UNIVERSITÉ DE STRASBOURG

ÉCOLE DOCTORALE PHYSIQUE ET CHIMIE-PHYSIQUE (ED 182)

Inserm UMR 1121 Biomatériaux et Bioingénierie

THÈSE

Présentée par :

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Soutenue le : **09 Mars 2018**

Pour obtenir le grade de : **Docteur de l'université de Strasbourg**

Discipline/ Spécialité : Biophysique

Une alternative pour les antibiotiques conventionnels : un nouveau peptide antimicrobien dérivé de la chromogranine A

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Acknowledgements

First and foremost, I would like to show my sincere gratitude to the almighty for providing me with the greatest support of all with whom I would not go any further in this work. And for their continuous guidance and support, their belief and trust, I would like to thank my supervisor **Prof. Youssef Haikel** for giving me the opportunity to work in a highly esteemed environment and for his moral and financial support. Not only that he provided me with an enormous support but he has been very encouraging and kind. I also appreciate his valuable advice, effective feedback, and his productive discussions that contributed to my work. It has been an immense honor to have him as a supervisor.

I extend my thanks and appreciation to all committee members of my PhD (**Prof. Pierre Farge, Dr. Mohammed Amiche, Dr. Catherine Tomasetto** and **Dr. Youssef Anouar**) for having kindly agreed to be examiners and to evaluate my work.

Also, my deepest heartfelt appreciation goes to **Prof. Céline Marban** for the continuous sufficient help of my PhD study and related research, for her patience, motivation, fruitful comments and immense knowledge. She sharp intellect inspired me to push beyond the boundaries of the utmost possibilities.

Moreover, I offer my deep gratitude to **Dr. Marie Hélène Metz-Boutigue**, for her fruitful advice and her guidance helped me in all the time of research and writing of this thesis. I could not have imagined finishing this work without her.

I am deeply and truthfully grateful for **Cosette Betscha, Sophie Helle, Camille Bergthold, Eric Mathieu, Claire Ehlinger, Pauline Dartevelle, Sabestian Baixe, Fadoua Daouad, Leyla Kocgozlu and Joseph Hemmerlé** for their welcoming and prestigious environment for which I would not be able to do this work, their enormous help and continuous flourishing comments. I am indeed indebted with the many blissful opportunities to meet and work with such people who I deeply respect and honor and whose ideas, professional guidance and assistance have contributed to the development of my work. My sincere thanks for them to provided me with an opportunity to join their team, and who gave me access to the laboratory and research facilities. Without their precious support, it would not be possible to conduct this research.

Also, I would also like to extend huge, warm thanks to all my friends and all Libyan community in France specially in Strasbourg for their love and sincerity.

Besides, I thank my friends in the following institution Faculty of Medical Technology, Zawia university, Libya in particular, I am grateful to them for enlightening me the first glance of research.

Last but not the least, I am endlessly indebted to my beloved parents, who raised me with a love of science and supported me in all my pursuits. Also, for my brothers and my sisters especially the twins **Tasneem** and **Hadeel** for supporting me spiritually throughout my PhD study and for their sincerity and encouragement. And for those whom may have unintentionally excluded, I'm grateful beyond measure for their help.

Ministry of Higher Education of Libya is highly appreciated for their endless support during my study in France. Given this opportunity, this expanded my knowledge and established extended attitudes towards life.

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Abbreviations

A

AAC: Acetyltransferase

ABC: ATP-binding cassette

AFM: Atomic Force Microscope

AMPs: Antimicrobial peptides

ANT: Adenylytransferases

APH: Phosphotransferases

Arg: Arginine

ATR-FTIR: Attenuated Total Reflection: Fourier Transform Infrared

C

Caco-2: Human intestinal epithelial cell line

CAT: Catestatin

CgA: Chromogranin A

CgB: Chromogranin B

CgC: Chromogranin C (Secretogranin II)

Cgs: Chromogranins

CHR: Chromofungin

Chrom: Chromacin

CPPs: Cell Penetrating Peptides

CRPs: Cysteine-rich peptides

Ctl: Cateslytin

CTSL: Cathepsin L

Cys: Cysteine

D

D-Ctl: Cateslytin D form

DCs: Dendritic Cells

E

E. coli: *Escherichia coli*

ERK-1/2: Extracellular Signal-Regulated Kinase-1/2

F

F. nucleatum: *Fusobacterium nucleatum*

FDA: Food and Drug Administration

FIC: Fractional Inhibitory Concentration

G

G-: Gram-negative

G.I: Gastrointestinal

G+: Gram-positive

Gly: Glycine

GPs: General physicians

GRPs: Glycine-rich peptides

H

HDPs: Host defense peptides

HGF: Human gingival fibroblasts

His: Histidine

HPLC: High Performance Liquid Chromatography

L

L-Ctl: Cateslytin L form

LC-SRM: Liquid Chromatography-Selected Reaction Monitoring

LPS: Lipopolysaccharide

M

MALDI-TOF: Matrix Assisted Laser Desorption Ionization - Time of Flight

MAPK-p38: Mitogen activated protein kinase-p38

MATE: Multidrug and toxic-compound extrusion

MDR: Multidrug resistance

MFS: Major facilitator superfamily

MIC: Minimal Inhibitory Concentration

MRSA: Methicillin resistance *staphylococcus aureus*

MSSA: Methicillin sensitive *staphylococcus aureus*

MTT: [3(4,5-dimethylthiazol-2-yl)-2,5 diphenyl tetrazolium bromide]

N

NMR: Nuclear Magnetic Resonance

P

P. intermedia: *Prevotella intermedia*

P. micra: *Parvimonas micra*

PBMCs: Peripheral blood mononuclear cells

PBPs: Penicillin-binding proteins

Pro: Proline

Prochrom: Prochromacin

Q

QT interval: Time from electrocardiogram Q wave to the end of the T wave corresponding to electrical systole

R

RND: Resistance nodulation division

S

S. aureus: *Staphylococcus aureus*

STAT-3: Signal transducer and activator of transcription-3

T

TNF-alpha: Tumor Necrosis Factor- alpha

Trp: Tryptophan

TFA: Trifluoroacetic acid

V

VEGF: Vascular Endothelial Growth Factor

VS-I: Vasostatin-I

W

WAKs: Cell wall – associated kinases

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Thesis abstract in English

The discovery of antibiotics to treat infectious diseases is one of the greatest achievements of modern medicine. Antibiotic therapy remains the prophylactic and curative practice most commonly used to fight against infections. However, antibiotic resistance acquired by numerous microorganisms is a major public health issue associated with additional costs for healthcare organizations. Indeed, excessive use of antibiotics causes accumulation of multi-resistance phenotypes in many bacterial strains. Infections caused by these resistant microorganisms often no longer meet the conventional treatments, lengthen the duration of illness related to infection and may even lead to patient death. However, for the past 25 years, no new classes of antibiotics have been discovered (Silver L, 2011). Moreover, the widespread use of antibiotics in modern medicine promotes the development and spread of antibiotic-resistant bacteria and thus the occurrence of nosocomial infections (Bereket W et al., 2012).

The development of new alternatives to conventional antibiotics is urgent to prevent the emergence of resistance phenomena. As such, the WHO even mentions a possible "post antibiotic era" where certain infections (such as a simple angina) could become fatal (http://www.who.int/fr).

Host defense peptides (HDPs)/ Antimicrobial peptides (AMPs) constitute a major component of innate immunity in most multicellular organisms and more specifically the first line of defense against infections. They emerged as excellent candidates in the development of new antibiotics because, they offer many advantages over conventional antibiotics. Indeed, AMPs are able to rapidly kill a broad spectrum of microorganisms, significantly reducing the problems of resistance. Also, they are not toxic towards host cells (Hancock R. E. and Lehrer R, 1998; Hancock R. E. and Sahl H. G, 2006; Marr A. K et al., 2006). Moreover, some AMPs have a great therapeutic potential because they are able to activate the immune system (Nijnik A et al., 2009; Haney E. F. and Hancock R. E, 2013; Hilchie A. L et al., 2013). AMPs are generally amphipathic with a net positive charge. They have a broad spectrum of activity against many pathogens (bacteria, viruses, fungi, parasites). In mammals, the AMPs play a key role as they are able to directly kill pathogens but also quickly trigger a modulation of the immune response (Hancock H. G. and Sahl H. G, 2006; Haney E. F. and Hancock R. E, 2013).

Among all isolated and characterized AMPs, natural peptides derived from chromogranins (Cgs) are of particular therapeutic interest. The chromogranin family predominantly consists of three members: chromogranin A (CgA), chromogranin B (CgB) and secretogranin II (CgC) (Helle K, 2004). These acidic proteins are stored in the secretory vesicles

of numerous cells from the neuroendocrine and immune systems. Cgs will undergo endogenous proteolysis degradation and release AMPs by exocytosis into the circulation where they can be detected in many body fluids where they can play their antimicrobial and immunomodulating roles (Metz-Boutigue M.H et al., 1993; Zhang D et al., 2009; Shooshtarizadeh P et al., 2010; Vandry H. and Metz-Boutigue M. H, 2010). Indeed, they are linear and very small (less than 20 residues), and therefore easier to synthesize for a minimal cost. In addition, these peptides are stable at high temperature, acidic pH or in the presence of serum.

Cgs-derived peptides are highly conserved among species, have antimicrobial activity against a broad spectrum of pathogens and are not toxic towards host cells. Some of them act as immonomodulators by activating neutrophils (Zhang D et al., 2009). Thus, regarding their exceptional biological and physicochemical properties, AMPs derived from Cgs are excellent candidates for the development of new antibiotic molecules.

Regarding to peptides derived from chromogranins (Cgs), Cateslytin (Ctl) represents a new interesting antimicrobial molecule (Briolat J et al., 2005; Postma T.M. and R.M.J. Liskamp, 2016). In fact, Ctl which is corresponding to bovine L-cateslytin (L-Ctl) and D-cateslytin (D-Ctl) CgA344-358 (RSMRLSFRARGYGFR) and its molecular weight 1860 Da. The Ctl is a fragment of Catestatin (CAT) corresponding to CgA344-364 (RSMRLSFRARGYGFRGPGLQL) with 2426 Da of molecular weight. In addition to its antibacterial properties, Ctl is also a potent antifungal agent. Moreover, many functions of bioactive products due to peptide cleavage such as immune systems, cardiovascular and endocrine (Postma T.M. and R.M.J. Liskamp, 2016). What cause the cell death is that the negatively charged aggregated to disrupt the membrane of cell, that because of the Ctl antiparallel β-sheets did not form pores (Postma T.M. and R.M.J. Liskamp, 2016).

The aim of my thesis is to characterize the epipeptide D-Ctl, where all L-residues replaced by D-residues with keeping the same sequences of L-Ctl. Thus, the efficiency of antimicrobial properties of L-Ctl and its stability were improved.

Different technics were performed such as antimicrobial assays, cells viability assays, cytokine release evaluation, reverse phase high-performance liquid chromatography (HPLC), mass spectrometry, epifluorescence optical microscopy, attenuated total reflection: fourier transform infrared (ATR-FTIR) spectroscopy and atomic force microscope (AFM) measurements.

Antimicrobial assays were performed to compare D-Ctl with L-Ctl against wide range of bacterial strains such as *Staphylococcus aureus* (MSSA), *S. aureus* methicillin resistance

(MRSA), *Parvimonas micra*, *Prevotella intermedia, Fusobacterium nucleatum*, *Escherischia coli* (wild type) and *E. coli* K-12 mutant E2146. The Minimal Inhibitory Concentration (MIC) was determined by using a mathematical model. As results of these experimental, D-Ctl MICs were from 2 to 15 times lower than L-Ctl with 8 to 24µg/mL in range. Then, D-Ctl was also compared with numerous conventional antibiotics. However, the MICs obtained for D-Ctl were still higher than the once of the conventional antibiotics tested, except Ampicillin and Kanamycin where the efficiency were similar than D-Ctl. Thus, D-Ctl could be a substitute to Ampicillin or Kanamycin in treatment of *E. coli* related infection. In order to decrease the dose of antibiotic prescribed for the patient, combination assays were performed to high light the synergistic or/and additive affects.

Besides, cells viability and immune assays were performed using MTT [3(4,5-dimethylthiazol-2-yl)-2,5 diphenyl tetrazolium bromide] and colorimetric assays on several cell lines and primary cells treated with D-Ctl and L-Ctl. D-Ctl shows no cytotoxicity to some types of cell lines and is unable to induce the release of inflammatory cytokines. For more interest, resistance assay was performed to compare the use of Ampicillin or Cefotaxim and D-Ctl to treat *E. coli* infections, where *E. coli* was cultured over 24 days with subMIC concentration of D-Ctl or Ampicillin or Cefotaxim. Thus, D-Ctl does not trigger resistance in *E. coli* and it was stable over 24 days. Unlike Ampicillin or Cefotaxim were multiply three times of MIC. More than that, the stability of D-Ctl was tested on the bacterial supernatant by using the HPLC, and the results demonstrated that the D-Ctl was stable for all the bacterial supernatant tested.

In addition to that, with the collaboration of UMR 7564, Nancy, France. The bacterial model *E. coli* MDR used for the physicochemical analysis such as epifluorescence microscopy, ATR-FTIR spectroscopy and atomic force microscopy, herein, D-Ctl and its conformer L-Ctl were compared to characterized the biological and mechanical properties. The results showed that the bacterial membrane was damaged by D-Ctl while was not damaged by L-Ctl. Finally, D-Ctl can be considered as a potent candidate for an alternative to conventional antibiotics, safe and stable as well as D-Ctl is not suffer of any microbial resistance.

In parallel of these data, new data obtained in our team (Pauline Dartevelle thesis in preparation) demonstrate that the D-Ctl has the efficiency also on the *Candida albicans.* And that it was not degraded by saliva and it is not toxic toward human gingival fibroblasts.

Finally, D-Ctl may be used to the development of new antimicrobial material and a patent has been deposited EP16306539.4 “New D- configured cateslytin peptide”.

Résumé de thèse en français

La découverte des antibiotiques dans le but de traiter les maladies infectieuses est une des plus grandes réussites de la médecine moderne. L'antibiothérapie est une pratique curative et prophylactique utilisée pour combattre les infections. Cependant, une utilisation excessive des antibiotiques cause une augmentation des phénotypes multi-résistants d'un grand nombre de microorganismes. Les infections causées par ces pathogènes résistants peuvent allonger la durée de la maladie et parfois même causer la mort du patient. Il s'agit d'un problème de santé majeur, avec d'importantes conséquences au niveau social et financier.

Durant les dernières 25 années, aucune nouvelle classe d'antibiotiques n'a été découverte (Silver L, 2011). De plus l'utilisation des antibiotiques à large spectre dans la médecine moderne, induit le développement de bactéries résistantes et ainsi l'apparition d'infections nosocomiales (Bereket W et al., 2012).

Dans ce contexte, le développement de nouvelles alternatives aux antibiotiques conventionnels est urgent afin de combattre ce système de résistance. La « World Health Organization » évoque même une possible « époque post-antibiotique », dans laquelle certaines infections (même une simple angine) pourrait devenir fatale (http://www.who.int/fr).

Les peptides de la défense de l'hôte (PDHs) ou peptides antimicrobiens (PAMs) sont une pièce majeure de l'immunité innée dans la plupart des organismes multicellulaires et plus spécifiquement font partie de la première ligne de défense contre les infections. Ils pourraient être d'excellents candidats pour le développement de nouveaux antibiotiques par leurs nombreux avantages comparés aux antibiotiques conventionnels. En effet, les PAMs sont capables de tuer rapidement un spectre large de microorganismes. De plus, les bactéries trouvent difficilement la parade à leur effet antimicrobien et ces peptides ne sont pas toxiques pour les cellules de l'hôte (Hancock R. E. et Lehrer R. 1998 ; Hancock R. E. et Sahl H. G. 2006 ; Marr A. K. et al., 2006). En complément de leurs effets antimicrobiens directs, certain PAMs activent les cellules du système immunitaire (Nijnik A. et al. 2009 ; Haney E. F. et Hancock R. E. 2013 ; Hilchie A. L. et al. 2013). Les PAMs sont amphipathiques et chargés positivement. Ils ont un spectre large d'activité contre de nombreux pathogènes (bactéries, virus, champignons, parasites). Chez les mammifères, les PAMs joue un rôle important car ils sont capables de tuer rapidement les pathogènes, mais également de provoquer rapidement une

modulation de la réponse immunitaire (Hancock R. E. et Sahl H. G. 2006 ; Haney E. F. et Hancock R. E. 2013).

Parmi les PAMs isolés et caractérisés, les peptides antimicrobiens dérivés de la chromogranine A (CGA) ont un intérêt thérapeutique particulier. La famille des chromogranines (CGs) correspond à des glyco-phospho protéines acides, qui sont stockées dans les granules de sécrétion de nombreuses cellules intra-granulaires du système nerveux endocrinien et immunitaire. Les CGs vont subir une dégradation protéolytique et libérer des PAMs par exocytose dans la circulation où ils peuvent jouer un rôle antimicrobien (Metz-Boutigue M.H. et al., 1993 ; Zhang D. et al., 2009 ; Shooshtarizadeh P. et al., 2010 ; Vaudry H. et Metz-Boutigue M.H, 2010). Ce sont des peptides courts et linéaires qui peuvent être synthétisés pour un moindre coût. De plus, ces peptides sont stables à haute température, pH acide et en présence de sérum.

Les PAMs dérivés des CGs sont très bien conservés tout au long de l'évolution. Certains d'entre eux agissent comme immuno-modulateurs par activation des neutrophiles (Zhang D. et al., 2009). Ainsi, de par leurs propriétés biologiques et physicochimiques exceptionnelles, ils sont d'excellents candidats pour le développent de nouveaux antibiotiques, capables de s'opposer au développement de microorganismes résistants.

Après analyse des PAMs dérivés des CGs, la cateslytine (Ctl) représente la molécule antimicrobienne la plus intéressante (Briolat J. et al., 2005 ; Postma T. M. et Liskamp R. M. J, 2016).

En fait, les Cateslytines utilisées sont, la L-Cateslytine (L-Ctl) et la D-Cateslytine (D-Ctl) d'origine bovine. La Ctl correspond à CgA344-358 (RSMRLSFRARGYGFR) avec un poids moléculaire de 1860 KDa. Elle est un fragment de la Catestatine (CAT), qui correspond à CgA344-364 (RSMRLSFRARGYGFRGPGLQL) dont le poids moléculaire est 2426 KDa.

En plus de ses propriétés antibactériennes, la Ctl est aussi un agent antifongique. Ce qui cause La mort cellulaire des microorganismes est due à l'agrégation du peptide à la surface de la membrane de la cellule (Postma T. M. et Liskamp R. M. J, 2016).

Dans ce contexte le but de ma thèse est de caractériser l'épipeptide D-Ctl où tous les acides aminés en conformation L sont remplacés par des acides aminés en conformation D. Plusieurs techniques ont été utilisées faisant appel à la microbiologie, la biochimie et la biophysique. Il s'agit de tests antimicrobiens, d'analyse de la viabilité cellulaire, de tests de biologie cellulaire sur les cellules immunitaires, ainsi que des tests concernant la stabilité du peptide dans le surnageant de microorganismes et des études du mécanisme d'action.

J'ai personnellement pratiqué (1) les tests antimicrobiens sur différentes souches pour déterminer les concentrations minimales inhibitrices (CMIs) des peptides étudiés, (2) l'évaluation de la libération de cytokines par les cellules immunitaires et (3) la phase inverse en HPLC. Les techniques de spectrométrie de masse, d'épifluorescence en microscopie optique, de spectrométrie par infra- rouge (ATR-FTIR), ainsi que des mesures en microscopie à force atomique (AFM) ont été réalisées en collaboration avec des laboratoires spécialisés (Laboratoire de Spectrométrie de Masse Bio-Organique Département des Sciences Analytiques, Institut Pluridisciplinaire Hubert Curien, UMR 7178 à Strasbourg et le LCPME - CNRS UMR7564 à Nancy).

Des analyses antimicrobiennes ont été effectuées pour comparer les activités de la D-Ctl et de la L-Ctl sur une large gamme de souches bactériennes telles que *Staphylococcus aureus* (MSSA), *S. aureus* methicillin resistance (MRSA), *Parviromonas micra*, *Prevotella intermedia*, *Fusobacterium nucleatum*, *Escherichia coli* (type sauvage) et *E. coli* K-12 mutant E2146. Un modèle mathématique permettant de calculer la CMI, a été utilisé. L'activité de la D-Ctl a une CMI entre 2 et 15 fois plus faible que la L-Ctl (de 8 à 24µg/mL). La D-Ctl a aussi été comparée à un certain nombre d'antibiotiques. Cependant, la concentration minimale inhibitrice obtenue pour la D-Ctl est plus élevée que celle des antibiotiques conventionnels testés, à l'exception de l'Ampicilline et de la Kanamycine, pour lesquelles leur effet est similaire. La D-Ctl est aussi utilisée en combinaison avec des antibiotiques, dans le but d'analyser un possible effet synergique et/ou additif, qui permettrait de diminuer la dose d'antibiotiques prescrite aux patients.

En outre, la viabilité des cellules traitées avec la D-Ctl et la L-Ctl a été évaluée en utilisant la technique colorimétrique MTT sur plusieurs lignées cellulaires et des cellules primaires. La D-Ctl ne montre aucune toxicité sur plusieurs lignées de cellules et n'est pas capable d'induire de réactions inflammatoires par la libération de cytokines. Par la suite, des tests de résistance des microorganismes au traitement par les agents antimicrobiens ont été réalisés pour comparer l'utilisation d'Ampicilline, de Cefotaxime et de D-Ctl pour traiter les infections à *E. coli*. D-Ctl n'a pas déclenché de résistance microbienne à 3 MIC contrairement à l'antibiotique et était stable sur 24 jours. De plus, en utilisant l'HPLC il a été montré que D-Ctl est stable dans tous les surnageants bactériens testés.

Les études biophysiques du mécanisme d'action (microscopie par épifluorescence, spectrométrie par infra-rouge (ATR-FTIR) et microscopie à force atomique (AFM) ont été développées sur le modèle bactérien de *E. coli*. Les effets de D-Ctl et L-Ctl ont été comparées

pour caractériser les propriétés biologiques et mécaniques. Les résultats montrent que les membranes bactériennes ont été endommagées par D-Ctl alors que L-Ctl ne les a pas endommagées.

Finalement, D-Ctl peut être considéré comme un candidat innovant pour une alternative aux antibiotiques conventionnels, car il est efficace, stable et n'induit pas de résistance microbienne.

En parallèle, de nouveaux résultats ont été obtenus dans le cadre de la Thèse de Pauline Dartevelle, qui est en cours de préparation. Il a été démontré que D-Ctl était efficace aussi sur *Candida albicans*, qu'il n'a pas été dégradé dans la salive et qu'il n'est pas toxique pour les fibroblastes gingivaux humains.

Pour conclure, la D-Ctl pourrait être utilisée pour l'élaboration de matériaux antimicrobiens et un brevet a été déposé en 2016 au niveau européen EP16306539.4 « Nouveau peptide Catestlytine en conformation D ».

PART-I
INTRODUCTION

Conventional antibiotics and bacterial resistance

1.1-Conventional antibiotics and bacterial resistance

The patients' protection of burden and suffering from infectious diseases can be treated by antibiotics, which saved the human race with no doubt. Without antibiotics drugs, a lot of people will capitulate to infectious diseases. But, sadly after a piece of time, antibiotics were introduced clinically and prescribed to treat diseases, it was noted that antibiotics have become to lose their effectiveness due to the growing number of antibiotic-resistant pathogens. Effective antibiotics represent big challenges in the future of modern medicine. The urgent need for antibiotics is essential and vital to reduce death rates that associated with infectious diseases, especially childhood mortality.

In spite of the successes investigation and production of variety of antibiotics, the search and discover for novel classes of antibiotics, is one of the greatest achievements and it has become an imperative in the modern medicine to solve the challenges related to resistance issue.

The world health organization (WHO) expressed concerns because of the increasing of antibiotics resistant and we may reach to the point that it no longer has the efficiency of antibiotics treatments and that could be the post-antibiotic era (World Health Organization, 2016). Therefore, the national organizations push for necessary implementation of applications to address antimicrobial resistant (UK Five Year Antimicrobial Resistance Strategy 2013 to 2018; White House, 2015).

1.1.1-General overview of antibiotic/antimicrobial

Antibiosis is the original word of the antibiotic term, the prefix “anti” means killing or fighting. As for “bios” it means life, which is originally a Greek word. Therefore, the term literally means life killing. Previously, the antibiotics were produced by one microorganism, and are considered as organic compounds (Russell A, 2004; Denyer S et al., 2004) or of biological origin (Schlegel H, 2003) that able to inhibit the growth or kill the other microorganisms at low concentration (Russell A, 2004). However, in the modern era, the term or the definition of antibiotic has been modified to antimicrobial, which includes anti-bacterial, anti-fungal, anti-parasitic and anti-viral drugs.

To struggle infections and the illness, antimicrobials are one of the medication classes, which are used to fight the microorganisms (Campbell S, 2007). Because of the term of antibiotics is technically used, thus not only antibiotics termed as antimicrobial but also synthetically formed compound. Therefore, the expression antimicrobial and antibiotic are used

interchangeably (Scott G, 2009).

Most of the antibiotics are prescribed by a general physicians (GPs) (Rokstad K and Straand J, 1997) and more than 85% of these antibiotics were prescribed to treat the several infectious diseases outside of hospital (Molstad S et al., 1994).

1.1.2-Historical overview

Historically, first natural antibiotic is Penicillin, which was discovered by Alexander Fleming, in 1928 (Fleming A, 1929). After a few years in 1932, Prontosil was discovered by Klarer and Mietzsch and it became available for the patients during the world war II. Then, many classes of antibiotics had been discovered in the mid of 20 century. Afterward, in the 1940s, Alexander Fleming, along with Howard Florey and Ernst Chain, brought penicillin into the therapeutic use. As a result, the three scientists won the Nobel Prize for medicine in 1945 (Brown K, 2004). As being called the "Golden Age of antibiotics" began to blossom with the appearance of penicillin and streptomycin in 1943, it led to a swift loss of interest in the therapeutic potential of natural host antibiotics such as lysozyme (Fleming A, 1922). and the significance of this immune defense strategy (Bentley R, 2009; Zaffiri L et al., 2012) [Figure 1].

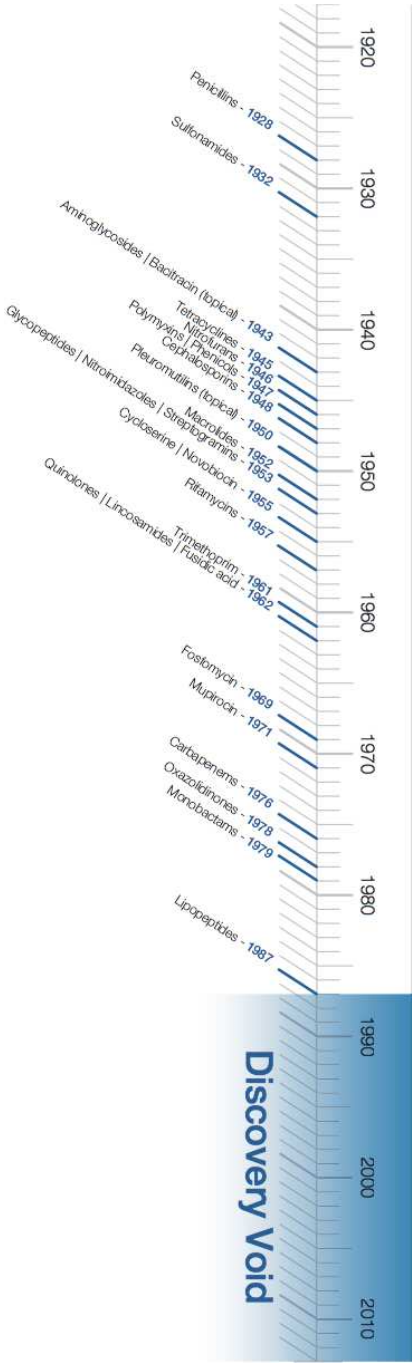

Figure 1: **Antibiotics discovery dates, during the period 1920-2010.**
It is important to point out the absence of new antibiotics for the period 1990-2010. Timeline taken from the (World Economic Forum, Global Risk Report 2013; Silver, L.L. 2011. Challenges of bacterial discovery. In Clinical Microbiology Reviews, 24: 71-109).

1.2- Classification of antibiotics

The antibiotics can be classified according to their molecular structure, mechanism of action and spectrum of action (Calderon C and Sabundayo B, 2007). Some common classes of antibiotics based on chemical or molecular structures include Beta-lactams (Penicillins and Cephalosporins), Aminoglycosides, Quinolones (Fluoroquinolones), Tetracyclines, Macrolides, Sulfonamides, and Glycopeptides (van Hoek A et al., 2011; Frank U and Tacconelli E, 2012; Adzitey F, 2015) [Table I].

Table I: Classification of Antibiotics

Class of Antibiotics	**Example of generation**	**Microorganisms**
Penicillins	Methicillin, Ampicillin, Amoxicillin, Oxacillin, Nafcillin, Carbenicillin, Piperacillin, Ticarcillin, Mezlocillin	Gram-negative bacteria (*Treponema pallidum and Meningococci*) Gram-positive bacteria (*Streptococci*) (Calderon C and Sabundayo B, 2007; Adzitey F, 2015; Jacob J, 2015; Boundless, 2016)
Cephalosporins	1st Cefalexin 2nd Cefoxitin 3rd Cefotaxime 4th Cepirome 5th Ceftaroline	Gram-positive bacteria (*Staphylococci, Streptococci, Neisseria klebsiella pneumoniae, haemophilus influenza, Proteus mirabilis, and Enterobacter aerogenes*) (Calderon C and Sabundayo B, 2007; Adzitey F, 2015; Jacob J, 2015)
Macrolides	Clarithromycin, Erythromycin, Azithromycin	Mostly Gram-positive bacteria (*Staphylococcus Pneumoniae*) Gram-negative bacteria (Hamilton-Miller J, 1973; Calderon C and Sabundayo B, 2007; Adzitey F, 2015; Jacob J, 2015)
Aminoglycosides	Strptomycin, Spectinomycin, Kanamycin, Neomycin, Gentamicin	Mostly Gram-negative bacteria Some of Gram-positive bacteria *Mycobacterium Tuberculosis* (Calderon C and Sabundayo B, 2007; Adzitey F, 2015; Jacob J, 2015)
Quinolones	Nalidixic, Ciprofloxacin, Levofloxacin, Sitafloxacin	Some anaerobic gram-negative bacteria Aerobic gram-positive bacteria (*M.tuberculosis*) (Domagala J,1994)

Tetracyclines	1st Oxytetracycline, Demeclocycline, Chlortetracycline, 2nd Methacycline, Minocycline, Meclocycline, Doxycycline, Rolitetracycline, Lymecycline, 3rd Tigecycline	Gram-negative bacteria Gram-positive bacteria (Walsh C, 2003; Fuoco D, 2012)
Sulfonamides	Sulfamethizole, Trimethoprim	Gram-negative bacteria Gram-positive bacteria (*Staphylococcus, Streptococcus, and Salmonella*) (Calderon C and Sabundayo B, 2007; Stawinski J et al., 2013; Xu F et al.,2014; Adzitey F, 2015; Jacob J, 2015)
Glycopeptides	Vancomycin, Teicoplanin	Gram-positive bacteria (Calderon C and Sabundayo B, 2007; Adzitey F, 2015; and Jacob J, 2015)

(Adzitey F, 2015; Ebimieow E and Ibemologi A, 2016; Boundless, 2016)

1.2.1- Penicillins

As mentioned above, the penicillin was the first antibiotic discovered by Alexander Fleming in the year of 1928. However, until 1938 the penicillin was not used clinically (Lewis K, 2013), and later on the penicillin found to be one of many other antibiotic compounds which called penicillins (McGeer A et al., 2001). Members of penicillins include Penicillin G and V, Methicillin, Ampicillin, Amoxicillin, Oxacillin, Nafcillin, Carbenicillin, Piperacillin, Ticarcillin, and Mezlocillin (Calderon C and Sabundayo B, 2007; Adzitey F, 2015; Jacob J, 2015; Boundless, 2016). Penicillins are beta-lactam classes and their structures include nucleus of 6- animopenicillanic acid (lactam plus thiazolidine) and other side chains (Zahner H and Maas W, 1972). These side chains allow the antibiotic to escape from specific enzymes produced by special bacterial strains and that could cause degradative ability. The natural penicillin, which is penicillin G has a narrow spectrum of activity that can work just between 30-60 minutes. It can be effective against gram-negative bacteria (*Treponema pallidum*), *meningococci* as well as gram-positive bacteria (*streptococci*) (Talaro K and Chess B, 2008). Whereas, new classes of penicillins have a broad activity, which fights many gram-negative bacteria such as *E. coli* and H. influenza as well as infections of the genitourinary tract, lower

respiratory tract, throat, and nose (Miller E, 2002).

1.2.2- Cephalosporins

In 1945, Guiseppe Brotzu discovered the first generation of cephalosporins, which had been isolated from fungus. Cephalosporins are subdivided into 5 generations with abroad spectrum of activity. They include cefalexin (1^{st} generation), cefoxitin (2^{nd} generation), cefotaxime (3^{rd} generation), cefpirome (4^{th} generation) and ceftaroline (5^{th} generation) (Calderon C and Sabundayo B, 2007; Adzitey F, 2015; Jacob J, 2015). Cephalosporins are containing a nucleus of 7-aminocephalosporanic acid as well as other ring side chain 3,6-dihydro-2 H-1,3- thiazine. This class of antibiotics is administered in the treatment of infections acquired and also to treat diseases due to penicillinase-producing, which include *Streptococci* and *Staphylococci*, some *E. coli, Neisseria, klebsiella pneumoniae*, *Haemophilus influenza*, *Enterobacter aerogenes,* and *Proteus mirabilis* (Pegler S and Healy B, 2007).

1.2.3- Macrolides

Erythromycin the first class of macrolides was discovered by J.M. McGuire in 1949 and by the year of 1951 was introduced clinically (Lewis K, 2013). Erythromycin was isolated from the metabolic products of *Streptomyces erythraeus* fungus (Moore D, 2015). The members of macrolides include Clarithromycin, Erythromycin, and Azithromycin (Hamilton-Miller J, 1973; Calderon C and Sabundayo B, 2007; Adzitey F, 2015; Jacob J, 2015).

Macrolides are effective against a wide spectrum of bacteria including gram-positive bacteria (*Staphylococcus*), as well as some gram-negative bacteria strains. In addition, they possess activity more than penicillins and are normally prescribed for allergic patients to penicillin (Moore D, 2015).

1.2.4- Aminoglycosides

Streptomycin was first aminoglycosides class discovered in 1943 and introduced clinically by the year of 1946 (Lewis K, 2013; Mahajan G and Balachandran L, 2012). Examples of aminoglycosides classes include Streptomycin, Spectinomycin, Kanamycin, Neomycin, and Gentamicin (Calderon C and Sabundayo B, 2007; Adzitey F, 2015; Jacob J,

2015). Aminoglycosides are positively charged and commonly they are composed of 3 amino sugars linked by glycosidic bonds. Furthermore, they have wide spectrum of action against most gram-negative strains and some gram-positive strains of bacteria.

1.2.5- Quinolones

In the early of 1960s, the quinolones were discovered as nalidixic acid and they were derived from quinine, which is the essential chemical structure of fluoroquinolones. Fluoroquinolones are subdivided into four generations: nalidixic acid, ciprofloxacin, levofloxacin, and sitafloxacin (Domagala J, 1994). Two rings that represent general structure of quinolones. However, to improve and increase their efficiency, some modifications have been made to the basic structure. Thus, the new generations possess an additional ring to their structure. These classes of antibiotics display bactericidal effect and they are fighting against some anaerobic gram-negative, aerobic gram-positive as well as *M. tuberculosis*. They are also capable of killing double mutants in related strains. However, they have limited uses for treat the infections of urinary tract (Andersson M and MacGowan A, 2003).

1.2.6- Tetracyclines

Tetracycline was discovered by Benjamin Duggar in 1945 and it was derived from Streptomyces (Sanchez A et al., 2004). The chlortetracycline (Aureomycin) was the first member of this class and clinically introduced in 1952. Tetracyclines include three generations: (1) early members Oxytetracycline, Demeclocycline, and Chlortetracycline; (2) other semi-synthesis members include Methacycline, Minocycline, Meclocycline, Doxycycline, Rolitetracycline, and Lymecycline (Walsh C, 2003) and (3) Tigecycline (Fuoco D, 2012). They are lipophilic nonionized molecules containing four hydrocarbon rings.

Tetracyclines are bacteriostatic and they have a wide range of action against gram-negative and aerobic gram-positive bacteria. Thus, the infections such as rickettsia, malaria, amoebic parasites, and elephantiasis can be treated by them. Taking into account, this class of antibiotics prescribed only for the patients who are more than 8 years old due to teeth discoloration side effect (Sanchez A et al., 2004).

1.2.7- Sulfonamides

In 1932 the prontosil was discovered and considered as the first sulfonamide (Lewis K, 2013). However, sulfonamides were reported and introduced clinically by the year of 1936. This class of antibiotics include Sulfamethizole and Trimethoprim (Calderon C and Sabundayo B, 2007; Adzitey F, 2015; Jacob J, 2015). Sulfonamide and Para-aminobenzoic acid have the similar structures. Therefore, sulfonamides have a wide range of activity to fight against bacteria.

Sulfonamides are commonly referred as bacteriostatic rather than bactericidal, they also can treat *E. coli, Streptococcus, Staphylococcus*, and *Salmonella*. Moreover, numerous studies reported that cancerous cell agents can be impeded by sulfonamides (Stawinski J et al., 2013; Xu F et al., 2014).

1.2.8- Glycopeptides

In 1952 the first identified glycopeptide was Vancomycin and nevertheless, clinically it was reported in 1958 (Lewis K, 2013). Two other members of glycopeptides are Vancomycin and Teicoplanin (Calderon C and Sabundayo B, 2007; Adzitey F, 2015; Jacob J, 2015). Usually, 7 amino acids linked to two sugars, which form the main structure of glycopeptides (Reynolds P, 1989; Kang H-K and Park Y, 2015). In 2014 Yim and his associates had been explained and described the different structural forms of glycopeptides.

1.3- Mechanisms of action of antibiotics

Antibiotics were found and prescribed to fight against infections; antibiotics can save lives by cut out the physiological mechanisms of bacterial cells. Therefore, all the antibiotics work in one of two ways of mechanisms; bacteriostatic agents, which inhibit bacteria from reproducing but doesn't otherwise kill them. Whereas, bactericidal agents actively kill bacterial cells (Kohanski M et al., 2010). There are three mechanisms of action used by antibiotics agent: inhibition of cell wall synthesis, inhibition of DNA and protein synthesis (Madigan M and Martinko J, 2006; Talaro K and Chess B, 2008; Wright G, 2010; Hills T, 2010) [Figure 2].

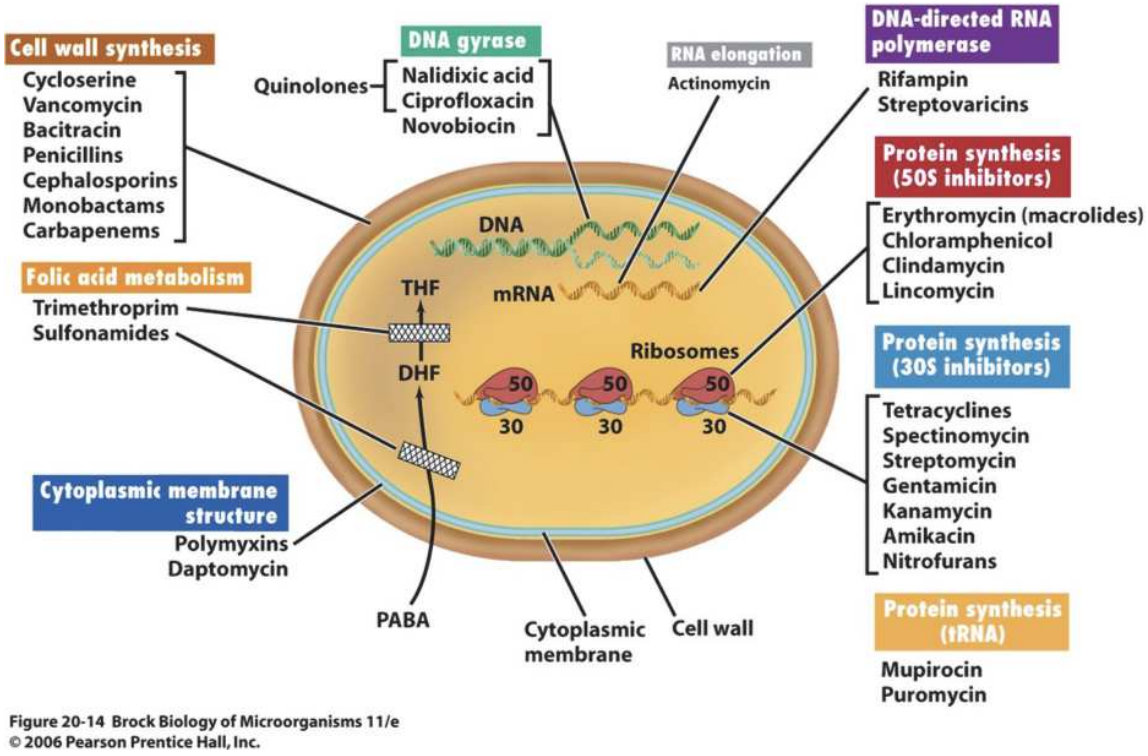

Figure 2: **Mechanisms of action of antibiotics classes** (Labnotesweek 4, 2013).
Cell wall synthesis inhibitors include Glycopeptide`s and β-lactams (Penicillin's, Cephalosporin's, Monobactam`s and Carbapenem`s). Protein synthesis inhibitors that interact with ribosomal subunits the 50S and 30S include Tetracycline's, Aminoglycosides, Macrolides, Clindamycin, and Chloramphenicol. DNA gyrase and topoisomerase IV inhibitors include Quinolones and Novobiocin. Also, Trimethoprim and Sulfonamides can inhibit the folic acid synthesis.

1.3.1- Inhibition of cell wall synthesis

The bacterial cells are surrounded by a rigid structure of peptidoglycan layers, unlike the human cells, which do not own these layers. This could be an advantage. It is due to that the antibiotics only will target the bacterial cell wall (Hills T, 2010). The basic structure of the peptidoglycan layers is p-(1-4) -N- acetyl Hexosamine (Bugg T and Walsh C, 1992; Holtje J, 1998).

Some classes of antibiotics such as (beta-lactam) like penicillins and cephalosporins (Kotra L and Mobashery S, 1998) are able to inhibit the synthesis of peptidoglycan layer of bacterial cell wall (Marshall W and Blair J, 1999; Butler M and Buss A, 2006). This process can be done by binding the peptidoglycan units to penicillin-binding proteins (PBPs) enzymes (Josephine H et al., 2004; Kohanski M et al., 2010) [Figure 3]. Therefore, the bacterial were killed as a result of weakened and damage of cell wall synthesis, which is called osmotic lysis.

As well as Vancomycin, which is considered as glycopeptide class, it interacts with cell wall and block transglycosylase and transpeptides activity (Nagarajan R, 1991; Kahne D et al., 2005).

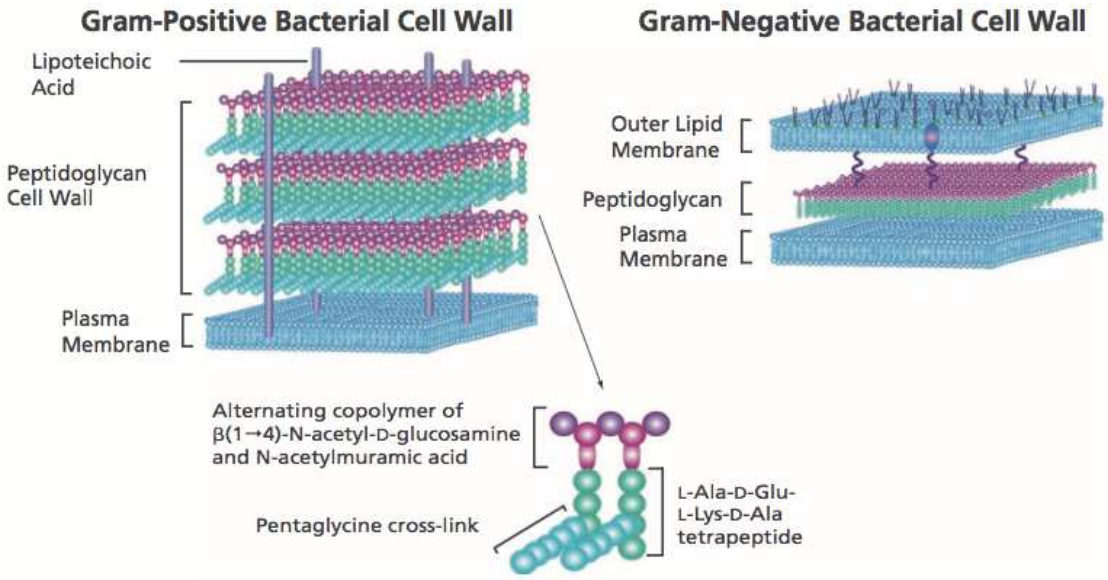

Figure 3: Inhibition of Gram-positive and Gram-negative bacterial cell wall biosynthesis. (BioFiles 4, SIGMA-ALDRICH 2006)

1.3.2- Inhibition of DNA synthesis

Fluoroquinolones induce the bacterial death act by inhibiting the activity both DNA gyrase and topoisomerase IV enzymes (Chatterji et al., 2001) by blocking the DNA replication and repairing (Bearden D and Danziger L, 2001; Hooper, 2001; Walsh C, 2003). In the most gram-negative bacteria, the DNA gyrase (topoisomerase II) is the primary fluoroquinolones target e.g. *Neisseria gonorrhoeae* and *E. coli* (Drlica K and Snyder M, 1978; Kohanski M et al., 2010). Moreover, topoisomerase IV is targeted by fluoroquinolones in the gram-positive bacterial strains such as *Staphylococcus*. As a result, blocking the DNA to recombine at the stage of DNA cleavage disrupts the bacteria replication (Chen C et al.,1996). Thus, the bacteria cell will die due to damage of DNA (Karch A, 2008). In addition, specific antibiotics such as Trimethoprim and Sulfamethoxazole can also inhibit folate synthesis, RNA and DNA synthesis.

1.3.3- Inhibition of protein synthesis

The inhibition of protein synthesis occurs during the phases of protein synthesis (elongation, initiation and termination) at the ribosome particularly on the 50S and 30S subunits. Based on the site of the target the inhibition of protein synthesis can be varied. Some classes of antibiotics work actively on bacterial ribosomes by inhibiting the 50S subunit: Clindamycin, Linezolid and highly effective class such as Chloramphenicol (Douthwaite S, 1992; Katz L and Ashley G, 2005), whereas, tetracyclines, aminoglycosides, and macrolides are the inhibitors for 30S subunit (Hooper D, 2001; McKee E et al., 2006; Hills T, 2010; Hong W et al., 2014). By binding 50S ribosome, the macrolides which are bacteriostatic can kill the microorganisms by inhibiting the protein synthesis. Also, they prevent the addition of amino acid to polypeptide chains during protein synthesis (Mazzei T et al., 1993; Tenson T et al., 2003).

Aminoglycosides were able to inhibit the protein synthesis and to act against bacteria by targeting and binding to 30S ribosomal subunits (Peterson L, 2008). Moreover, inhibition of the protein synthesis can be done due to the interaction between the positive charged aminoglycosides and the negative charged lipopolysaccharides (LPS) on the cell wall of bacteria (Jana S and Deb J, 2006). Furthermore, tetracyclines target the 30S subunit ribosome (Sloan B and Scheinfeld N, 2008) and also cause damage to polypeptide chains by preventing the addition of amino acids during protein synthesis (Medical News Today, 2015). Thus, the growth of bacteria can be interrupted *via* these classes and by blocking the access of aminoacyl-tRNAs to the ribosomes (Chopra I and Roberts M, 2001). The action between 16S rRNA and aminoglycosides cause mistranslation for proteins. Due to this interaction, there are changes happened in the complex between aminoacyl-tRNAs and mRNA (Pape T et al., 2000).

1.4- Problematic issue

The uses of antibiotics are very important to control and prevent developing infection risks during or after all types of surgical operations. However, as a result of excessive use of antibiotics for human therapy, most of pathogenic strains of bacteria became resistance and led to a major public health issue. It has been observed that the number of cases, which are infected by multidrug-resistant bacteria, is globally increased. Therefore, the infections have become a

reality threat to the world and human health which have listed in World Economic Forum Global Risks reported as one of the greatest threats (Walker D and Fowler T, 2011; World Economic Forum. Global Risks, 2013 and 2014).

Expanding commonness of resistance has been accounted for in numerous pathogens through out the years in various districts of the world (Byarugaba D, 2005). This has been credited to change microbial qualities, selective pressures of antimicrobial use, and societal and mechanical changes that improve the advancement and transmission of medication resistant organisms.

Since 1940s, researchers introduced the antibiotics into medicine, the uses of antibiotics have been expanded from treating infections to safeguard tumor patients and with patients who are suffering from immune deficiency diseases, as well as it has been used in agriculture for animal`s food (Walsh C, 2000). For this reason, the farm animal's species can be a concern for a prevalence of antibiotic resistant pathogens which has captured the attention of health organizations, governments, researchers as well as all stakeholders (Adzitey F, 2011). The period between the 1950s to the 1960s was the first period of discovering the antibiotics resistant phenomena among enteric bacteria such as *Shigella, E. coli*, and *Salmonella* (Watanabe T, 1963; Olarte J, 1983; Levy S, 2001).

Therefore, one of the most global public health problems especially in the developing countries is that resistant to antibiotics, which make the therapy unstable, costly and unsuccessful (www.who.int/drugresistance/en/) (Levy S, 2002).

In addition, some other diseases might be acquired by the patient who's under the hospital treatment termed "Nosocomial" (Khan H, 2015) which is kind of infection which must be taken into account as a major risk factor threatens patients' health and may cause to death (Brusaferro S et al., 2015). This phenomenon can be found in the developing countries that may reach 75% of the patients (Obiero C. et al., 2015). When such pathogens found at a sterile body site like cerebrospinal fluid or blood, or in the body fluids might be deemed an infection (Murray P et al., 2005). These infections considered as nosocomial when acquired by visitors and healthcare staff at the hospital (Lolekha S et al., 1981).

1.4.1- Mechanisms of bacterial resistance

Basically, how some bacteria resistant to antibiotics and how do acquire such resistant? There are two groups of bacteria, which are gram-positive, and gram-negative, they differ in cell wall composition. However, in the case of gram-positive has thick peptidoglycan layer forms about 50% of the cell wall material. Unlike, gram-negative, which has thin peptidoglycan layer, forms only 5-10% of the cell wall.

The penicillin will be very effective against gram-positive because the penicillin will target the protein membrane. However, the penicillin is not very effective against gram-negative bacteria because they possess an outer layer membrane on the way also the peptidoglycan layer quite thin.

The genetic material of the bacteria includes a circle DNA and plasmid are carrying the resistance genes (Bennett PM, 2008). The plasmid makes these resistance things usually it has to corporate itself to main DNA also the plasmid is synthesis the RNA in particular mRNA. Thereby, the mRNA has been synthesis for the plasmid; the mRNA will be read by the ribosome to make polypeptide to make proteins. An existence of bacteria alive can be translated by frustrating the effect of the antibiotics that is the genetic basis of resistance.

Several ways of mechanisms have evolved in bacteria to describe which can be genetic material or be gaining of new genetic material and these mechanisms can either chemically modify the antibiotics and these mechanisms as following: Target modifications, alteration of metabolic pathway, and reduced drug accumulation [Figure 4].

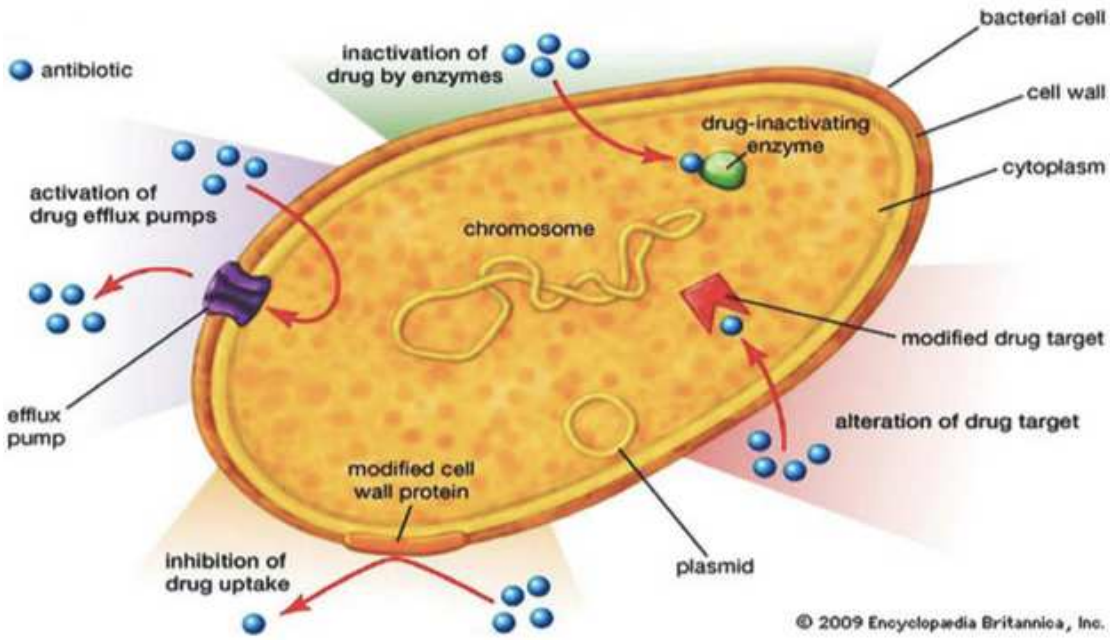

Figure 4: **Mechanisms of bacteria resistance.**
(Todar K, 2011; Wilcox S, 2013; Encyclopædia-Britannica, 2013)
Several mechanisms which lead to bacterial resistance toward conventional antibiotics are reported. These mechanisms include: alteration of drug target, inactivation of drug by enzymes, activation of drug efflux pumps, and inhibition of drug uptake.

1.4.1.1- Target modifications

Promote resistance to several classes of antibiotics as a result of changing in the targets of antibiotics that may conflict with or limit the activity of antibiotics drugs. Moreover, these changes in the target can prevent or decrease the effectiveness of bactericidal or bacteriostatic. For instance, the mutations of the target site such as ribosomal can lead resistant to streptomycin which is one of the aminoglycosides class (Wright G et al., 1998). Quinolones inhibited the activities of DNA gyrase and topoisomerase IV, which are very important for viability of bacteria. Therefore, mutations chromosomal like genes encoding the protein targets (gyrA and parC) are often involved in quinolone resistance (Hooper D, 2000; Fabrega A et al., 2009). On the other hand, the low-production of penicillin-binding protein (PBP2a) in *Staphylococcus aureus* (MRSA) can lead to methicillin resistance and to all beta-lactams classes in particularly for the same reason (Hartman B and Tomasz A, 1984; Chambers H, 1997; Hakenbeck R et al., 1999; Katayama Y et al., 2000; Fisher J et al., 2005). The bacteria can be resistant to macrolides also due to the modification of their target site on the ribosome, which is the most commonly

among the resistance mechanisms, that occur in domain V of the 23S rRNA in adenine residue (Leclercq R and Courvalin P, 1991a; Weisblum B, 1995; Schmitz F. et al., 2000). As a result of mutations in the RNA polymerase beta subunit (*rpoB*) gene cause resistant to rifampicin in *Mycobacterium tuberculosis* (Taniguchi H et al., 1996; Goldstein B, 2014) and *E. coli* (Jin D and Gross C, 1988).

1.4.1.2- Antibiotics modification and degrading enzymes

Degradation or alteration of the effectiveness of an antibiotic compound is very important for antibiotics to be resistant, thereby, the principal mechanism of resistance to beta-lactams remains beta-lactamases enzymes which are capable to break and hydrolyzing the beta-lactam ring of the molecule (Thomson K and Smith M, 2000; Livermore D, 2008; Nordmann P et al., 2011; Woodford N et al., 2011; Voulgari E et al., 2013). Beta-lactam rings are found in penicillin (Chain Epae, 1940). Also, the cephalosporins are affected by the same mechanism of resistance (Tortora G et al., 2010). Four beta-lactamases classes have been discovered and include: Class A penicillinases, Class B metallo-b-lactamases, Class C cephalosporinases and Class D oxacillinases (Thomson K and Smith M, 2000). Basically, three types of modifying enzymes that are responsible for making a chemical modification of aminoglycosides to be the resistance of bacteria, acetyltransferase (AAC), phosphotransferases (APH) or adenylyltransferases (ANT) (Wright G et al., 1998; Wright G, 1999; Ramirez M and Tolmasky M, 2010). Likewise, "chloramphenicol resistance is afforded by the enzyme of chloramphenicol acetyltransferase cat gene (Shaw W, 1966; Shaw W and Brodsky R, 1968), which is able to transfer an acetyl group from acetyl coenzyme A to chloramphenicol, that interrupting binding of chloramphenicol to the ribosomal subunit" (Murray I and Shaw W, 1997).

1.4.1.3- Efflux pumps

Certain bacteria can often become resistant to antibiotics through a mechanism known as efflux. Efflux pumps are found in the cell wall membrane, and it is essentially a channel that actively exports the antibiotics out of the cell. The antibiotic enters the bacterial cell through the channel term porin and the antibiotic pump out of the bacteria by efflux pump. Basically, all antibiotics classes are affected by efflux pumps. Particularly, tetracyclines, fluoroquinolones, and macrolides because the inhibition of protein and DNA biosynthesis can

be done by all these antibiotics thereby to have the effect unless they are inside the bacteria cell. The specificity of efflux pumps is varying as well as their mechanism (Nikaido H and Zgurskaya H, 1999; Webber M and Piddock L, 2003) [Figure 5].

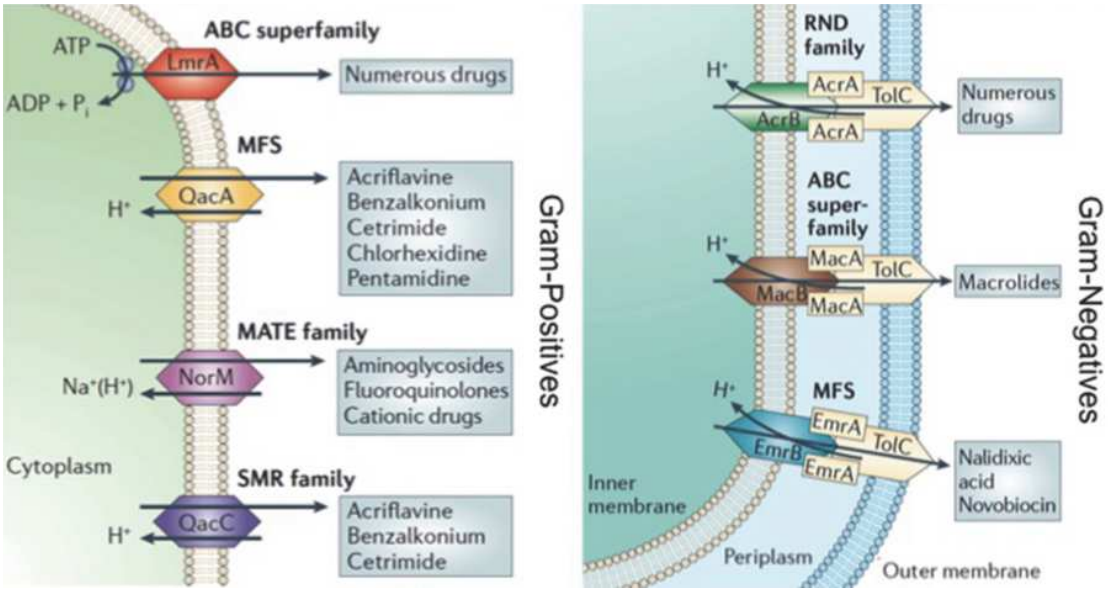

Figure 5: **Different types of efflux pumps in Gram-negative and Gram-positive bacteria.** The five major families have been illustrated of efflux pumps as follows: the major facilitator superfamily (MFS), ATP-binding cassette (ABC) superfamily, the small multidrug resistance (SMR) family, the multidrug and toxic-compound extrusion (MATE) family, and the resistance nodulation division (RND) family (Piddock L, 2006b; Modified by Munita JM & Arias CA, 2016).

In early of the 1980s was the first time for describing how the *E. coli* efflux system could pump the tetracycline out of the cytoplasm (McMurry L and Levy S, 1978; McMurry L et al., 1980; Nelson M and Levy S, 2011). As result of extruding a toxic compound out of the bacterial cell due to the production of complex bacterial machinery can lead antibiotic resistance (McMurry L et al., 1980). Numerous of efflux pumps classes in both pathogens whether gram-positive bacteria or gram-negative bacteria have been described.

Efflux pumps are currently classified into 5 major families (Pao S et al., 1998; van Veen H and Konings W, 1998), [Figure 5] including; (1) The major facilitator superfamily (MFS), (2) The resistance-nodulation-cell division family (RND), (3) The adenosine triphosphate ATP-binding cassette family (ABC), (4) The multidrug and toxic compound extrusion family (MATE), (5) The small multidrug resistance family (SMR). Whereas, these families vary in terms of energy exporter, structural conformation, where they can be found in which type of bacteria, and range of substrates they are able to fling (Piddock L, 2006a).

The efflux pumps of tetracycline are indicated as TetA and classifies into different classes including TetA(A), TetA(B), TetA(C), TetA(D), etc., occasionally, pointed out to simply as Tet(A), Tet(B), Tet(C) and Tet(D), respectively (Mendez B. et al., 1980; Curiale M and Levy S, 1982; Hickman R and Levy S, 1988; Levy S, 1989; Varela M and Griffith J, 1993). However, such as Tet pumps have narrow substrate specificity, also for some other efflux pumps, However, some transports have a large range of variation structurally substrates and these efflux pumps are named, multidrug resistance (MDR), these efflux pumps are presented in all bacteria and new pumps still have been describing. In the last few years, these have included LmrS in *S. aureus*, FuaABC in *Stenotrophomonas maltophilia*, KexD in *K. pneumoniae* and MdeA in *Streptococcus mutans* (Floyd J et al., 2010; Hu R et al., 2012; Ogawa W et al., 2012; Kim C et al., 2013). May also, some single bacteria possess different classes of efflux transporters. The evidence has indicated that the primary determinant of *Pseudomonas aeruginosa* resistance to aminoglycoside is MexXY multidrug efflux system.

A review was provided by Morita on a variety of bacteria such as MexXY pump`s *P. aeruginosa* and other efflux pumps for aminoglycoside (Morita Y et al., 2012). Moreover, numerous of studies made by some researchers, which clarified the intricacy of multidrug efflux systems regulation (Usui M et al., 2013; Deng Z et al., 2013). But, study for Baucheron et al, showed that may be overdone for the importance of multidrug efflux system for certain organism or particular antibiotics (Baucheron S et al., 2014).

However, only a little number of efflux pumps have been observed which able to pump the aminoglycoside out of the cell (Poole K, 2005; Poole K, 2012). AcrAD which is the major aminoglycoside efflux pump in gram-negative bacteria, and this pump responsible for multidrug transporter and it is one of efflux pump family (RND). The term AcrAD came out to characterize 3 component system which extends to the envelope of the cells; “i) AcrD spans the innermost cellular membrane and functions as a drug-proton antiporter, ii) AcrA is a membrane fusion protein found in the periplasm, iii) TolC is the outer membrane component of the pump” (Nikaido H, 2011). Efflux pumps (AcrAD-TolC) have been found in wide species of gram-negative bacteria such as *S. enterica* (Blair J et al., 2015), *E. coli* (Rosenberg E et al., 2000; Aires J and Nikaido H, 2005), *P. aeruginosa* (MexXY-OprM) (Morita Y et al., 2012), *A. baumannii* (AdeABC and AdeDE) (Coyne S et al., 2011) and *Burkholderia pseudomallei* (AmrAB-OprA and BpeAB-OprB) (Moore R et al., 1999).

1.4.2- Side effects of antibiotics

The required of antibiotics activities are to destroy or to inhibit the growth of degrading pathogenic bacteria. But, some of these antibiotics may affect the host defense in a detrimental way. [Table II] In general, antibiotics side effects can be divided into groups as following: 1- the microbial population`s changes; 2- a hypersensitivity as a result of changing in drug tolerance; 3- changes of functions of tissues or organs because of toxicity.

Unintended consequences of microbiological aspects and allergy are factors that complicate the use of the plurality of the antibiotic in present use. The higher incidence hypersensitivity reactions and microbial resistance may occur when the excessive use any sort of antibiotic. Therefore, it is very important to know how these antibiotics are working. e.g., gastrointestinal (G.I) distress with any oral antibacterial drug is the most side effects which are popularized between the majority of the antibiotics. But, several antibiotics are linked with side effects [Table II].

Table II: Side effects of antibiotics

Antibiotic class	Antibiotic member	Treatment purpose (Against)	Common side effect
Penicillins		G+ & G-	Skin rash, hypersensitivity reaction, anaphylaxis
//	Ampicillin	G+ & G-	Gastrointestinal
Cephalosporins		G+	Nausea, Vomiting, Diarrhea
//	Cefdinir and Cefepime	G+	Nausea, Rash, Diarrhea,
Tetracycline	Doxycycline	G+ & G-	Gastrointestinal
//		G+ & G-	Tooth discoloration
//	Tigycycline	G+ & G-	Nausea, Vomiting
Glycopeptides	Vancomycin	G+	Red neck syndrome, Ototoxicity
Macrolides	Erythromycin	G+ & G-	Nausea, Vomiting, Diarrhea, and Abdominal cramps
//	Clarithromycin	G+ & G-	Nausea, Diarrhea, and Abdominal pain, headache and metallic taste
//	Azithromycin	G+ & G-	Nausea, Diarrhea, and mild stomach pain
Aminoglycosides	Kanamycin, Neomycin, Amikacin, Strptomycin, Tobramycin and Gentamicin	G+ & G-	Nephrotoxicity, Ototoxicity, Photosensitivity, anaphylactoid reaction, Neuromuscular blockade
Chloramphenicol		G-	Plastic anemia, Grey baby syndrome, Vasomotor collapse, Cyanosis, Abdominal distention, Bone marrow suppression, Leukopenia, and Thrombocytopenia
Clindamycin	Clindamycin	G+& G-	Nausea, Anorexia, Bitter taste, Vomiting, Abdominal distention, and Flatulence
Fluoroquinolones		G+& G-	Nausea, Vomiting, Diarrhea, Dizziness, Headache, Pruritus, Skin rash, Retinopathy, and Chondorotoxicity
//	Sparfloxacin, Grepafloxacin	G+& G-	Prolongation of QT interval in cardiovascular
Sulfonamides	Sulfamethoxazole	G+& G-	Vomiting, Anorexia, Nausea, Hypersensitivity, Leukopenia, Anemia and Thrombocytopenia.

(Manten A, 1981 ; Alison E. Barnhill et al., 2012 ; Salma J and Rafik K, 2015 ; Adzitey F, 2015)

1.4.2.1-Penicillins

Food and Drug Administration (FDA) classifies penicillins as the safest antibiotic which is prescribed to breastfeeding women, as well as the penicillins, could be the most secure antibiotic among the others. However, there are some side effects related to penicillins such as skin rash, which is an allergy symptom. G.I is considered to be one of the most side effects, which it has been associated with Ampicillin. In addition, in the case of using penicillins the candidiasis is prevalent. Moreover, allergy and hypersensitivity reactions related to penicillins therapy, which could cause anaphylaxis (Miller E, 2002).

1.4.2.2-Cephalosporins

Diarrhea, nausea, and vomiting are the most common side effects that are related to cephalosporins. Due to cephalosporins, 1 to 3% of the patients have an allergic reaction. A light increase in hepatic transaminases enzymes owing to cephalosporins which are temporary between 1 to 7% of patient ratio (Marshall W and Blair J, 1999). Also, in 1997 FDA confirmed that cefdinir and cefepime which are a member from cephalosporins and they are secreted by kidneys and their activity really short which around 1.5 to 2 hours (Guay D, 2002). Diarrhea can be caused by both of them. Moreover, rash and nausea are associated with cefepime antibiotic.

1.4.2.3-Vancomycin

Rapidly expansion of vancomycin resistance owing to unlimited uses. The reason for hypotension and pruritus related to rapid infusion of vancomycin, that could be also associated with red man or red neck syndrome, a non-immunological. Other antibiotics can stimulate the histamine release might result in red man syndrome, these antibiotics include: Ciprofloxacin, amphotericin B, Rifampicin, and Teicoplanin. More than that, drug fever and skin rash, which are hypersensitivity reactions, considered being side effects for vancomycin. As well as ototoxicity related to vancomycin, which is being occurred in the ear (Hermans P and Wilhelm M, 1987). Numerous studies have been mentioned that most of the young patients especially in children have a severe reaction (Korman T et al., 1997).

1.4.2.4-Erythromycin

Owing to use of erythromycin therapy some of the side effects that appear on the patient are diarrhea, vomiting, nausea as well as abdominal cramps (Alvarez-Elcoro S and Enzler M, 1999). And it can also increase intestinal peristalsis (Pilot M et al., 1984; Catnach S and Fairclough P, 1992).

1.4.2.5-Clarithromycin

The main side effects of clarithromycin are diarrhea, nausea, abdominal pain, and headache and metallic taste. However, when the dose is less than 2000 mg the clarithromycin could be good and well tolerated. In this antibiotic, the CYP450 enzymes were inhibited by drug interaction due to clarithromycin (Alvarez-Elcoro S and Enzler M, 1999).

1.4.2.6-Azithromycin

G.I symptom e.g. diarrhea, nausea and mild stomach pain are the most common side effects related to azithromycin and all these side effects are not severe, they are mild to moderate (Drew R and Gallis H, 1992).

1.4.2.7-Clindamycin

Usually, nausea, anorexia, bitter taste, vomiting, abdominal distention, transient increase in hepatocellular enzymes and flatulence are the side effects of clindamycin (Dhawan V and Thadepalli H, 1982; Kasten M, 1999).

1.4.2.8-Aminoglycosides

Aminoglycosides cause nephrotoxicity (Rougier F et al., 2004; Pannu N and Nadim M, 2008) due to the accumulate of aminoglycosides in the renal tubules, and might cause ototoxicity (Selimoglu E, 2007; Guthrie O, 2008) is either the cochlear or the vestibulum (Palomar G et al., 2001) that could be serious reversible or losing hear owing to cochlear hair cells degeneration or dysequilibrium which are very common side effects that related to amino-

glycosides (Begg E and Barclay M, 1995). Premature infants and children more liable to ototoxicity especially during the development of the inner ear (Johnson R et al., 2010). However, this can be happening and increase when combining this drug with another. Another common serious side effects such as photosensitivity (Lankerani L and Baron E, 2004) and anaphylactoid reaction (Johannes C et al., 2007) as well as neuromuscular blockade associated with aminoglycoside (Parsons T et al.,1992; Pasquale T and Tan J, 2005). 20% of the patients who receiving such antibiotics related to aminoglycosides including Kanamycin, Neomycin, Amikacin, Streptomycin, Tobramycin, and Gentamicin have ototoxic side effects (Forge A and Schacht J, 2000; Selimoglu E, 2007).

1.4.2.9- Fluoroquinolones

Some mild and reversible side effects are very common related to fluoroquinolones, such as nausea, diarrhea, and vomiting which are G.I diseases as well as other side effects that affected the central nervous system (dizziness and headache) and also skin rash and pruritus (Ball P and Tillotson G, 1995; Bertino J and Fish D, 2000). Moreover, retinopathy (Wiebe V and Hamilton P, 2002; Velissariou I, 2006), chondrotoxicity (Hayem G et al.,1994; Stahlmann R et al.,1998; Simonin M et al., 1999) and ruptured tendons (Seeger J et al., 2006) are associated with fluoroquinolones and these side effects can be noted in juvenile patients (Leibovitz E, 2006). Due to the side effects of fluoroquinolones, they are not prescribed anymore to paediatric patients (Zhanel G et al., 2002). The side effects which occur in the cardiovascular due to sparfloxacin and grepafloxacin cause prolongation of QT interval (Time from electrocardiogram Q wave to the end of the T wave corresponding to electrical systole). For this reason, these antibiotics were withdrawn from the pharmacies (Zhanel G et al., 2002).

1.4.2.10-Tetracyclines

A group of side effects such as diarrhea, vomiting, candidiasis and nausea deemed as most common. Moreover, doxycycline, which is a class of tetracyclines can cause G.I side effects. One of the most side effects is very common that when the tetracycline is prescribed to the patients cause tooth discoloration (yellowness teeth) (Schwachman H and Schuster A, 1956; Sanchez A et al., 2004), this side effect can be found in children and adults too. This yellowness can happen during pregnancy also (Sloan B and Scheinfeld N, 2008), for this reason, the physic-

ians recommend prescribing this antibiotic to the patients who are more than 8 years old and not for pregnant women (Cunha B, 1985). Vomiting and nausea are related to tigecycline which is one of tetracyclines generation (Noskin G, 2005; Agwuh K and MacGowan A, 2006).

1.4.2.11-Sulfamethoxazole

The G.I diseases such as vomiting, anorexia, nausea, and hypersensitivity skin reactions are the most side effects related to sulfamethoxazole antibiotic (Connor E, 1998; Masters P et al., 2003). Moreover, sulfamethoxazole cause leukopenia, anemia, and thrombocytopenia. Sulfonamide can cause dermonecrolytic Stevens-Johnson syndrome (Roujeau J et al., 1995; See S and Mumford J, 2001).

1.4.2.12-Chloramphenicol

The use of chloramphenicol only in situations of life-threatening. Therefore, with being present the other classes of safer antibiotics than chloramphenicol wherefore the use of chloramphenicol is limited due to its toxicity. All the side effects which related to chloramphenicol can be fatal such as aplastic anemia (Krakoff I et al., 1955; Cruchaud A et al., 1963a; Cruchaud A et al., 1963b; Trevett A and Naraqi S, 1992) but this side effect cannot be visible during the first period of treatment. Thereby, it can take place either after a few weeks or months, another side effect can happen in newborns or infants which are gray baby syndrome, with vasomotor collapse, cyanosis and abdominal distention. Also, the bone marrow suppression is one of the severe toxicity that associated to dose (Laferriere C and Marks M, 1982; Smilack J et al., 1991). In addition, during the therapy with chloramphenicol occasional leukopenia and thrombocytopenia can be observed (Yunis A and Bloomberg G, 1964; Scott J et al., 1965; Turton J et al., 2006).

Alternative to conventional antibiotics: Antimicrobial peptides

2.1- Alternative to conventional antibiotics: Antimicrobial peptides

Host defense peptides (HDPs) or antimicrobial peptides (AMPs) are one of the most important key components of innate immune system (Zasloff M, 2002; Hancock R et al., 2012). They are the first line of defense against infectious agents. They are usually from 12 to 50 residues (Lai Y and Gallo R, 2009); they are able to kill the bacterial cell by interacting with the negative charge on the cell membrane (Zasloff M, 2002).

AMPs are present in all forms of life, including bacteria, fungi, plants, insects and mammals (Hancock R and Sahl H, 2006; Diamond G et al., 2009; Fjell C et al., 2012; Di Francesco A et al., 2013; Steckbeck J et al., 2014) with wide range of action against fungi, bacteria, protozoa, and some viruses (Reddy K et al., 2004; Marr A et al., 2006; Lai Y and Gallo R, 2009; Guani-Guerra E et al., 2010; Wilson S et al., 2013; Wilmes M and Sahl H, 2014). Furthermore, more than 2800 AMPs have been registered, which are occurring synthetic and naturally (Wang G, 2015; Wang G et al., 2016). Even though, with this huge number of AMPs in nature (the AMPs database lists more than 2800 unparalleled peptides from six kingdoms), (http://aps.unmc.edu/AP/). Until May 2017, there are 296 AMPs isolated from bacteria, 4 from archaea, 8 from protists, 13 from fungi, 343 from plants and 2137 from animals).

AMPs are commonly cationic in nature and the positively charged can be from +2 to +9, which according to amino acids that have positive charge (Arginine and Lysine) (Hancock R and Chapple D, 1999). Moreover, the positive charge residues carry an average of 40 to 50% hydrophobic residues (Yeaman M and Yount N, 2003; Hancock R and Sahl H, 2006; Pasupuleti M et al., 2012). Likewise, few of AMPs are negatively charged.

Historically, early work had been done on plants (Stec B, 2006) in 1896 showing that a substance lethal found in wheat flour which able to kill the bread yeasts (Jago W and Jago W, 1926). Some authors considered that lysozyme which was discovered by Alexander Fleming at end of 1920s (Fleming A and Allison V, 1922) represents an instance of a peptide with antimicrobial activity. By the year of 1939, the antimicrobial substances were isolated from *Bacillus brevis*, and named Gramicidins (Dubos R and Cattaneo C, 1939), at that time, they found this antimicrobial substance have activity against wide range of Gram-positive bacteria. Later on, another antimicrobial substance was discovered and isolated from certain *Escherichia coli* strain that was colicin. Moreover, the colicin was described by Grander by the year of 1950 (Gardner J, 1950). Various AMPs have been discovered and isolated since then.

As mentioned above AMPs exist as short, cationic amphipathic peptides that have diverse sequences generated *via* a multitude of tissues and cells that are in ever-complex life form (Hancock R and Diamond G, 2000). They have the import role of responding to inflammation (Lai Y and Gallo R, 2009; Wang S et al., 2015) and infection. Without host defense peptides, humans for example, would not be able to fight off infection leading to possible death. The eyes protection is also done by the tears which have wealthy source of AMPs such as lysozyme and cathelicidins (McDermott A, 2013). There are many immunomodulatory effects of AMPs (Hancock R and Diamond G, 2000; Zasloff M, 2002; Yang D et al., 2002, 2004; Fjell C et al., 2012). Some of which can create obliteration of bacteria through several methods and mechanisms. The diverse processes and actions of AMPs like wound healing and maintenance of microbiota, will be explored via close examination of research and literature pertaining to infection and immunomodulatory effects (Zasloff M, 2002; Wilmes M and Sahl H, 2014; Mangoni M et al., 2016). And many of AMPs are presently being examined in clinical trials (Fox J, 2013). Thus, the AMPs could be used as promising alternative to conventional antibiotic (Li Y et al., 2012; Xiao H et al., 2013 a, b; 2015 a, b; Yoon J et al., 2013, 2014; Yi H et al., 2014; Wang S et al., 2016).

2.2- Structural of antimicrobial peptides

While AMPs exhibit a grand multiplicity of primary/secondary structures, the majority of them share numerous shared properties that are pertinent to their activity. Such physicochemical and structural parameters are charge, hydrophobicity, and amphipathicity.

2.2.1- Charge

Many see cationicity as an indispensable feature for antimicrobial activity. The negative charged surface of the bacterial membrane provides an understanding of the initial process of AMPs activity concerning electrostatic interaction among some amino acids' positive charge present and this negative charge in the sequence. Lending from this interpretation, the existence of positively charged residues like lysine and arginine presents as a basic characteristic of AMPs' primary structures.

Numerous studies have demonstrated a strong correlation between cationicity and peptides' host defense activity (Bessalle R et al., 1992; Matsuzaki K et al., 1996; Dathe M et

al., 1996) for α-helix and β-sheet (Dathe M et al., 2001; Schibli D et al., 2002). Consequently, it appears the quantity of positively charged residues remains a pertinent characteristic to keep in mind. Nevertheless, it is a non-linear association, and the absence of a direct link has been backed by examination into model membranes (Tossi A et al., 2000; Yeaman M and Yount N, 2003; Toke O, 2005). The pattern/arrangement of charged amino acid residues along the density charge (sequence) plays also a significant role for the antimicrobial activity.

2.2.2- Amphipathicity

Probably the amphipathicity is a notable feature for AMPs such as bola- amphipathicity (Ali H, 2007), facial amphipathicity (Vandenburg Y et al., 2002) and radial amphipathicity (Xiong M et al., 2015). When looking amphipathicity, it is the peptide's ability to structure all hydrophobic residues onto one side plus on the opposite side, all hydrophilic residues. Due to the membrane's amphipathic nature, peptide amphipathicity becomes a significant parameter for the activity of AMPs (Fernandez-Vidal M et al., 2007). When peptides interact within insertion and membrane bilayer, the peptide's hydrophobic residues interact with a polar membrane lipid tails. This creates a clustering action, whereas hydrophilic residues interact with polar head groups of the aqueous environment of the membranes. The trouble in computing the amphipathicity has hindered the purpose of a correlation between peptide amphipathicity and activity (Eisenberg D et al., 1984). Anticipated the hydrophobic moment otherwise known as $M_{H,}$ as the vectorial sum of what is termed, 'individual amino acid hydrophobicities' regulated to a perfect α-helix as a quantifiable measure for peptide amphipathicity.

2.2.3- Hydrophobicity

Hydrophobicity presents as the percentage of a peptide's hydrophobic residues and its around 50% (Tossi A et al., 2000). Certainly, it works as an elemental feature indicating the level of peptide communication with the bilayer's core. Research on modulation of peptide hydrophobicity demonstrates that when peptide hydrophobicity increased (Zelezetsky I et al., 2005) it can increase the antimicrobial activity (Huang Y et al., 2010), so does the binding affinity to ever kind of cell membrane increased, minimizing the selectivity among membrane types (Wieprecht T et al., 1997a). Consequently, AMPs normally have a reasonable number of

hydrophobic residues and express higher attraction to microbial cell membranes (Wieprecht T. et al., 1997b; Chen Y. et al., 2007).

2.3-Antimicrobial peptides rich in amino acids

Most of AMPs are small molecules and they are between 12 to 50 amino acids residues (Lai Y and Gallo R, 2009). Numerous peptides are cationic and have +2 to +9 positive charge. Several AMPs are rich in specific amino acids, specially, Cysteine (Cys) (Selsted M et al., 1985), Histidine (His) (Oppenheim F et al., 1988), Proline (Pro) (Agerberth B et al., 1991), Glycine (Gly) (Park C et al. 2000), Tryptophan (Trp) and Arginine (Arg) (Chan D et al., 2006) and/or about <30% of hydrophobic amino acids. Moreover, α and β structures are usually rich in these amino acids which have been mentioned (Hancock R and Lehrer R, 1998; Zhang G and Sunkara L, 2014).

2.3.1-Cysteine-rich peptides

Cysteine-rich peptides (CRPs) are a group of amino acids that related to development and plant physiology. These peptides showed the encouragement for the growth and defence of plants, reproduction of plant and plant–bacteria symbiosis (Marshall E et al., 2011; Aalen R, 2013).

The structure of CRPs is very forked among groups. But, all of them have three general characteristics: (i) with less than 160 amino acids thus they are small in size, (ii) the region of N-terminal is protected which release peptide signal and (iii) normally 4 to 16 cysteine residues form up C-terminal of the CRPs domain.

Marine mollusks from salt water are risky from microorganism's pathogens in their surrounding environment. Therefore, to protect their selves to fight these microbial, they evolved highly active mechanisms which could be part of their innate immunity (Tincu J and Taylor S, 2004). Hence the most important components of the innate immune system are AMPs in marine mollusks (Destoumieux D et al., 1997; Mercado L et al., 2005; Arenas G et al., 2009; De Zoysa M et al., 2009). Within this diversity of natural AMPs, cysteine residues which making up intramolecular disulfide bonds that are very familiar (Dimarcq J et al., 1998; Bulet P et al., 2004; Reddy K et al., 2004; Yount N et al., 2006). CRPs and other defences showed

wide spectrum of activity against fungi and bacterial (Charlet M et al., 1996; Mitta G et al., 1999 a, b; Seo J et al., 2005; Gueguen Y et al., 2006; Gestal C et al., 2007).

2.3.2-Histidine rich peptides

Histatins peptides constitute a family of cathelicidins rich in residues of histidine which are present in human saliva (Oppenheim F et al., 1988; Van der Spek J et al., 1989; Van der Spek J et al., 1990; Brewer D et al., 1998; Tsai H and Bobek L, 1998; Helmerhorst E et al., 1999; Bals R, 2000). In 1988, the first histatin was discovered and isolated from human salivary gland (Oppenheim F et al., 1988) and submandibular glands secretions. However, the studies have not been confirmed that the histatin can be found also in airway secretions. Histatins rich polypeptides have a wide spectrum of action, not only against bacteria but also against fungi (Van, T.H et al., 1997).

According to chemical amino acids sequences, there are numerous histatins families: histatins 1 and 3 with the revealed lengths of 38 and 32 amino acids, histatin 5 is the most potent among the other family members and possess 24 amino acids (Sabatini L and Azen E, 1989; Raj P et al., 1990; Troxler R et al., 1990). Furthermore, histatins 1 and 3 are encoded by the genes which are present in humans HTN1 and HTN3 (Van, T.H et al., 1997). As a matter of fact, histatins are defense peptides for human being which became used in clinical trials especially bio-dental (Siqueira W et al., 2012; Khurshid Z et al., 2016a).

2.3.3-Proline rich peptides

PR-39 and protegrins, the most studied peptides among cathelicidins family (Zhao C et al., 1995; Linde C et al., 2001) are rich in arginine (Gennaro R et al., 2002). The cationic peptide PR-39 firstly isolated from pig intestine is rich in residues of arginine and proline (Agerberth B et al., 1991), afterwhile PR-39 isolated from the neutrophils (Storici P and Zanetti M, 1993). It plays important role in fighting against bacteria (Agerberth B et al., 1991). Proline-rich peptides were introduced to act by binding to heat shock proteins (Otvos L et al., 2000). Moreover, the immune response can be more effective due to the PR-39 functions. It can produce the synthesis of syndecans and also lead the wound healing *via* presence of heparin sulfate proteoglycans on the cell surface (Gallo R et al.,1994), likewise, for PR-39 owns characteristics for anti-inflammatory (Shi J et al., 1996) and chemotactic action towards neutro-

phils too (Huang H et al., 1997).

The development of the myocardial infarction can be also slowed down *via* PR-39 (Hoffmeyer M et al., 2000) and also, PR-39 can induce angiogenesis (Li J. et al., 2000).

2.3.4-Arginine rich peptides

Because of the guanidinium group position, that makes arginine the most basic amino acid among all, the first step of attracting the AMP to the target membranes helped by the arginine positive charges, which can create hydrogen bonds with negatively charged components. Furthermore, the CPPs can act by the head group of guanidinium, which is crucial for its uptake. The guanidinium ion on arginine is capable to shape bidentate H-bonds with a group of phosphate on a lipid head, but also H-bond can be formed with the lipid glycerol groups (Sun D et al. 2014).

2.3.5-Tryptophan rich peptides

The tryptophan (Trp) side peptide is a part of larger group related to AMPs that offer powerful activity against microorganisms. Tritripticin is a 13 residues Trp-rich AMP with a broad spectrum, due to unique biochemical properties of tryptophan to insert into biological membranes. It plays an important role against wide range of bacteria (Selsted ME et al., 1992), protozoa (Aley S et al., 1994), HIV-1 viruses (Robinson W et al., 1998; Yasin B et al., 2000) and fungi (Selsted ME et al., 1992; Falla T et al., 1996; Robinson W et al., 1998). Indolicidin was isolated from neutrophil granules of bovine and several tryptophan rich peptides can pass to membranes of bacteria without imperiling their integrity and act intracellularly, which refers to interactions with enzymes and nucleic acids. A hairpin structure can be adopted by indolicidin (Ladokhin A et al., 1999).

Other several factors in addition to the presence of Trp residues are important for antimicrobial activity. These factors include peptides residues position, close to N-terminus or carboxyl terminus. The location of Trp residues with this sequence is very important because its function is determined by its location. The Trp presence at the carboxyl terminus can inhibit the antimicrobial activity, while its presence at the amino terminus will improve this activity. Therefore, the activity of the antimicrobial needs cationic residues at the C-terminus and strong hydrophobic residues at the N-terminus (Bi X et al., 2013).

The efficiency of the AMPs can be affected by the location of Trp residues (Walrant A et al., 2011; Le´corche P et al., 2012; Rydberg H et al., 2012).

The biochemical properties of Trp complement the cationic Arg to obtain potent antimicrobial activities: Lactoferricin, tritrpicin, lysozyme, indolicidin (Pellegrini A et al. 1997; Ibrahim H et al. 2001) and puroindolines (Phillips R et al. 2011; Alfred R et al. 2013). Some other peptides are designed with similar structure properties by combinatorial libraries or rational design like combi-1 and combi-2 (Blondelle S et al. 1995), PAF26 (Lopez-Garcıa B et al. 2002), Pac-525 (Wei S-Yet al. 2006), the (RW)n series (Liu Z et al. 2007; Gopal R et al. 2012) and D5-NH2 (Saravanan R et al. 2014).

2.3.6- Glycine rich peptides

By the year of 1986, Condit and Meagher (Condit C and Meagher R, 1986) discovered the first Glycine rich peptide. Glycine has been found in the cell wall of many higher plants and more than 60% of Gly can be found in different tissues of eukaryotic species. Furthermore, three new Gly-rich peptides have been discovered and termed Ctenidin 1, 2, and 3 (Tommy Baumann et al., 2010) which had been isolated from spider *Cupiennius salei* hemocytes with antimicrobial activity against *E. coli*. In addition, Gly-rich peptides which isolated from guava seeds have a wide ring of activity against gram negative bacterial such *E. coli, Klebsiella sp and Proteus sp* (Pelegrini P et al., 2008). Moreover, Gly-rich peptides are capable to inhibit the growth of fungi and viruses (Brogden KA, 2005). Gly-rich peptides can be isolated from numerous taxonomic groups such amphibians (El Amri C and Nicolas P, 2008), arthropods (Otvos L Jr., 2000; Lorenzini D et al., 2003; Herbiniere J et al., 2005; Sperstad S et al., 2009) and plants (Park CJ et al., 2000; Egorov T et al., 2005).

According to the primary structure, Gly-rich domains are arranged in (GGGX) repetitions (Brogden K, 2005; Mangeon A et al., 2010). Thus, the content of Gly-rich peptides can be classified into three different main classes, first group has a 70% or more of Glycine residues and include that isolated from *Arabidopsis thaliana* and *Brassica napus* (Ringli C et al., 2001). Second group, which possess fewer Gly residues are present in saltbush and tomato (Ringli C et al., 2001). Last group, correspond to Glycine-rich peptides which possess increasingly content of Gly but not Glycine-rich domains (Ringli C et al., 2001).

Unlike to other studies by Sachetto-Martins and Fusaro, that mentioned Gly-rich peptides had been found in different eukaryotic species tissues that possess more than 60% of

Glycine. In addition, they classify the Gly-rich peptides in four main classes according to a primary structure too; Class I to class IV (Sachetto-Martins G et al., 2000; Fusaro A et al., 2001). Class I, which is also termed as "classic class"; a single peptide in this class might be connected to a Glycine-rich region with GGGX repeats. Because of the cell wall localization for this class the structure function is attributed to proteins (Cassab GI, 1998). Class II, characteristic of a cysteine-rich region connected at its C-terminus, which probably or might not have a signal peptide. AtGRP-3 one of this class family, and Cysteine rich residue interacts with cell wall to receptor kinases (cell wall–associated kinases) (WAKs) (Park AR et al., 2001). Class III, in fact, this class possesses proteins (Oleosins) that basically show a great diversity of structure due to lower number of Glycine residues compared to other classes. Class IV, are RNA-binding GRPs which may possess numerous of motifs besides the glycine-rich region such as RNA-recognition motif, cold-shock domain and zinc fingers (Fusaro A et al., 2001). Moreover, most of Gly-rich peptides are hydrophobic and they possess tyrosine and phenylalanine residues (Ringli C et al., 2001).

2.4- Classification of antimicrobial peptides

What can be defined as AMPs, they are molecules typically made of less than 50 amino acid residues (Lai Y and Gallo R, 2009) frequently existing within their common 'L' configuration. They can be separated into numerous subtypes that follow various criteria. Such criteria are: size, structure, origin, biological action, amino acid sequences, and finally, mechanism of action.

2.4.1- Secondary structure

Experts believe numerous AMPs adopt an extended/non-structured conformation within a water environment (Dathe M et al.,1996). This is different to others that acquire explicit configurations due to existence of intermolecular hydrogen bonds. These can be β-sheet peptides (Oishi O et al., 1997). In any case, peptides experience major conformational variations when binding to target cells. Although there are various criteria, to sort them following meaning criterion, it would fall to only secondary structure (Epand R et al., 1999; Van`t Hof W et al., 2001). Therefore, experts have proposed 4 types of AMPs. These are: extended and loop peptides, β-sheet, and α- helical (Hancock R et al., 1998; Zasloff M, 2002;

Takahashi D et al., 2010 ; Nguyen L et al., 2011; Pasupuleti M et al., 2012; Steckbeck J et al., 2014).

Nevertheless, the majority of AMPs contain two domains: α- helical, β-sheet, for example (Uteng M et al., 2003). Some cannot be classified. Again, most AMPs have cationic peptides or a net positive charge making them rich in lysine and/or arginine (basic amino acids). A minor quantity of AMPs that fall into a subgroup consisting of anionic peptides not covered in this text, does exist (Brogden K et al., 1996; Lai R et al., 2002; Von Horsten H et al., 2004). The AMPs have the ability to fold into amphipathic conformations to attract negatively charged phospholipids and to adopt an amphiphilic structure (hydrophilic sequences present on one side that are aligned on the opposite side with hydrophobic sequences).

The significance of antimicrobial activity in relation to secondary structure has been widely examined to extract structure-function relationships. For example, an α-helix peptide known as pardaxin displays lytic activity to both mammalian and microbial cells. With the incorporation of D-amino acids into the sequences, the α-helix conformation transformed to a β-structure inducing a loss of hemolytic activity. But, the maintaining of antimicrobial activity (Oren Z et al., 1999). Likewise, the cyclic peptide θ-defensin demonstrated 300% greater activity when related to open-chain analogue (Tang Y et al., 1999). These two cases indicated that alteration of secondary structure allows the dissociation of hemolytic and antimicrobial activity; the loss of a stable, rigid conformation can cause a drop-in activity of any active peptide.

2.4.1.1- α-helical antimicrobial peptides

AMPs belonging to the α-helical peptides family are considered the biggest, the most commonly found in nature, and the most researched within the class of cationic peptides (Zasloff M, 2002; Haney E et al., 2009a). Researchers and scientists have identified invertebrates, vertebrates and plants with these peptides. The subgroup is known to gain roughly 250 linear peptides containing antimicrobial activity, normally made of less than 40 amino acid residues and not containing cysteine (Brogden K, 2005).

Possessing a tertiary structure, α-helical peptides present a prominent amphipathic behavior (Mihajlovic M and Lazaridis T, 2010 a, b), being highly positively charged with a hinge/kink in the center (Gennaro R et al., 2000; Tossi A et al., 2000). Such peptides remain unstructured within an aqueous solution (Yeaman M and Yount N, 2003; Pasupuleti M et al.,

2012), folding into what is termed, 'α-helical configuration' when binding with bacterial membrane that either leads to insertion into its surface or absorption onto it. Researchers established a direct correlation between antibacterial activity and α-helical conformation (Park CB et al., 2000; Haney E et al., 2009b).

Furthermore, α-helical AMPs have other delegates such as Melittin (Fennell J et al.,1968; Terwilliger T and Eisenberg D, 1982a and 1982b) and Cecropins (Hultmark D et al., 1980; Steiner H et al., 1981) present in insects, (Magainins) Frogs (Zasloff M, 1987; Gesell J et al.,1997) [Figure 6], (Fowlicidins) Chickens (Xiao Y et al., 2006; Bommineni Y et al., 2007; Xiao Y et al., 2009). α-helical peptides being found just in primates and rodents (Patil A et al., 2004) [Figure 6].

Figure 6: **α-helical structured; Magainin-2 (PDB code 2MAG)** (Gesell J et al., 1997).

2.4.1.2- β-sheet antimicrobial peptides

The β-sheet subgroup includes numerous peptide with β-hairpin structure which are structured as β-sheet peptides conformationally stabilized by the presence of disulfide bridges (Brogden K, 2005; Yount N et al., 2006). β-sheet peptides may also adopt a cyclic conformation (Tossi A and Sandri L, 2002). α-helical and β-sheet peptides are unrivaled to vertebrates' animals. β-sheet peptides mostly exist in every classes of mammalian that had been inspected (Patil A et al., 2005) [Figure 7].

Conformation of β-sheet peptides within an aqueous solution can be further stabilized as they bind the bacterial membrane (Yeaman M and Yount N, 2003). The quantity of disulfide bridges has an influence on the general structure and on the peptide's activity. The cyclic structure may be essential for antimicrobial activity (Matsuzaki K et al., 1997; Rao A, 1999).

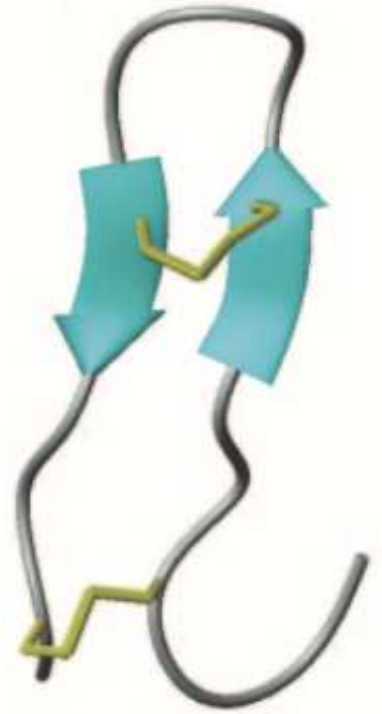

Figure 7: **β-sheet structured; Polyphemusin (PDB code 1RKK)** (Powers J et al., 2004).

2.4.1.3- Extended antimicrobial peptides

With cationic linear extended AMPs, they present what some may deem, odd amino composition. In terms of structure, these peptides are linear in shape, characterized by an overexpression an amino acid or more. This subgroup comprises of almost 90 peptides not presenting any secondary structure either in β-sheet or in α-helix [Figure 8]. A number of these peptides possess a high quantity of histidine residues, similar to histatin (Brewer D et al., 1998; Tsai H and Bobek L, 1998) originating in human saliva. PR-39 however, is abundant in arginine and proline residues (Takahashi D et al., 2010; Nguyen L et al., 2011). Prophenin is abundant in phenylalanine and proline. A notable trait of these peptides is their flexibility in solution. However, Extended peptides are neither bactericidal nor bacteriostatic in spite of being anti-parasitic and fungicidal (Luque-Ortega J et al., 2008). Thereby, most of these peptides are not membrane active.

Figure 8: **Extended structured peptide; Bos taurus indolicidin, secreted by neutrophils (PDB code 1G89)** (Rozek A et al., 2000).

2.4.1.4- Loop antimicrobial peptides

In this subgroup, proline-arginine abundant peptides do not have the ability to form amphipathic structures due to the overexpression of proline residues. The alternative then becomes adoption of a polyproline helical type-II structure (Boman H et al., 1993; Cabiaux V et al., 1994) [Figure 9].

Interestingly, any living organism possesses the capability of generating various classes of the previously mentioned AMPs (Hancock R and Diamond G, 2000). In a review stated that are at minimum, four likely reasons for such structural diversity existing among AMPs. The first important point to note about AMPs is while the activity spectrum of AMPs remains relatively broad, AMPs will not remain active against each pathogen encountered by the host. This then leads to a diverse plethora of AMPs with distinct, yet overlying activities that enable increase of the host's natural defense systems *versus* pathogens. The second is that AMPs demonstrating dissimilar structure might work together to act synergistically. Thirdly, AMPs possess interesting non-antimicrobial features like pro-inflammatory or chemotactic activities, that vary between classes. Fourth and lastly, various cell types yield various kinds of AMPs, complementing each other.

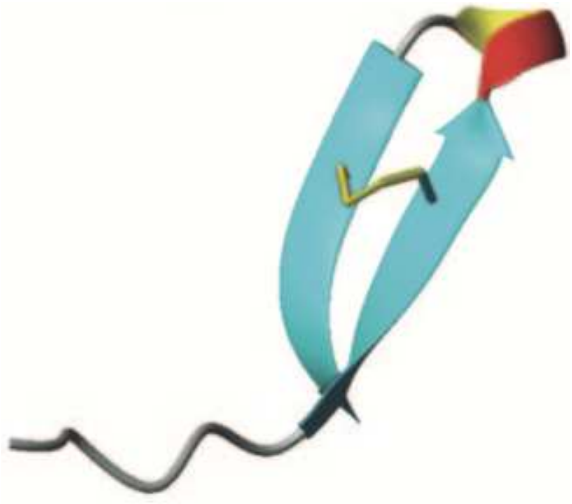

Figure 9: **Loop structured; Thanatin (PDB code 8TFV)** (Mandard N et al., 1998).

2.5-Biological activities of antimicrobial peptides

AMPs are broadly connected with host defense moreover by applying direct antimicrobial activities otherwise by moderating the function of immune cells (Hancock R and Sahl H, 2006).

2.5.1- Directly activation of antimicrobial peptides

2.5.1.1- Anti-tumoral

AMPs have the ability to directly target and obliterate infected cells and microbes (Ganz T et al., 1985; Garcia J et al., 2001b; Harder J et al., 1997, 2001; Ouellette A and Selsted M, 1996; Zaiou M et al., 2003). This direct action encompasses weakening of microbial membrane. Constructed on this sort of interaction they can either be anticancerous or antimicrobial. The antimicrobial nature side can be credited to negatively charged membranes of parasites, viruses, bacteria, and fungi (Hancock R and Diamond G, 2000; Zasloff M, 2002; Bader M et al., 2003; Mader J and Hoskin D, 2006; Nijnik A and Hancock R, 2009; Fjell C et al., 2012; Afacan N et al., 2012; Hancock R et al., 2012). Like this manner of action, AMPs can distinguish between cancer cells and healthy cells, because of selective demonstration of negative charge on its surface. Selective demonstration of negative charge may arise because of demonstration of a few anionic molecules (phosphatidylserine) on the surface (Utsugi T et al., 1991; Dobrzynska

I et al., 2004). Occasionally this recognition can be potentiated *via* selective demonstration of O-glycosylated saccharide mechanisms (Yoon J et al.,1997; Burdick M et al., 1997). Along with selective presentation, the growth of cancer compared to normal cells is greater with unusually distorted orientation that reduces them to being more susceptible to AMPs (Chan S et al.,1998; Hoskin D and Ramamoorthy A, 2008; Schweizer F, 2009).

2.5.1.2- Antiviral

Interaction with heparin sulfate makes AMPs effective against RNA/DNA viruses because heparin sulfate is a necessary component for viral access into cells (rabbit alpha defensin NP-1 and lactoferricin) (Sinha S et al., 2003; Jenssen H et al., 2006). NK-18 is a type of peptide that can instantaneously disrupt microbial membrane as well as interact with microbial genome, eventually potentiating and furthering antimicrobial activity (Yan J et al., 2013). Another interesting aspect of AMPs is how they can interact with several targets to block innumerable biological processes leading to an inhibition of microbial growth such as cell wall synthesis, RNA, DNA, and protein synthesis (Boman H et al., 1993; Zasloff M, 1992 and 2002). Chromogranin A (CgA) derived peptides (chromofungin and catestatin) can lead to the destabilization of the membrane, bind calmodulin and inhibit calmodulin enzymes dependent (Zhang D et al., 2009). In addition, catestatin inhibit plasmepsin in *Plasmodium falciparum*. (Akaddar A et al., 2010).

2.5.2- Antimicrobial peptides modulate immune system

AMPs typically are very effective in exhibition of direct antimicrobial activity to modulate the innate immune system (Scott M and Hancock R, 2000; Selsted M and Ouellette A, 2005; McPhee J and Hancock R, 2005; Bowdish D et al., 2005; Nijnik A and Hancock R, 2009; Afacan N et al., 2012; Hancock R et al., 2012). This is from the basis of effective concentration and direct contact to pathogen (Nelson A et al., 2009; LaRock C et al., 2015). Though, in many cases such concentration may not lead to the effective killing of overwhelming infectious agents. Furthermore, non-physiological state, like high saccharides and salt concentration can diminish the AMPs effectiveness (Bals R et al.,1998; Bowdish D and Hancock R, 2005; Diamond G et al., 2009). AMPs' immunomodulatory activities may assist in overcoming this problem. These events include employment of cellular proliferation, endotoxin

binding, activation of adaptive immune cells and innate immune cells.

2.5.2.1- Innate immune cells recruitment

AMPs can activate selective production of pro and anti-inflammatory cytokines and in the end, leukocyte chemotaxis (Choi K et al., 2012). A good example of this is LL-37 and its ability to attract neutrophils, mast cells, T cells, and monocytes (Chertov O et al., 1996; Niyonsaba F et al., 2002; Chen Q et al., 2004; Kurosaka K et al., 2005; Ciornei C et al., 2005). Selective degranulation of phagocytes is induced by the production of N-formyl peptide like receptors and G-protein coupled receptors (De Y et al., 2000; Mookherjee N and Hancock R, 2007). The same LL-37 behaves synergistically with IL-1β, enhancing creation of proinflammatory cytokines (MCP-1, MCP-3, IL-6 and IL-10) (Scott M et al., 2002; Mookherjee N et al., 2006 a; Yu J et al., 2007; Choi K et al., 2012). In *vitro*, a chromogranin-A derived peptide (Catestatin, CgA 344-364) can attract neutrophils, penetrate into cells and induce exocytosis (Shooshtarizadeh P et al., 2010). Defensins possess the ability to interact with MIP-3a receptor existing on inflammatory cells, regulating inflammatory response (Mookherjee N and Hancock R, 2007). IDR1002, which is an innate defense regulator, modulates inflammation induce by IL-1β in synovial fibroblasts (Turner-Brannen E et al., 2011).

2.5.2.2- Endotoxin binding

The important component of gram negative bacterial of outer membrane is LPS, which also called endotoxin (Alexander C and Rietschel E, 2001; Raetz C and Whitfield C, 2002). When an infection occurs, a vast quantity of endotoxins is produced inducing the inflammatory response. Inflammation can be both beneficial and life threatening when surpassed by a defined limit (Mookherjee N and Hancock R, 2007). Indolicidin, LL-37 and cecropins help moderate inflammatory response *via* binding to endotoxins safeguarding from endotoxemia (Bowdish D and Hancock R, 2005; Mookherjee N et al., 2006 b). Additionally, these AMPs may inhibit expression of genes (proinflammatory) induced by endotoxin. As earlier mention, LL-37 has the capacity to stop tumor necrosis factor-alpha (TNF-alpha) (Feldmann M and Maini R, 2003) production facilitated through endotoxins (Bowdish D and Hancock R, 2005; Mookherjee N et al., 2006 b).

2.5.2.3- Cellular proliferation and differentiation induced by antimicrobial peptides

AMPs have many talents, one of which is modulation of differentiation and proliferation of phagocytic cells. For example, neutrophils through inhibition of apoptosis, leading to activation of mast cells and wound healing. Such a process leads to the promotion of angiogenesis (Koczulla R et al., 2003; Chen X et al., 2006; Nagaoka I et al., 2006), LL-37 has the capability of improving expression of dendritic cells (DCs) *via* distinctive immune cells and help in what is called 'antigen presentation' (Tokumaru S et al., 2005; Mookherjee N and Hancock R, 2007). Neutrophil β-defensins and cathelicidin may turn on Mitogen-Activated Protein Kinase (MAPK-p38) as well as Extracellular Signal-Regulated Kinase (ERK-1/2) pathways in keratinocytes, mast cells, and monocytes (Bowdish D et al., 2004; Niyonsaba F et al., 2005; Chen X et al., 2006; Niyonsaba F et al., 2006; Choi K et al., 2014). Additionally, AMPs can control transcription *via* regulation of signal transducer and activator of transcription factor (STAT-3) (Tokumaru S et al., 2005).

2.5.2.4- Activation of adaptive immune cells

Other than the characteristic immune response modulation, there are other activations happening with AMPs in terms of adaptive immune cells (Mookherjee N and Hancock R, 2007). Various AMPs, like human β-defensins, neutrophil defensins, and cathelicidin (LL-37 and PR-39) express DCs receptor and can lead to T and B lymphocyte activation (Chertov O et al.,1996; Huang H et al., 1997). Moreover, LL-37 also moderates dendritic cells differentiation (Chen K et al., 2014), including dendritic cells, made T-cells polarization (Davidson D et al., 2004; Semple F and Dorin J, 2012). In terms of proliferation, defensins also increase production of T cells (Brogden K et al., 2003) made by IL-6, IL-10 and IFN-g (Lillard J et al., 1999a; Lillard J et al., 1999b). Recently, a study has made which noted that, taking the LL-37 *via* oral can excite T-cell according to antigen-specific antibody mucosal responses primarily *via* a 17-skewed pathway, that can be interacting with receptors on M cells (Kim S et al., 2015).

2.6-The mechanism of action of the antimicrobial peptides

Most AMPs have a mode of action that destroy bacteria consisting of membrane disruption, pore construction on the nanometer measure (Meroueh S et al., 2006; Vollmer W et al., 2008), followed by membrane depolarization. Researchers propose the following general model for mechanism of action: 1. AMP-membrane attraction, 2. attachment of the AMP onto the membrane and 3. insertion of the AMP into the membrane leading to disruption and then leakage of metabolites and ions. The most studied AMPs are α-helical peptides. β-sheet peptides regarding the mechanism of action (Breukink E and de Kruijff B, 1999; Mathew R and Nagaraj R, 2017) by which they permeabilize the membrane.

Many studies have focused on the net charge of AMPs and the direct correlation with their interaction or attraction with the bacterial membrane (Hancock R and Rozek A, 2002). Cationic AMPs have a +2 to +9 positive net charge and bacteria present an exceedingly negatively charged 'outer membrane' because of the existence of phosphate groups in LPS (Ruiz N et al., 2006), concerning gram-negative bacteria (Lai Y and Gallo R, 2009; Ebenhan T et al., 2014). For gram-positive bacteria, the lipoteichoic acids play a similar role (Jenssen H et al., 2006; Malanovic N and Lohner K, 2016). AMPs attract electrostatic interactions between the outer membrane (of bacteria) and themselves then allowing for the bringing of the two moieties together (Dathe M et al., 2001).

It has been established that, up to a defined threshold rate, the higher the positively charged AMP, the more improved the antibacterial selectivity and activity. In relation to attachment insertion, AMPs exist near the bacterial surface. The preliminary electrostatic interactions result in a nonspecific and actual relation of the AMP with bacterial membranes (Yeaman M and Yount N, 2003; Giuliani A et al., 2007; Yeung A et al., 2011; Ebenhan T et al., 2014). Thanks to the negative charge formed by the membrane, AMPs can distinguish between bacteria and host cell.

AMPs have the ability to aggregate, generating a cluster that deposits onto the membrane. Research focusing on *in vitro* studies disclosed that contingent on some parameters like the nature of the AMP, the peptide/membrane concentration, AMPs bind to the surface of bacteria *via* two dissimilar states. These are an 'I and S state' I stands for Insertion (Huang H, 2000), S signifies Surface. Fundamentally, at a low peptide-to-lipid ratio, peptides have the tendency to be adsorbed onto a surface, leading to adoption of an orientation parallel to bilayers.

Existing in a functionally inactive "S" state encouraging a stretching or thinning of the membrane.

When the peptide-to-lipid ratio rises reaching a brink reliant generally on the lipid arrangement of the bilayer, the alignment of AMP fluctuates, becoming perpendicular to the bilayers (Brogdan K, 2005; Melo M and Castanho M, 2012). In other words, AMPs shift to the "I" state starting the process of inset into the membrane, ultimately concluding to pore formation. Subsequently the binding of the bacterial membrane, an AMPs will undergo conformation alterations to adopt dynamically promising secondary structures verbalized by hydrophobicity. Meaning, α-helical peptides will espouse directions perpendicular or to the membrane (Dennison S et al., 2007). Furthermore, membrane permeation exists as a rigorous process concerning bunches of AMPs, as it is dynamically negative for the passing of an α-helical peptide through the membrane, behaving as a monomer.

In relation with the peptide structure, several modes of action founded on this model have been suggested: toroidal pore model, barrel-stave model, and carpet model (Brogdan K, 2005; Melo M et al., 2009) [Figure 10]. However, a little discussion on the mechanisms of action for AMPs which covered the dynamic processes and the detailed atomistic events (Epand R and Vogel H, 1999; Shai Y, 2002; Guilhelmelli F et al., 2013; Lee T et al., 2016). The three models differ chiefly in the attachment-insertion phase. The in *vivo* mechanisms along with the exact description of AMP-membrane interactions remain contentious and it is vital to state membrane disruption is a multifaceted wonder involving a mixture of intricate mechanisms. Like the reporting of some peptide-related cases where it does not act on the membrane rather accrues inside the bacteria cell.

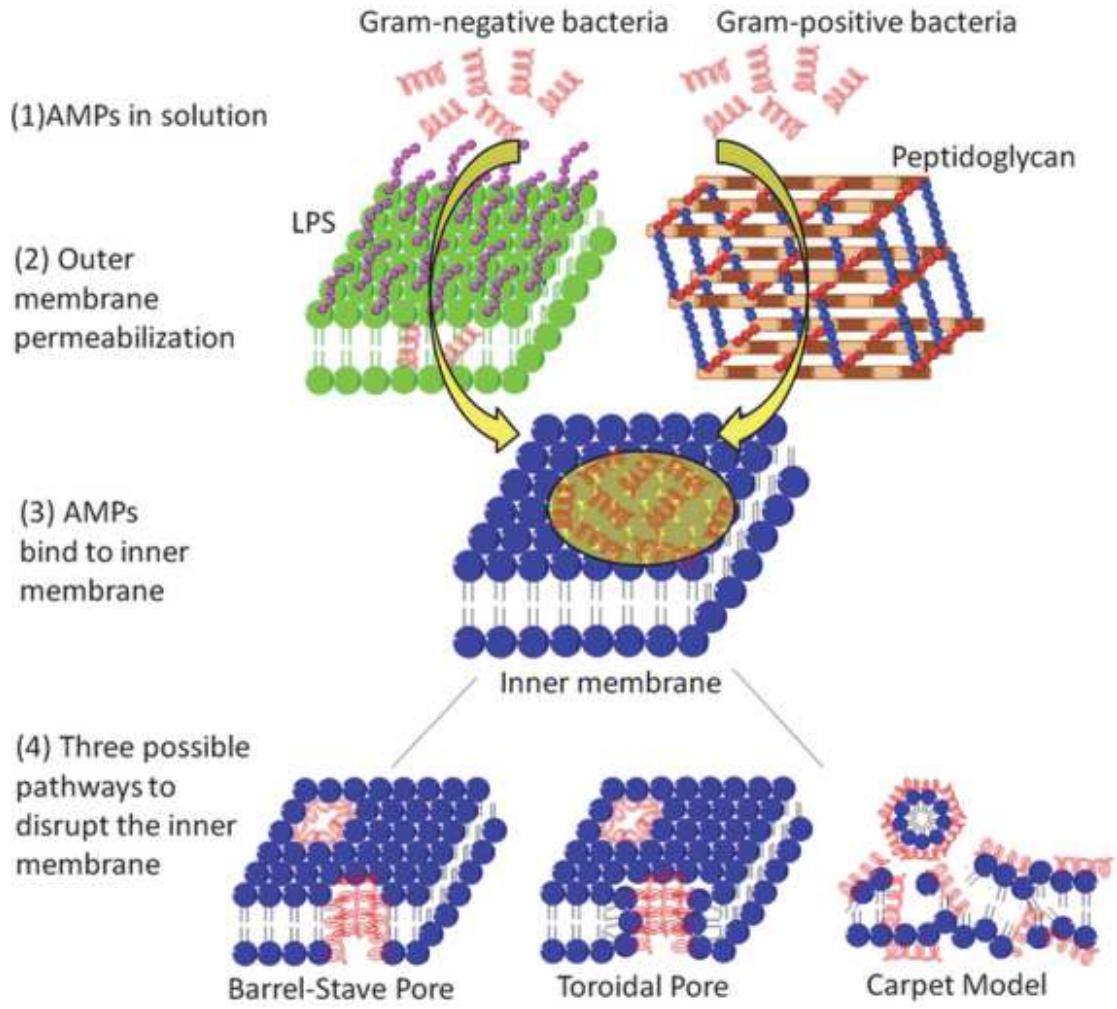

Figure 10: **The mechanisms of action of antimicrobial peptides** (Li J et al., 2017).
Barrel-Stave model, the AMPs insert themselves perpendicularly into the membrane. Toroidal pore model which looks like Barrel-stave model, but, the phospholipid head group of the membrane constantly contact with AMPs. Carpet model, in this model the hydrophobic sides of AMPs cover small areas of the membrane which lead pores in the membrane.

2.6.1- The barrel-stave model

The first mechanism that would explain the AMP ability to kill bacteria was proposed by Ehrenstein et al (Ehrenstein G and Lecar H, 1977). Barrel-stave model formation is derived by hydrophobic match. AMPs accrue as monomers on the surface of bacteria, then creating circle patterns. When binding, they adopt a direction that is parallel to lipid bilayer, coating local areas in a carpet-like fashion (Pouny Y et al., 1992). The following action entails the AMPs reorienting perpendicularly, inserting into the bacteria membrane's lipid core resulting in a form like a barrel whose laths are the α-helical AMPs (Yang L et al., 2001). Throughout this development, AMPs experience conformational point shift: The AMP's hydrophobic surf-

aces face outward, near the membrane's acyl chains consequently making parallel with the bilayer's lipid core, while hydrophilic regions create the pore's interior and face each other (Brogden K, 2005). Increasingly, new AMPs are enlisted and *via* a self-aggregation process, the pore size rises as more AMPs assume a trans-membrane formation (Christensen B et al., 1988; Westerhoff H et al., 1989; Duclohier H et al., 1989; Kagan B et al., 1990; Juvvadi P et al., 1996; Yang L et al., 2001; Porcelli F et al., 2004). The size of pore based on different factors, such as the composition of lipids and lipid/the peptide ratio. Different pores can be structurally formed by maculation which had been mentioned in AMPs previously studies (Wang Y et al., 2016). According to the pore shape, the AMPs including pardaxin, alamethicin, and dermcidin motivate barrel stave pores (Laver D,1994; Porcelli F et al., 2004; Song C et al., 2013), whereas, the other AMPs motivating toroidal pore (Matsuzaki K, 1998; Sokolov Y et al., 1999; Yang L et al., 2001; Henzler Wildman K et al., 2003; Sengupta D et al., 2008; Lee M et al., 2013). Within this model, one can see a membrane neither bent nor deformed during insertion process. Undeniably, the AMP inserts within the bilayer *via* the action of "drilling" the membrane. Moreover, the importance of hydrophobic and electrostatic interaction to the AMPs molecules due to the interaction that occurred with both the head groups and lipid tails (Mihajlovic M and Lazaridis T, 2010 a, b; Bertelsen K et al., 2012) [Figure 10].

2.6.2- The toroidal pore model

In 1999, another model, proposed by Hancock et al (Hancock R and Chapple D, 1999). helped to combine the actions of two models (carpet and barrel-stave). AMPs molecules break through deeper into the membrane. The AMPs amassed on the membrane in a similar fashion to the carpet model with a perpendicular insertion into it, causing its distortion (Matsuzaki K et al., 1996). Different from the barrel-stave model, lipids are interpolated in the transmembrane channel with the AMPs in formation. Protegrins, magainins, and melittin are AMPs's examples which are forming toroidal pore (Matsuzaki K et al., 1996; Yang L et al., 2001; Henzler Wildman K et al., 2003; Hallock K et al., 2003; Brogdan K, 2005). The composition of the pore is phospholipid and peptide (Matsuzaki K et al., 1996; Ludtke S et al., 1996; Huang H, 2000; Yang L et al., 2001; Hallock K et al., 2003) and this model was founded according to some studies related to magainin peptides (Matsuzaki K et al., 1995; Ludtke S et al., 1996).

AMPs interaction with the membrane allows them to an α-helical structure, as well as direct themselves parallel to the surface of the membrane allowing the binding to take place

amid the AMPs polar face and head group of the lipids. This then causes the bending of the membrane (Yamaguchi S et al., 2002). The membrane's positive curvature bends of phosphorlipids and toroidal pore formation (Matsuzaki K et al., 1998; Hallock K et al., 2003) so that the pore is lined by the lipid head groups and the AMPs (Sengupta D et al., 2008). Whereas interaction of the lipid tails occurs with the AMP's hydrophobic surface (Yang L et al., 2001; Mihajlovic M and Lazaridis T, 2010 a, b; Bertelsen K et al., 2012). The proposed of toroidal pores are to take shape of subsequent to peptide induced membrane thinning (Heller W et al., 2000; Huang H, 2000; Chen F et al., 2003; Lee M et al., 2004) that can lead to breakthrough of short peptides to form a peptide/lipid pore. Ultimately, the forming of toroidal pores in the membrane will lead to disruption of said membrane. The variance with the other models is the time of insertion, when the AMPs remain eternally bound to the membrane's LPS moieties [Figure 10].

2.6.3- The carpet model

In 1992, Pouny et al., studied a cationic amphipathic α-helical peptide isolated from frog skin named dermaseptin S and established that the communication of this AMP with membranes evidently deviated from what was discovered in the barrel-stave model. They then proposed a new mechanism, the carpet model (Pouny Y et al., 1992) [Figure 10]. Like the barrel-stave model (Ehrenstein G and Lecar H, 1977), the carpet model posits aggregation of AMPs onto the bilayer surface. While true to its predecessor, the main difference is how the AMPs keep a parallel position to the membrane surface while the action took place (Bechinger B, 1999).

Sometimes, it is difficult to identify or distinguish between carpet and toroidal pore mechanisms. For example, the human LL-37 intercalates parallel to the membrane surface (Henzler Wildman K et al., 2003). Although the carpet and channel forming model are different in some aspects. However, they are sharing some of the same characteristics.

Bound to the bacterial surface (hydrophobic side facing exterior) the peptides continue acting as clusters (Oreopoulos J et al., 2010; Polyansky A et al., 2010; Wadhwani P et al., 2012; Scheinpflug K et al., 2015); ultimately coating the bacterial surface just as a carpet would. As the concentration of peptides surges, the membrane becomes weakened from unfavorable energetics becoming more likely to interpolate into it as what can be termed 'detergent-like fashion' causing the breakup and dissolving of the membranes into micelles. Such a mechanism

does not encompass pore formation. Here is an instance where there is no insertion into the membrane by the AMPs. An efficient mode of action would mean, there must be a high concentration of AMPs to get 50% cell killing (Lohner K, 2009) as they have to cover the entire bacterial membrane (Rotem S and Mor A, 2009). Notably, contrast to the toroidal pore and barrel-stave model, it is not necessary for AMPs to adopt an explicit structure like α-helical for permeabilization of the membrane.

2.6.4-Intracellular targets

Research indicates membrane disruption is frequently not adequate to generate bacteria death. At times membrane disruption is not required for bacteria death. Evidence suggests the targeting of intracellular parts (Yeaman M and Yount N, 2003). For example, Buforin II is a proline α-helical AMP. This AMP does not permeabilize the outer membrane of the bacteria, it penetrates and accumulates within the cytoplasm, applying cytotoxic activity (Park CB et al., 2000). A concerted action is involved in the mechanism of translocation when it comes to other AMPs. Those abundant in arginine can translocate across nuclear and cellular membranes, enabling interaction with RNA and DNA. They may also inhibit synthesis pathways (Cudic M and Otvos L, 2002; Nicolas P, 2009) by interacting with proteins (Futaki S et al., 2001). While no general scheme exists to detail such mechanisms, when the AMP is in the cytoplasm, it can interact in various ways: RNA and protein synthesis, inhibition of cell-wall synthesis, binding to DNA, inhibition of DNA (Brogden K, 2005; Straus S and Hancock R, 2006; Nicolas P, 2009; Hilpert K et al., 2010), inhibition of enzymatic activity (Yeaman M and Yount N, 2003; Brogden K, 2005; Yount N et al., 2006; Nguyen L et al., 2011), activation of autolysin, and changing cytoplasmic membrane. Lastly, it is important to point out that the action of AMPs might be different based on conditions of a test and also can be affected by outer factors such as osmolarity, temperature, and media PH (Yeaman M and Yount N, 2003).

2.7- The antimicrobial peptides derived from chromogranin A (CgA)

Several new AMPs are released by the secretory vesicles of the bovine adrenal medulla chromaffin cells. Highly conserved within humans, the corresponding sequences have a main cleavage site located in 78-79 of bCgA. The following removal of K77 and K78 (two basic residues) by the carboxypeptidase H (Metz-Boutigue MH et al., 1993) yields vasostatin-I (VS-

I; bCgA1-76) (Lugardon K et al., 2000) and prochromacin (Prochrom; bCgA79-431) (Strub J et al., 1996), two antimicrobial fragments. C- and N- terminal domains containing antimicrobial activities make up many shorter active fragments. They have been identified as: For VS-I, bCgA1-40 (N CgA; NCA) (Maget-Dana R et al., 2002) and bCgA47-66 (chromofungin; CHR) (Lugardon K et al., 2001), for ProChrom, bCgA173-194 (Chromacin; Chrom) (Strub J et al., 1996) bCgA344-364 (Catestatin; CAT) (Briolat J et al., 2005) and bCgA344-358 (Cateslytin; Ctl). CgA's unique disulfide bridge remains existent in NCA and VS-I arrangements. Two post-translational variations are significant for antibacterial activity expression of Chrom. Chrom possess the O-glycosylation of S186 and phosphorylation of Y173 (Strub J et al., 1996). Besides, worthy of mention is dimerization motif GXXXG akin to what was reported for Glycophorin A (Brosig B and Langosch D, 1998) exists in the Chrom sequence (G184-G188) [Figure 11].

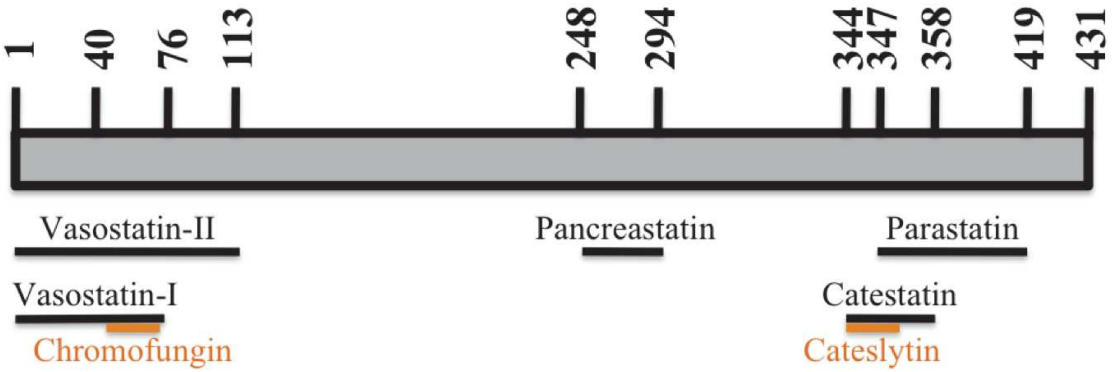

Figure 11: **Chromogranin A derived peptides.**
Bovine chromogranin A-derived peptides with the complete sequence (431 amino acids). Black color represents the natural cleavage sites and the other generated peptides are in orange color.

2.7.1-Vasostatins

Vasostatins I and II otherwise known as CgA1–76 (Aardal S and Helle K, 1992) and CgA1–113 characterize CgA's N-terminal fragments, exerting a great spectrum of cardiovascular homeostatic activities (Tota B et al., 2010) that include vasodilation, antimicrobial activities (Lugardon K et al., 2000; Aslam R et al., 2012), and inhibition of parathyroid hormone secretion (Russell J et al.,1994). Furthermore, neurotoxic effects in neuronal/microglial cell co-cultures (Ciesielski-Treska J et al., 1998), and finally, modulation of cell adhesion (Gasparri A et al., 1997) were also reported. In rat heart the process of conve-

rsion to vasostatin peptides occurs in the extracellular matrix and at the cell membrane level of cardiomyocytes (Glattard E et al., 2006). CgA1–76 which can be cleaved from CgA by plasmin (Colombo B et al., 2002) act as a vascular smooth muscle dilatating agent (Aardal S and Helle K, 1993) and CgA1–113 are structurally comparable inducing similar effects while acting through endocrine, autocrine, and paracrine, mechanisms (Helle K and Aunis D, 2000).

So far, researchers have not identified high-affinity, classical receptors. Although antimicrobial action/membrane perturbation have been hypothesized in heart and endothelium (Cerra M et al., 2008; Fornero S et al., 2012). Vasostatins have been linked to vasculogenesis and remodeling (Mazza R et al., 2010). Vasostatin I inhibits vascular endothelial growth factor (VEGF) induced endothelial cell proliferation and migration and the formation of capillary-like structures (Belloni D et al., 2007), as well as vasostatin I is capable to prevent cytoskeletal reorganization included by TNF-α, pertussis toxin and thrombin (Ferrero E et al., 2004; Blois A et al., 2006). Yet, vasostatin I display vasorelaxant attributes, exerting negative inotropic while also having lusitropic effects on the heart (Corti A et al., 2004; Imbrogno S et al., 2004; Cerra M et al., 2006), chiefly in the company of strong adrenergic stimuli. Cardiosuppressive effects (Imbrogno S et al., 2004) of this nature may be due to a non-competitive counter-action stimulated by the b-adrenergic-mediated positive inotropism (Tota B et al., 2008). All of this then suggests both vasoactive and cardiotropic attributes of vasostatins offer evidence that peptides may act as the cardiovascular system's homeostatic stabilizers. Specifically, under circumstances of sympathetic overstimulation, like when under a stress response (Helle K and Aunis D, 2000; Tota B et al., 2010).

Along with cardiovascular effects, there exists also a regulatory role within the immune system. Research shows vasostatin controls the innate immunity by encouraging calcium entry into the neutrophils of humans (Zhang D et al., 2009). Additionally, vasostatin directly impedes growth of fungi, yeast, and bacteria through penetration of into their membranes. Lastly, vasostatins assist in modulating pro-adhesive communication of smooth muscle cells and fibroblasts with extracellular matrix proteins (Ratti S et al., 2000) as well as employ parathyroid hormone secretion autocrine inhibition in parathyroid cells (Angeletti R et al., 2000).

2.7.2-Chromofungin

Human and bovine chromofungin (CgA47-66) has a similar sequence (RILSILRHQNLLKELQDLAL). When endoprotease Glu-C from *Staphylococcus aureus* was

used to digest VS-I (Metz-Boutigue MH et al.,1993), (a generated peptide), CHR, (shortest active) was the VS-I-derived peptide corresponding to the sequence Arg^{47} –Leu^{66} (Lugardon K et al., 2001). It displays antifungal and yeast cells activity (Lugardon K et al., 2001; Taylor C et al.,2000). While such a peptide was produced post digestion of existing material in chromaffin secretory vesicles, it may be theorized that it may be moduled as infections occur by *S. aureus*.

CHR's three-dimension structure has been observed and confirmed in water–trifluoroethanol (50:50) (Lugardon K et al., 2001) *via* the use of 1H-NMR (Nuclear Magnetic Resonance) spectroscopy. This investigation discovered the amphipathic sequence 53–56's helical character, while the segment 48–52 lends to a hydrophobic character (Lugardon K et al.,2001). Significance of the amphipathic sequence in relation to antifungal activity was established from loss of this kind of activity against *Neurospora crassa* with substitution of two proline residues for L61 and L64 respectively, allowing for the disruption of helical structure.

2.7.3-Catestatin

Catestatin (CAT) is a 21-amino acid peptide corresponding to bovine CgA344-364 and human CgA352-372, acting along nicotinic cholinergic receptors in chromaffin cells (Mahata S et al., 1997). CAT inhibit the release of catecholamines. The targeted ablation in a mouse model of CgA locus generates severe hypertension, which can only be resolved through administration of CAT. Interestingly, hypertensive patients show increased CgA (Chen Y et al., 2010) along with diminished CAT plasma levels (O'Connor D et al., 2002; Meng L et al., 2011). This means CAT deficiency could play a part in formation of hypertension (Rao F et al., 2007; Mahapatra R, 2008). CAT's pathogenesis has a major neurogenic element built on a continued over action of the sympathetic nervous system. Also, it can regulate blood pressure (Bassino E et al., 2011; Biswas N et al., 2012; Liao F et al., 2015) is sympatho-inhibitor and attenuates sympathetic barosensitivity (Rao F et al., 2007; Schillaci G et al., 2011; Gaede A and Pilowsky P, 2012).

CAT may be influenced by individual genetic profile. Moreover, CAT's genetic variant Gly364Ser appears to provide protection against hypertension development (Rao F et al., 2007), while the CgA dispensation to CAT seems to efficient in women *versus* men (Fung M et al., 2010). The ability of CAT to encourage cardiovascular responses at both local and systemic levels is noteworthy (Friese R et al., 2010). Specifically, induction of both antihypertensive and

vasorelaxant effects histamine release induction from mast cells (Kennedy B et al., 1998; Kruger P et al., 2003; Angelone T et al., 2008; Aung G et al., 2011; Biswas N et al., 2012). It can also exhibit marked vasculogenic and angiogenic activities, as it encourages production and migration of endothelial cells stimulating chemotaxis of vascular smooth muscle cells (Guo X et al., 2011). Moreover, CAT can mark several diseases, such as myocardial infarction, carcinoid tumors of intestine and heart failure (Ceconi C et al., 2002; Prommegger R et al., 2003; Conlon J, 2010; Zhu D et al., 2011; Liu L et al., 2013; Meng L et al., 2013; Liao F et al., 2015). Effects such as migration, proliferation and anti-apoptosis in endothelial cells comparable to that of VEGF were identified in *vitro* in tube formation assays (Kirchmair R et al., 2004; Theurl M et al., 2010).

CAT's role in inflammation has lately been emphasized in terms of induction and chemotaxis of pro-inflammatory cytokines (Egger M et al., 2008; Aung G et al., 2011). Such evidence proposes action in the neurodegenerative disease, because CgA signifies a significant component of the plaques found in people with Alzheimer's disease (Rangon C et al., 2003) and the resulting CAT generates a chemotactic effect on monocytes (Egger M et al., 2008) surrounding and invading plaques (Lechner et al., 2004). Furthermore, bovine CAT is very potent agent and it can directly inhibit growth of bacteria, yeast, and fungi, including Gram-negative and positive (Briolat J et al., 2005; Radek K et al., 2008; Akaddar A et al., 2010; Shooshtarizadeh P et al., 2010; Metz-Boutigue MH et al., 2010), likely due to its highly cationic nature, a typical aspect of antibacterial compound (Aslam R et al., 2013).

2.7.4-Cateslytin

The short active form of CAT is Cateslytin (bCtl; bCgA344-358). It corresponds to the antimicrobial domain (Briolat J et al., 2005; Biswas N et al., 2009). The N-terminal sequences bCgA344-351 and bCgA 348-358 are active. However, the C-terminal sequences bCgA352-358 is not active. Ctl has immune regulation properties (Zhang K et al., 2006) and it can also regulate smooth muscle cell proliferation (Guo X et al., 2011). Endogenous construction of CAT is further processed by cysteine protease membrane cathepsin L (CTSL) by an extra cleavage R358-G359 of catestatin in chromaffin secretory vesicles (Lee J et al., 2003). The interaction of Ctl with a fungal membrane, *via* adoption of an accumulated antiparallel beta-sheet structure occurs right at membrane interfaces (Jean-Francois F et al., 2009). Such a mechanism often leads to the separation of rigid and fluid membrane structures referring to the

fungal membrane, on models that contain ergosterol, ultimately leading to the membrane's disruption (Jean-Francois F et al., 2009). The utilization of zwitterionic biomembranes shows development of beta-sheets, generating 1 nm diameter pores (Jean-Francois F et al., 2008b).

Ctl through the use of NMR studies demonstrated conversion into anti-parallel beta-sheets gathering at bacterial membranes', negatively charged surface (Jean-Francois F et al., 2007; Jean-Francois F et al., 2008a). The significance of Arginine residues in binding to negatively charged lipids is notable (Postma T.M. and R.M.J. Liskamp, 2016). Subsequent Ctl interaction lead various thicker and more rigid membrane domains (Jean-Francois F et al., 2008a). Ctl can be regarded as an extremely potent AMP with ability to inhibit yeast, bacterial, and fungal growth observed at micromolar concentrations (Postma T.M. and R.M.J. Liskamp, 2016). Additionally, with mammalian cells it is nontoxic (Briolat J et al., 2005). Heightened activity of Ctl comparatively to other Cgs-derivative peptides is attributed thanks in part to the small size (15 residues) and +5 net charges (R344, R347, R351, R353, R358) and 5 hydrophobic residues (M346, L348, F360, Y355, F357), offering better communication between negatively charged bacterial membrane. Lastly, deeper microbial membrane penetration can be attributed to a high arginine ratio.

Combination peptides and conventional antibiotics

3.1- Combination peptides and conventional antibiotics

Innate immunity with AMPs and their mechanisms of action are well considered during evolution. However, the knowledge of their pharmacodynamic is still very confined in *vitro* and in *vivo* as well.

A huge revolution had occurred in the medical field when different categories of antibiotics have been discovered (Fleming A, 1929). AMPs also have been offered and being accepted as a novel antibiotic, due to offering a wide spectrum of activities against various bacterial strains (Zasloff M, 2016), and they are a very important component of immune defences in multicellular organisms (Johnston P and Rolff J, 2013; Johnston P et al., 2014).

Due to excessive use of antibiotics, the resistance phenomena have been acquired by numerous microorganisms (Magiorakos A et al., 2012), then impairs our capacity to treat infections, posing a growing challenge for global public health (Levy S and Marshall B, 2004; Bergstrom C and Feldgarden M, 2008; Smith R and Coast J, 2013). Therefore, to address this bacterial resistance, many solutions are currently under scrutiny.

The combination strategy is one of the suggestions to take advantage of the full possibility of existing antibiotics. Another way to search for possibility to find natural antimicrobial agents such as AMPs that have antimicrobial activity against wide range of pathogenic microbes and they might support the present antibiotic power. Some AMPs available commercially which are now prescribed and used clinically as alternative to the conventional antibiotics or accompany with them (Giuliani A et al., 2007). Moreover, in the natural conditions, these AMPs are supposed to be less to produce resistance and mutagenesis, although, it can obtain resistance strains in the laboratory under intensive selections (Perron G et al., 2006; Rodriguez-Rojas A et al., 2014; Dobson A et al., 2014).

In the last few decades, the clinical successes confirmed the combination therapy which is multi-advantageous and useful (Zimmermann G et al., 2007). The interaction or the cooperation between two or more antimicrobial agents, that can produce effects of their combination such as synergistic, additive and antagonist (Greco W et al., 1995; Chou T, 2006; Cokol M et al., 2011; Imamovic L and Sommer O., 2013). During the combination of the agents, the results of effects could be stronger, equal and/or weaker than the antimicrobial agent individually (Loewe S, 1953; Chou T, 2010). One of the notable advantage through the combination treatment is synergistic effects that can probably retard the drug resistance development, minify the individual drugs dosage, elimination of resistance strains, and toxicity,

therefore it can reduce side effects (Hegreness M et al., 2008; Lehar J et al., 2009; Cokol M et al., 2011; Tamma P et al., 2012; Imamovic L and Sommer O, 2013; Worthington R and Melander C., 2013). Therefore, the only common strategy to develop the new antibiotics that are to treat and to eliminate multidrug-resistant bacterial infections is synergistic drug combination.

The definition of synergy that can occur between the agents of antimicrobial is a greater than 2 log rises in the activity of bactericidal in *vitro* when compared with the activity of bactericidal of each agent alone (Klastersky J et al., 1977; Klastersky J and Zinner S, 1982; Giamarellou H et al., 1984; Giamarellou H, 1986; Den Hollander J et al., 1997).

To increase the chance of treatment, along with antibiotics combination, natural AMPs have been combined with antibiotics (McCafferty D et al., 1999; Brumfitt W et al., 2002; Hancock R and Sahl H, 2006; Naghmouchi K et al., 2012; Bahar A and Ren D, 2013). AMPs has shown several mechanisms of action which can be listed into two types: membrane lysis and no membrane lysis (intracellular targets) (Shah P et al., 2016).

Commonly, numerous of AMPs dependent on concentration, and dual mechanisms are notified for them. The membrane lysis caused by AMPs at high concentration, whereas at low concentration the membrane will not lysis (Cudic M and Otvos L, 2002). Therefore, AMPs as excellent candidates in the development of future antimicrobial agents' due to selective target, wide spectrum, diverse mode of action and lower toxicity (Hancock R and Patrzykat A, 2002).

3.1.1- Analysis of the antimicrobial peptides of the combination (Antibiotic / AMPs)

Combination of peptides with conventional antibiotics will be calculated taking into account the minimal inhibitory concentration (MIC) of the antibiotic and the MIC of the peptide. Then evaluated the Fractional Inhibitory Concentration (FIC).

The microdilution concentration assays are used. (Sueke H et al., 2010) Therefore, to calculate the FIC index must divide the MIC of peptide/antibiotic within combination by the single peptide/antibiotic. Moreover, with this FIC of the antibiotic and the FIC of the peptide, the determination of the FIC index corresponding to the addition of both FIC. Finally, depending on the FIC index, it could be identified the effect of the combination.

The FIC index for the combination of different two antimicrobial agents (peptide/antibiotic) was calculated according to the following equation:

FIC index= (MIC of Peptide X in combination) / (MIC of Peptide X alone)

+ (MIC of antibiotic Y in combination) / (MIC of antibiotic Y alone)

FIC index was interpreted as follows: $\leq$0.5: synergistic activity, 0.5–1: additive activity, 1–4: indifference, >4: antagonism (Sueke H et al., 2010).

Finally, several studies support combinations of AMPs with antibiotics for increasing the activities with a fabulous synergistic effect (McCafferty D et al.,1999; Brumfitt W et al., 2002; Naghmouchi K et al., 2012). Hence, by combining AMPs with conventional antibiotics a new hope for facing the battle with large spectrum of microorganisms appeared.

PART-II
MATERIALS AND METHODS

1-Purification of synthetic antimicrobial peptides

The synthetic peptides were purchased from Proteogenix (Schiltigheim, France): Ctl corresponds to the L and D forms of bovine L-cateslytin (L-Ctl) and D-cateslytin (D-Ctl) bCgA344-358 (RSMRLSFRARGYGFR). They were obtained with >95% of purity with a molecular weight of 1860 Da.

In order to verify if peptides were oxidized during storage, they were chromatographed by using a Dionex HPLC system (Ultimate 3000; Sunnyvale, CA USA) on a Macherey Nagel Nucleosil RP 300-5C18 column (10 × 250 mm; particle size 5 µm and pore size 100 nm). The fraction containing synthetic peptides were then detected at 214_{nm} of absorbance. The identification is based on the retention time and the absorbance $A214_{nm}$. The elution of material was obtained with a gradient which consisted of (Solvent A) 0.1% (v/v) Trifluoroacetic acid (TFA) in water and (Solvent B) 0.09% (v/v) TFA in 70% (v/v) acetonitrile-water. The rate of the elution flow was 700µL/min with the gradient of solvent B as indicated on chromatograms.

Then the different peaks containing peptides were analysed by Matrix Assisted Laser Desorption Ionization-Time of Flight (MALDI-TOF) mass spectrometry (UltraflexTM TOF/TOF (BrukerDaltonics, USA) (Sizova D et al., 2007) and by automated Edman sequencing (Briolat J et al., 2005) on an Applied Sequencing System Procise (Applied Biosystems, Foster City, USA) (Metz-Boutigue MH et al., 1998) in order to evaluate the concentration of synthetic peptides. The peptides used for the different assays correspond to non-oxidized forms.

2-Antimicrobial activity analysis

Numerous microorganisms of gram-positive (*Staphylococcus aureus* methicillin sensitive, *S. aureus* methicillin resistance and *Parvimonas micra*), gram-negative bacteria (*Prevotella intermedia, Fusobacterium nucleatum, Escherichia coli* wild-type and *E. coli* K-12 mutant E2146), as well as *candida albicans* were tested to determine the antimicrobial activities of peptides and conventional antibiotics too. Antibacterial and antifungal activities were performed to evaluate the antimicrobials.

S. aureus methicillin sensitive (ATCC® 25923™) (MSSA) was purchased from ATCC and *S. aureus* methicillin resistance (MRSA) S1 strain was kindly provided by Dr Gilles Prévost (Institute of Bacteriology EA7290, Strasbourg). Other oral cavity pathogens such as:

Parvimonas micra (ATCC® 33270™), *Prevotella intermedia* (ATCC® 49046™), *Fusobacterium nucleatum* (ATCC® 49256™) were purchased from ATCC (Manassas, USA). *Candida albicans* (ATCC® 10231™) and *Escherichia coli* (ATCC® 25922™) were also purchased from ATCC and *Enterococcus feacalis* (CCM 2541) was provided by the Czechoslovac Collection of Microorganisms. Furthermore, *E. coli* K-12 mutant E2146 was kindly provided by the Institut Pasteur of Paris. This strain was constructed from *E. coli* MG1655 (*E. coli* genetic stock center CGSC#6300) and is resistant to ampicillin, chloramphenicol and kanamycin (Francius G et al., 2011).

2.1- Antibacterial and antifungal assays

E. coli and *S. aureus* were precultured aerobically at 37°C according to the instructions. *E. coli* strains were cultured in Luria Bertani broth pH 7.0± 0.2 (Sigma, Le pont de Claix, France), *S. aureus* strains were cultured in Mueller Hinton broth pH 7.3± 0.1 (Difco, USA). In addition, *P. micra, F. nucleatum and P. intermedia* were cultured anaerobically in Anaerobe Basal Broth pH 6.8± 0.2 (Oxoid, Hampshire, England).

All the strains above mentioned were firstly plated on the agar plates and cultivated for 24h at 37°C. After incubation, one colony per isolate was transferred to 5 mL of culture medium and incubated with shaking for overnight at 37°C. The cultures were suspended at absorbance of OD_{600} = 0.001 in specific media according to bacteria strain or fungi. OD_{600} was evaluated with a spectrophotometer (BIO-RAD Smatspec™ plus).

All the strains mentioned above were treated with different concentrations of synthetic peptides or conventional antibiotics. They were incubated in 96-wells plates for 24h at 37°C with shaking. As a positive control Tetracycline (10µg/mL) and Cefotaxime (0.1µg/mL) was used to evaluate the percentage of growth inhibition of strains. All the assays were evaluated by the OD_{620} with a Multiskan EX microplate spectrophotometer (Thermo Fisher Scientific) and performed in triplicate.

2.2-Minimum inhibitory concentration (MIC) determination

After a statistical analysis of the lowest concentration of antimicrobial agents (peptides/antibiotics) capable to inhibit 100% of the inoculum, the MIC value was identified for each molecule and its action against a specific strain. This MIC was determined from a mod-

ified Gompertz function as described in (Lambert R and Pearson J, 2000).

2.3- Combination of peptides with antibiotics

In order to decrease the concentration of the antibiotics administered, a combination of D-Ctl with conventional antibiotics may allow to highlight the synergistic or additive effects. Therefore, the D-Ctl could potentiate the antimicrobial effect of numerous antibiotics, such as Cefotaxime which prescribed to treat *E. coli* resistance strains infections, Vancomycin and methicillin which were used to fight against *S. aureus* infections as well as Amoxicillin which was recommended to treat infections related to oral cavity pathogenic.

After determining the antibiotics and D-Ctl MICs for all strains, antimicrobial assays were performed as previously described in (Section 2.1). The strains were incubated in 96-wells plated and treated with combination of the antibiotics with D-Ctl as following formula; ½ MIC of D-Ctl + ½ MIC of antibiotic, ½ MIC of D-Ctl + ¼ MIC of antibiotic and ½ MIC of D-Ctl + 1/10 MIC of antibiotic. Then, ¼ MIC of D-Ctl + ½ MIC of antibiotic, ¼ MIC of D-Ctl + ¼ MIC of antibiotic and ¼ MIC of D-Ctl + 1/10 MIC of antibiotic.

Then calculate a parameter called FIC which is:

$$FIC_{antibiotic} = MIC_{antibiotic} \text{ in combination} / MIC_{antibiotic} \text{ alone}$$

$$FIC_{D\text{-}Ctl} = MIC_{D\text{-}Ctl} \text{ in combination} / MIC_{D\text{-}Ctl} \text{ alone}$$

With the FIC of the antibiotic and the FIC of the peptide, we determined the FIC index corresponding to the addition of both FICs. Finally, depending on the FIC index, it could identify the effect of the combination.

$$\text{FIC Index} = FIC_{antibiotic} + FIC_{D\text{-}Ctl}$$

3-Peptides stability assays by using HPLC

In order to evaluate the stability of synthetic peptides in different medium, experiments were assessed in the supernatant of different strains of bacteria and human saliva which were collected from healthy volunteers by using HPLC according to the methods previously reported (Section 1).

3.1-Stability against bacterial virulence factors

Bacterial supernatants were analyzed by using HPLC and the supernatants were prepared for all the bacterial strains which have been mentioned in the (Section 2). First of all, the bacteria were precultured in 5mL of culture medium as indicated above (Section 2.1) and incubated for 24h at 37°C. Secondly, the culture was centrifuged at 10000g for 1 min and the supernatant filtered by using a 0.22µm MillexH-GV (Millipore, Carrigtwohill, Ireland) to eliminate the presence of bacteria. Then 1mL of each supernatant for was incubated 48h at 37°C to check the absence of bacteria. Finally, the peptides D- and L-Ctl were incubated in the bacterial supernatant previously prepared during 24h at 37°C and the analysis of the peptides stability is obtained by using a Dionex HPLC system (Ultimate 3000; Sunnyvale, CA USA) on a Nucleosil reverse-phase 300–5C18-column (46250 mm; particle size: 5 mm; porosity, 300 Å) (Macherey Nagel, Hoerdt, France) according to the method previously reported (Section 1).

3.2-Stability against saliva

In this assay samples of saliva 100µL were collected from 4 men and 7 women healthy volunteers from the Odontology Faculty of the University of Strasbourg, France. These samples were collected according to European legislation and incubated with or without D-Ctl or L-Ctl (230µg/mL=123µM) in presence of water 100µL. Then, the samples were incubated for 24h at 37°C, centrifuged for 5min at 14000g at 4°C. Afterward, these samples were diluted 3 times in water with 0.1% (v/v) formic acid.

In order to evaluate the stability of each sample 2µL of treated peptide was analysed by using the LC-SRM (Liquid Chromatography-Selected Reaction Monitoring) technology in collaboration with the laboratory LSMBO (Strasbourg, France) (MacLean B et al., 2010).

4-Evaluation cytotoxicity of peptides for mammalian cells

Basically, to determine the cytotoxicity of L- and D-Ctl peptides several experiments were performed on different human cell lines and primary cells as well: human gingival fibroblasts (HGF-1) cell line, human intestinal epithelial cell line (Caco-2) and peripheral blood mononuclear cells (PBMCs). The cytotoxicity was determined by MTT [3(4,5-dimethylthiazol-2-yl)-2,5 diphenyl tetrazolium bromide] (Sigma-Aldrich, Oregon, USA) assays.

4.1- Cytotoxicity for HGF-1

HGF-1 cell line (ATCC® CRL-2014™) were cultured by Pauline Dartevelle in DMEM medium (Sigma-Aldrich) at 37°C in a 5% CO2 humidified incubator, and supplemented with 10% foetal bovine serum (Gibco) and 1% penicillin/streptomycin (Prepared in the laboratory). Then the cells were cultivated into 96-well plates at concentration 10^6 cellules/mL for 24h at 37°C before being treated with serial dilution of peptide at different concentration (0-100µg/ mL) for 24, 48 or 72 h.

Finally, MTT was added at final concentration of 0.25mg/mL to each well. Then for approximately 3h of incubation the cells at 37°C in a 5% CO2 humidified incubator, the cells were lysed with isopropanol/HCL (96:4, v/v). Afterward, the cells were incubated for 15min at room temperature with shacking and by using Multiskan™ EX microplate spectrophotometer (Thermo Fisher Scientific) the cells viability was assessed by optical density OD_{570nm}. Experimental was performed in triplicate.

4.2- Cytotoxicity for Caco-2

The Caco-2 cell line (ATCC® HTB-37™) was kindly provided by Dr Benoit Frisch (UMR 7199 CNRS University of Strasbourg) and these cells were cultured in Eagle`s Minimum Essential Medium (Thermo Fisher Scientific) (MEM (1X) + GlutaMAX™-1) (Gibco, UK) at 37°C in a 5% CO2 humidified incubator, and supplemented with 20% bovine calf serum and 1% penicillin/streptomycin. During the confluence of the cells or in their exponential phase growth that can estimate the good number of adherent cells, these cells were cultivated into 96-well plates at cells concentration $1X10^6$ cells/mL for 24h at 37°C before being treated with serial dilution of L-Ctl and D-Ctl. After 72h, MTT was added at final concentration of 0.25mg/mL to each well. Then for approximately 2 h of incubation the cells at 37°C in a 5% CO2 humidified incubator, the cells were lysed with isopropanol/HCL (96:4, v/v). By using a Multiskan™ EX microplate spectrophotometer (Thermo Fisher Scientific), the cytotoxicity of the cells was assessed by optical density OD_{570nm}. Experimental was performed in triplicate.

4.3- Cytotoxicity for PBMCs

PBMCs picked up from healthy volunteers were obtained from the blood transfusion centre of Strasbourg (Etablissement Français du Sang, Strasbourg) and these cells were isolated by density gradient centrifugation using Lymphoprep™ (Stemcell Technologies). PBMCs were then maintained in AIM V® medium (Thermo Fisher Scientific) at 37°C in a 5% CO2 humidified incubator. PBMCs were treated directly with serial dilution of L-Ctl and D-Ctl for 72h. Then MTT was added to each well at final concentration of 0.25mg/mL. The following steps which performed to determine the cytotoxicity as such that performed for Caco-2 cells.

4.4-Haemolysis assays

The lysis of red blood cells was monitored by the release of hemoglobin to the extracellular environment. Whole blood from one healthy volunteer was obtained from the blood transfusion centre of Strasbourg (Etablissement Français du Sang, Strasbourg) and washed twice with PBS (800g, 10 min). Red blood cell suspensions were then incubated with D-Ctl or L-Ctl at different concentrations (0-100 µg/mL) for 1h at 37°C. As a positive control, total lysis of red blood cells was obtained by incubating the cells with 0.1% SDS. After the incubation, cells were centrifuged at 800 g for 10 min and diluted with 1 mL of PBS. Haemoglobin released was determined by optical density OD_{420} using a Multiskan™ EX microplate spectrophotometer (Thermo Fisher Scientific, USA).

5-Inflammatory effects

Human PBMCs were prepared as previously described and treated for 24h with 60 µg/mL D-Ctl or L-Ctl. Supernatants were then filtered and assessed for cytokine dosage according to the manufacturer's instructions. The following cytokines were measured using the Bio-Plex® Multiplex Immunoassay system (Bio-Rad): G-CSF, GM-CSF, IFN-γ, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12, IL-13, IL-17, MCP-1, MIP-1β, TNF-α (Panezai J et al., 2017). This experimental was in realized collaboration with Dr. Gilles Prévost (Institute of Bacteriology EA 7290, Strasbourg).

6-Acquired resistance assays

To compare the use of conventional antibiotics such Ampicillin or Cefotaxime and D-Ctl to treat *E. coli* (ATCC® 25922™), resistance assays were performed. *E. coli* was cultured over 24 days at ½ MIC. The changes in the MICs values of the peptides and antibiotics against the inoculum were determined each 3 to 4 days.

One colony of *E. coli* was precultured in 3mL of LB medium and incubated with ½ MIC of antimicrobials (Ampicillin, Cefotaxime, D-Ctl) for 24h at 37°C. Then medium was changed every 24h with adding the same concentration (½ MIC) for each antimicrobial compound and 30µL of culture bacteria. To determine the new MIC for each antimicrobial compound, the cultures were centrifuged at 13.2*1000^{rpm} for 1 min. After getting rid of the supernatant, fresh medium was added with same volume at the beginning. Then the bacteria were suspended at absorbance of OD_{600}=0.001and bacteria were plated in 96-well plated in the present of different concentration of antibacterial compound which had mentioned above. After 24h of incubation the bacteria were assessed by optical density OD_{620} using a Multiskan EX microplate spectrophotometer (Thermo Fisher Scientific) (Ling L et al., 2015).

7-Analysis of interaction between D-Ctl and planktonic *E. coli* 2146

The bacterial model used for the physicochemical analysis (AFM, infrared spectroscopy and epifluorescence microscopy) is *E. coli* MDR. Bacteria were cultured in Luria Broth (Miller, Fluka) at 25 g/L (LB) or at 6.25 g/L (LB/4) in deionized water (Purelab Option, ELGA). All the cultures were incubated in a water bath shaker (Inova 3100, New Brunswick Scientific) at 37 ± 1°C and under continuous agitation at 160rpm. After an overnight subculture (16h, with ampicillin and kanamycin), bacteria were cultured in 200 mL of LB medium (without antibiotics) with an initial optical density at 600 nm (OD_{600}, measured with a cell density meter Biochrom AG, Fisherbrand) of 0.050 ± 0.005.

For epifluorescence and infrared spectroscopy analyses, the antimicrobial assays against planktonic *E. coli* MDR were performed in sterile 96-well plates (Nunc) in a final volume of 200 mL. When the optical density of the bacterial culture reached an OD_{600} value between 0.5 and 0.6 (bacteria were at the end of the exponential phase), the suspension was diluted in LB or LB/4 to give an OD_{600} = 0.10 ± 0.01. The necessary volume of the stock solution of the peptide at 1 g/L was spotted in the bacterial suspension. Sterility and growth controls were ster-

ile LB and LB/4, and a bacterial suspension without peptide, respectively. The plate was incubated for 20h at 22°C.

7.1-Epifluorescence optical microscopy

To analysis planktonic bacteria by fluorescence microscopy using *Bac*Light™ bacterial viability kit (L7012, Molecular Probes, Eugene, USA) was used. This kit allows to determine the permeability of the sessile cells in case of present and absence of the AMP. The kit used a mixture of SYTO-9 green-fluorescent nucleic acid stain (Excitation/emission maxima: 480/500nm) and the red- fluorescent nucleic acid stain propidium iodide (excitation/ emission maxima: 490/635nm). Therefore, bacteria with intact membranes fluoresce green, while bacteria with damaged membranes fluoresce red. After 20 hours of incubation, 200 µL of the 24 hours-old bacterial suspension were mixed with 300 µL of *Bac*Light™ solution (15 µL of the reconstructed *Bac*Light™ solution as described by the manufacturer in 300 µL of sterile water), and stained for 20 min in the dark at 22 ± 1°C. The suspension was then filtrated with 0.2 µm black filters (Millipore, GTBP04700) and rinsed three times with sterile water to eliminate excess *Bac*Light™. The sample was mounted in *Bac*Light™ mounting oil as described by the manufacturer. Both fluorescences were viewed simultaneously with the 100X oil immersion objective of an Olympus BX51 microscope equipped with an Olympus XC50 camera.

7.2-ATR-FTIR spectroscopy

The recording for ATR-FTIR spectra was between 4000 and 800 cm^{-1} on a Bruker Vertex 70v spectrometer equipped with a KBr beam splitter and a DTGS detector and driven by the OPUS 7.5 software. Single beam spectra for the resolution was 4 cm^{-1}. For getting spectra, A nine-reflection diamond ATR accessory was used (DurasamplIRTM, SensIR Technologies, incidence angle: 45°). The number of bidirectional double-sided interferogram scans was 200, which corresponds to a 2min accumulation. By using Blackman-Harris three-term apodization function and Mertz phase correction mode, all interferograms were Fourier processed. There was no ATR correction performed. In an air-conditioned room and at 21 ± 1°C the measurements were assessed. 50µL of the bacterial suspensions in their culture media

was put on the ATR crystal. To eliminate the spectral background, centrifuged a half of the suspension at 8000rpm during 5 min. Subtraction of water steam was performed when needful.

7.3-AFM mechanical properties measurements

Using a MFP3D-BIO instrument, AFM experiments were performed (Asylum Research Technology, Oxford Instruments Company, Mannheim, Germany). Conical shape of silicon nitride cantilevers was bought from Asylum Research Technology (Olympus TR400 PSA, Mannheim, Germany). The spring constants of the cantilevers measured using the thermal noise method were found to be 0.02-0.03 nN/nm. In PBS and at room temperature these tests were performed in triplicate. The method that used to define Young's modulus was the nanoindentation that determines the force versus indentation curves. It can be obtained mechanical properties during the recording a grid map of 50-by-50 force curves, which can be done on numerous of bacterial clusters that include at least 10 bacteria electrostatically immobilized onto PEI coated glass substrate. The approach rate was 2 µm/s, the maximal loading force was 4 nN and the piezodrive was fixed to 2 µm. According to the Sneddon model, the analysis of the approach curves was estimated by the histograms which corresponding to the statistic distribution of the Young modulus (Sneddon I, 1965; Gavara N and Chadwick R, 2012) where δ is the indentation depth, *R* is the curvature radius of AFM-tip apex, ν the Poisson coefficient and *fBECC* the bottom effect correction described by Gavara et Chadwick (Gavara N and Chadwick R, 2012). By mean of an automatic Matlab algorithm, all the FVI were analyzed and they are described elsewhere (Polyakov P et al., 2011). Bacteria were exposed in presence of different concentration of D-Ctl (8, 40 and 80 µg/mL) as well as in different concentration L-Ctl (8, 150 and 750 µg/mL) which are assessed in PBS buffer for 20h at 22 °C. Mechanical properties were measured by AFM in force mapping mode at indentation rate of 2 µm/s and the average values correspond to at least 500 force curves taken from at least 10 bacteria. After exposure to the peptide, bars labelled with * and ** the corresponding values were obtained after only 3 and 0.8 hours, respectively. In fact, all bacteria were extremely damaged after these periods of exposure and not enough for relevant measurements.

8- Time-lapse videomicroscopy of interaction peptide and *Candida albicans*

To investigate the interaction between *Candida albicans* and D-Ctl, time-lapse video microscopy has been used. This study analyzed by Pauline Dartevelle.

II- Matériels et méthodes en français

1-Purification de peptides antimicrobiens synthétiques

Les peptides synthétiques ont été achetés auprès de Proteogenix (Schiltigheim, France): Ctl correspond aux formes L et D de la L-cateslytine bovine (L-Ctl) et de la D-cateslytine (D-Ctl) bCgA344-358 (RSMRLSFRARGYGFR). Ils ont été synthétisés avec une pureté > 95% et leur poids moléculaire est de 1860k Da. Afin de vérifier si les peptides ont été oxydés pendant le stockage, ils ont été chromatographiés en utilisant un système HPLC Dionex.

Ensuite, les différents pics contenant des peptides ont été analysés par spectrométrie de masse MALDI-TOF (UltraflexTM TOF / TOF (BrukerDaltonics, USA) (Sizova D et al., 2007) et par séquençage Edman automatique (Briolat J et al., 2005) sur le système de séquençage Procise (Applied Biosystems, Foster City, USA) (Metz-Boutigue MH et al., 1998) afin d'évaluer la concentration en peptides synthétiques. Les peptides utilisés pour les différents dosages correspondent à des formes non oxydées.

2-Analyse d'activité antimicrobienne

De nombreux micro-organismes à Gram positif (*Staphylococcus aureus* sensible à la méthicilline, *S. aureus* résistant à la méthicilline et *Parvimonas micra*) et à Gram négatif (*Prevotella intermedia, Fusobacterium nucleatum, Escherichia coli* sauvage et E2146 mutant E. coli K12), ainsi que *Candida albicans* ont été testés pour déterminer les activités antimicrobiennes des peptides et des antibiotiques conventionnels. Les activités antibactériennes et antifongiques ont été réalisées pour évaluer les caractériser peptides antimicrobiens.

Toutes les souches mentionnées ci-dessus ont été traitées avec différentes concentrations de peptides synthétiques ou d'antibiotiques conventionnels. Ils ont été incubés dans des plaques à 96 puits pendant 24 heures à 37°C sous agitation. La tétracycline (10 µg/mL) et la céfotaxime (0,1 µg/mL) ont été utilisées en tant que témoin positif pour évaluer le pourcentage d'inhibition de croissance des souches. Tous les dosages ont été évalués et réalisés en triple exemplaire avec un spectrophotomètre de microplaques Multiskan EX (Thermo Fisher Scientific). La lecture s'est faite à la Densité optique de 620 nm (DO 620 nm).

2.1-Détermination de la concentration minimale inhibitrice (CMI)

Après une analyse statistique de la plus faible concentration d'agents antimicrobiens (peptides / antibiotiques) capable d'inhiber 100% de l'inoculum, la valeur de la CMI a été identifiée pour chaque molécule et son action contre une souche spécifique. Cette CMI a été déterminée à partir d'une fonction modifiée de Gompertz, comme décrit dans (Lambert R et Pearson J, 2000).

2.2-Combinaison de peptides avec des antibiotiques

Afin de diminuer la concentration des antibiotiques administrés, une combinaison de D-Ctl avec des antibiotiques conventionnels permet de mettre en évidence les effets synergiques ou additifs. Pour calculer un indice appelé FIC la formule suivante a été appliquée :

$$FIC_{antibiotic} = MIC_{antibiotic} \text{ en combinaison} / MIC_{antibiotic} \text{ seul}$$

$$FIC_{D\text{-}Ctl} = MIC_{D\text{-}Ctl} \text{ en combinaison} / MIC_{D\text{-}Ctl} \text{ seul}$$

$$\text{Indice FIC} = FIC_{antibiotic} + FIC_{D\text{-}Ctl}$$

3-Stabilité contre les facteurs de virulence bactérienne

Les différents surnageants bactériens (Section 2) ont été analysés en utilisant l'HPLC. Tout d'abord, les bactéries ont été pré-cultivées dans 5 ml de milieu de culture comme indiqué ci-dessus (Section 2.1) et incubées pendant 24 h à 37°C. Deuxièmement, la culture a été centrifugée à 10000 g pendant 1 min et le surnageant a été filtré en utilisant un MillexH-GV de 0.22µm (Millipore, Carrigtwohill, Irlande) pour éliminer la présence de bactéries. Ensuite, 1mL de chaque surnageant a été incubé 48 h à 37°C pour vérifier l'absence de bactéries. Enfin, les peptides D- et L-Ctl ont été incubés dans le surnageant bactérien préalablement préparé pendant 24 h à 37°C.

3.1-Stabilité contre la salive

Dans cette étude, des échantillons de salive de 100µL ont été prélevés chez 4 hommes et 7 femmes volontaires sains de la Faculté d'Odontologie de l'Université de Strasbourg, France.

4-Cytotoxicité d'évaluation des peptides pour les cellules de mammifères

Pour déterminer la cytotoxicité des peptides L- et D-Ctl, plusieurs expériences ont été réalisées sur différentes lignées cellulaires humaines des et cellules primaires : fibroblastes gingivaux humains (HGF-1), cellule épithéliale humain de l`intestin (Caco-2) et cellules mononuclées du sang périphérique (PBMC). La cytotoxicité a été déterminée par dosages de MTT [3 (4,5-diméthylthiazol-2-yl) -2,5 diphényltétrazolium bromure] (Sigma-Aldrich, Oregon, USA).

4.1-Tests d'hémolyse

La lyse des globules rouges a été contrôlée par la libération d'hémoglobine dans l'environnement extracellulaire.

5-Effets inflammatoires

Des PBMC humaines ont été préparées comme décrit précédemment et traitées pendant 24 h avec 60 µg/mL de D-Ctl ou de L-Ctl. En utilisant Bio-Plex® Multiplex Immunoassay system (Bio-Red). Cette expérimentation a été réalisée en collaboration avec le Dr Gilles Prévost (Institut de Bactériologie EA 7290, Strasbourg) (Panezai J et al., 2017).

6-Essais de résistance acquis

Pour comparer l'utilisation d'antibiotiques conventionnels, tels que l'ampicilline ou la céfotaxime et le D-Ctl pour traiter *E. coli* (ATCC® 25922™), des dosages de résistance ont été réalisés. *E. coli* a été cultivé pendant 24 jours à ½ MIC. Les changements dans les valeurs de CMI des peptides et des antibiotiques contre l'inoculum ont été déterminés tous les 3 à 4 jours (Ling L et al., 2015).

7-Analyse de l'interaction entre D-Ctl et *E. coli* planctonique 2146

Le modèle bactérien utilisé pour l'analyse physico-chimique (AFM, spectroscopie infrarouge et microscopie à épifluorescence) est *E. coli* MDR. Pour les analyses d'épifluorescence et de spectroscopie infrarouge, les tests antimicrobiens contre la MDR planctonique de *E. coli* ont été réalisés dans des plaques stériles de 96 puits (Nunc) dans un volume final de 200 mL.

7.1-Microscopie optique à épifluorescence

L'analyse des bactéries planctoniques par microscopie à fluorescence, a été réalisée en utilisant un kit de viabilité bactérienne *Bac*Light™ (L7012, Molecular Probes, Eugene, USA). Ce kit permet de déterminer la perméabilité des cellules sessiles en présence et en absence de l'AMP. Le kit utilise un mélange d'acide nucléique fluorescent vert SYTO-9 (maximum d'excitation / émission : 480 / 500nm) avec l'iodure de propidium fluorescent rouge-acide de propidium (maximum d'excitation / émission : 490 / 635nm).

7.2-Spectroscopie ATR-FTIR

L'enregistrement pour les spectres ATR-FTIR était compris entre 4000 et 800 cm^{-1} sur un spectromètre Bruker Vertex 70v équipé d'un séparateur de faisceau KBr et d'un détecteur DTGS et piloté par le logiciel OPUS 7.5. Les spectres à faisceau unique pour la résolution étaient de 4 cm^{-1}. Pour obtenir les spectres, un accessoire ATR diamant à neuf reflets a été utilisé (DurasamplIRTM, SensIR Technologies, angle d'incidence: 45°). Le nombre de balayages d'interférogrammes bidirectionnels recto-verso était de 200, ce qui correspond à une accumulation de 2 minutes. En utilisant la fonction d'apodisation à trois termes de Blackman-Harris et le mode de correction de phase de Mertz, tous les interférogrammes ont été traités par les séries de Fourier.

7.3-AFM mesures des propriétés mécaniques

Des expériences d`AFM (Asylum Research Technology, Oxford Instruments Company, Mannheim, Allemagne) ont été réalisées en utilisant un instrument MFP3D-BIO. La forme

cônique des cantilevers en nitrure de silicium a été achetée chez Asylum Research Technology (Olympus TR400 PSA, Mannheim, Allemagne). Les constantes de ressort des porte-à-faux mesurées à l'aide de la méthode du bruit thermique se sont révélées être 0,02-0,03 nN / nm. Dans du PBS et à température ambiante, ces tests ont été réalisés en triple exemplaire. La méthode utilisée pour définir le module de Young était la nano-indentation qui détermine la force par rapport aux courbes d'indentation.

8-Videomicroscopie Time-lapse de l`interaction peptide -*Candida albicans*

La vidéo-microscopie accélérée a été utilisée pour étudier l'interaction entre *Candida albicans* et D-Ctl. Cette étude a été réalisée par Pauline Dartevelle.

PART-III
RESULTS

Thesis objectives

The general aims of this study are to find an alternative to the conventional antibiotics (antimicrobial agents) in order to prevent microbial resistance. Therefore, this study focuses on new antimicrobial peptides (AMPs) to develop new antimicrobial agent. AMPs are involved to treat pathogenesis of bacterial infections. They are important parts of innate immunity system in most multicellular organisms. Moreover, they can represent a deposit of new families of anti-infectious agents or compete in combination with conventional antibiotics.

The present thesis concerns a new potent antimicrobial peptide derived of bovine chromogranin A sequence (344-358) corresponding to the D-isomer of Cateslytin (Ctl). This study includes two parts relative to the antimicrobial activities of D-Ctl and its combination with calcium hydroxide Ca(OH2).

The first part concerns the characterization of D-Ctl compared to L-Ctl, in which all L amino acids have been replaced by D amino acids with the same sequence, but in dextrogyre conformation. Moreover, some of the conventional antibiotics were also compared with the two peptides tested. In this point of view, several technics approaches were used including microbiology (broth microdilution assays), cell biology (cells viability and inflammatory effects assays) and microscopy (ATR-FTIR spectroscopy, epiflorescence microscopy, atomic force microscopy). The results are as follow: D-Ctl acts on Gram-negative and Gram-positive bacteria and compared to L-Ctl (measurement of MICs). Moreover, D-Ctl can potentiate some antibiotics within the combination. More than that, the efficiency of D-Ctl as same as Ampicillin and it is more efficient than Kanamycin. D-Ctl does not generate cytokine release and is not haemolytic too. It is not toxic towards cell line and primary cells. Furthermore, D-Ctl is not degraded by the pathogen's virulence factors as well as D-Ctl does not trigger resistance on *E. coli*. Moreover, D-Ctl acts against *E. coli* MDR (atomic force microscopy and ATR-FTIR microscopy) after high damage of the cell wall of bacteria and the elasticity of bacterial cell wall was also decreased.

The second part focused on the role of D-Ctl in combination with calcium hydroxide Ca(OH)2 therapeutics in the root canal pathogens. A description of antimicrobial activity was conducted on *E. faecalis* which suggesting increased stability and antibacterial effect superior to calcium hydroxide alone. This combination also presented a low level of toxicity toward human gingival fibroblasts compared to Ca(OH)2 alone.

Introduction to manuscript I

D-Cateslytin, a new antimicrobial peptide with therapeutic potential

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Published by Scientific reports journal

Peptides are needful for human health. They have been vastly used for development new therapeutic agents to treat infections in general, cancers, cardiovascular, metabolic disease and immune system. Up to date, in the universal market pharmaceutical, there are more than 70 of these peptides used as commercial drugs. Nevertheless, 30% of these peptides or less are natural peptides. However, the proteases degrade the natural peptides easily because inducing losing of bioactivity. (Liu M et al., 2016). Therefore, the thing which is interesting for researchers newly, that improve the biological stability, binding activity of natural bioactive peptides and specificity of peptides which can be done by using the transformations of chemical structural. There are some main strategies used for chemical structural transformation of peptides: 1-Replace with an unnatural amino acid such as D-amino acids, b-amino acids and N-methyl-a-amino acids, 2-modification of C- or N- terminal regions, 3-retro-inverso or mirror-image phage- display peptides, 4-the sequence of peptide cyclization, 5-isosteric, or not, amide bond replacement between two amino acids (Vlieghe P et al., 2010).

Among these strategies replacement with D-amino acid is uncommonly potent. Therefore, in this study, we aimed to develop new antimicrobial peptides with therapeutic potential which is D-Ctl. D-Ctl is a derivative of L-Ctl, D-Ctl was modified to improve its efficiency to fight against bacterial infections. This D-Ctl and L-Ctl were tested in parallel on the large panel of microorganism and the results showed that D-Ctl displays a rapid direct killing of bacteria even in low concentration, compared to L-Ctl which was less efficient than D-Ctl with different in ranging of MICs starting from 1.7 (MSSA) to 17.9 folds (*E. coli* MDR). D-Ctl and L-Ctl showed also the ability to be stable in the supernatant of bacteria, except, L-Ctl was degraded by (*E. coli* wild type and *E. coli* MDR). Moreover, the data observed that D-Ctl and L-Ctl have not any toxicity to immune cells and other types of a human cell line. Interestingly, the results showed that D-Ctl does not trigger resistance to *E. coli* even some oth-

er conventional antibiotics such as Ampicillin and Cefotaxime were 3-fold MIC increased for 24 days. One more data for D-Ctl which was stable also in the human saliva. Moreover, the results show also the D-Ctl could potentiate the antibacterial effect of several antibiotics when they are combined together. Lastly with more interest, in collaboration with the group of UMR 7564 Nancy, France and their results demonstrated that after using infrared analysis *E. coli* metabolism were poorly impacted when was treated by D-Ctl and the bacterial cell wall is highly destroyed.

Manuscript-I

D-Cateslytin, a new antimicrobial peptide with therapeutic potential

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D-Cateslytin, a new antimicrobial peptide with therapeutic potential

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Received: 27 June 2017
Accepted: 20 October 2017
Published online: 09 November 2017

The rise of antimicrobial resistant microorganisms constitutes an increasingly serious threat to global public health. As a consequence, the efficacy of conventional antimicrobials is rapidly declining, threatening the ability of healthcare professionals to cure common infections. Over the last two decades host defense peptides have been identified as an attractive source of new antimicrobials. In the present study, we characterized the antibacterial and mechanistic properties of D-Cateslytin (D-Ctl), a new epipeptide derived from L-Cateslytin, where all L-amino acids were replaced by D-amino acids. We demonstrated that D-Ctl emerges as a potent, safe and robust peptide antimicrobial with undetectable susceptibility to resistance. Using Escherichia coli as a model, we reveal that D-Ctl targets the bacterial cell wall leading to the permeabilization of the membrane and the death of the bacteria. Overall, D-Ctl offers many assets that make it an attractive candidate for the biopharmaceutical development of new antimicrobials either as a single therapy or as a combination therapy as D-Ctl also has the remarkable property to potentiate several antimicrobials of reference such as cefotaxime, amoxicillin and methicillin.

The discovery of antimicrobials to treat infectious diseases is one of the greatest achievements of modern medicine. However, excessive and inappropriate use of antimicrobials fosters the emergence and spread of antimicrobial-resistant microorganisms. Indeed, infections caused by antimicrobial-resistant microorganisms also known as "superbugs" often no longer respond to conventional treatments, thereby extending the duration of the disease related to infection and even lead to patient death[1,2]. Antimicrobial-resistant microorganisms, including multidrug-resistant types, are often responsible for healthcare-associated infections and constitute a serious threat to public health worldwide, specifically among vulnerable populations such as critically ill patients[3]. Infections caused by Gram-negative bacteria are a particular concern for public health because these microorganisms are so versatile that they can exchange genetic material and rapidly deploy an arsenal of resistance mechanisms, particularly under selective pressure[4]. Especially, this phenomenon resulted in a drastic increase in the prevalence of *Escherichia coli* multidrug-resistant (*E. coli* MDR) strains and the onset of healthcare-associated urinary tract or bloodstream infections[5–8].

Novel classes of antimicrobials were rare in the past thirty years and of sharp administration. Specifically, the discovery of fluoroquinolones in the 1970s brought to an end the portfolio of antimicrobials against Gram-negative bacteria[9]. Nevertheless, antimicrobial therapy remains the prophylactic and curative practice most commonly used to fight against infections in the city and the hospital. However, due to the emergence of selected antimicrobial-resistant microorganisms and the lack of new antimicrobials on the market, we are now facing the possibility of a future without effective antimicrobials for treating bacterial infections. As a consequence, there is a persisting and urgent medical need to develop new antibacterial compounds.

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Pathogen	Gram	Respiratory type	MIC (peptide)		Antibiotic of reference	
			L-Ctl (μg/mL)	D-Ctl (μg/mL)	Name	(μg/mL)
Escherichia coli (ATCC 25922)	−	Facultative anaerobe	75	8.0	Ampicillin	7.0
					Kanamycin	21
Escherichia coli (MDR) (K-12)	−	Facultative anaerobe	150	8.4	Cefotaxime	0.1
Fusobacterium nucleatum (ATCC 49256)	−	Obligate anaerobe	125	22	Amoxicillin	0.6
Prevotella intermedia (ATCC 49046)	−	Obligate anaerobe	149	10	Amoxicillin	0.5
Parvimonas micra (ATCC 33270)	+	Obligate anaerobe	120	23	Amoxicillin	0.5
Staphylococcus aureus (MSSA) (ATCC 25923)	+	Facultative anaerobe	40*	24	Methicillin	1.2
Staphylococcus aureus (MRSA) (S1)	+	Facultative anaerobe	37*	18	Vancomycin	0.8

Table 1. Antibacterial activity of D-Ctl compared to L-Ctl. The percentage of growth inhibition of the indicated pathogens in the presence of different concentrations of D-Ctl or L-Ctl was determined by broth microdilution assays. Each MIC, defined as the lowest concentration of a drug able to inhibit 100% of a bacterial inoculum, was determined using a modified Gompertz function. Experiments were performed with biological replicates. *Values obtained from Aslam *et al.*[18].

Over the last two decades, host defence peptides (HDPs) have emerged as new attractive candidates in the development of novel anti-bacterial treatments, specifically for antimicrobial-resistant infections[10]. The benefits of using HDPs are that they act by disrupting the bacterial membranes, a mechanism that is fast and non-specific. Therefore bacteria are not prone to develop high-level resistance towards these compounds in the same extent as towards conventional antimicrobials[11]. Moreover they display a broad-spectrum of pathogens, including multidrug resistant Gram-positive and negative bacteria[12]. HDPs are usually rather short (12–50 amino acids), cationic and amphiphilic with a broad diversity in their secondary structure and well preserved during evolution. HDPs are naturally present in tissues frequently exposed to pathogens, such as the skin, lungs, and gastrointestinal tract. Besides their broad spectrum of antimicrobial properties, they also exhibit significant immunomodulatory effects[13].

Among all isolated and characterized HDPs, Cateslytin (Ctl) constitutes an excellent candidate for the development of a new class of antimicrobials. Indeed, Ctl is short and linear (15 amino acids) and therefore very easy to synthesize for a minimal cost. Moreover, it is stable at high temperature and low pH. Ctl results from the proteolysis of chromogranin A, an acidic protein stored in the secretory vesicles of numerous neuroendocrine and immune cells and is released upon stress in most of the body fluids[14–17]. In addition to its antibacterial properties, Ctl is also a potent antifungal agent[18,19].

In the present study, we report the biological characterization of D-Ctl, a new epipeptide derived from L-Ctl, where all L-amino acids were replaced by D-amino acids (patent application: EP16 306539.4). Using various approaches including microbiology (broth microdilution assays), cell biology (viability and cytokine release assays) and microscopy (atomic force microscopy, epiflorescence microscopy, ATR-FTIR spectroscopy), we characterized the biological and mechanical properties of D-Ctl compared to its conformer L-Ctl. Overall, D-Ctl emerges as a potent, safe and stable antimicrobial that damages bacterial cell walls and still not suffer of any microbial resistance.

Results

D-Ctl is an efficient antimicrobial agent against various bacterial strains. One of the downfalls of the use of therapeutic peptides relies on their lack of proteolytic stability towards proteases. One way of controlling the stability of a therapeutic peptide is to synthesize its epimer, which has the same sequence as the parent peptide with all levogyre (L) amino acids replaced by dextrogyre (D) amino acids. Such peptides are more resistant to proteolysis, hence increasing their half-lives and bioavailability. Therefore, we synthesized D-Ctl and compared its respective antibacterial efficiency with L-Ctl. To this aim, we used a panel of Gram-negative strains: *Escherichia coli* wild type, *Escherichia coli* multidrug resistant (*E. coli* MDR), *Prevotella intermedia*, *Fusobacterium nucleatum* and Gram-positive strains: *Staphylococcus aureus* Methicillin Sensitive (MSSA), *Staphylococcus aureus* Methicillin Resistant (MRSA), *Parvimonas micra*. This panel includes facultative and strict anaerobes (Table 1). The antibacterial activity of D-Ctl versus L-Ctl was assessed by the measurement of their MIC (Minimal Inhibitory Concentration) defined as the lowest concentration of peptide able to inhibit 100% of the inoculum. Depending on the bacterial species, the MIC of D-Ctl ranged between 8 and 24 μg/mL (Table 1 and Supplementary Figure S1). D-Ctl was specifically efficient against *P. intermedia* with a MIC of 10 μg/mL and *E. coli* with a MIC of 8.0 μg/mL for *E. coli* wild type and 8.4 μg/mL for *E. coli* MDR. Overall, the MIC of D-Ctl was 2 to 18 times lower than the one of L-Ctl (Table 1 and Supplementary Figure S1).

We then compared the MIC of D-Ctl with the MIC of antimicrobials of reference. Interestingly, the antimicrobial activity of D-Ctl on *E. coli* was comparable to that of ampicillin and kanamycin and could therefore constitute an alternative treatment for *E. coli* infections (Table 1 and Supplementary Figure S2). Regarding the others species tested, the antimicrobials of reference were still more efficient than D-Ctl (Table 1 and Supplementary Figure S2).

D-Ctl is a potentiator for numerous antimicrobials of reference. We then investigated whether D-Ctl could potentiate the antibacterial effect of several antimicrobials of reference, specifically methicillin and vancomycin extensively prescribed to treat *S. aureus* infections, amoxicillin recommended in numerous

Pathogens	Combination	MIC $_{alone}$ (μg/mL)	MIC $_{combination}$ (μg/mL)	FIC	FICI	Effect
Escherichia coli MDR	D-Ctl	8.4	4.2	0.5	1.0	Additive
	Cefotaxime	0.1	0.05	0.5		
Fusobacterium nucleatum	D-Ctl	22	11	0.5	1.0	Additive
	Amoxicillin	0.6	0.3	0.5		
Prevotella intermedia	D-Ctl	10	2.5	0.25	0.5	Synergistic
	Amoxicillin	0.5	0.125	0.25		
Parvimonas micra	D-Ctl	23	5.8	0.25	0.5	Synergistic
	Amoxicillin	0.5	0.125	0.25		
Staphylococcus aureus (MSSA)	D-Ctl	24	12	0.5	0.75	Additive
	Methicillin	1.2	0.3	0.25		
Staphylococcus aureus (MRSA)	D-Ctl	18	18	1	2	Indifferent
	Vancomycin	0.8	0.8	1		

Table 2. Antibacterial activity of D-Ctl in combination with conventional antimicrobials. The percentage of growth inhibition of the indicated pathogens in the presence of different concentrations of antimicrobials was determined by broth microdilution assays. The MICs of each drug were used to calculate the FIC index of each combination. Each experiment was performed at least in duplicate.

infections including periodontal infections and cefotaxime often used as second intention treatment against *E. coli* resistant strains. According to the European Committee on Antimicrobial Susceptibility Testing[20], the effect of a combination between two antibacterial compounds can be evaluated by their FICI (Fractional Inhibitory Concentration Index). The FICI consists of the sum of the FICs of both antibacterial agents: FIC index = $FIC_{antimicrobial} + FIC_{D\text{-}Ctl}$. For each compound, the FIC was determined as the ratio between the MIC of the compound in combination ($MIC_{combination}$) and the MIC of the compound acting alone (MIC_{alone}). On the basis of their FIC index, each combination was categorized as synergistic (≤ 0.5), additive (>0.5 to 1), indifferent (>1 to <4) or antagonistic (≥ 4).

For each strain, the MICs of D-Ctl and the antimicrobial of reference were evaluated (MIC_{alone}) (Table 2 and Supplementary Figures S1 and S2). Then, different combinations of D-Ctl and the antimicrobial of reference were tested in order to determine the $MIC_{combination}$. The FICI was then calculated as described above. We observed a synergistic effect between D-Ctl and amoxicillin for *P. micra* and *P. intermedia* and an additive effect for D-Ctl and cefotaxime, methicillin and amoxicillin on *E. coli* MDR, MSSA and *F. nucleatum*, respectively (Table 2 and Supplementary Figure S3). Regarding MRSA, no potentiator effect was highlighted between D-Ctl and methicillin (Table 2 and Supplementary Figure S3). Altogether, D-Ctl also emerges as an effective potentiator for several antimicrobials currently prescribed in clinic to fight severe bacterial infections.

Unlike ampicillin and cefotaxime, D-Ctl does not trigger resistance in *E. coli*. To assess whether *E. coli* would develop resistance under a selective pressure, we cultured *E. coli* wild type in the presence of sub-MIC concentrations of D-Ctl (½ MIC), ampicillin or cefotaxime for 24 days. Interestingly, *E. coli* failed to generate mutants of resistance as its MIC remained stable for the whole duration of the culture (Fig. 1). In contrast, the MICs of ampicillin and cefotaxime, two antimicrobials of reference used to treat *E. coli* infections, rapidly increase over the course of the culture to reach 3x MIC at day 24 (Fig. 1).

D-Ctl is not cytotoxic and does not elicit cytokine release. In order to investigate whether D-Ctl would be a good lead compound for the development of a new antimicrobial, we assessed several safety issues such as its haemolytic activity, cytotoxicity and immunogenicity through cytokine release.

One of the major side effects of conventional antimicrobials, but also several HDPs, is to alter the intestinal homeostasis by damaging the intestinal epithelial barrier[21]. To verify whether D-Ctl affects the integrity of the intestine epithelium, we assessed the cytotoxicity of D-Ctl towards Caco-2 cells, a human intestinal epithelial cell line. As shown in Fig. 2A, no cytotoxicity was measured after 72 hours of incubation with neither D-Ctl nor L-Ctl at concentrations up to 100 μg/mL.

In order to be administered as a systemic therapy, antimicrobials should not interfere with blood cells homeostasis. Subsequently, we assessed whether D-Ctl was toxic towards human erythrocytes but also human peripheral blood mononuclear cells (PBMCs). For haemolytic assays, D-Ctl or L-Ctl was incubated with human erythrocytes at concentrations ranging from 0 to 100 μg/mL. No cell lysis was observed at all, demonstrating that neither D-Ctl nor L-Ctl is haemolytic, even at concentrations higher than its MICs (Fig. 2B). Similarly, no cytotoxicity was detected in PBMCs following an exposure of 72 hours with D-Ctl or L-Ctl at concentrations up to 100 μg/mL (Fig. 2C and D).

In addition, an antimicrobial drug candidate should not trigger immunogenicity. To verify whether D-Ctl influences the immune system, we performed a cytokine release assay. To this aim, human PBMCs were treated with D-Ctl for 24 hours and cytokines were quantified after 24 hours in the cell supernatant using the Bio-Plex® technology (Bio-Rad). As indicated in Fig. 3A and B, no significant cytokine release was observed following D-Ctl or L-Ctl treatment. As a control, PBMCs were treated with LPS in the same conditions, resulting in the release of a broad spectrum of pro-inflammatory cytokines such as TNFα, G-CSF and IFNγ but also the anti-inflammatory

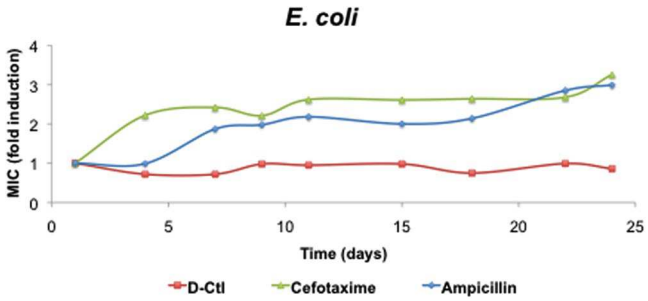

Figure 1. Resistance acquisition assay of *E. coli* in the presence of D-Ctl compared to ampicillin and cefotaxime. The *E. coli* wild-type strain was cultured in the presence of ½ MIC of the antibacterial agent for 24 days. The fold change in MIC was evaluated at the indicated days.

cytokine IL-10 (Fig. 3C). This result indicates that neither D-Ctl nor L-Ctl is associated with major cytokine release.

D-Ctl is more resistant to degradation by secreted bacterial proteases than L-Ctl. Linear L-peptides with α-helical structures are usually susceptible to proteolysis. As an example, V8 and aureolysin, two proteases secreted by *S. aureus* are responsible for the cleavage of the host defence peptide LL-37 and therefore contribute to bacterial survival[22]. The specific spatial configurations of the cleavage sites for these enzymes are not present in D-peptides although these peptides might be cleaved by non-specific hydrolysis during enzymatic digestion. Subsequently, we assessed the sensitivity of D-Ctl to secreted bacterial proteases by HPLC. To this aim, different bacterial supernatants were incubated with D-Ctl (or L-Ctl as a control) for 24 hours at 37 °C. As depicted in Fig. 4, D-Ctl was not degraded in none of the bacterial supernatants tested (Fig. 4B,D,F,H,J,K and L). In contrast, L-Ctl was degraded in the presence of secreted proteases from *E. coli* wild type (Fig. 4A) and MDR (Fig. 4C) but not *F. nucleatum* (Fig. 4E), *P. intermedia* (Fig. 4G) and *P. micra* (Fig. 4I). Of interest, in a previous study, we demonstrated that L-Ctl was also stable in the supernatant of MSSA and MRSA[18]. Consequently, the change in conformation between L-Ctl and D-Ctl does not affect their sensitivity towards secreted bacterial proteases, except for *E. coli* wild type and MDR.

D-Ctl dramatically damaged the cell wall of *E. coli* MDR. To have a first insight on the mechanism of action of both peptides, suspensions of *E. coli* MDR ($DO_{600} = 0.1$, ~6×10^6 bacteria/mL) were subjected or not (as a control experiment) to the action of L-Ctl and D-Ctl during 20 hours at several initial concentrations (0.05x MIC, 1x MIC, 5x MIC, and 10x MIC). Figure 5(a and g) shows the infrared spectra of the bacteria cultivated in LB and LB/4 media without peptide. The spectral fingerprints are characteristic of live bacteria[23]. In LB/4, the additional biosynthesis of glycogen can be observed (red arrows, Fig. 5g) probably due to a lack of some nutrients with respect to carbon[24]. The corresponding epifluorescence images (next to the infrared spectra) after *Bac*Light™ staining show a green fluorescence suggesting intact cell membranes. The average elasticity assessed by AFM force measurements was 310 ± 71 kPa (Fig. 6) that was in line with previous data obtained on the same strain[25].

At 8 μg/mL for both enantiomers, the infrared spectral features were very similar to those recorded for the untreated bacteria (Fig. 5a,b and c), suggesting that the metabolic activity of the bacteria was not or poorly modified. However, some differences in the corresponding epifluorescence images were observed. Whereas bacteria treated by L-Ctl showed only a green fluorescence, those treated with D-Ctl at the same concentration showed some green bacteria but also a lot of orange/red bacteria. This result suggested that the membranes of the bacteria were not damaged by L-Ctl but were damaged by D-Ctl for a lot of bacteria. The mechanical properties of the bacteria reported in Fig. 6 showed that L-Ctl did not significantly impact the cell wall elasticity (320 ± 46 kPa). Consequently, the integrity of the bacterial membrane was preserved in spite of the presence of L-Ctl, in accordance with the epifluorescence results. Conversely, the treatment with D-Ctl at the same concentration dramatically reduced by a factor of 3.7 the average elasticity of the bacterial cell wall (83 ± 48 kPa). This loss of elasticity suggested that D-Ctl strongly damaged the bacterial membrane as it was already reported in the literature for other antimicrobial peptides[26–28]. These results emphasized that the action of the two enantiomers were very different at the same concentration. Whereas D-Ctl showed a very strong activity against *E. coli* MDR, this was not the case for L-Ctl. Indeed, for the latter the concentration was only 0.05x MIC instead of MIC for D-Ctl.

When the bacteria were treated at the MIC of L-Ctl (150 μg/mL), the infrared spectrum of the bacteria left after the treatment was very similar to the one of the non-treated bacteria (Fig. 5a and d). This result suggested that as for D-Ctl at the MIC, the treatment with L-Ctl at the MIC did not or slightly modify the bacterial metabolism. The epifluorescence images after BacLightTM staining show a mixture of green and orange/red bacteria. It suggested that the bacterial membranes were damaged for some bacteria as it was previously observed for D-Ctl at its MIC. The calculated average elasticity was reduced by a factor of 3 with respect to the untreated bacteria (105 ± 69 kPa, see Fig. 6). The action of both enantiomers was almost the same on the membrane elasticity at their MICs.

For higher concentrations of L-Ctl and D-Ctl (at 750 μg/mL and above 40 μg/mL, respectively) no infrared spectra could be recorded (Fig. 5e,f and h). This result was in accordance with epifluorescence images. Only very few bacteria were observed on the filters. The bacteria were almost completely lysed. In the case of L-Ctl, AFM

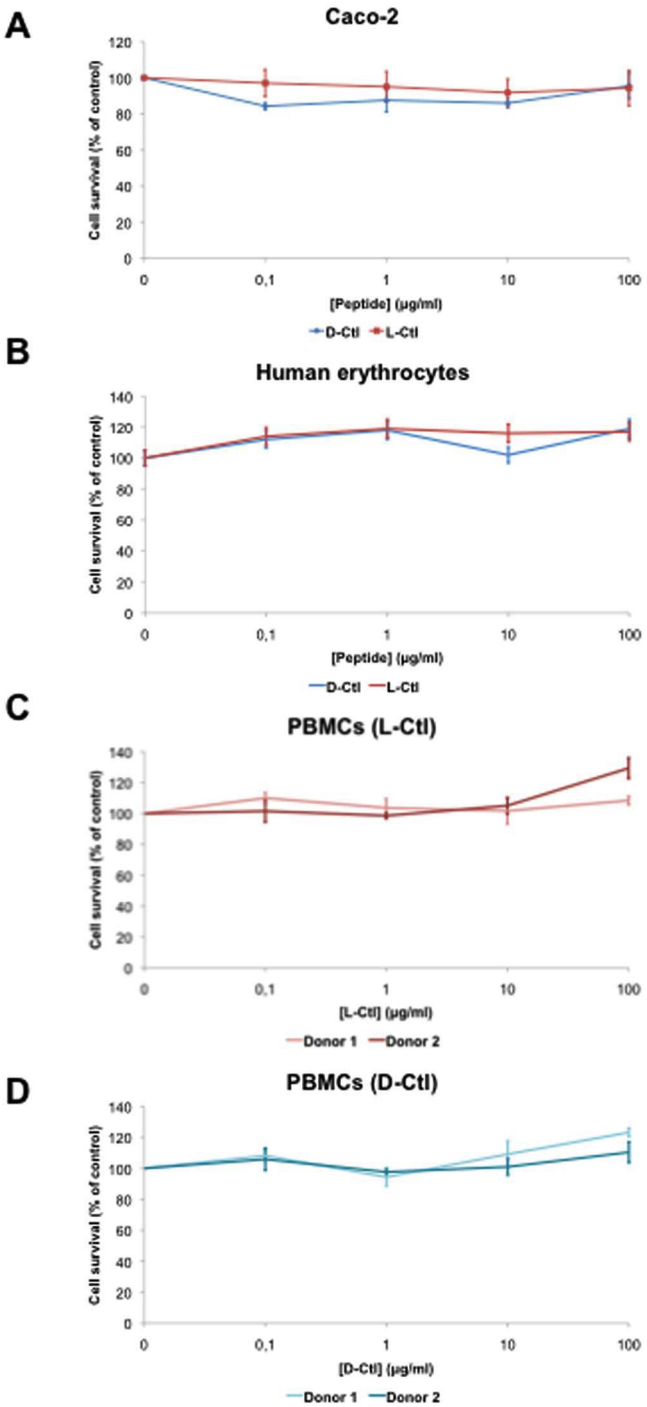

Figure 2. Cytotoxicity assays of D-Ctl and L-Ctl. The cytotoxicity of D-Ctl and L-Ctl on Caco-2, a human intestinal epithelial cell line (**A**) and PMBCs (**C** and **D**) was assessed at the indicated concentrations for 72 hours. Red blood cells haemolysis was evaluated after a one-hour treatment with the indicated concentrations of D-Ctl or L-Ctl (**B**). Each figure corresponds to a mean of at least two independent experiments.

measurements show no significant difference between the treatments performed at the MIC and at 5x MIC in terms of elasticity (112 ± 56 kPa for the latter concentration). For D-Ctl at 40 and 80 µg/mL, the bacterial elasticity could be measured only as soon as at 3 hours and 0.8 hours, respectively, because no bacteria were left after 20 hours of treatment. The average elasticity was already reduced by a factor 7 to 8 (44 ± 37 kPa and 28 ± 21 kPa, respectively, Fig. 6). Conversely to the action of L-Ctl above the MIC, the damages that occurred on the bacteria were reached earlier with D-Ctl, and they were dramatic for the cell integrity.

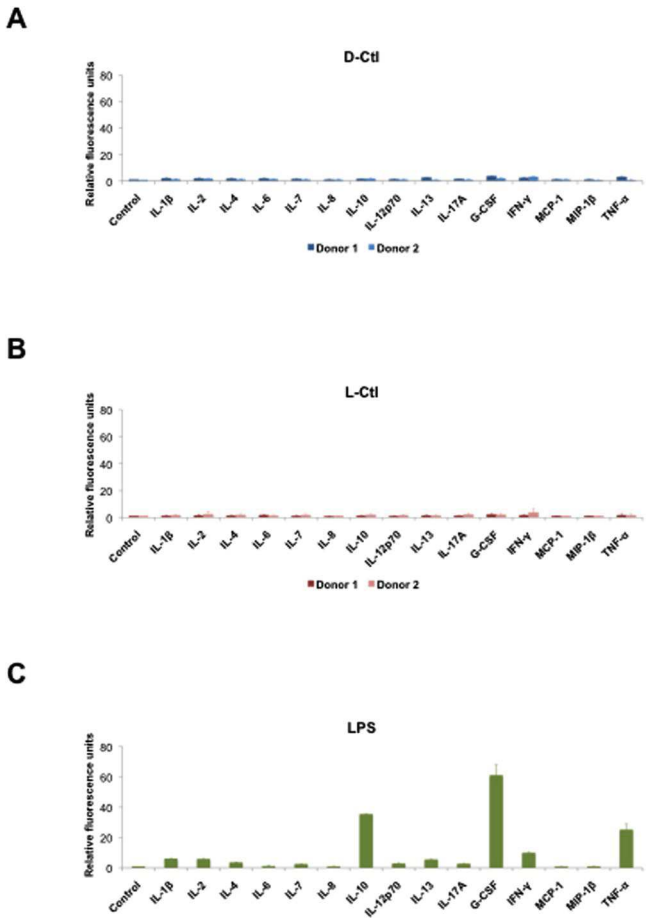

Figure 3. Cytokine release assay following treatment of PBMCs with D-Ctl or L-Ctl. Cells from healthy volunteers were treated with D-Ctl (**A**), L-Ctl (**B**) or LPS (**C**) for 24 hours and the indicated cytokines levels were evaluated in the cell supernatant using the Bio-Plex® technology.

Discussion

The rise of antimicrobial resistant microorganisms constitutes an increasingly serious threat to global public health. As a consequence, the efficacy of conventional antimicrobials is rapidly declining, threatening the ability of healthcare professionals to cure common infections[1,2]. Hence, the development of new antibacterial compounds with less potential to trigger resistance constitutes a public health challenge.

In the last two decades, host defence peptides have been proposed as a potential source of novel antimicrobials[12]. Although more efficient antimicrobials are currently on the market[29], host defence peptides display numerous advantages over conventional antimicrobials, such as an incomparably broad spectrum of action, a fast mode of action and most importantly, a very low potential to induce resistance. In this study, we report the antibacterial properties of D-Ctl on a large panel of bacteria including Gram-positive and Gram-negative pathogens but also obligate and facultative anaerobes. D-Ctl is a derivative of L-Cateslytin (L-Ctl), already known for its antimicrobial properties, specifically against *S. aureus*. D-Ctl consists of the same sequence as L-Ctl with all levogyre (L) amino acids replaced by dextrogyre (D) amino acids. By introducing these modifications, we intended to increase the stability of the peptide towards bacterial proteases, as liability is the Achilles' heel of peptide therapeutics. Indeed, in contrast to L-Ctl, D-Ctl cannot be degraded by cellular proteases. In accordance, our results demonstrated that D-Ctl is stable in all bacterial supernatant tested (MSSA and MRSA, *E. coli wild* type and MDR, *P. micra*, *P. intermedia* and *F. nucleatum*). Remarkably, L-Ctl was already a robust compound, resistant to the degradation by secreted proteases from *S. aureus* MSSA and MRSA[18], *P. micra*, *P. intermedia* and *F. nucleatum* but degraded in the supernatant of *E. coli* wild type and MDR.

As expected, D-Ctl was much more efficient than L-Ctl with a difference in the MIC ranging from 1.7 (MSSA) to 17.9 folds (*E. coli* MDR). Active against both Gram-positive and Gram-negative bacteria, D-Ctl could be considered as a broad-spectrum antimicrobial. However, a larger panel of pathogens remain to be screened to validate such an assumption. Nevertheless, D-Ctl was specifically efficient on *E. coli* wild type and MDR with a MIC of 8.0 µg/mL and 8.4 µg/mL, respectively. Overall, the MICs of D-Ctl were comparable with the ones of LL-37 and its truncated mimetics KE-18 and KR-12 (8.4 to 19.3 µg/mL for *S. aureus* and 2.1 to 9.8 µg/mL for *E. coli*)[30] but also of human β-defensins 2 and 3, which ranged between 1.4 µg/mL and >250 µg/mL depending on the bacterial strain[31]. When compared to the antimicrobial of reference for each pathogen, antimicrobials were still more efficient than D-Ctl except for *E. coli* wild type where the efficiency of D-Ctl (MIC = 8.0 µg/mL) was comparable

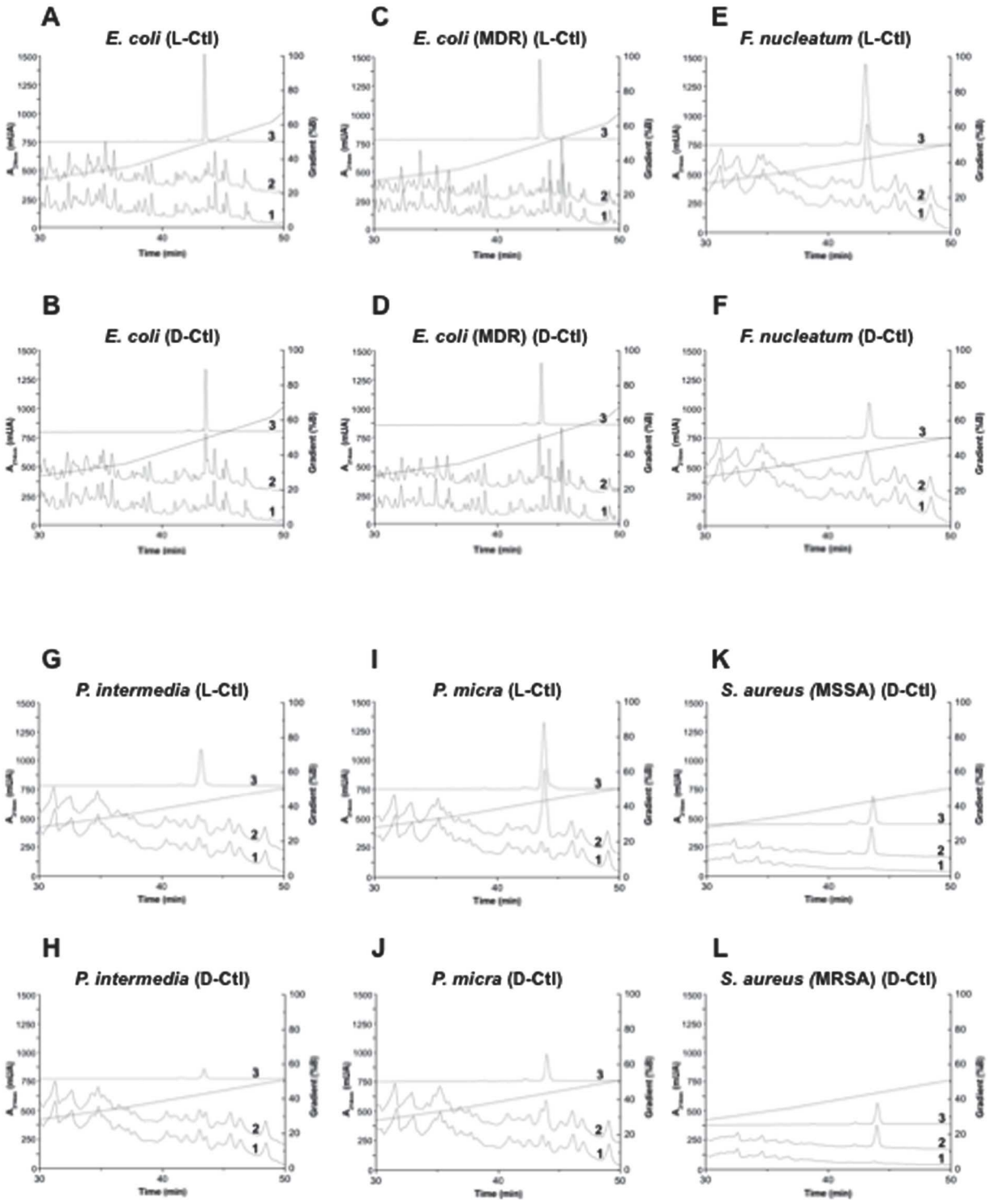

Figure 4. Stability of D-Ctl and L-Ctl towards proteases secreted by different bacterial strains. Supernatants from *E. coli* wild type (**A** and **B**), *E. coli* MDR (**C** and **D**), *F. nucleatum* (**E** and **F**), *P. intermedia* (**G** and **H**), *P. micra* (**I** and **J**), *S. aureus* methicillin sensitive (MSSA) (**K**), *S. aureus* methicillin resistant (MRSA) (**L**) were incubated with D-Ctl or L-Ctl, as indicated, for 24 hours. Peptide stability was then assessed by HPLC. Chromatograms 1 correspond to supernatant only, chromatograms 2 correspond to supernatant and peptide and chromatograms 3 corresponds to peptide only.

with ampicillin (MIC = 7.0 μg/mL) and much higher than kanamycin (MIC = 21.6 μg/mL). However and of high interest, the potential for *E. coli* to develop resistance to D-Ctl under selective pressure was not detectable for D-Ctl, unlike ampicillin and cefotaxime (three fold MIC increase for both antimicrobial over 24 days).

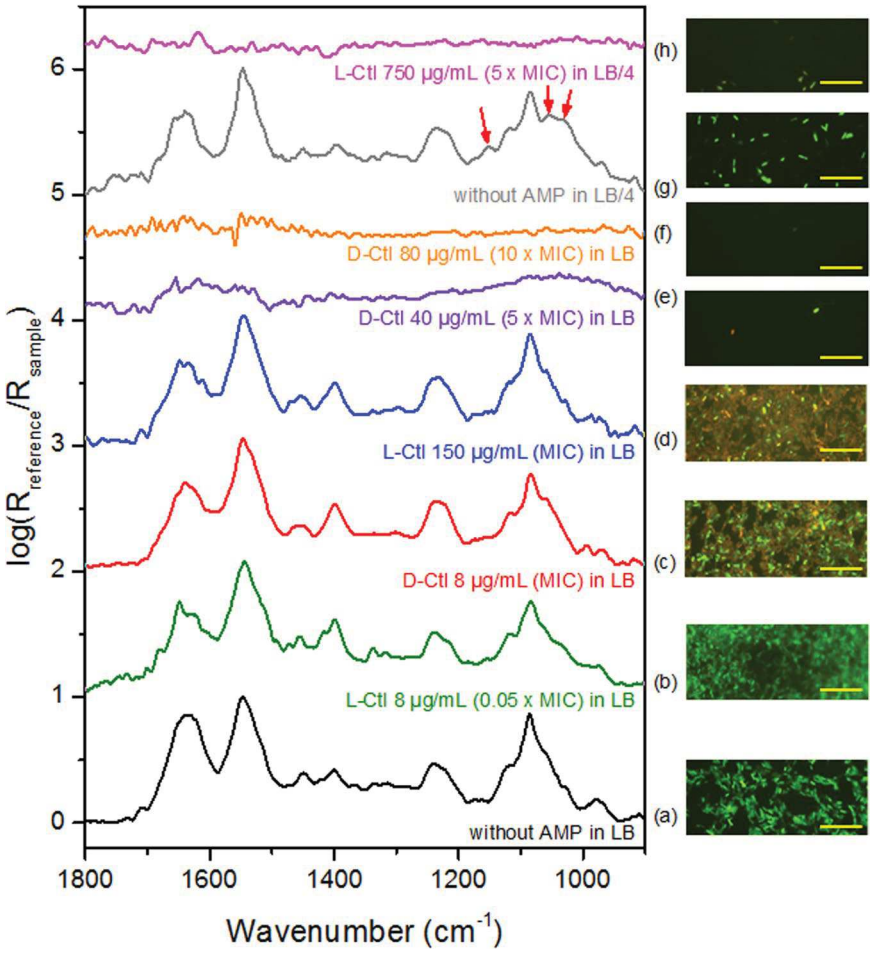

Figure 5. Spectral fingerprints of *E. coli* MDR. Left panel: IR-ATR spectra of planktonic *E. coli* MDR incubated with or without L and D conformers of Ctl during 20 hours. The spectra are normalized to one with respect to the Amide II band. Offsets of spectra are used for clarity. Right panel: Corresponding representative epifluorescence images of *E. coli MDR* after incubation with or without L and D conformers of Ctl during 20 hours. Bar: 20 µm.

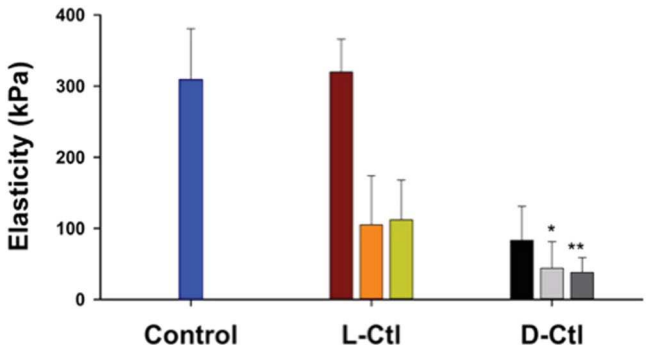

Figure 6. Elasticity of *E. coli* MDR treated with D-Ctl or L-Ctl for 20 hours. *And **refer to data obtained after only 3 hours and 0.8 hours of treatment, respectively. Bars for L-Ctl correspond to the average elasticity of bacteria subjected to antimicrobial peptide treatments performed at concentrations of 8, 150 and 750 µg/mL, respectively. For D-Ctl, the bars correspond to the average elasticity of bacteria subjected to the peptide at concentrations of 8, 40 and 150 µg/mL, respectively.

Combination antibacterial therapy is frequently used to prevent or delay the emergence of resistance[32]. Interestingly, D-Ctl is not only a strong antimicrobial candidate against *E. coli*, but it can also be used in conjunction with conventional antimicrobials to enhance their antibacterial activity against other pathogens. As a

matter of fact, here we report the synergistic effect of D-Ctl and amoxicillin against *P. micra* and *P. intermedia*. Furthermore, D-Ctl in combination with cefotaxime, methicillin or amoxicillin displayed an additive antibacterial effect against *E. coli* MDR, *S. aureus* and *F. nucleatum*, respectively. As a result of these associations, the concentration of conventional antimicrobials could be remarkably decreased from a factor two to four with potential implications on bacterial resistance.

Remarkably, the antibacterial activity of D-Ctl was not associated with cellular toxicity and does not interfere with the production of cytokines from LPS-stimulated PBMCs. These toxicology outcomes constitute a valuable point towards the use of D-Ctl as a new antimicrobial against *E. coli* infections. Indeed, the powerful antibacterial activity of most antimicrobials currently on the market is balanced by detrimental side effects. Specifically, fluoroquinolones, the antimicrobials of reference against *E. coli* infections are associated with immunomodulation, severe nephrotoxicity and tendinopathies[33,34]. Besides, D-Ctl was insensitive to proteases secreted by targeted pathogens. This property of D-Ctl was expected, as there is no L-amino acid within its structure.

Mechanism by which D-Ctl exerts its antibacterial activity was deciphered by physico-chemical methods. From the infrared data, it is suggested that the bacterial metabolism was not or poorly impacted. However the bacterial membrane was permeabilized as it was shown by the epifluorescence images after *Bac*Light™ staining. From the drastic decrease of the cell wall elasticity, it can be also suggested that the bacterial cell wall is highly damaged, and action of D-Ctl leads to loss of cytosol until the bacterial lysis and the death of the bacteria. Here we showed that the rate of the antimicrobial action and the minimum amount of peptide molecules necessary to reach the cell lysis are strongly dependent on the conformation of the peptide. Surprisingly, our results demonstrated that the D-conformer had the most efficient action for the lowest MIC (by a factor of around 20), contrary to previous studies that did not show such a significant difference in antimicrobial activity of L- and D-conformers[35,36].

In the last decade, there have been a few HDPs entering clinical trials, specifically cathelicidins and defensins natural peptides or derivatives such as LL-37, MBI-226 (studies NCT00211523, NCT00211497 and NCT00027248 for the prevention of central venous catheter-related bloodstream infections and acne) and PMX-30063 (study NCT01211470 for acute bacterial skin and skin-structure infection). However, the clinical and commercial development of these peptide-based drugs has some limitations such as high cost of production, susceptibility to proteases and cytotoxicity. For example, the human cathelicidin LL-37 enhances apoptosis of epithelial cells, smooth muscle cells and T cells at levels above 10 µM[37]. Besides being cytotoxic, LL-37 is also sensitive to protease cleavage, leading to the abolishment of its antimicrobial properties[38]. Defensins have also been extensively considered as an alternative to classical antimicrobials. However, the main limitation to their use as therapeutics is the lack of efficient production methods due to their complex secondary and tertiary structures[39,40]. In this context, D-Ctl presents many assets compared to other peptide-based drugs. Indeed, D-Ctl is short (15 amino acids) and linear, which makes it really easy to produce. Moreover, the use of a D-peptide emerges as a fruitful strategy to avoid degradation by secreted bacterial proteases. To put it in a nutshell, D-Ctl emerges as a potent, safe and robust antimicrobial with undetectable susceptibility to resistance, which makes it an attractive candidate for biopharmaceutical development. However, for an eventual entry into humans, a full assessment of safety pharmacology and drug toxicology will have to be conducted.

Methods

Peptide synthesis. The chemically synthesized peptides corresponding to L-Cateslytin (L-Ctl) and D-Cateslytin (D-Ctl) (RSMRLSFRARGYGFR, purity >95%) were purchased from Proteogenix.

Microorganisms and mammalian cell cultivation. *Escherischia coli* (ATCC® 25922™), *Staphylococcus aureus* (ATCC® 25923™), *Fusobacterium nucleatum* (ATCC® 49256™), *Prevotella intermedia* (ATCC® 49046™) and *Parvimonas micra* (ATCC® 33270™) were purchased from ATCC. *E. coli* K-12 mutant multidrug resistant (MDR) was kindly provided by the Institut Pasteur of Paris. This strain was constructed from *E. coli* MG1655 (*E. coli* genetic stock center CGSC#6300). It is resistant to specific antimicrobials such as ampicillin, chloramphenicol, and kanamycin[25]. The *S. aureus* Methicillin Resistant (MRSA) S1 strain was kindly provided by Dr Gilles Prévost (University of Strasbourg)[18]. Microorganisms were cultured according to the manufacturer's or the owner's instructions in their respective media: Luria Bertani broth (Sigma) was used for *E. coli* strains, Mueller Hinton broth (Difco) for *S. aureus* strains and Anaerobe Basal broth (Oxoid) for *F. nucleatum*, *P. intermedia* and *P. micra*.

The Caco-2 cell line (ATCC® HTB-37™) was kindly provided by Dr Benoît Frisch (UMR 7199 CNRS University of Strasbourg) and cultured at 37 °C in a 5% CO2 humidified incubator in Eagle's Minimum Essential Medium (Thermo Fisher Scientific) supplemented with 20% bovine calf serum and 1% penicillin/streptomycin. Human Peripheral Blood Mononuclear Cells (PBMC) from healthy volunteers were obtained from the blood transfusion centre of Strasbourg (Etablissement Français du Sang, Strasbourg) and isolated by density gradient centrifugation using Lymphoprep™ (Stemcell Technologies). PMBC were then maintained in AIM V® medium (Thermo Fisher Scientific) at 37 °C in a 5% CO2 humidified incubator.

Minimum inhibitory concentration (MIC) determination. The MIC was determined by broth microdilution. An overnight culture of each bacterial strain was diluted (approximately to $OD_{600} = 0{,}001$) and microorganisms were plated in 96-well plates in the presence of different concentrations of antimicrobials, D-Ctl or L-Ctl alone or in combination. Three technical replicates were performed for each condition. After 24 hours of incubation, the microorganism growth was assessed by optical density OD_{600} using a Multiskan™ EX microplate spectrophotometer (Thermo Fisher Scientific). The MIC, defined as the lowest concentration of a drug alone or in combination able to inhibit 100% of the inoculum, was determined from a modified Gompertz model as described in Lambert *et al.*[41]. Each experiment was performed with at least three biological replicates.

Haemolytic assays. The lysis of red blood cells was monitored by the release of haemoglobin to the extracellular environment. Whole blood from one healthy volunteer was obtained from the blood transfusion centre of Strasbourg (Etablissement Français du Sang, Strasbourg). Cells were then washed twice with PBS (800 g, 10 min), resuspended in 1 mL of PBS and incubated with D-Ctl or L-Ctl at different concentrations (0–100 μg/mL) for 1 hour at 37 °C. As a positive control, total lysis of red blood cells was obtained by incubating the cells with 0.1% SDS. For each condition, three technical replicates were performed. After the incubation, cells were centrifuged at 800 g for 10 min and the level of haemoglobin released in the supernatant was determined by optical density OD_{420} using a Multiskan™ EX microplate spectrophotometer (Thermo Fisher Scientific).

Cell viability assays. The MTT [3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyl tetrazolium bromide] assay was used to assess the cytotoxicity of D-Ctl and L-Ctl. Cells in their exponential phase of growth were seeded into a 96-well plate at 1×10^6 cells/mL prior being treated with a tenfold serial dilution of D-Ctl or L-Ctl. Three technical replicates were performed for each condition. After 72 hours incubation, MTT (Sigma-Aldrich) was added to each well at a final concentration of 0.25 mg/mL. Cells were then incubated for an additional 2 hours at 37 °C in a 5% CO2 humidified incubator and lysed with isopropanol/HCl (96:4, v/v). Cell cytotoxicity was then assessed by optical density OD_{570} using a Multiskan™ EX microplate spectrophotometer (Thermo Fisher Scientific). Each experiment was performed with at least three biological replicates.

Cytokine release assays. The following cytokines: G-CSF, GM-CSF, IFN-γ, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12, IL-13, IL-17, MCP-1, MIP-1β, TNF-α were measured using the Bio-Plex® Multiplex Immunoassay system (Bio-Rad). In brief, human PBMCs were prepared as previously described and treated for 24 hours with D-Ctl (60 μg/mL), L-Ctl (60 μg/mL) or LPS (5 μg/mL). Three technical replicates were performed for each condition. Supernatants were then filtered and assessed for cytokine dosage according to the manufacturer's instructions.

Resistance acquisition assays. An *E. coli* (ATCC® 25922™) culture was sequentially diluted every day in the presence of the different antibacterial compounds: D-Ctl, ampicillin or cefotaxime at ½ MIC during 24 days. The changes in the MICs values were determined as previously described by broth microdilution at the indicated times. The experiment was performed with three technical replicates.

Peptide stability assays towards secreted bacterial proteases. Bacterial supernatant was prepared as follows: a single colony of each strain was resuspended in 5 mL of culture medium as indicated above and incubated at 37 °C overnight. The culture was then centrifuged at 10000 g for 1 min and the supernatant was filtered using a 0.22 mM MillexH-GV (Millipore, Carrigtwohill, Ireland). An aliquot of each supernatant was incubated at 37 °C for 48 hours. Absence of growth was interpreted as lack of viable microorganism. 400 μL of supernatant was then incubated with or without each peptide of interest at 37 °C for 24 hours. As a control, each peptide was incubated in water at 37 °C for 24 hours. Samples were then separated using a Dionex HPLC system (Ultimate 3000; Sunnyvale, CA USA) on a Nucleosil reverse-phase 300–5C18-column (46250 mm; particle size: 5 mm; porosity, 300 Å) (Macherey Nagel, Hoerdt, France). Absorbance was monitored at 214 nm and the solvent system consisted of 0.1% (v/v) TFA in water (solvent A) and 0.09% (v/v) TFA in 70% (v/v) acetonitrile-water (solvent B). Elution was performed at a flow rate of 700 mL/min with a gradient of solvent B as indicated on the chromatograms.

Planktonic *E. coli* suspensions for physicochemical analysis. The bacterial model used for the physicochemical analysis (AFM, infrared spectroscopy and epifluorescence microscopy) is *E. coli* MDR. Bacteria were cultured in Luria Broth (Miller, Fluka) at 25 g/L (LB) or at 6.25 g/L (LB/4) in deionized water (Purelab Option, ELGA). All the cultures were incubated in a water bath shaker (Inova 3100, New Brunswick Scientific) at 37 ± 1 °C and under continuous agitation at 160 rpm. After an overnight subculture (16 hours, with ampicillin and kanamycin), bacteria were cultured in 200 mL of LB medium (without antimicrobials) with an initial optical density at 600 nm (OD_{600}, measured with a cell density meter Biochrom AG, Fisherbrand) of 0.050 ± 0.005.

For epifluorescence and infrared spectroscopy analyses, the antimicrobial assays against planktonic *E. coli* MDR were performed in duplicate in sterile 96-well plates (Nunc) in a final volume of 200 mL. When the optical density of the bacterial culture reached an OD_{600} value between 0.5 and 0.6 (bacteria were at the end of the exponential phase), the suspension was diluted in LB or LB/4 to give an $OD_{600} = 0.10 \pm 0.01$. The necessary volume of the stock solution of the peptide at 1 g/L was spotted in the bacterial suspension. Sterility and growth controls were sterile LB and LB/4, and a bacterial suspension without peptide, respectively. The plate was incubated for 20 hours at 22 °C.

Epifluorescence optical microscopy. Planktonic bacteria were analysed by fluorescence microscopy using the *Bac*Light™ stain kit (L7012, Molecular Probes, Eugene, USA) in order to determine the permeability of the cells in the absence and presence of the peptide. This kit contains two nucleic acids dyes: SYTO 9 (excitation/emission maxima: 480/500 nm) that penetrates all the cells, and propidium iodide that penetrates only cells with damaged membranes (excitation/emission maxima: 490/635 nm). Therefore, bacteria with intact membranes fluoresce green, while bacteria with damaged membranes fluoresce red. After 20 hours of incubation, 200 μL of the 24 hours-old bacterial suspension were mixed with 300 μL of *Bac*Light™ solution (15 μL of the reconstructed *Bac*Light™ solution as described by the manufacturer in 300 μL of sterile water), and stained for 20 min in the dark at 22 ± 1 °C. The suspension was then filtrated with 0.2 μm black filters (Millipore, GTBP04700) and rinsed three times with sterile water to eliminate excess *Bac*Light™. The sample was mounted in *Bac*Light™ mounting oil as described by the manufacturer. Both fluorescences were viewed simultaneously with the 100x oil immersion objective of an Olympus BX51 microscope equipped with an Olympus XC50 camera.

ATR-FTIR spectroscopy. ATR-FTIR spectra were recorded between 4000 and 800 cm^{-1} on a Bruker Vertex 70 v spectrometer equipped with a KBr beam splitter and a DTGS detector, and driven by the OPUS 7.5 software. The resolution of the single beam spectra was 4 cm^{-1}. A nine-reflection diamond ATR accessory (Durasampl*IR*™, SensIR Technologies, incidence angle: 45°) was used for acquiring spectra. The number of bidirectional double-sided interferogram scans was 200, which corresponds to a 2 min accumulation. All interferograms were Fourier processed using the Mertz phase correction mode and a Blackman-Harris three-term apodization function. No ATR correction was performed. Measurements were performed at 21 ± 1 °C in an air-conditioned room. 50 µL of the bacterial suspensions in their culture media was put on the ATR crystal. Half of the suspension was centrifuged at 8000 rpm during 5 min and the supernatant was used to remove the spectral background. Water vapour subtraction was performed when necessary.

AFM mechanical properties measurements. AFM experiments were carried out using a MFP3D-BIO instrument (Asylum Research Technology, Oxford Instruments Company, Mannheim, Germany). Silicon nitride cantilevers of conical shape were purchased from Asylum Research Technology (Olympus TR400 PSA, Mannheim, Germany). The spring constants of the cantilevers measured using the thermal noise method were found to be 0.02–0.03 nN/nm. Experiments were performed in triplicate in PBS at room temperature. The nanoindentation method was used to determine the Young's modulus from the force vs. indentation curves. Mechanical properties were obtained by recording a grid map of 50-by-50 force curves on several bacterial clusters containing at least 10 bacteria electrostatically immobilized onto PEI coated glass substrate. The maximal loading force was 4 nN, the piezodrive was fixed to 2 µm and the approach rate was 2 µm/s. The histograms corresponding to the statistic distribution of the Young modulus were estimated from the analysis of the approach curves according to the Sneddon model[42,43] where δ is the indentation depth, ν the Poisson coefficient, R is the curvature radius of AFM-tip apex and f_{BECC} the bottom effect correction described by Gavara et Chadwick[42]. All the FVI were analysed by mean of an automatic Matlab algorithm described elsewhere[44]. Bacteria were then exposed to various L-Ctl concentrations (8, 150 and 750 µg/mL) and also to various D-Ctl concentrations (8, 40 and 80 µg/mL) in PBS buffer at 22 °C for 20 hours. Mechanical properties were measured by AFM in force mapping mode at indentation rate of 2 µm/s and the average values correspond to at least 500 force curves taken from at least 10 bacteria. For bars labelled with * and ** the corresponding values were obtained after only 3 and 0.8 hours of peptide exposure, respectively. Of notice, beyond these exposure periods all bacteria were too damaged and not enough for relevant measurements.

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Acknowledgements

This work was supported by the Marie Curie Research Grants Scheme, CIG (Career Integration Grant) attributed to C.M.

Author Contributions

Study conception and design: C.M. and M.H.M.B., acquisition of data: A.Z., P.D., F.D., C.E., F.Q., G.F., C.Bo., C.Be., analysis and interpretation of data: C.M., M.H.M.B., A.Z., F.Q., G.F., B.F., G.P., P.L., F.S., Y.H., drafting of manuscript: C.M., M.H.M.B. F.Q., G.F. All authors review the manuscript.

Additional Information

Supplementary information accompanies this paper at https://doi.org/10.1038/s41598-017-15436-z.

Competing Interests: The authors declare that they have no competing interests.

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Introduction to manuscript II

Calcium hydroxide and D-Cateslytin: a combination therapy to eradicate root canal pathogens

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Submission to Journal of Endodontics

I had contributed with our group for another study concerning the use of combination of D-Ctl with calcium hydroxide Ca(OH)2 for trying to inhibit the growth of *Enterococcus faecalis* which cause the most infections in the root canal. The aim of this combination was to improve the antimicrobial activity of Ca(OH)2 and to prevent its secondary effects.

Our results show that the combination completely inhibit the growth of *Enterococcus faecalis* when we combined 0.85mg/mL of Ca(OH)2 and ½ MIC of D-Ctl. By comparison, Ca(OH)2 alone can only inhibit 58%(± 5%) of this bacteria. Moreover, this combination was tested also on several oral cavity pathogenic strains such *as P. micra, P. intermedia, F. nucleatum* and *Candida albicans* too. The results demonstrated that this combination was active against all these microorganisms at the concentration range of 0.85mg/mL of Ca(OH)2 and ½ MIC of D-Ctl. Furthermore, the peptide D-Ctl remains stable in this combination and not degraded by the *Enterococcus faecalis* supernatant. It is important to point out that this combination has a low level of toxicity toward human gingival fibroblasts compared to Ca(OH)2 alone.

To conclude, my contribution to this study is focused on the numerous antimicrobial assays and I brought my knowledge to D-Ctl for discussion of the data.

Manuscript-II

Calcium hydroxide and D-Cateslytin: a combination therapy to eradicate root canal pathogens

Calcium hydroxide and D-Cateslytin: a combination therapy to eradicate root canal pathogens

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Keywords: D-Cateslytin, calcium hydroxide, *Enterococcus faecalis*, Endodontic treatment, root canal filling material, root canal therapy

Acknowledgment: The authors deny any conflicts of interest.

Calcium hydroxide and D-Cateslytin: a combination therapy to eradicate root canal pathogens

ABSTRACT

Introduction: The success of endodontic treatments depends on the eradication of intracanal pathogens. Since irrigation and instrumentation can only partially eliminate bacteria, the use of intracanal medication has been suggested to improve the eradication of root canal pathogens. Even though calcium hydroxide is one of the most popular intracanalar dressings, its antimicrobial properties, particularly against *Enterococcus faecalis*, are still debated. Host defense peptides are immunomodulatory components with antimicrobial properties, and potentially excellent candidates to enhance the endodontic antimicrobial properties of calcium hydroxide. **Methods:** In this study, we combined D-Cateslytin (D-Ctl), a host defense peptide efficient against *E. faecalis*, together with calcium hydroxide to test for enhanced antimicrobial properties using antimicrobial assays. In addition, stability and cytotoxicity assays were performed to address the safety of the combination. **Results:** A saturated solution of calcium hydroxide was able to inhibit 58% (±5%) of bacterial growth but the combination of both was able to fully inhibit *E. faecalis* growth (50% of calcium hydroxyde and ½ MIC of D-Ctl). This combination was not degraded by the proteases secreted by *E. faecalis* and showed a low level of toxicity on human gingival fibroblasts. Besides *E. faecalis*, this combination was also effective in killing other endodontic pathogens: *P. micra, P. intermedia, F. nucleatum* and *C. albicans*. **Conclusions:** Hence, a combined treatment of calcium hydroxide and D-Ctl eradicates endodontic pathogens and could be used as an innovative therapy to reduce root canal treatment failures.

INTRODUCTION

The success of an endodontic treatment depends on the eradication of the bacteria responsible for this disease (1). Although chemo-mechanical disinfection has been shown to be effective in reducing intracanal microorganisms, most of the root canal system is inaccessible to mechanical instrumentation and irrigating solutions (2, 3). Since endodontic pathogens exist not only in the root canal itself but also in the whole dentinal structure (3), viable bacteria have been isolated in 50 to 70% of an infected root canal after instrumentation and antibacterial irrigation (4). As chemo-mechanical disinfection cannot eradicate dental bacteria, temporary dressings have been proposed as a feasible complementary strategy (5). Intracanalar dressings are predicted to have effective antimicrobial properties, blocking reinfection while exhibiting low periapical tissue toxicity.

Calcium hydroxide ($Ca(OH)_2$) is a common intracanalar medication used in endodontics. Among its remarkable properties, $Ca(OH)_2$ is the best medication to dry a canal with apical exudates before obturation (6). Currently, $Ca(OH)_2$ is the only medication capable of efficiently inactivating bacterial endotoxins responsible for inflammatory reactions involved in apical periodontitis (6). However, aside from its anti-inflammatory actions, the antibacterial properties of $Ca(OH)_2$ are still debated. While studies have confirmed its antimicrobial effectiveness against endodontic pathogens including *Fusobacterium nucleatum, Prevotella nigrescens, Actinomyces israelii*, *Porphyromonas endodontalis, Prevotella intermedia* and *Porphyromonas gingivalis* (7, 8), *Enterococcus faecalis* is resistant to $Ca(OH)_2$ (9-12).

E. faecalis is a gram-positive bacteria frequently associated with endodontic failure. These bacteria invade dentinal tubules and remain viable despite chemo-mechanical

disinfection of the root canal. Recontamination of the canal by the residual bacteria may explain why *E. faecalis* can cause periapical disease in endodontically treated teeth (13). The complete elimination of *E. faecalis* with an intracanal medication will without any doubt improve the success rate of endodontic treatments (14).

Therefore, a current challenge is to identify an antimicrobial agent that could be combined with $Ca(OH)_2$ to eradicate root canal pathogens. Host defense peptides are good candidates for this purpose. These small peptides are part of the innate immune response and are released into the circulation shortly after an infection (15). They have a strong antimicrobial activity against a broad spectrum of pathogens and play a crucial role in modulating the immune response. These peptides induce less resistance than antibiotics and are non-toxic to host cells (15, 16). Among all host defense peptides, chromogranin A (CgA)-derived peptides have been studied for the development of new antibiotics because they are short and stable in a wide range of pH and temperature (17). Specifically, Cateslytin (Ctl) displays strong antimicrobial activity against a large spectrum of pathogens (18, 19). Interestingly, we recently observed that by substituting all L-amino acids from Ctl with D-amino acids, we could drastically improve its antibacterial efficiency against a large range of bacteria including several oral pathogens such as *Parvimonas micra*, *Prevotela intermedia* and *Fusobacterium nucleatum* (20).

In the present study, we combined D-Ctl with $Ca(OH)_2$ in order to improve its bacterial efficiency and to develop a new non-toxic and stable combination therapy efficient against endodontic pathogens, including *E. faecalis.*

MATERIALS AND METHODS

Antimicrobial agents

The following peptides were purchased from Proteogenix: Chromofungin (CHR ($bCgA_{47-70}$: RILSILRHQNLLKELQDLAL), bovine Catestatin (CAT, $bCgA_{344–364}$: RSMRLSFRARGYGFRGPGLQL), bovine L-Cateslytin and D-cateslytin (L-Ctl and D-Ctl ($bCgA_{344–358}$: RSMRLSFRARGYGFR).

Preparation of calcium hydroxide solutions

$Ca(OH)_2$ was purchased from Sigma-Aldrich. A saturated solution of $Ca(OH)_2$ was obtained by dissolving 170mg of $Ca(OH)_2$ in 100mL of water (100%, 1.7mg/mL) and diluted to ½ (50%, 0.85mg/mL) and ¼ (25%, 0.425mg/mL).

Microorganisms and mammalian cell line

Fusobacterium nucleatum (ATCC© 49256™), *Prevotella intermedia* (ATCC© 49046™), and *Parvimonas micra* (ATCC© 33270™) were purchased from ATCC. *Enterococcus faecalis* (CCM 2541) was obtained from the Czechoslovac Collection of Microorganisms**.** Bacteria were cultured in Anaerobe Basal Broth (Oxoid) at 37°C in anaerobic conditions. *Candida albicans* (ATCC© 10231™) was cultured in Sabouraud medium (BD), supplemented with tetracycline (10µg/mL) and cefotaxime (10µg/mL).

The mammalian cell line HGF-1 (ATCC® CRL-2014™) was commercially obtained from ATCC and cultured at 37°C and 5% CO_2 in Dulbecco's Modified Eagle's Medium (Thermo Fisher Scientific) supplemented with 10% (v/v) bovine calf serum (Dutscher) and 1% (v/v) penicillin-streptomycin (Thermo Fisher Scientific).

Broth dilution assays

An overnight culture of each pathogen was diluted (OD_{600nm} = 0.001) and incubated at 37°C in 96-plates in the presence of different concentrations of antimicrobial agents. After 24h incubation, the OD_{600nm} was evaluated with a spectrophotometer (Multikan EX, ThermoScientific). Each assay was done at least in triplicate.

Determination of the Minimal Inhibitory Concentration (MIC)

The MIC, defined as the lowest concentration of peptide able to inhibit 100% of the inoculum, was calculated using a modified Gompertz model as described in Lambert et Pearson (21).

Cytotoxicity assays

The cytotoxicity of the antimicrobial agents was examined by MTT [3(4,5-dimethylthiazol-2-yl)-2,5 diphenyl tetrazolium bromide] assays (Sigma Aldrich) using HGF-1 cells as a model. Cells were incubated for 24h in a 96-wells plate before being treated with various concentrations of $Ca(OH)_2$ alone or supplemented with the peptide. Untreated cells were used as a control. After 24h, 48h and 72h incubation, cells were carefully washed with PBS and treated with MTT at a final concentration of 0.25mg/mL. HGF-1 cells were then incubated for 2h at 37°C before being lysed with isopropanol/HCl (48:2, v/v). Cell viability was assessed by reading the OD_{540nm} with a Multiskan EX microplate spectrophotometer (Thermo Fisher Scientific).

Stability assays of D-Ctl in the supernatant of *E. faecalis*

The stability of D-Ctl was assessed either in the supernatant of *E. faecalis* or in $Ca(OH)_2$. The supernatant of *E. faecalis* was prepared as follows: a single colony of

E. faecalis was suspended in 5mL of Anaerobe Basal Broth and incubated at 37°C overnight. The culture was then centrifuged at 10,000g for 1min, filtered (0.2µm) and the supernatant was incubated at 37°C for 24h alone or with D-Ctl (300µg/mL). As a positive control, D-Ctl (300µg/mL) was also incubated in similar conditions. The samples were analyzed by HPLC (Dionex, Ultimate 3000) using a Nucleosil reverse-phase 300-5C18-column (4.6x250mm; particle size: 5µm; porosity, 300Å) (Macherey Nagel). The two solvents used were: 0.1% (v/v) TFA in water (Solvent A) and 0.09% (v/v) TFA in 70% (v/v) acetonitrile-water (solvent B). Absorbance was measured at 214nm (A_{214nm}). The flow rate for elution was 0.7mL/min with a gradient of solvent B as indicated on the chromatograms.

Stability assays of D-Ctl in calcium hydroxide

D-Ctl was incubated in a buffered solution of $Ca(OH)_2$ solution (pH=9) at 37°C for 24h. The pH of the solution was the same as for the broth dilution assays. As a control, D-Ctl was also incubated in water at 37°C for 24h. The samples were analyzed by HPLC (Dionex, Ultimate 3000) using a Nucleosil reverse-phase 300-5C18-column (4.6x250mm; particle size: 5µm; porosity, 300Å) (Macherey Nagel). The two solvents used were: 0.1% (v/v) TFA in water (Solvent A) and 0.09% (v/v) TFA in 70% (v/v) acetonitrile/water (solvent B). Absorbance was measured at 214nm (A_{214nm}). The flow rate for elution was 0.7mL/min with a gradient of solvent B as indicated on the chromatograms.

Statistical analysis

Each assay was done at least in triplicate. For broth dilution and cytotoxicity assays, standard deviations were determined**.**

RESULTS

A saturated solution of $Ca(OH)_2$ does not inhibit *E. faecalis* growth

The efficiency of a saturated solution of $Ca(OH)_2$ (100% = 1,7mg/mL, pH=9) was assessed by broth dilution assays on *E. faecalis* and *F. nucleatum*. Our results confirmed previous studies showing that a saturated solution of $Ca(OH)_2$ was unable to completely inhibit the growth of *E. faecalis*. In our hands, the inhibition rate was only 58% (± 5%). In addition, as previously described (7), we confirmed that a saturated solution of $Ca(OH)_2$ was able to inhibit 100% (± 1%) of the growth of *F. nucleatum* **(Figure 1)**.

D-Ctl displays antimicrobial properties against *E. faecalis*

We then tested the antibacterial efficiency of several CgA-derived peptides such as Chromofungin (CHR), Catestatin (CAT) and Cateslytin (L-Ctl or D-Ctl) against *E. faecalis* using broth dilution assays. All peptides were tested at 200µg/mL. Our results demonstrated that all natural peptides derived from CgA (CHR, CAT and L-Ctl) have no antimicrobial activity against *E. faecalis.* The only peptide able to achieve bacterial growth inhibition was D-Ctl **(Figure 2A)**. D-Ctl was therefore chosen as the best candidate to combine with $Ca(OH)_2$. Its MIC on *E. faecalis,* determined by broth dilution assays, was 156µg/mL **(Figure 2B)**.

D-Ctl is stable in a saturated solution of $Ca(OH)_2$

To inquire whether D-Ctl was stable in $Ca(OH)_2$, we tested the stability of D-Ctl at the MIC in a saturated solution of $Ca(OH)_2$ (100% = 1.7mg/mL, pH=9) by HPLC **(Figure 3A)**. Under our experimental conditions, D-Ctl was eluted after 38min **(Figure 3A,**

chromatogram 1). The same peak was clearly identified in a saturated solution of $Ca(OH)_2$, suggesting that D-Ctl at the MIC remains stable under these conditions **(Figure 3A, chromatogram 2)**.

D-Ctl remains stable in the supernatant of *E. faecalis* *E. faecalis* can overcome the innate immune system response and trigger persistent infections. One of its mechanisms of resistance is the degradation of antimicrobial peptides (22). In order to counteract an endodontic infection, D-Ctl should therefore not be degraded by the proteases secreted by *E. faecalis*. For this reason, the stability of D-Ctl in the supernatant of *E. faecalis* was assessed by HPLC **(Figure 3B)**. In our experimental conditions, D-Ctl was eluted at 38min **(Figure 3B, chromatogram 1)**. The same peak was still observed when D-Ctl was incubated with the bacterial supernatant **(Figure 3B, chromatogram 2)**. Notably, the other peaks on the chromatogram correspond to proteins in the media but also proteases secreted by the bacteria **(Figure 3B, chromatogram 3)**. In conclusion, D-Ctl is resistant to the degradation by the virulence factors of *E. faecalis*, allowing a prolonged action of the peptide against this pathogen.

$Ca(OH)_2$ is cytotoxic for HGF-1 at high concentration

The cytotoxicity of a saturated solution of $Ca(OH)_2$ (100% = 1.7mg/mL), but also diluted solutions (50% and 25%) were assessed by MTT assays on human gingival fibroblasts (HGF-1) grown for 72h in culture. Cell viability was assessed and expressed as a percentage of the control. At 50% and 25% saturation, $Ca(OH)_2$ showed a toxicity below 4% (±7%), even after three days of incubation. Meanwhile, a saturated solution of $Ca(OH)_2$ was able to kill 50% (±6%) of the bacteria after 72h of

incubation **(Figure 4)**. According to these results, diluted solutions of $Ca(OH)_2$ constitute better choices than a saturated solution for new efficient endodontic treatments.

Combination of D-Ctl and $Ca(OH)_2$ inhibits key endodontic pathogens

To identify the most efficient combination of $Ca(OH)_2$ and D-Ctl to inhibit the growth of *E. faecalis*, broth dilution assays were performed with different concentrations of $Ca(OH)_2$ and D-Ctl. Specifically, a saturated solution of $Ca(OH)_2$, as well as diluted solutions of $Ca(OH)_2$ (50% and 25%) were combined with the MIC, ½ MIC and ¼ MIC of D-Ctl **(Figure 5A)**.

As depicted, the combination using the lowest concentration of $Ca(OH)_2$ and D-Ctl able to kill an inoculum of *E. faecalis* was a 50% saturated solution of $Ca(OH)_2$ (0.85mg/mL) with ½ MIC of D-Ctl (78µg/mL). The stability of D-Ctl in such a solution was confirmed by HPLC **(Figure 5B)**. Indeed, D-Ctl eluted at 38 min **(Figure 5B, chromatogram 1)** and was stable in a 50% saturated solution of $Ca(OH)_2$ **(Figure 5B, chromatogram 2)**. We also assessed the cytotoxicity of this combination towards HGF-1 with MTT assays **(Figure 5C)**. The combination showed a mild toxicity of about 17% over 72 hours.

Finally, we verified that the combination was also efficient on other endodontic pathogens. To this aim, we performed broth dilution assays with *Parvimonas micra, Prevotella intermedia, Fusobacterium nucleatum* and *Candida albicans* **(Figure 5D)**. Our results show that 50% $Ca(OH)_2$ was able to inhibit 87% (±2%) of *C. albicans* growth; 15% (±6%) of *P. micra* growth, 77% (±6%) of *P. intermedia* growth and 30% (±10%) of *F. nucleatum*, whereas the combination inhibited respectively 97% (±1%), 98 (±2%), 98 (±3) and 97% (±5%) of the pathogen's growth. Therefore, the

combination of D-Ctl/$Ca(OH)_2$ is not only efficient against *E. faecalis*, but also against other key endodontic pathogens like *C. albicans*, *P. micra*, *P. intermedia* and *F. nucleatum*.

DISCUSSION

Currently, calcium hydroxide is considered as the first choice for intracanal dressing. Its popularity is due to its physical, chemical and biological properties. Calcium hydroxide acts as a barrier, preventing the ingress of bacteria into the endodontic system. Furthermore, it limits the space for bacterial multiplication (23). The antimicrobial activity of $Ca(OH)_2$ is directly attributed to its chemical properties. Indeed, the elevation of the pH induced by the release and diffusion of hydroxyl ion, damages the microbial cytoplasmic membrane, leading to cellular death (6). Nevertheless, enterococci and yeast have been shown to tolerate an alkaline environment and are known to be resistant to $Ca(OH)_2$, even *in vitro* (24). For this reason, innovative therapies must be introduced to find better antimicrobials than calcium hydroxide.

Several combination therapies have been developed using well-known intracanalar medications such as chlorhexidine (25), Ledermix (26) or more unusual medications such as liquorice (9) to eradicate microorganisms from the root canal. Chlorhexidine is an endodontic irrigant, known for its antimicrobial properties, particularly against *E. faecalis* (27). Even though chlorhexidine is a very effective irrigant killing endodontic pathogens, clinical trials failed to prove its effectiveness as an intracanal medication *in vivo* (28, 29). Moreover, in a recent review, the mixture of $Ca(OH)_2$ with chlorhexidine showed no synergistic or additive antimicrobial effect (25). Similarly, liquorice extract alone or combined with $Ca(OH)_2$ showed a better antimicrobial effect than $Ca(OH)_2$ against *E. faecalis*, but these properties were not increased in combination (9). The use of local antibiotics is a tempting solution to eradicate root canal bacteria. However, the combination of Ledermix and $Ca(OH)_2$ did not show any

additive or synergistic effect. This study provides encouraging results towards the development of an innovative treatment to eradicate root canal microorganisms. Indeed, the combination of $Ca(OH)_2$ (50% saturation) and D-Ctl (½ MIC) could inhibit *E. faecalis* growth. D-Ctl has recently emerged as a potent, safe and robust antimicrobial peptide with low susceptibility to resistance (20).

Besides drug resistance, pathogens can overcome the innate immune response by degrading host defense peptides. As an example, proteases secreted by *E. faecalis* can degrade LL-37, a host defense peptide known for its antimicrobial properties (22). As D-Ctl strictly consists of D-amino acids, it is not sensitive to bacterial proteases (20) including E*. faecalis*.

Another reservation concerning the efficacy of $Ca(OH)_2$ *in vivo,* is the buffering capacity of the dentin (30). Indeed, several studies found that the buffering effect of the dentin and the reduced diffusion of hydroxyl ions which maintain an alkaline pH (pH=8) in the dental tubules (even in the presence of $Ca(OH)_2$), might explain its low efficiency in an extracted tooth model (31, 32). In this study, the antimicrobial and stability tests were also conducted in an alkaline environment (pH=9) due to the buffering capacity of the bacterial cultures. Thus, our experimental conditions mimic the environment of the dentin, suggesting that the antimicrobial activity of the combination therapy between $Ca(OH)_2$ and D-Ctl could well be observed *in vivo*.

The absence of cytotoxicity is also an important characteristic for an efficient intracanal dressing. From this point of view, $Ca(OH)_2$ and chlorhexidine are the most acceptable intracanal medicaments while others, like phenol and formocresol are highly cytotoxic (33). In our study, neither 50% $Ca(OH)_2$ alone, nor in combination with D-Ctl was toxic to human gingival fibroblasts. This result is in accordance with a previous study that demonstrated that $Ca(OH)_2$ was not toxic to human dental pulp

cells (34). In addition, another study conducted with human periodontal ligament fibroblasts, also concluded that the effect of $Ca(OH)_2$ on cell viability and cytokine release was minimal (35). This body of evidence seems to confirm a mild cytotoxicity of $Ca(OH)_2$.

Even though *E. faecalis* titers strongly correlate with endodontic failure, several other pathogens are involved in endodontic infection. Here, we showed that the combination between D-Ctl and $Ca(OH)_2$ is also efficient against four other endodontic pathogens: *Parvimonas micra, Prevotella intermedia, Fusobacterium nucleatum* and *Candida albicans*. These encouraging results suggest that this combination therapy could eradicate endodontic biofilms. However, further investigations are needed to test the efficiency of this combination on biofilms.

ACKNOWLEDGMENTS:

This work was supported by a CIG (Career Integration Grant) PEPTHIV618601 attributed to C.M. (Marie Curie Research Grants Scheme).

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FIGURE LEGENDS

Figure 1: Inhibition of *E. faecalis* and *E. nucleatum* growth by a saturated solution of $Ca(OH)_2$

Broth dilution assays were performed on *E. faecalis* and *F. nucleatum* in the presence of a saturated solution of $Ca(OH)_2$. Results are expressed in percentage of growth inhibition and represent a mean of at least three independent experiments. For each set of assays, standard deviations were determined.

Figure 2: Activity of antimicrobial peptides against *E. faecalis*

A) Broth dilution assays were performed on *E. faecalis* in the presence of the indicated peptides at a final concentration of 200µg/mL. Results are expressed in percentage of growth inhibition and correspond to a mean of at least three experiments done independently. For each set of assays, standard deviations were determined.

B) The MIC of D-Ctl on *E. faecalis* was determined by broth dilution assays in the presence of increasing amounts of D-Ctl and determined using a modified Gompertz model. For each set of assays, standard deviations were determined.

Figure 3: Stability of D-Ctl in a saturated solution of $Ca(OH)_2$ and in the supernatant of *E. faecalis*

A) D-Ctl at the MIC was incubated in a saturated solution of $Ca(OH)_2$ (100% = 1.7mg/mL, pH=9) for 24h. The samples were then analyzed by HPLC. The chromatograms 1 and 2 correspond to D-Ctl and D-Ctl diluted in a saturated solution of $Ca(OH)_2$ (pH=9), respectively.

B) The supernatant of *E. faecalis* was incubated with or without D-Ctl at 37°C for 24h and the samples were analyzed by HPLC. The chromatograms 1, 2 and 3 correspond to D-Ctl, D-Ctl diluted in the supernatant of *E. faecalis* and the supernatant of *E. faecalis* alone, respectively.

Figure 4: Cytotoxicity of $Ca(OH)_2$ towards human gingival fibroblasts

HGF-1 cells were incubated with $Ca(OH)_2$ 100%, 50% or 25% and MTT assays were performed after incubations of 24h, 48h and 72h. Results were expressed in percentage of cell viability in comparison to the control and represent a mean of at least three independent experiments. For each set of assays, standard deviations were determined.

Figure 5: Antimicrobial activity, stability and cytotoxicity of the combination between D-Ctl and $Ca(OH)_2$

A) The most efficient combination of $Ca(OH)_2$ and D-Ctl was determined by broth dilution assays. The different combinations tested are indicated in the graph. Results correspond to a mean of at least three experiments done in triplicate and are expressed in percentage of *E. faecalis* growth inhibition. For each set of assays, standard deviations were determined.

B) The stability of the combination was determined by HPLC. The chromatograms 1 and 2 correspond to D-Ctl and the combination of 50% $Ca(OH)_2$ and ½ MIC of D-Ctl diluted mili-Q water (pH=8,5), respectively.

C) The cytotoxicity of the combination between 50% $Ca(OH)_2$ and ½ MIC of D-Ctl on HGF-1 cells was assessed with MTT assays. Data represent a mean of at least three

independent experiments and are expressed in percentage of viability. Standard deviations were determined for each condition.

D) The efficacy of the combination between 50% $Ca(OH)_2$ and ½ MIC of D-Ctl compared with 50% $Ca(OH)_2$ was assessed on four other main endodontic pathogens: *Fusobacterium nucleatum, Parvimonas micra, Prevotella intermedia* and *Candida albicans*. Results are expressed in percentage of growth inhibition and correspond to a mean of at least three independent experiments, each performed in triplicate.

Figure 1

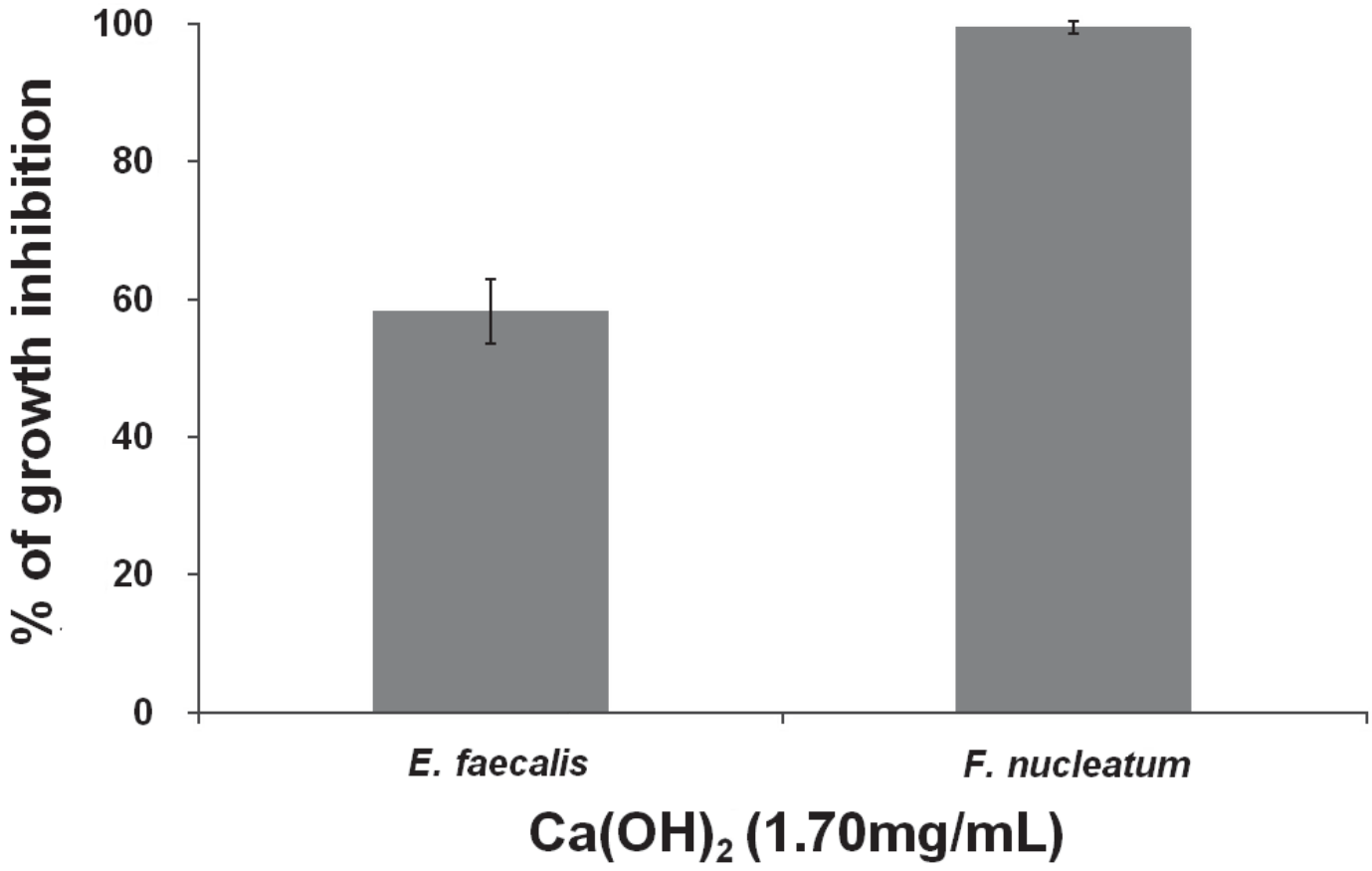
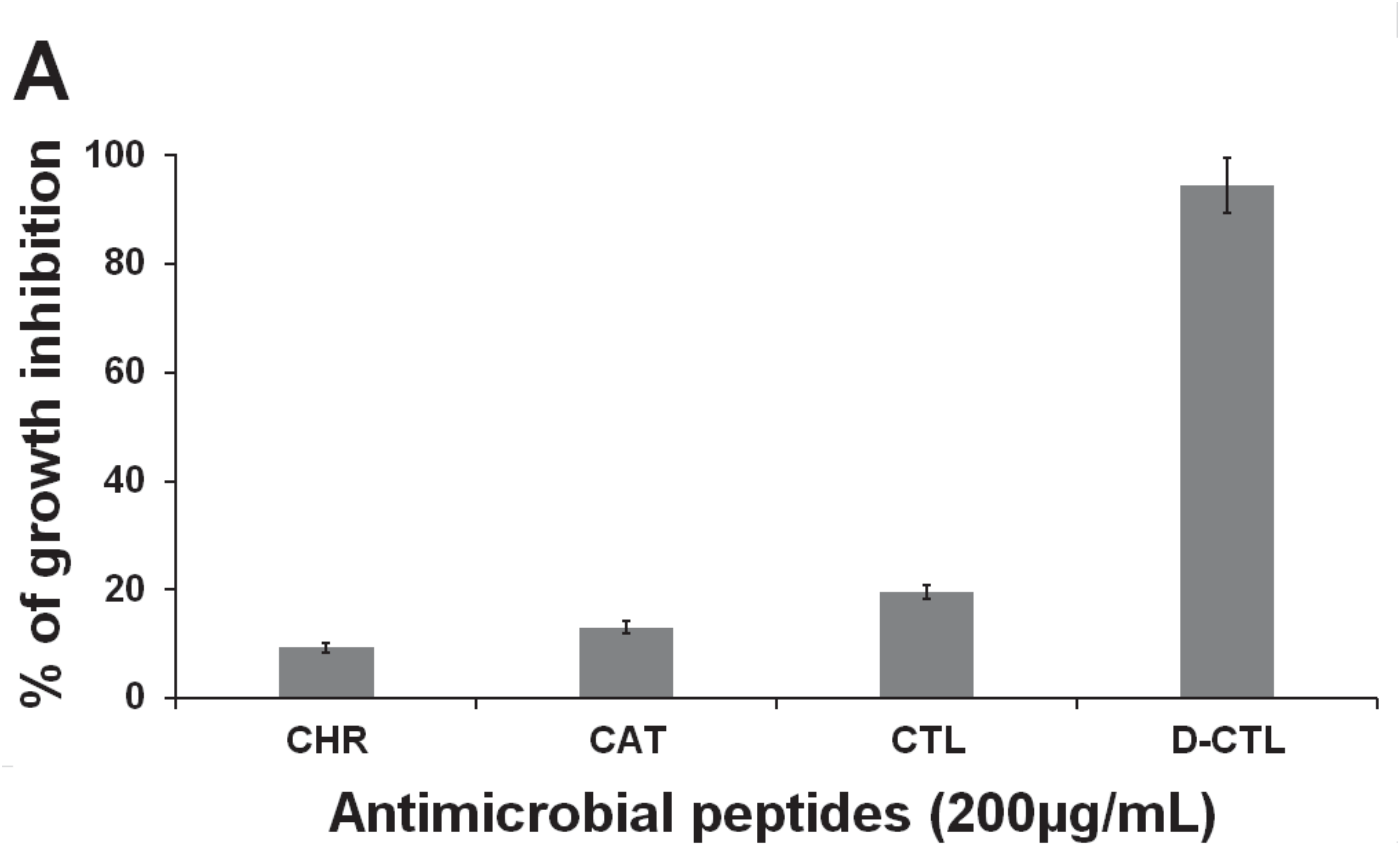

100
80
60
40
20
0
% of growth inhibition
E. faecalis
F. nucleatum
Ca(OH)2 (1.70mg/mL)

Figure 2.


A
% of growth inhibition
100
80
60
40
20
0
CHR
CAT
CTL
D-CTL
Antimicrobial peptides (200μg/mL)

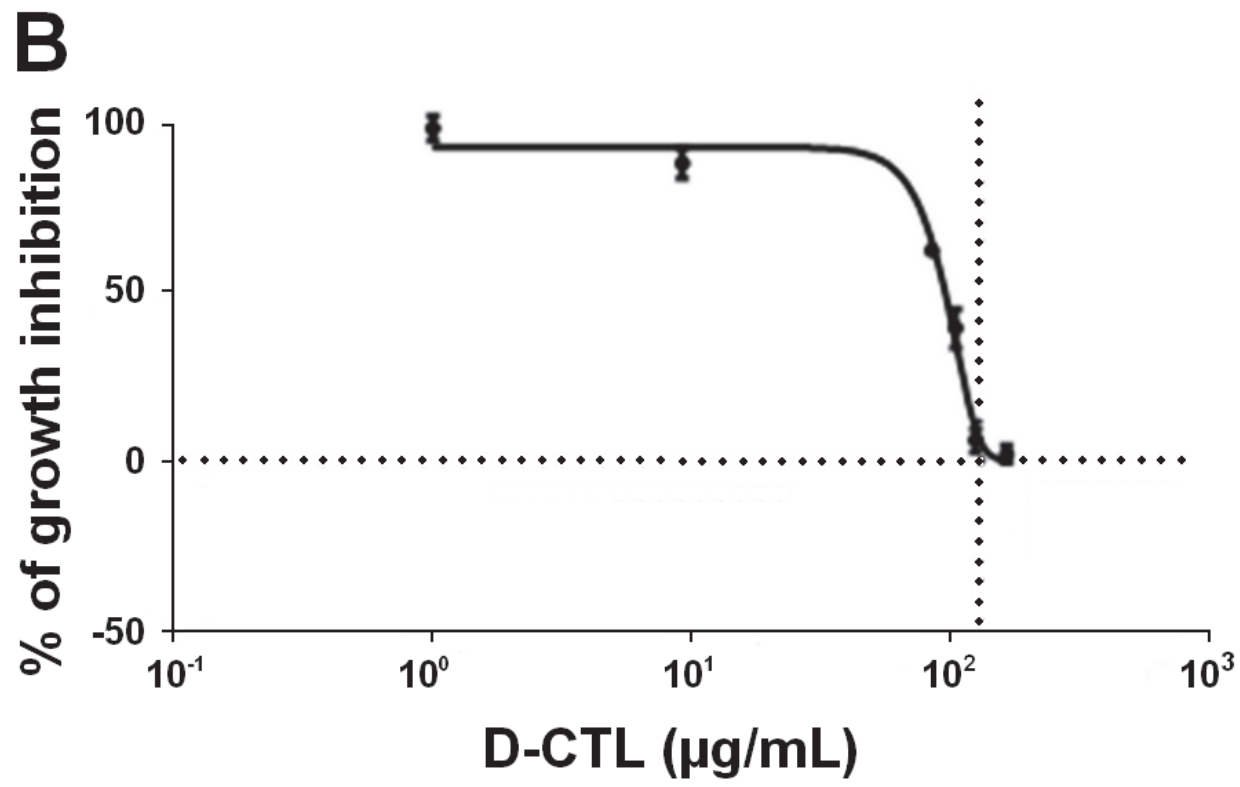

B
% of growth inhibition
100
50
0
-50
10-1
100
101
102
103
D-CTL (μg/mL)

Figure 3.

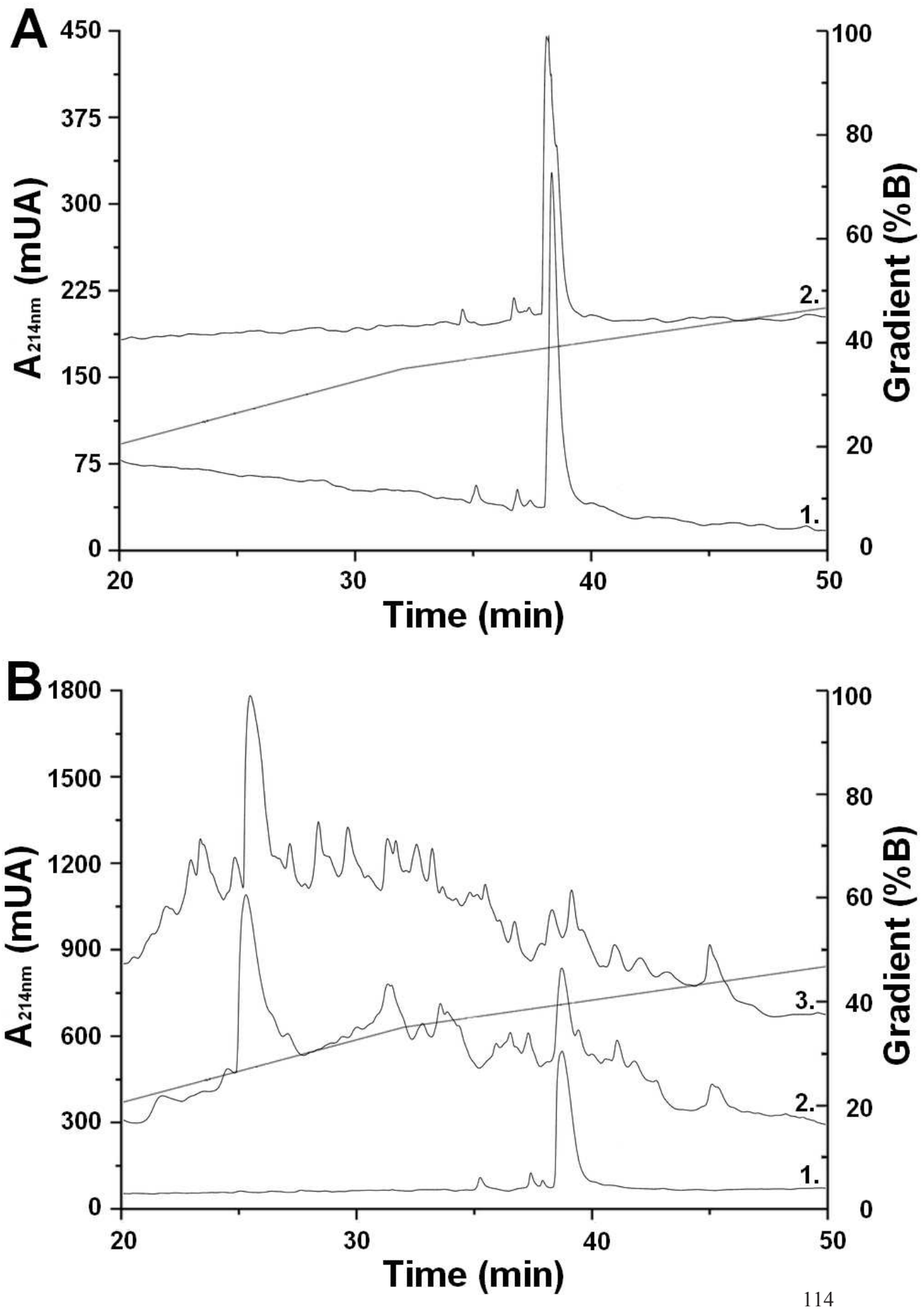

A
450
375
300
225
150
75
0
A214nm (mUA)
100
80
60
40
20
0
Gradient (%B)
2.
1.
20
30
40
50
Time (min)
B
1800
1500
1200
900
600
300
0
A214nm (mUA)
100
80
60
40
20
0
Gradient (%B)
3.
2.
1.
20
30
40
50
Time (min)

Figure 4 :

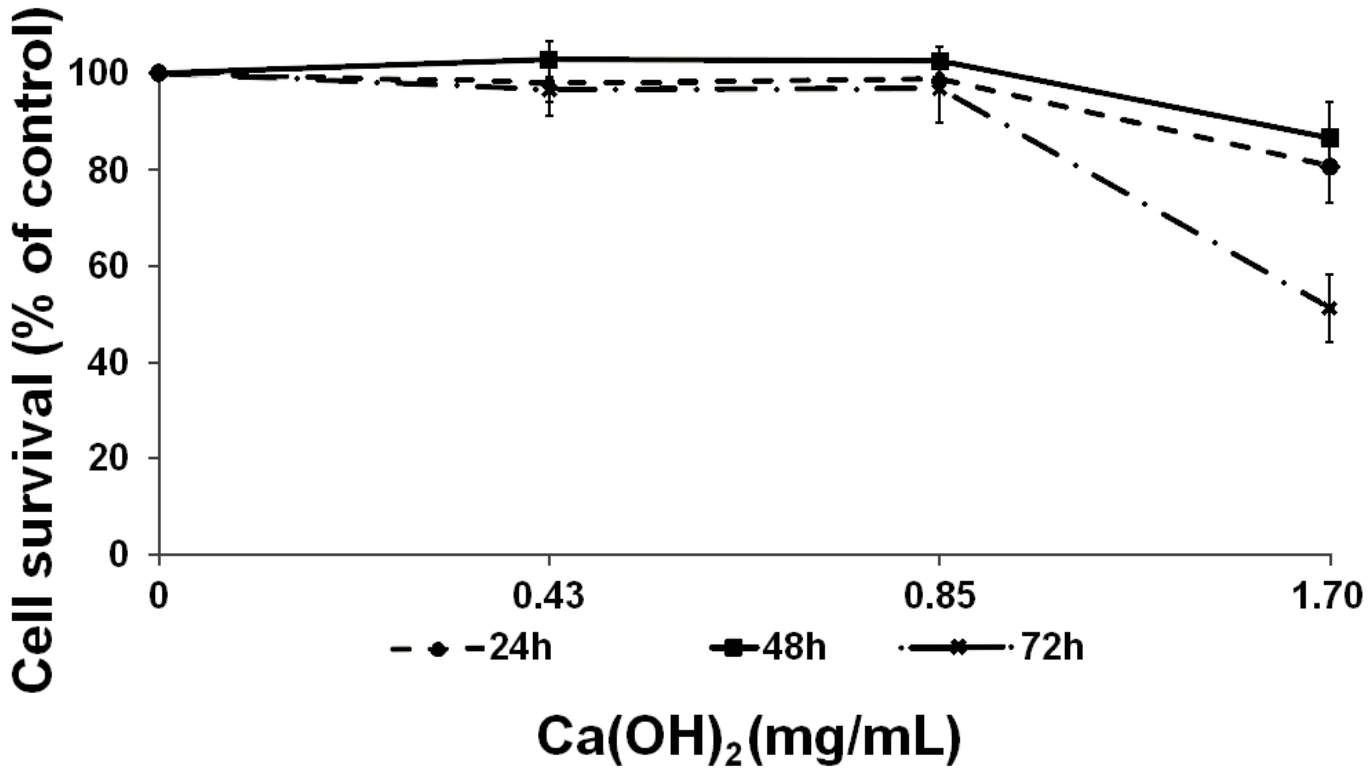
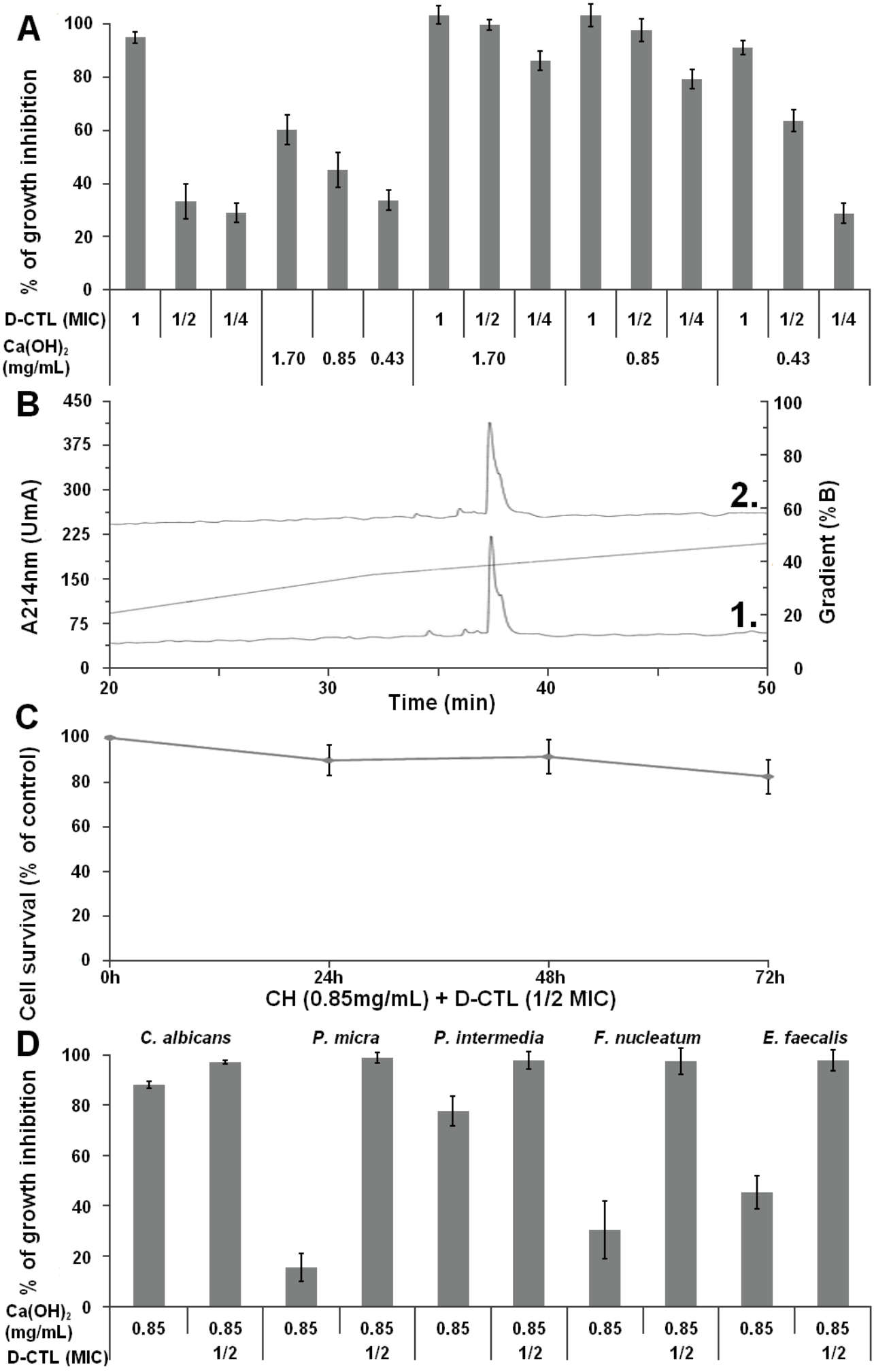

Cell survival (% of control)
100
80
60
40
20
0
0
0.43
0.85
1.70
24h
48h
72h
Ca(OH)2(mg/mL)

Figure 5.


A
% of growth inhibition
100
80
60
40
20
0
D-CTL (MIC)
1
1/2
1/4
1
1/2
1/4
1
1/2
1/4
1
1/2
1/4
Ca(OH)2 (mg/mL)
1.70
0.85
0.43
1.70
0.85
0.43
B
A214nm (UmA)
450
375
300
225
150
75
0
2.
1.
Gradient (% B)
100
80
60
40
20
0
20
30
40
50
Time (min)
C
Cell survival (% of control)
100
80
60
40
20
0
0h
24h
48h
72h
CH (0.85mg/mL) + D-CTL (1/2 MIC)
D
% of growth inhibition
C. albicans
P. micra
P. intermedia
F. nucleatum
E. faecalis
100
80
60
40
20
0
Ca(OH)2 (mg/mL)
D-CTL (MIC)
0.85
0.85
1/2
0.85
0.85
1/2
0.85
0.85
1/2
0.85
0.85
1/2
0.85
0.85
1/2

Publications and communications

Publications and Communications

- Publications

1- **Abdurraouf Zaet**, Pauline Dartevelle, Fadoua Daouad, Claire Ehlinger, Fabienne Quilès, Grégory Francius, Christian Boehler, Camille Bergthold, Benoît Frisch, Gilles Prévost, Philippe Lavalle, Francis Schneider, Youssef Haïkel, Marie-Hélène Metz-Boutigue, Céline Marban. ***D-Cateslytin, a new antimicrobial peptide with therapeutic potential.*** Scientific Reports 7:15199 (2017) DOI: 10.1038/s41598-017-15436-z

2- Claire Ehlinger, Pauline Dartevelle, **Abdurraouf Zaet**, Kurashige Yoshihito, Youssef Haïkel, Marie-Hélène Metz-Boutigue, Céline Marban. ***Calcium hydroxide and D-Cateslytin: a combination therapy to eradicate root canal pathogens.*** **(Submitted)**

3- Pauline Dartevelle, Claire Ehlinger, **Abdurraouf Zaet**, Morgane Rabineau, Benoit Westermann, Jean-Marc Strub, Sarah Cianferani, Youssef Haïkel, Marie-Hélène Metz-Boutigue, Céline Marban. ***D-Ctl: a new antifungal agent for the treatment of oral Candida albicans associated infections.*** **(Under process)**

- Communications

Poster at:

International Conference on Advances in Biomedicine and Biomedical Engineering

6th International Conference on Biotechnology and Bioengineering in September 26-28, 2017 Offenburg, Germany.

PART-IV
DISCUSSION AND PERSPECTIVES

Discussion and Perspectives

The resistance of bacterial strains to the antibiotics considered as one of the eventual fears which affect the humans being healthy as well as the animals in all region of the world. Thereby, excessive use of these antibiotics leads to the emergence and spread of bacterial resistance, which cause well-known infections such urinary tract, blood-stream, and pneumonia therefore these infections could become fatal. Besides, misuse of antibiotics will lead the situation worse. Hence, continue of keeping the efficiency of these antibiotics and operate them for human health interest is something extremely important (Chang Q et al., 2015).

Intriguingly, the development of new molecules that could be alternative to conventional antibiotics is critical need to curb the emergence of resistance phenomena. Correspondingly, all the drugs which are used as an antimicrobial agent are containing natural peptides that can limit or prevent resistance phenomena against microorganisms including parasites, fungi, bacteria as well as viruses.

As a matter of fact, the major component of innate immunity system is AMPs which have been offered as a novel source for antibiotics (Zasloff, 2002). They display a rapid direct antimicrobial action and they modulate the response of immune cells (Zhang L and Gallo R, 2016).

Within my thesis I reported the design and characterization of a new antimicrobial agent to be alternative to conventional antibiotics. In fact, the basic study corresponds to the analysis of new molecules of AMPs which supposed to have connections in the dynamic of host-pathogen interactions. Several antimicrobial assays were performed to compare the efficiency between D-Ctl and conventional antibiotics on wide broad of obligate and facultative anaerobes bacterial strains such as *S. aureus* MSSA and MRSA, *E. coli wild* type and MDR, as well as some oral cavity pathogenic such as *F. nucleatum*, *P. intermedia* and *P. micra.* These bacterial strains are responsible for several serious diseases that can release many of virulence factors.

D-Ctl is derivative of L-Cateslytin (L-Ctl) and it was modified and with keeping the same sequences of L-Ctl but in dextrogyre configuration of (D) amino acids. The interest in D-peptides have increased over recent years. Comparable with L-peptide, D-peptide is able to extend the plasma half-life, increase the stability of enzymes, improve binding activity and specificity with the receptor or target proteins and improve oral bioavailability (Liu M et al., 2016). Therefore, D-peptides are stable more than L-peptides toward proteolysis, and they used

synthetic vaccines and as immunomodulators in T-cell responses due to its great potential (Van Regenmortel MH and Muller S, 1998).

We deliberated in this study to examine the antimicrobial activity of D-Ctl and L-Ctl as a control in parallel, as well as the different conventional antibiotics had been tested. Remarkably, our results manifested in that the efficiency of D-Ctl is still higher more than L-Ctl, and the range of MIC was varied between 1.7 in MSSA to 17.9 folds in *E. coli* MDR. D-Ctl is active against wide range in both grams of bacteria. Thereby, D-Ctl deemed to be an antimicrobial agent. In turn, when the efficiency of D-Ctl was compared with the antibiotics which prescribed for each pathogen, the results demonstrated that the efficiency of antibiotics agents were still higher than the D-Ctl. Except, the efficiency of Ampicillin which is for *E. coli* wild type (MIC=7.0 µg/mL) that could be the same of D-Ctl efficiency (MIC= 8.0 µg/mL) and also much higher than Kanamycin (MIC= 21.6 µg/mL). All together these data are protected by a patent EP16306539.4 "New D- configured cateslytin peptide". Furthermore, to verify such a hypothesis a large panel of bacterial strains [Table III] were screened in collaboration with a company (Atlangram, Atlantic Group for Research on Anti-Microbials, Nantes, France). To continue this study, we have received a financial support from the SATT Conectus for a "preincubation project". And the data will be integrated in a new paper [Table IV].

Table III: List of strains tested by Atlangram

Strains	WT	ESBL	AmpC (high level)	Carbapenemase		
				OXA 48	KPC	VIM
Escherichia coli	3	3	3	3		
Klebsiella pneumoniae	3	3			3	
Enterobacter cloacae	3	3	3	3		
Enterobacter aerogenes	3	3	3			
Serratia marcescens	3		3			
Morganella morganii	3		3			
Citrobacter freundii	3		3			
Methicillin-susceptible *Staphylococcus aureus* (MSSA)	3					
Methicillin-resistant *Staphylococcus aureus* (MRSA)	3					
Pseudomonas aeruginosa	3		3			3
Candida albicans	3					
Total (78 isolats)	33	12	21	6	3	3

Table IV: The MICs (µg / mL) of the 4 molecules are obtained by Atlangram

Strains	Type			**Isoform D**	**Control D**	**Isoform L**	**Control L**
Esherichia coli	Wild type	-	ATCC 6 (25 922)	32	>128	128	>128
		-	Ec 4	16	128		
		-	Ec 204	32	128		
	BLSE	CTXM	Ec 46 (C11)	16	128	>128	>128
		CTXM	Ec 47 (C12)	16	>128		
		CTXM	Ec 70	64	>128		
	Amp C	-	Ec 73	128	>128	>128	>128
		-	Ec 74	16	128		
		-	Ec 195	8	64		
	OXA 48	BLSE	Ec 71	16	128		
		-	Ec 197	16	64		
		-	Ec 198	16	128		
Klebsiella pneumoniae	Wild type	-	B-24	32	>128	>128	>128
		-	B-73 (C3)	32	128		
		-	B-75 (C4)	32	128		
	BLSE	CTXM	B-49 (C2)	32	128	128	>128
		-	B-50	32	>128		
		CTXM	B-68 (C1)	32	>128		
	KPC	-	B-97	>128	>128		
		BLSE	B-101	32	>128		
		-	B-102	128	>128		
Enterobacter cloacae	Wild type	-	B-141	128	>128	>128	>128
		-	B-142	32	128		
		-	B-144	64	>128		
	BLSE	-	B-43	32	>128	>128	>128
		-	B-57	32	128		
		-	B-167	32	>128		
	Amp C	-	B-38	32	>128		
		-	B-44	32	>128		
		-	B-96	32	128		
	OXA 48	BLSE	B-112	64	128		
		BLSE	B-113	32	128		
		BLSE	B-118	32	128		
Enterobacter aerogenes	Wild type	-	B-145	32	128	128	>128
		-	B-146	32	128		
		-	B-147	32	128		
	BLSE	-	B-168	32	>128	>128	>128
		-	B-169	64	128		
		-	B-170	32	128		
	Amp C	-	B-148	32	128		
		-	B-149	32	128		
		-	B-150	32	128		

Serratia marcescens	Wild type	-	B-151	>128	>128	>128	>128
		-	B-152	>128	>128		
		-	B-153	128	>128		
	Amp C	-	B-154	>128	>128	>128	>128
		-	B-155	128	>128		
		-	B-156	128	>128		
Morganella morganii	Wild type	-	B-157	>128	>128	>128	>128
		-	B-158	>128	>128		
		-	B-159	>128	>128		
	Amp C	-	B-59	>128	>128	>128	>128
		-	B-160	>128	>128		
		-	B-161	>128	>128		
Citrobacter freundii	Wild type	-	B-162	32	128	128	>128
		-	B-163	32	128		
		-	B-164	32	128		
	Amp C	-	B-65	64	128	>128	>128
		-	B-165	32	128		
		-	B-166	64	128		
MSSA	Wild type	-	ATCC 1 (29213)	64	>64	>128	>128
		-	SA 100	64	>128		
		-	SA 112	32	>128		
MRSA	Wild type	-	ATCC 21	64	>128	>128	>128
		-	SA 111	64	>128	>128	>128
		-	SA 166	64	>128		
Pseudomonas aeruginosa	Wild type	-	P122	>128	>128	>128	>128
		-	P129	128	>128		
		-	P131	128	>128		
	Amp C	-	P124	128	>128	>128	>128
		-	P125	128	>128		
		-	P85	128	>128		
	VIM	-	P144	128	>128		
		-	P-149	64	>128		
		-	P-150	128	>128		
Candida albicans	Wild type	-	L1	256	512	512	512
		-	L2	256	512		
		-	L3	256	512		

Importantly, in order to decrease the concentration of the antibiotics used to fight infection, antimicrobial combinations are administered to prevent or delay the emergence of resistance.

Notably, our results showed that in a combination of D-Ctl and Amoxicillin there is a synergistic effect against *P. micra* and *P. intermedia*. In addition, D-Ctl offered additive effect in combination with Methicillin, Amoxicillin and Cefotaxime fighting against *F. nucleatum*, *E. coli* MDR and *S. aureus*, respectively.

More added to the list of interest which is the stability of L-Ctl and D-Ctl towards bacterial proteases. Interestingly, the results demonstrate that D-Ctl is stable in all bacterial supernatant examined. Previous data of our group show that L-Ctl also was stable and resistance to degradation by virulence factors of *S. aureus* MSSA and MRSA (Aslam et al., 2013). Our present data show that L-Ctl was not degraded by *P. micra*, *P. intermedia* and *F. nucleatum* virulence factors. In contrast, L-Ctl was degraded by *E. coli* wild type and MDR virulence factors.

For more benefit with more interest, resistance assays of *E. coli* were performed to highlight the bacterial resistance toward the D-Ctl, Ampicillin and Cefotaxime to treat *E. coli* infections. The results showed that D-Ctl does not trigger resistance on *E. coli*, unlike to Ampicillin and Cefotaxime from which MICs were multiplied by 3-fold over 24 days. As known, the main compound of the gram-negative bacteria membrane is that LPS which also termed as an endotoxin, the releasing of LPS occurs during cell death, bacteria cell division as well as during the treatment of gram-negative bacterial infections *via* antibiotics.

Moreover, LPS is a strong inducer of the human`s innate immune system. Large amounts of cytokines pro-inflammatory can be produced by phagocytic and monocytic cells which caused by LPS when is released into the blood system. Due to these cytokines can damage multiple organs, for example, septic shock syndrome (Evans M and Pollack M, 1993; Papo N and Shai Y, 2005; Rosenfeld Y et al., 2006; Trent M et al., 2006). D-Ctl and L-Ctl do not trigger cytokine release. Also, PBMCs were treated with LPS as a control with the same conditions. Data show that LPS induce the release of wide range of anti-inflammatory cytokines such as IL-10, even more, it released pro-inflammatory cytokines such as TNFα, IFNγ and G-CSF. [Figure 3 in manuscript 1]. Furthermore, the results of our experiments concluded that D-Ctl and L-Ctl are not haemolytic and no lysis of cells were observed at all. Also, there are no cytotoxic towards PBMCs after 72h of treatment even the peptides concentration up to

100μg/mL [Figure 2 in manuscript 1]. With these results of toxicology can be emphasized to employ D-Ctl as a new antimicrobial against *E. coli* infections.

By physico-chemical methods, the mechanism for antimicrobial activity against *E. coli* was deciphered. By using infrared analysis, we show that after treatment by D-Ctl the metabolism of *E. coli* is poorly impacted [Figure 5 in manuscript 1]. Nevertheless, the permeabilized for the membrane of bacterial were exhibited by the images of epifluorescence after stained by *Bac*light ™ [Figure 6 in manuscript 1]. Losing cytosol causes lysis and death bacteria due to action of D-Ctl, and when the elasticity of bacterial cell wall decreases drastically, it can be suggested that the bacterial cell wall is highly destroyed [Figure 6 in manuscript 1]. Herein, the results presented the tiniest quantity of peptide molecules also the proportion of the antimicrobial action needed to arrive at the point of lysis of cell which is robustly based on the conformation of the peptide. Amazingly, even though of the lowest concentration of D-conformer is still most efficient action (by a factor of around 20) that is confirmed by our results. which dissimilar to other lately studies which have not presented such a remarkable variance in antimicrobial activity of L-and D-conformers (Chen Y et al., 2006; Wang C et al., 2016).

Lately a few years ago, some natural peptides such as cathelicidins or other derivatives peptides like MBI-226 and LL-37 have been introduced to clinical trials. (studies NCT00211523, NCT00211497 and NCT00027248 for the prevention of central venous catheter-related bloodstream infections and acne) and PMX-30063 (study NCT01211470 for acute bacterial skin and skin-structure infection). Because of the rising price of peptides production, a capability to cytotoxicity and proteases the development of these peptides in clinical and trading are limited. For instance, over 10 μM of LL-37 (human cathelicidin) can promote smooth muscle cells, T cells and apoptosis of epithelial cells (Oudhoff M et al., 2010). On top of that, LL-37 is sensitive to protease cleavage that induce abolishment of its antimicrobial properties (Koneru L et al., 2016). Widely believed defensins have observed as another choice to classical antimicrobials. Nevertheless, due to the complicated secondary and tertiary structures, there are no qualified methods of production of large amount, thus it is the main barrier to use them as therapeutics (Marr A et al., 2006; Corrales-Garcia L et al., 2011).

The accumulated evidence provides convincing support for D-Ctl which shows plentiful of properties compared to other peptide-based drugs. As a matter of fact, D-Ctl is linear, short (15 amino acids) and stable at high temperature with a wide range of pH, which makes its products really accessible. Furthermore, using D-peptide appears as a prolific approach to elim-

inate degradation by secreted bacterial proteases. Succinctly, what makes D-Ctl works as an appealing factor for biopharmaceutical development is its potential and safety aspects; in addition to that, it has remarkable efficiency with undetectable susceptibility to resistance. Furthermore, in order to achieve accessibility for mankind, there should be a safe and secure pharmacology and drug toxicology. Now this study needs to be extended *in vivo* such as animals (rats) and the challenge is to set up pharmacology studies because are very important prior to clinical trials.

Discussion et perspectives en français

La résistance des souches bactériennes aux antibiotiques est considérée comme un risque majeur pour les humains en bonne santé, ainsi que les animaux dans toutes les régions du monde. En effet, l'utilisation excessive des antibiotiques conduit à l'émergence et à la propagation de la résistance bactérienne, ce qui provoque des infections sévères pouvant devenir fatales. Par conséquent, il faut veiller à maintenir l'efficacité de ces antibiotiques en les utilisant dans l'intérêt de la santé humaine (Chang Q et al., 2015). Une autre alternative concerne le développement de nouvelles molécules antibiotiques pour freiner l'émergence des phénomènes de résistance. Ainsi, les PAMs peuvent limiter les phénomènes de résistance contre les microorganismes (les parasites, les champignons, les bactéries ainsi que les virus). (Zasloff, 2002). Ils présentent une action antimicrobienne directe rapide et modulent la réponse des cellules immunitaires (Zhang L et Gallo R, 2016).

Dans ma thèse, j'ai exposé la conception et la caractérisation d'un nouveau PAM comme alternative aux antibiotiques conventionnels. Plusieurs essais été réalisés pour comparer l'efficacité de D-Ctl et des antibiotiques conventionnels, sur une large gamme de souches bactériennes anaérobies, telles que *S. aureus* MSSA et MRSA, *E. coli* wild type et MDR, ainsi que certains pathogènes de la cavité buccale tels que *F. nucleatum, P. intermedia et P. micra*. Ces souches bactériennes sont responsables de plusieurs maladies graves, et peuvent libérer beaucoup de facteurs de virulence.

D-Ctl est un dérivé de la L-Cateslytine (L-Ctl) qui a été modifié en conservant la même séquence que L-Ctl, mais avec des acides aminés en configuration dextrogyre (D). L'intérêt pour les peptides D a augmenté au cours de ces dernières années. Par comparaison avec le L-peptide, le D-peptide est capable d'allonger la demi-vie plasmatique, d'augmenter la stabilité du peptide face aux enzymes, d'améliorer l'activité de liaison et la spécificité des protéines cibles et d'améliorer la biodisponibilité orale (Liu M et al., 2016). Par conséquent, les D-peptides sont plus stables que les L-peptides vis-à-vis de la protéolyse. En effet, ils sont utilisés depuis longtemps en tant que vaccins synthétiques et immunomodulateurs dans la réponse des lymphocytes T en raison de leur grand potentiel (Van Regenmortel MH et Muller S, 1998).

Nos résultats se sont avérés remarquables en ce qui concerne l'efficacité de D-Ctl qui est supérieure à celle de L-Ctl. La CMI est précisément 1,7 fois plus élevée pour L-Ctl que pour D-Ctl sur *E. coli* MSSA, et 17,9 fois plus sur *E. coli* MDR. Toutefois, lorsque l'efficacité de D-Ctl a été comparée aux antibiotiques prescrits pour chaque agent pathogène, les résultats ont

démontré que la majorité des agents antibiotiques était plus efficaces. Seuls l'ampicilline sur *E. coli* wild type possède une efficacité proche (CMI = 7,0 µg/mL, *vs* 8,0 µg/mL pour D-Ctl), et la kanamycine une efficacité moins élevée (CMI = 21,6 µg/mL). Toutes ces données sont protégées par un brevet EP16306539.4 "Nouveau peptide de cateslytine en configuration D". De plus, pour vérifier une telle hypothèse, un large panel de souches bactériennes [Tableau III] a été testé en collaboration avec une société (Atlangram, Groupe Atlantique de Recherche sur les Anti-Microbiens, Nantes, France).

Pour poursuivre cette étude, nous avons reçu un soutien financier de la SATT Conectus pour un « projet de préincubation». Et les données sont intégrées dans un autre document [Tableau IV].

Afin de réduire la concentration des antibiotiques utilisés pour combattre l'infection, des combinaisons antimicrobiennes sont évaluées pour prévenir ou retarder l'émergence de résistance. Nos résultats ont montré que dans une combinaison de D-Ctl et d'amoxicilline, il existe un effet synergique contre *P. micra* et *P. intermedia*. En outre, D-Ctl présente un effet additif, respectivement en le combinant avec la méthicilline, l'amoxicilline et la céfotaxime pour lutter contre *F. nucleatum*, *E. coli* MDR et *S. aureus*.

La stabilité L-Ctl et D-Ctl vis-à-vis des protéases bactériennes représente un intérêt majeur que nous avons étudié. De manière intéressante, nos résultats démontrent que D-Ctl est stable dans tous les surnageants bactériens examinés. Les données antérieures de notre laboratoire ont montré que L-Ctl était également stable et résistait à la dégradation par les facteurs de virulence de *S. aureus* MSSA et MRSA (Aslam et al., 2013). Nos données actuelles montrent que L-Ctl n'a pas été dégradé par les facteurs de virulence de *P. micra, P. intermedia et F. nucleatum*. En revanche, L-Ctl a été dégradée par des facteurs de virulence de *E. coli* wild type and MDR.

Des tests de résistance de *E. coli* ont été effectués pour mettre en évidence la résistance bactérienne envers le D-Ctl, l'ampicilline et le céfotaxime. Les résultats ont montré que D-Ctl ne provoque pas de résistance à *E. coli*, contrairement à l'ampicilline et à la céfotaxime dont les CMI ont été multipliées par trois en 24 jours.

Le composé principal de la membrane des bactéries Gram-négatives est une endotoxine, le LPS. Sa libération se produit pendant la mort cellulaire, la division cellulaire bactérienne, ainsi que pendant le traitement des infections bactériennes Gram négatif *via* des antibiotiques. De plus, le LPS est un puissant activâtes de l`immunité innée chez l'humain. De grandes quantités de cytokines pro-inflammatoires peuvent être produites par des cellules phagocytaires

et monocytaires, activées par le LPS lorsqu'il est libéré dans la circulation. Ces cytokines peuvent endommager plusieurs organes et provoquer par exemple, le syndrome de choc septique (Evans M et Pollack M, 1993, Papo N et Shai Y, 2005, Rosenfeld Y et al., 2006, Trent M et al., 2006). D-Ctl et L-Ctl ne déclenchent pas la libération de cytokines. De plus, les PBMCs ont été traitées avec du LPS comme témoin dans les mêmes conditions. Les données montrent que le LPS induit la libération d'une large gamme de cytokines anti-inflammatoires comme l'IL-10, et même de cytokines pro-inflammatoires telles que le TNFα, l'IFNγ et le G-CSF. [Figure 3 dans le manuscrit 1]. Les résultats de nos expériences démontrent que D-Ctl et L-Ctl ne sont pas hémolytiques, aucune lyse de cellules n'ayant été observée. En outre, il n'y a pas de cytotoxicité envers les PBMCs après 72 h de traitement, même avec une concentration de peptides élevée à 100 μg/mL [Figure 2 dans le manuscrit 1]. Avec ces résultats de toxicologie, D-Ctl pourrait être employé comme un nouvel antimicrobien contre les infections à *E. coli*.

Par des méthodes physico-chimiques, le mécanisme de l'activité antimicrobienne contre *E. coli* a été déchiffré. En utilisant l'analyse infrarouge, nous montrons qu'après traitement par D-Ctl, le métabolisme d'*E. coli* est impacté [Figure 5 dans le manuscrit 1]. La perméabilité de la membrane bactérienne est mise en évidence par les images d'épifluorescence après coloration par *Baclight* ™ [Figure 6 dans le manuscrit 1]. La perte du cytosol provoque la lyse et la mort des bactéries par l'action de D-Ctl, et lorsque l'élasticité de la paroi cellulaire bactérienne diminue considérablement, on peut penser que la paroi bactérienne est majoritairement détruite [Figure 6 dans le manuscrit 1]. Étonnamment, nos résultats montrent que la CMI de D-Ctl est 20 fois place faible que celle de L-Ctl. Ce n'est pas le cas d'autres études récentes qui n'ont pas mis en évidence de différences aussi importantes dans l'activité antimicrobienne des conformères L et D (Chen Y et al., 2006, Wang C et al., 2016).

Il y a quelques années, certains peptides naturels tels que les cathélicidines MBI-226 et LL-37 ont été introduits dans des essais cliniques (études NCT00211523, NCT00211497 et NCT00027248 pour la prévention des infections de la veine centrale liées au cathéter veineux central et de l'acné) et PMX-30063 (étude NCT01211470 pour l'infection aigue de la peau). En raison du coût élevé de la production de peptides, de leur capacité de cytotoxicité et de leur diminution d'efficacité à cause du clivage par des protéases la commercialisation de ces peptides en clinique est encore limitée. Par exemple, à une concentration supérieure à10 μM LL-37 favoriser l`apoptose des cellules épithéliales, des cellules musculaires lisses, des lymphocytes T (Oudhoff M et al., 2010). De plus, LL-37 est sensible au clivage des protéases qui induisent l'abolition de ses propriétés antimicrobiennes (Koneru L et al., 2016).

Les défensines sont largement considérées comme un autre choix d'antimicrobiens classiques. Néanmoins, en raison de leurs structures secondaires et tertiaires complexes, il n'existe pas encore de bonnes méthodes de production en grande quantité. C'est donc le principal obstacle à leur utilisation comme agent thérapeutique (Marr A et al., 2006 ; Corrales-Garcia L et al., 2011).

L'ensemble des qualités de D-Ctl qui ont été montrées dans ma thèse prouve sa supériorité par rapport à d'autres PAMs. En effet, D-Ctl est linéaire, court (15 acides aminés), stable à haute température sur une large gamme de pH et dans de nombreux surnageants bactériens.

Pour résumer, le potentiel et la non toxicité in *vitro* de D-Ctl en font un facteur attrayant pour son développement biopharmaceutique. Afin d'assurer son utilisation l'accessibilité chez l'homme, il est nécessaire que ses propriétés pharmacologiques soient démontrées in *vivo* sur un modèle animal (rat) avant la mise en place des essais cliniques.

PART-V
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Une alternative pour les antibiotiques conventionnels : un nouveau peptide antimicrobien dérivé de la chromogranine A

Résumé

Les peptides antimicrobiens (PAMs) représentent des composants importants de l`immunité innée. Ils sont présents dans la plupart des organismes multicellulaires et constituent la première ligne de défense contre les infections. Ils possèdent un large éventail d`activités, une non-toxicité contre les cellules de l`hôte et des effets synergiques avec les antibiotiques conventionnels. Par conséquent, ils peuvent être d`excellents candidats dans le développement de nouveaux antibiotiques pour lutter contre la résistance de microorganismes.

Concernant les PAMs dérivés de la chromogranine A (CgA), la cateslytine (Ctl) présente des activités antimicrobiennes directes et des propriétés immunomodulatrices. Dans ma thèse, j`ai cherché à caractériser l`épipeptide D-Ctl, où tous les résidus en conformation-L ont été remplacés par des résidus en conformation-D. Tout d`abord, la stabilité dans les surnageants bactériens et des dosages de l`activité antimicrobienne ont été réalisés, ainsi que l`analyse de viabilité des cellules et des dosages des cytokines libérées par les cellules immunitaires. L`efficacité de D-Ctl a été comparée à celle de L-Ctl contre des souches bactériennes, puis les CMIs ont été déterminées et comparées dans le cas de combinaisons avec des antibiotiques conventionnels, afin de montrer un effet synergique et/ou additif. De plus, D-Ctl ne déclenche pas de résistance chez *E. coli*. Des tests de cytotoxicité ont été effectués sur plusieurs types de lignées cellulaires et de PBMCs. Les effets inflammatoires aussi ont été testés. Ensuite, le modèle bactérien *E. coli* MDR a été utilisé pour des analyses physico-chimiques, telles que la microscopie à épifluorescence, la spectroscopie ATR-FTIR et la microscopie à force atomique. Enfin, le brevet D-Ctl a été déposé en 2016 sous le numéro EP 16306539.4 « Nouveau peptide de cateslytine en conformation D ».

En conclusion, D-Ctl est capable de tuer rapidement un large spectre de micro-organismes, et il pourrait potentialiser l`effet antimicrobien de plusieurs antibiotiques.

Mots-clés : Peptides antimicrobiens, Antibiotiques, Chromogranine A, Cateslytine, D-Ctl et L-Ctl.

Abstract

Antimicrobial peptides (AMPs) represent important components of innate immunity. They are present in most multicellular organisms and constitute the first line of defense against infections. They exhibit a large spectrum of activities, a non-toxicity against host cells and synergistic effects with conventional antibiotics. Therefore, they can be as excellent candidates in the development of new antibiotics to fight pathogens resistance.

Concerning to AMPs derived from chromogranin A (CgA), Cateslytin (Ctl) represents a new antibiotic, which displays direct antimicrobial activities and immunomodulatory properties. In my thesis, I aimed to characterize the epipeptide D-Ctl, where all (L-conformation) residues were replaced by (D-conformation) residues. Firstly, antimicrobial assays were performed, cells viability, immune assays, and the stability in bacterial supernatant was tested. The efficiency of D-Ctl was compared with L-Ctl against bacterial strains, then MICs were determined and compared with combinations in presence of classical antibiotics in order to show synergistic or/and additive effect. Moreover, D-Ctl does not trigger resistance in *E. coli*. Also, cytotoxicity assays were performed on several types of cell line and PBMCs. Inflammatory effects were tested too. Then, bacterial model *E. coli* MDR was used for physicochemical analysis such as epifluorescence microscopy, ATR-FTIR spectroscopy and atomic force microscopy. Finally, D-Ctl patent has been deposited in 2016 under the number EP 16306539.4 “New D-configured cateslytin peptide”.

To conclude: D-Ctl is able to rapidly kill a broad spectrum of microorganisms, and it could potentiate the antimicrobial effect of several antibiotics.

Keywords: Antimicrobial peptides, Antibiotics, Chromogranin A, Cateslytin, D-Ctl and L-Ctl.