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Posters

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3-**Ahmed Khassaf Al Atya**, Karima Drider-Hadiouche, Anne Vachee and Djamel Drider. Potentialization of β-lactams with colistin: in case of extended spectrum β-lactamase producing *Escherichia coli* strains isolated from children with urinary infections. GDR. 26-27 October 2015. Orleans. France

4-Delphine L. Caly, Mickaël Chevalier, Christophe Flahaut, Benoît Cudennec, **Ahmed Khassaf Al Atya**, Romain D'Inca, Eric Auclair and Djamel Drider. Characterisation of DD14, an enteroccin with anti-*Clostridium perfringens* activity. Colloque Adebiotech. 16-17 March 2016. Paris. France.

ABSTRACT

In this study, we isolated one hundred and seven (107) bacteria with Gram positive staining and devoid of catalase activity, arguing thereof of their belonging to the group of lactic acid bacteria (LAB). These LAB strains were isolated from samples of meconium of six blind donors at Roubaix hospital (North of France). All these LAB strains were identified by MALDI-TOF as *Enterococcus faecalis*. Nevertheless, only six isolates including *E. faecalis* 14, *E. faecalis* 28, *E. faecalis* 90, *E. faecalis* 97 and *E. faecalis* 101 (obtained from donor 3) and *E. faecalis* 93 (obtained from donor 5) exhibited antagonism against Gram negative bacteria (GNB) and mainly Gram positive bacilli (GPB). This antagonism was attributed to the production of lactic acid and bacteriocins, which are ribosomally synthesized antimicrobial peptides. The identification of these antagonistic LAB strains was completed by the sequencing of their 16S rDNAs. Besides their antagonism capabilities, these antagonistic strains were studied for a set of characteristics such as antibiotic resistance, hemolytic activity, virulence factor, aggregation and hydrophobicity to design any possibility of probiotic grade.

Bacteriocins produced by *E. faecalis* 28 and *E. faecalis* 93, designed as enterocins DD28 and DD93, which are issued from two distinct donors, revealed anti-*Staphylococcus aureus* activity and even anti-methicillin-resistant *Staphylococcus aureus* (MRSA) activity. They were then purified by a simplified two-step purification procedure including an ion exchange chromatography and reversed-phase high-performance liquid chromatography (RP-HPLC). Their molecular masses were determined by mass spectrometry and were shown to be 5,204.48 Da and 5,203.90 Da respectively. Interestingly, these molecular masses do fit with those usually reported in the literature particularly those of enterocins L50A, L50B, MR10A and MR10B. To establish the DNA sequences of enterocins DD28 and DD93, we have amplified the total DNA from the producing strains *E. faecalis* DD28 and *E. faecalis* DD93, using the forward (5'-ATG GGAGCA ATC GCA AAA TTA GTG A-3') and reverse (5'-TTA ATG TCT TTT TAG CCA TTT TCA ATT TG-3') primers, that were previously designed to amplify DNA coding for enterocins MR10A and MR10B (Liu et al., 2011).Taking advantage of these primers and PCR conditions, we have obtained DNA fragments (amplicons) from each of the aforementioned strains, cloned into pGEM-T Easy vector and sequenced them at Eurofins MWG operon, Ebersberg (Germany).The obtained DNA sequences displayed perfect alignment (100% identity) with DNA coding for enterocins L50A, L50B and MR10A, MR10B.

As mentioned above, the enterocins DD28 and DD93 were remarkable for their activity against *S. aureus;* a dreadful food poisoning agent and human pathogen. This activity was also evidenced even slightly against methicillin resistant *S. aureus* (MRSA). The inhibition of MRSA was performed with

enterocins DD28 and DD93 alone or in combination with erythromycin and kanamycin. The inhibition of MRSA was studied against planktonic cells using the kill curves and checkerboard assays. The inhibition study unveiled a clear synergistic effect. Furthermore, this anti-MRSA activity was also studied and highlighted against biofilm formation using AISI 304 L steel slide and glass devices.

Related to bacteriocins and antibiotics associations, we have also established that enterocin DD14, another enterocin obtained in the frame of this project, and which DNA sequence is identical to those of enterocins DD28 and DD93 was able to potentialize colistin, an antibiotic used in the veterinary and human medicines to treat infections caused by GNB. The potentialization of colistin was observed against a set of *Escherichia coli* strains from swine origin. Indeed, the combination of enterocin DD14 and colistin appeared able to decrease *E. coli* counts in planktonic and biofilm cultures.

Given the importance of the data we have obtained on the potentialization of colistin with enterocins DD14, we investigated whether colistin was also able to potentialize other antibiotics such as those of the β-lactams group. To this end, we tested the combinations of colistin and ticarcillin or colistin and cefotaxime, two antibiotics of β-lactams group to which six *E. coli* strains from infant origin were resistant. Indeed these non-nosocomial *E. coli* strains were isolated at Roubaix hospital from children with urinary infections and were considered based on their antibiogrammes as extended spectrum β–lactamase (ESBL) *E. coli* strains. The combinations tested here (colistin-ticarcillin or colistin cefotaxime) have led to synergistic effects, as supported by the FIC index (fractional inhibitory concentration) determinations and killing curves experiments. Indeed, the MIC values of ticarcillin has dropped below its breakpoint, in the case of isolates S2, S3 and S5, while it has almost reached the breakpoint for cefotaxime in the case of isolate S2 and the isolate S1. The data gathered from this ESBL study unveiled a possibility of novel treatment of severe *E. coli* infections.

RESUME

Les bactéries lactiques appartiennent au groupe des firmicutes et sont caractérisées par une coloration différentielle positive et l'absence d'une enzyme respiratoire, la catalase. Le principal objectif de cette thèse était d'isoler et identifier des bactéries lactiques antagonistes à partir de meconium. Ainsi, nous avons isolé 107 bactéries répondant aux critères biochimiques suscités, à partir de six échantillons de méconium, issus de nouveaux nés, à l'hôpital de Roubaix (Nord de France). Toutes les bactéries ont été identifiées par MALDI-TOF comme *E. faecalis*. Toutefois, seuls les isolats *E. faecalis* 14, *E. faecalis* 28, *E. faecalis* 90, *E. faecalis* 97 et *E. faecalis* 101 obtenus à partir du donneur 3, et *E. faecalis* 93 obtenu à partir de donneur 5, avaient une activité antagoniste contre un panel de bactéries à Gram négatif et à Gram positif. Cet antagonisme est attribué à la production d'acide lactique et de bactériocines, qui sont des peptides antimicrobiens de nature protéique, synthétisés par voie ribosomique. L'identification de ces bactéries antagonistes a été confirmée par séquençage du gène 16S (ADNr 16S). Les souches antagonistes ont été plus amplement étudiées pour établir quelques critères probiotiques. Ainsi les six souches antagonistes sont dépourvues d'activité hémolytique, sensibles aux antibiotiques utilisés pour le traitement des infections à entérocoques, caractérisées par l'absence de facteurs de virulence, majeurs et possèdent des scores d'agrégation et hydrophobie très élevés.

Les bactériocines produites par *E. faecalis* 28 et *E. faecalis* 93 issues de deux donneurs différents, possèdent une activité anti-*S. aureus*, y compris anti-*S. aureus* resistant à la méthycilline (SARM). Ainsi, les bactériocines produites par ces souches et appelées entérocines DD28 et DD93, ont été purifiées par chromatographie échangeuse d'ion et HPLC en phase inverse. Leurs masses moléculaires sont respectivement 5 204,48 Da et 5 203,90 Da. Elles sont compatibles avec celles décrites dans la littérature plus particulièrement celles des entérocines L50A et L50B, MR10A et MR10B. Ainsi, des essais d'amplification des gènes codant pour les entérocines DD28 et DD93 ont été réalisés par des amorces 5'-ATG GGAGCA ATC GCA AAA TTA GTG A-3' et 5'-TTA ATG TCT TTT TAG CCA TTT TCA ATT TG--3' en utilisant le programme PCR suivant : une dénaturation initiale de 5 min à 95 °C, suivie par 30 cycles de 1 min à 95 °C, 1 min à la température de fixation des amorces, et une extension de 10 min à une température de 72 °C.

Ces amplifications ont permis d'obtenir des fragments d'ADN (amplicons) de 290 pb. Ces amplicons ont été clonés dans le vecteur pGEM-T Easy vector et séquencés (Eurofins MWG operon, Ebersberg, Germany). L'analyse des séquences nucléotidiques, a permis de montrer, un parfait alignement (100% d'identité) avec les séquences nucléotidiques des entérocines MR10A et MR10B.

Comme indiqué, les entérocines mises en évidence dans cette étude se distinguent par un large antagonisme incluant des souches comme *S. aureus;* une bactérie pathogène d'intérêt alimentaire et médical. Il faut noter que les entérocines et plus particulièrement les entérocines DD28 et DD93 issues de deux donneurs différents, avaient une activité contre les souches SARM. Pour concevoir de nouveaux concepts thérapeutiques tout en gardant l'efficacité des antibiotiques existants, nous avons réalisé des tests anti-SARM combinant les entérocines DD28 et DD93 avec l'érythromycine et la kanamycine. En effet, après un criblage d'activité anti-Staphylocoque (*epidermidis*, *aureus*, SARM), nous avons choisi comme modèle une souche de SARM, appelée SARM-S1, isolée chez un patient âgé de 80 ans à l'hôpital de Strasbourg. Cette souche "SARM-S1" nous a été donnée par le Docteur Gilles Prévost afin de valider l'activité de nos molécules. Par ailleurs, la souche SARM-S1 est résistante à la kanamycine et aux entérocines testées mais elle est sensible à la rifampicine et la vancomycine. Les combinaisons entérocines-kanamycine ou entérocines-érythromycine ont montré un effet synergique avec des index FIC de 0.3. Toutefois, ces effets synergiques sont confirmés par les courbes de bactéricidie avec une perte de population SARM-S1 de plus de 2 Log_{10}, après 3 heures d'incubation avec les antibiotiques suscités. Des essais de protection (fonctionnalisation) de surfaces de types acier ou verre, par les entérocines DD28 et DD93, les antibiotiques et les combinaisons entérocines-antibiotiques ont montré un effet anti-installation de biofilms. Les études ont été appuyées par des observations microscopiques (épifluorescence et microscopie électronique à balayage).

Il faut noter que les entérocines isolées dans le cadre de ce travail, à l'instar des entérocines DD28 et DD93, ne sont pas dotées d'une activité contre les bactéries à Gram négatif. Toutefois, notre étude a permis de montrer que l'association de l'entérocine DD14, une bactériocine produite par l'isolat 14 (*E. faecalis* 14) dont la séquence nucléotidique est identique à celles des entérocines DD28 et DD93, est capable de potentialiser l'activité de la colistine; un antibiotique de nature lipopeptidique utilisé en médecines humaine et vétérinaire pour traiter les infections causées par les bacilles à Gram négatif. Dans cette partie du travail, nous avons établi que la combinaison entérocine DD14-colistine avait un effet synergique sur des souches *d'E. coli* d'origine porcine. Ces souches appartiennent à la collection Resapath et ont été généreusement données par le Docteur Isabelle Kempf (ANSES, Ploufragan). Certaines des souches étudiées ont une résistance à la colistine, ce qui constitue un défi à relever dans le traitement futur des infections impliquant ces souches. Les effets synergiques observés *in vitro* sont consolidés par les courbes de bactéricidies et une perte de la population d'environ 2 Log_{10}. Enfin, les souches *E. coli* utilisées comme cible dans cette partie expérimentale ont été caractérisées par la technique de RAPD (Random amplified polymorphic DNA) afin d'établir une éventuelle relation clonale.

Enfin dans le cadre de la potentialisation des antibiotiques d'importance majeur comme la colistine, nous avons regardé si la colistine elle-même était capable d'avoir un effet sur d'autres antibiotiques

comme ceux de la famille des β-lactamines. Ainsi, des souches d'*E. coli* d'origine infantile ont été isolées à l'hôpital de Roubaix, à partir de patients (enfants) ayant des infections urinaires. Ces souches ne sont pas des souches nosocomiales. Dans un premier travail, nous avons regardé par la technique de RAPD le lien génétique entre ces souches (lien clonal), puis établi leurs antibiogrammes. Ces souches se sont révélées produire une β-lactamase et avoir une résistance à ce groupe d'antibiotiques. Des essais combinant la colistine et la ticarcilline ou encore la colistine et la céfotaxime ont montré des activités synergiques *in vitro* avec des diminutions des CMI respectives de ces antibiotiques en dessous des points critiques. Celles-ci ont été confirmées et consolidées par les courbes de bactéricidies avec une perte de la population bactérienne d'au moins $2Log_{10}$. Les données de cette étude permettent d'espérer de nouvelles possibilités de traitement des infections à *E. coli*.

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ABBREVIATIONS

ATCC…….. American type culture collection

AMPs …… Antimicrobial peptides

B. infantis ……..... *Bifidobacterium infantis*

BLIS…… Bacteriocin like inhibitory substance

C-section ……..…… Caesarian section

CFS.....Cell free supernatants

CFU …….....Colony-forming-unit

CIP …….. Collection de l'Institut Pasteur

CMV ……..….Cytomegalovirus

CO2 ……..…….... Carbon dioxide

Da……..... Dalton

DNA ……..... Dexo ribonucleic acid

E. coli……..... *Escherichia coli*

E. faecalis ……..... *Enterococcus faecalis*

ECDC……..... European Centre for Disease Prevention and Control

ESBL……Extended-Spectrum beta-lactamases

GIT……..... gastrointestinal tract

GPB……..... Gram positive bacteria

GNB……..... Gram negative bacteria

GRAS……..... Generally recognized as safe

H ……..... hour

H_2O_2……Hydrogen peroxide

HPV …….... human papillomavirus

Kb ……..... Kilo base

LAB ……..... Lactic acid bacteria

MAS……..... Meconium Aspiration Syndrome

MDR……..... Multidrug resistant

Min ……..... Minute

MRSA …….....Methicillin-resistant *Staphylococcus aureus*

PBS…….…..... Phosphate buffered saline

PCR ……..…..… Polymorphic chain reaction

PFGE……..…. Pulsed field gel electrophoresis

RAPD……..…… Random amplified polymorphic DNA

St. thermophilus ……..…. *Streptococcus thermophilus*

UTIs……..... Urinary tract infections

WHO……..... World Health Organization

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General introduction

Bacteriocins have been widely used in many fields such as food preservation and have been proposed for medical applications such as the treatment of infections caused by the multidrug-resistant (MDR) bacteria (Karpiński and Szkaradkiewicz, 2016). In the past 70 years, significant developments in the diagnosis and treatment of infectious diseases have been achieved and conducted to major reduction of the associated morbidity and mortality (Magnussen et al., 2004). Since the discovery of penicillin, and until recent past, antibiotics have been relevant and efficient for the treatment of different bacterial infections (Gould and Bal, 2013; Tenover, 2006). Nevertheless, several studies reported a high number of bacterial pathogens exhibiting resistance to antibacterial agents. Moreover, organizations such as the European Centre for Disease Prevention and Control (ECDC), and the World Health Organization (WHO), are considering infections caused by MDR bacteria as a major public health concern and social burden (Roca et al., 2015). Many pathogens including *Staphylococcus aureus*, *Enterococcus* spp, *Enterobacteriaceae*, *Pseudomonas aeruginosa*, and *Acinetobacter baumanii* are considered as MDR bacteria because of their aptitude of resistance to various antibiotics (Magiorakos et al., 2012). The mechanisms of antibacterial resistance are complex. Moreover, scientists stated that microorganisms embedded in biofilms could cause many infections in animals and humans, leading to complex clinical profiles. The relationship between the biofilm formation and antimicrobial resistance have been established in many types of pathogenic bacteria such as *E. coli*, *Klebsiella pneumoniae*, *Moraxella catarrhalis*, *Proteus mirabilis* and *P. aeruginosa* (Franci et al., 2015).

Remarkably, there are many community-acquired and nosocomial infections caused by resistant bacteria such as those involved in the respiratory tract infections, gastro-intestinal infections (GTIs) and urinary tract infections (UTIs) (Gupta et al., 2011; Lee et al., 2013). UTI are important cause of death (Raghubanshi et al., 2014). The most common causes of UTIs are the extended-spectrum β-lactamase (ESBL) producing bacteria, including *E. coli* and *K. pneumoniae* but also *K. oxytoca*, and *Proteus* spp. These bacteria were shown to be resistant to antibiotics of different groups including penicillins, cephalosporins and monobactams (Kumar et al., 2015). The occurrence of ESBL-producing bacteria has increased worldwide (Lee et al., 2007; Briongos-Figuero et al., 2012). The most ESBL- production is occurring in *E. coli* strains, which are responsible for over 80% of children UTIs (Yoon et al., 2011). Despite the emergence of infections caused by antibiotic-resistant bacteria, there are many attempts to find effective treatments that could have essential role in the inhibition of the growth of the aforementioned bacteria. These inhibitions could be conducted through potentialization of existing antibiotics, determining novel insights in the pharmacokinetics and pharmacodynamics. The concepts of antibiotics combinations provide social outcomes by reasonable use of antibiotics, control of their toxicity and

management of resistance development (Cassir et al. 2014). Nowadays, there is a need for novel molecules or alternative therapeutics to treat bacterial infections mainly those ascribed to GNB (Albers et al., 2014). To this end, chemistry and genomics have been increasingly applied to uncover new potential therapeutics by using antimicrobial peptides (AMPs) (Anthouard and DiRita, 2015; Lombardi et al., 2015). AMPs, such as bacteriocins have been suggested to be a valid alternative to "traditional" antibiotics (Balciunas et al., 2013; Cotter et al., 2013; Cavera et al., 2015). Bacteriocins are proteinaceous ribosomally synthesized, which are mainly active against closely related species (Ge et al., 2016).

Notably, all classes of bacteriocins are ribosomally synthesized, and their coding DNA are usually organized in operon structures containing the structural gene, as well as genes coding for immunity proteins, ABC transporters and accessory proteins. In the case of lantibiotics (class I bacteriocins), they are secreted onto the extracellular medium through an ATP Binding Cassette, formed by proteins encoded by *lanf*, *lanE* and *lanG* genes. Notably, *lanI* gene codes for a lipoprotein (immunity protein), which binds to the outer surface of the membrane and interacts thereof with the lantibiotic to prevent pores forming (Franz et al., 2007). Genetic and regulation of non lantibiotics bacteriocins showed some similarities with lantibiotics group including the arrangement of the structural, transport, regulatory and immunity genes. However, the immunity system for the non-lantibiotic bacteriocins is specific (Drider et al., 2006), Genes coding for bacteriocins are either located on the chromosome or DNA plasmids (Miller et al., 2005). Bacteriocins belonging to class IIa group are produced under immature form with the leader peptide, then the gene coding for the pre-bacteriocin is co-transcribed with the immunity gene. Moreover a second operon including genes coding for proteins involved in the transport of bacteriocin, and a third one, located nearby comprises the genes of the regulatory system, which are necessary to transport and secrete the mature bacteriocin (Miller et al., 2005). Class IIb bacteriocins are leaderless peptides. Seemingly, there is a specific immunity system allocated this group. In this case, the immunity was conferred by the ABC transporter system, by pumping the bacteriocin out of the cell (Cintas et al., 2000; Criado et al., 2006b).

Another group of bacteriocins have particular structure, including cyclic or circular bacteriocins, as the well studied enterocin AS-48, with head-to-tail ligation and are genetically organized in operon comprising the gene coding for the peptide, and those coding for the immunity proteins, ABC transporter system and hydrophobic basic proteins located at the membrane (Ito et al., 2009). In the case of enterocin AS-48, the complete expression of fully operative bacteriocin and immunity require not less than 10 ORFs (*as-48A, B, C, C1, D, D1,E, F, G* and *H*) (Franz et al.,2007)

Recent studies have shown that bacteriocins produced by LAB might be active against unrelated species such *Campylobacter jejuni*, *E. coli* and *Salmonella* Thyphimurium (Embaby et al., 2014; Rumjuankiat et al.,

2015) . LAB found naturally in the human microbiota are generally considered as safe and could be thereof considered as serious candidate for probiotic applications. Bacteriocins produced by enterococci, so far called enterocins, interesting models because of their antagonism against food-spoiling or pathogens. Enterocins are synthesized by different *Enterococcus* species mainly *E. faecalis*, *E. faecium*, *E. mundtii*, *E. avium* and *E. hirae* strains, which are isolated from different sources (Birri et al., 2010; Goto and Yan, 2011; Saavedra et al., 2004; Sánchez et al., 2007). Moreover, several studies indicated that many enterocins, such as enterocin AS-48 produced by *E. faecalis* AS-48, displayed antagonism against GNB and GPB (Grande Burgos et al., 2014). Moreover, several studies underlined the possibilities of using enterocins in human and animal health and also in food preservation (Birri et al., 2010; Todorov et al., 2010). Related to this, the combination of antibiotics and bacteriocins offers a promising strategy to design novel therapeutic options, at time when the potency of traditional antibiotics is fading (Naghmouchi et al., 2013). The combination of antibiotics have been always used to treat severe infections. However, the combinations of antibiotics with bacteriocins are becoming interesting alternatives aiming at reducing the amount of antibiotics used in both human and veterinary medicine. These novel formulations are anticipated to reduce the overall general level of bacterial resistance. Potential achievements with combination of antimicrobial compounds, compared to monotherapy, include a broader antibacterial spectrum, synergistic effects and reducing risk for emerging resistance (Tängdén, 2014). Because of the increasing resistance to broad-spectrum β-lactams in Gram-negative bacilli and absence of no new antibiotics in the pipeline, we assume that combination of antimicrobials therapy should be further explored, as previously suggested by G. Weiss (2003). Antimicrobial agents already used including colistin, which has been recently reintroduced in the human therapy as drug of last resort. Indeed, colistin was combined to different classes of antibiotics including carbapenems, tigecycline, or aminoglycosides, leading thereof to successful treatments on clinical cases (Bassetti and Righi, 2015). The combination of colistin with other antibiotics against MDR strains appeared to be successful (Kumar et al., 2010; Traugott et al., 2011). Moreover, the combination of colistin with azithromycin, doxicycline and rifampin, has revealed a synergistic activity against *P. aeruginosa* isolated from an intensive care unit (López-Fabal et al., 2008). Another report combining colistin and nisin, which is a class I bacteriocin produced so far by *Lactococcus lactis* (nisin A, Z, F) and *Streptococcus uberis* (nisin U) exhibited synergistic action against a set of GNB, among which *E. coli* O157, *P. aeruginosa* and *S.* Thyphimurium. Besides, the antagonism displayed by this combination, the use of colistin and nisin formulation, has led to the drop of colistin cytotoxicity on mammalian Caco-2 cells (Naghmouchi et al. 2013). Interestingly, enterocin AS-48, which is described as a perfect bacteriocin was tested in combination with 2-nitro-1-propanol (2NPOH) against *S. aureus* strains in oat and soya drinks (Burgos et al., 2015). Huang *et al*. (2013) reported that enterocin RM6 produced by *E. faecalis* was active against MRSA. In direct line, Cotter *et al*. (2013) and Allen *et al*. (2014) considered the combination of bacteriocins and phenolic compounds as good concepts for the control of resistant strains in the food chain. Data involving bacteriocins in the medical field as novel

therapeutic agents (Cavera et al., 2015) are in continuing enhancement, which advocates their potential and timely utility to help in overcoming the antibiotic resistance burden. Recent study reported that bacteriocin use as nisin in combination with conventional antibiotics such as ceftriaxone triggers the outer membrane permeation of *Salmonella spp* (Singh et al., 2013). Currently, bacteriocins stand as viable and sustainable alternative to traditional antibiotics in the infection treatments (Cotter et al., 2013)

Main objectives of this project

Use of bacteriocin as alternative therapeutics to treat bacterial infections when the potency of antibiotics is fading is indeed an interesting option that needs to be deeply studied. The data related to the use of bacteriocins as safe antibacterial agents to replace the traditional antibiotics or enhance their activity must pay attention of the public authorities, agencies of regulation and governments. Different options have been designed to fight against the phenomenon of bacterial resistance. These options include the use of probiotics, essential oils, phage therapy (Wang et al., 2015), and more recently the use of bacteriocin (Ghodhbane et al., 2015). The bacterial resistance is a scaring situation that we need to master and avoid to threaten the next generations. Any concept and any evidence aiming at diminishing the bacterial resistance should be welcome in human and veterinary medicine. To this end, different objectives were designed and achieved throughout the present PhD project. The present manuscript is organized into the following chapters:

Chapter 1 is a dissertation on the microbial content and diversity of the meconium. Here, the role of lactic acid bacteria, in particular that of *Enterococcus* genus is highlighted.

Chapter 2 is a research chapter dedicated to isolation and characterization of LAB mainly those displaying antagonism and probiotic properties.

Chapter 3 is a research chapter focused on the purification of enterocins DD28 and DD93, determination of their molecular masses and coding DNA sequences. In this chapter, the anti-MRSA activity is unveiled on planktonic and biofilm formation.

Chapter 4 is research chapter focused on the potentialization of colistin by enterocin DD14; another bacteriocin purified and characterized in our laboratory. The activity of colistin and enterocin DD14 was tested against a set of *E. coli* strains from swine origin, which were grown under planktonic and biofilm cultures.

Chapter 5 is research chapter dealing, in turns, with the potentialization of antibiotics of β-lactams group by colistin. This potentialization was performed on the extended spectrum β-lactamase (ESBL) producing *E. coli* strains, isolated from children with urinary infections.

Chapter 6 is a general conclusion and draws some prospects.

Chapter I: Literature Review

1. What is meconium?

Meconium comes from the Greek word “meconiumarion” meaning poppy juice or opium-like (Romero et al., 2013). It is the first intestinal discharge or the earliest feces of infants, and consists of material ingested or secreted by the gastrointestinal tract (GIT) during the time that the fetus spends in the uterus (Kumagai et al., 2007). It is a sticky dark green substance containing epithelial cells, blood, amniotic fluid, lanugo, bile, bile acid, pancreatic juice, water, and mucus (Swarnam et al.2012). The green coloration of meconium is due to the bile pigments, which are products of heme catabolism. It is the first to be detected in the bile of the fetus after 14 weeks of gestation, then the concentrations of bile increase during gestational period (Blumenthal et al., 1980; Yamaguchi and Nakajima, 1995). Several reports indicated that many drugs are metabolized in the liver, secreted into the bile and thereafter enter into the small bowel, and therefore can be detected in the meconium of the neonate at the time of birth (Wingert et al., 1994). At birth the newborn will evacuate about 60-200 g of meconium (Lee and Kim, 2013). In some cases, the meconium passes when the fetus is still *in-utero*, staining the amniotic fluid. Meconium stained amniotic fluid is seen in 20% of deliveries and likely to develop respiratory distress (Mundhra, 2013). Because meconium is seldom found in the surrounding amniotic fluid and when a newborn baby breathes a mixture of meconium and amniotic fluid, Meconium Aspiration Syndrome (MAS) generally occurs in term or post-term infants and may be associated with severe respiratory failure (Dargaville, 2012; Haakonsen Lindenskov et al., 2015).

2. Gastrointestinal microbiota of newborn infants

Microorganisms found in the meconium are closely related to the microbiota of the gastrointestinal tract (GIT) of newborns. GIT is a complex ecosystem influenced by many biotic and abiotic factors playing a fundamental role in maintaining homeostatis equilibrium. Ninety per cent of the human body is composed of prokaryotic cells, and this group of microorganisms is defined as microbiota (Tsabouri et al., 2014). The GIT is the largest source of microbes, as the human gut microbiome contains up to 10^{14} bacteria, which is 10 fold the number of cells in the human body (Munyaka et al., 2014). The bacteria living in the GIT are known as gut microbiota or gut flora and play an important role in human health through promoting the intestinal homeostasis, the development and the maturation of the immune system and protecting against pathogens (Gerritsen et al., 2011; Hattori and Taylor, 2009). Several studies realized formerly demonstrated dynamic changes in the bacterial composition in the gut during pregnancy and childhood development (Johnson and Versalovic, 2012; Palmer et al., 2007). Other studies suggested that the GIT of infants is sterile and the initial bacterial colonization starts after baby transit through the birth canal (Thum et al., 2012) or immediately after the birth (Biasucci et al., 2008). After a vaginal delivery, the GIT of the infant is colonized by the vaginal and fecal

microbiota of the mother. Infants born by caesarean section are exposed to the skin microbiota of the mother instead. However, the first microbial exposure of newborn delivered by caesarean section perhaps arise from the surrounding environment, hospital staff, and/or other neonates (Satokari et al., 2009; Schwiertz et al., 2003). Studies on intestinal microbiota should include analysis of the microbial ecology and the complexity of the metabolism of the microbial community, as various host–microbial interactions occur at the interface between microbes and host intestine (Ley et al. 2006). Gut microbes start their colonization as soon as the infant's oral mucosa is exposed to the environment (Mackie et al. 1999). More recently, especially in relation to bacteria colonizing the placenta, evidence has been presented suggesting spread of microorganisms from the mother to the amniotic cavity (Fardini et al., 2010). Some data reported that C-section delivered newborns have lower gut microbial diversity, compared to the vaginally delivered infants (Song et al., 2013). Recently, Groer *et al*. (2014) demonstrated that bacterial strains from the maternal gut were transmitted onto child intestine during the pregnancy (Groer et al. 2014). It is further admitted that the microbial colonization starts with facultative anaerobic bacteria belonging to genus of *Enterococcus* or *Streptococcus* and *Enterobacteriaceae*, which are followed by anaerobic bacteria such as *Lactobacillus*, *Bifidobacterium*, *Clostridium, Bacteroides* (Makino et al., 2011).

2.1. Factors affecting the development of gut microbiota in infants

The first colonization of intestinal microbiota in newborns appeared to be influenced by external and internal factors, which may consequently affect the infant's health. The type and number of microbial species of infants GIT were not determined by chance, but by a combination of factors such as mode of delivery, gestational age, host genetics, diet, antibiotic use, and environmental factors (Musso et al., 2010). Some factors, more than others, could affect the colonization of GIT and the composition of the gut microbiota in newborns (Munyaka et al. 2014).

2.1.1. Mode of delivery

The bacterial community colonizing the GIT of infant is from the environmental origin, mainly from the mother. The mode of delivery (vaginally or Caesarian section) determines the nature of infant gut microbes (Thum et al., 2012). Through normal vaginal delivery, an infant is exposed to the mother's vaginal and fecal microbes, which results in the colonization of bacteria such as *Bifidobacterium*, *Lactobacillus*, *Enterococcus* and *E. coli* (Marques et al., 2010; Morelli, 2008). Makino *et al*. (2013) showed that several Bifidobacteria originating from the mother origin were transmitted and resulted in the colonization of the infant's intestine shortly after the birth through vaginal delivery. It has been reported that vaginally delivered newborns acquired bacterial communities closely related to their own mother's vaginal microbiota, dominated by *Prevotella, Lactobacillus* and *Sneathia spp* (Goulet, 2015). On the other hand, a number of studies have described altered intestinal

microbiota profiles in infant delivered by Caesarian section (Scott et al., 2015). Caesarian-section babies harbor microbes that are similar to those of the skin including, *Corynebacterium*, *Staphylococcus*, and *Proionibacterium* (Dominguez-Bello et al., 2011; Song et al., 2013). (Table 1)

Table 1. The factors affecting colonization and diversity of infant's microbiota (Munyaka et al., 2014)

Factors affecting colonization of gut microbiota before birth	**Factors affecting colonization of gut microbiota during or at birth**	**Factors affecting colonization of gut microbiota after birth**
1-Intra-uterine environment	1-Mode of delivery (Caesarean section vs vaginal delivery)	1- Breast feeding vs formula feeding
2-Maternal exposures or practices such as stress, antibiotic use and smoking	2-The environment at the time of delivery	2-Weaning or food supplementation
3-Length of gestation period (term vs preterm)	3-Contact with the mother or health care staff	3-Antibiotic exposure
		4-Home structure (contact with the mother and other family members including siblings and close contact relative)

2.1.2. Diet

The mother needs a diet during pregnancy period and breastfeeding to provide adequate energy and nutrients to support her metabolism and the fetus development, and subsequent milk production. Diet is considered as one of the primary drivers shaping the changes in the structure of the microbiome during infancy, and eventually in the establishment of the adult one, as it has been shown for the microbiome after the exposure to the components of food (Voreades et al., 2014). Moreover, dynamic balance between the host physiology, diet, and GIT microbiota, directly influences the stability of the gut ecosystem and microbial diversity (Munyaka et al., 2014). Previous studies underlined the differential exposure of the infant to fecal, vaginal and skin bacteria from the mother. The mode of birth, and the type of feeding during the first months of life (breastfeeding or formula), are major factors affecting the richness, composition and diversity of the GIT microbial community (Vallès et al., 2014). Furthermore, a recent publication advocated that the type of feeding influences the microbiota composition directly, through providing the substrates for bacterial proliferation and functions (Le Huërou-Luron et al., 2010). It has been reported an increase of more than twice of the number of bacterial cells in breastfed newborns, compared to formula-fed ones (Guaraldi and Salvatori, 2012). The origin of found Lactobacilli and Bifidobacteria or their DNA in the placenta remains speculative. Lactobacilli and Bifidobacteria are also found in the breast-milk (Repa et al., 2015). Notably, *Bifidobacterium bifidum*, *B. longum*,

B. breve and *B. adolescentis* were isolated from both breast-fed and formula-fed infants, whereas *B. infantis* is typical of breast-fed, and *Bacteroides fragilis* of formula-fed infants (Fallani et al., 2011; Mackie et al., 1999).

2.1.3. Antibiotics

The GIT microbiota composition of neonates varies between diseased and healthy individuals. Thus, the use of antibiotics during pregnancy could lead to a delayed colonization or reduced abundance of beneficial bacteria (Of et al., 2002; Russell et al., 2012). Indeed, antibiotics can cause an imbalance in the gut microbiota resulting in the repression of both pathogenic and beneficial species, permitting thereof the overgrowth of antibiotic-resistant strains. However, it is now established that effects of antibiotic treatments on GIT microbiota can differ with the dose and type of antibiotic administered (Voreades et al., 2014). The reduction of microbial diversity is often detected after ingestion of antibiotics in infants of less than one year, and the complete recovery of the initial bacterial composition is seldom achieved (Ferrer et al., 2013). Several studies indicated that the use of antibiotics in the prenatal period (during pregnancy) were associated with a delayed colonization by some microbes especially those belonging to *Bifidobacteria* and *Lactobacillus* genera (Zwielehner et al., 2011). Moreover, the antibiotic exposure, which is common in preterm infants, extremely decreases the microbial diversity and promotes the growth of pathogens such as *Klebsiella*, *Clostridium* and *Veillonella* spp, which are associated with infants sepsis (Madan et al., 2012).

2.2. Microbial diversity in meconium

Historically, the fetus in the intrauterine environment was considered as sterile, with the initial microbial exposure occurring at the birth by vaginally delivering or Caesarian section through contacting maternal vaginal or skin microbiota (Dominguez-Bello et al., 2010; Palmer et al., 2007). However, the presence of microorganisms in meconium suggests that an internal route may transport them to the fetal GIT during pregnancy. Such route might proceed through the bloodstream of the placenta, from where bacteria could pass into the amniotic fluid or fetal circulation (DiGiulio, 2012; Jiménez et al., 2008). During the different stages of pregnancy, the infants swallow large quantities of amniotic fluid, suggesting that the maternal digestive tract may be the origin of microbes found in the amniotic fluid and leads to microbial colonization of the fetal gut (Dasanayake et al. 2005; Jiménez et al. 2005). However, numerous studies reported the presence of different microbes in the umbilical cord, amniotic fluid and placenta (DiGiulio et al., 2008; Hitti et al., 1997; Oh et al., 2010) **(Figure 1**).

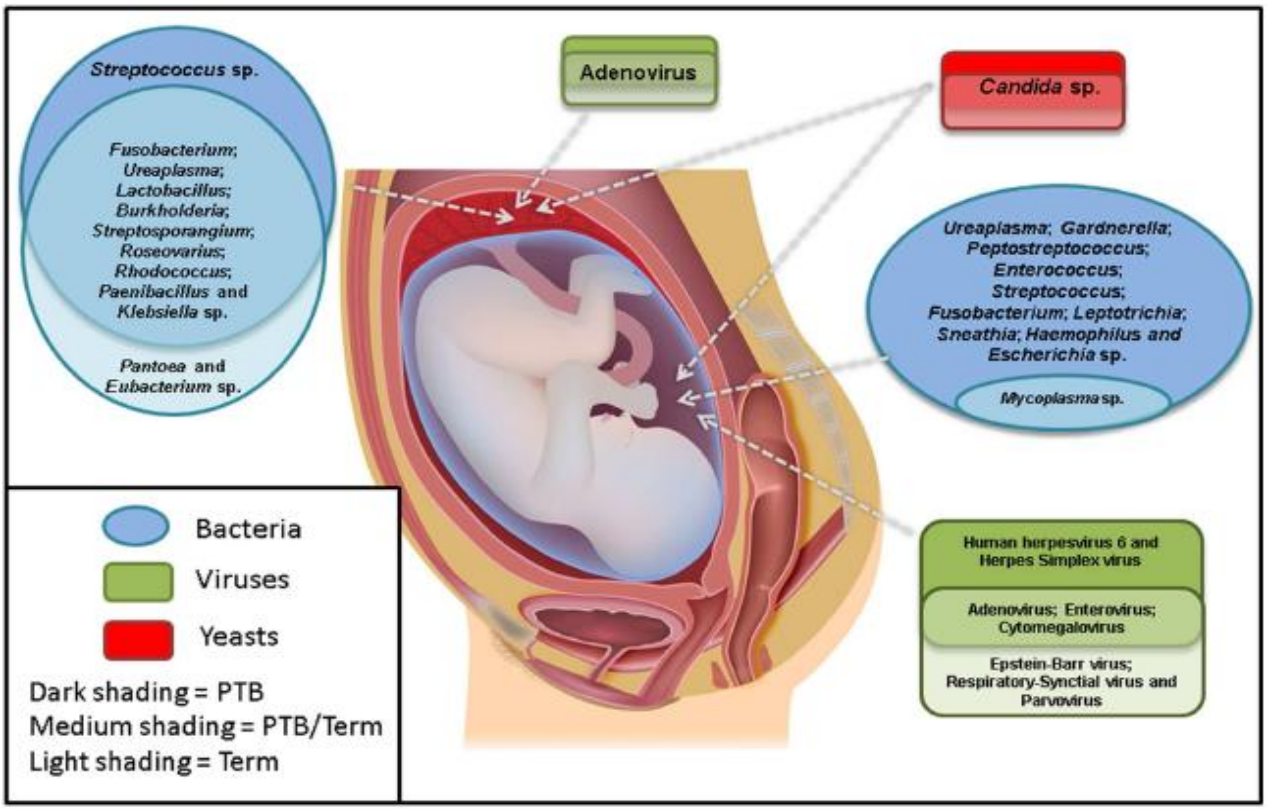

Figure 1. The most commonly detected microorganisms in the amniotic fluid and placenta from preterm and term pregnancies (Payne and Bayatibojakhi, 2014)

In fact, several reports have shown that meconium is not sterile and gut colonization may start before the birth (Jiménez et al., 2008; Madan et al., 2012). On the other hand, meconium microbiota differs from those present in the feces of adults (vaginal and skin), but presents similarities to the fecal microbiota samples from young infants (Gosalbes et al., 2013),although most of studies have focused on the bacterial diversity in the amniotic fluid and meconium. Furthermore, studies reported the presence of fungi, viruses, and protozoa in these two specific environments (Zauli et al., 2013). Remarkably, the eukaryotic microorganisms can be found in the intestinal microbiota. Analysis of the fungal diversity in the GIT unveiled that a majority of fungal phylotypes belongs to Basidiomycota and Ascomycota, including *Candida* and *Saccharomyces* genera (Ott et al., 2008; Scanlan et al., 2008). Different species belonging to the *Candida* genus appeared in the amniotic fluid (Kim et al., 2010). *Candida albicans* is certainly the predominant species recovered directly from the amniotic fluid, but *C. parapsilosis*, was reported to cause fetal infection, and *C. glabrata* was isolated from the amniotic cavity (DiGiulio, 2012). In addition, some authors highlighted the presence of many types of viruses in placenta and amniotic fluid such as human papillomavirus (HPV) and cytomegalovirus (CMV) (Srinivas et al., 2006). Moreover, Villanueva et al. (2000) reported that the presence of CMV in the meconium of infected neonates can be detected by PCR. Other viral taxa were also detected by PCR in amniotic fluid including adenovirus, including enterovirus, Epstein-Barr virus, and respiratory syncytial virus (Baschat et al., 2003). However, the

most recent studies have focused on the bacterial diversity in the meconium of healthy and ill neonates. The presence of pathogenic species, or absence of beneficial species, in early childhood has been suggested to play an important role in the initiation of preterm birth, autism or other immunological deficiency, and development of asthma or eczema allergy (Vaishampayan et al., 2010; Wang et al., 2011; Yap et al., 2011). Another evidence that supports the *in-utero* origin of the meconium microbiota is the fact that microbial composition in feces changes particularly during the first week from the birth, including many types of bacteria such as *Serratia*, *Klebsiella* and *Lactobacillus* appearing (Moles et al., 2013). In a previous study the microbial composition of meconium showed high concentrations of staphylococci, enterococci, and enterobacteria colonizing the GIT of vaginally and caesarean-delivered term and preterm neonates even from the first day of life (Adlerberth et al., 2006; Borderon et al., 1996). Furthermore, bacterial transfer from the mother to the fetus was established on a mice model. Oral administration of labeled bacteria such as *E. faecium* to pregnant mice allowed the isolation of the same bacteria from the fetal meconium after birth by caesarian section (Jiménez et al., 2008). After birth, the microbiota of the infants gut resembles to the maternal vaginal microbiome and skin, with *Enterococcaceae*, *Streptococcaceae*, *Closteridiaceae*, *Lactobacillaceae*, and *Bifidobacteraceae* predominating bacteria taxa (Aires et al., 2011; Lahtinen et al., 2009). A report suggested that the composition of oral microbes of pregnant women showed that some bacteria, such as *Actinomyces naselundii*, were associated with low birth weight and preterm delivery, while others, such as lactobacilli, were related to a higher birth weight and term delivery date (Dasanayake and Li, 2005). An additional illustration of the link between the meconium microbiota composition and infant health has been provided by Gosalbes *et al*. (2013), who found several types of meconium microbiota in healthy newborns containing various potential pathogens as *Staphylococcus*, *E. coli*, and *Shigella spp.*, but dominated by LAB including *Enterococcus spp.*, *Leuconostoc spp.*, and *Lactococcus spp.* Moreover, some studies showed that *E. faecalis* strains were predominant in the meconium (Al Atya et al., 2015). Interestingly *Bifidobacteria* have been detected in healthy, full- term birth babies (Lewis et al., 2013). The composition and diversity of the GIT microbiota play an important role in the human health, providing efficient defense against pathogens by various mechanisms such as colonization resistance and production of antimicrobial compounds (Gerritsen et al., 2011).

3. Lactic acid bacteria (LAB)

3.1. General characterization of lactic acid bacteria

LAB are Gram-positive non-spore-forming, catalase-negative, anaerobic or facultative aerobic cocci or rods bacteria, producing lactic acid as the main fermentation metabolite derived from carbohydrates (O'Sullivan et al. 2002). LAB are found in different ecological niches including soil (Chen and Yanagida, 2006), swine (Mélançon and Grenier, 2003) and water (Hwanhlem et al., 2014). Some LAB isolated from another sources such as fermented foods, plants and the gastrointestinal tracts of many animals and humans (Rubio et al., 2014), where some species can live as commensal microorganisms (Steidler and Rottiers, 2006). LAB species are indigenous microorganisms in many food-related habitats, including plant (fruits, vegetables, and cereal grains) and milk, they are also naturally present in the mucosal surfaces of animals such small intestine, colon, and vagina (Makarova et al., 2006) (**Figure 2**). Moreover, it is widely known that LAB can excrete different metabolites. Metabolism of LAB is associated with the production of many beneficial compounds like antimicrobial compounds, organic acids and enzymes implicated in bioconversion of complex organic compounds into simple functional compounds (Corsetti et al., 1998). The lack of catalase in LAB causes an accumulation of hydrogen peroxide (H_2O_2), which is considered as the one of the most important inhibitory metabolites acting against both bacteria and fungi (Caplice, 1999). Kang *et al*. (2005) reported that some strains of *Lactobacillus fermentum* were able to inhibit the growth of enterotoxigenic *E. coli* that causes diarrhea in piglets by producing hydrogen peroxide.

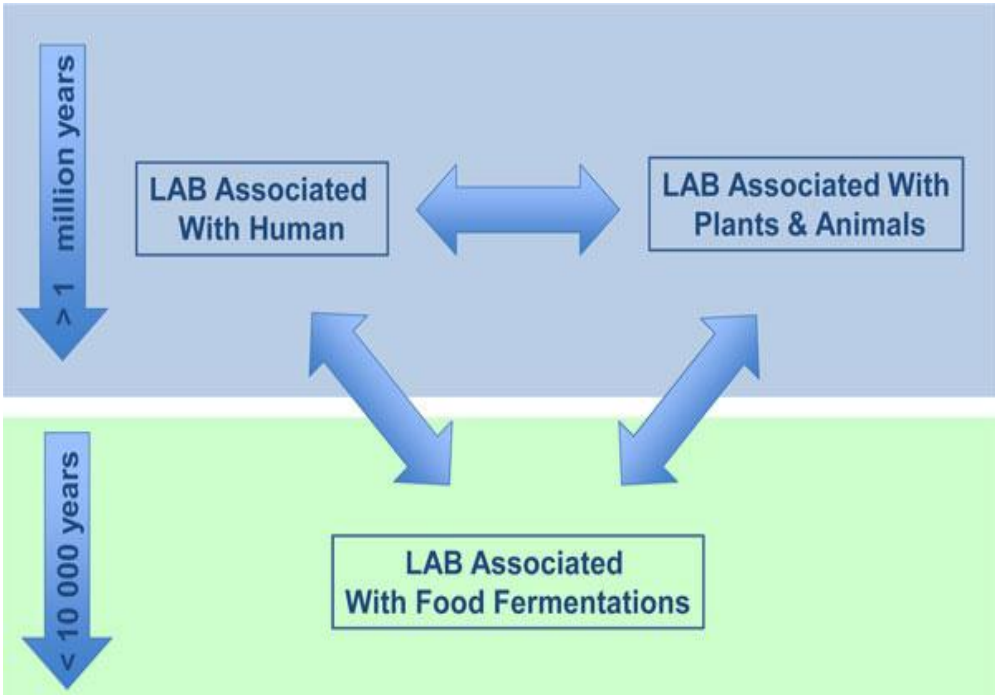

Figure 2. Overview of LAB associations with plants and animals, human and foods (Douillard and Vos 2014).

The main source of energy for bacterial growth is carbohydrates. Metabolisation of carbohydrates by LAB into different useful compounds (mainly lactic acid), is achieved by fermentation. Based on the end-product formed during the fermentation of glucose, LAB can be mainly divided into two main groups (**Figure 3**): (i) Homofermentative LAB, which produce lactic acid as the major or sole end-product of glucose fermentation, such as *Lactococcus*, *Streptococcus, Pediococcus*, and some of lactobacilli. (ii) Heterofermentative LAB, which produce equimolar amounts of lactate, CO_2 and ethanol from glucose *via* the hexose monophosphate or pentose pathway, like *Weissella* and *Leuconostoc* and some lactobacilli (Prückler et al., 2015) (**Figure 3**). The production of organic acids (lactic acid, acetic acid, propionic acid) by LAB has an antimicrobial effect against pathogenic bacteria through the inhibition of active transport processes, enzymes-leaded reactions and modification of their membrane potential (Cleveland and Montville, 2001). Production of organic acid, such as lactic acid, causes a decrease of the pH, contributing to the food preservation by inhibiting the growth of most food spoilage microorganisms (Yang et al. 2012).

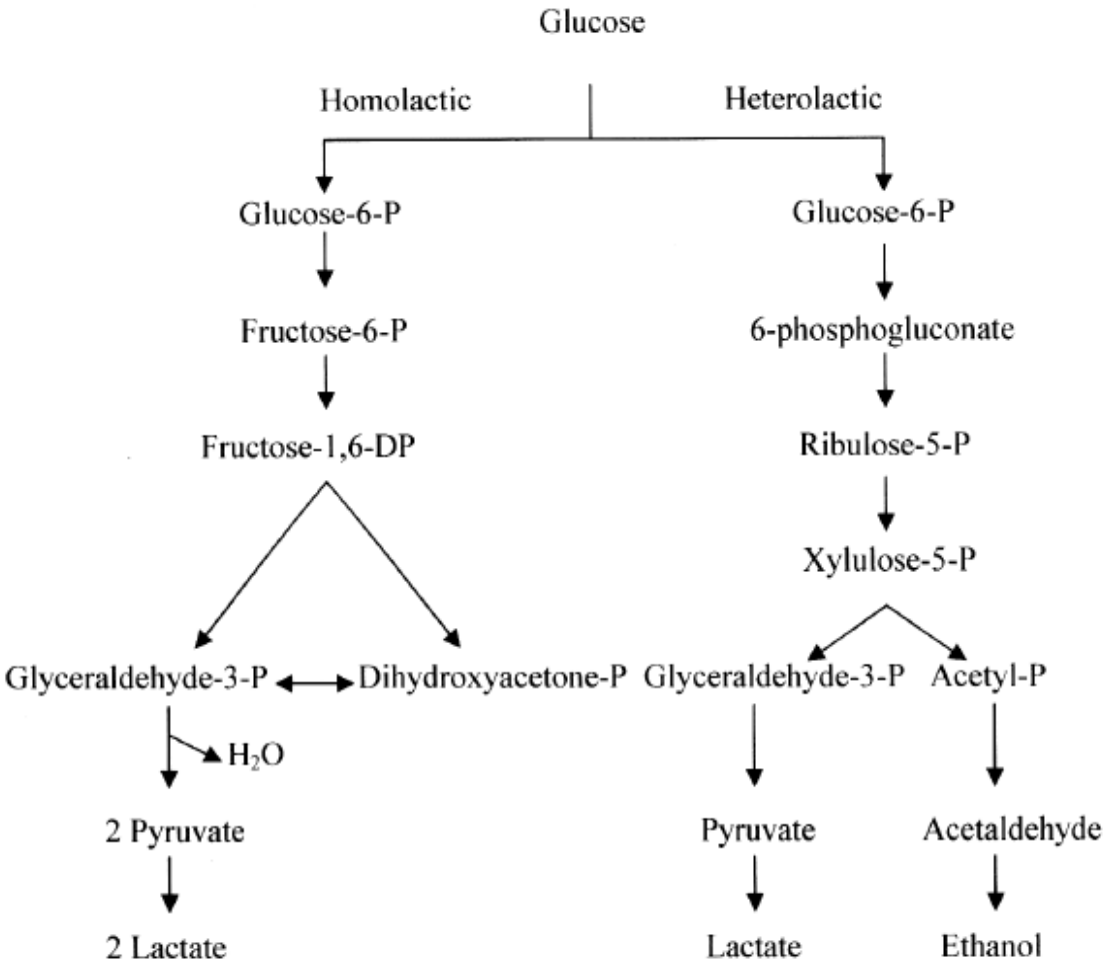

Figure 3. Generalized scheme for the fermentation of glucose in LAB (Caplice 1999).

3.2. Classification of lactic acid bacteria

The organization of LAB taxonomy into different genera was proposed based on their morphologies, growth at different temperatures, glucose fermentation aptitudes, ability to grow at high salt concentrations, acid or alkaline tolerance and configuration of the lactic acid produced. Recently, classification of LAB has suggested new genera, which now include the following ones: *Carnobacterium, Dolosigranulum, Aerococcus, Alloiococcus, Enterococcus, Globicatella, Lactococcus, Oenococcus, Tetragenococcus, Vagococcus, and Weissella. Lactobacilli, Carnobacteria* and some *Weissella* are rods while the remaining genera are cocci (Liu et al., 2009). Another interesting study was performed by Stiles and Holzapfel (1997), who defined four major phylogenetic groups of LAB (**Figure 4**):

- The first group consists of *Streptococcus* and *Lactococcus*. In the *Streptococcus* genus, one specie (*Streptococcus thermophilus*) is used in the food industry.
- The second group contains *Oenococcus*, *Leuconostoc* and *Weissella* (Collins et al., 1993). These bacteria are widely used in industry food for wine production (*Oenococcus oeni*), and fermentation of certain vegetables (*Leuconostoc citreum*).
- The third group includes the genera *Enterococcus*, *Tetragenococcus*, *Melissococcus*, *Carnobacterium*, *Vagococcus*, *Aerococcus*, and *Lactosphaera*
- The fourth group includes *Lactobacillus* and *Pediococcus*.

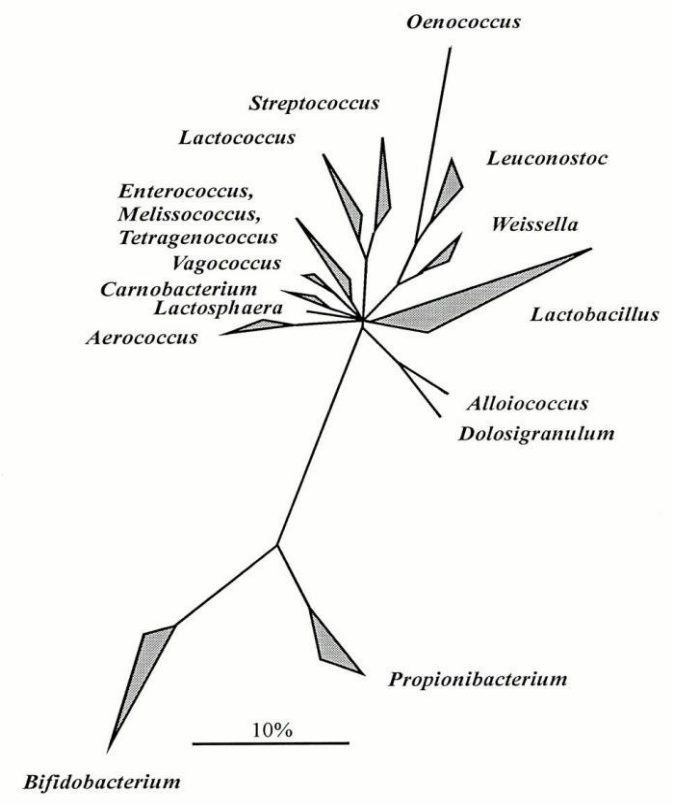

Figure 4. Phylogenetic tree of LAB (Schleifer and Ludwig, 1995).

The morphology is considered as a major characteristic in bacterial taxonomy, and it is still important in the current description of LAB genera (Menconi et al., 2014). Thus, LAB can be divided into rods (*Lactobacillus* and *Carnobacterium*) and cocci (all other genera). However, the genus *Weissella* can include cocci and rods (Collins and Samelis, 1993). Recently, molecular based approaches, such as 16S rDNA sequencing were developed and allowed a more accurate identification of bacteria (Tamang et al., 2008). Calo-Mata et al. (2007) identified genera *Carnobacterium*, *Enterococcus*, *Tetragenococcus* and *Vagococcus* upon the sequencing of their genes coding for ribosomal RNA 16S and 23S.

3.2.1. *Enterococcus*

The *Enterococcus* genus is composed of bacteria exhibiting morphologies such as cocci, occurring alone or in pairs (**Figure 5**). They are Gram-positive, facultative anaerobic, catalase-negative, oxidase-negative, growing at 10°C, 45°C and able to grow in 6.5% NaCl (Devriese et al., 1993; Facklam and Elliot, 1995)**.** Most species of *Enterococcus* can hydrolyze esculin in the presence of 40% bile salts (Oulquié Moreno et al., 2006). The heat resistance of enterococci depends not only on the temperature but also on the growth phase (Martinez et al., 2003). Enterococci are usually homo fermentative, the lactic acid is the end product of glucose fermentation, without production of gas (Klein, 2003).

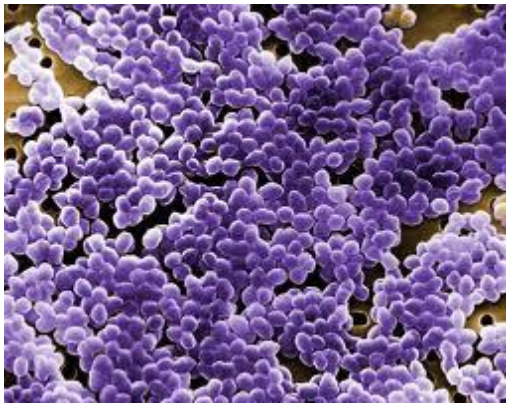

Figure 5. The shape of *Enterococcus spp.*

Until 1984, *Enterococcus spp*. were classified as Group D streptococci, with *Streptococcus faecalis* and *Streptococcus faecium* as major species. Considering physical and biochemical characteristics, these species were classified as Enterococci rather than Streptococci (Schleifer and Kilpper-Balz, 1984). Reclassification of the species belonging to the Streptococci into genera *Streptococcus*, *Lactococcus*, and *Enterococcus* was based on genetic, chemical and biochemical analyses (Devriese et al., 1995; Schleifer and Kilpper-Balz, 1984). Recent reports have focused on the identification of the *Enterococcus* species, by using biochemical analysis and molecular identification techniques. Based on 16 rRNA gene similarities, there are 37 species of *Enterococcus*, which fall into seven species groups (Table 2).

Table 2. *Enterococcus* species and their allocation into species groups (Franz et al., 2011)

Species group based on16S rRNA gene similarity	Species
E. avium group	*E. avium*, *E. devriesei*, *E. gilvus, E. malodoratus*, *E. pseudoavium.* *E. raffinosus*
E. cecorum group	*E. cecorum*, *E. columbae*
E. dispar group	*E. dispar*, *E. asini*, *E. canintestini*, *E. hermanniensis, E. Pallens*
E. faecalis group	*E. faecalis*, *E. caccae*, *E. haemoperoxidus*, *E. moraviensis*, *E. silesiacus*, *E. termitis*, *E. ureasiticus*, *E. quebecensis*
E. faecium group	*E. faecium*, *E. canis*, *E. durans*, *E. hirae*, *E. mundtii*, *E. phoeniculicola*, *E. ratti*, *E. villorum*, *E. thailandicus*
E. gallinarum group	*E. gallinarum*, *E. casseliflavus*
E. saccharolyticus group	*E. saccharolyticus*, *E. acquimarinus*, *E. camelliae*, *E. italicus*, *E. sulfurous*

Another study reported that the genus *Enterococcus* includes more than 40 identified different species (Santagati et al., 2012). DNA relatedness, assessed by fingerprints approach allowed the identification and differentiation of the enterococcal species (Baele et al., 2000). The phylogenetic position in *Enterococcus* species group was determined by 16S rDNA sequençing. For example, the phylogenetic analysis of *E. pseudoavium* showed the closest relatedness with *E. avium* species group (Švec et al., 2005). The phylogenetic analysis based on 16s rDNA gene sequencing also provided evidence that *Enterococcus* strains are more closely related to the *Tetragenococcus*, *Vagococcus* and *Carnobacterium* rather than to *Streptococcus* and *Lactococcus* (Holzapfel et al., 2001).

3.2.1.1. Habitat

Enterococci are ubiquitous bacteria found in the environment, they are found in a wide variety of environments such as water, soil, sewage, plants, and animals (Goto and Yan, 2011; Layton et al., 2010). However, several studies on ecology of *Enterococcus* species showed predominance of *E. faecium* and *E. faecalis* from different sources like fish, cheese, sausages, minced beef and pork (Foulquié Moreno et al., 2006; Klein, 2003). Interesting work performed by Leclerc *et al.* (1996) established the presence of *E. faecalis*, *E. faecium*, *E. durans*, *E. hirae* and *E. cecorum* in the gastrointestinal tract of animals including poultry, cows, sheep, and pigs. Enterococci are also considered as a part of the natural gastrointestinal microbes of healthy human (Ben Said et al., 2015). High concentrations of *Enterococcus* species are present in the human feces, reaching about 10^4 to 10^6 of bacteria per gram of wet weight (Layton et al., 2010). In consequence enterococci are used as indicators of drinking water fecal contamination. Some data reported that the species of *E. faecalis* and *E. faecium* may be more abundant in the feces of human than other species of enterococci, while *E. casseliflavus* and *E. mundtii* may be more prevalent than the other species in the environmental biotopes like plants (Ferguson et al., 2005; Wheeler et al., 2002). Many strains of enterococci colonize the human body, especially the GIT, vagina, skin, oral cavity, the upper respiratory tract as “normal” commensals (Hayashi et al., 2005; Jamet et al., 2012). Sometimes these bacteria can be involved in the nosocomial infections located in the urinary tract, blood stream, abdomen, biliary tract, endocardium, and surgical site (Peel et al., 2012). Many *Enterococcus* species are intrinsically resistant to different classes of antibiotics, including penicillins, cephalosporins, and lincosamides, to a lesser extent to aminoglycosides. In some cases, the therapeutic option becomes limited due to the increasing levels of acquired resistance to multiple antibiotics in the *Enterococcus* species such as *E. faecalis* and *E. faecium* (Heuer et al., 2006). Thus, it is essential to investigate not only the antibiotic resistance profile but also the assessment of the virulence genes of enterococci isolates from the environment and those which are used in the fermentation of meat and vegetable products or used as dairy starter cultures, there are many types of virulence genes found in enterococcal species (Carlos et al., 2010).

The virulence factors described in enterococci include cytolysins (*cylB* and *cylA*), gene encoding an aggregation protein involved in adherence to eukaryotic cells (*agg*), gene responsible for a cell wall protein (*esp*), adhesins ($efaA_{fs}$ and $efaA_{fm}$) involved in binding to the host cell and biofilm formation, sex pheromones (*cpd*, *cob*, *ccf* and *cad*), gelatinase (*gelE*), serine protease, collagen and other compounds (Eaton and Gasson, 2001; Foulquié Moreno et al., 2006; Franz et al., 2003; Valenzuela et al., 2009). Despite some enterococci species being responsible for nosocomial infections in humans, there are many of them which are used in dairy (cheeses and milk) and meat products (Franz et al. 2003). High resistance endurance to different concentrations of salts and survival in the presence of variable pH make these LAB species versatile and well adapted to several food systems. *Enterococcus spp.* was shown to play an important role in the preparation of various fermented milk and meat products in the Mediterranean region (Franz et al., 2011; Giraffa, 2002). Several reports pointed out that the use of enterococci in foods, contributing thereof to flavors, aroma and the ripening of fermented foods, was safe (Antonio M Martín-Platero et al., 2009; Ogier and Serror, 2008).

Moreover, the use of *Enterococcus* as starter culture in the cheeses product testifies the positive role in this bacteria (Giraffa, 2003). Most commonly isolated enterococci from cheese are *E. faecalis* and *E. faecium*, followed by *E. durans* (Antonio et al., 2009). It also has been noticed that the presence of LAB in high numbers, especially enterococci, in fermented products contributed to the development of flavor due to the metabolism including proteolytic, glycolytic, and lipolytic activities (Hugas, 1998; Settanni and Corsetti, 2008).

3.2.1.2. Enterococcus species as Probiotics

According to FAO/WHO (2002), probiotics are defined as “live microorganisms which, when administered in adequate amounts, confer a health benefit on the host”. The term of probiotic increases, along with the understanding of the mechanisms by which they influence human and animal health (Lakshminarayanan et al., 2014; Uyeno et al., 2015). LAB are found naturally in the human intestine, and are generally considered to be safe. For this reason, they have been developed for commercial purposes as probiotics (Fernández et al., 2003; Gu et al., 2008).

LAB genera such as *Bifidobacterium*, *Lactobacillus*, and *Enterococcus* are the most promising probiotic strains (Buntin et al., 2008). Likewise, the representative species include *Bifidobacterium breve*, *B. longum*, *B. bifidum*, *B. infantis*, *Lactobacillus acidophilus*, *L. johnsonii*, *L. casei*, *L. gasseri*, *L. plantarum*, *L. rhamnosus*, *E. faecalis* and *E. faecium* (Kaur et al., 2002). A number of potential probiotic strains of Lactobacilli, Bifidobacteria, or Enterococci isolated from animals like dogs and cats (Biagi et al., 2007; Sauter et al., 2005). Notably, Enterococci strains play a fundamental role in food fermentation and nowadays these bacteria are frequently used as probiotics (Lačanin et al., 2015). These strains showed probiotic characteristics and appeared to be beneficial to the host when they are ingested in adequate quantities (Nami et al., 2015a). The colonization of the

gastrointestinal tract by probiotic strains prevents growth of harmful bacteria by competitive exclusion or by production of antimicrobial compounds (Pieniz et al., 2014). The different species of *Enterococcus* exhibited properties such as lipolytic, proteolytic, esterolytic and other enzymatic activities, and in addition they are able to produce bacteriocins. These characteristics are relevant to make these strains suitable and selectable for application in food fermentations or as probiotics (Belgacem et al., 2010; Foulquié Moreno et al., 2006). On the other hand, the hemolytic activity Enterococci could be considered as a major drawback, as well as the expression of virulence factors such as cytolysin genes, or their resistance to vancomycin. These criteria are those considered for their use as probiotics or starter cultures (İspirli et al., 2015). Recently, it was reported that the *E. faecalis* UGRA10 strain has several probiotic properties based on its survival in the GIT and gut colonization. These include resistance to acid pH, bile tolerance, bile salt hydrolysis, adhesion to different human cellular lines, and biofilm formation (Cebrián et al., 2012). Other studies unveiled the potential of Enterococci as probiotics in animal and human leading thereof to a possible health-promoting option through the intestinal microbial balance (Barbosa et al., 2014; Franz et al., 2011). Another interesting work by Zeyner and Boldt (2006) showed that *E. faecium* DSM 10633 strain could be considered as useful for probiotic applications in piglets to decrease the severity of diarrhea. Further, studies pointed out the safety, beneficial properties and successful applications of *Enterococcus* strains as probiotics. In spite of these promising studies, the FAO/WHO is still unfavorable to give universal approval of *Enterococcus* as probiotic based on their potential acquisition and dissemination of antibiotic resistance, especially to vancomycin (Henning et al., 2015)

3.3. Bacteriocins

Bacteriocins are small ribosomally synthesized antimicrobial peptides that have antibacterial activity towards closely related or non related strains to the producer strain. A great number of GNB and GNB produce such compounds (Cleveland et al., 2001; De Vuyst and Leroy, 2007). Bacteriocins have specific activity towards specific range of targets, generally to closely related bacteria to the producing strain. Notably, the producing bacteria, are protected against their own bacteriocins by specific immunity proteins (Yang et al., 2014). These antimicrobial peptides were found almost in all major groups of bacteria, and many species are anticipated to produce different types of bacteriocins (Riley and Wertz, 2002). The first bacteriocin was described by Gratia in 1925 as antimicrobial peptide produced by *E. coli* and called colicin. Since 1969, the nisin was approved worldwide as food additive E234 and commercially available as Nisaplin (Balciunas et al., 2013). In the last decades, several studies focused on the purification and characterization of bacteriocins, especially those from LAB, and their potential applications as natural food preservatives or therapeutic agents (Van Heel et al., 2011; Cotter et al., 2013). It should be noted that LAB producing bacteriocins are generally recognized as safe (GRAS) (Cleveland and Montville, 2001). The production of bacteriocins may positively affect the ability of a probiotic strain to compete with other microbes in the GIT and plays a fundamental role in the gut microbiota

composition and on the host immune system (Dobson et al., 2012). Bacteriocins production can contribute to microbial survival in the human GIT. One study reported that the intestinal isolate *B. longum*, which produces a bacteriocin, that it might be an important factor for GIT survival (Lee et al., 2008). The bacteriocin antimicrobial activity can be explained by killing or inhibiting growth of competing strains or pathogens (Majeed et al., 2011). Most of bacteriocins from LAB are small (<10 kDa) cationic, amphiphilic, heat-stable, and membrane permeabilizing peptides (Zacharof and Lovitt, 2012). According to Klaenhammer (1993), bacteriocins can be classified into four classes. Class I bacteriocins, called lantibiotics, are thermostable peptides, with very low molecular weight (<5 kDa), represented by nisin. The class II bacteriocins are small thermostable peptides (<10 kDa) which are subdivided into three subgroups: class IIa (pediocin and enterocin), class IIb (lactocin G) and class IIc (lactocin B). Class III bacteriocins are heat labile peptides and are represented by a high molecular weight (>30 kDa) such as helveticin J. The class IV bacteriocins are large peptides linked to lipids or carbohydrates. After gathering novel insights on bacteriocins structures, Drider *et al*. (2006) proposed a classification including only three classes based on their biochemical and genetic DNA organizations. According to Fujita *et al*. (2007), when novel bacteriocin sequences become available, the classification system may eventually change. Nisin belongs to class I bacteriocins, it has been largely used in the food industry as antibiotic agent and is the most extensively studied LAB bacteriocin. Nisin comprises 34 amino acids and exhibits a wide-spectrum antimicrobial action against GNB such as *Listeria monocytogenes*, *Bacillus cereus*, *Clostridium spp.* and *S. aureus* (Rilla et al., 2004). Many reports indicated that bacteriocins might be active against fungi and GNB (Ahmad et al., 2014; Heredia-Castro et al., 2015). Related to this, Smaoui *et al*. (2010) have also found that the bacteriocin BacTN635, produced by *L. plantarum* inhibits the growth of four GNB, *Salmonella enterica* ATCC 43972, *P. aeruginosa* ATCC 49189, *Hafnia sp*. and *Serratia* sp. and the pathogenic fungus *C. tropicalis* R2 CIP203.

The synthesis of bacteriocins usually occurs as biologically inactive pre-peptides that include an N-terminal leader peptide attached to the C-terminal pro-peptide (Cotter et al., 2005) Bacteriocins are antimicrobial peptides synthesized through the ribosomal machinery involving thereof transcription and translation and the genes coding for bacteriocin production and immunity, which are usually organized in operon groups and located either on the chromosome or on the plasmids (Drider et al., 2006; Franz et al., 2007). There are four genes required for the production of Class II bacteriocins including, the pre-bacteriocin structural gene, the immunity gene, the gene that encodes the ABC secretion system for the bacteriocin and its induction gene (Alvarez-Cisneros and Espuñes, 2011)(**Figure 6**). Some genetic determinants of bacteriocin such as enterocin L50A and B produced from *E. faecium* L50 are located on the 50-kb plasmid pC17 (Criado et al., 2006a). The genes coding for enterocin-1071 are situated on a 50-kb conjugative plasmid (Balla et al., 2000). Enterocin X produced by *E. faecium* KU-B5 is composed of two peptides, designed as EntXα and EntXβ (Hu et al., 2010).

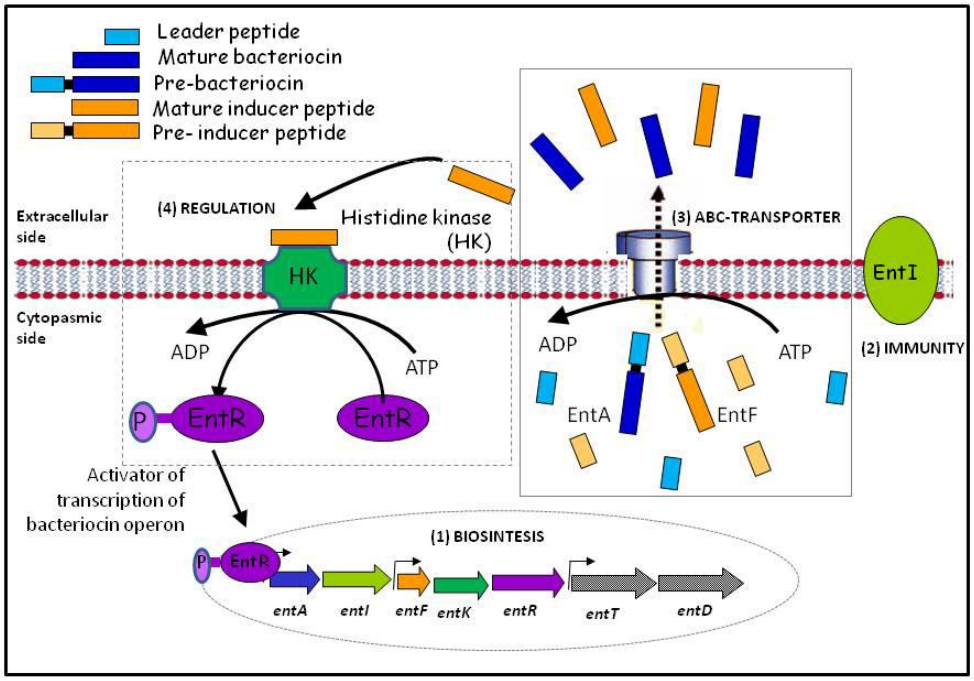

Figure 6. Schematic presentation of the biosynthesis of enterocins (Alvarez-Cisneros and Espuñes, 2011).

The mode of action of bacteriocins is class and subclass dependent. Thus, the class I, such as nisin, has a dual mode of action (**Figure 7**). Indeed, nisin might cause a cell death by preventing correct cell wall synthesis through binding to lipid II. Nisin may employ lipid II as a docking molecule to initiate a process of membrane insertion and pore formation (Gillor et al., 2008). Class II bacteriocins inhibit the growth of bacteria by pore forming, resulting thereof in the loss of the proton-motive force and depletion of ATP (Chikindas et al., 1993).

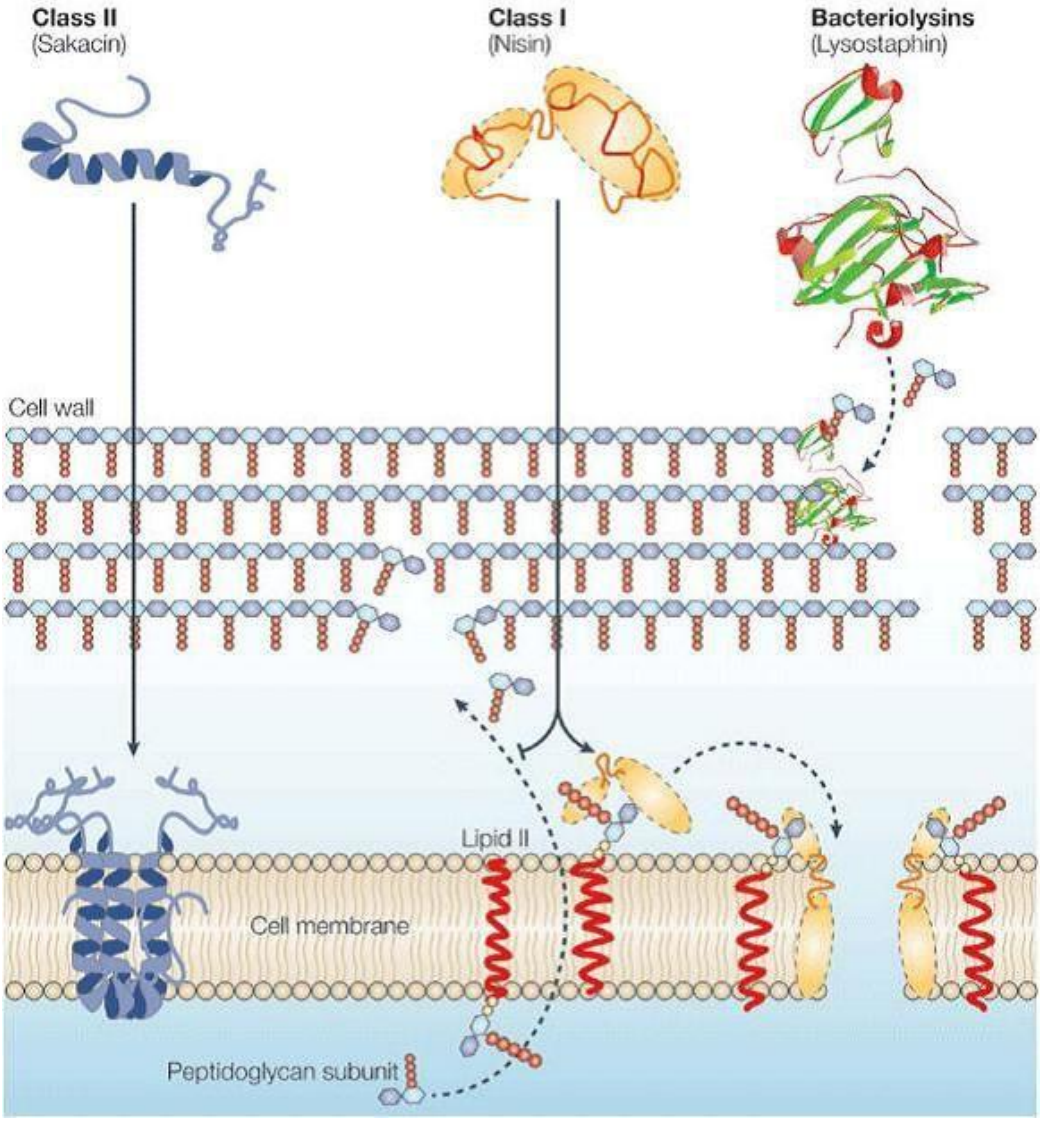

Figure 7. Mode of action of bacteriocins produced by LAB (Cotter et al. 2005).

The majority of class III (Bacteriolysins) kills bacteria by affecting directly the cell wall of GPB leading to lysis of the target cell and its death (Bastos et al. 2010).

Bacteriocins have some similarities with traditional antibiotics. Indeed, they can both act on the bacterial cell wall, protein biosynthesis, DNA replication and transcription, and more commonly disruption of the bacterial membrane (Collin et al., 2013; Garg et al., 2014) (**Figure 8**). A number of bacteriocin inhibits the growth of GPB and GNB by physiochemical effects on the outer membrane of bacteria such as lacticin Q (Yoneyama et al., 2011). Another study reported that enterocin AS-48 interacts with the targeted membrane and led to a rapid decrease in the membrane potential of bacteria (Grande Burgos et al., 2014)

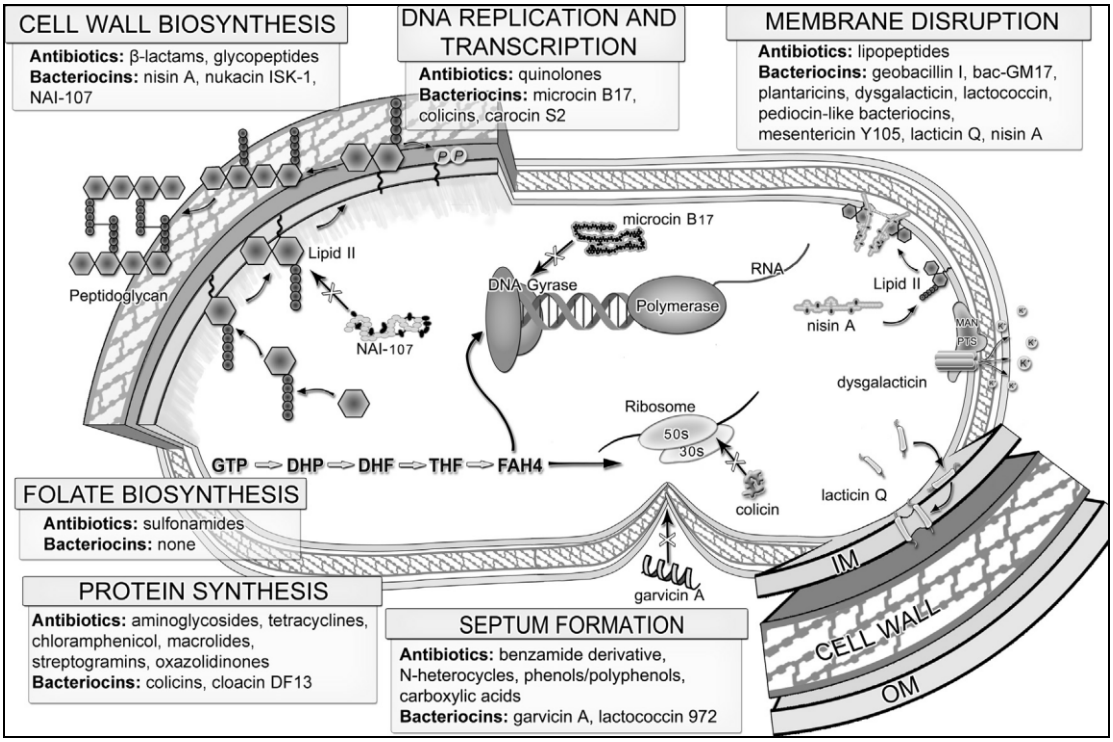

Figure 8. Targets of both antibiotics and bacteriocins, their general location, and examples of each capable of inhibition of these targets (Cavera et al., 2015)

3.3.1. Enterocins

Bacteriocins produced by Enterococci are called enterocins. As a part of bacteriocins specifically produced by this genus, these peptides share common characteristics as they are ribosomally synthesized proteins with activity that is directed usually against closely related species (Huang et al., 2013). These peptides are synthesized by different strains of Enterococcal species, mainly *E. faecalis* and *E. faecium* strains isolated from different sources (Goto and Yan, 2011), but enterocins were also isolated from *E. muntii*, *E. avium*, and *E. hirae* (Birri et al., 2010; Saavedra et al., 2004; Sánchez et al., 2007). During the past decade, the number of characterized enterocins has importantly increased (Foulquié Moreno et al., 2003). Studies carried out on enterocins showed bactericidal activity against spoilage microorganisms and pathogens such as *L. monocytogenes*, *S. aureus*, *Clostridium sp*, *Bacillus cereus*, *Vibrio cholera*, and *E. coli* (Alvarez-Cisneros and Espuñes, 2011; Nishie et al., 2012).

Table 3. Comparison of classification systems for bacteriocins (Franz et al., 2007).

Class	Example
Class I. Lantibiotic enterocins	Cytolysin
Class II enterocins Class II.1 Enterocins of the pediocin family Class II.2 Enterocins synthesized without a leader peptide Class II.3 Other linear non-pediocin-like enterocin	Enterocin A Enterocin L50A Enterocins B
Class III. Cyclic antibacterial	Enterocin AS-48
Class IV. Large proteins	Enterolysin A

Franz *et al*. (2007) suggested a classification scheme specific to enterocins, including four classes: Class I enterocins (lantibiotic enterocins), Class II enterocins (small, non lantibiotic peptides), Class III enterocins (cyclic enterocins) and Class IV enterocins (large proteins) (Table 3). Enterococci, producing enterocins, are isolated from waste, food, and gastrointestinal tract of humans and animals, but may also be isolated from other sources such as fermented foods and feces of healthy babies. There are many enterocin producers strains, among which *E. faecalis* and *E. faecium* from clinical origin; for example, bacteriocin 31 (Tomita et al., 1996) and bacteriocin 41 (Tomita et al., 2008) from *E. faecalis*, and from *E. faecium* bacteriocin 43 (Todokoro et al., 2006), bacteriocin 32 (Nes et al., 1996), and bacteriocin 51 (Yamashita et al., 2011).

Cytolysin is the most thoroughly characterized Enterococcal lantibiotic; it has the ability to inhibit a broad range of GPB, and it is lethal for a broad range of prokaryotic and eukaryotic cells, such as erythrocytes from various animals, it is therefore often referred as a hemolysin (Coburn and Gilmore, 2003). The class II of enterocins including subclass IIa enterocin such as enterocin A and P, subclass IIb such as enterocin L50 and Q (Enterocin L50A/B and Enterocin Q), and subclass IIc as enterocin B (Nami et al., 2015b). Enterocin AS-48 is a cyclic antibiotic peptide produced by *E. faecalis* AS-48; this peptide is active against both GPB and GNB (Grande Burgos et al., 2014). Conversely, enterocin 4 produced by *E. faecalis* INIA 4, which has globally same structural characteristics as enterocin AS-48, was in turns devoid of antagonism against GNB (Joosten et al., 1996). Enterolysin A is a class IV bacteriocin with a broad inhibitory spectrum activity against different species of GPB and GNB. Several studies indicated that the enterolysin A is produced by many species of enterococci such as *E. faecalis* LMG 2333 and DPC5280 (Hickey, 2003). Enterolysin A showed antimicrobial activity on various foodborne and clinical pathogens (Khan et al. 2013).

3.3.1.1. Enterocin AS-48

Enterocin AS-48 is a circular cationic bacteriocin produced by *E. faecalis* AS-48 strain and has a wide inhibitory spectrum against many GPB including *Bacillus cereus* and *L. monocytogenes* (Baños et al., 2016) (**Figure 9**). Some GNB were also sensitive to enterocin AS-48 such as *E. coli* (Grande Burgos et al., 2014).

Number of *E. faecalis* strains, isolated from different sources, like milk and traditional chesses, produce enterocin AS-48 (Folli et al., 2003). Recently, enterocin AS-48 became subject of industrial and pharmaceutical interest and genetic engineering of the producer strains of this bacteriocin to improve and optimize the production of the enterocin AS-48 was explored (Cebrián et al., 2014).

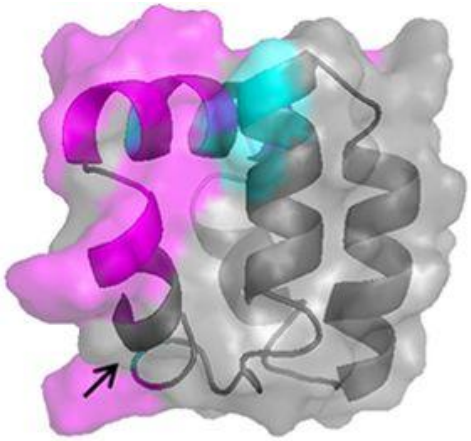

Figure 9. 3-D structure of Enterocin AS-48 (Gabrielsen et al., 2014)

3.3.1.2. Enterocin L50A *and* L50B

The bacteriocins belonging class IIb are produced by several strains of enterococci, such as enterocins L50A and L50B produced by *E. faecium* L50 (Cintas et al., 2000), enterocins MR10A and MR10B produced by *E. faecalis* MRR 10-3 (Martín-Platero et al., 2006). Enterocin L50 (L50A and L50B) is consisting of two leader-less antimicrobial peptides with a 72% sequence identity (Criado et al., 2006c) Moreover, enterocins L50A and L50B are also produced by other strains, like *E. faecium* IT62 isolated from ryegrass in Japan. These bacteriocins are active against GPB but also against few GNB (Izquierdo et al., 2008). It has been reported that enterocin L50A and L50B isolated from *E. faecalis* SL-5 have antimicrobial activity against *Propionibacterium acnes* (Kang et al., 2009). Previous study showed that enterocins L50A and L50B inhibit the growth of beer spoilage bacteria and may be used as a beer biopreservative (Basanta et al., 2008). The antimicrobial activity of enterocin L50A and enterocin L50B was similar, but some differenced could be observed as enterocin L50A more active against GPB than enterocin L50B (Cintas et al., 1998).

3.3.2. Application of enterocins

Recently, many studies dedicated to the use of bacteriocins such as enterocins in food preservation and clinical cases, have been reported (Tables 4 and 5). Indeed, several studies reported that enterocin A, B, P (Chahad et al., 2012) and enterocin AS-48 can be easily applied in foods (Nes et al., 2006).

Tables 4 Applications of enterocins in human and animal health

Strains	Enterocins	Application	Target organisms	References
E. faecalis SL-5	Bacteriocin ESL5	Acne vulgaris	*Propionibacterium acnes*	(Kang et al., 2009)
E. faecium	Enterocin A	Otitis media	*Streptococcus pneumonia* *Streptococcus pyogenes*	(Kandricáková and Lauková, 2012)
Lactococcus lactis	Enterocin A mutants A24P and T27G	Nosocomial infection	Vancomycin-resistant enterococci	(McClintock et al., 2015)
E. faecium E50-52	Enterocin E50-52	Systemic infection	*Salmonella sp.* *Campylobacter jejuni* *Clostridium sp*	(Svetoch et al., 2008)
E. faecium LWP760	Enterocin 760	Respiratory infection in mice	*Bacillus anthracis*	(Svetoch et al., 2011)
E. faecium T8	Bacteriocin T8	Viral infection	Human immunodeficiency virus	(De Kwaadsteniet et al., 2006)
E. faecium ST5Ha	Bacteriocin ST5Ha	Viral infection	Herpes virus type 1	(Todorov et al., 2010)

Tables 5 . Applications of enterocins in natural or fermented food products

Strains	Enterocins	Application	Target organisms	References
E. faecalis OSY-RM6	Enterocin RM6	Cottage cheese	*L. monocytogenes*	(Huang et al., 2013)
E. faecium CTC492	Enterocin A and B	Cooked ham	*L. monocytogenes,* *Lactobacillus sakei*	(Marcos et al., 2008)
E. faecalis A-48-32	Enterocin AS-48	Oat and soya drinks	*S. aureus*	(Burgos et al., 2015)
E. faecium HZ	Enterocins HZ	Ultra-high temperature processing milk	*L. monocytoegnes*	(Yildirim et al., 2014)
E. faecium L50	Enterocin L50A/L50B	Beers	*Lactobacillus brevis,* *Pediococcus damnosus*	(Batdorj et al., 2006)
E. faecalis N1-33	Enterocin N1-33	Custard cream	*Bacillus cereus*	(Hata et al., 2009)
E. casseliflavus IM 416K1	Enterocin 416K1	Vegetables and fruit	*L.. monocytogenes*	(Anacarso et al., 2011)
E. faecium CN-25	Enterocin CN-25	Fermented fish products	*L. monocytogenes*	(Sonsa-Ard et al., 2015)

Chapter 2. LAB from meconium and assessment of their probiotic properties

This chapter was aimed at unrevealing the LAB content of meconium samples collected at Roubaix Hospital in the north of France. For this prospective study, only six samples were obtained and investigated. It should be pointed out that studies focusing on the bacterial burden and particularly that of LAB from meconium sources are not so available in the literature. Indeed, the isolation of LAB from this matrix and particularly those exhibiting antagonism could be considered as innovative and original. The samples of meconium, collected at Roubaix hospital, were designed as S1, S2, S3, S4, S5 and S6. The samples were serially diluted in saline water and platted onto MRS agar medium (de Man et al., 1960). After incubation under 5% CO_2 at 37°C for 24–48 h, the plates were inspected. Afterwards, the colonies leading to Gram positive staining bacteria and devoid of catalase activity were considered as LAB. Remarkably, all these colonies were identified by MALDI-TOF and when necessary by 16S rDNA sequencing. Both methods (MALDI-TOF and 16S rDNA sequencing) were in perfect agreement.

The number of colonies corresponding to LAB that was obtained per sample is depicted in Table 6. Thus, this first screening permitted the isolation of 107 LAB from the six distinct samples of meconium (Table 6).

Table 6. Numbers of LAB isolates in the meconium samples

Samples	LAB isolates
1	20
2	12
3	26
4	12
5	17
6	20

The MALDI-TOF SM (**Figure 10**) identification of these isolates concluded to the presence of only *E. faecalis.* This lack of diversity could be explained, at least technically, by the use of MRS as the only medium of LAB isolation. Moreover, Fisher and Phillips (2009) associated the lack of diversity to the presence of virulence factors in this species. In this study, we have established that a set of putative genes coding for virulence factors could be encountered at least in the antagonistic strains (Table 7). These genes included those coding for cytolysins (*cylB* and *cylA*), which are aggregation proteins involved in the adherence to eukaryotic cells. There are also genes coding for cell wall protein synthesis (*esp*), for adhesins ($efaA_{fs}$ and $efaA_{fm}$), which bind to

the host cell and also reported in the biofilm formation, for sex pheromones (*cpd*, *cob*, *ccf* and *cad*), gelatinase (*gelE*), for serine protease, for collagen (Foulquié Moreno et al., 2006; Valenzuela et al., 2009).

Table 7. Amplification of putative genes coding for known virulence determinants

Isolates	Putative genes coding virulence factors
E. faecalis 14	*agg, gelE, efaAs, ccf, ace*
E. faecalis 28	*agg, gelE ,efaAs, cpd, ccf, ace*
E. faecalis 90	*agg, gelE, efaAs, ccf, ace*
E .faecalis 93	*agg, gelE, efaAs, cpd, ccf, ace*
E. faecalis 97	*agg, gelE, efaAs, cpd, ccf, ace, cylM, cylA*
E. faecalis 101	*agg, gelE, efaAs, cpd, ccf, ace, cylM, cylA*

Putative genes coding for virulence factors as detected by PCR were *cyl*, cytolysin; *gelE*, gelatinase; *agg*, aggregation substance *esp*, enterococcal surface protein; *ace*, collagen adhesion; *efaA*, enterococcal antigen; *cpd*, *cob*, *ccf*, sex-pheromones.

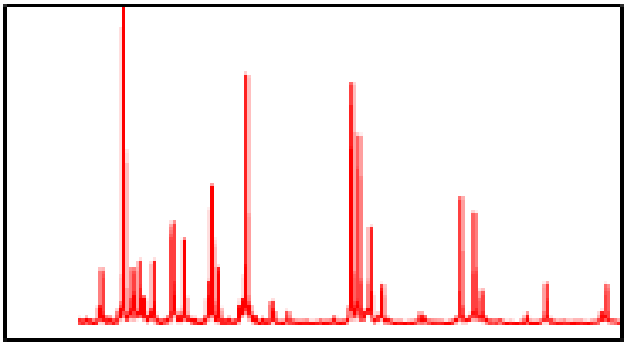

Figure 10. Typical MALDI-TOF Mass Spectrometer obtained for the LAB isolates

Moreover, using different approaches, we established the inhibitory activities of *E. faecalis* 14, *E. faecalis* 28, *E. faecalis* 90, *E. faecalis* 93, *E. faecalis* 97 and *E. faecalis* 101. Importantly, isolates *E. faecalis* 14, *E. faecalis* 28, *E. faecalis* 90, *E. faecalis* 97 and *E. faecalis* 101 were obtained from donor 3 (sample 3), whilst isolate *E. faecalis* 93 was obtained from donor 5 (sample 5). This antagonism was ascribed to both production of lactic acid and bacteriocins named thereof enterocins. The identification of these antagonistic strains was confirmed by 16S rDNA sequencing. To establish any genetic relatedness or clonal relationship between these

antagonistic strains, we extracted and analyzed their DNA by repetitive element palindromic-PCR (Rep-PCR) technology (Al Kassaa et al., 2014). The dendrograms resulting from the Rep-PCR amplification led us thinking that these antagonistic isolates have a quite similar DNA patterns.

Besides their antagonisms, isolates *E. faecalis*14, *E. faecalis* 28, *E. faecalis* 90, *E. faecalis* 93, *E. faecalis* 97 and *E. faecalis* 101 displayed more interesting features such as their high scores of aggregation and hydrophobicity, the absence of hemolytic activity, and their sensitivity to all nearly antibiotics used to treat Enterococci infections. Taken together these data render the newly isolated strains as potential candidates for probiotic applications.

To gain more insights on the inhibitory activities of *E. faecalis* 14, *E. faecalis* 28, *E. faecalis* 90, *E. faecalis* 93, *E. faecalis* 97 and *E. faecalis* 101, we established that concomitant production of lactic acid and enterocins inhibited the growth of GNB and mainly GPB, including the methicillin-resistant *S. aureus* (MRSA). MRSA was reported as a frequent cause of infection in the pediatric and adult populations (Machuca et al., 2014). In this chapter, we unveiled the potential of *E. faecalis* 28 and *E. faecalis* 93 isolated, as previously stated, from two different donors, to inhibit the growth of *S. aureus* ATCC 33862. The co-culture of *E. faecalis* 28 or *E. faecalis* 93 with *S. aureus* ATCC 33862 has led to a drastic decrease, 3 Log_{10} of CFU/ml, of *S. aureus* ATCC 33862.

As conclusion, the work performed in this chapter showed that Enterococci are present, not necessarily as dominant species, in meconium. Importantly, Enterococci isolated from this matrix appeared to be safe and producing potent antimicrobial substances such as enterocins. Besides their antagonistic activities, the probiotic characteristics revealed such as their antibiotic susceptibility, absence of hemolytic activity and virulence factors, high hydrophobicity and aggregation scores and high inhibitory activities, render these strains as serious candidates for probiotic applications. The data gathered from this study are in good agreement with those recently reported by Lačanin *et al*. (2015), who are supporting the use of this species as probiotics.

Probiotic potential of *Enterococcus faecalis* strains isolated from meconium

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Abstract

107 bacterial isolates with Gram positive staining and negative catalase activity, presumably assumed as lactic acid bacteria, were isolated from samples of meconium of six donors at Roubaix hospital, in the north of France. All these bacterial isolates were identified by MALDI-TOF mass spectrometry as *Enterococcus faecalis*. However, only six isolates among which *E. faecalis* 14, *E. faecalis* 28, *E. faecalis* 90, *E. faecalis* 97 and *E. faecalis* 101 (obtained from donor 3) and *E. faecalis* 93 (obtained from donor 5) were active against some Gram negative bacteria (GNB) and Gram positive bacteria (GPB), through production of lactic acid and bacteriocin like inhibitory substances (BLIS). The identification of these isolates was confirmed by 16rDNA sequencing and their genetic relatedness was established by REP-PCR and pulsed field gel electrophoresis (PFGE) methods. Importantly, the aforementioned antagonistic isolates were sensitive to various classes of antibiotics tested, exhibited high scores of coaggregation and hydrophobicity, and were not hemolytic. Taken together, these properties render these strains as potential candidates for probiotic applications.

Keywords: **meconium, *Enterococcus faecalis*, antagonism, anti-*Staphylococcus* activity, Enterocins**

1. Introduction

Human microbiome is undergoing dynamic changes in bacterial content in the gut during pregnancy and development of childhood (Dominguez-Bello et al., 2010). In early childhood, the presence of pathogenic species, or absence of beneficial ones, leads to adverse effects such as initiation of preterm birth (DiGiulio et al., 2008), and development of asthma, allergy, and autism (Hong et al., 2010; Johansson et al., 2011; Wang et al., 2011). Microbes colonizing the gastrointestinal tract of the infant are originated from the mother and surrounding environment during the birth and shortly thereafter (Mshvildadze et al., 2010). The fetus, as well as the intrauterine environment, have been considered sterile, but the presence of microbes was reported in amniotic fluid (Hitti et al., 1987), fetal membranes (Steel et al., 2005), and meconium (Gosalbes et al., 2013). Meconium is the first intestinal discharge of newborns, which consists of a viscous, sticky, dark green substance. It might contain materials ingested during time that the infant has spent in the uterus; these could include epithelial cells, mucus, bile acid, blood, lanugo and amniotic fluid (Cleary and Wiswell, 1998; Gosalbes et al., 2013). Newborns seem to acquire their first microbiota at birth and maternal vaginal or skin bacteria colonize newborns delivered vaginally or by caesarian section (C-section) (Alicea-Serrano et al., 2013). Hu et al. (2013) confirmed that meconium is not sterile and does contain diversified microbiota. Moles et al (2013) characterized the microbiota from meconium and fecal samples obtained during the first 3
weeks of life from 14 donors. They showed that bacilli and other Firmicutes were important in meconium whilst Proteobacteria dominated in the fecal samples based on molecular method. *Staphylococcus* predominated in meconium while *Enterococcus* and GNB were more abundant in fecal samples (Moles et al., 2013). Makino et al. (2013) showed that several *Bifidobacterium* strains transmitted from the mother are colonizing the infant's intestine shortly after birth. Thus, the gut of infant might contain different species such as Enterococci, Bifidobacteria and Lactobacilli that protect mucus of infants from pathogenic species through production of inhibitory substances including hydrogen peroxide, organic acids and bacteriocins (Vizoso-Pinto et al., 2006; Rodríguez et al., 2012). Enterococci , particularly *E. faecalis* and *E. faecium* are involved in the reduction or prevention of gastro intestinal tract infections (Franz et al., 2011). Enterococci belong to the group of LAB,

which is known to produce lactic acid as the end product of sugar fermentation, and antimicrobials, which are active against pathogens including *Staphylococcus aureus* and *Listeria monocytogenes* (Campos et al., 2006). Further, many strains of *E. faecalis* produce bacteriocins named enterocins, a family of safe (Belguesmia et al., 2010), and ribosomally synthesized antimicrobial peptides (AMP) (Drider and Rebuffat, 2011). This study aimed at studying and taking advantage of the LAB isolated from meconium sampled at Roubaix hospital in the North of France

2. Materiels and methods

2.1. Preparation of samples, isolation and identification of lactic acid bacteria

Samples of fresh meconium were collected, in sterile dry plastic containers, from six donors(newborn infants), at Roubaix hospital in the North of France. Samples of meconium were stored at 4$_{o}$C until to be processed. One gram of meconium was resuspended in 9 ml of0.9% (w/v) of sterile saline solution, and was serially diluted from 10^{-1} to 10^{-6}. One ml of each dilution was poured onto Petri dishes and melt MRS (de Man-Rogosa-Sharpe) agar medium (Biokar, France) (de Man et al., 1960) was poured gently. After agar solidification at room temperature, plates were incubated under 5% CO_2 at 37 $_{o}$C for 24-48 h. The grown colonies were Gram-stained and checked for catalase activity. Presumptive LAB strains were selected, maintained at -80$_{o}$C in MRS with 25% glycerol as stock culture, until use. The presumptive LAB isolates were identified by the VITEK MS v2.0 MALDI-TOF mass spectrometry according to manufacturer's instructions.

2.2. Antimicrobial activity

The antibacterial activity was assessed against the GNB and GPB listed in Table 1, using the well known agar diffusion test (Naghmouchi et al., 2006). The cell free supernatants (CFS) used for antibacterial activity measurement were obtained by centrifuging (9000 x *g*, 10 min,4$_{o}$C), overnight cultures of *E. faecalis* grown at 37 °C for 18 to 24 h, on MRS broth. Wellswere performed in solid agar and 50 μl of each CFS or neutralized cell-free supernatant(NCFS) (pH6.5) were poured into the wells. The Petri plates were left at room temperature,in sterile conditions, for 1 h before incubation for 18 h at adequate temperature. After thisperiod of

incubation, the antibacterial activity was detected by observing the inhibitionzones around the well containing the CFS or NCFS. Importantly, the impacts of temperature and pH variations as well as proteinase K, papain, trypsin, and -chymotrypsin at 1 mg/ml(Sigma-Aldrich Germany) were determined towards *Listeria innocua* CIP 103982.

2.3. Molecular characterization of the antagonistic isolates

Total DNA was extracted from each antagonistic isolates using Wizard® Genomic DNAPurification Kit (Promega, USA). For 16S rDNA analysis, total DNA was amplified with

16S forward 5'-AGAGTTTGATCMTGGCTCAG-3' and 16S reverse 5'-GGMTACCTTGTTACGAYTTC-3´ primers (Drago et al. 2011), and the following PCRprogramme : 94$_o$C/3 min, 29 cycles at 94$_o$C/40s, 55$_o$C/50s and 72$_o$C/2 min; and finally 72$_o$C/10 min. For REP-PCR analysis, total DNA was amplified with 5'- $(GTG)_5$-3 primer and the following programme: 95°C/5min, 30 cycles at 94°C/1min, 40°C/1min, 72°C/8min, and finally 72°C/16 min. Amplicons were separated on 1% agarose gel. Electrophoresis was performed at 100V for 2h using 1X Tris-borate-EDTA. The gels were stained with GEL RED (Biotium, Canada), and visualized by GelDoc (Bio-Rad, France). Dendrograms were generated automatically using the DIVERSILAB® software (Biomérieux, France).The amplicons for 16S rDNAs were purified with the pJET1Kit (Fermentas, France), andsequenced at Eurofins MWG Operon (Germany). Partial 16S rDNAs sequences werecompared to that of *E. faecalis* ATCC 19433 using ClustalW2 software (http://www.ebi.ac.uk/Tools/msa/clustalw2/).

2.4. Challenge tests : *Staphylococcus aureus* ATCC33862 VS. antagonistic *E.faecalis.*

E. faecalis was grown in MRS and *S. aureus* ATCC33862 in Brain Heart Infusion (BHI)(10_5 CFU/ml) at 37°C for 18h. After this period, the cultures had reached 10_8 CFU/ml for *E. faecalis*, and 10_5 CFU/ml for *S. aureus* ATCC33862. Afterwards, 0.1 ml of each culture was added to 20 ml of BHI containing leading to 5. 10_5 CFU/ml (*E. faecalis*) and 5. 10_2 CFU/ml (*S. aureus* ATCC33862). One ml of this coculture was withdrawn at 0, 4, 8, 12, 18 and 24 h, and was serially diluted from 10^{-1} to 10^{-6}, in 0.9 % (w/v) of saline water. Then 1 ml of each dilution was plated on Plates dishes containing MRS agar for *E. faecalis*, and Chapman agar for *S. aureus* ATCC33862. The number of colonies was determined after 24 h of incubation at 37°C.

2.5. Lactic acid quantification

Lactic acid was quantified by HPLC spectra system P1000XR (Thermo, USA). Isolates *E. faecalis* 28 and *E. faecalis* 93 were grown in MRS broth at 37$_o$C, samples were withdrawnafter 0, 4, 8, 12, 18 and 24 h of incubation, centrifuged (10,000 x *g*, 10 min, 4$_o$C), andsterilized by filtration using Millipore filter (0.2 µm). The CFS were divided into threesamples. Sample 1 was used to measure pH, sample 2 to assess the antibacterial activity against *S. aureus* ATCC33862 by the well diffusion method (Naghmouchi et al., 2006), and sample 3 to determine the concentration of lactic acid. All these experiments were performed in triplicate.

2.6. Autoaggregation assay

Autoaggregation assays were performed according to initial protocol of Del Re et al. (2000) and modified by Al Kassaa et al. (2014). Thus, isolates of interest were cultured on MRS for 18 h at 37$_o$C, then cells were centrifuged (5,000 × *g*, 4$_o$C, 15 min), washed three times with phosphate saline (PBS) buffer (pH 7.0) and were resuspended in sterile PBS to obtain a viable cell count of 10$_8$ CFU/ml. The bacterial suspension of 4 ml was mixed by vortexing for 10s and incubated at room temperature for different time intervals (0, 1, 2, 3, 4 or 5 h). At each interval, the growth was measured at OD_{600nm}. The percentage of autoaggregation was expressed as follows :

% autoaggregation = [(OD_1-OD_2) / (OD_1)]× 100.

Where OD_1 represents the optical density at time (1, 2, 3, 4 or 5 h) and OD_2 the data at time 0h. All experiments were performed in triplicate.

2.7. Hydrophobicity

The hydrophobicity was determined using *in vitro* method to detect the bacterial adhesion to hydrocarbons (Rosenberg et al., 1980). Each antagonistic LAB isolate was grown in MRS broth at 37$_o$C for 18 h. The bacterial cells harvested by centrifugation (5,000 x *g*, 4$_o$C, 15 min) were washed twice with PBS (pH7.0), resuspended in the same solution and the OD_{600nm} was determined. One milliliter of xylene (Fluka, Germany) was added to 3 ml of cell suspension and vortexed for 2 min after 10 min of incubation at room temperature. The aqueous phase was removed after 2 h of incubation at room temperature and the OD_{600nm} was determined. The percentage of

hydrophobicity was calculated using the formula given below. All experiments were performed in triplicate. % hydrophobicity = [(OD_{600nm} reading 1–$OD_{600\ nm}$ reading 2)/ OD_{600nm} reading 1] x 100.

2.8. Hemolytic activity

The hemolytic activity was determined by streaking Enterococcal isolates on Columbia blood agar supplemented with 5% (v/v) of human blood or Sheep blood. The plates were incubated at 37°C for 48 h. The presence or absence of zones of clearing around the colonies was interpreted as -hemolysis (positive hemolytic activity) or -hemolysis (negative hemolytic activity), respectively. When observed, greenish zones around the colonies were interpreted as -hemolysis and taken as negative for the assessment of hemolytic activity (Semedo et al., 2003).

2.9. Antimicrobial susceptibility

Study of antibiotic susceptibility was performed by three independent methods: disk diffusion method, minimal inhibitory concentrations (MICs) using E-test (Bio-Mérieux, France), and VITEK 2 system (Bio-Mérieux, France). Related to this, AST-P606 card wasused for "Enterococci" encompassing nearly all important antibiotics among which ampicillin, gentamicin, kanamycin, streptomycin, levofloxacin, moxifloxacin, erythromycin, clindamycin, linezolid, teicoplanin, vancomycin, tetracycline, nitrofurantoin chloramphenicoland trimethoprim-sulfamethoxazole. Antibiotic susceptibility and MICs were determined and analyzed according to the French Committee on Antimicrobial Susceptibility Testing (CA-SFM 2013).

3. Results

3.1. Scavengenging of lactic acid bacteria and elucidation of their antagonism

107 LAB isolates were isolated from six samples of meconium newborns infants (six donors) obtained at Roubaix hospital in the North of France. The number of LAB isolates per sample of meconium/donor was as follows: sample 1 (20 LAB isolates), sample 2 (12 LAB isolates),sample 3 (LAB 26 isolates), sample 4 (LAB 12 isolates), sample 5 (LAB 17 isolates), andsample 6 (LAB 20 isolates). In addition to their Gram positive staining, and absence of catalase activity, all these 107 LAB isolates were able to hydrolyze esculine. Importantly, allof them were identified by MALDI TOF mass spectrometry (Biomérieux, France), with high score (>99 %), as *E.*

faecalis. Among these *E. faecalis* species, only six isolates designed as *E. faecalis* 14, *E. faecalis* 28, *E. faecalis* 90, *E. faecalis* 93, *E. faecalis* 97 and *E. faecalis* 101 resulted to be antagonistic. Partial 16S ribosomal DNA sequences of these isolates as well as those of E. faecalis ATCC19433 were aligned using ClustalW2 software (http://www.ebi.ac.uk/Tools/msa/clustalw2/).This alignment shows a percentage of similarity higher than 99.7% to *E. faecalis*, confirming phylogenetic identification of our isolates. When the 16S rDNA sequence of *E. coli* ATCC 11229 was compared to that of enterococci, the percentage of similarity was estimated to 76.33%, which clearly is very low. These antagonistic isolates were able to produce lactic acid and BLIS and consequently to inhibit growth of GNB and GPB (Table 1). This antagonism was restricted to GPB when the pH of CFS was adjusted to 6.5 (Table 1). The BLIS produced by the aforementioned antagonistic strains resulted to be insensitive to pH and temperature variations but sensitive to proteases. Remarkably, only *E.faecalis* 90 and *E. faecalis* 101 were active against *Pseudomonasfluorescens* (Table 1).

3.2. Genetic patterns of the antagonistic strains

Genetic patters gathered by REP-PCR permitted to create two main groups. Indeed, group 1 contains isolates *E. faecalis* 14, *E. faecalis* 28, *E. faecalis* 90, *E. faecalis* 97 and *E. faecalis* 101, whereas group 2 contains only isolate *E. faecalis* 93 (**Figure 1**). Based on their genetic patterns and analysis of the dendrogram and PFGE (data not shown here), we assume that *E. faecalis* 14 and *E. faecalis* 28, then *E. faecalis* 90 and *E. faecalis* 93, *E. faecalis* 97 and *E. faecalis* 101 have similar DNA patterns.

3.3. Highlights on anti-*S. aureus* ATCC33862 activity

The data gathered from the challenge tests indicate that inhibition of *S. aureus* ATCC 33862 by *E. faecalis* 28 and *E. faecalis* 93 was due to production of lactic acid and bacteriocin. The number of *S. aureus* ATCC33862 cells has decreased of about 5.5 Log, 5 Log and 2 log in presence of *E. faecalis* 28, *E. faecalis* 93 and *E. faecalis* ATCC29212 (**Figures 2A, 2B,2C**).

3.4. Production of lactic acid and bacteriocins like inhibitory substances

E. faecalis 28 and *E. faecalis* 93 isolates produced up to 7.06 g/l of lactic acid, after 24h of culture. As shown on **figures 3A & 3B**, the drop of pH observed during coculture, was correlated to increase of lactic acid production.

The anti-staphylococcal activity was ascribed to both lactic acid and bacteriocins, and this activity appeared to increase until 18h of co-culture.

3.5. Cell surface properties of the antagonistic isolates

Table 2 shows the scores of hydrophobicity and autoaggregation of antagonistic isolates.The highest scores were registered for *E. faecalis* 14; that was isolated from donor 3.

3.6. Antagonistic strains are sensitive to antibiotics and are not hemolytic

As indicated in Tables 3A & 3B, antagonistic strains are sensitive to antibiotics tested. To be noted that *E. faecalis* 14 and *E. faecalis* 93 are resistant to erythromycin, whilst isolates *E. faecalis* 28, *E. faecalis* 90, *E. faecalis* 97 and *E. faecalis* 101 exhibit intermediate resistance to this drug. Further, all antagonistic isolates are sensitive to ampicillin, gentamicin, kanamycin, streptomycin, levofloxacin, moxifloxacin, linezolid, teicoplanin, vancomycin, tetracycline, nitrofurantoin and chloramphenicol but are resistant to clindamycin and trimethoprim-sulfamethoxazole. The data analysis was performed

according to "Antibiogram committee of French Microbiology of Society" based on theEuropean Committee on Antimicrobial Susceptibility Testing recommendations. Remarkably, these strains were not hemolytic (data not shown).

3.7. Accession numbers

The accession numbers were KP057871 (*E. faecalis* 14), KP057872 (*E. faecalis* 28*),* KP057873, *E. faecalis* 90*),* KP057874 *E. faecalis* 93*)*, KP057875 (*E. faecalis* 97), KP057876 (*E. faecalis* 101), NR_115765.1 (*E. faecalis* ATCC 19433) and GQ340751.1 *Escherichia coli* ATCC 11229.

4. Discussion

This study aimed at strictly isolating LAB and particularly bacteriocinogenic LAB from meconium of newborns infants. Thus, 107 bacterial isolates were obtained from meconium of six donors at Roubaix Hospital in the north of France. All the bacterial isolates were identified as *E. faecalis*. Interestingly, Borderon et al. (1996); Adlerberth et al. (2006) showed that *E. faecalis* colonizes the gut of vaginally and caesarean-delivered term and preterm neonates Clinical isolates of enterococci are weakly diverse comparatively to those obtained from the

environment, but *E. faecalis* remains the dominant species (Kuhn et al., 2003). According to Fisher and Phillips (2009), this weak diversity could be linked to the virulence factors present in this species. Isolates *E. faecalis* 14, *E. faecalis* 28, *E. faecalis* 90, *E. faecalis* 93, *E. faecalis* 97 and *E.*

faecalis 101 produce inhibitory compounds that comprise lactic acid and BLIS. The concomitant production of these antimicrobials inhibited a set of GNB and GPB, including the robust methicillin-resistant *S. aureus* (MRSA), which is frequently encountered in hospital environment. Remarkably, this antagonism was abolished when the CFS was neutralized, highlighting thereof the role of BLIS. Vancomycin is the standard antibioticused for the treatment of MRSA infections. Recently, Liang et al. (2014), suggested daptomycin as a reasonable alternative for treating MRSA mainly for osteoarticular infections (OAIs). The activity of these antibiotics could be potentialized with bacteriocins, which are overall safe (Belguesmia et al., 2010), ribosomally synthesized antimicrobial peptides (Drider and Rebuffat, 2011). Genotyping of these isolates performed by REP-PCR and PFGE (data not shown) concluded to three different strains. Percent Identity Matrix based on 16S ribosomal DNA confirms a high score of similarity of enterococci sequences and a low score of similarity with *E. coli* ATCC 11229 used as different bacterial taxon. In this study, *E. faecalis* 28 and *E. faecalis* 93, obtained from two different sources, were the most actives isolates. In their presence, the counts of *S. aureus* ATCC33862 has drastically decreased. Further, the antagonistic isolates displayed high scores of autoaggregation and hydrophobicity. These two characteristics might be important for the interplay of these isolates with *S. aureus* ATCC33862. Clewell et al. (2002) reported that production of aggregation substance (Agg) on the donor cell-surface facilitates contact with recipient cell by binding to enterococcal binding substance. Importantly, these antagonistic strains were not hemolytic and resulted to be sensitive to antibiotic usually used to treat enterococci infections. The resistance to clindamycin and Trimethoprime/sulfamethoxzole is thought to be a species characteristic. According to the opinion of the FEEDAP panel on the updating of criteria used in the assessment of bacteria for resistance to antibiotics of human and veterinary importance, strains carrying an acquired resistance to antimicrobial(s) should not be used as feed additives, unless it can be demonstrated that it is a result of chromosomal mutation(s) (EFSA 2005). This study unveiled the presence of only *E. faecalis*

species in samples of meconium obtained from six donors at Roubaix hospital in the north of France. Off 107 *E. faecalis* isolates, only *E. faecalis* 14, *E. faecalis* 28, *E. faecalis* 90, *E. faecalis* 93, *E. faecalis* 97 and *E. faecalis* 101 resulted to be bacteriocinogenic. In the best of our knowledge, this is the first report dealing with bacteriocinogenic LAB from meconium. The number of antagonistic isolates is relatively low (5.60%) compared to the frequency usually reported in the literature (Nes et al. 2014). The high hydrophobicity and aggregation scores registered for the aforementioned antagonistic isolates, as well as absence of hemolytic activity and sensibility to various antibiotic render these strains as potential candidates for probiotic applications.

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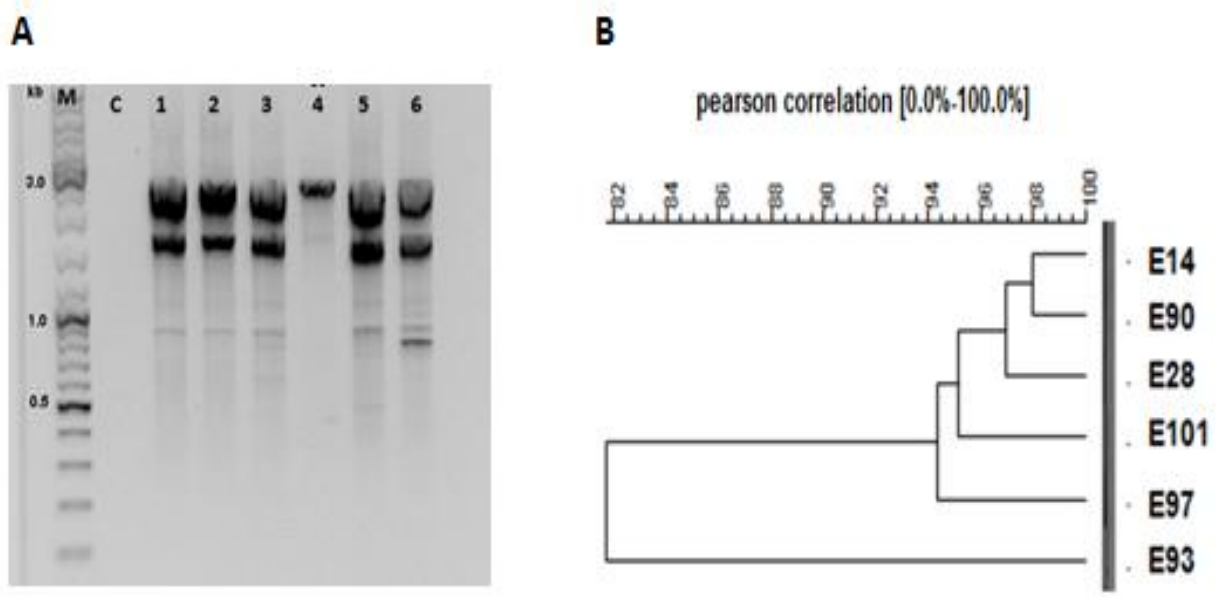

Figure 1. Genetic relatedness of antagonistic strains. (A) REP-PCR product were separated on 1% agarose gel. Lane M corresponds to the DNA markers (thermo, USA), Lane C corresponds to the PCR control (PCR carried without DNA template), lanes 1. 2, 3, 4, 5, 6 corresponds to genetic patterns of *Enterococcus faecalis* 14 (Ef14), *E. faecalis* 28 (Ef28), *E. faecalis* 90 (Ef90), *E. faecalis* 93 (EF93), *E. faecalis* 97 (Ef97), and *E. faecalis* 101 (Ef101). (B) Dendrogram outlining Pearson correlation of antagonistic *E. faecalis* 14 (Ef14), *E. faecalis* 28 (Ef28), *E. faecalis* 90 (Ef90), *E. faecalis* 93 (EF93), *E. faecalis* 97 (Ef97), and *E. faecalis* 101 (Ef101).

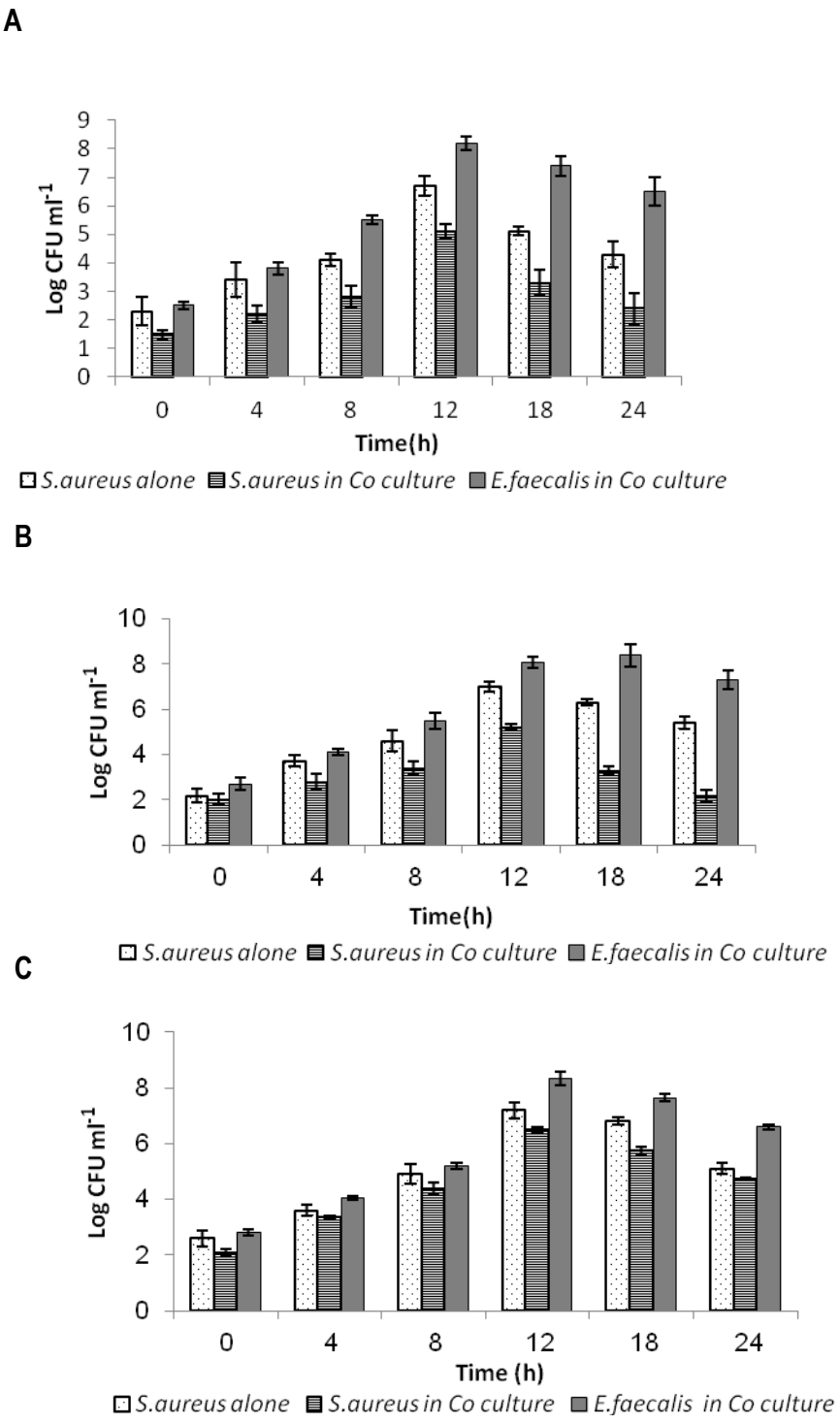

Figure 2. Interplay between *Staphylococcus aureus* ATCC33862 and enterococci during coculturure .Samples were withdrawn at regular intervals of time and plated onto agar brain heart for counting of *S. aureus* and on MRS for counting of enterococci *E. fecalis* 28(A), *E. fecalis* 93(B) and *E. fecalis* ATCC29212(C). The value expressed in log CFUT ml^{-1} (±SD) is the average of least three independent experiments.

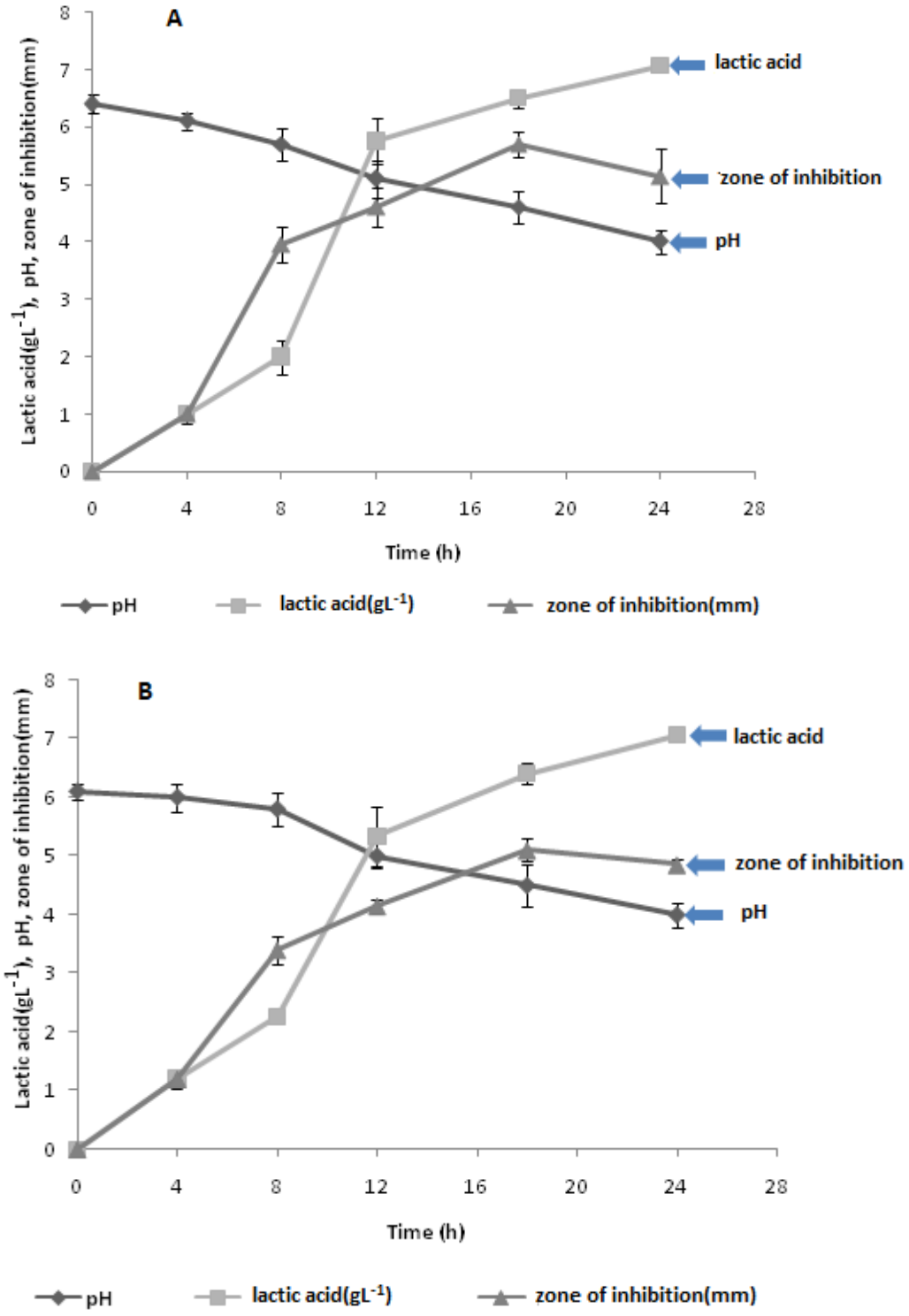

Figure 3. Measurement of pHs, amount of lactic acid (g L^{-1}) and inhibitory activity of the cell free supernatants (CFS) prepared from *E. faecalis* 28 (A) and *E. faecalis* 93 after different times of growth. The data are the average of at least three independent experiments.

Table 1. Inhibitory activity before neutralization of the cell-free supernatant (BN) and after the neutralization of the cell-free supernatant (AN).

Indicator strain	*Enterococcus faecalis* 14		*E. faecalis* 28		*E. faecalis* 90		*E. faecalis* 93		*E. faecalis*97		*E. faecalis*10 1	
	AN	BN	AN	BN	AN	BN	AN	BN	AN	BN	AN	BN
Listeria monocytogenes ATCC3512	5.8±0.4	7.15±0.2	3.6±0.84	6.0±0.2	3.5±08	5.9±0.5	4.0±0.1	6.6±0.1	3.70±0.6	6.25±0.4	3.3±0.24	6.5±0.5
Listeria innocua CIP103982	6.3±0.4	7.65±0.2	4.8±0.56	6.1±0.1	4.3±0.61	6.2±0.2	4.1±0.5	5.7±0.1	4.20±0.2	6.3±0..9	5.6±0.8	7.5±0.7
Bacillus subtilis ATCC6633	6.2±0.1	7.7±0.4	4.7±0.3	6.8±0.2	4.8±0.3	7.3±0.2	3.8±0.1	6.2±46	2.60±0.5	5.5±0.7	3.3±0.24	7.2±0.2
Staphylococcus aureus ATCC33862	3.6±0.8	4.8±0.3	5.6±0.4	6.0±0.2	5.5±0.6	6.1±0.1	4.9±0.9	5.7±0.9	4.50±0.5	4.6±0.21	3.6±0.3	4.8±0.2
Methicillin-resistant *S. aureus* (MRSA)	0	2.6±0.7	0	3.2±0.8	0	2.8±0.5	0	2.5±0.4	0	2.3±0.55	0	2.4±0.6
Enterococcus faecalis	6.1±0.8	7.6±0.56	4.2±0.6	5.2±0.4	4.2±0.6	5.1±0.3	4.3±1.0	5.8±3	5.10±0.9	4.7±0.14	5.0±0.80	4.50±0.4
Escherichia coli CIP103982	0	6.4±0.2	0	4.85±0.2	0	4.5±0.5	0	4.3±0.7	0	3.9±0.25	0	4.15±0.
Klebisella oxytoca	0	6.65±0.3	0	5.65±0.3	0	4.7±0.1	2.1±0.3	4.6±0.4	0	3.75±0.1	2.55±0.20	4.70±0.1
*Proteus mirabilis**	0	5.4±0.3	0	5.95±0.5	0	6.25±0.	0	3.9±0.1	0	4.45±0.4	0	5.0±15
Salmonella Heidbelberg*	0	5.7±0.3	0	5.15±0.1	0	4.7±0.7	0	5.3±0.7	0	4.55±0.	0	4.15±0.
*Pseudomonas fluorescens**	0	5.8±0.4	0	5.0±0.25	2.5±0.3	5.6±0.5	0	5.5±0.5	0	4.4±0.15	2.6±0.20	6.20±0.1
Candida albicans ATCC10231	0	0	0	0	0	0	0	0	0	0	0	0
*Saccharomyces cerevisiae***	0	0	0	0	0	0	0	0	0	0	0	0

The diameter of inhibition zone is measured in millimeters. *Ef: Enterococcus faecalis.* The value "0" designs the absence of inhibitory activity under the experimental conditions used in this work. The SD designs the stander diviation. The data are the means of at least three independent assay. Each assay consisted in three measures. *Designs strains obtained from our laboratory collection, while ** designs strains generously provided by lesaffre company.

Table 2. Autoaggregation and hydrophobicity percentages of antagonistic meconium enterococci

Isolates	% of autoaggregation	% of hydrophobicity
E. faecalis 14	49±0.47	47±0.8
E. faecalis 28	44±1.6	45.6±0.47
E. faecalis 90	35±1.24	41±1.2
E. faecalis 93	44.6±1.8	46.3±1.2
E. faecalis 97	41±1.24	34±1.4
E. faecalis 101	45±1.6	43±0.94
E. faecalis ATCC29212	45.6±0.1.2	42.3±0.9

Table 3 A. Antibiotic susceptibility of antagonistic isolates

Antibiotics	*E. faecalis* 14	*E. faecalis* 28	*E. faecalis* 90	*E. faecalis* 93	*E. faecalis* 97	*E. faecalis* 101
Ampicillin	S (≤ 2)	S (≤ 2)	S (≤ 2)	S (≤ 2)	S (≤ 2)	S (≤ 2)
Gentamycin	S	S	S	S	S	S
Kanamycin	S	S	S	S	S	S
Streptomycin	S	S	S	S	S	S
Levofloxacin	S(1)	S (1)	S (1)	S (1)	S (1)	S (1)
Moxifloxacin	S (≤ 0.25)	S (≤ 0.25)	S (≤ 0.25)	S (≤ 0.25)	S (≤ 0.25)	S (≤ 0.25)
Clindamycin	R (≥ 8)	R (≥ 8)	R (≥ 8)	R (≥ 8)	R (≥ 8)	R (≥ 8)
Linezolid	S (2)	S (2)	S (2)	S (2)	S (2)	S (2)
Teicoplanin	S (≤ 0.5)	S (≤ 0.5)	S (≤ 0.5)	S (≤ 0.5)	S (≤ 0.5)	S (≤ 0.5)
Vancomycin	S (1)	S (1)	S (1)	S (2)	S (1)	S (2)
Tetracyclin	S (≤ 1)	S (≤ 1)	S (≤ 1)	S (≤ 1)	S (≤ 1)	S (≤ 1)
Nitrofuranation	S (≤ 16)	S (≤ 16)	S (≤ 16)	S (≤ 16)	S (≤ 16)	S (≤ 16)
Chloramphenicol	S (8)	S (8)	S (8)	S (8)	S (8)	S (8)
Trimethoprime/sulfamethoxzole	S (≤ 10)	S (≤ 10)	S (≤ 10)	S (≤ 10)	S (≤ 10)	S (≤ 10)
Erythromycin	R (≥ 8)	I (4)	I (4)	R (≥ 8)	I (4)	I (4)

R: Resistant, S: Sensitive, I: Intermediate determined by Vitek 2; MIC, Minimal inhibitory concentration determined by Vitek 2 system.

Table 3 B. Antibiotic susceptibility of antagonistic isolates

Antibiotics	*E. faecalis* 14	*E. faecalis* 28	*E. faecalis* 90	*E. faecalis* 93	*E. faecalis* 97	*E. faecalis* 101
Ampicillin	S (0.75)	S (0.75)	S (0.75)	S (0.75)	S (0.38)	S (0.38)
Gentamycin	S	S	S	S	S	S
Kanamycin	S	S	S	S	S	S
Streptomycin	S	S	S	S	S	S
Levofloxacin	S(1)	S (1)	S (1)	S (1)	S (1)	S (1)
Moxifloxacin	S	S	S	S	S	S
Clindamycin	R	R	R	R	R	R
Linezolid	S (2)	S (1)	S (1)	S (1.5)	S (1.5)	S (2)
Teicoplanin	S (0.25)	S (0.25)	S (0.25)	S (0.25)	S (0.25)	S (0.25)
Vancomycin	S (2)	S (1)	S (1)	S (1)	S (2)	S (2)
Tetracyclin	S	S	S	S	S	S
Nitrofuranation	S	S	S	S	S	S
Chloramphenicol	S	S	S	S	S	S
Trimethoprime/sulfamethoxzole	S	S	S	S	S	S
Erythromycin	R (2)	I (2)	I (1)	R (1.5)	I (1)	I (1.5)

R: Resistant, S: Sensitive, I: Intermediate determined by Vitek 2; MIC, Minimal inhibitory concentration determined by E test.

Chapter 3. Anti-MRSA potency of enterocins DD28 and DD93 and inhibition of biofilm formation

As reported in chapter 2, *E. faecalis*14, *E. faecalis* 28, *E. faecalis* 90, *E. faecalis* 93, *E. faecalis* 97 and *E. faecalis* 101 are active against a wide range of GPB including MRSA, which has become a worrisome superbug worldwide. The anti-MRSA activity detected in the neutralized supernatants of *E. faecalis* 28 and *E. faecalis* 93 was the highest ones based on the diameters of the inhibition zones. Importantly, this anti-MRSA activity has drastically increased upon concentrating the supernatant. (**Figure 11**).

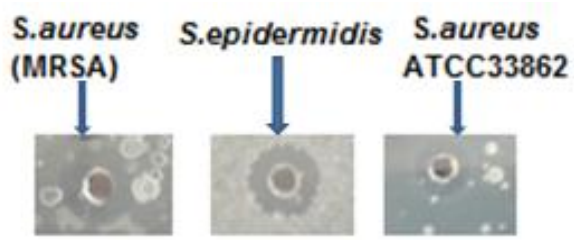

Figure 11. Antibacterial Staphylococcal activity of enterocin DD28

Afterwards, we purified the bacteriocins produced by *E. faecalis* 28 and *E. faecalis* 93 using a simplified two-step purification procedure consisting of cation exchange chromatography followed by a reversed-phase high-performance liquid chromatography (Abriouel et al., 2003). The masses of enterocins DD28 and DD93, determined by mass spectrometry, were 5,205.21 Da and 5,204.89 Dalton, respectively. These masses match with those usually reported in the literature for enterocins. Thus, PCR amplification of total DNA from *E. faecalis* 28 and *E. faecalis* 93 was performed with the reverse 5'-TTAATGTCTTTTTAGCCATTTTCAATTTG-3' and forward 5'-ATGGGAGCAATCGCAAAATTAGTAG-3' primers designed by Lui et al .(2011) to amplify class IIb enterocins L50A and L50B. The PCR programm consisted in initial denaturing step of 5 min at 95 °C, followed by 30 cycles of 1 min denaturing at 95 °C, 1 min annealing at a specific temperature for the primers for each of the known enterocin gene, and 10 min extension at 72°C. The PCR fragments rising from our amplifications were of 287 bp each. They were cloned into pGEM-T Easy vector (Promega Corp., Madison, WI, USA) and transformed into *E. coli*. The recombinant plasmids were extracted using GeneJET Plasmid DNA Purification Kit (Thermo Scientific Fermentas). The PCR fragments were then sequenced (Eurofins MWG operon, Ebersberg, Germany) and the resulting sequences were aligned with sequences of

known bacteriocin and were found identical with MR10A and MR10B enterocins (Martín-Platero et al., 2006)

To gain more insights on the antibacterial potencies of these bacteriocins, we assessed their activities against different *Staphylococcus* strains, including *S. epidermidis* (kindly provided by Dr. Anne Vachée, Roubaix Hospital, France), *S. aureus* ATCC 33862, MRSA ATCC 43300, and MRSA-S1 and MRSA-S2 (MRSA-S1 and MRSA-S2 were kindly provided by Dr. Gilles Prévost, Strasbourg School of Medicine, France). This anti-*Staphylococcus* activity was determined by MIC using the microtiter plates. The data gathered, from this experiment, underline a MIC value twice higher for MRSA isolates compared to *S. epidermidis* and *S. aureus* ATCC 33862. (Table 8). Regarding this activity which appeared to be strain-dependent, we elected to pursue our investigations using MRSA-1 as a target strain.

Table 8. Determination of minimum inhibitory concentration (MIC)

Strains	MIC (µg/ml) of Enterocin DD28	MIC (µg/ml) of Enterocin DD93
S. aureus ATCC33862	100	100
S. epidermidis	100	100
MRSAATCC 43300	200	200
MRSA-S1	200	200
MRSA-S2	200	200

ATCC: American Type Culture collection

The susceptibility of MRSA-S1 strain was determined, at Roubaix hospital using Vitek-2 technology, against nearly all antibiotics families. MRSA-S1 strain appeared to be resistant to kanamycin and erythromycin but sensitive to oflaxacin, rifampin and vancomycin. The susceptibility and resistance features were attributed according to the European Committee on Antimicrobial Susceptibility Testing Breakpoint tables (EUCAST).

Assays of combinations of enterocins DD28 and DD93 with kanamycin and erythromycin were performed on planktonic culture. The resulting data unveiled the potential of these bacteriocins to potentialize the anti-bacterial activities of erythromycin and kanamycin. Indeed, the MICs values of erythromycin have decreased from 16 to 1 mg/L, whilst that of kanamycin has decreased from 32 to 4 mg/L. Both MICs were below the breakpoints of erythromycin and kanamycin, rendering thereof the target MRSA-S1 strain sensitive to these antibiotics in the presence of enterocins. The calculation of FIC index, as suggested by Patterson *et al*. (2006) was below 0.5, advocating a clear synergistic effect. To confirm this achievement, we carried out different kill curves kinetics combining enterocins DD28 and DD93 with erythromycin and kanamycin. These combinations have led to a drop of 3 Log_{10} of MRSA-S1 within the first 3 hours, confirming thereof the synergistic effects, registered for the planktonic

culture. After 3 hours of treatment and the important drop seen, the cell counts were stable and this stability could be ascribed to the half-life of the enterocins, which was very likely over.

To gain more insight on the anti-MRSA-S1 activity, we have set up biofilm on AISI 304L stainless steel and glass slides. Importantly, when these devices were treated free of antibacterial agents, biofilm could be formed as supported by the SEM data. However, the addition of enterocins DD28 and DD93, or antibiotics such as vancomycin and rifampin to which the strain MRSA-S1 was sensitive, the biofilm formation was compromised, conversely to the addition of antibiotics such as kanamycin, and erythromycin to which MRSA-S1 was resistant. The cells counts performed upon assays of biofilm formations of AISI 304L stainless steel slides were supported by epifluorescence images.

Anti-MRSA activities of Enterocins DD28 and DD93 and evidences on their role in the inhibition of biofilm formation

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Abstract

Methicillin-resistant *Staphylococcus aureus* (MRSA), along with other antibiotic resistant bacteria, has become a worrisome superbug worldwide. This work was aimed at studying the efficacies of two class IIb bacteriocins, enterocins DD28 and DD93, against MRSA-S1 grown in planktonic culture and embedded in biofilms. These bacteriocins were purified, from the cultures supernatants of *Enterococcus faecalis* 28 and 93, using a simplified purification procedure consisting in a cation exchange chromatography and a reversed-phase high-performance liquid chromatography. The anti-Staphylococcal activity of these bacteriocins was shown *in-vitro* by the assessment of the minimal inhibitory concentration (MIC). Afterwhich, a checkerboard and time-kill kinetics permitted unveiled a synergistic effect of these bacteriocins in combination with erythromycin and kanamycin against a clinical MRSA-S1; a clinical isolate. Furthermore, these bacteriocins alone or in combination with erythromycin and kanamycin have impeded the formation of MRSA-S1 biofilms on both stainless steel and glace devices as supported by the microbial cell counts, epifluorescence and Scanning Electron Microscope analyses.

Introduction

Staphylococcus aureus is among the 5 top pathogens found as normal resident of the skin and nasal flora in at least 25 to 30% of healthy humans, and it is associated with hospital acquired (HA-MRSA) and community acquired (CA-MRSA) infections ranging from superficial wound infections to life-threatening deep infections such as septicemia, endocarditis and toxic shock syndrome. Thus, antibiotic resistance abilities and biofilm-forming capacity contribute to the success of *Staphylococcus aureus* as a harsh human pathogen in healthcare as well as in community settings. It should be noted that the last decade has seen a welcome increase in the number of agents available for the treatment of MRSA including antibiotics such as fluoroquinolones, linezolid, rifampin and antimicrobial peptides such as daptomycin, tigecycline and mainly vancomycin. Susceptibility to vancomycin was first reported in 1996 in Japan, leading to emergence of heterogeneous resistance to vancomycin phenotype[1]. Furthermore, MRSA with reduced susceptibility to vancomycin have also been reported in the ocular infections, and there has been a rise in *S. aureus* resistance to new and old generation fluoroquinolones that are commonly

used for prophylaxis after intravitreal injections and intraocular surgeries[2]. Daptomycin which is considered drug of last resort after vancomycin breakdown for the treatment of MRSA infections[3] has shown non inferiority to vancomycin in the treatment of MRSA bacteremia[4] is threatened because of the emergence of daptomycin resistance, especially in deep-seated infections[3]. In spite of these anti-MRSA agents, there is a need of alternatives and better therapeutic options to fight MRSA related infections than currently recommended agents.

Antimicrobial peptides (AMPs) are largely admitted as sustainable alternatives to traditional antibiotics in order to combat the scaring and increasing bacterial infections. AMPs can be gathered by different pathways including chemical synthesis, controlled enzymatic digestion of proteins and production from all living cells. Bacteria are known to be a great source of AMPs, including lipopeptides and bacteriocins. Conversely to lipopeptides, bacteriocins are proteinaceous and ribosomally synthesized AMPs produced mainly by lactic acid bacteria (LAB)[5]. LAB, belonging to *Enterococcus* genus are known to produce enterocins, among which a great number has been purified and characterized such as enterocin A, B, P, and AS-48[6,7]. Enterocins-producing strains were isolated from a wide range of sources, including fermented food, environmental and clinical[8]. Notably, enterocins are produced by different Enterococcal species mainly *Enterococcus faecalis* and *E. faecium*[9]. Enterocins produced by other species including *E. muntii*, *E. avium, E. durans*, and *E. hirae* strains were also reported in the literature[10-13]. Multiple enterocins-producing enterococci strains have been characterized for their large range of activities, inhibiting thereof the growth of many undesirable bacteria[8]. A previous report undersigned the potential of *E. faecium* L50 to produce three different enterocins, among which enterocins L50A and L50B, P, and Q which act synergistically and inhibit growth of many Gram-positive bacteria[14]. Remarkably, enterocins are also produced by enterococci from gastrointestinal tract origins of humans, animals, human infection sites and healthy babies feces[15,16,17]

Recently, a compilation of studies underpinned that enterocins, as enterocin E-760, are active against Gram-positive and Gram-negative bacteria, impeding thereof the growth of *Salmonella enterica*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Campylobacter jejuni* and *Staphylococcus aureus* strains[18]. As above-indicated, pathogens as MRSA are responsible of diverse infections, especially in healthcare system. The increasing resistance of Gram-positive bacteria to the broad-spectrum antibiotics and the lack of new molecules expected to become available in the near future strengthens the discovery of novel anti-MRSA agents and design of novel therapeutic options[19]. The combinations of antibiotics represent promising management strategies for these life-

threatening infections[20,21]. Besides the antibiotics could be combined to chemical or physical treatments[16,22]. Several studies have highlighted successful *in vitro* associations of bacteriocins and antibiotics to inhibit the growth of bacteria with resistant phenotypes[23-29].

In the present study, enterocins DD28 and DD93, produced by *E. faecalis* 28 and *E. faecalis* 93 respectively, were purified and their DNA codifying sequences were determined. Furthermore, the anti-MRSA activity was determined against MRSA-S1 used as a model. In spite of their relatively high MICs values, enterocins DD28 and DD93 were able to synergize with kanamycin and erythromycin two antibiotics to which the target MRSA-S1 strain was initially resistant. Assays to evaluate the anti-MRSA-S1 biofilm formations conducted on AISI 304L stainless steel and glass devices have proven the efficacies of these bacteriocins as anti-MRSA biofilm agents.

Results

Assessment of Enterocins DD28 and DD93 amounts upon purification process. The first step of the purification procedure permitted to enhance the specific activity recovered from the cell-free supernatant (CFS), from 86.20 to 3,902.44 AU/mg (enterocin DD28) and 96.15 to 2,318.84 AU/mg (enterocin DD93) (Table 1). The second purification step involving a separation on RP-HPLC column permitted to purify these enterocins to homogeneity and evaluate their runtime to 42.5 min. Importantly, the specific activity has increased to 67,368.42 AU/mg for enterocin DD28 and 77,108.43 AU/mg for enterocin DD93 (Table 1). The purified enterocins DD28 and DD93 were analyzed by mass spectrometry and appeared to have very close molecular masses of respectively 5,205.21 Da and 5,204.89 Da (Fig. 1).

Enterocins DD28 and DD93 were active against Staphylococci. Different *S. aureus* strains were used to demonstrate the enterocins antibacterial activity. Thus strains *S. epidermidis*, *S. aureus* ATCC 33862, MRSA ATCC 43300, MRSA-S1 and MRSA-S2 were used as target strains. The MICs values were similar for *S. epidermidis* and *S. aureus* ATCC 33862, but they were two times higher for MRSA ATCC 43300, MRSA-S1 and MRSA-S2. For this study, we have then proceeded with MRSA-S1 strain that was previously isolated from the blood of an 83 years old patient and studied for its susceptibility to chromagranin-derived peptides[30].

Resistance profile of MRSA-S1 to traditional antibiotics. The susceptibility of MRSA-S1 as determined by VITEK2 method showed resistance to erythromycin and kanamycin with MICs values of ≥ 8 µg/ml and 32 µg/ml,

respectively. In turns, this strain exhibited sensibility to ofloxacin (0.5 µg/ml), rifampin (0.03 µg/ml) and vancomycin (1 µg/ml) (Table 2). Notably, both enterocins DD28 and DD93 showed inhibition against MRSA-S1 and *S. aureus* ATCC 33862 used as controls (Table 3). The MICs values were 100 µg/ml for *S. aureus* ATCC 33862, and 200 µg/ml for MRSA-S1 (Table 3).

Checkerboard assays revealed a synergistic effect of enterocins DD28 and DD93 with antibiotics. When erythromycin and kanamycin were used in combination with enterocin DD28 or enterocin DD93, the MICs values observed were lower than those obtained when these molecules were tested individually. These combinations permitted a synergetic effect regarding the FIC values of 0.31 registered for both enterocins DD28 and DD93, in combination with erythromycin and kanamycin on MRSA (Table 4). As evidenced from this assay, the MICs values of erythromycin and kanamycin have dropped under their breakpoint points[31] when they were associated to enterocins DD28 and 93.

Killing curves kinetics confirmed the synergistic effects. The killing curves experiments realized on MRSA-S1 strain treated with erythromycin and kanamycin and their combinations with enterocins DD28 or DD93 have confirmed the synergistic effect anticipated by the FIC values. As expected, the aforementioned antibiotics are devoid of any inhibitory activity against the planktonic MRSA-S1 culture. The population number as means of CFU/ml remained identical in the untreated samples, as well as in those treated with erythromycin and kanamycin alone portraying this lack of activity. Nevertheless, the combination of enterocins DD28 and DD93 with erythromycin and kanamycin reduced the CFU/ml counts of MRSA-S1 by at least 2 to 3 logs during 3 to 24 h of incubation, leading to a killing of 99 to 99.9% of the CFU/ml of the initial bacterial populations (Fig. 3, Table 5).

Sequence alignments and blast interpretation. The amplification of total DNA from *E. faecalis 28* and *E. faecalis 93* with the reverse and forward primers designed by Cintas et al.[32] to amplify class IIb enterocins L50A and L50B permitted in this study to obtain 287 bp DNA fragments. Afterwards they were successfully cloned into the pGEM-T plasmid and sequenced using 7T and SP6 primers. The resulting sequences were blasted on blastn pubmed database and showed complete alignment with the sequences of enterocin MR10A and MR10B (Fig. 2), which are two class IIb bacteriocins produced by *E. faecalis* MRR 10-3[33].

Biofunctionalization of stainless steel and glass slides on MRSA biofilm formation. MRSA-S1 exhibited resistance to erythromycin. Thus, when the AISI 304L stainless steel slides were conditioned with 8 mg/L erythromycin, the biofilm formation, as supported by epifluorescence microscopy, occurred normally. These results were comparable to those obtained with the untreated stainless slide (Fig. 4, Table 6), advocating that MRSA-S1 biofilm formation was not affected by this antibiotic. Enterocins DD 28 or DD93 alone, at 200 µg/ml, delayed the MRSA-S1 biofilm formation compared to the control test (Fig. 4, Table 6). As a consequence, this result is similar to those obtained with vancomycin (1 µg/ml) and rifampin (0.03 µg/ml), for which the MRSA-S1 exhibited susceptibility (Table 1), and thereof used as positive controls. Similarly, the combination of erythromycin and enterocin DD28 at 1 µg/ml and 50 µg/ml, respectively has led to similar feature as that obtained with antibiotics.

SEM microscopy analysis showed lower number of adherent MRSA-S1 cells on glass devices after conditioning with vancomycin (1 µg/ml) and combination of enterocin DD28+erythromycin (50/1 µg/ml), comparatively to the unconditioned glass slide (Fig. 5). However, the biofilm formation on a glass slide treated with enterocin DD28 alone (200 µg/ml) showed more limited effect on the colonization of the surface compared to an untreated glass slide (Fig. 5).

Discussion

Staphylococci are commonly found in healthy people, usually in the lining of the nose. These bacteria can colonize other areas *via* the hands, and particularly the wet parts of the body such as the armpits or the genital area[34]. *S. aureus* is the most often species encountered in the infectious diseases, generally as benign ones, but it can be involved in more serious infections as pneumonia, bacterial meningitis and gastroenteritis. Moreover, this bacterium is also implied in the nosocomial infections, sometimes affecting bloodstream, joints, bones, lungs or heart, resulting in very complicated infections, even with fatal epilogue[35].

Among other *Staphylococcus* species, those grouped under the term of coagulase negative staphylococci (CNS) such as *S. epidermidis*, *S. hominis* and *S. saprophyticus*, are leading infections and act as opportunistic bacteria [35]. However these CNS remain much less virulent for humans than *S. aureus* strains[3]. Overall community strains of *Staphylococcus* are sensitive to penicillin M molecules such as methicillin and oxacillin[37]. They are also sensitive to macrolides, aminoglycosides, fluoroquinolones and synergistines. Multidrug resistance

(MDR) to antibiotics, including methicillin and aminoglycosides, is commonly found in *S. epidermidis* and *S. haemolyticus*, frequently isolated in hospital and healthcare units[35]. This environment is the main source of MRSA because of their multiple antibiotic resistances, rendering it frequently responsible of nosocomial infections[37].The methicillin-resistance is associated with a resistance to all β-lactams and some other antibiotics including aminoglycosides and related macrolides, synergistines, fluoroquinolones and fosfomycin. Nevertheless, some molecules remain active against these bacteria like glycopeptides, rifampin and fusidic acid[35, 37]

Besides MDR, staphylococci are able to form biofilms on abiotic surfaces such as stainless steel, catheters and polystyrene material[26]. The biofilm lifestyle stands as a hurdle to antibiotic treatments that need to be overcome. The *icaADBC* genes, encoding the synthesis of the exopolysaccharide of polymer matrix were reported to be involved in biofilm formation[38]. Further, this matrix acts as a physical barrier, limiting thereof drugs penetration and protecting bacteria embedded in biofilms. The ability to form biofilm was also observed for other staphylococci species, including *S. epidermidis*[38, 39].

The present study permitted to purify enterocins DD28 and DD93, determine their masses and DNA codifying sequences. The easy purification procedure used in this study was inspired from the protocol described by Abriouel et al [40]. The masses of enterocins DD28 and DD93, as determined by mass spectrometry, were 5,205.21 Da and 5,204.89 Dalton, respectively (Fig. 1). These masses are perfectly matching with those yet reported for enterocins of class IIb as enterocins L50A and L50B produced by *E. faecium* L50[14], enterocins MR10A and MR10B produced by *E. faecalis* MRR 10-3[40] and enterocins A5-11A and A5-11B produced by *E. durans* A5-11[10]. The sequence alignments of putative DNA codifying enterocins DD28 and DD93 with MR10A, MR10B ones, allowed to conclude that all these bacteriocins are likely similar, resulting in the perfect alignment of the whole coding sequences.

The MIC values obtained for enterocins DD28 and DD93 turned to be strain dependent. As expected the highest MIC value was observed from MRSA-S1 and the lowest ones were observed for *S. epidermidis* and *S. aureus* ATCC 33862. The anti-Staphylococcal activities as revealed by the MICs values remain relatively elevated compared to those obtained with different antibiotics. It should be noted that semi-purified peptides were used for these experiments. Further experiments aiming at revealing the anti-MRSA with enterocins DD28 and DD93 chemically synthesized and from their derivative peptides will certainly help improving and understanding this anti-MRSA-S1 activity. Furthermore, the main drops observed in the killing curves

experiments, when any of these enterocins was combined to erythromycin or kanamycin occurred in the first 3 h before no longer decrease. This curve feature is typically observed in the synergistic killing curve assays. We assume that the mainly activity ascribed to these bacteriocins is taking place during the first hours before seeing a decay of their activities. To improve the half-lives of bacteriocins activity, different possibilities could be regarded. Indeed, we can use preferably circular bacteriocins to avoid the adverse effect of proteases or to proceed with bacteriocins encapsulation and coating. Notably, MRSA from hospital settings, are known to be resistant to erythromycin and kanamycin but remain sensitive to vancomycin and rifampin, which are widely used as treatment for MRSA infections[41, 42, 43]. The experiments performed here provide novel evidence on the treatment of MRSA carrying resistance to erythromycin and kanamycin by their combinations with bacteriocins such as enterocins DD28 and DD93. These synergistic treatments stand as a promising strategy in the management of antibiotics. Besides the anti-MRSA-S1 activity observed on the planktonic culture, our study identified a potential application of these combinations against anti-MRSA-S1 biofilm formation. Staphylococci were reported to form biofilms on different biomaterials [44] rendering them as responsible of recurrent infections in the healthcare units[45]. Mature biofilms are extremely harsh to eradicate[46]. In our study the conditioning of AISI 304L stainless steel and glass slides with different antimicrobials including enterocin DD28 alone, antibiotics alone or their combinations, permitted fruitful data on MRSA-S1 biofilm formation. The data focusing on the anti-MRSA-S1 biofilm formation on both slides have similar features to those obtained on planktonic culture. Importantly, the combination of enterocins DD28 and DD93 with erythromycin or kanamycin has significantly reduced the MRSA-S1 population of about 3 Log_{10} CFU/ml, within the first 3 h of *in vitro* treatment. Similarly, the MRSA-S1 population was reduced of about 2 Log_{10} CFU/ml, on AISI 304L stainless steel slides. These anti-biofilm formation data were confirmed by epifluorescence and SEM analyses.

Materials and Methods

Strains and growth conditions and antibacterial susceptibility. *E. faecalis* DD28 and *E. faecalis* DD93 recently isolated from meconium[47] were grown in MRS (de Man, Rogosa and Sharpe) medium at 37°C for 16 to 18h[48]. *S. epidermidis* (Kindly provided by Dr. Anne Vachée, Roubaix hospital, France), *S. aureus* ATCC33862, and Methicillin Resistant *S. aureus* (MRSA) strains including MRSA ATCC 43300, MRSA-S1 and MRSA-2

(kindly provided by Dr. Gilles Prévost, Strasbourg university, France) were grown in Brain Heart Infusion (BHI) broth at 37°C for appropriate experimental time.

Antibiotic susceptibility was performed by VITEK 2 system (Bio-Mérieux, France), encompassing nearly all important antibiotics. Antibiotic susceptibility and MICs were determined and analyzed according to the French Committee on Antimicrobial Susceptibility Testing (FCAST)[49]

Purification of bacteriocins and determination of their masses. Purification of enterocins DD28 and DD93 was performed at room temperature using two-step methods adapted from Abriouel et al.[40]. Briefly 1,000 ml of the cell-free culture supernatant of *E. faecalis* 28 and *E. faecalis* 93, were adjusted to pH 6.3 using 5M NaOH. Then Carboxymethyl Sephadex CM-25 (GE Healthcare, Sweden) gel slurry equilibrated in distilled water was added to culture supernatants (1:40, v/v). The obtained mixtures were held under stirring for 30 min and decanted for another 30 min. The supernatants were removed, and the sediment gel slurry containing bacteriocins activities, captured by cation interactions with the matrix were loaded onto a 10×50 cm glass column. The gels were washed with one volume (1V) of distilled water and 2V of 0.5 M NaCl, followed by 2V of 1.5 M NaCl to elute the adsorbed bacteriocins. The obtained fractions were filtrated through 0.22 µm pore size low protein binding filters (Millex GV; Millipore Corp., Belford, MA-USA) and tested for bacteriocin activity.

The active fractions from the cation exchange chromatography were applied onto a reversed-phase high-performance liquid chromatography (RP-HPLC) by using a column C-18 (5µm, 250 x 3mm, VYDAC 218 TP53; Grace, Deerfied, Illinois-USA) previously equilibrated in solvent A (10 mM trifluoroacetic acid, TFA; Fluka), at a flow rate of 5 ml/min. Non-adsorbed material was eliminated by washing the column with solvent A until the UV absorbance of the effluent at 210 nm reached baseline. The material retained in the column was eluted with a gradient ranging from 0 to 40% of solvent B [isopropyl alcohol/acetonitrile (2:1, v/v) in 40 mM TFA] in over 5 min, then followed by increasing of solvent B gradient from 40 to 100% for over 25 min at a flow rate of 2.5 ml/min. Fractions of the column effluent were collected according to their UV light absorbance, lyophilized and dissolved again in distilled water before being tested for bacteriocin activity.

Antimicrobial activity was tested along the purification procedure on the cell-free supernatant, partly purified peptide (upon cation exchange step) and purified peptide (upon Reversed Phase-HPLC step). Arbitrary units (AU) were calculated based on the spot method[27]. Thus, the resulting sample was serially diluted twofold with

filter-sterilized phosphate buffer. 10 µl of each diluted sample was spotted onto the plate of BHI medium containing *Listeria innocua* CIP80.11 as indictor strain. The plates were then incubated at 37°C overnight, and the titer was defined as the reciprocal of the highest dilution (2^n) that resulted in inhibition of the indicator lawn. The AU of antibacterial activity per milliliter was defined as 2^n ×1000 µl/10µl. The protein concentration was determined by BCA assay using Sigma-Aldrich BCA kit (USA).

Purified enterocins were analyzed with an Ultraflex MALDI-ToF/ToF mass spectrometer (Bruker, Bremen, Germany) equipped with a smart beam laser. Samples were analyzed using an accelerating voltage of 25 kV and matrix suppression in deflexion mode at m/z 1000. The laser power was set to just above the threshold of ionization (around 60%). Spectra were acquired in reflector positive mode in the range of 3 000 at 10 000 Da. Each spectrum was the result of 1 000 laser shots per m/z segment per sample delivered in 10 sets of 50 shots distributed in random locations on the surface of the matrix spot. The instrument was externally calibrated in positive reflector mode using Bradykinin (1-7) $[M+H]^+$ 757.3991, Angiotensin II $[M+H]^+$ 1 046.5418, Angiotensin I $[M+H]^+$ 1 296.6848, Substance P [M+H]+ 1347.7354, Bombesin $[M+H]^+$ 1619.8223, ACTH (1-17) $[M+H]^+$ 2 093,0862. For analysis, a mixture of 1 µl of purified enterocin and 1 µl of α-cyano-4-hydroxycinnamic acid (10 mg/ml 70:30 water/acetonitrile with 0.1% TFA) was spotted onto a MALDI-TOF MTP 384 target plate (Bruker Daltonik GmbH, Leipzig, Germany) according to the procedure of the dried-droplet preparation.

Minimal inhibitory concentration (MICs) and Checkerboard experiments. A pure colony of each *Staphylococcus* strains used in this study was grown overnight in BHI medium at 37°C. Afterwards 10 µl of each overnight culture were added to the wells of bioassay microplates of 96 well cell culture plate (Cellstar) containing different concentrations of enterocins DD28 and DD93, ranging from 50 to 800 µg/ml of each bacteriocin. The MIC is defined as the lowest concentration of an antibiotic that will inhibit the visible growth of a microorganism after overnight incubation.

Antimicrobial agent interactions were determined using checkerboard assay. The concentrations used for enterocins DD28 and DD93 were comprised between 25 to 400 µg/ml, while those used for erythromycin and kanamycin were ranging from 0.25 to 64 µg/ml. Microplates were inoculated with MRSA-S1 strain at ~10^6 CFU/ml, in a final volume of 200 µl per well, and incubated overnight at 37°C. The fractional inhibitory concentration index (FICI) was calculated for each combination using the following formula: FICA + FICB = FICI,

where FICA = MIC of drug A in combination/MIC of drug A alone, and FICB = MIC of drug B in combination/MIC of drug B alone. The FICI was interpreted as follow: synergism = FICI ≤ 0.5; indifference = 0.5 < FICI ≤ 4; antagonism = FICI > 4 [50].

Killing curves experiment. This experiment was realized on the MRSA strain. Tubes containing BHI supplemented with enterocin DD28, enterocin DD93, erythromycin, kanamycin or combination of bacteriocin and antibiotic, at previously defined concentrations during checkboard assay, were inoculated with MRSA-S1 to a density of about $5x10^5$ CFU/ml in a final volume of 5 ml and incubated at 37°C for 24h. The killing kinetics of the enterocin DD28 alone, enterocin DD93 alone, erythromycin alone, kanamycin alone and enterocin DD28 or enterocin DD93 in combination with erythromycin or kanamycin was assessed against MRSA-S1 using standard time-killing experiments and viable bacterial counts on agar plates. The final concentration of enterocin DD28 and enterocin DD93 was 50 mg/l, erythromycin 1 µg/ml, kanamycin 4 µg/ml and for combination of bacteriocins with antibiotics the concentrations used were 50 µg/ml for enterocin DD28 and DD93 and 1µg/ml or 4 µg/ml, respectively for erythromycin and kanamycin.

Aliquots were removed at different times 0, 3, 6, 9 and 24 h of incubation, and then serially diluted in saline for determination of viable counts. Diluted samples (100 µl) were plated on Tryptone-Soya-Agar (TSA) plates and colonies were counted after overnight incubation at 37°C. Bactericidal activity was determined as 3 $\log_{10}$ CFU/ml reduction in the colony count relative to the initial inoculums[51]

DNA extraction and PCR amplification of enterocins codifying DNA. Total DNA from *E. faecalis* 28 and *E. faecalis* 93 were extracted following the same procedure as described in Al Atya et al.[47]. The following forward: ATGGGAGCAATCGCAAAATTAGTAG and Reverse: TTAATGTCTTTTTAGCCATTTTTCAATTTG primers described by Liu et al. [52] were used to amplify the two genes encoding enterocin L50A and L50B. The PCR conditions consisted of an initial denaturing step of 5 min at 95 °C, followed by 30 cycles of 1 min denaturing at 95 °C, 1 min annealing at a temperature specific for the primers for each of the known enterocin gene, and 10 min extension at 72 °C. Polymerase chain reaction (PCR) was done using the PCR Master Mix (2X) (Thermo Scientific Fermentas, Villebon sur Yvette, France) as a mixture of Taq DNA polymerase. DNA extraction was performed using the Wizard® Genomic DNA Purification Kit (Promega Corp., Madison, WI, USA). Ligation of PCR products was done into pGEM-T Easy vector (Promega Corp., Madison, WI, USA). Plasmid

extraction was carried out using GeneJET Plasmid DNA Purification Kit (Thermo Scientific Fermentas). Restriction endonucleases were supplied by Thermo Scientific Fermentas. Ligation of inserts to different vectors was effected using the DNA Ligation Kit <Mighty Mix> from Takara (Ozyme, Saint Quentin en Yvelines, France). Recovery of DNA from agarose gels was performed with GeneJET Gel Extraction kit (Thermo Scientific Fermentas).

In all cases, the instructions of the suppliers were followed. All the construction sequences were verified by DNA sequencing (Eurofins MWG operon, Ebersberg, Germany).

Functionalization of AISI 304L stainless steel and glass slides with antimicrobial compounds. To study the effect of treatment of stainless steel slide, reproducing abiotic surfaces used in healthcare units and industries environment, we used the protocol adapted from Ait Ouali *et al*.[53]. Briefly 2 ml of antimicrobials at their MIC values: enterocin DD28 (200 µg/ml), enterocin DD93 (200 µg/ml), erythromycin (8 µg/ml), ofloxacin (0.5 µg/ml), vancomycin (1 µg/ml), rifampin (0.03 µg/ml) and combination of enterocin DD28 with erythromycin (50/1 µg/ml) were added on the surface of each AISI 304L slide placed in sterile Petri plates, and incubated for 2 h at 37 °C. During this step a conditioning film on the AISI surface may be formed. Sterile Tryptone Soy Broth – Yeast Extract (TSB-YE) was added in an additional AISI304L slide as a control for this conditioning step. After this step the antimicrobial compounds and the TSB-YE were removed and replaced by 2 ml of MRSA-S1 suspension at 10^7 CFU/ml. After 1 h of incubation time, the supernatant containing non-adherent MRSA-S1 cells was removed and replaced by 2 ml of sterile TSB-YE medium on the surface of each slide, and the incubation was conducted for 0, 3, 6, and 24 h to survey the installation of MRSA-S1 biofilm. After each time of incubation, the slides were washed twice with 30 ml of Phosphate Buffered Saline (PBS) (pH 7.0). Finally, the AISI 304L stainless steel slides were immersed individually in 30 ml phosphate buffer and sonicated. The detached MRSA-S1 cells were enumerated by plating the bacteria on Tryptone Soy Agar (TSA) after growth at 37 °C for 24 h. Additional slides were prepared and served for epifluorescence observation after their staining with live/dead components as explained below.

For SEM analysis, sterilized glass slides of 1cm² were deposited in wells of sterile 24-well tissue culture plates (BD Falcon, USA). Then, as previously, the glass slides were treated with 2 ml of these following antimicrobial compounds : enterocin DD28 (200 µg/ml), vancomycin (1 µg/ml) and combination of enterocin

DD28 with erythromycin (50/1 µg/ml), or TSB-YE medium as control, during 2 h at 37 °C. After this conditioning step of the glass slides, the antimicrobial compounds solutions were removed and replaced by 2 ml of MRSA suspension at 10^7 CFU/ml. The following steps are exactly the same applied on the AISI304L stainless steel slides; however incubation was conducted for 5 consecutive days, changing the TSB-YE medium each 24 h. Once the incubation ended, the glass slides were prepared for SEM analysis.

Epifluorescence and SEM microscopy analyses. Biofilm cells were stained with a BacLight LIVE/DEAD bacterial viability staining kit according to the manufacturer's instructions (Molecular Probes, Invitrogen, France). After dilution of 1.5 µl of each reagent with 1 ml of physiological water (0.85% m/v NaCl), the obtained mixture was gently deposited on the upper face of the slide where the biofilm development may occurred. Following the incubation (15 min in the dark) of the slides, the staining solution was aspirated and biofilms were observed using an epifluorescence microscope (Nikon Optiphot-2 EFD3, Japan).

Conclusion

Here, we purified and determined the masses of enterocins DD28 and DD93, two class IIb bacteriocins, produced by *E. faecalis* DD28 and *E. faecalis* DD93, recently isolated from meconium. Afterwards, we determined their DNA codifying sequences. The antagonism experiments unveiled anti-SARM activity on planktonic and biofilm cultures.

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Author contribution

SS and RB realized SEM analysis, AV realized Vitek-2antibiograms, AKA realized most of the experiments, YB contributed to MIC determinations and sequences analyses, GC realized the mass analysis, RR and DD supervised this work. All authors read and approved the manuscript

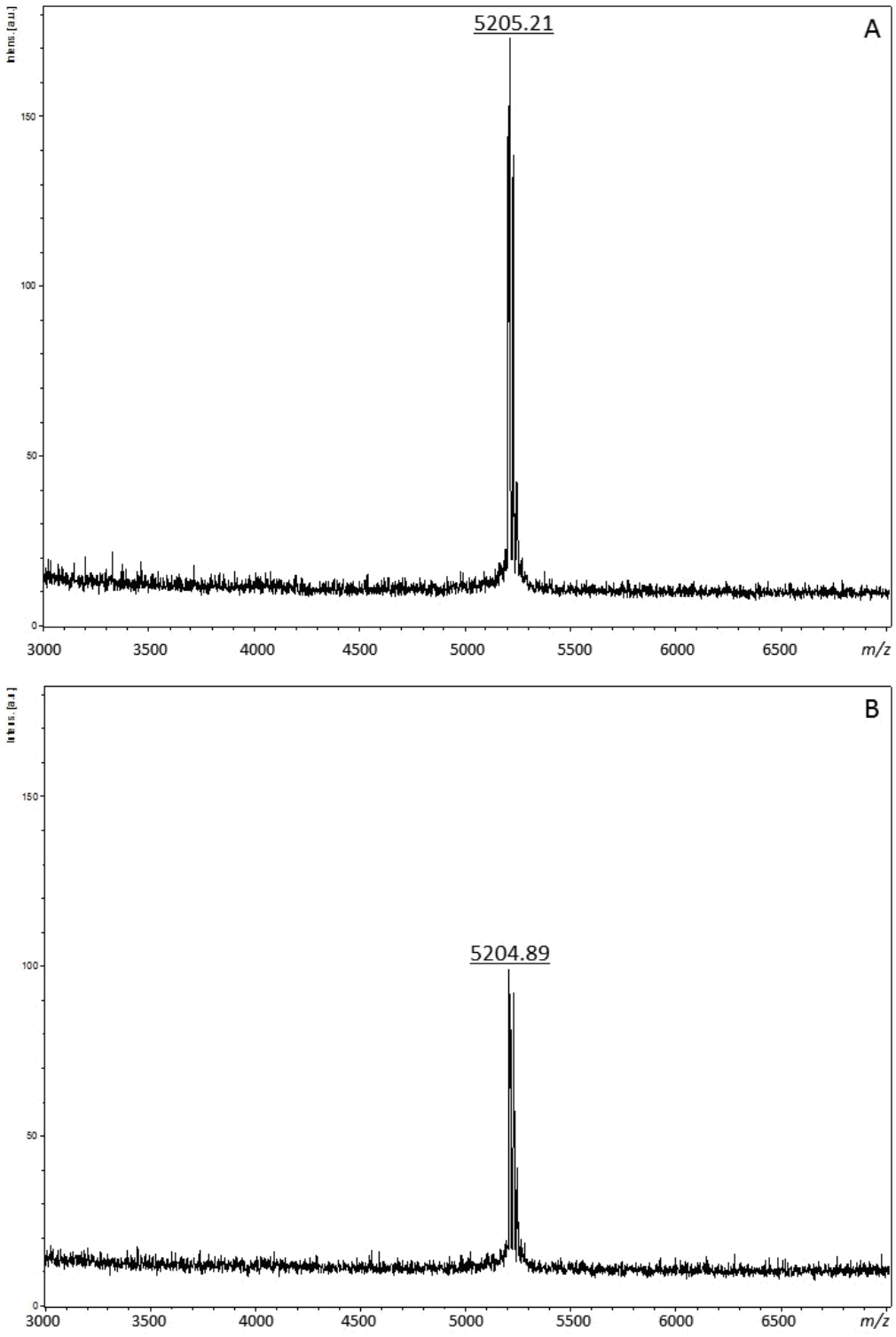


Figure 1. Mass spectrometry analyses of purified enterocin DD28 (A) and enterocin DD93 (B)

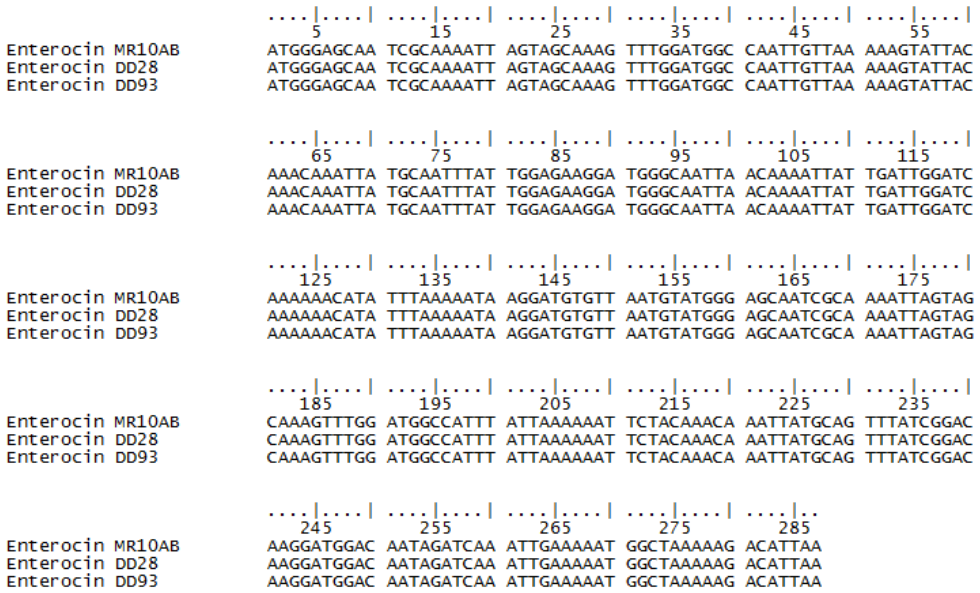
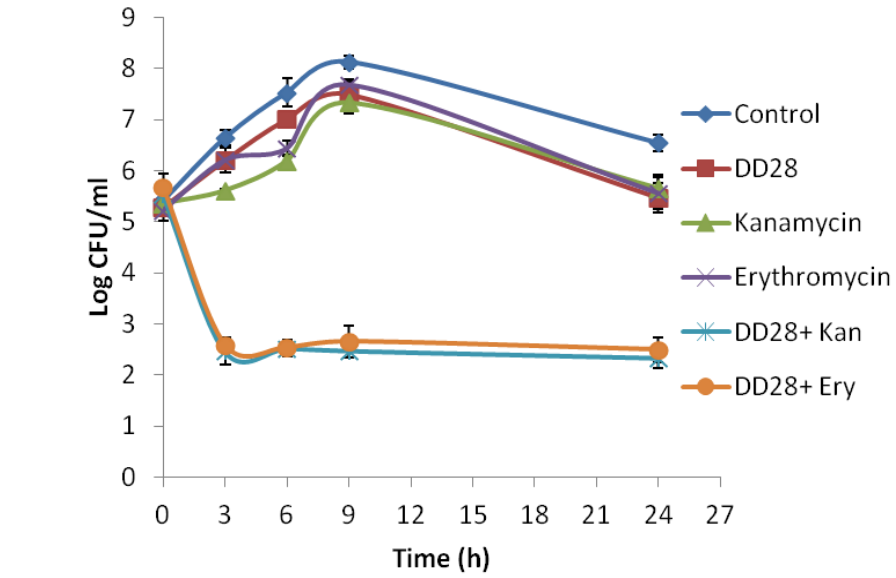
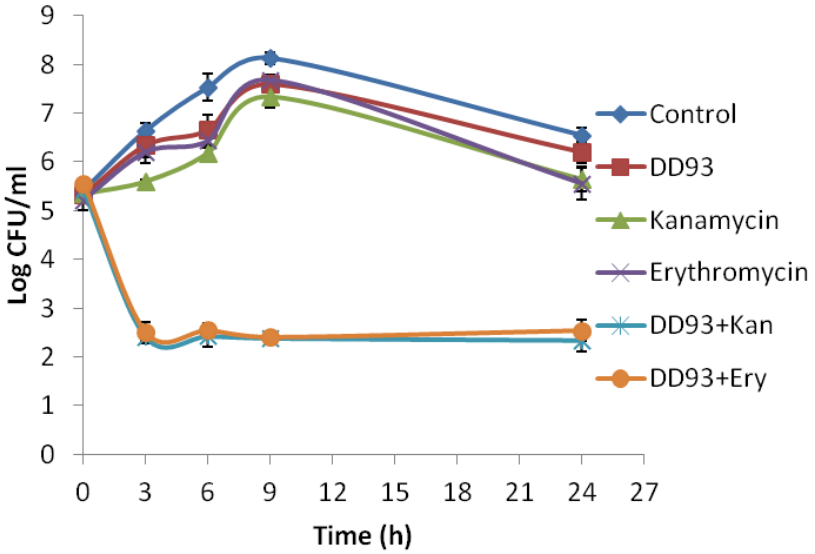


Figure 2. Alignment of putative enterocins DD98 and DD93 DNA sequences using ClustalO (http://www.ebi.ac.uk/Tools/msa/) software. (.) Sign indicates same nucleotide in the considered position for all the sequences aligned.



A



B

Figure 3. Time-killing kinetics on planktonic cultures of MRSA-S1 at 0, 3, 6, 9, and 24 h in the presence of enterocins DD28 and DD93 (50 µg/ml) alone or combined with kanamycin (4 µg/ml) and erythromycin (1 µg/ml) against SARM-S1. Panel A shows the effect of enterocin DD28+Kanamycin at 50 µg/ml-4 µg/ml and enterocin DD28+erythromycin at 50-1 µg/ml against MRSA-S1. Panel B shows the effect of enterocin DD93+kanamycin (50-4 µg/ml) and enterocin DD93+erythromycin (50-1 µg/ml) against MRSA-S1. The data (±SD) are the average of at least three independent experiments. In each experiment, three measures were performed.

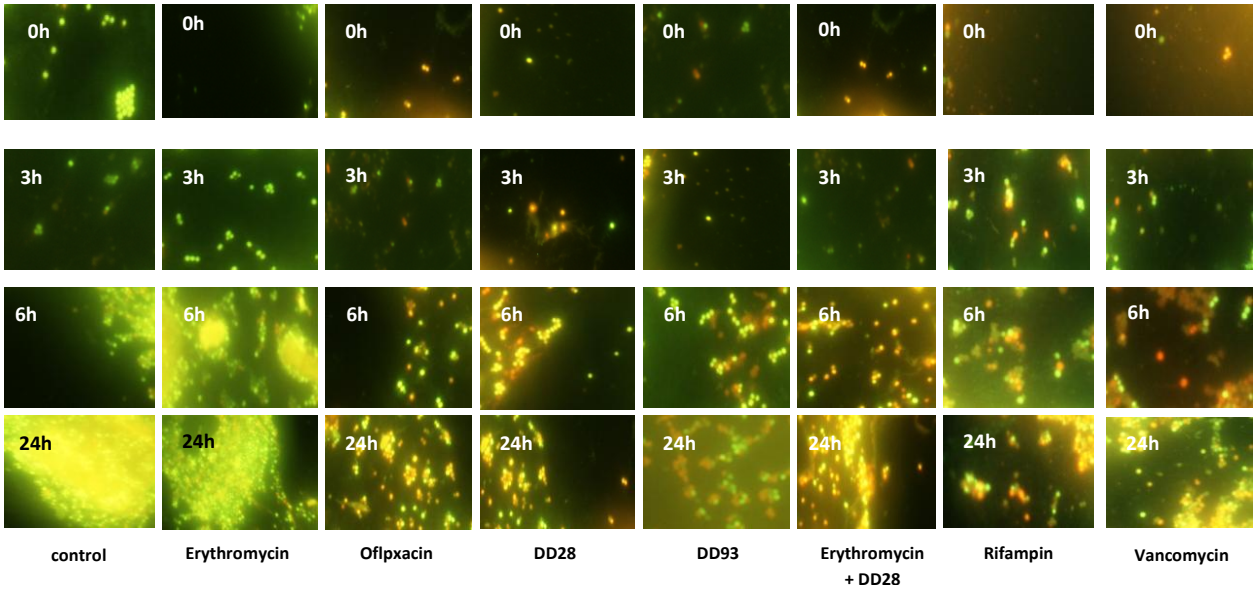


Figure 4. Biofilms formation by MRSA-S1 on AISI 304L stainless steel slides conditioned with antimicrobial agents for 2 h and then washed and inoculated with 2 ml of.10 ^{7} CFU/ml MRSA-S1 culture before removing, washing and adding TSB-YE medium for 0, 3, 6 and 24 h of incubation at 37 °C. Concentrations of antimicrobial agents used were 8 µg/ml for erythromycin, 0.03 µg/ml for rifampin, 200 mg/L for enterocin DD28, 50 µg/ml + 1 µg/ml for enterocin DD28 + erythromycin, respectively. Biofilms were stained with the BacLight Live/Dead Viability Kit and imaged by epifluorescence microscopy after staining pattern for live cells (green) and dead cells (red). The experiments were performed at least twice and representative images are shown.

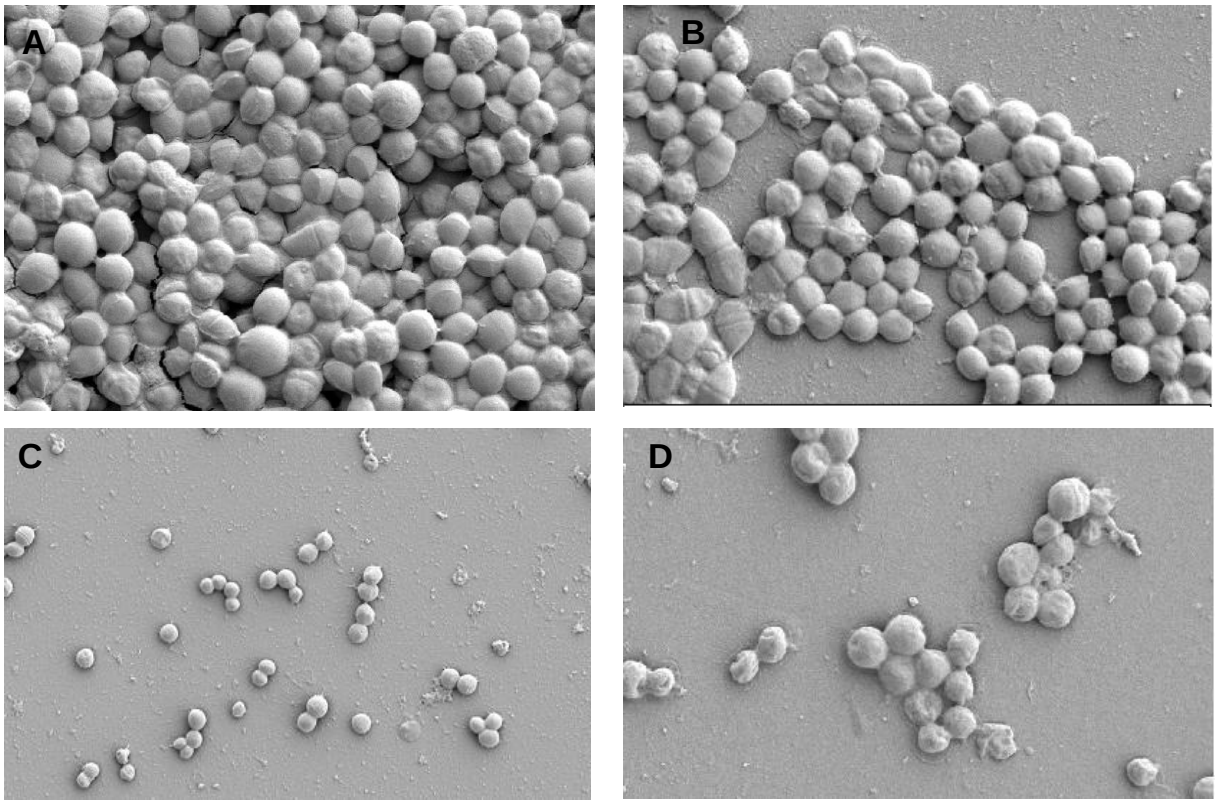


Figure 5. **SEM of biofilms formation by MRSA-S1 on glass slides**. Panel A corresponds to the untreated sample (control) carrying 5 days old biofilm. Panels B, C and D show the samples conditionned with enterocin DD28 (200 µg/ml), Erythromicin+enterocin DD28 (1-50 µg/ml) and vancomycin (1 µg/ml), respectively.

Table 1. Purification of Enterocins DD 28 and DD93

Bacterial strains	Purification Stages	Volume (ml)	Activity AU/ml	Protein (mg/ml)	Total protein (mg)*	Total activity	Specific activity** (AU/ml)	Purification factor
E. faecalis 28	Supernatant	1,000	800	9.28	9,280	800,000	86.2069	1
	Sephadex CM 25	50	3,200	0.82	41	160,000	3,902.439	45.2
	C18 RP-HPLC	1	6,400	0.095	0.095	6,400	67,368.42	17.2
E. faecalis 93	Supernatant	1,000	800	8.32	8 320	800,000	96.15385	1
	Sephadex CM 25	50	1,600	0.69	34.5	80,000	2,318.841	24.1
	C18 RP- HPLC	1	6,400	0.083	0.083	6,400	77,108.43	33.2

*Total protein concentration refers to protein concentration per milliliter multiplied by the volume (ml). It was determined by BCA assay.** Specific activity is determined by dividing the arbitrary units (AU) by the total amount of proteins.

Table 2. Antibiotic susceptibility of MRSA-S1

Antibiotics	**MIC (mg/L)**	**Antibiotics**	**MIC (mg/L)**
Benzylpenicillin	R 0.25	Linezolid	S 2
Oxacillin	R 1	Teicoplanin	S ≤ 0.5
Gentamicin	S ≤ 0.5	Vancomycin	S 1
Kanamycin	R 32	Tetracyclin	S ≤ 1
Tobramycin	R ≥ 16	Fosfomycin	S ≤ 8
Ofloxacin	S ≤ 0.5	Nitrofurantoin	S ≤ 16
Erythromycin	R ≥ 8	Fusidic acid	S ≤ 0.5
Lincomycin	R ≥ 16	Rifampin	S ≤ 0.03
Pristinamycin	S 1	Trimethoprim-sulfamethoxazole	S ≤ 10

R. Resistant; S. Sensitive; MIC, Minimal inhibitory concentration determined by Vitek 2 system

Table 3. Determination of minimum inhibitory concentration (MIC)

Strains	MIC (µg/ml) of Enterocin DD28	MIC (µg/ml) of Enterocin DD93
Staphylococcus aureus ATCC33862	100	100
Staphylococcus epidermidis	100	100
MRSAATCC 43300	200	200
MRSA-S1	200	200
MRSA-S2	200	200

Table 4. Effects of antimicrobials combinations against MRSA-S1

Strain	DD28 (µg/ml)	Kanamycin (µg/ml)	Erythromycin (µg/ml)	DD28-Kanamycin (µg/ml)	FIC	DD28- Erythromycin (µg/ml)	FIC
MRSA-S1	200	32	16	50/4	0.375	50/1	0.31
	DD93 (µg/ml)	Kanamycin (µg/ml)	Erythromycin (mg/l)	DD93-Kanamycin (µg/ml)	FIC	DD93- Erythromycin (µg/ml)	FIC
MRSA-S1	200	32	16	50/4	0.375	50/1	0.31

Fractional Inhibitory Concentration (FIC) index .The data (±SD) are the average of at least three independent experiments. (MIC given in mg/L). Kanamycin sulfate (K4000-5G) and Erythromycin (E 5389-5G) were obtained from Sigma-Aldrich.

Table 5. Effect of DD28-Kanamycin, DD28-Erythromycin, DD93-Kanamycin and DD93-Kanamycin combination against MRSA-S1

Combination	0h	3h	6h	9h	24h
Control	5.41±0.08	6.64±0.15	7.52±0.26	8.12±0.12	6.54±0.16
DD28-Kanamycin (50-4 µg/ml)	5.52±0.14	2.45±0.26	2.52±0.15	2.47±0.12	2.33±0.20
DD28-Erythromycin (50-1 µg/ml)	5.6±0.26	2.58±0.05	2.53±0.07	2.65±0.32	2.49±0.24
DD93-Kanamycin (50-4 µg/ml)	5.42±0.03	2.41±0.08	2.43±0.22	2.38±0.05	2.34±0.23
DD93-Erythromycin (50-1 µg/ml)	5.55±0.07	2.50±0.2	2.54±0.13	2.40±0.07	2.54±0.22

Log_{10} colony count lower than that at time Zero without antimicrobial agent (control).-1=Δ 1 Log_{10} CFU/ml = 90% killing; -2 =Δ 2 Log_{10} CFU/ml = 99% killing; -3= Δ 3 Log_{10} CFU/ml = 99.9% killing.

Table 6. Effect of antimicrobials on MRSA-S1 biofilm formation at different incubation times on AISI 304L stainless steel slides.

Time (h)	0h	3h	6h	24h
Control	5.55±0.08	7.25±0.15	7.81±0.18	8.85±0.11
Erythromycin (8 µg/ml)	5.25±0.25	7.09±0.19	7.39±0.29	8.57±0.12
Ofloxacin (0.5 µg/ml)	3.12±0.12	5.24±0.22	5.58±0.18	6.45±0.13
Vancomycin (1 µg/ml)	3.10±0.08	5.29±0.19	5.20±0.14	6.10±0.03
Rifampin (0.03 µg/ml)	3.11±0.10	5.17±0.11	5.28±0.17	6.06±0.04
Enterocin DD28 (200 µg/ml)	3.29±0.1	5.51±0.1	5.62±0.13	6.54±0.12
Enterocin DD93 (200 µg/ml)	3.35±0.21	5.59±0.1	5.58±0.19	6.58±0.17
Erythromycin / DD28 (1/50 µg/ml)	3.35±0.1	5.32±0.17	5.15±0.25	6.13±0.1

The data (Log_{10} CFU/ml) (±SD) are the means of at least three independent experiments.

Chapter 4. Potentialization of colistin by enterocin DD14: application to *E. coli* strains from swine origin

Enterocin DD14 is produced by *E. faecalis* 14, isolated from meconium in the frame of this PhD project. This isolate does produce a bacteriocin designed enterocin DD14, which was purified and extensively studied in our laboratory by Dr. Delphine Caly. The data related to this topic will be soon submitted for publication. Independently of that work, enterocin DD14 was tested for its capabilities to potentialize or not colistin, which is a lipopeptide of polymixins group. Polymixins are lipopeptides antibiotics forming 7 groups named polymyxins A, B, C, D, E, M and P (Martin et al., 2003; Niu et al., 2013; Stansly, 1949) . Polymyxin E is known as colistin, polymyxin A known as aerosporin, whereas polymyxin M is known as mattacin.

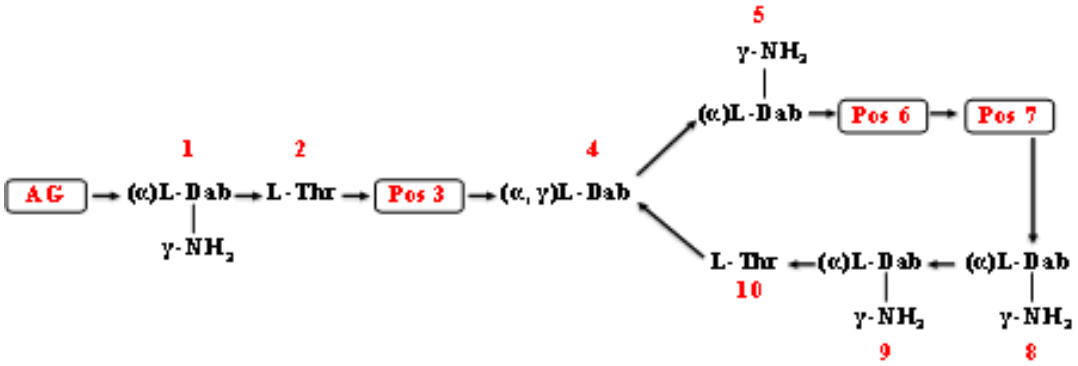


Figure 12. Structure of polymyxins

As depicted on **figure 12**; polymyxins are composed of heptapeptide cationic cyclic linked to lipidic acid moiety by amide liaison (Katz and Demain, 1977). Each polymyxin group is itself divided into several subgroups carrying changes in the lateral chain (lipidic acid). Notably, the polymyxins antibiotics are known to exert their bactericidal activities through direct interaction involving the lipid A pattern of lipopolysaccharide (LPS). Importantly, LPS is defined as a structural component of the external membrane harbouring also antigens (antigen O), polysaccharide and lipid A. The lipid A is carrying negative charges because of for its phosphate molecules (Raetz and Whitfield, 2002).

Thus, polymyxin can link tightly to LPS and induce a cell permeability change, inside of the internal membrane, leading to the cell death as shown in **Figure 13** (Storm et al., 1977). Different modes of actions have been so far proposed based on biophysical studies (Velkov et al., 2010). These models are arguing that polymyxins are acting as tensioactive agents and lipid A molecule is a great target in this mechanism (Velkov et al., 2010).

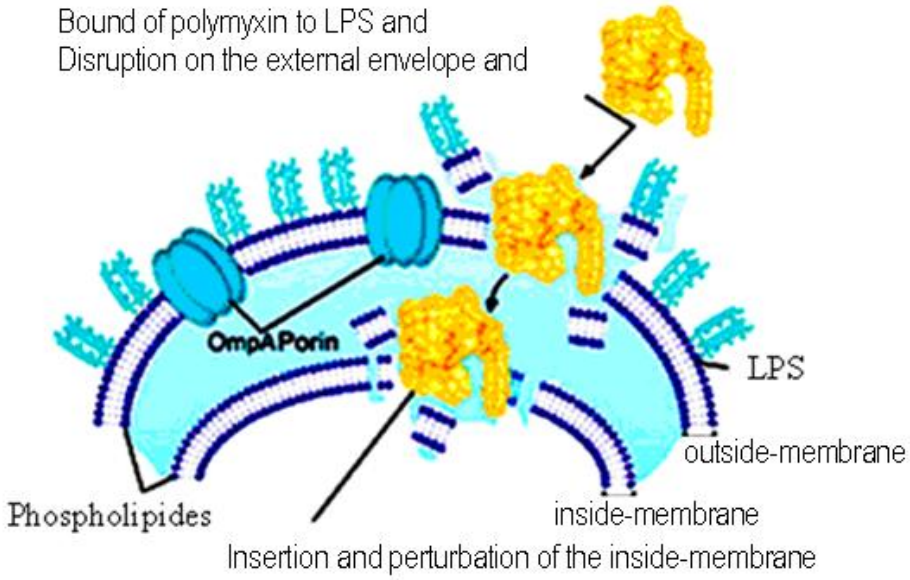
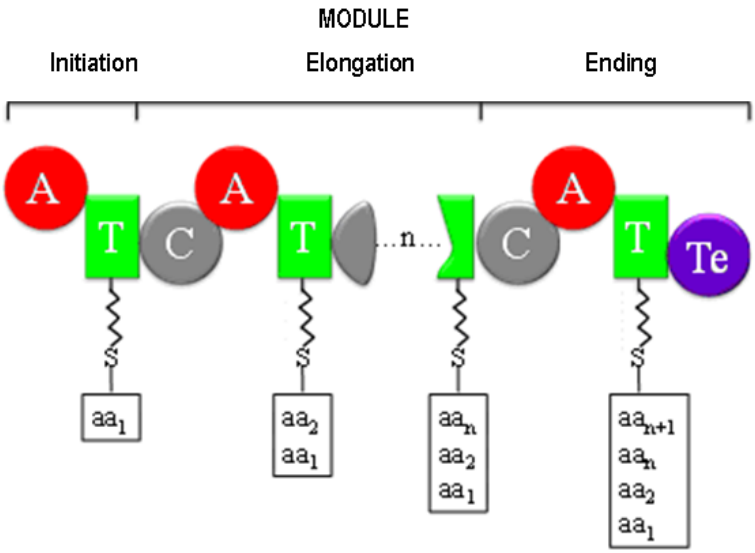


Figure. 13 Mode of action of polymyxin B against GNB targeting the external envelope (Velkov et al., 2010)

In terms of their spectra, polymyxins are known to be active mainly against GNB, most of *E. coli*, *Klebsiella sp*., *Enterobacter sp*., *P. aeruginosa*, and *Acinetobacter sp*. isolates (Landman et al., 2008). Nevertheless, inhibition of *Salmonella sp., Shigella sp., Pasteurella sp., Haemophilus sp., Bordetella pertussis* and even *Legionella pneumophila* were also detected (Landman et al., 2008). It should be noted that species belonging to *Proteus*, *Providencia*, *Serratia*, *Brucella*, *Neisseria*, *Chromobacterium*, and *Burkholderia* displayed resistance to polymyxins (Falagas and Kasiakou, 2005). Polymyxins are devoid of antagonism when the target strains are Gram negative cocci, and against aerobic GPB, anaerobic bacteria, and fungi (Hermsen et al., 2003; Storm et al., 1977).

Polymyxins are synthesized through the non-Ribosomal Peptide Synthetase (NRPS) machinery, which involve multienzymatic complexes organized in different modules. During peptide synthesis, several genes coding for synthetases are entailed. These genes are usually genetically organized in operon (Schwarzer et al., 2003). Each synthetase is composed of different modules usually ranging from 2 to 20 units (Stachelhaus and Marahiel, 1995). Each module is responsible of the incorporation of a specific monomer within the peptidic chain. Inside of each module, enzymatic domains are present and implied in the incorporation or modification of amino-acids during peptide synthesis. Modules are themselves subdivided onto catalytic domains allowing activation of monomers (adenylation A domain), elongation of peptidic chain (thiolation T domain), formation of peptidic bonds (condensation C domain) and then peptide release (thioesterase Te domain) (Sieber and Marahiel, 2005) (Figure 14).



A : adenylation domain, T: Thiolation domain, C: condensation domain,Te: Thioesterase domain,aa: aminoacid

Figure 14. Modular structure of NRPS with different domains (adapted from Strieker et al., 2010)

The experiments carried out on this part permitted to test the activity of enterocin DD14 against different strains of *E. coli* obtained from the Resapath network (ANSES, Ploufragan). These *E. coli* strains are from swine origin and characterized for their resistance to colistin and non-clonality relationship. Thus, enterocin DD14 was not active against these target strains, but its combination with colistin has led to synergism.

Indeed *E. coli* strains of swine origin with resistance to colistin were grown under planktonic and biofilm cultures, and then treated with bacteriocins (enterocin DD14 and nisin) alone or in combination with colistin. Consequently, colistin alone reduced of about 50% the growth of the *E. coli* wild-type strain, conversely to the bacteriocins alone, which almost had no effect (<5%). In Europe the percentage of resistance to colistin in *E. coli* strains isolated from the digestive tract microbiota of healthy animals remains below 1% (Kempf et al., 2013).

Of particular importance, the combination of both bacteriocins with colistin conducted to the eradication of planktonic and disruption of biofilms of *E. coli* cells even if cells are carrying resistance to colistin. Indeed, nisin and enterocin DD14, which are not active against the Gram negative *E. coli* permitted to eradicate planktonic cells and disrupt biofilms when they are associated to colistin. Mechanistically, colistin which targets the LPS and bacteriocins will have further inside inhibitions. Naghmouchi *et al*. (2013) showed a synergistic effect of colistin in combination with nisin against GNB and the possibility of reducing or eliminating the toxicity of this drug by use of low concentrations.

Effects of colistin and bacteriocins combinations on the *in vitro* growth of *Escherichia coli* strains from swine origin

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Abstract

Escherichia coli strains from swine origin either sensitive or resistant to colistin were grown under planktonic and biofilm structures, and then treated by bacteriocins (enterocin DD14 and nisin) alone or in combination with colistin. Colistin alone was able to reduce of about 50% the growth of the *E. coli* wild-type strain, while bacteriocins alone showed very slight inhibitory effect (<5%). Interestingly, the combination of one bacteriocin and colistin significantly affected the exponential growth phase of the tested *E. coli* strains, whereas the concomitant use of the three studied antimicrobials agents permitted to eradicate the growth of the aforementioned target *E. coli* strains.

Keywords

Bacteriocins, *E. coli*, planktonic, biofilms, colistin

1. Introduction

The indiscriminate use of antibiotics in the livestock production has been implicated in the development and dissemination of drug resistance in livestock [1, 2, 3]. Antibiotics have been extensively dispensed to improve the animal health and welfare. However, whether they have undeniably contributed to reduce post-weaning pathologies, concomitantly they also induced concerns regarding bacterial resistance and their dissemination. This alarming situation led to ban their use as growth promoters in Europe in 2006 . Recently, it was recommended to restrict the use of some particular molecules for animals, e.g. fluoroquinolones, and 3rd and 4th generation cephalosporins, advocating a more reasonable use of antibiotics, and especially the ones which are considered of major importance for human health. Among the antibiotics, colistin, belonging to the polymyxin group (polymyxin E), is commonly used in human therapy as drug of last resort to treat infections in patients with cystic fibrosis caused by Gram negative bacilli [4]. Remarkably, colistin is used in pig farming to control infections caused by Gram negative bacilli such as *Escherichia coli.* The infections caused by the pathogenic bacteria *E. coli* are sources of severe drawbacks including diarrhoea, septicaemia and colibacillosis, mainly in young piglets [5]. Antibiotics resistant *E. coli* isolates are sometimes recovered from pathological cases, particularly in piglets [6]. In Europe the percentage of resistance to colistin in *E. coli* strains isolated from the digestive tract microbiota of healthy animals remains <1% [6]. Although this percentage is considered low, the use of colistin should only be reserved for treatment of clinically affected animals and no longer for prophylaxis of diseases [7]. Additionally, the development of *E. coli* Extended Spectrum -Lactamase (ESBL)-producing isolates increases concern about the resistance to antibiotics [8, 3]. Overall, these examples contribute to understand how a drastic reduction of the use of antibiotics in this field should be a worldwide guideline.

The ongoing research on alternatives to antibiotics are focused on finding a real solution to this problem. Related to this, the combination of antibiotics and antimicrobial peptides could be a sustainable, efficient and affordable approach. Bacteriocins are ribosomally synthesized peptides that are secreted by

both Gram negative and Gram positive bacteria [9]. These peptides are able to inhibit the growth of closely related species through several mechanisms, i.e. the formation of pores within the bacteria cell membrane [10]. Bacteriocins produced by Gram positive bacteria, such as nisin and enterocin, are usually active against phylogenetically related strains but not against Gram negative bacteria. In this study, we demonstrate that the combination of colistin and bacteriocins, i.e. nisin and enterocin, could have inhibitory effects against *E. coli* strains from swine origin organized in planctonic and biofilms models.

2. Materials and methods

2. 1. E. coli strains used in this study

The *E. coli* strains 147, 184, 242, 289, 436, E1A4, E2A8, E3A3, E4A4 and E5A16, used in this work were obtained from the French Résapath network, which is dedicated for the surveillance of antimicrobial resistance in pathogenic bacteria of animal origin (https://www.resapath.anses.fr/). The strains referred as E1A4, E2A8, E3A3, E4A4 and E5A16 were isolated from feces of healthy pigs.

2. 2. Genetic relatedness of E. coli strains from swine origin

Total DNA was extracted from each strain using the wizard® Genomic DNA purification kit (Promega, USA). Total DNA was then quantified with a Nanodrop Lite (Biowave II, Biochrom WPA, Cambridge, UK). For RAPD analysis, total DNA was amplified with M13-core primer (5'-GAG GGT GGC GGT TCT-3') or DAF4 primer (5'-CGG CAG CGC C-3'). according to the protocol described by Vogel et al. [11]. PCR products were separated by electrophoresis on a 2% agarose gel, run at 80V for 2 h, using 1X Tris-borate-EDTA. The gels were then stained with GelRed (Biotium, Canada), and visualized by GelDoc (Bio-Rad, France). The RAPD patterns were then analyzed visually.

2.3. Antimicrobials used in this study

Nisin was purchased from Sigma-Aldrich (USA). The stock solution (50 mg/ml) was prepared by dissolving nisin powder in acidified distilled water (pH 2) as reported [12]. The solution was then diluted in appropriate volume to a working concentration of 1 mg/ml.

Colistin was purchased from Sigma-Aldrich (USA). The stock solution of colistin (50 mg/ml) was prepared by dissolving colistin sodium methanesulfonate powder in distilled water. The solution was diluted in appropriate volume to a working concentration of 1 mg/ml.

Enterocin DD14 was shown to be produced by *E. faecalis* DD14, a strain recently isolated from meconium and characterized for its antibacterial spectra [13]. Enterocin DD14 was semi-purified using the protocol described by Abriouel et al. [14]. Briefly, was added to carboxymethyl Sephadex CM-25 (GE Healthcare) gel slurry equilibrated in distilled water to culture supernatant (1:40,v/v) adjusted previously to pH 6.3. The mixture was held under stirring for 30 min and decanted for another 30 min. The supernatant was removed, and the sediment gel slurry containing bacteriocin activity was loaded on a 10×50 cm glass column. The gel was washed with one volume of distilled water and two volumes of 0.5 M NaCl, followed by two volumes of 1.5 M NaCl in distilled water in order to elute the adsorbed bacteriocin protein. Eluted fractions were filtrated through 0.22 µm pore size low protein binding filters (Millex GV; Millipore Corp., Belford,MA, USA) and tested for bacteriocin activity. The protein concentration was determined by BCA assay (Sigma-aldrich. USA).

The activity of bacteriocin (UA/ml) was measured according to Naghmouchi et al. [15], while its quantification was determined by BCA assay (Sigma-Aldrich. USA).

2. 4. Planctonic and biofilms growth conditions

To setup the planktonic growth models, the *E. coli* strains were grown overnight at 37°C in Tryptone soya broth (TSB) (Sigma- Aldrich, USA). Bacterial suspension of about 1.0×10^8 CFU/ml was distributed

(200 µl/well) in triplicate on 96-well polystyrene microtiter plates (Cellstar, Greinerbio-one, Germany). The *E. coli* planktonic cells were then submitted to antibacterial treatment. In the presence of antimicrobial agents, the total volume of bacterial broth and antibacterial solutions were adjusted to 200 µl/well. The microtiter plates were then incubated at 37°C for 2 h, followed by serial dilutions in sterile saline solution and plating in triplicate on Tryptone soya agar (TSA). Viable cells count obtained after 24h incubation at 37°C were used to calculate the average of CFU/well.

To setup biofilm cultures, each bacterial suspension was inoculated in TSB and distributed (200 µl/well) in triplicate on 96-well polystyrene microtiter plates. The plates were incubated at 37°C for 24 h to allow biofilm formation. Then, the culture broths were discarded and the biofilms formed on the microtiter plates were washed with 200 µl of sterile saline solution to remove loosely associated bacterial cells. Single or combined antimicrobials were added to the wells in the same conditions than those used for planktonic cells assays. The plates were further incubated at 37°C for 2h. After treatments, the bacterial broth and antimicrobial solutions were removed and the wells were washed with 200 µl of phosphate buffered saline (PBS, pH 7.2), followed by serial dilutions in sterile saline solution and plating in triplicate on TSA. Viable cells count obtained after 24 h incubation at 37°C were used to calculate the average CFU/well. Statistical ANOVA analysis ($P<0.05$) supported this data.

2.5. Antibacterial susceptibility

The *E. coli* strains were tested for their susceptibility to a set of antibiotics (Table 1) based on the determination of the minimal inhibitory concentrations (MICs) using E-test (Bio-Mérieux, France) according to the producer's instructions and VITEK 2 system (Bio-Mérieux, France). The results were interpreted according to CA-SFM [16]. The *E. coli* ATCC 25922 was used as a control.

3. Results

3.1. E. coli genotype diversity

The RAPD carried out with two distinct primers as suggested by Vogel et al. [11] on *E. coli* strains did not lead to major genotype discrepancies or any clonality relationship between the tested strains (Figs 1A & 1B). For the purpose of this study which consisted to evaluate the efficacy of bacteriocins and colistin combinations towards *E. coli* strains from swine origin, we have used only *E. coli* strains 184 and 289 whose RAPD patterns are slightly different.

3. 2. Antibacterial resistance of E. coli strains revealed resistance to colistin

The resistance of the 10 aforementioned *E. coli* strains was strain dependant manner. Indeed, various resistant profiles were observed including resistance to penicillins, cephalosporins, quinolones, tetracyclines, chloramphenicol, nitrofurantoin and trimethoprime-sulfamethoxazole. Importantly, the *E. coli* 184, *E. coli* 289, *E. coli* 147, *E. coli* 436 and *E. coli* 242 strains were resistant to colistin showing MICs higher than 16 mg/l (Table 1).

3.3. Planktonic and biofilms structures of E. coli strains were disrupted by combinations of bacteriocins and colistin

The treatment with nisin or enterocin DD14 showed a slight decrease effect on the formation of planktonic and biofilms by *E. coli* strains; this minor effect could be attributed to the detergent effect of these bacteriocins (Tables 2 and 3). Because of the high inoculum concentration and resistant

phenotype, the treatment of the targeted bacteria was realized with 1 mg/l of colistin alone and permitted a significant ($P<0.05$) decrease of *E. coli* counts, mainly against S phenotype for the planktonic and biofilm cultures (Tables 2 and 3). Moreover, the combination of colistin with enterocin DD14 displayed the same impact on the growth of *E. coli* wild-type in planktonic and biofilm cultures (Tables 2 and 3). However, the combination of colistin and enterocin DD14 resulted in an advantage due to its impact on the growth of resistant *E. coli* phenotypes (Table 1), showing a reduction of CFU/ml of about 56% and 45% for the planktonic and biofilms grown cells, respectively. Similarly, the combination of colistin with nisin led to comparable results. Indeed, nisin alone or nisin combined with enterocin DD14 were ineffective advocating that Gram negative bacteria are not the appropriate target strains (Tables 2 and 3). The combination of nisin with colistin reduced of about 75% and 70% the growth of *E. coli* wild-type in planktonic and in biofilm cultures, respectively (Tables 2 and 3). The use of the latter combination also demonstrated a clear impact on the *E. coli* strains with colistin-"R" phenotypes since their counts was diminished of about 60% for planktonic and 55% for biofilms structures (Tables 2 and 3). Remarkably, the combination of both bacteriocins, i.e. enterocin DD14 and nisin, with colistin conducted to the eradication of planctonic and disruption of biofilms of *E. coli* strains even against colistin-resistant strains (Tables 2 and 3).

4. Discussion

The indiscriminate use of antibiotics on farms raising domestic animals destined for food is regarded as source of generating and disseminating antimicrobial resistance (AMR) organisms and determinants through food and environmental circuits [17]. Thus, organisms with antimicrobial resistance are able to grow in the gut microbiota of wild mammals both when the animals consume antimicrobials present in feed and water and as a result of exposure to farm waste [18, 19]. A key point to be considered is that bacteria that populate the intestinal tract of animals and humans are very similar, thus the resistant to

antibiotics capability could be genetically transferred, advocating that when resistance occurs in animals, it can affect human intestinal bacteria [20].

In the developing countries, the use of antibiotics in veterinary medicine must be revisited and better controlled to avoid development and dissemination of bacterial resistance strains though the food chain. This storyline will be the worst scheme to leave for the next generations. The misuse of antibiotics in veterinary medicine conducted to emergence of bacterial resistance. Notably, the microorganism *E. coli* is a commensal bacteria of warm-blooded animals. This bacteria is often used as indicator for the presence of AMR in monitoring and surveillance programs due to the availability of simple and the efficient isolation procedures [21,22]. Recently Kempf et al. [6] reported the resistance of *E. coli* strains from swine origin to colistin that is an antibiotic recently returned as final therapeutic option to treat infections caused by multidrug-resistant (MDR) including *Pseudomonas aeruginosa*, *Acinetobacter baumannii* and *Enterobacteriaceae* (e.g., *E. coli*, *Klebsiella pneumoniae*), for which mortality can be high [7]. Another contemporary concern is related to the presence of ESBL producing *E. coli* isolates. Indeed, Changkaew et al. [8] reported the presence of ESBL-producing *E. coli* harbouring class 1 integrons, which clearly may become a health risk if these integrons are transmitted to humans. Gao et al. [3] indicated the prevalence of genetically similar ESBL-producing *E. coli* in swine manure and agricultural soil. These examples denote the need to find sustainable and effective alternatives to antibiotics in veterinary medicine but also in human medicine.

Antibiotics of polymyxin group that are considered since their come back as drugs of last line of defence to fight infections caused by Gram negative bacilli will shortly enter in a tumultuous era. The resistance to polymyxins, which probably occurs thought chromosomal mutations, is compromised. Indeed, Liu et al. [23] reported the emergence of the first plasmid-mediated polymyxin resistance mechanism, MCR-1, in *E. coli*. This finding is clearly heralding the breach of last hope group of antibiotics against Gram negative bacilli. Thus, alternatives aiming to reduce the amount of colistin in veterinary medicine are more then needed. The data gathered here led us to suggest that bacteriocins

could be a good adjuvant to augment antibiotic efficacy, in particularly in combination with colistin. Indeed, nisin and enterocin DD14, which are not active against the Gram negative *E. coli* permitted to eradicate planctonic cells and disrupt biofilms when they were associated to colistin. Mechanistically, colistin should target the LPS and bacteriocins will have further inside inhibitions.

Recent reports highlighted the potency of combination of colistin and nisin or colistin and pediocin AcH to inhibit the growth of Enteropathogenic *E. coli* strains and diminish the cytotoxicity of colistin [24]. Likewise, the combination of polymyxins and bacteriocins has also been shown to inhibit growth of Gram positive bacteria [25, 26]. The role of bacteriocins as adjuvant has become convincing for these applications. Besides their capabilities to potentialize antibiotics, this subcategory of antimicrobial peptides are usually devoid of toxicity against eukaryotic cells [27, 28].

5. Conclusion

This study unveiled the bacteriocins claims as enhancers of polymyxins antibiotics against the pathogen *E. coli* from swine origin. In this study, even the bacteriocins used (nisin and enterocin DD14) were not initially active against *E. coli* strains, their association to colistin conducted to effective formulation supporting the potential of these molecules considered as novel wave of antibiotics [29]. Further bacteriocins will be tested in the future against human isolates and demonstrate their potency in animal models.

Conflict of interest

No conflict of interest to declare

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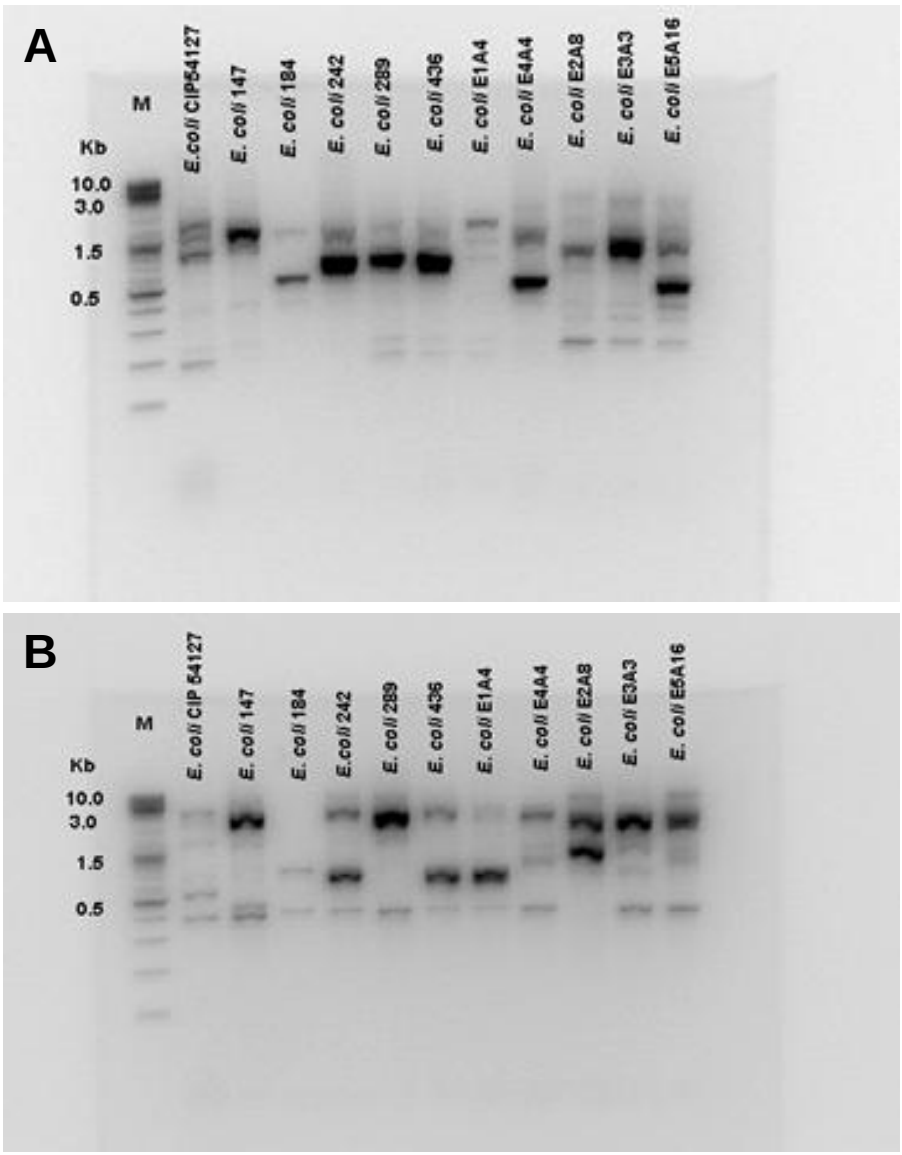

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Fig. 1. RAPD profiles of *E. coli* strains from swine origin used in this study. Electrophoresis was realized on 2% agarose gel. In both gels, lanes M show O'GeneRuler DNA Ladder Mix, (Thermo, USA). RAPD profiles in panel A were obtained with primer DAF4 primer (5'-CGG CAG CGC C-3'), whilst those presented in panel B were obtained with primer M13-core primer (5'-GAG GGT GGC GGT TCT-3').

Table 1. Antibiotic susceptibility of *Escherichia coli* (*E. coli*) strains from swine origin

Antibiotics	*E. coli* 184	*E. coli* 189	*E. coli* 147	*E. coli* 436	*E. coli* 242	*E. coli* E5A16	*E. coli* E1A4	*E. coli* E4A4	*E. coli* E3A3	*E. coli* E2A8	Break-points (CA-SFM2013 mg/l)
Ampicillin	S(≤2*)	R(≥32)	R(≥32)	S(4)	R(≥32)	R(≥32)	R(≥32)	R(≥32)	R(≥32)	S(≤2)	4-8
Amoxicillin/ Clavulanic acid	S(≤2)	S(4)	I(8)	S(≤2)	R(≥32)	S(4)	I(8)	S(4)	S(4)	S(≤2)	4/2-8/2
Ampicillin/ sulbactam	S(≤2)	S(≤2)	R(16)	S(≤2)	R(16)	R(16)	R(16)	R(16)	R(16)	S(≤2)	4/8-8/8
Ticacillin	S(≤8)	R(≥128)	R(≥128)	S(≤8)	S(≤8)	R(≥128)	R(≥128)	R(≥128)	R(≥128)	S(≤8)	8-16
Ticacillin/ Clavulanic acid	S(≤8)	S(≤8)	R(32)	S(≤8)	I(16)	I(16)	I(16)	I(16)	I(16)	S(≤8)	8/2-16/2
Piperacillin	S(≤4)	R(≥128)	R(≥128)	S(≤4)	S(8)	R(≥128)	R(≥128)	R(≥128)	R(≥128)	S(≤4)	8-16
Piperacillin + Tazobactam	S(≤4)	S(≤4)	S(≤4)	S(≤4)	S(≤4)	S(≤4)	S(≤4)	S(≤4)	S(≤4)	S(≤4)	8/4-16/4
Cefalotin	S(4)	S(4)	I(16)	S(8)	R(≥64)	I(16)	I(16)	S(4)	I(16)	S(4)	8-32
Cefuroxime	S(4)	S(≤1)	S(4)	S(4)	R(16)	S(4)	S(4)	S(4)	S(4)	S(4)	8-8
Cefoxitin	S(≤4)	S(≤4)	S(8)	S(≤4)	I(32)	S(≤4)	S(≤4)	S(≤4)	S(≤4)	S(≤4)	8-32
Cefixim	S(≤0.25)	S(≤0.25)	S(0.5)	S(0.5)	R(≥4)	S(≤0.25)	S(≤0.25)	S(≤0.25)	S(0.5)	S(≤0.25)	1-2
Cefotaxim	S(≤1)	S(≤1)	S(≤1)	S(≤1)	S(≤1)	S(≤1)	S(≤1)	S(≤1)	S(≤1)	S(≤1)	1-2
Ceftazidim	S(≤1)	S(≤1)	S(≤1)	S(≤1)	S(≤1)	S(≤1)	S(≤1)	S(≤1)	S(≤1)	S(≤1)	1-4
Ceftriaxon	S(≤1)	S(≤1)	S(≤1)	S(≤1)	S(≤1)	S(≤1)	S(≤1)	S(≤1)	S(≤1)	S(≤1)	1-2
Cefepime	S(≤1)	S(≤1)	S(≤1)	S(≤1)	S(≤1)	S(≤1)	S(≤1)	S(≤1)	S(≤1)	S(≤1)	1-4
Aztreonam	S(≤1)	S(≤1)	S(≤1)	S(≤1)	S(≤1)	S(≤1)	S(≤1)	S(≤1)	S(≤1)	S(≤1)	1-8
Ertapenem	S(≤0.5)	S(≤0.5)	S(≤0.5)	S(≤0.5)	S(≤0.5)	S(≤0.5)	S(≤0.5)	S(≤0.5)	S(≤0.5)	S(≤0.5)	0.5-1
Imipenem	S(≤0.25)	S(≤0.25)	S(≤0.25)	S(≤0.25)	S(≤0.25)	S(≤0.25)	S(≤0.25)	S(≤0.25)	S(≤0.25)	S(≤0.25)	2-8
Meropenem	S(≤0.25)	S(≤0.25)	S(≤0.25	S(≤0.25)	S(≤0.25)	S(≤0.25)	S(≤0.25)	S(≤0.25)	S(≤0.25)	S(≤0.25)	2-8
Amikacin	S(≤2)	S(≤2)	S(≤2)	S(≤2)	S(≤2)	S(≤2)	S(≤2)	S(≤2)	S(≤2)	S(≤2)	8-16
Gentamicin	S(≤1)	R(≥16)	R(≥16)	S(≤1)	S(≤1)	S(≤1)	S(≤1)	S(≤1)	S(≤1)	S(≤1)	2-4
Tobramycin	S(≤1)	R(≥16)	R(≥16)	S(≤1)	S(≤1)	S(≤1)	S(≤1)	S(≤1)	S(≤1)	S(≤1)	2-4
Nalidixic acid	S(≤2)	R(≥32)	R(≥32)	S(≤2)	R(8)	S(≤2)	S(≤2)	S(≤2)	S(≤2)	S(≤2)	8-16
Ciprofloxacin	S(≤0.25)	S(≤0.25)	S(≤0.25)	S(≤0.25)	S(≤0.25)	S(≤0.25)	S(≤0.25)	S(≤0.25)	S(≤0.25)	S(≤0.25)	0.5-1
Levofloxacin	S(≤0.12)	S(≤1)	S(0.5)	S(≤0.12)	S(≤0.12)	S(≤0.12)	S(≤0.12)	S(≤0.12)	S(≤0.12)	S(≤0.12)	1-2
Moxifloxacin	S(≤0.25)	S(0.5)	S(0.5)	S(≤0.25)	S(≤0.25)	S(≤0.25)	S(≤0.25)	S(≤0.25)	S(≤0.25)	S(≤0.25)	0.5-1
Ofloxacin	S(≤0.25)	R(2)	S(0.5)	S(≤0.25)	S(≤0.25)	S(≤0.25)	S(≤0.25)	S(≤0.25)	S(≤0.25)	S(≤0.25)	0.5-1
Minocycline	R(≥16)	S(4)	S(≤1)	R(≥16)	R(≥16)	S(4)	S(4)	I(8)	S(2)	I(8)	4-8
Tetracycline	R(≥16)	R(≥16)	R(≥16)	R(≥16)	R(≥16)	R(≥16)	R(≥16)	R(≥16)	R(≥16)	R(≥16)	4-8
Tigecycline	S(≤0.5)	S(≤0.5)	S(≤0.5)	S(≤0.5)	S(≤0.5)	S(≤0.5)	S(≤0.5)	S(≤0.5)	S(≤0.5)	S(≤0.5)	1-2
Nitrofurantoin	S(64)	S(64)	S(64)	S(64)	S(32)	S(64)	S(64)	S(≤16)	S(≤16)	R(256)	64-64
Chloramphenicol	S(4)	R(≥64)	R(≥64)	S(4)	S(≤2)	S(4)	S(4)	S(8)	R(≥16)	S(4)	8-8
Colistin	R(≥16)	R(≥16)	R(≥16)	R(≥16)	R(≥16)	S(≤0.5)	S(≤0.5)	S(≤0.5)	S(≤0.5)	S(≤0.5)	2-2
Trimethoprim	S(≤0.5)	R(≥16)	R(≥16)	R(≥16)	R(≥16)	R(≥16)	R(≥16)	R(≥16)	S(≤0.5)	S(≤0.5)	2-4
Trimethoprim/ sulfamethoxazole	S(≤20)	R(≥320)	R(≥320)	R(≥320)	R(≥320)	R(≥320)	R(≥320)	R(≥320)	S(≤20)	S(≤20)	2/38-4/76

Interpretation (R : resistant, I : intermediate ; S : susceptible), MIC (µg/ml) as obtained by vitek.

Table 2

Effect of Enterocin DD14 in combination with colistin and Nisin on planktonic cells of *Escherichia coli (E. coli)* strains from swine origin.

Antimicrobials	Log CFU /ml *E. coli* CIP54127	Log CFU /ml *E. coli* 184	Log CFU /ml *E. coli* 289
None (Control assay)	8.1± 0.03 ^{a}	8.0±0.05^{a}	8.1±0.05^{a}
Enterocin DD14 (25µg/ml)	7.7±0.09 ^{a}	7.6±0.2^{ab}	7.7±0.24^{ab}
Enterocin DD14 (50µg/ml)	7.4±0.18 ^{ab}	7.5±0.17^{ab}	7.4±0.14^{bc}
Colistin (1 mg/ml)	3.9±0.03 ^{d}	7.3±0.10^{bc}	7.3±0.12^{bc}
Colistin (1 mg/ml) + Enterocin DD14 (25µg/ml)	3.6±0.4 ^{d}	4.7±0.3^{d}	4.6±0.3^{e}
Colistin (1 mg/ml) + Enterocin DD14 (50µg/ml)	3.1±0.07 ^{d}	3.5±0.26^{e}	3.4±0.12^{f}
Nisin (1 mg/ml)	7.5±0.28 ^{ab}	7.4±0.1^{b}	7.5±0.09^{bc}
Nisin (1 mg/ml)+ Enterocin DD14 (25µg/ml)	7.0±0.03 ^{bc}	7.1±0.02^{bc}	7.1±0.05^{cd}
Nisin (1 mg/ml) + Enterocin DD14 (50µg/ml)	6.7±0.08 ^{c}	6.8±0.17^{c}	6.7±0.08^{d}
Nisin (1 mg/ml)+ Colistin (1 mg/ml)	2.1±0.09 ^{e}	3.3±0.19^{e}	3.4±0.06^{f}
Nisin (1 mg/ml)+ Colistin (1 mg/ml)+ Enterocin DD14 (25µg/ml)	0	0	0
Nisin (1 mg/ml)+ Colistin (1 mg/ml)+ Enterocin DD14 (50µg/ml)	0	0	0

Means without a common letter are significantly different ($p<0.05$) using one way-ANOVA, and Tukey post-hoc Test.

Table 3

Effect of bacteriocin DD14 in combination with colistin and Nisin on biofilm of *Escherichia coli* (*E. coli*) strains from swine origin

Antimicrobials	Log CFU /ml *E. coli* CIP54127	Log CFU /ml *E. coli* 184	Log CFU /ml *E. coli* 289
None (Control assay)	8.1± 0.07^{a}	8.1±0.06^{a}	8.1±0.05^{a}
Enterocin DD14 (25µg/ml)	7.8±0.17^{a}	7.7±0.04^{ab}	7.6±0.2^{b}
Enterocin DD14 (50µg/ml)	7.6±0.26^{ab}	7.5±0.1^{bc}	7.6±0.13^{bc}
Colistin (1 mg/ml)	4.1±0.1^{d}	7.4±0.16^{bc}	7.4±0.18^{bcd}
Colistin (1 mg/ml) + Enterocin DD14 (25µg/ml)	4.0±0.1^{d}	5.0±0.01^{e}	5.1±0.09^{f}
Colistin (1 mg/ml) + Enterocin DD14 (50µg/ml)	3.6±0.4^{d}	4.4±0.14	4.6±0.11^{g}
Nisin (1 mg/ml)	7.6±0.13^{ab}	7.5±0.2^{bc}	7.4±0.08^{cd}
Nisin (1 mg/ml) + Enterocin DD14 (25µg/ml)	7.1±0.09^{bc}	7.2±0.01^{cd}	7.2±0.05^{d}
Nisin (1 mg/ml) + Enterocin DD14 (50µg/ml)	6.8±0.10^{c}	6.9±0.12^{d}	6.7±0.06^{e}
Nisin (1 mg/ml) + Colistin (1 mg/ml)	2.5±0.45^{e}	3.6±0.4^{f}	3.5±0.07^{h}
Nisin (1 mg/ml) + Colistin (1 mg/ml) + Enterocin DD14 (25µg/ml)	0	0	0
Nisin (1 mg/ml) + Colistin (1 mg/ml) + Enterocin DD14 (50µg/ml)	0	0	0

Means without a common letter are significantly different ($p<0.05$) using one way-ANOVA, and Tukey post-hoc Test.

Chapter 5. Potentialization of β-lactams by colistin in the case of extended spectrum β-lactamase *E. coli* producing strains from infant origin

UTIs of child caused by the extended spectrum β-lactamase (ESBL)- producing *E. coli* has became an emerging phenomenon worldwide during the last decades. Our studies showed the combination of the colistin with two β-lactams family antibiotics, ticarcillin and cefotaxime, allowed to obtain a synergetic effect on five ESBL-producing bacteria, identified as *E. coli* and isolated from children with urinary infections hospitalized at Roubaix hospital in the north of France. Most of UTIs are caused by Extended Spectrum β-lactamases (ESBLs) producing Enterobacteriaceae, especially *E. coli* and *K. pneumoniae,* participating in antibiotics resistance dissemination (Yadav et al., 2015).

The DNA genotypes of these non-nosocomial isolates were determined by Random Amplified Polymorphic DNA (RAPD) method. Further, their DNA plasmids content revealed the presence of two distinct plasmids for S1, S2, S3 and one plasmid for S4 and S5. To overcome resistance of *E. coli* ESBL isolates to β-lactams, we conducted assays combining ticarcillin/colistin, and cefotaxime/colistin. Colistin, also called polymyxin E, is a polypeptide antibiotic. These combinations permitted synergistic interactions as the FIC index were under 0.5. Importantly, the combination ticarcillin/colistin has reached the breakpoint for isolates S1 and S4, whilst it was below the breakpoint for isolates S2, S3 and S5 offering consequently novel option to fight against infections caused by ESBL producing bacteria. On the other hand, the combination of cefotaxime-colistin has drastically reduced the MIC value of cefotaxime and the breakpoint was almost reached for isolate S2. In this case the susceptibility of the strain was not reached. Based on our data, the MIC of colistin could reasonably be reduced 4 fold, when administrated with ticarcillin and cefotaxime, which could be given by injection. Remarkably, the kill curves analyses were carried out with only isolates S1 and S2. Besides the synergistic effect, this combination could ensure the drop of cytotoxic effects of colistin. Although the colistin/β-lactam combination therapy was already provided as useful therapeutic option for treatment of ESBL producing *E. coli* UTI in children. The data gathered from our research portray the application of these combinations in the case of infections caused by carbapenem-resistant strains. Recent study revealed the synergistic effect of colistin in combination with some antibiotics such as meropenem and tigecycline against MDR clinical *K. pneumoniae* isolates (Stein et al., 2015)

Potentialization of β-lactams with colistin : in case of extended spectrum β-lactamase producing *Escherichia coli* strains isolated from children with urinary infections

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Abstract

Five strains producing ESBL bacteria, identified as *Escherichia coli*, were isolated from children with urinary infections at Roubaix hospital in the north of France. The clonal relationship of these isolates was determined by rep-PCR. Besides their genetic relatedness, these ESBL bacteria appeared to contain at least two DNA plasmids. The antibacterial susceptibility of these ESBL bacteria was assessed towards multiple antibiotics, mainly those of β-lactams family. The strains displayed different patterns of resistance. The ESBL producing bacteria were resistant to ticarcillin and cefotaxime but the combination of these antibiotics with colistin has dropped the MIC of ticarcillin below its breakpoint (isolates S2,S3 and S4), and has almost reached the breakpoint for cefotaxime (isolate S2). Thus, kill curves analyses carried out with only isolates S1 and S2, strengthened the bactericidal activity of the combinations of colistin-ticarcillin and colistin-cefotaxime against ESBL *E. coli*. Indeed, reduction of 3 log10 colony count were observed after 24 h of incubation.

Keywords : β-lactams, colistin, potentialization, ESBL, *E. coli*

1. Introduction

Infections with ESBL producing bacteria are considered as the most serious with high rates of mortality, morbidity and likely healthcare expenditure. ESBLs have been reported in *Escherichia coli* and also in other genera of the Enterobacteriacea [1-2]. Nevertheless, the most ESBL-production is occurring in *E. coli* strains [3]. Thus, *E. coli* strains are the most important causes UTIs [4-5] and are responsible for over 80% of children UTIs [6], followed by *Klebsiella pneumoniae*, *Proteus* spp. and *Staphylococcus* [7]. The increasing prevalence of pathogens producing extended-spectrum-lactamases (ESBLs) is reported worldwide in both hospitalized patients and in out-patients [8]. Antibiotic treatments of ESBL bacterial infections could include different antibiotics such as carbapenems [9- 10], fluoroquinolones, nitrofurantoin, amoxicillin and ampicillin [11-12]. Most of resistant bacteria are not susceptible to second and third generation of cephalosporins, and constitute a major problem regarding their ability to transfer antibiotics-resistance [13]. Urinary tract infections (UTIs) are potential sources of ESBLs infections. UTIs constitute the most prevalent bacterial infections during the childhood in the world. UTIs are increasingly due to bacteria with multiresistance to common antibiotics, and this situation stands as hurdle for treatment of these infections which are occurring when potency of antibiotics is fading and therapeutic options are limited [14-15]. ESBL producing strains are clearly a major public concern. To overcome this threat, the physicians consider the use of colistin as drug of last resort. Colistin is an old antibiotic of polymyxins group, which was largely used to fight against infections caused by Gram negative bacteria such as diarrhea, urinary tract infection and other diseases like otic and ophthalmic infections. Polymyxins were withdrawn from therapeutic circuit because of their nephro- and neuro- toxicity and also because other antibiotics including the third generation cephalosporins were developed and marketed. The nephrotoxicity of colistin is currently considered as less important than it was debated [16]. This study aimed at characterizing clinical strains of ESBL *E. coli* strains isolated at Roubaix hospital (north of France) with urinary infections. The susceptibility of these strains to a set of family antibiotics was

determined and discussed. Besides, the impact of antibiotic combinations including tandem colistin - -lactams were performed in order to unveil novel therapeutic options.

2. Material and methods

2. 1. Bacterial isolates

The *E. coli* ESBL producing strains used in this work were isolated from children with urinary infections hospitalized at Victor Provo hospital (Roubaix, France) between September 2014 to February 2015. The ESBL *E. coli* isolates were identified by the VITEK MS v2.0 MALDI-TOF mass spectrometry according to manufacturer's instructions (Bio-Mérieux, France). The ESBL *E. coli* strains were stored at -80 o95 C in Brain-Heart-Infusion (BHI) with 25% glycerol as stock culture, until use.

2.2. Antimicrobial susceptibility

Study of antibiotic susceptibility was performed by three independent methods: disk diffusion method, minimal inhibitory concentrations (MICs) using E-test (Bio-Mérieux, France), and VITEK 2 system (Bio-Mérieux, France), encompassing nearly all important antibiotics. Antibiotic susceptibility and MICs were determined and analyzed according to the French Committee on Antimicrobial Susceptibility Testing.

2.3. RAPD analysis of ESBL E. coli *isolates*

Total DNA was extracted from each ESBL *E. coli* isolateusing the wizard® Genomic DNA purification kit (Promega,Madison, WI, USA). For RAPD analysis, total DNA wasamplified with M13-core primer (50-GAG GGT GGC GGTTCT-30) or DAF4 primer (50-CGG CAG CGC C-30). Ampli-fication was performed as described by Vogel et al. [17]. The PCR products were separated by electrophoresis on a 2%agarose gel, run at 80 V for 2 h, using 1X Tris-borate-EDTA.The gels were then stained with GELRED (Biotium, Canada), and visualized by GelDoc (Bio-Rad, France). The RAPD patterns were then analyzed visually.

2.4. Plasmid DNA extraction

The ESBL *E. coli* isolates S1, S2, S3, S4, S5 were grown for 18h at 37°C in Brain- Heart-Infusion (BHI) medium until they reach the OD600nm of 0.8-1, the cells were then harvested by centrifugation (5,000 rpm, 10 min, 4°C), and plasmids extracted by the GeneJET Plasmid Miniprep Kit protocol (Thermo scientific). The quality of plasmid DNA was checked on 1.0% agarose gel labeled with 0.5‰ (v/v) GelRed for 1 h at 100V. Gels were analyzed with a Gel-Doc 2000® (Biorad, Hercules, CA, USA).

2.5. Minimal inhibitory concentration (MICs)

A pure colony of each ESBL *E. coli* isolate was grown overnight in BHI medium at 37°C. Afterwards 10 µl of each overnight culture of ESBL *E. coli* isolate were added to the first wall of bioassay microplate of 96 well cell culture plate (Cellstar) containing 0.5 µg/ml of colistin, then 10 µl were added to the next wall containing 0.25 µg/ml of colistin. The concentrations of colistin tested were ranking from 0.031 to 0.5 µg/ml µg/ml. In case of ticarcillin the concentrations tested were ranking from 4 µg/ml to 512 µg/ml, whilst the range tested for cefotaxime was comprised between 4 µg/ml to 256 µg/ml. The MIC is defined as the lowest concentration of an antibiotic that will inhibit the visible growth of a microorganism after overnight incubation.

2. 6. Checkerboard assays

Antibiotics interactions were determined using checkerboard assay as previously described [18]. The concentration used for colistin were 0.01 - 0.25 µg/ml, while those used for ticarcillin and cefotaxime were 4 -128 µg/ml and 4 - 64 µg/ml, respectively. Microplates were inoculated with each ESBL *E. coli* isolate to yield ~10^6 CFU/ml in a 200 µl final volume and incubated overnight at 37°C. The fractional inhibitory concentration index (FICI) was calculated for each combination using the following formula: FICA + FICB = FICI, where FICA = MIC of drug A in combination/MIC of drug A alone, and FICB = MIC of drug B in combination/MIC of drug B alone. The FICI was interpreted as follow: synergism = FICI ≤ 0.5; indifference = 0.5 < FICI ≤ 4; antagonism = FICI > 4 [19].

2.7. Kill curves assays

Tubes containing BHI supplemented with colistin, ticarcillin or cefotaxime (respective antibiotics) were inoculated with ESBL *E. coli* to density of ~5x10^5 CFU/ml in a final volume of 5 ml and incubated at 37°C. The killing kinetics of the colistin alone, colistin in combination with ticarcillin, and colistin in combination with cefotaxime was assessed against each of the ESBL *E. coli* isolates using standard time-killing assay and viable bacterial counts. The final concentration of colistin and ticarcillin were 0.063-8 µg/ml, (isolate S2), and 0.063-16 µg/ml (isolate S1). The final concentration of colistin and cefotaxime were 0.063-8 µg/ml (isolate S1) and 0.63-4 µg/ml (isolate S2). Aliquots were removed at time 0, 3, 6, 9 and 24 h post incubation, serially diluted in saline for determination of viable counts. Diluted samples (100 µL) were plated on Tryptone-soya-agar agar plates and colonies were counted after overnight incubation at 37°C. Bactericidal activity was determined as 3 log10 CFU/ml reduction in the colony count relative to the initial inoculum [20].

3. Results

3.1. RAPD analysis and DNA plasmid content of ESBLstrains

The RAPD data showed the genetic patterns of ESBL *E.coli* isolates. As evident from Fig. 1A and B, ESBL isolates S1and S2 displayed different RAPD patterns when their totalDNA was amplified with DAF4 and some similarities whentheir DNA was amplified with M13 core primer. Similarly,ESBL *E. coli* isolates S3 and S5 presented some RAPD sim-ilarities with primer M13-core, and differences with primerDAF4. Finally, the isolate S4 presented overall differentRAPD patterns from the other isolates. As conclusion, wecould consider that profiles of these strains S1, S2, S3, S4 andS5 are distinguishable and suggest that the strains are notidentical.Furthermore, the ESBL strains S1, S2, S3 appeared toharbor at least two plasmids of different molecular weight,whilst the ESBL strains S4 and S5 revealed the presence ofonly one plasmid (Fig. 1B).

3. 2. Antibiotic resistance profiles

The MIC values are presented in Table 1. The MICs are interpreted according to the recommendations of EUCAST and Antibiogram board of the French society of microbiology. Overall, isolate 1 was resistant to almost all β-lactams tested except for carbapenems (imipenem and ertapenem). Isolate S1 displayed resistance to aminoside (tobramycin) and cyclines (tetracycline). In turns, isolate S2 displayed resistance as well to β-lactams except for cefepime, ceftazidime, imipenem and ertapenem. Isolate S2 was resistant to aminosides (tobramycin and gentamicin), tetracylines (tetracycline), quinolones (nalidix acid, ofloxacin) and cotrimoxazole. Isolates S3, S4 and S5 were nevertheless resistant to a less number of β- lactams (Table 1).

3.3. Inputs of antibiotics combinations

The checkerboard analysis showed synergistic interactions between colistin and β- lactams (Table 2). The MIC of ticarcillin in combination with colistin has diminished below the breakpoint for isolates S2, S3 and S5. For all isolates, the MIC of cefotaxime in combination with colistin has diminished significantly 194 but remained above the breakpoint. The time kill assays were determined for isolates S1 and S2, by combining colistin and ticarcillin, and on the other hand, colistin and cefotaxime. These combinations allowed the reduction of about 3 log10 CFU/ml in the colony count after 24 h of incubation (Table 3, Fig. 3). It should be noted that the MIC of colistin allowing potentialization of β-lactams was equaled to 0.063 µg/ml. This is quite important for the management of toxicity of this antibiotic.

4. Discussion

Urinary tract infections (UTIs) are deemed as major clinical concern in childhood, and ESBL producing organisms are the leading cause of healthcare-related UTIs. *E. coli* remains as one of the most important bacteria causing infections in pediatrics and ESBLs producing making them resistant to β-lactam antibiotics [21]. The recognition of ESBL-producing organisms as risk factor contributing to elevated death rates and the increasing resistance to antibiotics warrant further studies that could help to determine new recommendations to master UTIs and also to better manage antibiotic use in children and

adolescents. Overall, the increasing number of incidences in resistance of gram-negative bacilli against even newer antibiotic including carbapenem has gathered interest in the old antibiotics, such as colistin, which is considered nowadays as salvage therapy in the treatment of multidrug resistant infection. Besides, the re-habilitation of colistin, potentialization and implementation of bioengineering strategies to enhance the functional characteristics of existing antibiotics could be considered as serious therapeutic options. In this study, the non-nosocomial ESBL-*E. coli* strains were isolated from children hospitalized at Roubaix

hospital for urinary infections. These strains were characterized for their possible clonality relationship by studying theirtotal RAPD DNA patterns and looking for the presence of anyDNA plasmid, which is considered as a mean of bacterial resistance dissemination [22]. The ESBL producing strainsstudied here seem to carry at least two natural plasmids, but the implication of these DNA supports in ESBL function remains to be elucidated. Related to ESBL DNA supports, Hong et al.[23] reported that genes coding for β-lactamase in *Pseudomonas aeruginosa* could be found on integrons, transposons, plasmids, or on the chromosome. Notably, genetic subtypes of ESBLs were reported since 1980s have so far exceeded 200 [24]. To be noted that most of ESBLs belong toTEM, SHV, and CTX-M types [2,25,26]. Remarkably, TEM is the main type of b-lactamase, and the TEM-1 group is the most common ones. Further CTX-M enzymes was a new group of plasmid-mediated ESBLs which has become the predominant ESBLs reported in Europe from last decade and has since increased dramatically in many countries [25e27].As evident from Table 1, the susceptibility data indicated that the ESBL producing strains are resistant to most b-lactams and to other antibiotics, including quinolones and amino-glycosides. Similar resistance patterns were recently revealed for ESBL *E. coli* strains isolated in China [24]. To overcome resistance of *E. coli* ESBL isolates to b-lactams, we conducted assays combining ticarcillin/colistin, and cefotaxime/colistin. Colistin, also called polymyxin E, is a polypeptide antibiotic.These combinations permitted synergistic interactions as the FIC index were under 0.5. Importantly, the combination ticarcillin/colistin has reached the breakpoint for isolates S1 and S4, whilst it was below the breakpoint for isolates S2, S3 and S5 offering consequently novel option to fight against infections caused by ESBL producing bacteria. On the other hand, the combination of cefotaxime-colistin has drastically reduced the MIC value of cefotaxime and the breakpoint was almost reached for isolate S2. In this case the susceptibility of the strain was not reached. Based on our data, the MIC of colistin could reasonably be reduced 4 fold, when administrated with ticarcillin and cefotaxime, which could be given by injection. Besides the synergistic effect, this combination could ensure the drop of cytotoxicity effects of colistin. Although the colistin/b-lactam combination therapy was already provided as useful therapeutic option for treatment of ESBL producing *E. coli* UTI in children, the data gathered from our research portray the application of these combinations in the case of infections caused by carbapenems

resistant strains. In direct line, Zusman et al. (2013) [28] have reviewed the main *in-vitro* interactions of polymyxins and carbapenems towards a wide range of GNB included in the ESKAPE pathogens group. The authors unveiled the high synergy rates and low resistance development when carbapenem/polymyxin combination especially against *Acinetobacter baumannii*. Recently, we highlighted the efficacy of colistin antimicrobial peptides combinations against Gram negative bacteria [29,30]. In light of checkerboard and kill curves analyses, we report the *in vitro* potency of colistin and b-lactams, mainly colistin-ticarcillin combination against ESBL *E. coli* strains isolated from urinary infections. The hypothesis of mode of action of these antibiotics could be that colistin triggers the anti-*E. coli* activity and then the b-lactams pursueeasily their activity. In direct line, Kempf et al. [31] demonstrated synergistic activity of colistin combination with sulbactam against *A. baumannii*. The re-utilization of colistin, an old antibiotic in combination with other antibiotics may offerrational use of antibiotics. This study shows the pertinence of the colistin/b-lactams combination to fight against strains resistant to carbapenems. More ESBL *E. coli* isolates from children origin will be tested to strengthen this prospective study and then we have to proceed with validation in the animal models. Furthermore, combinations of colistin with penicillin and colistin-cephalosporins will be tested.

Conflict of interest

No conflict of interest declared.

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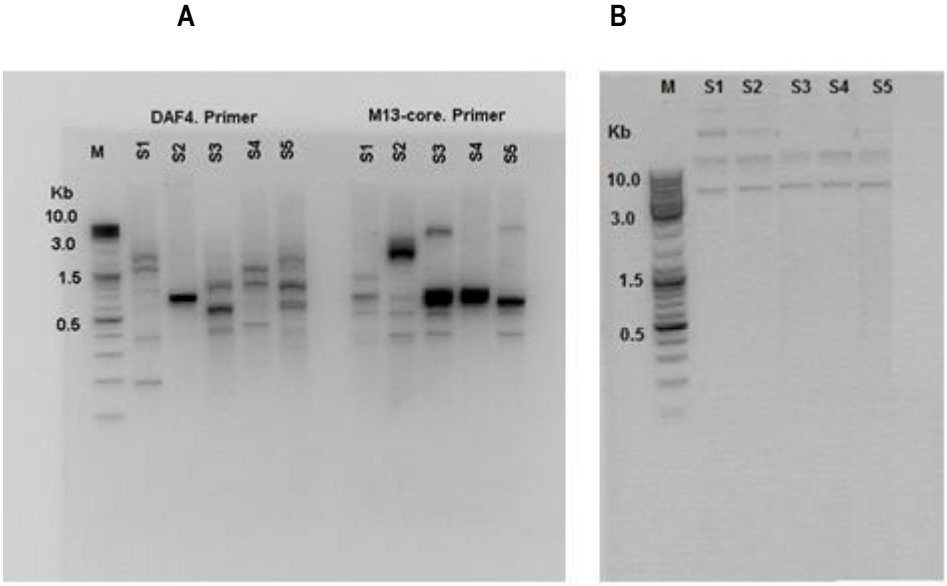

Fig. 1. A: RAPD patterns of the ESBL *E. coli* isolates used in this study. Electrophoresis was performed on 2% (w/v) agarose gel. Lanes S1, S2, S3, S4 and S5 represent the RAPD DNA patterns of *E. coli* isolates 1, 2, 3, 4 and 5 upon amplification with DAF4 and M13-core primers. B: Plasmid contents of ESBL *E. coli* isolates *E. coli* 1, 2, 3, 4 and 5 used in this study. Electrophoresis was performed on 1% (w/v) agarose gel. In both gels (A, B) lanes M show O'GeneRuler DNA Ladder Mix, (Thermo, USA).

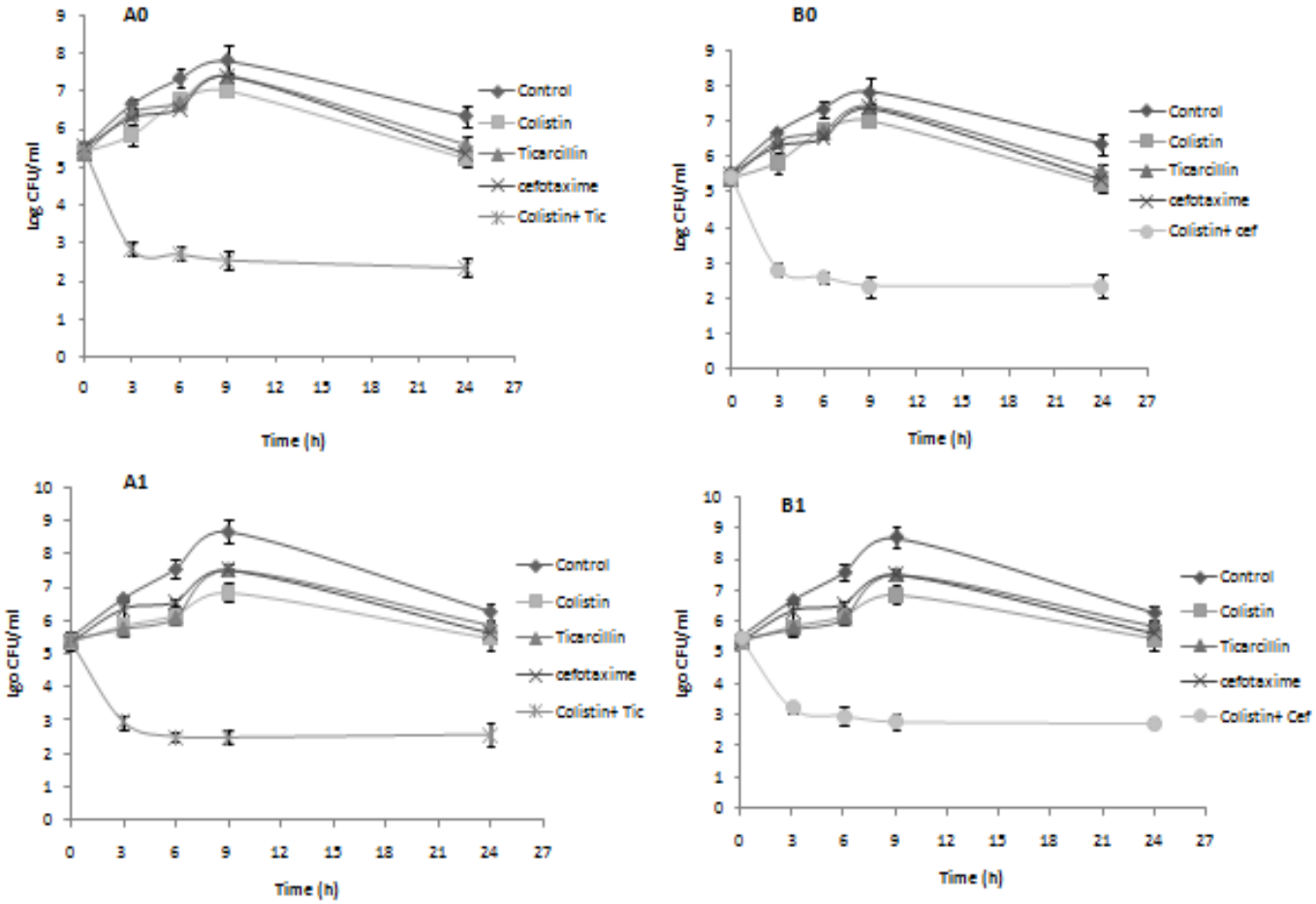

Fig. 2. Time-kinetics of colistin-ticarcillin (Tic) and colistin-cefotaxime (Cef) against ESBL-producing *E. coli* strains at 0, 3, 6, 9, and 24 h of incubation. A0:Effect of colistin-ticarcillin combination against *E. coli* isolate S1; B0: Effect of colistin-cefotaxime combination against *E. coli* isolate S1; A1: Effect of colistin-ticarcillin combination against *E. coli* isolate S2; B1: Effect of colistin-cefotaxime combination against *E. coli* isolate S2. The data (±SD) are the average of at leastthree independent experiment. In each experiment, three independent measures were performed.

Table 1. Antibiotic susceptibility of ESBL *Escherichia coli* (*E. coli*) strains isolated from the children with urinarytract infection

Antibiotics MIC: mg/l	*E. coli* S1	*E. coli* S2	*E. coli* S3	*E. coli* S4	*E. coli* S5
Ampicillin	R (≥ 32)	R (≥ 32)	R (≥ 32)	R (≥ 32)	R (≥ 32)
Amoxicillin + Clavulanic acid	R (≥ 32)	R (16)	R (≥ 32)	I (8)	R (16)
Ticarcillin	R (≥ 128)	R (≥ 128)	R (≥ 128)	R (≥ 128)	R(≥ 128
Ticarcillin + Clavulanic acid	R (≥ 128)	R (≥ 128)	R (≥ 128)	I (16)	R (≥ 128)
Piperacillin	R (≥ 128)	R (≥ 128)	R (≥ 128)	R (≥ 128)	R (≥ 128)
Piperacillin + Tazobactam	R (≥ 128)	R (≤ 4)	R (≥ 128)	S (≤ 4)	S (≤ 4)
Cefalotin	R (≥ 64)	R (≥ 64)	R (≥ 64)	R (≥ 64)	R (≥ 64)
Cefoxitin	S (≤ 4)	S (≤ 4)	S (≤ 4)	S (≤ 4)	S (≤ 4)
Cefixim	R (≥ 4)	R (≥ 4)	R (≥ 4)	R (≥ 4)	R (≥ 4)
Cefotaxime	R (≥ 64)	R (≥ 64)	R (≥ 64)	R (≥ 64)	R (≥ 64)
Ceftriaxone	R (≥ 64)	R (≥ 64)	R (≥ 64)	R (32)	R (≥ 64)
Cefepime	R (≥ 64)	I (2)	I (2)	I (2)	I (2)
Ceftazidime	R (16)	I (4)	I (≤ 1)	S (≤ 1)	S (≤ 1)
Aztreonam	R (≥ 64)	R (16)	I (2)	I (2)	I (4)
Imipenem	S (≤ 0.25)	S (≤ 0.25)	S (≤ 0.25)	S (≤ 0.25)	S (≤ 0.25)
Ertapenem	S (≤ 0.5)	S (≤ 0.5)	S (≤ 0.5)	S (≤ 0.5)	S (≤ 0.5)
Tobramycin	R (≥ 16)	R (4)	S (≤ 1)	S (≤ 1)	S (≤ 1)
Gentamicin	S (≤ 1)	R (≥ 16)	S (≤ 1)	S (≤ 1)	S (≤ 1)
Amikacin	I (16)	S (≤ 2)	S (≤ 2)	S (≤ 2)	S (≤ 2)
Tetracycline	R (≥ 16)	R (≥ 16)	S (≤ 1)	S (≤ 1)	S (≤ 1)
Tigecycline	S	S	S	S	S
Minocycline	S (2)	S (2)	S (≤ 1)	S (≤ 1)	S (≤ 1)
Nalidixic acid	S (≤ 2)	R (8)	S (≤ 2)	S (≤ 2)	S (≤ 2)
Ofloxacin	S (≤ 0.25)	R (2)	S≤ (0.25)	S (≤ 0.25)	S (≤ 0.25)
Ciprofloxacin	S (≤ 0.25)	S(≤ 0.5)	S (≤ 0.25)	S (≤ 0.25)	S (≤ 0.25)
Nitrofurantoin	S (32)	S (≤ 16)	S (≤ 16)	S (32)	S (≤ 16)
Cotrimoxazole	S (≤ 20)	R (≥320)	S (≤ 20)	S (≤ 20)	S (≤ 20)
Colistin	S (≤ 0.5)	S (≤ 0.5)	S (≤ 0.5)	S (≤ 0.5)	S (≤ 0.5)

R. Resistant; S. Sensitive; I, Intermediate; MIC, Minimal inhibitory concentration determined by Vitek 2 system

Table 2. Antibiotic combinations against ESBL *Escherichia coli* strains isolated from children with urinary tract infection.

Isolate	Colistin	Ticarcillin	Cefotaxime	Colistin/ Ticarcillin	FIC	Colistin/ Cefotaxime	FIC
E. coli S1	0.25	512	256	0.063/16	0.28	0.063/8	0.28
E. coli S2	0.25	512	256	0.063/8	0.26	0.063/4	0.26
E. coli S3	0.25	512	256	0.063/8	0.26	0.063/8	0.28
E. coli S4	0.25	512	256	0.063/16	0.28	0.063/8	0.28
E. coli S5	0.25	512	256	0.063/8	0.26	0.031/8	0.15

Fractional Inhibitory Concentration (FIC) index determined as previously described [19]. The data (±SD) are the average of at least three independent experiments. (MIC given in µg/ml). Breakpoint value of ticarcillin according to EUCAST and CA-SFM is 16, whereas that of cefotaxime is 2. Colistin sodium methanesulfonate (27655-5G), ticarcillin disodium salt (T5639-1G) and cefotaxime sodium salt (C7912-1G) were obtained from Sigma-Aldrich.

Table 3. Effect of colistin-ticarcillin and colistin-cefotaxime combinations against ESBL-producing *Escherichia coli* isolates S1 and S2.

Isolates	Combination	Control	0h	3h	6h	9h	24h
E. coli S1	Colistin-Ticarcillin	5.54±0.05	5.42±0.20	2.83±0.17	2.71±0.19	2.55±0.23	2.34±0.33
	Colistin-Cefotaxime	5.54±0.05	5.41±0.12	2.81±0.21	2.58±0.17	2.34±0.28	2.33±0.32
E. coli S2	Colistin-Ticarcillin	5.48±0.20	5.35±0.09	2.94±0.21	2.5±0.16	2.48±0.21	2.57±0.36
	Colistin-Cefotaxime	5.48±0.20	5.46±0.10	3.31±0.16	2.98±0.28	2.79±0.26	2.72±0.15

Log10 colony count lower than that at time Zero without antimicrobial agent (control). -1=Δ 1 Log10 CFU/ml = 90% killing; -2 =Δ 2 Log10 CFU/ml = 99% killing; -3= Δ 3 Log10 CFU/ml = 99.9% killing.

General conclusion and perspectives

Our study has permitted some remarkable achievements in the field of antimicrobials. Indeed, this is the first work dedicated to isolation of LAB with antagonism from meconium. This ecosystem was seldom targeted and subjected for such studies. Thus, we have isolated 107 bacterial isolates from meconium of six distinct and blind donors generously provided by Dr. Sylvie Rousseau (Roubaix hospital in the north of France). All these isolates were identified, by mass spectrometry MALDI-TOF, as *E. faecalis*, a strain of major importance in the hospital environment but also in the food industries.

The screening of antagonism permitted to find that *E. faecalis* 14, *E. faecalis* 28, *E. faecalis* 90, *E. faecalis* 93, *E. faecalis* 97 and *E. faecalis* 101 are antagonistic through the production of organic acids (lactic acid) and bacteriocins (enterocins).The identification of the antagonistic strains was confirmed by sequencing their 16S rDNA. Their Rep-PCR analysis showed a genetic relatedness but not necessarily the same clone. The challenge tests performed with *E. faecalis* 28 and *E. faecalis* 93 demonstrated the necessity of both lactic acid and bacteriocins for the control of the hardy *S. aureus* ATCC33862. The bacteriocins produced by *E. faecalis* 28 and *E. faecalis* 93, called enterocins DD28 and DD93, were purified during this study by a simplified two-step purification procedure. These enterocins appeared to have a molecular mass, determined by mass spectrometry of 5204.48 Da and 5203.90 Da respectively and seem to belong to class IIb bacteriocins. Besides their antagonism, the strains antagonistic strains appeared to be as non-hemolytic, sensitive to different antibiotics and mainly against those used for the treatment of enterococci infections. These strains were also exhibiting high scores of hydrophobicity and aggregation.

. Further, the combinations of enterocins DD28 and DD93, with two antibiotics, kanamycin and erythromycin, resulted in a synergetic effect on *Staphylococcus* strains including MRSA-S1 strain with high resistance to many antibiotics. The killing curves experiments performed with bacteriocins and enterocins on MRSA-S1 strain has led to promising data. Indeed these combinations permitted to shift the phenotypes of this strain MRSA-S1 from resistance to kanamycin and erythromycin to sensibility to these antibiotics. The killing curves kinetics were remarkable with a drop in the cell-counts of MRS-S1 strain. This drop has at least 2 to 3 Log_{10} reduction, meaning that 90 to 99% of the initial population was reduced after 3 hours of exposure.

These combinations have also expressed an important feature as they impede the MRS-S1 to be embedded in biofilm lifestyle. The inhibition of biofilm formation was supported by microbial cell counts and epifluorescence and SEM microscopy.

Another important finding resulting from this work is the activity of enterocin DD14 against *E. coli* from swine origin. As clearly discussed above, colistin is a drug of major importance. It is still used in the veterinary medicine to fight against severe GNB infections. It is also given to piglets as "preventive medicinal food" when the piglets are separated from sow. Colistin is also back to human medicine after a ban attributed to its cytotoxicity. Thus, this antibiotic is used in veterinary and human medicines but the last superbug found by a Chinese group (Liu et al., 2015) opens new avenue in terms of utilization of this antibiotic.

Related to that, assays conducted here with bacteriocins (nisin and enterocin DD14) against *E. coli* from swine origin were unsuccessful but their combination with colistin permitted to diminish the cells numbers of these *E. coli* strains from swine origin even those carrying resistance to this antibiotic.

Finally, the potential of colistin was also studied deeply here. We targeted five *E. coli* strains isolated from infants with urinary infections and characterized ESBL-producing strains *E. coli*. The results showed that the MIC of ticarcillin in combination with colistin has diminished below the breakpoint for *E. coli* isolates S2, S3 and S5.For technical reasons, the kill-curves kinetics were determined only for *E. coli* isolates S1 and S2, by combining colistin and ticarcillin, or colistin and cefotaxime. These combinations allowed the reduction of about 3 Log_{10} CFU/ml in the colony count after 24 hours of incubation.

Overall, the data gathered from this PhD project supports the role of bacteriocins as novel wave of antibiotics. In spite of the regulation and different hurdles anticipated to their practical use, we assume that a better management of antibiotics, which are fading, has to be associated in the first step with their combinations with antimicrobial peptides such as bacteriocins. The data showed that GNB as *E. coli* from swine origin can be treated efficiently with colistin and bacteriocins. Such concept will help to save the amount of antibiotics to be used and limit the development of bacterial resistance. This view is also true for the treatment of harsh infections caused by GPB such as MRSA. The data unveiled that despite their weak activity against MRSA, the enterocins DD28 and DD93 permitted a significant reduction of the growth of this robust pathogen and impeded its evolution to biofilm lifestyle.

To keep working on this fascinating area, the next indications will be a better exploration of the meconium matrix to really finger its diversity. In addition to the meconium, it will be interesting to look at other sources and proceed with novel screenings of bacteriocins with anti-SARM activity and anti-*E. coli* activity. The possibilities of development of such niches are the hospital environment and also the patients.

Major points to be undertaken in the future are :

- Genetically engineered enterocins. As models enterocin DD28 or DD93 can be easily genetically modified by site-directed mutagenesis, in order to understand which amino-acids are necessary for the

anti-SARM activity. Afterwards, this activity could be improved by incorporating more appropriate aminoacids, as this has already be done for other bacteriocins and other targets.

-Hybrid antimicrobial peptides. This concept is of major interest. It will be easy to combine parts of AMPs (C-terminal part from one AMP to N-terminal part of other AMP) in order to increase the anti-MRSA activity. This can be done obviously on bacteriocins.

Of course, the studies anticipated in the future, will not be limited to the *in vitro* assessment but they have also to be carried out in animals.

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