoring of the screening data to identify the optimum concentrations. It should be noted that there are small nuances in the variation of biofilm from one bacterium to another. This clearly explains why each bacterium has a different resistance capacity. Based on what can be seen in Figure 4, 4M6 appears to be more resistant than the other two bacteria. TC14, on the other hand, shows sensitivity to the combined molecules, except that at high concentrations (200 µM), it seems to have a relatively weak effect. This aspect will be better explained in the next parts of our study dealing with EC50 values. The potentiation of the compounds' antibiofilm effect by the screening method should call on other experimental data to better appreciate the molecules' efficacy. In addition to the results obtained in Figures 3 and 4, we have calculated EC50 values, which are the concentrations likely to eliminate 50% of the effect.

Highlighting the potentiation of the anti-biofilm effect of natural compounds by determining median effective concentrations (EC50)

To better assess the potentiation of the antibiofilm effect of natural compounds (OB1, AS1984, AS162) by their combination with coumarin derivatives, EC50s were determined. The lower the median effective concentration (EC50), the more effective the compound. The synergistic effect of the combinations was very marked with both OB1 and AS194 on biofilm formation in P. ulvae TC14. In combination, these two compounds significantly reduced their baseline EC50 in P. ulvae TC14. This EC50 decrease was marked by reduction rates ranging from 69.2 to 75.3% for OB1 and from 65.4 to 77% for AS194 (Table 1). This indicates the high activity of both compounds in the presence of coumarin derivatives. This case of synergism is all the more interesting as we are dealing here with two types of compounds with different targets. On the one hand, natural antibiofilm compounds target biofilm formation, and on the other, coumarin derivatives potentially target efflux pumps. Similar results were found in the work of Ebrahimi et al. (2018). However, these authors' work was more in the medical field. They found cases of synergism between different compounds but also established an isobolographic approach to determine limiting values. Isobologram calculation could be an interesting approach in this study, but the main objective was to assess the potentiation of effects. This is a method increasingly used in the medical field, and less similar work has been done on marine biofilms.

Combinations with AS162 revealed a relatively low EC50 reduction in *P. ulvae* TC14. In the case of *P. lipolytica* TC8 and *Paracoccus spp.* 4M6, the synergistic effect proved highly significant, with OB1 EC50s showing a sharp reduction in both bacteria (in excess of 54%). This synergistic effect was also felt on AS162 in *P. lipolytica* TC8, where the EC50 reduction was also over 54% in the presence of each of the coumarins. In *Paracoccus spp*. 4M6, EC50s corresponding to combinations including AS194 revealed less marked synergism than those containing OB1 and AS162 (Table 1). In the previous part of our study, antagonism was noted in 4M6 at high concentrations of antibiofilm compounds. In the case of EC50s, there were no cases of antagonism, although there were cases of slight

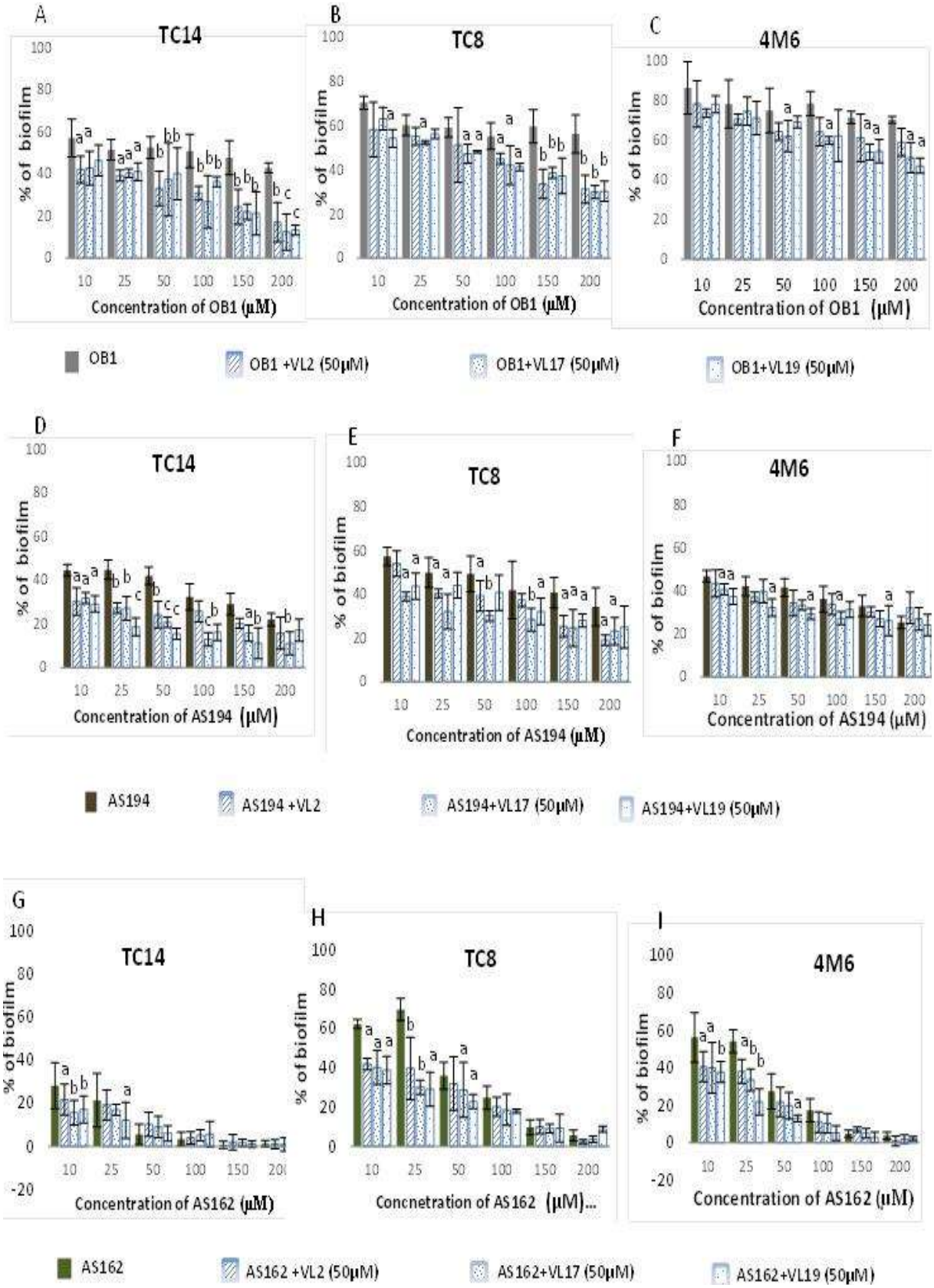


Figure 4. Effect on biofilm formation in TC14, TC8 and 4M6. Trials were performed three times in replicates in 96-well transparent microplates. Lettered assays are significantly different ($P<0.05$) from the control (bacterial cultures with analog alone).

Table 1. Determination of EC50s of combinations on strain biofilm and their reduction rates.

Strains	Compounds and combination	CE50 (µM) of biofilm formation	CE50 Reduction rate (%)
TC14	OB1	69.85±5.9	
	OB1+VL2 (50 µM)	21.5±3.1[c]	69.2
	OB1+VL17 (50 µM)	17.5±4.0[c]	75.0
	OB1+VL19 (50 µM)	17.3±3.5[c]	75.3
	AS194	24.8±3.9	
	AS194+VL2 (50 µM)	5.7±0.8[c]	77.0
	AS194+VL17 (50 µM)	7.5±1.9[c]	69.8
	AS194+VL19 (50 µM)	8.59±5.7[c]	65.4
	AS162	4.4±1.3	
	AS162+VL2 (50 µM)	2.4±1.1[b]	45.5
	AS162+VL17 (50 µM)	2.7±1.2[b]	38.6
	AS162+VL19 (50 µM)	3.6±0.8	18.2
TC8	OB1	127.9±3.6	
	OB1+VL2 (50 µM)	47.1±2.9[c]	63.2
	OB1+ VL17 (50 µM)	48.5±2.7[c]	62.1
	OB1+ VL19 (50 µM)	50.9±3.1[c]	60.2
	AS194	48.8±5.0	
	AS194+ VL2 (50 µM)	24.0±2.9[b]	50.6
	AS194+ VL17 (50 µM)	13.8±2.5[c]	71.7
	AS194+ VL19 (50 µM)	25.4±2,4[b]	48.0
	AS162	29.37±4.0	
	AS162+ VL2 (50 µM)	9.4±2.1[c]	68.0
	AS162+ VL17 (50 µM)	10.4±1.0[c]	64.6
	AS162+ VL19 (50 µM)	13.4±1.5[c]	54.4
4M6	OB1	328.0±9.5	
	OB1+ VL2 (50 µM)	140.6±4.8[c]	57.1
	OB1+ VL17 (50 µM)	134.3±7.0[c]	59.1
	OB1+ VL19 (50 µM)	172.9±6.9[b]	47.3
	AS194	26.91±4.6	
	AS194+ VL2 (50 µM)	13.6±0.9[b]	49.4
	AS194+ VL17 (50 µM)	17.8±2.0[c]	33.8
	AS194+ VL19 (50 µM)	20.5±1.9[a]	23.8
	AS162	18.8±2.1	
	AS162+ VL2 (50 µM)	6.4±1.9[c]	66.0
	AS162+ VL17 (10 µM)	9.181±1.1998[b]	51.01
	AS162+ VL19 (5 µM)	10.34±1.77115[a]	45.2

Affected letter values (a, b and c) are significantly different from controls (natural compounds used alone).

reduction, particularly in 4M6. EC50 calculations give a good idea of the potentiation of the antibiofilm effect of natural marine compounds.

Conclusion

This study evaluated the potentiation of the anti-adhesive and antibiofilm effects of three natural marine compounds. This potentiation was made possible by combining these compound with coumarin derivatives known to be efflux pump inhibitors. It was thus possible to identify another pathway for modulating efflux pumps in marine bacteria, with the aim of enhancing the antibiofilm effect of thecompounds.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Denaturing gradient gel electrophoresis (DGGE): An alternative culture independent method for bacterial screening in bovine milk sample

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Bovine mastitis is a disease that has a great impact on Brazilian livestock production. Some bacteria described as mastitis-causing agents are not easily cultivable in conventional media, making their diagnosis difficult. The aim of the present study was to detect bacteria present in milk from mastitic and non-mastitic quarters of dairy cattle in Rio de Janeiro-Brazil, using a culture-independent and culture-dependent method. Milk samples were collected from healthy and mastitic quarters. Blood agar medium was used to isolate bacteria. Polymerase Chain Reaction - Denaturing Gradient Gel Electrophoresis (PCR-DGGE) followed by gene sequencing was used to detect bacterial species. Bacteria were isolated from 12 milk samples and *Staphylococcus aureus* was the only species identified. Bands unique to mastitis were detected in some animals, signaling possible causative agents of the disease in the herd. Species of the genus *Streptococcus* identified in these samples could not be isolated in culture medium. The present study concluded that the DGGE technique proved to be efficient in detecting bacteria that have difficulty growing in culture medium.

Key words: Bovine mastitis, polymerase chain reaction, *Staphylococcus aureus*, *Streptococcus*.

INTRODUCTION

In 2017, Brazil ranked fourth in the world milk production, behind the United States, India and China. However, the number of lactating cows reached the second position, behind only India, revealing the low milk yield of our herd. So Brazilian productivity (1,525 L/cow/year) is far surpassed by the United States, China, the United Kingdom, Turkey, New Zealand, France, Russia and Germany. These data are worrisome since Brazil is a country with large dairy herds but Brazilian cows are considered as low production potential (FAOSTAT, 2017).

Factors related to this reduction in productivity include mastitis, an inflammatory disease with a multifactorial etiology that interferes with the number of somatic cells

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and the physical and chemical characteristics of the milk, leading to a reduction in quality. Mastitis is considered the greatest economic impact disease on milk production, responsible for condemning tons of milk per year in Brazil and responsible for reaching about 72% of the Brazilian herd (Oliveira et al., 2013).

Considering the clinical aspect, it is classified into clinical or subclinical mastitis. In clinical mastitis, there are obvious signs of inflammation, such as edema, temperature increase, hardening and pain in the mammary gland, and/or appearance of lumps, pus or any change in milk characteristics, and systemic symptoms such as depression, dehydration and decrease of food intake. These symptoms can be verified by physical examination of the udder by inspection and palpation, in addition to the collection of the first jets of milk to perform a test of the screened mug to detect alterations in the appearance of milk (Dürr, 2005).

Subclinical mastitis is prevalent in Brazilian herds, responsible for reaching 90% of mastitic cows. There are no evident changes in the udder of the cows, and their spread is related to the moment of milking, due to inadequate hygiene conditions, either by handlers or milking equipment. The diagnosis can be made through the somatic cell count (CCS) in milk, where its increase reflects the increase of leukocytes consequent to mammary infection. Thus, CCS reflects the health status of the mammary gland and measures the risk of non-physiological changes in milk composition, constituting an essential tool in the evaluation and monitoring of udder health (Blowey and Edmondson, 2010). However, the most common test for this diagnosis is the California Mastitis Test (CMT). The CMT is a qualitative test that indicates the presence of somatic cells to a greater or lesser degree in milk.

This consists in collecting milk from the mammary quarters, individually, in a suitable tray, adding a neutral anionic detergent, which acts by breaking the leucocyte membrane, releasing the nucleic material (DNA), which presents a gelatinous mass. Both tests must be confirmed by microbiological diagnosis (Langoni et al., 2009). Mastitis can evolve to spontaneous cure or, in most cases, to a chronic condition, making it necessary to identify the causative agent to adjust the control and treatment measures (Brasil, 2012).

Several bacteria are incriminated in cases of subclinical mastitis in dairy cattle. *Staphylococcus aureus*, *Streptococcus agalactiae*, *Corynebacterium bovis* and *Streptococcus dysgalactiae* are among the main microorganisms involved. In Brazil, *S. aureus* is the main species (Oliveira et al., 2013). In addition to *S. aureus*, some coagulase-negative species are also isolated from mastitis samples as causative agents (Abril et al., 2020).

Microbial culture is still considered standard method to assay the etiology of mastitis. However, some bacteria related as mastitis causing pathogens are not easily cultivable in conventional media which compromises its accuracy. Another issue that must be taken into account when using isolation and identification procedures is that many mastitis-related bacteria are also considered commensal, hindering the precise diagnosis (Schukken et al., 2009). Molecular techniques have been used to characterize bovine mastitis isolates and allowed us to know the genetic profiles of these agents and to cross-reference such information to understand the diversity of the circulating clones in the region studied, an essential factor for the proper development of prevention programs and successful therapies (Marques et al., 2013).

This study was developed to analyze the bacterial diversity presented in the milk of cows with subclinical mastitis. To achieve this goal the bacterial populations from mastitic and non-mastitic mammary glands were compared through culture and identification procedures. Also, the culture-independent technique, Denaturing Gradiente Gel Electrophoresis (PCR-DGGE) followed by sequencing, was used to identify the prevalent bacterial species in the milk samples.

MATERIALS AND METHODS

Sampling

The present study was carried out in a dairy farm located in the South Fluminense area of Rio de Janeiro, Brazil. Before the milking, the California Mastitis Test (CMT) was performed to diagnose subclinical mastitis. The somatic cell count (CCS) was performed by flow cytometry at ESALQ-USP/Piracicaba, SP. Milk presenting a ≤ 200,000 $SC.ml^{-1}$ counting was considered normal (Langoni et al., 2011). The results of the present study were compared to the Brazilian Normative Instruction 62 which recommended a maximum value of 500,000 $cells.ml^{-1}$ (Brasil, 2011). Ten animals presenting both healthy and mastitic mammary quarters were selected. Two samples of each animal comprising healthy and ill mammary quarters were collected, totaling 20 samples. The collections were performed in the morning, by manual and individual milking (Fonseca and Santos, 2000).

Cultivation dependent methodology

Milk samples were previously incubated at 37°C for 6 h and subsequently inoculated onto Blood agar (HiMedia® base agar with 5% ram blood) for the primary isolation. After 37°C incubation for 24 h, the resulting colonies were submitted to Gram staining. According to the characteristics, the isolates were processed for phenotypic identification (Koneman et al., 2008).

To confirm phenotypic identification, the isolates were submitted to MALDI-TOF MS technique. The spectra of each sample were generated in a mass spectrometer (MALDI-TOF LT MicroflexBruker, Bruker, Billerica, MA) equipped with a 337 nm nitrogen laser in the linear mode controlled by the FlexControl 3.3 program (Bruker Daltonics). The spectra were collected in the mass range between 2,000 and 20,000 $m.s^{-1}$ and later analyzed by the MALDI Biotyper 2.0 (Bruker) program, with the standardized configurations for bacterial identification. It was considered as acceptable the identification that presented values equal to or greater than two in a scale ranging from zero to three (Motta et al., 2014).

Cultivation independent methodology

Bacterial total DNA extraction was performed according to Tiago et al. (2015). The first PCR used the primers 27f (Suzuki and Giovannoni, 1996) and 1512r (Kane et al., 1993). The products of this reaction were used as template for the second PCR using primers that amplify the V3 region of 16S rDNA, GC-338f and 518r (Ovreâs et al., 1997). For PCR reactions, *S. aureus* controls from clinical samples belonging to the genetic material bank of the Veterinary Bacteriology Laboratory were used. The products of the second PCR reaction were evaluated on an 8% polyacrylamide gel and a concentration gradient between 40 and 70% defined from the mixture of urea and deionized formamide solutions. In this step, the PCR products are separated by induced denaturation, adopting different positions along the gel according to the different types of gene sequence and molecular weights of the microorganisms. Electrophoresis was performed at 70 V and 60°C for 18 h in a DcodeTM "Universal Mutation Detection System" (BIO-Rad, Richmond, USA). The gels were photographed and the images analyzed with the Bionumerics software (AppliedMaths, Saint-Martens-Latem).

DGGE bands were excised from the gel and transferred to microtubes containing 5 µl of water and subsequently incubated at 4°C for 12 h. Two microliters of eluted DNA were submitted to a PCR reaction using 338f and 518r primers. The PCR products were purified using the Exo-Sap Kit (USB Corporation, Cleveland, Ohio) as recommended by the manufacturer. Both strands were sequenced on Applied Biosystems ABI 3130xl sequencer at Helixxa Bases for Life (Campinas/SP). The sequences were edited in the DNA Sequence Assembler v4 program (2013), and then compared with other sequences in the NCBI GenBank database (www.ncbi.nlm.nih.gov/) using the BLAST algorithm for species inference (Altschul et al., 1997).

RESULTS

Of the 20 milk samples collected, 12 presented microbial growth when inoculated onto blood agar medium (Table 1). The only species detected was *S. aureus*. It was not possible to detect bacterial growth in eight samples from four animals (3, 6, 9, 10), even in the milk samples collected from quarters with positive California Mastitis Test (CMT).

By means of DGGE gel analysis it was possible to notice differences in bacterial population between milk samples from healthy and mastitic quarters of the same animal. This difference considered not only the presence or absence of the band, called the Operational Taxonomic Unit (OTU), but also the intensity of the bands (Figure 1).

There is no relationship of CCS with the number of bands in the gel or with the intensity of its bands, but exclusive bands were detected in mastitic milk samples of the following animals: 3 (band 4 - *Enterococcus faecium*), 4 (band 6 - *Bacillus* species), 6 (band 13 - *S. agalactiae*) and 7 (band 17 - *Streptococcus uberis*) as shown in Figure 1 indicated with red arrows and shown in Table 2. Other exclusive bands are present in non-mastitic milk samples of the following animals: 4 (band 8 - *Bacillus* spp.), 5 (band 11 - *Amphibacillus* species), 6 (band 14 and 15 - both *S. aureus*) and 7 (band 18 - *S. aureus*) (Table 2).

Bands were detected in both mastitic and non-mastitic milk samples. Some bands have higher intensity in mastitic milk samples as 1, 2, 3, 10, 12 (*S. aureus*, *S. aureus, Enterococcus faecalis, S. aureus, S. aureus,* respectively). Otherwise, some have higher intensity in non-mastitic milk samples as 5, 7, 16, 19, 21, 22 and 23 (*S. aureus, Staphylococcus haemolyticus, S. aureus, S. aureus, S. aureus, Bacillus* spp.) (Figure 1 and Table 2).

DISCUSSION

The use of independent of cultivation methods techniques based on nucleic acids such as Denaturing Gradient Gel Electrophoresis (DGGE) allows the evaluation of a larger number of samples at the same time and a broader and faster detection of the potential pathogens presented in the samples, increasing the expected results. It was noticeable in this study that culture-dependent methods did not allow for a more comprehensive assessment of the organisms presented in both healthy and mastitic milk samples, and that even spending a higher cost for individual and specific evaluations of these micro-organisms was not so sensitive in detecting the bacterial diversity.

The only species isolated by culture-dependent methodology with blood agar medium was *S. aureus*, which is predictable since it is widely disseminated in dairy environment and consequently considered the main agent related to bovine mastitis (Lazzari et al., 2014). It is important to consider that the presence of an agent is not a sufficient criterion to attribute the etiology of a disease (Viana et al., 2014). Bacteria such as *S. aureus* are also considered part of the microbiota of the cows. Other criteria should be taken into account such as, agent concentration, pathogenicity of the strain and immune status of the animal (Schukken et al., 2009).

Four samples presented no microbial growth in blood culture medium, reinforcing the difficulties in the adoption of culture-dependent protocols, since it is not possible to state that the absence of growth is due to specific bacterial requirements or even to some inhibitory agent, biotic or abiotic, interfering in the growth of species present in the sample, or even if the inflammatory process detected by CMT was caused by bacterial agent. It is important to point out that the protocol used for bacterial isolation is standardized for type-of-sample analysis (Blagitz et al., 2011).

The evaluation of the intensity of the bands is a semi-quantitative analysis that suggests an increased DNA concentration in the sample. This technique had already been used to characterize the surface microbiota of mammary gland in a study of mastitis bacterial diversity (Braem et al., 2012) and also in the diagnosis of bacteria

Table 1. Distribution of bacterial species isolated from milk samples in blood agar medium.

Animal	CCS (SC.ml^{-1})	CMT/quarter	Sample	Isolates
1	1,429,000	Positive	1P	*Staphylococcus aureus*
		Negative	1N	*Staphylococcus aureus*
2	897,000	Positive	2P	*Staphylococcus aureus*
		Negative	2N	*Staphylococcus aureus*
3	1,071,000	Positive	3P	NBG
		Negative	3N	NBG
4	9,999,000	Positive	4P	*Staphylococcus aureus*
		Negative	4N	*Staphylococcus aureus*
5	1,618,000	Positive	5P	*Staphylococcus aureus*
		Negative	5N	*Staphylococcus aureus*
6	100,000	Positive	6P	NBG
		Negative	6N	NBG
7	273,000	Positive	7P	*Staphylococcus aureus*
		Negative	7N	*Staphylococcus aureus*
8	346,000	Positive	8P	*Staphylococcus aureus*
		Negative	8N	*Staphylococcus aureus*
9	269,000	Positive	9P	NBG
		Negative	9N	NBG
10	793,000	Positive	10P	NBG
		Negative	10N	NBG

CCS = Counting somatic cells; SC = somatic cells; CMT = California mastitis test; NBG= no bacterial growth.
Source: Authors

that present difficulties to be cultured (Kuang et al., 2009). The most bands present in both milk samples are from *Staphylococcus* genus. It is worth noting that the lack of *S. aureus* growth in some samples may be due to the possibility of intermittent secretion of this agent (Abril et al., 2020). *S. aureus* may be considered as the most important species within the study, being detected in most samples. In a general sense it is possible to assume that the greater its presence in the mammary glands, the greater the probability of occurrence of the disease, the risk to health and the loss in production.

It was possible to detect the presence of exclusive OTU obtained from non-mastitic milk samples in three animals. *Amphybacillus* species were detected only in healthy samples and may represent part of commensal microbiota. This kind of microorganism is very important in balancing the effect of pathogenic bacteria on the mammary gland. Samples presenting these bacteria showed no signs of disease or pathogenic bacteria. Similarly, *Bacillus* spp. can be considered a commensal organism and in this study was identified in both samples' types.

A very important finding was the detection of *Streptococcus* spp. only by DGGE technique in samples where there was no microbial growth in the culture medium. Bacterial culture in blood agar has for many years been the standard method for identification of mastitis pathogens. Depending on the culture medium used, the inoculum volumes applied and the specific analyses, the sensitivity of *Streptococcus* detection ranges from 20.5 to 78% (Keefe, 1997). The samples containing *Streptococcus* spp. were the same where *S. aureus* could not be cultured by the conventional method. This suggests a possible interference mechanism acting on these bacteria.

Streptococcus spp. and *Enterococcus faecium* were

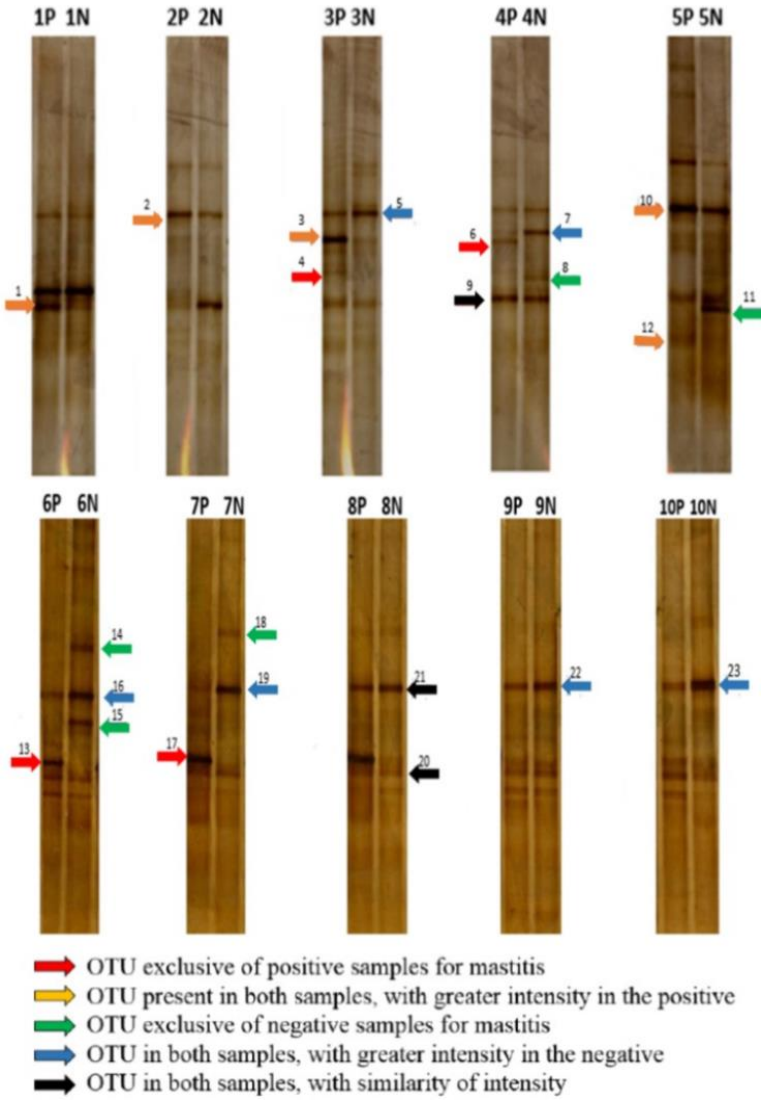

Figure 1. Denaturing Gradient Gel Electrophoresis of 16S rDNA gene of bacteria from quarter-milk samples with positive (P) and negative (N) California Mastitis Test. P = California Mastitis Test (CMT) positive, N = CMT negative.
Source: Authors

detected only in mastitic quarters, and could represent a correlation with the disease since they were not found in healthy quarters. *Enterococcus faecalis* was found in both samples, but with more intensity in the positive samples, suggesting a pathogenic potential. Although the study focused on subclinical mastitis, another interesting result was the detection of *Streptococcus uberis* in samples of mastitic milk once this bacterium is known to be implicated in clinical mastitis (Abureema et al., 2014).

The DGGE gel observation indicated differences between the bacterial microbiota in both milk samples. The OTU sequencing from DGGE is a tool that could differentiate the commensal from the potential pathogenic microbiota. As a matter of fact, it is relevant to point out that the control of this disease is related to the implementation of milking good practices, sanitary management and the comprehension of mastitis impact on production. When this concept is fully established the consequent economic problems and the prevalence of these bacteria in the herds tend to diminish.

Conclusion

The DGGE technique proved to be efficient in detecting bacteria that have difficulty growing in the culture medium

Table 2. Distribution of bacterial species in milk samples from mastitic and non-mastitic quarters by culture-independent methodology.

Bands no.	Animal	Sample*	Bands characteristics	Specie inference
1	1	1P	OTU in both samples, with higher intensity in positive mastitic quarters	*Staphylococcus aureus*
2	2	2P	OTU in both samples, with higher intensity in positive mastitic quarters	*Staphylococcus aureus*
3	3	3P	OTU in both samples, with higher intensity in positive mastitic quarters	*Enterococcus faecalis*
4	3	3P	OTU in both samples, with higher intensity in positive mastitic quarters	*Enterococcus faecium*
5	3	3P	OTU in both samples, with higher intensity in negative mastitic quarters	*Staphylococcus aureus*
6	4	4P	Exclusive OTU from positive mastitic quarters	*Bacillus* spp.
7	4	4N	OTU in both samples, with higher intensity in negative mastitic quarters	*Staphylococcus haemolyticus*
8	4	4N	Exclusive OTU from negative mastitic quarters	*Bacillus* spp.
9	4	4P	OTU in both samples, with similar intensity	*Staphylococcus aureus*
10	5	5P	OTU in both samples, with higher intensity in positive mastitic quarters	*Staphylococcus aureus*
11	5	5N	Exclusive OTU from negative mastitic quarters	*Amphibacillus* spp.
12	5	5P	OTU in both samples, with higher intensity in positive mastitic quarters	*Staphylococcus aureus*
13	6	6P	Exclusive OTU from positive mastitic quarters	*Streptococcus agalactiae*
14	6	6N	Exclusive OTU from negative mastitic quarters	*Staphylococcus aureus*
15	6	6N	Exclusive OTU from negative mastitic quarters	*Staphylococcus aureus*
16	6	6P	OTU in both samples, with higher intensity in negative mastitic quarters	*Staphylococcus aureus*
17	7	7P	Exclusive OTU from positive mastitic quarters	*Streptococcus uberis*
18	7	7N	Exclusive OTU from negative mastitic quarters	*Staphylococcus aureus*
19	7	7N	OTU in both samples, with higher intensity in negative mastitic quarters	*Staphylococcus aureus*
20	8	8P	OTU in both samples, with similar intensity	*Enterococcus faecalis*
21	8	8P	OTU in both samples, with higher intensity in negative mastitic quarters	*Staphylococcus aureus*
22	9	9P	OTU in both samples, with higher intensity in negative mastitic quarters	*Bacillus* spp.
23	10	10P	OTU in both samples, with higher intensity in negative mastitic quarters	*Staphylococcus aureus*

*P = California Mastitis Test (CMT) positive, N = CMT negative.
Source: Authors

and presented itself as a diagnostic alternative for mastitis control in milk productions.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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