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Apport des Nanoparticules d'alginate à la multifonction des peptides antimicrobiens, exemple de la bactériocine de classe IIb Entérocine DD14

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Finally, here came this last page. You don't even think of it when you start writing thesis work, then we look forward to it at the first lines, at the first breaks or at the end of a hard week of writing a manuscript, then you're like, "Already! I finished!! ". Without a doubt, this page represents the end of the most difficult task I have ever undertaken. However, the last page is only once plus a first page announcing the beginning of another adventure. Now is the time to thank all of those, close or far, who helped me during my unique adventure to make this work a reality. These acknowledgments are always challenging and I apologize, in advance, to those who could be forgotten.

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ABSTRACT

In this PhD project, we used alginate nanoparticles as carriers of a leaderless two-peptide bacteriocin, the enterocin14 designed EntDD14. This bacteriocin, previously isolated from *Enterococcus faecalis* 14 strain, was characterized for its potent anti-bacterial activity against a set of Gram-positive bacteria. Nanoparticles used here were obtained from alginate, which is considered as a safe and sustainable polymer, by a green method (ball mill). Afterwards, EntDD14 was adsorbed onto these alginate nanoparticles (AlgNPs) by a simple physical process. Then, anti-bacterial activity of this formulation was measured against pathogenic strains of *Clostridium perfringens* isolated from chicken died from necrotic enteritis (NE). As a matter of fact, the antibacterial activity registered for the formulation has increased based on the MIC values obtained, which were 4 times below that registered for EntDD14 alone.

Importantly, the activity of this newly developed formulation was then assessed under conditions mimicking those occurring in the gastro-intestinal tract of human as well as those form chicken. The activity remained thereof remarkably stable. Moreover, the safety of bacteriocin alone and adsorbed on the AlgNPs was established by studying the cytotoxicity on Caco-2 and HT-29 human cell lines models after 24 hours of contact. Further studies on the effect of EntDD14 alone, or EntDD14 loaded on alginate nanoparticles used at a sub-inhibitory concentration, were conducted on Clostridia strains. The expression of genes coding for toxins involved in the pathogenicity of this malevolent bacterium was determined by qPCR. The toxins targeted were α-toxin, enteritis toxin B-Like, collagen adhesion protein and thiol activated cytolysin. Remarkably, the expression of these genes was significantly down-regulated following the addition of EntDD14 loaded on nanoparticles, but not upon the addition of EntDD14 alone. Taken together these data showed that adsorption of EntDD14 on alginate nanoparticles

permitted to obtain a safe formulation, with enhanced activity against *Clostridium perfringens* and able to decrease the toxins genes expression involved in the pathogenicity.

To gain more insights and reveal further function attributable to EntDD14, we carried out a study on the synthesis of interleukins, mainly IL-6 and IL-8. This study consisted to induce inflammatory reaction by challenging eukaryotic Caco-2 cells with LPS. After induction of inflammation on Caco2 cells with LPS (50 µg/ml) from *E. coli* (LC control), the IL-6 secretion has reached 2.5 pg/ml, and has drastically reduced to around 0.5 pg/ml when treated with EntDD14 at 60 and 240 µg/ml. Regarding IL-8, the effect of the different treatments was clear with a decrease in the IL-8 secreted in all cases by at least two to three folds in comparison to untreated inflamed control (138.85 pg/ml).

In this study, the antiviral activity of EntDD14 alone or that of the formulation was also evaluated. Thus, Vero cells, isolated from African green monkey's kidney, infected with herpes simplex virus (HSV-1) at a multiplicity of infection (MOI) of 0.07, were incubated with different concentrations of EntDD14 ranging from 7.5 to 150 µg/ml. After to 24 h of incubation, the EntDD14 showed a significant anti-HSV1 activity at higher concentrations (>30 µg/ml).

In conclusion EntDD14 exhibits interesting multifunctional activities and showed good suitability as alternative or complement antibiotic treatments in association with nanoparticles in necrotic enteritis context.

RESUME

Dans ce projet de thèse, nous avons évalué l'apport des nanoparticules d'alginate sur la multifonction des bactériocines, en particulier l'entérocine DD14 (EntDD14). Cette bactériocine à deux peptides sans séquence leader, appartenant donc à la calsse IIb, a été caractérisée pour son activité antibactérienne contre des germes pathogènes comme *Clostridium perfringens*. Les nanoparticules d'alginate (AlgNPs) que nous avons utilisé ont été produites par un broyage physique, en utilisant un broyeur à billes (Retsch PM100), sans traitement chimique. Une fois les AlgNPs préparées, la bactériocine (EntDD14) est incorporée par adsorption physique à leur surface. Des analyses permettant d'établir l'activité antibactérienne contre des souches pathogènes de *C. perfringens*, isolées de poulets atteints d'entérite nécrotique, ont ensuite été effectuées. Ainsi, la concentration inhibitrice minimale (CMI) de la formulation, résultant de l'adsorption de l'EntDD14 sur les AlgNPs, a été diminuée d'un facteur de 4, par rapport à celle obtenue avec EntDD14 seule. Il convient de noter que la formulation mise au point dans le cadre de cette thèse reste active dans les conditions physiologiques du tractus gastrique de l'homme et du poulet reconstituées *in vitro*. Par ailleurs, nous avons également montré que l'EntDD14 seule, ou fixée sur les AlgNPs, ne présente aucune cytotoxicité cellulaire sur les modèles de cellules humaines appartenant aux lignées Caco-2 et HT-29 après 24 heures de contact.

Par la suite l'impact de l'EntDD14 seule et/ou adsorbée sur des AlgNPs, à des concentrations sub-inhibitrices, sur le niveau d'expression des gènes codant pour la synthèse des toxines (toxine α, β-Like toxine, la protéine d'adhésion du collagène et la cytolysine activée par le thiol) ont été déterminées. Ainsi, les niveaux d'expression de ces gènes ont été considérablement diminués après traitement de la culture avec les formulations EntDD14/AlgNPs mais pas l'adjonction de la bactériocine seule.

L'augmentation de l'activité de la bactériocine par les nanoparticules, la réduction de l'expression des gènes codant les toxines impliquées dans la NE et l'absence de cytotoxicité cellulaire montrent ainsi la viabilité de ce type de formulation comme alternative thérapeutique dans le traitement de cette pathologie.

Afin d'évaluer l'apport des nanoparticules d'alginate sur les capacités multifonctionnelles de l'EntDD14 nous avons étudié l'impact de cette molécule, seule ou adsorbée sur les AlgNPs (à 1xCMI et 4xCMI), sur la réponse inflammatoire des cellules Caco2. Nous avons ciblé particulièrement les interleukines IL-6 et IL-8, après induction de l'inflammation avec du lipopolysaccharide (LPS) (50 µg / ml) provenant d'*Escherichia coli*. Ainsi nous avons pu observer une réduction drastique du niveau de sécrétion de l'IL-6, passant de 2,5 pg/ml à moins de 0,5 pg/ml. Concernant l'IL-8, l'effet des différents traitements est également clairement établi, avec une diminution du niveau de sécrétion de cette interleukine d'au moins deux à trois fois en comparaison avec le contrôle inflammé mais non traité.

Nous avons également étudié l'activité antivirale de la bactériocine EntDD14 libre et fixée sur les AlgNPs. Nous avons évalué cette activité contre le virus de l'herpès (HSV-1), en utilisant comme modèle des cellules Vero, provenant de reins de singe vert d'Afrique, infectées à une multiplicité d'infection (MOI) de 0,07. Celles-ci ont été incubées ensuite en présence de différentes concentrations d'EntDD14 (7,5 à 150 µg/ml) et après 24 h d'incubation, une baisse significative du titre viral a été observée pour les concentrations les plus élevées (>30 µg/ml).

Ainsi ces travaux ont permis de mettre en évidence la multifonctionnalité de l'entérocine DD14 ainsi que sa pertinence comme altérnative ou complément aux traitements antibiotiques en association avec les nanoparticules dans le contexte de l'entérite nécrotique.

Table of Contents

General Introduction 1
Chapter I: Literature Review 8
1. *Clostridium perfringens* 8
1.1. Definition/Microbiology 8
1.2. Epidemiology 9
1.3. Clinical Infections, Causes and Symptoms 10
1.4. *C. perfringens* types and its Pthogenesis 11
1.4.1. Toxinotypes 12
1.4.1.1 *C. perfringens* type A 12
1.4.1.2. *C. perfringens* type B 13
1.4.1.3. *C. perfringens* type C 14
1.4.1.4. *C. perfringens* type D 15
1.4.1.5. *C. perfringens* type E 16
1.4.2. Toxins 17
1.4.1.1. α-toxin 17
1.4.1.2. Θ-toxin (perfringolysine) 19
1.4.1.3. CnaA toxin 19
1.4.1.4. Necrotic Enteritis B-like (NetB) toxin 20
1.5. Necrotic Enteritis 24
1.5.1. Symptoms 25
1.5.1.1. Clinical Signs 25
1.5.1.2. Subclinical Signs 26
1.6. Economic Impacts 26
1.7. Necrotic Enteritis Causes 27
1.8. Prevention and treatment of the Necrotic Enteritis 28
2. Antibiotic 29
2.1. Definition 29
2.2. History 30
2.3. Antibiotics classification 31
2.3.1. Antibiotics Consumption in France 36
3. Antibiotic Resistance 38

3.1. History 38
3.2. Antibiotic Resistance Definitions 42
3.3. Resistance Origins and its Relationship with Antimicrobial use 42
3.4. Mechanisms of the Antibiotic Resistance 43
3.5. Types of Antibiotic Resistance and the Mode of Transmission of Resistance Genes 45
3.5.1. Intrinsic Resistance (Natural Resistance) 45
3.5.2. Acquired Resistance 46
3.5.2.1. Transmission of Acquired Resistance by Mutations (vertical and hereditary transmission) 46
3.5.2.2. Transfer of Genetic Material (Horizontal Gene Transfer-HGT) 47
3.5.2.2.1. Transformation 47
3.5.2.2.2. Transduction 49
3.5.2.2.3. Conjugation 49
3.6. Antibiotics Resistance of *C. perfringens* 51
3.7. Strategies to Control Antimicrobial Resistance (AMR) 52
4. Alternatives to Conventional Antibiotics 54
4.1. New Classes of Antibiotics 55
4.1.1. Outer Membrane Protein Targeted Antibiotics (OMPTA) 55
4.1.2. Biphenylacetylene based LpxC Inhibitor 56
4.1.3. Odilhorhabdins (ODLs) 57
4.1.4. Mursamacin 57
4.2. Phage Therapy 58
4.2.1. Life Cycle of the Phage 58
4.2.2. Phage Therapy Advantages over Antibiotics 60
4.2.3. Overcoming the Challenges of Phage Therapy 60
4.2.4. Phage-antibiotic Combination 61
4.2.4.1. CRISPR-Molecular Tool 61
4.3. Vaccines 63
4.3.1. Bacterial Vaccines 64
4.3.2. Antiviral Vaccination's Impact on Antimicrobial Prescriptions 65
4.3.3. Vaccines of Future 65
4.3.4. The Limits of Vaccinations 66
4.4. Antimicrobial Peptides (AMPs) 67

4.4.1. Sources of Antimicrobial Peptides and their Current Status 68
4.4.2. Bacteriocins 69
4.4.2.1. Definition 70
4.4.2.2. Bacteriocins produced by Gram-positive Bacteria: 70
4.4.2.3. Modes of action of bacteriocins 74
4.4.2.4. The Potent Antimicrobial Activities of Bacteriocins expand their Applications 75
4.5. Nano-Objects 77
5. Nano-antibiotics 78
5.1. Definition 78
5.2. A Brief History of Nano-Objects 79
5.3. Development of Nano-Objects and Drug Delivery 82
5.4. Types and Applications of Various Nanoparticles used in the Field of Veterinary Medicine 83
5.4.1. Different Types of Nanoparticles 84
5.4.1.1. Non-Degradable Nanoparticles 84
A. Quantum Dots 84
B. Fullerenes 85
C. Metallic Nanoparticles 86
5.4.1.1.1. Toxicity of Non-Degradable Nanoparticles 87
5.4.1.2. Degradable Nanoparticles 88
Liposomes 88
Polymeric Nanoparticles 89
A. Starch Nanoparticles 90
B. Chitosan Nanoparticles 90
C. Alginate Nanoparticles 91
Chapter 2. Materials and Methods 96
Chapter 3. Alginate nanoparticles potentiate the anti-*Clostridium perfringens* activity of enterocin DD14 (EntDD14), and down-regulate the expression of genes coding for toxins in this pathogen. 123
Chapitre 4. Potent anti-inflammatory activity of a leaderless enterocin DD14 (EntDD14): Down regulation of IL-6 and IL-8 166
Chapter 5. Broadening and enhancing bacteriocins activities by association with bioactive substances. 186
Chapter 6. General conclusion and perspectives 210
References 214

ABBREVIATIONS

AGPs……..... Antibiotic growth promoters

AlgNPs……..…….…...Alginate nanoparticles

AMR……...…… Antimicrobial resistance

ANSES…….…… National social security administration

ANSM…….…..National agency for medicines and health products safety

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

Caco-2……..…....Human colonic adenocarcinoma cells

CDC……. Cholesterol-dependent cytolysine

E. coli.....*Escherichia coli*

EntDD14…….…… Enterocin DD14

ESVA ……..…...,….European Surveillance of Veterinary Antimicrobial Consumption

EFSA…… ….European Food Safety Authority

EMA ……..European Medicines Agency

ESBL……..Eextended-spectrum-β-lactamase

FDA……Food and drug administration

GIT……..... Gastrointestinal tract

GRAS…….Generally regarded as safe

GyrA……...Gyrase A gene

HCl…….…..…Hydrochloric acid

HDPs……..…..…..Host defense peptides

HGT ...……..…..…Horizontal Gene Transfer

Hib…….…..... *Haemophilus influenza b*

HNO_3…….……...… Nitric acid

HSV-1…….....…Herpes simplex virus 1

HT-29……..…...Human colorectal adenocarcinoma cells

IL ……..…..Interleukin

K……..Potassium

LA-MRSA…….…….….Livestock-associated methicillin resistant *Staphylococcus aureus*

LMH……...…….Hepato cellular carcinoma cells

LA-MRSA ……...……...livestock-associated methicillin resistant *Staphylococcus aureus*

LPS…….…….…Lipopolysaccharide

NCTC …….National Collection of Type Cultures

MBC……...….…Minimum bactericidal concentration

MIC…….…....Minimum inhibitory concentration

MOI……..…...… Multiplicity of infection

Na……....Sodium

NE……..….Necrotic enteritis

NetB……..… Necrotic Enteritis B-like toxin

NMR…….…….….Nuclear magnetic resonance

OMPs…….……Outer membranes proteins

PLC……..……..… Phospholipase-C

SOP……Standard operating procedure

S. epidermidis……*Staphylococcus epidermidis*

S. agalactiae…… *Streptococcus agalactiae*

SNE…….……..…..Subclinical necrotic enteritis

STM…….....….Scanning tunneling microscope

TACY..…….……..Thiol-activated cytolysine

TB…….……..Tuberculosis

WHO……..……World health organization

List of figures

Figure 1. Crystal structure of α-toxin 18
Figure 2. Modes of action of α-toxin on biological membrane 18
Figure 3. Crystal structure of NetB 22
Figure 4. A, binding ability of the mutants to LMH cells. 22
Figure 5. Surface representation of the rim domain of αHL (A) and NetB (B) in the same orientation 23
Figure 6. The different targets of antibiotics in bacterial cell 35
Figure 7. Antibiotics consuming by 30 countries participated to ESVAC 37
Figure 8. The years of market introduction of the major families of antibiotics 40
Figure 9. Deaths caused by AMR each year 41
Figure 10. The four major Mechanisms of Resistance of the Bacteria towards Antibiotics 45
Figure 11. A simple illustration of the vertical transfer gene. 46
Figure 12. The 3 steps of HGT transformation. 48
Figure 13. Principle of Transduction and the Bacteriophages Pathway 49
Figure 14. Conjugation diagram 50
Figure 15. Antimicrobial potential of the alternative strategies to prevent NE 52
Figure 16. Strategies to Fight against Antibiotic Resistance. 55
Figure 17. General structure of phage 59
Figure 18. The two reproduction cycles of phages 60
Figure 19. Challenges facing the use of phages therapy and its solutions. 61
Figure 20. Illustration of the mode of action of the new CRISPR. 62
Figure 21. Effect of vaccination on the emergence of pathogens 63
Figure 22. A comparison between antibiotics and vaccines. 64
Figure 23. Number of publications on antimicrobial peptides between 2004 and September 2016 69
Figure 24. Major phylogenetic groups of LAB 71
Figure 25. Mode of action of bacteriocins 75
Figure 26. The number of the surface atoms is inversely proportional to the nanoparticle diameter 78
Figure 27. A-Maya Blue; B- Lycurgus cup 80
Figure 28. Letters “IBM” wrote by xenon atoms shows the capability of the IBM STM in 1989 82
Figure 29. Types of various nanoparticles used in veterinary medicine. 83
Figure 30. Applications of Quantum Dots (QDs) 85
Figure 31. Schematic Representation of a Fullerene C_{60}. 86
Figure 32. Schematic representation of liposome structure 89
Figure 33. Schematic representation of the 5 modes of chitosan nanoparticles synthesis 91
Figure 34. Number of published papers in Scopus per year 93
Figure 35. Determination of the MIC using diffusion method. 103
Figure 36. A, Broadcast antibiogram MIC concordance curves 104
Figure 37. Amino acid sequence alignment of EntDD14A and EntDD14B 123
Figure 38. Sodium alginate structural data 124
Figure 39. Pleiotropiceffect of IL-6 167

Figure 40. Anti-HSV1 activity of EntDD14 at 5 different concentrations……..170

List of tables

Table 1. Medically important species of *Clostridium*..... 9
Table 2. *C. perfringens* types according to their prevalent toxins..... 12
Table 3. Location, biochemical and biological activity of *C. perfringens* toxin-encoding genes..... 17
Table 4. Antibiotics used in Broiler Chickens for NE prevention..... 28
Table 5. Example of preeminent antibiotics inhibiting the synthesis of peptidoglycan..... 32
Table 6. Antibiotics inhibitor activity on the proteins synthesis..... 34
Table 7. Multidrug Resistance of *C. perfringens*..... 51
Table 8. Examples of 5 Vaccines in clinical trials..... 66
Table 9. Classification of bacteriocins according to Klaenhammer (1993)..... 72
Table 10. Classification scheme for bacteriocins suggested by Cotter et al. (2005)..... 72
Table 11. Classification of LAB Bacteriocins according to Drider et al..... 72
Table 12. Spectrum of Antimicrobial Activity of Bacteriocins..... 76
Table 13. Examples of alginate nanoparticles in drug delivery..... 92
Table 14. Strains of *C. perfringens* used as target bacteria..... 98
Table 15. Example of standard assay set up table..... 101
Table 16. Primers designed and used in *C. perfringens* Clin1 for RT-qPCR..... 115
Table 17. Primers designed and used in Caco2 cells for RT-qPCR..... 121
Table 18.The minimum inhibitory concentration (MIC) values of EntDD14, alone or loaded on alginate nanoparticles (EntDD14 + Alg NPs)..... 125

General Introduction

Since their discovery, antibiotics are considered as a key therapy for the treatment of bacterial infections (Fleming 1929). These drugs have been classified into natural agents produced therefore by eukaryotic and prokaryotic cells, and as synthetic agents. Regarding their structure, antibiotics are divided into several groups including penicillins, tetracyclines, cephalosporins, quinolones, lincomycins, macrolides, sulphonamides and glycopeptides (Korzybski et al. 2013). A recent report showed that between 2000 and 2015, the consumption of antibiotics has significantly increased (about 65%) worldwide (E. Y. Klein et al. 2018). In 2017, 759 tons of antibiotics for human health and 514 tons of antibiotics for animal health were sold in France (Anses/ANSM 2018; ECDC/EFSA/EMA 2017). In addition to the wrong diagnosis, the misuses of antibiotics endowed with broad spectra, active therefore against Gram-positive and Gram-negative bacteria, conducted to the emergence of resistant bacteria and thus to the development of transmissible antibiotic resistance, which became a major problem for human and animal health. Currently, it is accepted that many evidences correlating the use of antibiotics in animals and intrusion antimicrobial resistance (AMR) in humans exist. This is not surprising as antibiotics used for animal health are the same than those used for humans, delineating the concept of "One health" which considers the antibiotic resistance under a global point of view.

Based on that, the frequent prescription of antibiotics started to limit their potency and new strategies allowing overcoming these health-threats are needed. Currently, several promising strategies are considered as potentially suitable to fight AMR are under consideration. These include new classes of antibiotics with activity against Gram-positive and Gram-negative multi-resistant bacteria (Stokes et al. 2020), phage therapy (Taati Moghadam et al. 2020);

vaccines(Kingwell 2018); nanoantibiotics (Khurana et al. 2018) and bacteriocins, which are steadily reported as a next class of antibacterial agents (Chikindas, et al., 2018).

Bacteriocins are antimicrobial peptides characterized by their diversity in terms of molecular weight, biochemical properties, spectra, and modes of action. Bacteriocins are naturally synthesized and produced, during the primary growth phase, by numerous self-immunized, Gram-positive or Gram-negative (Klaenhammer, 1993). Many schemes for bacteriocins classifications have been suggested, starting with that from Klaenhammerin 1988 (Klaenhammer T. R., 1988), which suggested structure and mode of actions of bacteriocins as key elements of classification (Klaenhammer, et al., 1993). In turn, Drider et al. in 2006 (Drider et al. 2006) classified bacteriocins according to their biochemical and genetic properties. Recently, further classification was conducted by Alvarez-Sieiro et al. (2016) and this one considered the biosynthesis mechanism and biological activity of bacteriocins as key elements for their classification (Alvarez-Sieiro, et al., 2016). Bacteriocins displayed different biological functions, besides their antibacterial activity. Related to their inhibitory function, it has been suggested that the natural concentration, sub-minimum inhibitory concentration, (sub-MIC) of bacteriocins, allowed to expel the intruders from the environment of the producing strain Therefore the primary functions of bacteriocins were expected to be signaling and repelling, not killing. Additional function of bacteriocins can be detected at higher concentrations (MBC: minimum bactericidal concentration) than at the natural levels. These lead to pore formation, disturbances of the membrane, or inhibition of the cell division process (Algburi et al., 2017; Chikindas et al., 2018).

Bacteriocins can be used to control bacterial infections like necrotic enteritis (NE), which is caused by *Clostridium perfringens*, a harmful pathogen affecting the GIT of poultry. NE is

considered as one of the most widespread disease, affecting the GIT of poultry and causing high economic losses worldwide (Tamirat et al. 2017). According to Keyburn et al. (2008), and Savva et al. (2013), the NetB toxin, which is a β-pore forming toxin produced by *C. perfringens* and able to form pores of about 1.7 nm diameter in leghorn chicken hepato cellular carcinoma cells (LMH) was considered as the main virulence factor for NE disease. Recent study revealed additional predisposing factors, including feed manipulation and coccidial infections facilitating the NE disease (Uzal et al. 2015; Prescott et al. 2016).

In our laboratory, Caly et al. (2017) revealed the anti-*Clostridium perfringens* activity of DD14, a leaderless class IIb bacteriocin, produced by *Enterococcus faecalis* 14, a strain isolated from a meconium sample a new born, recovered at Roubaix Hospital in the north of France.

In addition to their antibacterial activity, bacteriocins can also display activities against viruses (Cavicchioli et al., 2019). This is the case of enterocin B which exhibit activity against influenza viruses (H1N1, H3N2) (Ermolenko, et al., 2019). Also, subtolisin and enterocin CRL35 can act on the multiplication of poliovirus (PV-1) or herpes simplex virus 1 (HSV-1) by perturbing their late stages of replication (Cavicchioli et al., 2019; Wachsman et al., 2003).

Moreover, few studies have reported the anti-inflammatory activity of bacteriocins. For example plantaricin EF (PlnEF), a class IIb bacteriocin, can induce reduction of the colonic tumor necrosis factor alpha (TNF-α) and IL-6 levels in mice (Yin et al., 2018). Nisin was also shown to have anti-inflammatory activities, affecting the secretion of pro-inflammatory interleukins induced by pathogens under certain conditions (Małaczewska et al., 2019).

Despite numerous promising and potential applications, bacteriocins are nevertheless vulnerable for enzyme degradation, they are also poorly soluble, and exhibit low stability in the

GIT, and unwanted interactions with other molecules can reduce their bioavailability, and limit therefore their efficiency. To limit these adverse effects and improve the potency of bacteriocins, it is possible to proceed with nanotechnology. Indeed, nanoparticles are good and suitable tools to increase their stability, solubility, dispersibility and protect them from harsh conditions and thus enhance their absorption into the bloodstream (J. McClements, 2015).

Nanoparticles are ultrafine particles with size range from 1 to 100 nanometers in diameter (R. Boverhof, et al., 2015). As a matter of fact, nanoparticles have excellent characteristics relative to solid particles (micro- and macro-scale); these characteristics are influenced by the physicochemical properties of the nanoparticles such as size, shape and other properties (surface area, surface charge, etc). Size of nanoparticles plays an essential role in their interaction with cells and influence host response which affect their targeting ability, toxicity and their distribution in the body. Interestingly, nanoparticles can overcome the restrictive passage between blood and brain, which make them promising in blood-brain barrier transport system (BBB) (Betzer, et al., 2017) .

Due to the inverse correlation between particle size and the surface area to volume ratio, more the size is small more the surface area relative to volume is bigger therefore increases the reactivity of these particles (Rihn, 2018). Limbach et al,(2005) have studied the nature of the relationship between the cellular uptake and size of nanoparticles, the result showed that there is a size-dependent behavior, in certain range, in cellular uptake which is lower for smaller nanoparticles than for larger ones (Limbach, et al., 2005).

As for drug release, the size of nanoparticles is mainly influencing the rate of drug release. When the particle size is small, its surface area relative to volume gets larger (Redhead, et al.,

2001). In this case, a faster drug release takes place due to the presence of the drug at or near the surface of the particle. Unlike the larger particles, in which more drug molecules are loaded and thus slow release dominate (Redhead, et al., 2001). Also, the size of nanoparticles can affect its biological fate and determines its removal time period *in vivo*. Recent study, conducted by Jasinski et al, (2018) showed that small nanoparticles are less captured by microphages and more rapidly eliminated by organs, e.g. kidneys. In the other hand, larger size leads to longer half-life and bigger accumulation at organs (Jasinski, et al., 2018).

In addition to the size, there is also the shape of the particle that affects its physicochemical properties and the way it interacts with the biological components inside the body including blood circulation half-life, cellular uptake, targeting performance, the pathways to enter the cells, intestinal transport and ability to overcome biological barriers (Banerjee, et al., 2016; Moghimi, et al., 2001). Banerjee et al (2016) studied the effect of nanoparticle geometry in oral drug delivery, wherefore different shapes of nanoparticles were prepared (rod, disc and sphere-shaped nanoparticles). As a result, the cellular uptake of nanorods was higher compared to spheres (Banerjee, et al., 2016).

Main objectives of this project

The overuse of antibiotics has conducted to an ecological imbalance which has become evident in the spread of multidrug resistant of foodborne pathogens, like *C. perfringens* strains which display resistance to multiple antibiotics. Bacteriocins are steadily reported as safe antimicrobial peptides with capability to replace the aging antibiotics, which activity is decreasing. Here we investigated the inputs of polymeric nanoparticles (alginate) as carriers of bacteriocins and assess multiple functions like antibacterial activity, gene expression, safety towards eukaryotic cells, inflammation responses and even antiviral activity. Of note, EntDD14 is a leaderless bacteriocin characterized for its potent antibacterial activity. Thus, EntDD14 was loaded on alginate nanoparticles, leading to a new nano-antibiotic formulation with promissing therapeutic applications. This newly developed nano-antibiotic formulation was tested against spore-forming clostridia particularly *C. perfringens*, which is responsible for NE, a harmful altering chicks and birds. Below are indicated the different achievements chapters organization.

Chapter 1 is a dissertation part of this thesis. Overall this section describes the pathogenesis attributes and pathways of *C. perfringens* as well asthe different strategies to control the AMR phenomenon.

Chapter 2 is dedicated to the methods and materials used throughout this work.

Chapter 3 is a research chapter dealing withthe alginate nanoparticles and their inputs on the anti-*C. perfringens* activity of EntDD14. This was drawn in MIC values and supported by molecular studies based on the qPCR data. This work was published in "***Probiotics and Antimicrobial Proteins***".

Chapter 4 is a research chapter dealing with the anti-inflammatory and antiviral of both EntDD14 alone and EntDD14 loaded on alginate nanoparticles. This work was the subject of an article in progress.

Chapter 5 is a review underlining the multifunction of bacteriocins, mainly their synergism with other antimicrobial agents such as nanoparticles. This work was the subject of a publication in ***International Journal of Environmental Research and Publich Health***, under doi number: https://doi.org/10.3390/ijerph17217835

Chapter 6 is a general conclusion and draws the future direction for this project.

Chapter I: Literature Review

Breeding faces too many issues including those related to the infectious diseases, which result in heavy economic losses world wide. Amongst the causative agents of these infections the pathogenic bacteria which represent a major threat for livestock. For example Q fever, responsible for abortions and stillbirths in cattle, caused by Coxiella burnetii which is transmitted mainly by respiratory route (Agriculture gouv. 2010); Paratuberculosis (inflammation of the intestine) due to Mycobacterium avium subsp. paratuberculosis (Map) which mainly infects calves in utero (Anses, 2009), are one of the most documented disease. Beside the aforementioned infectious bacterial agents *Clostridium perfringens* responsible for necrotic enteritis (NE), is an acute enterotoxemia affecting the GIT of commercial broiler flocks worldwide (Tamirat et al. 2017).

1. *Clostridium perfringens*

1.1. Definition/Microbiology

The clostridia are a group of strictly anaerobic large (4–6 • ~1μm) Gram-positive bacilli containing ~ 203 species. They can be pleomorphic. Some tolerate small amounts of oxygen. They form **spores**, which can be centrally or terminally positioned causing bulging within the cell. They are soil **saprophytes** or normal commensals of the human and animal gut. However, they are capable of causing deadly diseases, which are invariably mediated by **potent exotoxins**.

The genus consists of a number of medically important pathogens (**Table 1**), including *Clostridium perfringens, C. tetani and C. botulinum*. They can be differentiated on the basis of their biochemical activities, including saccharolysis and proteolysis.

Table 1. Medically important species of *Clostridium*.

Species	Isolated area	Derived name of bacteria	Disease	Date of discovery	Name of finder	Ref.
C. botulinum	Raw ham, livers	Sausage poisoning in Latin	Botulism	1897	Van Ermengen	(Sakaguchi 1982)
C. perfringens	Digestive tract	*Bacillus welchii*	Several diseases including gas gangrene	1891	William H. Welch	(Lucey et al. 2004)
C. tetani	Digestive tract	Greek word tetanos (stretch)	Tetanus	1885	Arthur Nicolaier	(Parker 2006)

Some of these species are responsible for various diseases in humans and animals. The key virulence factors in pathogenic clostridia are the powerful toxins released from vegetating (growing) bacteria. *C. perfringens* causes gas gangrene and food-poisoning. *C. tetani* causes tetanus, *C. botulinum* causes botulism and *C. difficile* causes antibiotic-associated diarrhea (Labbe et al., 2017; Dennis L. Stevens et al., 2002; A. Albrecht, 2015).

1.2. Epidemiology

Clostridium perfringens is widely spread in air, sediments, sewage, liquid manure, carcasses, dust, plant surface and soil. Of note, healthy humans and animals can carry, in low amounts, in their digestive tract. This bacterium is frequently associated with beef meet, gravies, dried or pre-cooked food, raw meat and poultry (Bromberg et al. 2004). Remarkably a recent

study from Bhattacharya et al. (2020) pointed out leeks in the cheese sauce as a potential source of *C. perfringens* (Bhattacharya et al. 2020).

Clostridia are tedious microorganisms that require amino acids, carbohydrates and vitamins in order to grow (Jalbert 2008). *C. perfringens* species is endowed with saccharolytic and proteolytic enzyme activities, allowing to metabolize various sugars like glucose, maltose, sucrose and lactose, resulting thereof in gas production (Friedemann et al. 1941). *C. perfringens* can grow in a temperature range comprised between 15 and 50°C, with an optimum between 41 and 45 °C (Willardsen et al. 1978). Regarding the pH, the optimal growth was recorded at pH comprised between 5 and 9 (A. Albrecht 2015).

1.3. Clinical Infections, Causes and Symptoms

Most of cases related to foodborne illness reported in the USA were attributed to *C. perfringens;* it is thereof considered as the third food poisoning bacterium (Harry et al. 2009). Disease attributable to *C. perfringens* caused drastically thousands deaths and more than 70 million illnesses cases yearly (Mead et al. 1999). Food poisoning is a major health issue worldwide. The agents involved in this disease include parasites, viruses, prions, bacteria (including enterotoxinogenic *C. perfringens*).The resulting symptoms are diarrhea, abdominal pain, intestinal fluid loss and nausea (Mead et al. 1999; Rood et al. 2018). These symptoms are expressed less than one day after the consumption of contaminated food (Alzubeidi et al. 2018). Many studies demonstrated that *C. perfringens* strains can attach to a variety of surfaces including stainless steel, Teflon, polystyrene and biotic surfaces. The hydrophobicity of the surface material plays an important role in biofilm formation by this species. The major cause of infection held responsible due to the cross-contamination which occurs during the food

processing by contaminated stainless steel surfaces (Alzubeidi et al. 2018). Adherence of bacteria or their spores to these surfaces can be influenced by the cell surface hydrophobicity as well as the negative charges (Jindal and Anand 2018; Rosenberg 1984; 1984;Peng, Tsai, and Chou 2001). Spores from *Clostridium* species were shown to have an extraordinary hydrophobicity albeit the disinfection process, which is applied in the food industry, delineating thereof the capability of this malevolent organism to survive to toxic chemicals (Paredes-Sabja et al. 2008; Raju, Setlow et al. 2007; Setlow 2006). Decontamination of food by heat-treatment is useless, because spores that are produced by *C. perfringens* are resistant to high pressures, cold and high temperatures (Jihong Li et al. 2016). After ingestion, following food contamination, these spores find favorable growth conditions in the gastrointestinal tract causing enterotoxin (CPE), tone of the main toxin responsible for food poisoning (Skjelkvåle and Uemura 1977). The DNA coding for CPE can be located on a plasmid (P-*cpe*) or a chromosome (C-*cpe*), however foodborne illness is rather associated with P-*cpe* strains and seldom with C-*cpe* strains (Paredes-Sabja et al. 2008; Raju et al. 2007; Setlow 2006; Lahti et al. 2008).

1.4. *C. perfringens* types and its Pthogenesis

The *C. perfringens* strains can produce a **number of potent toxins;** the most predominant is the α-toxin (phospholipase C) which causes host cell lysis. All isolates of *C. perfringens* produce α-toxin. Various strains of the organism produce any of another 17 well-known toxins, including collagenase, proteinase, hyaluronidase and deoxyribunuclease. On the basis of these toxins, the *C. perfringens* strains are divided into five types, A-E based on their ability to produce any of the four major toxins (α, β, ε and ι), which are involved in the pathogenesis, as shown in **Table 2** (Niilo 1980).

Table 2. *C. perfringens* types according to their prevalent toxins.

Toxinotype of *C. perfringens*	α-toxin	β-toxin	ε-toxin	ι-toxin
A	++	-	-	-
B	+	++	+	-
C	+	++	-	-
D	+	-	++	-
E	+	-	-	++

++ = Produced as a predominant toxin + = Smaller quantities produced. - = Not produced

1.4.1. Toxinotypes

1.4.1.1 *C. perfringens* type A

The toxinotype A is the most widely distributed in nature produces α-toxin, also called phospholipase-C"PLC". To kill human muscle cells, *C. perfringens* uses these toxins, causing necrosis **(myonecrosis)**. Death of these cells creates an even more suitable anaerobic condition where the organism can grow rapidly and release gas, hence the disease is known as **'gas gangrene'**.

The role of PLC in the pathogenesis has been confirmed by studying *C. perfringens* isogenic strains, in which the gene coding for PLC has been genetically inactivated, and the resulting phenotype was discarded from gas gangrene (Awad et al. 1995), with main symptoms of massive local edema, fever and sudden onset with a prominent pain (Gonzalez 1998; Hoover et al. 2000). Importantly, it was reported that the gene (*plc*) coding for α-toxin is present in the chromosome of all strains of *C. perfringens* (Niilo 1980), however the amount of produced α-toxin is tightly dependent on the level of its expression (Bullifent et al. 1996). Interestingly, *C. perfringens* type A produces the highest amount of α-toxin (Niilo 1980). Infected and discolored blood inside the

wound and under the skin turns the infected area to black. Gas gangrene can be caused by other, less common clostridia, including *C. novyi, C. septicum, C. histolyticum, C. sporogenes, C. bifermentans, C. fallax* and *C. absonun* (Hatheway 1990; Gonzalez 1998; Hoover et al. 2000).

1.4.1.2. *C. perfringens* type B

The *C. perfringens* type B carries 3 genes coding for α-toxin (*plc*), β-toxin (*cpb*) and ε-toxin (*etx*). Most of infections caused by this toxinotype were described in limited geographical areas around the world. This is the case of South Africa, Middle East and Europe and even few cases in North and South America. Diseases caused by *C. perfringens* type B are mostly occurring in lambs and seldom in foals, calves and goats (Songer 1996). It should be noted that, lambs less than 14 days, a condition called "lamb dysentery", which is characterized by an abdominal pain and diarrhea, is also caused by this toxinotype. This disease affects as well older lambs causing thereof acute neurological signs like blindness, which is associated with brain lesions attributable to the ε-toxin (ETX). Calves aged less than 10 days can be affected by the disease, which is clinically similar to that reported in lambs. Of note, only rare cases have been described in older calves. However, it was reported that the percentage of recovery in calves is higher than in lambs (Stubbings 1990; Fernandez-Miyakawa et al. 2007; Gkiourtzidis et al. 2001; J. Li et al. 2013). Analyses of animals suffering from this disease showed that their intestine contains both β and ε-toxins (Uzal et al. 2016).

Nevertheless, the toxinotype B associated diseases occur usually in protein deficient people. Shortage of proteins food intake leads to low trypsin production consequently underpins the inability to inactivate the β-toxin. Recent studies indicated the sensitivity of the β-toxin to proteases like trypsin, conversely to ε-toxin which requires proteolytic activation. Some studies

showed that a part of the β-toxin was inactivated after trypsin treatment (Fernandez-Miyakawa et al. 2007). The passage of the β-toxin through blood circulation occurs once *C. perfringens* type B produces it in the intestine, causing enterotoxemia (Dworkin et al. 2006).

1.4.1.3. *C. perfringens* type C

According to the classification of *C. perfringens* toxinotypes done by Niilo (Niilo 1980), the type C strains are able to produce both α and β toxins. *C. perfringens* type C isolates are responsible for illness in both human and animals. In human, this microorganism causes "pigbel" (form of necrotizing enterocolitis). Until 1980s, *C. perfringens* type C was reported as the main agent of the death of Papua New Guinea Highlands children's and children from other countries (Johnson et al. 1997). There are many factors favoring the risk of being infected by pigbel, among which the low rate in trypsin production due to low protein food intake or consumption of high amount of foods containing trypsin inhibitors (Sayeed et al. 2008). *C. perfringens* type C is responsible for economic losses in livestock as it causes infections of many animals like cattle, sheep, dogs, horses, chickens, pigs and chickens in many European countries, Japan and USA (Songer 1996). This microorganism is frequently present in the gastrointestinal tract (GIT) of new born animals and could take advantage of an abnormal intestinal microbiota to provoke the associated disease (Timoney et al. 1988). In calves, especially new borns, *C. perfringens* type C causes hemorrhagic enterotoxaemia and necrotic enteritis, usually accompanied by abdominal pain. In sheep, β-toxin causes an enterotoxaemia, named struck, in which the adult sheep dies so rapidly giving the impression of has been hit by a lightning strike. In lambs, this toxinotype causes hemorrhagic enteritis. The multiplication of *C. perfringens* type C in the abomasums and small intestine leads to destruction of the mucosa of the gastrointestinal tract, often without

diarrhea or dysentery. The accumulation of the fluid within the thoracic cavity and peritoneum is an evidence of toxemia which occurs, usually, without any lesion in the gut (Songer 1996).

In addition to α and β-toxins produced by *C. perfringens* type C strain, this type can frequently produce β2-toxin, enterotoxin (CPE) and perfringolysin O (PFO) during log-phase growth (Fisher et al. 2006). A recent study revealed that β-toxin is responsible for enterotoxaemia disease in domestic animals as well as for necrotizing enteritis in human. Some studies showed that the toxinotype C becomes less pathogenic, following the inactivation of the *cpb*-encoding gene. On the other hand, the inactivation of genes coding for α-toxin and PFO did not show comparable changes in the lethality of the studied models (Uzal et al. 2009; Sayeed et al. 2008).

1.4.1.4. *C. perfringens* type D

Among five toxinotypes (A, B, C, D and E) of *C. perfringens*, the type D is known to produce large amount of ε-toxin. This toxin enables a pathogenic phenotype, even the upmost one, associated with diseases in ruminants. In addition of α- and ε-toxins, this bacterium can produce additional toxins like CPE, β2, PFO and λ-toxin (Uzal et al., 2010).

C. perfringens type D ubiquitously distributed in the environment is a normal resident of the intestinal microbiota of humans and animals. Nonetheless, under certain conditions and when the GIT microbiota is altered, this microorganism causes diseases in the GIT and enterotoxaemia in sheep and goats as well as in other animal species (Gonçalves et al. 2009). Albeit differences in the infection signs between sheep and goats, the last one is typically characterized by enterocolitis, in addition to commonly encountered symptoms, like hydropericardium, lung and brain edema (Uzal et al. 2008; Smith et al 2009). In the brain, endothelial cells are affected by

ETX which lead to perivascular edema, deterioration and necrosis of the surrounding cerebral parenchyma in animals (Buxton, et al., 1981). Limited information is available for the pathogenesis process of *C. perfringens* type D in humans. However, many cases of gas gangrene attributed to this toxinotype have been reported for humans (Morinaga et al. 1965).

1.4.1.5. *C. perfringens* type E

The *C. perfringens* type E is able to produce α- and ι-toxins, which are major toxins. In addition, to these aforementioned toxins, it has been established that genes coding λ and β2-toxins are present in some isolates (Jihong Li et al. 2007; Niilo 1980). This toxinotype, which is thought to be infrequent can cause enterotoxemia and enteritis in rabbits (Baskerville et al. 1980; L.M. Redondo et al. 2013). Moreover, *C. perfringens* type E disease can affect bovines and ovine causing hemorrhagic enteritis or sudden death. The observation done on the animal bodies displayed lesions and edema in the mucosa of the intestine and abomasums, along submucosal edema, foci of hemorrhage and acute inflammation. The first description of the disease, in calf and lambs, attributable to toxinotype E was reported more than 50 years ago in Britain (Hart and Hooper 2008). In France, two cased of *C. perfringens* type E were recently reported characterized as deathly for calf, and noticeably responsible for illness for a 60 years-old woman (Diancourt et al. 2019). Albeit, the lack of information on the pathogenesis of *C. perfringens* type E, the ι-toxin is, nevertheless recognized as the principal virulence factor of the toxinotype E (Diancourt et al. 2019).

Recent studies reported the ability of *C. perfringens* type E to produce metabolites inhibiting *C. perfringens* type A growth. The heat treatment (80°C for 10 min and 100°C for 30 min) of the inhibitory factor leads to antibacterial activity loss. The molecules were susceptible to trypsin

and protease treatments, indicating a proteinaceous nature of these molecules (Leandro M. Redondo et al. 2015; Vieco-Saiz et al. 2020). A similar strategy of micro-environment inhibition was observed for *C. perfringens* strains responsible for necrotic enteritis in poultry as a high dominance of pathogenic invaders over the intestinal microbiota was detected (Barbara et al. 2008; Leen Timbermont et al. 2009).

1.4.2. Toxins

1.4.1.1. α-toxin

The α-toxin is a zinc metallophospholipase that is activated by zinc. It is a 43 kDa protein encoded by the *plc* gene, which product is characterized by a phospholipase C/sphingomyelinase activity, and further lethal traits (**Table 3**). The crystal structure of α-toxin consists of two domains, which are the N-and C-domains. The first contains nine α-helices, and the second consists of eight antiparallel strands of β-sandwich motif (**Figure 1**) (J. Sakurai 2004). The C-domain (binding domain) enables this toxin to bind calcium and enter to phospholipid bilayers. Once the N-domain (active domain) is linked to the phospholipid head-groups present on the cell surface, the hydrolysis of phosphatidylcholine undergoes due to phospholipase activity of binding domain. The hydrolysis process produces diacylglycerol and trigger the activation of a variety of second messenger pathways (**Figure2**) (J. Sakurai 2004).

Table 3. Location, biochemical and biological activity of *C. perfringens* toxin-encoding genes.

Toxin	Gene	Genetic Determinant	Bio-chemical activity	Biological activity	References
A	*Plc*	Chromosome	Phospholipase C, Sphingomyelinase	Cytolytic, hemolytic	(Saint-Joanis, Garnier, and Cole 1989)
β	*Cpb*	Plasmid	Pore-forming activity	Cytolytic, Hemorrhagic necrosis of intestinal mucosa	(Hunter 1993)
E	*Etx*	Plasmid	Pore-forming activity	Oedema in various organs, lethal	(Petit et al. 1997)
ι (ia-ib)	*Iap-ibp*	Plasmid	*ADP-ribosylation-transferase*	Disruption of cell barrier integrity	(Perelle et al. 1993)

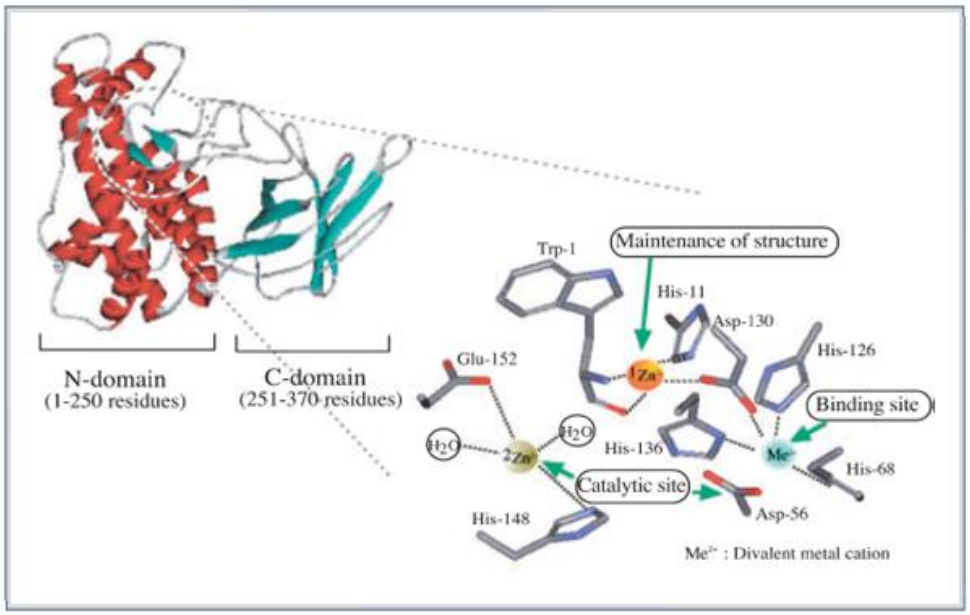

Figure 1. Crystal structure of α-toxin showing N- and C-domains and the relationship between divalent metal cations and amino acid residues (J. Sakurai 2004).

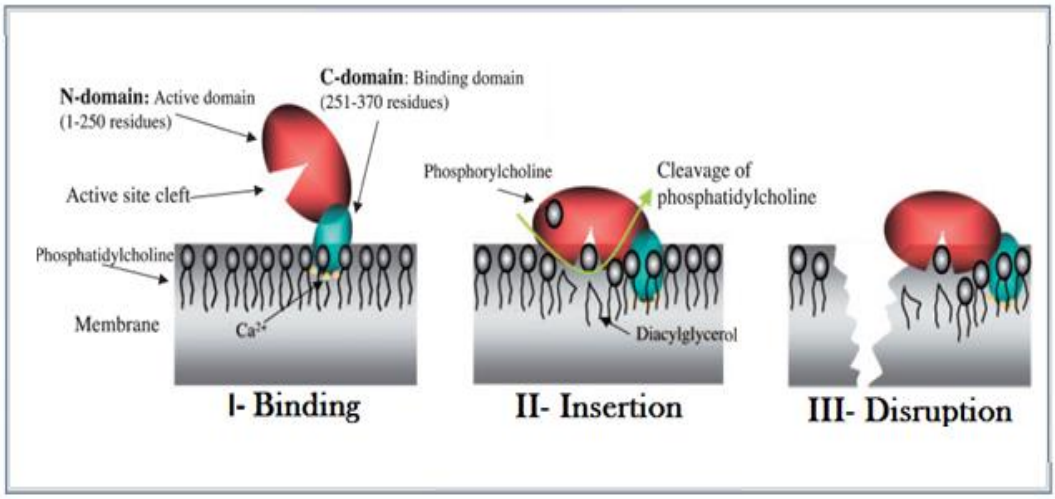

Figure 2. Modes of action of α-toxin on biological membrane (J. Sakurai 2004).

1.4.1.2. Θ-toxin (perfringolysine)

The perfringolysine (PFO) is a pore forming toxin belonging to two toxins family, cholesterol-dependent cytolysine (CDCs) and thiol-activated cytolysine (TACY) (Popoff2014; Billington et al. 2000). In addition of its lytic activity against the eukaryotic cells, many diseases are ascribed to the activity of PFO toxin. These include the targeting of the host inflammatory cells and tissues, dysregulation of the polymorpho nuclear leukocytes (PMNL)/endothelial cell adhesive interactions (Bryant et al. 1993). The Perfringolysine is a single chain polypeptide produced as a water soluble enzyme, constituted of 40-50 monomers (Billington et al. 2000; Shepard et al. 2000). The pore forming mechanism starts by the binding of the monomers to the membrane *via* the cholesterol receptor which activates each bounded monomer. The activation of monomers allows them to interact with each other. Once activated, the monomers start oligomerizing on the bounded surface to form a prepore structure which is converted to pore (diameter of 250–300 Å) after insertion of the transmembrane domain (Shepard et al. 2000; Morgan, Andrew, and Mitchell 1996).

1.4.1.3. CnaA toxin

Pathogenic microorganisms can use different strategies to overcome cells targeted barriers (Krogfelt 1991). The adhesion to the host surface cells is a critical first step that is required for bacterial colonization (Donnenberg 2000). Pathogenic bacteria produce molecules, which facilitate their adherence to surfaces. These molecules can be lypopolysaccharides, fimbriae and adhesins (Krogfelt 1991). The bacterial adherence brings into play a very selective interaction between the produced molecules and the host cell receptors (Klemm et al. 2000). The proliferation of *C. perfringen*s and deliverance of its toxins requires bacterial adherence to the

damaged tissue (Hitsumoto et al. 2014). With regard to that, the damaged tissue contains many proteins including fibronectin and collagen which represent the most super abundant protein in tissue. Recent studies underpinned accessory genome regions, which may play a significant role in a necrotic enteritis disease. Two chromosomal variable regions (VR-10B and VR-109) coding for the cell surface related proteins were identified among 63 *C. perfringens* strains. VR-10B, derived from VR-10 locus located downstream of the capsular polysaccharide locus, codes for the collagen adhesion and contains at least 7 genes. In direct line, some *C. perfringens* strains known to carry collagen adhesion gene (*cnaA*) (Jost et al. 2006). Of note, the VR-10B chromosomal region was frequently present in chicken suffering from necrotic enteritis (Hitsumoto et al. 2014; Konto-Ghiorghi et al. 2009; Wade et al. 2016). The CnaA, collagen adhesion protein, encoded gene allows *C. perfringens* to bind to collagen types III, IV and V at higher levels compared to non-disease producing strains, suggesting thereof high binding levels to the extracellular matrix molecules (ECMM), which enhance the virulence of necrotic enteritis (Martin et al. 2010).

1.4.1.4. Necrotic Enteritis B-like (NetB) toxin

The NetB toxin, produced by *C. perfringens* type A, is considered as a member of the β-pore forming toxins, which is able to form pores of about 1.7 nm diameter in leghorn chicken hepatocellular carcinoma cells (LMH) (Keyburn et al. 2008; Savva et al. 2013). The X-ray crystallography revealed the similarity between the crystal structure of NetB, and that of the αHL (α-Hemolysin, a Heptameric Transmembrane Pore). It does contain three domains, which are the β-sandwich domain composed of 2 β sheets that includes 11 β strands (1, 2, 3, 5, 9, 10, 11, 12, 14 and 15) and a single α-helix. In addition of the extended strands 5 and 12, the rim domain

consists of two other strands (4 and 13), the 3_{10} -helix and random coil. In contrast, the stem domain made up of 2 long, curved amphipathic strands (7 and 8). Interestingly, the stem and sandwich domains are connected by two short coils, making thereof a region similar to the members of pore forming toxins (Fig.3) (Savva et al. 2013; Song et al. 1996; Yamashita et al. 2011). The NetB was shown to have a limited protein sequence similarity with α-toxin that is produced by *Staphylococcus aureus* (31% identity), and β-toxin from *C. perfringens* (38% identity) (Keyburn et al. 2008).

A mutagenesis study, conducted by Savva et al. (2013), permitted to investigate the role of the residues along the rim domain in the NetB function. To this end, 11 NetB mutants were created along the rim domain. These variants are K77A, Y78A, Y79A, Y187A, H188A, Y191A, R200A, Y202A, W257A, E258A, and W262A, and found to be involved in the cell binding mechanism (Savva et al. 2013). The binding ability test of the NetB mutants, which was conducted on LMH cells, showed that Y191A, R200A and W262 derivatives have the lowest binding ability compared to the wild type toxin, whereas the mutant W262A has the lowest binding among the 11 NetB variants (**Figure 4A**). Also, these three variants have reduced cytotoxicity effect on LMH cells compared to wild type toxin with W262A displaying the biggest decrease (**Figure 4B**).

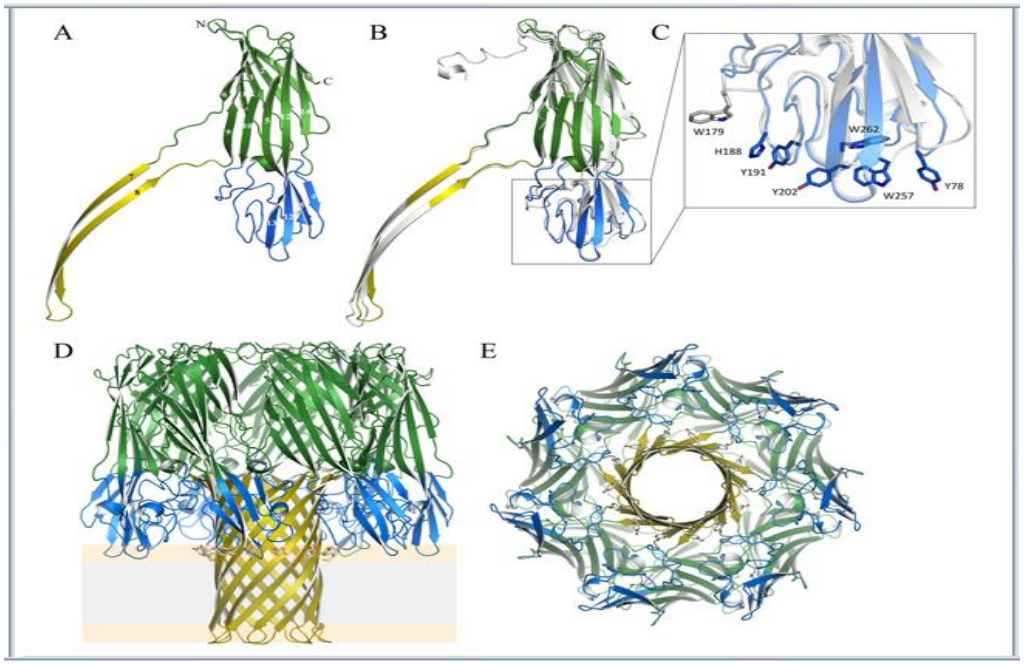

Figure 3. Crystal structure of NetB.A, illustration of an isolated NetB subunit. The three colored domains represent the sandwich domain (Green), rim domain (Blue) and stem domain (Yellow), in addition of the β strands which were described elsewhere. B, Comparison of the NetB subunit to the αHL subunit (light gray). C, represents a zoom view of the rim domain containing the residues of the NetB that were mutated and shown to affect the function. D and E show the representation of all the domains of the NetB (colored as in A) viewed from the side of the protein (D) and cytoplasmic aspects (E). (Savva et al. 2013).

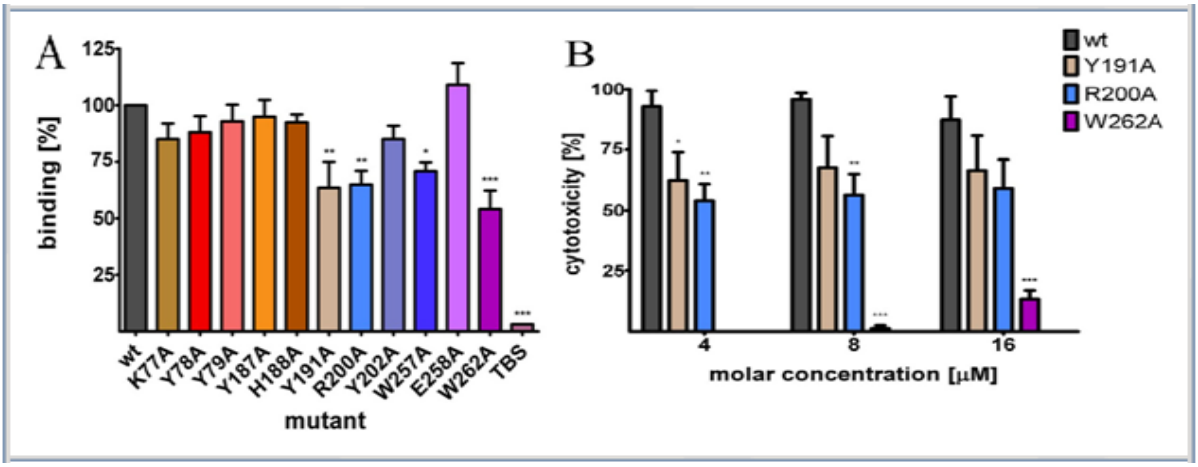

Figure 4. A, binding ability of the mutants to LMH cells.The binding ability is shown relative to wild type NetB (100%). Asterisks indicate the differences between them. B shows the cytotoxicity of NetB on LMH cells according to 2 fold double dilution and 2 hours of NetB contact (Savva et al. 2013).

Furthermore, it was known that the family of pore forming toxins binds to the membrane surface of the target cell through the rim domain, which contains aromatic residue loops that interact with the bilayer and destabilize the membrane (De and Olson 2011). For example, Arg-200 and Trp-179 residues in αHL, which are included in the rim loops, in addition to their role in forming the phospholipid binding pocket (**Figure 5A**), have shown to play a key role in toxin function (Monma et al. 2004; Walker et al. 1995). In NetB, although the phosphocholine binding pocket is absent, the Arg-200 is present and appeared to play a determinant role in the binding and the toxicity (**Figure 5B**). This suggests that the NetB binds to the membrane target cell through alternative interactions and the NetB is able to interact with the cholesterol despite the absence of other lipids (Savva et al. 2013).

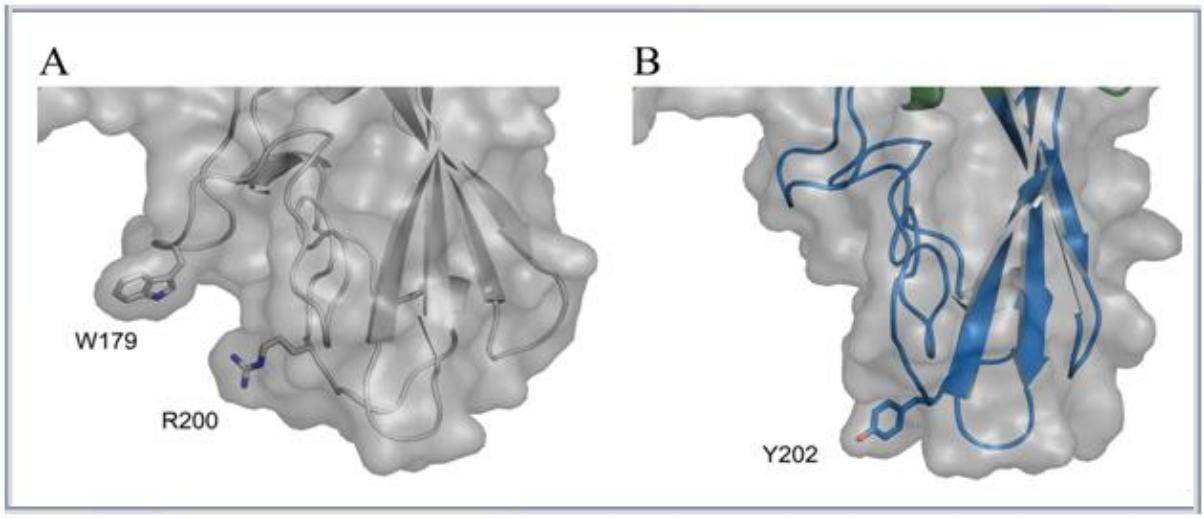

Figure 5. Surface representation of the rim domain of αHL (PDB ID 7AHL) (A) and NetB (B) in the same orientation.The phosphocholine binding pocket formed by Arg-200 and Trp-179 in αHL, is absent in NetB. However, tyrosine 202 in NetB affects binding and toxicity and could be involved directly in lipid binding. NB: Arginine 200 in NetB is absent from the model because of its disorder (Savva et al. 2013).

1.5. Necrotic Enteritis

The necrotizing soft tissue infection (NSTI) is a microbial infection, which induces a critical damage in the soft tissue of the body, leading to death (Misiakos et al. 2014; Hakkarainen et al. 2014). *C. perfringens* is one of the most frequently associated pathogen with this infection, namely the necrotic enteritis (Chapnick et al. 1996; Popoff 2014; Urschel 1999).

The necrotic enteritis (NE) is considered as one of the most widespread disease, affecting the GIT of poultry and causing high economic losses in the world (Tamirat et al. 2017). Recently, the use of the antibiotic growth promoters (AGPs) in the livestock production to increase the weight and feed efficiency allowed to limit the effects of necrotic enteritis in the poultry farming (Long et al. 1976; Prescott et al. 1978). However, the misuse of antibiotics as AGPs participates in the development and proliferation of resistant bacteria. Moreover, a number of these antibiotics are also used in the treatment of bacterial infections in both human and animals (Chatterjee et al.2019). Due to the prevalence of the antibacterial resistance, the European Commission (EC) has banned, since 2006, the marketing and the use of antibiotics as AGPs in livestock production (Anadón 2006). This decision enabled to prevent the use of antibiotics, which are requested for human health, and in animals and feed. Consequently, the limitation of AGPs has increased the risks of intestinal infection in chickens and the multiplication of the necrotic enteritis cases (Immerseel et al. 2004). Recent studies, using more than one million birds, were conducted on the commercial broiler farms in Canada to evaluate the consequences of using the drug-feed supplements, such as essential oils and water acidification, instead of the antibiotics growth promoters. According to the study, the broiler which has been subjected to this drug-free program showed increasing in feed conversion ratio,

decreasing in the weight gain and outstanding increase in the necrotic enteritis and the subclinical enteritis cases (Gaucher et al. 2015).

The NE often occurs in the birds aged between 2-6 weeks (Cooper et al. 2010), and more usually during October-March than April-September (Magne Kaldhusdal 1996). In contrast, the highest peak incidence of NE in United Kingdom occurred during winter in comparison to warmer seasons (Hermans et al. 2007).

1.5.1. Symptoms

1.5.1.1. Clinical Signs

The NE infection induces many clinical signs like a difficulty to move, loss of appetite, depression and diarrhea. Most often the clinical signs can be visible only during a short period of time, for only a few hours, after the birds die (M'Sadeq et al. 2015). The gut found swollen with gas and the intestinal mucosa is covered, fully or partially, with a variable membrane color (yellow, green and sometimes brownish-orange) which is made up of mucosal enterocytes surrounded by fibrin. Also, in some cases, the mucosa can be grayish and thick. In difficult cases, the epithelium can be partly detached (Williams 2005). In animal models infected experimentally, clinical signs, such as dilation of vessels of the *lamina propria* and onset of edema, can be visible after one hour of inoculation. Three hours later, the edema can be more clearly marked and finds in the intestinal lumen untied epithelial cells from the point of the villi. After 5 hours, the points of the villi will be necrotic, inducing the reduction of the villi. The tips will be completely necrotic after 8 to 12 hours. Interestingly, no cellular invasion signs were detected, which lead to believe that the cells are not invaded by the bacteria and the clinical signs appear because of the bacteria's toxins only (Jalbert 2008).

1.5.1.2. Subclinical Signs

Subclinical necrotic enteritis (SNE) was described by Kaldhusdal and Hofshagenin 1992 (M. Kaldhusdal et al. 1992). In this form of NE, neither clinical signs nor peak of mortality were present (L. Timbermont et al. 2011). Birds with SNE present focal lesions in their small intestinal mucosa and high numbers of *C. perfringens* with a reduced normal bacterial microbiota. In addition, the birds exhibited an increasing conversion ratio and weight losses compared to the healthy animals (M. Kaldhusdal et al. 1992). Necrotic lesions are mostly occurring in the duodenum, jejunum and ileum and often in the caeca (Immerseel et al. 2004; L. Timbermont et al. 2011).

The NE induces several modifications within the small intestine, like dilation, fragility, thin wall, hyperemic and filled with gas, and presence of tan-orange pseudomembrane covering the mucosal surfaces (Broussard et al. 1986; Olkowski et al. 2006). Also, the microscopic examination showed an accumulation of high numbers of inflammatory cells at the basal side of enterocytes and *lamina propria*, and the presence of heterophilic granulocytes within the junction (Long 1973; Olkowski et al. 2006; Al-Sheikhly et al. 1980).

1.6. Economic Impacts

The NE is one of the most common diseases registered in poultry, causing thereof heavy economic losses to the global poultry industry. It has been estimated that the cost for the chicken industry has reached around 6 billion US$/year in the world (Maria soriano 2019). In 2001, Lovland and kaldhusdal showed that after 2.5 years of retreat the in-feed antibiotic avoparcin, the index of NE infections increased, inducing producer's incomes reduction by 25 to 43%, due to impaired feed conversion ratio (FCR) and reduced weight gain (Lovland et al. 2001). In addition, the clinical form of the disease induces high death rates more than 50% and about 1% of flock

mortality per day. However, the real economic losses due to this disease are not due to the mortality of the infected birds, but due to birds suffering from the disease and surviving with SNE (Brockotter 2016). Despite the subclinical form is hard to be detected and not usually treated, this form is the most costly for the industry. SNE costs about 0.05$/broiler in United States, without taking into consideration the occasional losses induced by the decrease of the food conversion ratio (Brockotter 2016; Novus 2020).

1.7. Necrotic Enteritis Causes

For over 30 years, it was acknowledged that α-toxin produced by *C. perfringens* type A, is responsible for NE in poultry (Hofshagen et al. 1992). However, a study conducted by Keyburnet al. (2006) showed that α-toxin is not the main virulence factor in NE disease. The authors constructed α-toxin derivative mutants and assessed their virulence in chickens model and concluded that complete virulence has been retained (Keyburn et al. 2006). Other evidences in the non-implication of the α-toxin in this disease attributable to the fact that there is no any correlation between the lesions induced by NE and this toxin, but was linked to the cell proliferation of *C. perfringens* (Si et al. 2007). Keyburn et al. (2008) described a new virulence toxin, called the necrotic enteritis B-like toxin (NetB), which was considered as the main virulence factor for NE (Keyburn et al. 2008).

The feed manipulation (fishmeal diet) is also considered as one of the most widely predisposing factor for the occurrence of NE (Uzal et al. 2015; Prescott et al. 2016). On other hand, coccidial infections due to *Eimeria* was shown to play a major role in the development of NE in chickens (Prescott et al. 2016). The *Eimeria* infections are expressed in different ways including physical damage to the intestinal epithelium thereby promoting the colonization and

proliferation of *C. perfringens* (Williams et al. 2003; Williams 2005; Van Immerseel et al. 2009). The leakage of plasma protein resulting from the damaged epithelial cells provides a rich nutrient source for the growth of *C. perfringens* (Immerseel et al. 2004). Recently, Yang et al. (2019) assessed the effect of these two predisposing factors on the induction of NE. The authors performed an experimental reproduction model of the NE and reported that the use of NetB-positive *C. perfringens,* without fishmeal incorporation and *Eimeria* inoculation, did not show certain consistent induction of NE, which is consistent with the conclusion that the proliferation of *C. perfringens* requires predisposing factors.

1.8. Prevention and treatment of the Necrotic Enteritis

The outbreaks of NE have been effectively prevented by using antibiotics (repeated **doses** at **specific time** intervals) such as β-lactams (penicillin G, amoxicillin and ampicillin), polypeptides (bacitracin), aminoglycosides (neomycin and strepromycin), aminocyclitols (spectinomycin), macrolides (tylosin) and lincosamides (lincomycin) (**Table 4**) (Landoni, et al., 2015).

Table 4. Antibiotics used in Broiler Chickens for NE prevention (Landoni, et al., 2015).

Antibiotic	Type	Oral dose	Withdrawal time
Penicillin G	Bactericidal	300.000-400.000 IU/L	1 day
Amoxicillin	Bactericidal	10-20 mg/kg feed (100-200 ppm)	5 days
Ampicillin	Bactericidal	1.5 g/L	5 days
Bacitracin	Bactericidal	Prophylactic 55-110 mg/kg feed Therapeutic 200-400 mg/kg feed	0 days
Neomycin	Bactericidal	Prophylactic 9.6-19.1 mg/L. Therapeutic 35-80 mg/L or 35-226 g/ton	Canada 7 days
Streptomycin	Bactericidal	66-100 mg/L	4 days
Spectinomycin	Bactericidal	1 g/L	5 days
Tylosin	Bacteriostatic	800-1000g/ton; 500 mg/L	3-5 days
Lincomycin	Bacteriostatic	16 mg/L; 2 g/ton feed	0 days

2. Antibiotic

As indicated, antibiotics are the main means used to control the NE and preserve the health of animals and ensure economic income for livestock sector. But what are the antibiotics and their properties?

2.1. Definition

"Antibiotic" and "antibiotic agent" words were used for the first time by the American microbiologist Waksman in 1941. The name of antibiotic was given to any chemical substance produced by microorganisms, in the form of a dilute solution, which has the ability to inhibit the growth of other microorganisms and can destroy them (Waksman et al 1943; Waksman et al 1942; Waksman 1947). Waksman defined the word antibiotic only to natural substances produced by microorganisms and does not include synthetic or semi-synthetic antimicrobial compounds (Waksman 1947; A. Klein 2012). In 1957, Turpin and Velu have defined the term antibiotics: "any chemical compound, produced by a living organism or produced by synthesis, with a high chemotherapeutic coefficient whose therapeutic activity manifests itself at very low dose in a specific way, by the inhibition of certain vital processes, with regard to viruses, microorganisms or even certain multicellular beings" (Cohen et al. 2008; Le loir et al. 2009). Nowadays, the most accepted definition for antibiotics is "natural or synthetic chemicals used to treat bacterial infections (cystitis, pneumonia, bacterial angina, etc.)" (A. Klein 2012). Obviously, there are many other definitions of antibiotics, depending on their activity spectrum, toxicity and origin.

2.2. History

In the human history, there have been a lot of cultures which had remarkable medical knowledge and which have noticed the healing ability of some molds. For example, surgeons of antiquity and the middle ages were using moldy rags and put it on wounds to prevent infections (Keyes et al. 2003).

In 1877, Pasteur and Joubert observed that the growth of *Bacillus anthracis* was inhibited by saprophytic bacteria (Choudhary et al. 2015)

In 1893, BartolomeoGosio isolated a fungal metabolite, mycophenolic acid, from a mold of the *Penicillium* family, which showed inhibition activity on the anthrax bacteria. But at that time his results were not noticed worldwide (Bentley 2000).

Three decades before Alexander Fleming, in 1897, the French student Ernest Duchesne wrote his doctoral thesis on the antibiotic effect of molds. He observed that the stable boys who worked at the military hospital stored the saddles of the horses in a dark and damp room to encourage the formation of molds, because the wounds caused by the friction of the saddles healed more quickly (Pouillard 2002).

In 1929, the bacteriologist Alexander Fleming incubated a bacterial culture which was, the day after, inhibited by fungi. From this observation, he discovered by chance the antibacterial power of green mold known as *Penicillium notatum*, this mold was found to cause death of bacteria, but was toxic to humans and animals. Few years later, Dr. Chain and Flory published their research and announced the development of penicillin which called the “miracle cure of modern medicine” (Fleming 1929).

In 1935, the first sulfonamide was put on the market. It was particularly effective against streptococcal infections, such as scarlet fever, meningitis, or urinary tract infections (Domagk 1935).

2.3. Antibiotics classification

Each antibiotic has its particularity, its mechanism of action, contraindications, instructions for use, route of use, frequency of use, and other undesirable effects. Also, the antibiotics can be classified according to their origin: nature (produced by eukaryotic or prokaryotic) or synthetic. Their chemical structure, which often based on a basic structure, allowed the classification of the antibiotics in groups (penicillins, tetracyclines, cephalosporins, quinolones, lincomycins, macrolides, sulfonamides, glycopeptides) (Korzybski et al. 2013). According to their effect bacteria, antibiotics can be divided into two groups (de Lima Procópio et al. 2012; Korzybski et al 2013):

1- Bactericidal antibiotics, which cause the death of the bacteria and therefore an inhibition in the size of the bacterial population (aminoglycosides, β-lactamines, sulfonamide-trimethoprim combination and quinolones).

2- Bacteriostatic antibiotics, which stop bacterial multiplication and therefore growth interruption of the bacterial population (macrolides, tetracyclines, sulfonamides chloramphenicol and lincosamides).

In addition, the spectrum of antibacterial activity of the antibiotics differs from each other. Therefore, each antibiotic is associated with a specific list of bacterial species on which it could be effective. The spectrum of the antibiotic on the various bacterial species is an essential

element to take into-account when initiating antibiotic therapy (Shears and Hart 1997). Another classification of antibiotics takes in consideration their mode of action since each one can interact differently with the bacteria (Shears et al. 1997; Jean-Pierre FLANDROIS , 1997):

1- Inhibitors of bacterial envelope synthesis (B lactam such as: penicillins, fosfomycin, glycopeptide and carbapenem).
2- Protein synthesis inhibitors (phenicols, cyclin, fusidic acids and oxazolidinones).
3- Inhibitors of nucleic acid synthesis (quinolones, mupirocin and other).
4- Inhibitors of folic acid synthesis (sulfonamides)
5- Complex or uncovered mechanisms (nitrated products and tuberculosis).

Subgroups classifications of antibiotics according to their spectrum of antibacterial activity and their mode of action are depicted in **Tables 5, 6** and **Figure 6**.

Table 5. Example of preeminent antibiotics inhibiting the synthesis of peptidoglycan and their antibacterial spectrum (Jean-Pierre Flandrois, 1997; Yala et al. 2001; Stahl 2003; Leclercq et al. 2006).

Subgroups	Antibiotics (DCI)	Spectrum of antibacterial activity	Mode of action
Penicillin G and its derivatives	Parenteral: -Benzyl Penicillin (penis G)-Benzyl Penicillinprocaine-Bénéthamine-benzylpénicilline-Benzathine-benzyl penicillin Oral: - Phenoxy methyl penicillin (penicillin V) - Clometocillin	• Cocci Gram +: *Streptococci* (group A, C, G and B), Sensitive *pneumococci*. • Cocci Gram-: *Neisseria* (especially *meningococcs*) • Gram + bacilli: *Corynebacteriumdiphteriae*, *Bacillus anthracis*, *Listeria* monocytogenes , Anaerobes ……	✓ Bacterial wall, by selective toxicity: They act on the synthesis of peptidoglycan in inhibiting proteins binding penicillin (PLP). ✓ PLP inhibition results in the inhibition of bridge ✓ formation ✓ PLPs have an activity transpeptidasic, carboxypeptidasic and transglycolasic. ✓ Pentacyclic responsible for reticulated structure of the wall. ✓ The obtain of bizaroids forms lead to bacterial

			lyses
Penicillins M (Antistaphyloco-ccal)	- Methicillin - Dicloxacillin, - Flucloxacillin - Oxacillin - Isoxazolyl-penicillin	-*Staphylococcus* producer of penicillinase. -*Staphylococcus* MRSA-(sensitive with Oxacillin)	See the mode of action of penicilinsG and its derivatives
Carboxy-penicillins	- Carbenicillin, -Ticarcillin	- Enterobacteria producing cephalosporinases: *Citrobacter*, *Enterobacter*, *Serratia*, *Proteus* indole +. -*Pseudomonas aeruginosa*). -Gramsresistantto ampicillin.	See the mode of action of penicilinsG and its derivatives
Cephalosporins from 1st generation	Injectable, metabolicallyunstable : Cefalotine, Cefacetrile. Injectable, metabolically stable : Cefaloridine. Oral Cephalospori-ns :Cefalexine, Cefradine.	- *Staphylococci* MRSA - *Streptococci* (except *enterococci*) - *Haemophilus influenzae* - Some Gram -bacilli - (*E.coli*, *Proteus mirabilis*, *Salmonella*..) - Inactive on *Pseudomonas aeruginosa*	Bacterial wall, by selective toxicity: They act on the synthesis of peptidoglycan in inhibiting proteins binding penicillin (PLP). PLP inhibition results in the inhibition of bridge formation PLPs have an activity transpeptidasic, carboxypeptidasic and transglycolasic. Pentacyclic responsible for reticulated structure of the wall.
Cephalosporins from 2nd generation	Injectable: - Cefoxitin (Cephamycin) - Cefuroxime - Cefamandole	-*Staphylococcus* MRSA Group A *Streptococci* -*Streptococcus pneumoniae* -*H.influenzae* -Gram - bacilli -Inactive on *P.aeruginosa*	See the mode of action of cephalosporins from 1st generation
Cephalospo-rins from 3rd generation	Injectable : - Cefotaxim - Ceftriaxone - Latamoxef - Ceftazidime -Cefmenoxime - Cefpirome - Cefsulodin - Cefepime Oral: Cefixime	- Grams- bacilli - Gram+ cocci: *Pneumococcus*, *Streptococcus* (except *enterococci*) - Gram - cocci - Some are active on *Pseudomonas* (Ceftazidime)	See the mode of action of cephalosporins from 1st generation
Carbapenems	- Imipenem - Meropenem - Ertapenem - Faropenem	Gram – bacteria including *Pseudomonas aeruginosa*	See the mode of action of cephalosporins from 1st generation

Oxapenames or clavams (acid clavulanic β-lactamases inhibitors. Used in combination with a β-lactamine	- Amoxicillin + Clavulanic acid - Ticarcillin + Clavulanic acid	- Fermentative gram - bacteria - Oxidative gram - bacteria	See the mode of action of cephalosporins from 1st generation
Glycopeptides	-Vancomycine - Teicoplanine	Gram + bacteria and essentially: - MRSA + *staphylococci* - *Enterococci* - *Pneumococcus* resistant to penicillins	Bacterial wall, blocking the polymerization of peptidoglycan by a mechanism complex.

Table 6. Antibiotics inhibitor activity on the proteins synthesis, nucleic acids' synthesis, folate synthesis, membrane envelopes and their antibacterial spectrum (Stahl 2003; Bourgeois-Nicolaos et al. 2012; Jean-Pierre Flandrois, 1997; Adam et al. 2004; Chiquet et al. 2008).

Subgroups	Antibiotics	Spectrum of antibacterial activity	Mode of action
Macrolides-Lincosamides-Streptogramins (MLS)	True macrolides: 14 atoms:Erythromycin, Oleandomycin, Roxithromycin 15 atoms: Azithromycin 16 atoms: Josamycin, Spiramycin, midecamycin	- Gram + cocci: *Staphylococcus* MRSA-, *Streptococcus* - Cocci Gram- bacteria: *Neisseria*, *Moraxelles* - Gram + bacilli: *Corynebacterium diphteriae, Listeria monocytogenes, Bacillus* -Some Gram- bacilli: *Campylobacter*	MLS are inhibitors proteins synthesis. They act at the level s / unit 50S of the ribosome. They inhibit the growth of thechain Polypeptide in progress.
Polymyxins	- Polymyxin B - Polymyxin E or colistin	- Gram- bacilliexcept: *Proteus*, *Providencia*, *Serratiam arcescens* *Morganella morganii* and *Edward siellatarda* -Gram + bacteria And *mycobacteria* are naturally resistant.	They have a charge positive and act like surfactants agents. They act on the cell membrane by attaching to phospholipids from where barrier break osmotic.
Quinolones	Nalidixic acid, Acid pipemide, Acid oxolinic, Flumequin	*Enterobacteries* The Gram + are Resistant	Selective inhibition of the synthesis of the bacterial DNA by acting ontwo enzymes involved in this synthesis: gyrase DNA and topoisomerase IV.
Sulfonamides	Sulfapyridine,Sulfafurazole Sulfaméthoxydiazine Sulfaméthoxy pyridazine Sulfaméthoxazole	Gram- bacteria, but there are a lot of resistance to vis these antibiotics.	Inhibit synthesis of the folates, acids purines and nucleic acid laying down on the dihydropteroate synthetase (DHPS)

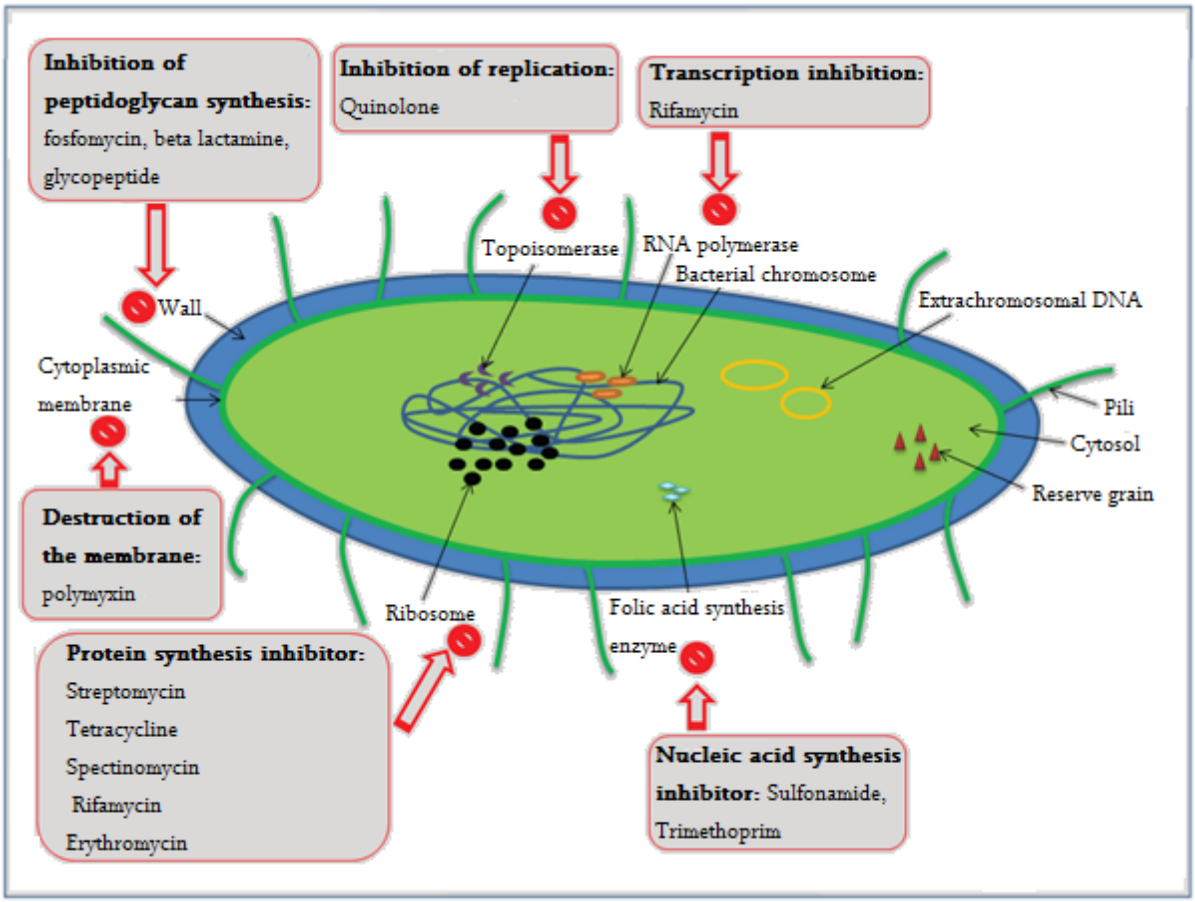

Figure 6. The different targets of antibiotics in bacterial cell (Carmen Chifiriuc, et al., 2016).

Global Antibiotic Consumption

According to Klein et al. (Klein et al. 2018), during sixteen years, between 2000 and 2015, a remarkable increase (about 65%) has occurred in the consumption of antibiotics in the world, arguing an increase from 21.1 to 34.8 billion daily doses. This data was the outcome of a study collected from 76 countries around the world. Interestingly, the countries which have low and middle income, showed a particular increase in antibiotic consumption and reached about 114% growth rate, which means 24.5 billion daily doses (in comparison with the standard value which is 21.1 billion). Notably, between 2000 and 2015, the consumption of antibiotics has increased

by 79% in China, 65% in Pakistan and has doubled in India. These three countries were considered as the largest antibiotics consumers among low and middle income countries.

2.3.1. Antibiotics Consumption in France

In 2017, 759 tons of antibiotics for human health and 514 tons of antibiotics for animal health were sold in France. However, the correlation between consumption in human and animal health is varied between different European countries (Anses/ANSM 2018; ECDC/EFSA/EMA 2017).

1. Human Health:

According to the Europe-wide network of national surveillance systems (ESAC-Net), the French consumption was of 2.16 doses/1,000 inhabitants/day in 2016 i.e. above 6.3% among the European average. According to this statistic, France ranked at the 8th number for consuming greater antibiotics (Data from 23 countries have been provided), while in 2006, it was placed on the 3rd rank (out of 16 countries that provided data) (Anses/ANSM 2018).

2. Animal Health:

In 2016, France was ranked in the 12th position of the most antibiotics consuming country out of 30 countries participated to European Surveillance of Veterinary Antimicrobial Consumption (*ESVAC*), which reported a low average consumption in contrast to other European countries (71.9 mg / kg *vs.* 124.6 mg / kg). In six years (between 2010 and 2016), the France consumption was decreased by 46%. Also, the average consumption for ESVAC members was decreased by 24% (**Figures 7** and **7.1**) (Anses/ANSM 2018).

The decrease in antibiotics consumption is due to the action taken by the European Union which banned in 2006 the use of antibiotics in animal feed to overcome the antibiotic resistance.

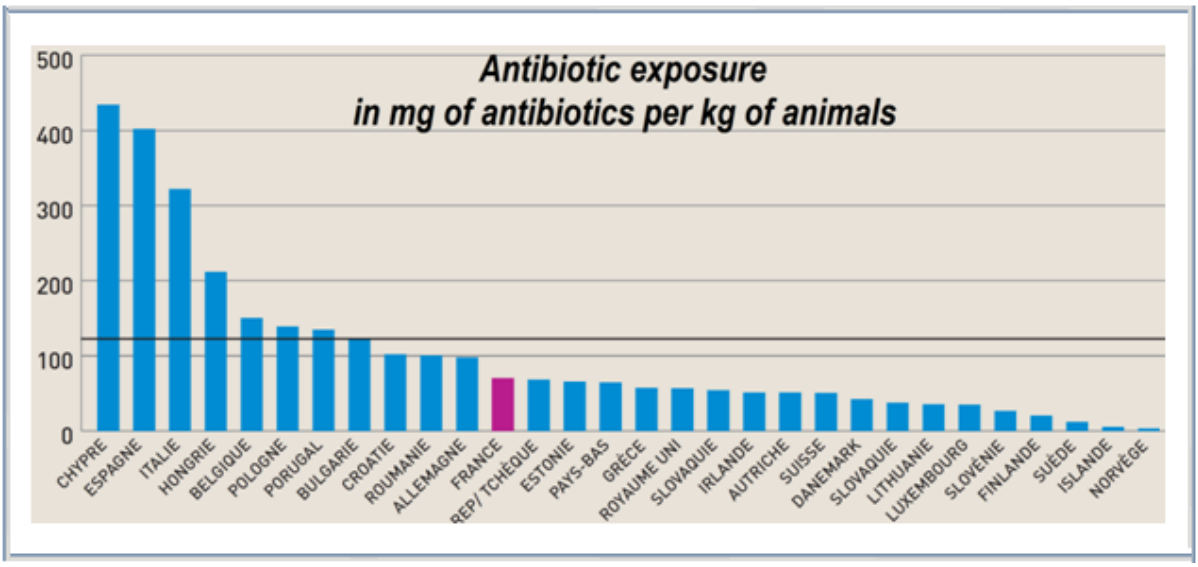

Figure 7. Antibiotics consuming by 30 countries participated to ESVAC (Source: ESVAC / Anses)

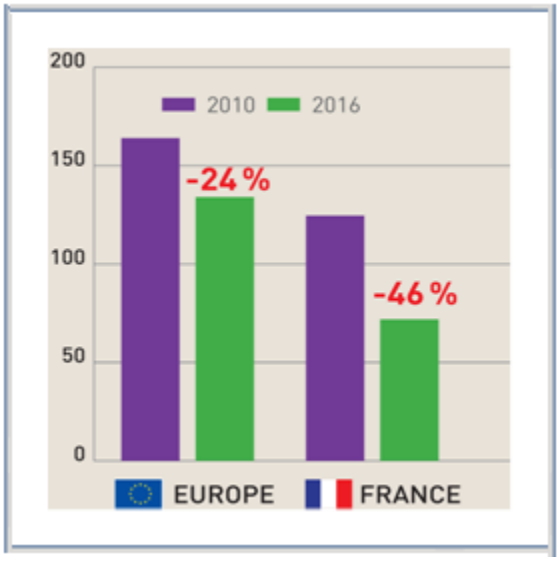

Figure 7. 1. Comparison of the decreasing in antibiotics consumption in Europe and France between 2010 and 2016(Source: ESVAC / Anses).

3. Antibiotic Resistance

Antibiotic resistance is considered as a major problem in terms of human and animal health worldwide. One of the leading causes of emergence of the resistant bacteria is using broad-spectrum antibiotics, active against both Gram-positive and Gram-negative bacteria. Moreover, the wrong diagnosis exacerbated this phenomenon. The appearance and rapid diffusion of bacterial strains resistant to antibiotics put a question mark on the effectiveness of these treatments both in humans and in animals. Based on that, a full integration and interaction of multiple skills and knowledge are required. One of the central sources of information which could be used to manage antibiotic resistance risks is controlling the sales of antibiotics for veterinary use (Fortané 2019).

3.1. History

Bacterial resistance is the ability to dare to the effects of antibiotics or biocides that are supposed to kill or control them (SCENIHR 2009). In 2011, a study conducted by D'Costa et al. (D'Costa et al. 2011) based on the analyses of the metagenomic of ancient DNA, taken from beringian permafrost sediments of 30,000 years, showed that the antibiotic resistance is an ancient phenomenon. During this study, a high number of genes, encoding resistance to glycopeptides, β-lactam and tetracycline antibiotics, have been identified. In addition, the evidence of the complexity, antiquity and similarity of the origins of the mechanisms of the resistance were highlighted. In 1940, the first bacterial antibiotic resistance to sulfonamides was identified. However, the consequences of this discovery have not been taken seriously due to regular discovery of new antibiotics (Singer et al. 2003; Ligon 2004). Few years later, in 1960, the US Surgeon General, Dr. William H. Stewart has announced "It is time to close the book on infectious diseases, and declare the war against pestilence won" (Spellberg et al. 2008). Despite

this announcement, the identification of antibacterial resistance has started to grow during the following years for indicating serious problems (**Figure 8**) (D'Costa et al. 2011; Spellberg et al. 2008; Montanari et al. 2003; "Santé Publique France" 2019; Fischbach et al. 2009; Cochetti et al. 2008)

Figure 8. The years of market introduction of the major families of antibiotics and concomitant history of the identification of resistances.

Antimicrobial resistance (AMR) has become one of the greatest threats humanity faces in today's world. In India, more than 60.000 babies die each year as a result of antibiotic resistance infections. In Thailand, AMR causes about 38.000 deaths each year and more than 23.000 deaths in USA (CDC Global Health 2018; Laxminarayan et al. 2013). A recent study conducted by O'Neil (2014), reported that the bacterial infections (TB, HIV and malaria) associated with antibiotic resistant strains cause the death of about 700.000 people each year, and both multidrug- resistant and extremely antibiotic-resistant tuberculosis (TB) kill about 200.000 people every year (O'Neill 2014).

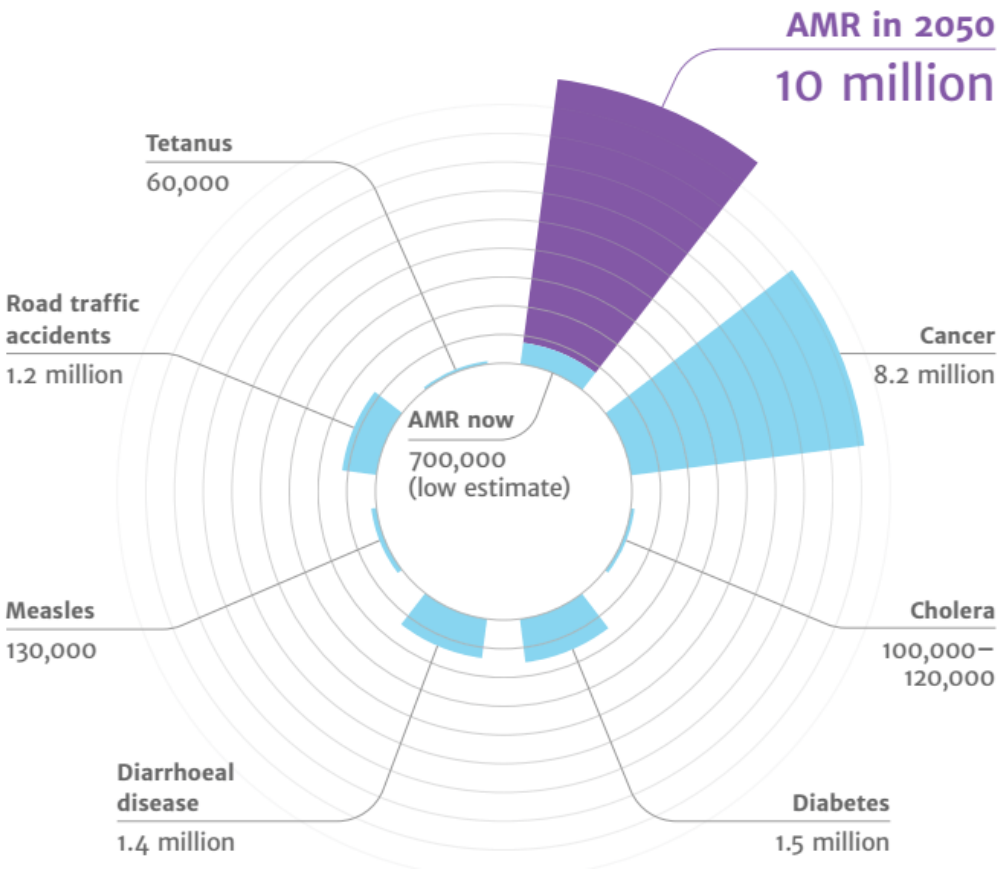

Figure 9. Deaths caused by AMR each year (O'Neill 2014)**.**

According to statistical analysis of increase of the antibiotic resistance for six pathogens, if no action has been taken, the number of deaths caused by AMR will exceed 10 million lives every year by 2050 (an average of one death each three seconds) (**Figure 9**), which is accompanied with loss of 100 trillion dollars in global economic output (O'Neill 2014). The delay in dealing with the emergence of AMR and the lack of its restraint, in addition to the lack of investment in the development of new antibiotics would lead to a health crisis not only in isolated areas, but on a world-wide level (O'Neill 2014).

3.2. Antibiotic Resistance Definitions

Several definitions of antimicrobial resistance can be taken into consideration depending on the domain in which it is studied (Guillemot 2006). Clinically, a bacterial strain is considered to be resistant in the event of therapeutic failure with the antibiotic used. For pharmacologists, a strain is qualified as resistant if the concentration which it reaches at the level of the site of action is lower than the minimum inhibitory concentration (MIC). In microbiology, a resistant strain is one that has a resistance mechanism allowing it to increase its MIC. Finally, in epidemiology, it is the variation of the MIC of a strain compared to that of the usual population which gives it the status of resistant.

3.3. Resistance Origins and its Relationship with Antimicrobial use

The overuse of antimicrobial agents in human (Goossens et al. 2006) and animal as medicine (Chantziaras et al. 2014) is the leading cause to emergence of bacterial resistance (Llor et al. 2014). Under normal conditions, each individual has few resistant bacteria among the billions of bacterial population present in its intestinal microbiota. Based on that, resistant bacteria cannot establish themselves easily compared to non-resistant bacteria. However, the

antibiotic treatment, used to inhibit the bacteria responsible of the infection, gives additional advantage of the resistant bacteria to survive and therefore to multiply and become predominant as compared to non-resistant bacteria ("Santé Publique France" 2019). This is how the improper use of antibiotics in humans and veterinary medicines has promoted the spread of resistance. Additionally, many evidences have correlated the antimicrobial use in animal intruding antimicrobial resistance (AMR) in humans since the antibiotics used in animal health treatment are the same in humans (One health). Furthermore, there are several transmission routes of the resistant bacteria such as direct contact with animals and indirect contact *via* its food consumption. Nonetheless, the spread of resistant bacteria can occur by transmission from animal to the environment (Linton 1977), like the transmission of the livestock-associated methicillin-resistant *Staphylococcus aureus* (LA-MRSA), potentially induced by pig farming in particular, to the farmers (de Neeling et al. 2007) and to the slaughterhouse workers after direct contact with infected pigs (Gilbert et al. 2012). In addition, pigs were also associated with the transmission of extended-spectrum-β-lactamase (ESBL) producing bacteria (Randall et al. 2014). But, it seems that the prevalence of ESBL producing *E. coli* in pigs is lower than that observed in poultry slaughter (Geser et al. 2012).

3.4. Mechanisms of the Antibiotic Resistance

There are four major mechanisms (**Figure 10**) allowing the bacteria to resist to antibiotics' actions (Correia et al. 2017; Tenover 2006):

- Prevent the antibiotic from entering into the bacteria by reducing the permeability of the cell wall (e.g. resistance to carbapenems in *Serratiam arcescens*)

- Modification of the target sites of antibiotic which become no longer recognized by the antibiotic (e.g. the ability of the *E. coli* strains to resist to fluoroquinolones by modification of the GyrA and ParC subunits of the DNA gyrase)
- Some bacteria, like *Pseudomonas*, have a system known as efflux pumps which allow the bacteria to expel ingested chemicals (e.g. resistance to fluoroquinolones and trimethoprim in *Pseudomonas aeruginosa*) or elimination of the antibiotic by the transport systems "porins" (loss of sensitivity to β-lactamines)
- Some microorganisms have shown their ability to produce enzymes which inactivate antibiotics (e.g. penicillinase that can inactivate penicillins)

Besides the aforementioned ones, other mechanisms could be involved in the resistance of the bacteria:

- Protection of the target from the action of the antibiotic (e.g. ribosome protection conferring resistance to tetracycline in *C. perfringens*).
- Modification of the antibiotic, unabling it to bind to its target (resistance to chloramphenicol or fosfomycin in *P. aeruginosa*).
- Degradation of the inactivated antibiotic (*Enterobacteriaceae* produce β-lactamases conferring resistance to the penicillins).

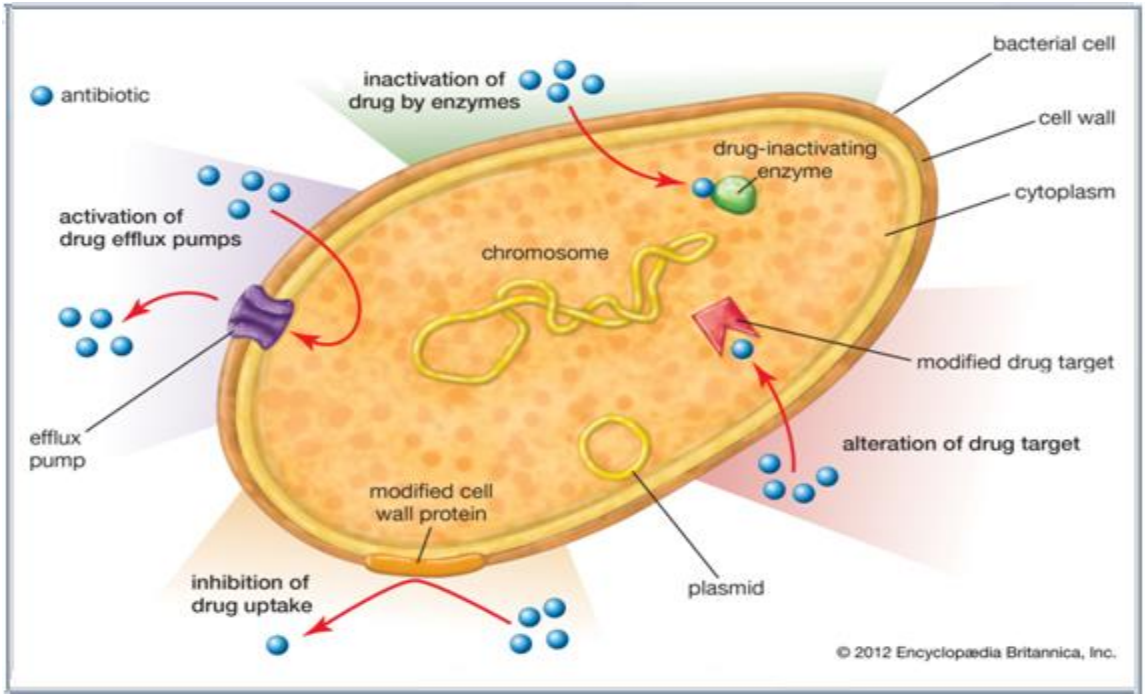

Figure 10. The four major Mechanisms of Resistance of the Bacteria towards Antibiotics. This figure was adopted from Britannica. https://www.britannica.com/science/antibiotic-resistance

3.5. Types of Antibiotic Resistance and the Mode of Transmission of Resistance Genes

Antibiotic resistance is a natural phenomenon, which is in frame with the law of natural selection. This resistance can be divided into two types: the intrinsic resistance and the acquired resistance (Coculescu, 2009).

3.5.1. Intrinsic Resistance (Natural Resistance)

The intrinsic resistance is the natural ability of bacteria to resist to the activity of a particular antibiotic agent. This natural resistance is the consequence of specific structural and functional characteristics of the bacteria. Antibiotic interacts with specific sites present in the bacteria structure; however some microorganisms naturally are devoid of such target sites and therefore are not susceptible to these agents. This resistance could also be due to the differences in the chemical structure of the antibiotic and bacterial membrane which confer low permeability to

that drug, especially for those interacting with intracellular targets. This phenomenon occurs mainly in Gram-negative bacteria which have complex outer layer where antibiotics have low penetration rate (Coculescu, 2009).

3.5.2. Acquired Resistance

In addition to the natural resistance, some microorganisms, after exposure to physical or chemical threat, acquire new molecular mechanisms conferring resistance to antibiotics normally active against them (Scott 2017). The acquired resistance can take place by two mechanisms:

3.5.2.1. Transmission of Acquired Resistance by Mutations (vertical and hereditary transmission)

The chromosomal mutations (modification of existing bacterial genes) take place inside the bacterial genome. This mutation, which can occur spontaneously and seldom, is promoted by the exposure of the bacteria to an antibiotic which exert an antibacterial pressure. During the bacterial reproduction, the mother cell can transmit the mutation to their daughter cells making the transmission exclusively hereditary (**Figure 11**) (Coculescu, 2009).

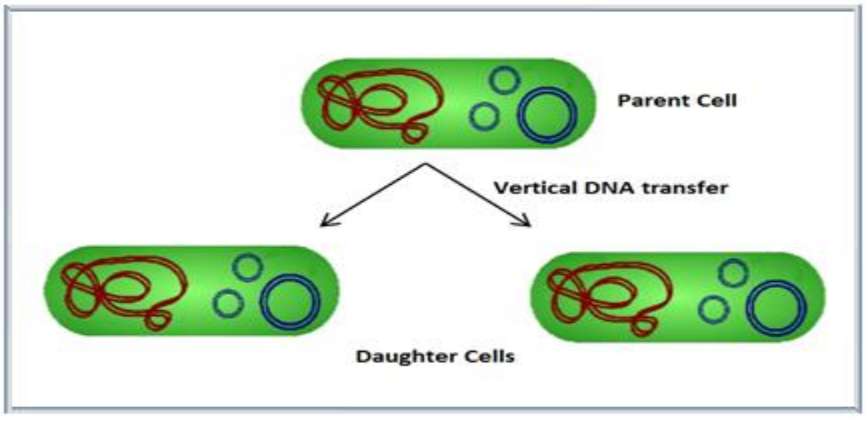

Figure 11. A simple illustration of the vertical transfer gene.

The bacteria which have a chromosomal resistance can defy one type of antibiotic. For example, the mutation of the gene encoding the ribosome protein S12 confers to *E. coli* strains, harboring this modification, resistance to streptomycin (Kone et al. 2019; "Santé Publique France" 2019).

3.5.2.2. Transfer of Genetic Material (Horizontal Gene Transfer-HGT)

It is well acknowledged that microorganisms have the ability to share the genetic material from each other, therefore acquiring of resistance. Transfer of genetic materials occurs horizontally through, horizontal gene transfer HGT, which is a rapid process than the intrinsic one. The other genetic transfers include transformation, transduction and the conjugation (Coculescu, 2009).

3.5.2.2.1. Transformation

The transformation process is dependent on the presence of donor and recipient cells (competent cells). The first step is the cut of DNA that is released by the donor cells which surrounds the competent recipient cells. The DNA fragments are then picked up and get inside by the cells and incorporated into the chromosome *via* recombination (**Figure 12**). Only the cells which are similar to the donor cells can acquire mutation conferring resistance *via* transformation. However, this transfer is partial and represents less than one percent of the bacterial genome therefore it is limited. Furthermore, the probability of this transfer is very low (about $10^{-4} – 10^{-6}$) (Danan 2006; Scott 2017).

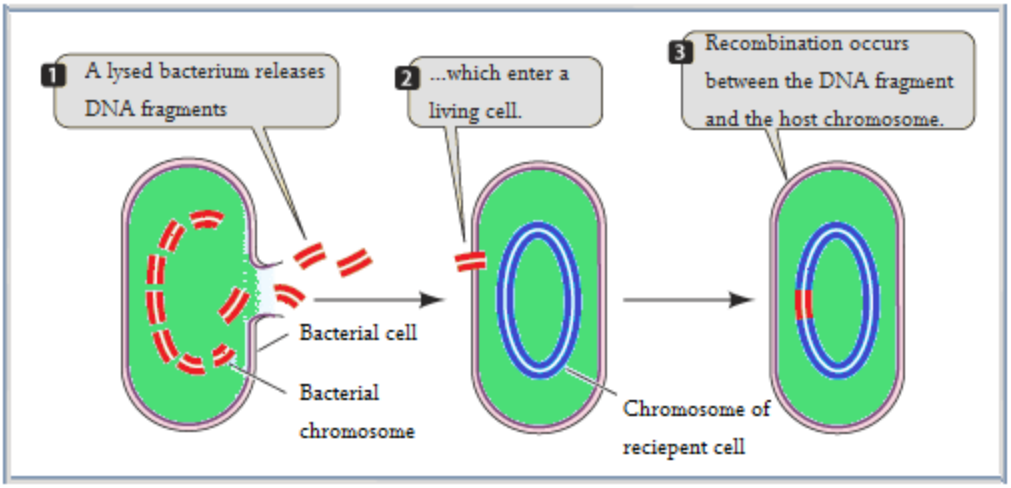

Figure 12. The 3 steps of HGT transformation (Manage, 2018).

In 1923, the bacteriologist Griffith identified two strains of Streptococci, one called S (smooth) strain which is virulent and causes pneumonia and the second one called R (rough) strain devoid of virulence. During his experiments, Griffith showed that the R strain can be converted into virulent S strain. During more than 20 years, scientists believed that the proteins were the key factor of this transformation. In 1944, three bacteriologists, Avery, MacLeod and McCarty, have analyzed the composition of the transforming factor which was shown to be precipitated after adding of alcohol (which means it is not a carbohydrate or polysaccharide nature) and was not destroyed in presence of protease neither lipases (which means it is not of proteinaceous or lipidic nature). Also, the substance remains active in presence of ribonuclease which digests RNA. After several experiments, they demonstrated that the transforming principle is only produced by the DNA (Méthot 2016).

3.5.2.2.2. Transduction

This transmission process is mediated by viruses, known as bacteriophages, which transfer the genetic material from the infected bacteria to the recipient bacteria (**Figure 13**). This phenomenon is linked to the infection cycle of the bacteriophages, which adhere to the recipient bacteria, inject their genetic material and start their reproduction. In case the genetic material, containing mutation conferring resistance, has recombined to the genome of the host bacterial cell (it depends to bacteria species), the recipient bacteria will acquire antibiotic resistance genes. The process which takes place directly from virus to bacteria is called "conversion". The efficiency of this mechanism is lower than that of conjugation. The frequency of this phenomenon is near to that one present in "transformation", however the injected material represent 1 to 2% of bacterial genome (Guillemot 2006; Maurin 2013; Scott 2017).

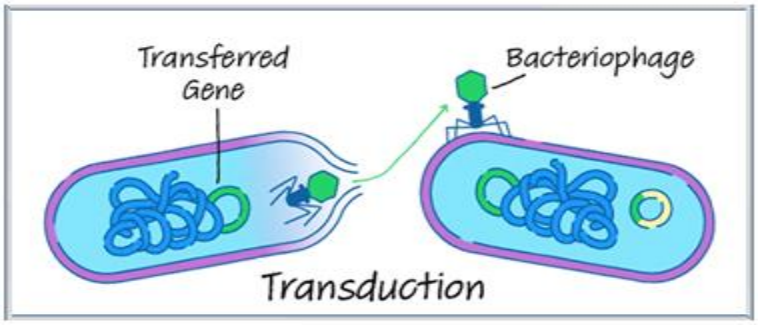

Figure 13. Principle of Transduction and the Bacteriophages Pathway to Transmit Genetic Materials from Bacteria to Host Bacterial Cell (Rebecca 2019).

3.5.2.2.3. Conjugation

Bacterial conjugation is an extra chromosomal mechanism by which the tansfer of genetic material occurs by a connection between two cells. In contrast to other mechanisms (transformation and transduction), this is the only mechanism that occurs by direct cell-to-cell

contact. During this mechanism, the donor cell transfers a plasmid, a small circular strand of DNA often present inside the bacterial cytoplasm, on which is present a gene called factor F that is responsible for the synthesis of sexual pilus providing the contact bewteen the donor cell and the recipient cell. The integration of the plasmid into the chromosome is only occuring when the plasmid is recombinant. If not, the plasmid remains free inside the bacterial cytoplasm until another transfer. Notably, this mechanism isoccuring in different species of bacteria (Guillemot 2006; Maurin 2013; Scott 2017). About 80% of acquired resistance belongs to the plasmid resistance which can affect more than one antibiotic; therefore, several families of antibiotics can be affected by such acquired resistance mechanism, called multi-resistance ("Santé Publique France" 2019). In addition tothe multi-resistance, which can be acquired by the conjugation, this process provides the transfer of vurilent genes from the donor bacteria to the recipient bacteria. HGT represents an exchange of 10 to 20% of the bacterial genome and more than 80% of the clinical resistance (Coculescu, 2009). This mechanism has been described in the graphic below (**Figure 14**):

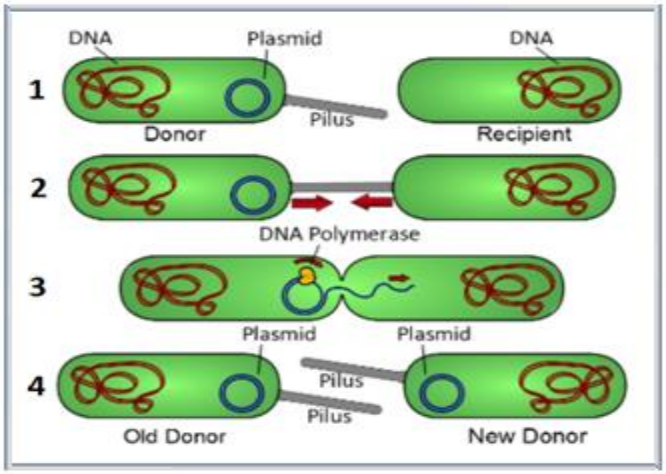

Figure 14. Conjugation diagram, 1. Donor bacteria produce bridge (pilus); 2. The bridge allows the connection between the two bacteria; 3. Transfer of a small strand of DNA from the donor cell to the recipient cell. 4: Both cells start synthesizing the complementary strand in order to produce a circular plasmid (double stranded), both bacteria are now donor or F-factor. http://2008.igem.org/Team:Heidelberg/Project/Killing_I

3.6. Antibiotics Resistance of *C. perfringens*

The uncontrolled uses of antibiotics provoked an ecological imbalance, which has become evident in the spread of multidrug resistant of foodborne pathogens, like *C. Perfringens* strains which display resistance to multiple antibiotics (**Table 7**). The development of bacterial resistance maybe acquired by genetic factors e.g. the tetracycline-resistant of *C. perfringens* originated from carrying these bacteria to tetracycline resistance genes, *tetA(P) and tetB(P).* The gene *TetA(p)* encodes a protein responsible for the active efflux of tetracycline from the cell, while *tetB(p)* encodes a protein providing a ribosomal-protection tetracycline-resistance mechanism (Lyras, et al., 1996). An earlier study showed that some *C. perfringens* strains are resistant to lincomycin. The lincomycin-resistant of *C. perfringens* originated from carrying these bacteria to lincomycin resistance genes, *lnu(A)* and *lnu(B).* Both resistance genes encode nucleotidyl transferases (Martel, et al., 2003).The continuous flow of resistance genes into the human gut microbiota *via* the contaminated food is one of the main problems human medicine is facing(Teuber, 1999).

Table 7. Multidrug Resistance of *C. perfringens*.

Origin	Antibiotic resistance	References
Pigs	Ceftiofur, enrofloxacin, erythromycin, lincomycin and tylosin	(Ngamwongsatit, et al., 2016)
Human and pigs	Tetracyclin, imipenem, metronidazole, penicillin, vancomycin and chloramphenicol	(Tansuphasiri, Wiriya, & Sangsuk, 2005)
Pigs	Tetracycline, erythromycin, lincomycin, and clindamycin	(Teuber, 1999)
Poultry, pigs and cattle	Bambermycin and flavomycin	(Devriese, Daube, Hommez, & Haesebrouck, 1993)

However, in 2006, the AGP bans with a particular focus on finding novel preventive treatments against NE including phage therapy, vaccination, bacteriocins and other related processes. The alternative strategies are based on the identification of *C. perfringens* pathogenicity factors as potential targets for NE prevention (**Figure 15**) (L. Caly, et al., 2015). These strategies and others are detailed elsewhere (section 4-alternatives to conventional antibiotics)

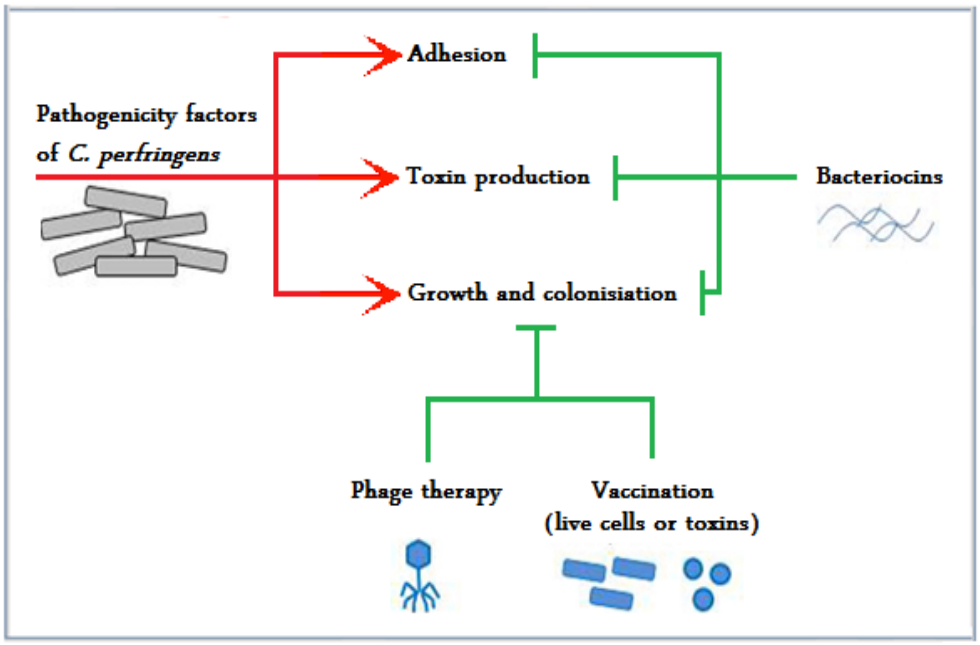

Figure 15. Antimicrobial potential of the alternative strategies to prevent NE *vs.* virulence and pathogenicity factors of *C. perfringens*. The green line represents the inhibition.

3.7. Strategies to Control Antimicrobial Resistance (AMR)

Antibiotics have been used for many years, their frequent prescription started to limit their effectiveness, therefore human and animal health has started to get closer to the danger zone of the treatable diseases. It is time to find another cure brings us again to the safe side, otherwise we

will return to the "dark ages" of medicine (Elhani, 2011).

The AMR is a global problem; consolidation of efforts is a first step in facing the threat. Only global strategy combining several parameters can fight the antibiotic resistance.

International, national and local strategies have been suggested for prevention and tackling AMR in human and animal (Ministère Agriculture 2017; Uchil et al. 2014). These strategies are based on following guidelines:

1) Promotion of good practices and rational use of antibiotics:

a) Good indication: Prescription of antibiotics only to treat infections caused by bacteria (not virus), or even their limitation to reduce the pressure of selection.

b) Good molecule: it is highly recommended to do bacteriological analysis with an antiobiogram to find out if the target bacteria have resistance to prescribe effective antibiotic.

c) Good dose: the dose of the prescribed antibiotic depends on several factors such as the infection type, age of the infected person and the weight…

d) Duration time: it should be always respected in order to prevent bacterial resistance.

2) Strengthen supervision and reduce risky practices: Aims to more understand the risks linked to the overuse of antibiotics and better control the consumption of critical antibiotics and eliminate the economic interests of antibiotics.

3) Raising awareness of all stakeholders (General public and professionals).

4) Develop alternatives to antibiotics: This fourth objective avoid the use of conventional antibiotics and shows the importance of investing in the basic research and health fields and trying to treat animal health without using antibiotics.

4. Alternatives to Conventional Antibiotics

Nowadays, several studies are dedicated to new strategies to overcome the health-threats of antibiotic resistant bacteria (**Figure 16**). Among these strategies, new classes of antibiotics evidenced to be effective against both Gram-positive and Gram-negative multi-resistant bacteria (Stokes et al. 2020; Qiu et al. 2019). Another alternative is the phage therapy, using viruses to kill pathogenic bacteria (Taati Moghadam et al. 2020; Romero-Calle et al. 2019) including *C. perfringens* (W. Miller, et al., 2010). A multivalent bacteriophage cocktail INT-401, designated by Miller et al.(2010) demonstrated future proof ability of bacteriophages to control necrotic enteritis, caused by *C. perfringens*, in broiler chickens (W. Miller, et al., 2010). Vaccines, which confer a high way for protection by preventing the infections to humans and animals, are also seen as an interesting strategy (Kingwell 2018) e.g. using a non-virulent NetB positive ($nvNetB^+$) strain *of C. perfringens* type A can be promising for preventing necrotic enteritis in chickens and turkeys (Mishra, et al., 2017). Of note, bacteriocins have shown to be a promising candidate to overcome the antimicrobial resistance and prevention of pathogenic infections (Chikindas, et al., 2018; Rea, et al., 2011; Meade, et al., 2020) such as the anti-*C. perfringens* activity of DD14 (L Caly, et al., 2017). Finally, a novel rational approach using nanotechnology is currently investigated, which has found to be an effective strategy to tackle the antibiotic resistant bacteria problem (Eleraky et al. 2020). Nowadays, numerable studies are focusing on nanoantibiotics as a potential targeted therapy (Khurana et al. 2018).

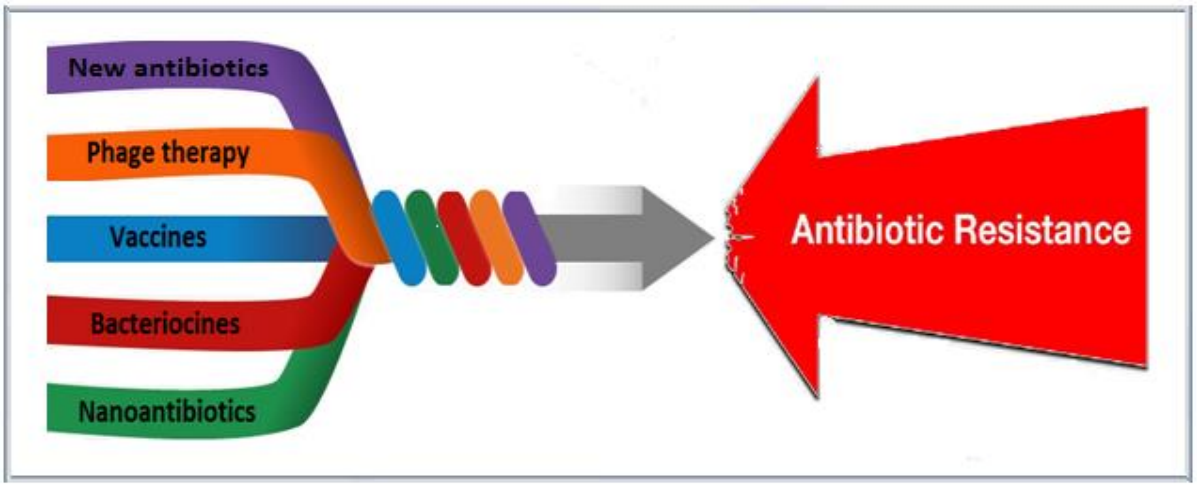

Figure 16. Strategies to Fight against Antibiotic Resistance.

4.1. New Classes of Antibiotics

Many companies as well as academic institutions are working on new anti-infection drugs, along with novel classes of antibiotics. With respect to that, new achievements enabled to discover antibiotics from untapped sources. These new antibiotic candidates displayed effective results and will be available in the market in near future. Here, we discuss five new classes of antibiotics with different mode of actions.

4.1.1. Outer Membrane Protein Targeted Antibiotics (OMPTA)

Gram-negative bacteria contain an outer membrane which serves as a protective barrier, and stands as check point for the uptake and export of nutrients and signaling chemical compounds. The outer membrane is an asymmetric bilayer, inner leaflet and outer leaflet, which contain glycerophospholipids and lipopolysaccharide (LPS), respectively, in addition to a large amount of integral β-barrel outer membrane proteins (OMPs), essential for biogenesis of the outer membrane (Konovalova et al. 2017).

New synthetic molecules (chimeric peptidomimetic antibiotics) containing a large number of cyclic peptides, related to murepavadin, exhibited activity against many Gram-negative multi-drug resistant bacteria among colistin resistant bacteria (Luther et al. 2019).

According to the study of Luther et al. (2019), the mode of action of this newly developed drug class was unrevealed by NMR spectroscopy, which localized the binding site of the new antibiotic compounds on OMP. These new antibiotic targets two important components, the first is a complex fat-like substances (lipopolysaccharides), the second is BamA, an essential protein for the β-barrel folding complex (BAM), which plays a role in folding and insertion of the β-barrel proteins inside the outer membrane of the Gram-negative bacteria (Konovalova et al. 2017; Luther et al. 2019).

4.1.2. Biphenylacetylene based LpxC Inhibitor

UDP-3-O-(R-3-hydroxymyristoyl)-N-acetylglucose amine deacetylase (LpxC) is one of the six essential enzymes in the lipid A biosynthetic pathway of the outer membrane of Gram-negative bacteria. In the past decades, many efforts have been made for identifying a safe compound targeting this enzyme, at effective dosage levels, but all attempts were unsuccessful. Lemaître et al. (2017) have reported a novel biphenylacetylene based LpxC inhibitor (LPC-069), a compound that treats severe Gram-negative bacterial infections, including a broad panel of multi-resistant and extremely antibiotic-resistant strains causing nosocomial infections such as *Yersinia pestis*, the causal agent of bubonic plague (Lemaître et al. 2017).

4.1.3. Odilhorhabdins (ODLs)

In February 2017, the world health organization (WHO) published a list of antibiotic resistant “priority pathogens” to draw up in a bid to promote development of new antibiotics. The list highlights the threat of Gram-negative bacteria, which became resistant to multiple antibiotics. Among carbapenem resistant Gram-negative bacteria, *Enterobacteriaceae* and *Acinetobacter baumanni* were at the top of the list (WHO, 2017). In 2017, in European Union and the European Economic Area (EEA), carabapenem-resistance rates for *Enterobacteriaceae* and *A. baumanni* were 7.2% and 33.4%, respectively (ECDC, 2019; ECDC, 2018).

The mode of action of ODLs is based on a new mechanism of inhibition of the bacterial ribosome. ODLs are considered unique, because they bind to a receptor on the ribosome that has never been exploited by the conventional antibiotics. Once the ODLs introduced into the bacteria, they affect the reading ability of the ribosome making it unable to create right proteins. This miscoding contributes to deformed proteins and leads to the death of the bacteria. NOSO-502 showed potent activity against nosocomial infections caused by *Enterobacteriaceae* (e.g. polymyxin and carbapenem-resistant *Enterobacteriaceae*) (Racine, 2018). A second generation of ODLs will be launched for clinical trials in 2022. NOSO-2G will be the first candidate for the treatment of ventilation-associated pneumonia and hospital-acquired pneumonia (VAP/HAP) (Nosopharm, 2019).

4.1.4. Mursamacin

A novel class of antibiotics, called mursamacin, was recently identified from soil-dwelling round worms. The producing bacteria, *Xenorhabdus griffiniae*, were isolated from insect-killing *Steinernema* roundworms of central Kenya. These bacteria showed effective inhibition against

methicillin-resistant *Staphylococcus aureus* (MRSA). Different fractions of cell-free supernatant were extracted at different times of fermentation i.e.180h to 355h showed different percentages of growth inhibition of MRSA. Furthermore, high performance liquid chromatographic (HPLC) analysis revealed that the heat stable supernatant contains two major compounds. Further researches should be done to characterize these two compounds (Awori et al. 2017).

4.2. Phage Therapy

Phages are viruses that infects bacteria, discovered many years before penicillin, by Frederick Twort and Felix d'Herelle in 1917 (Doss et al. 2017). The first use of the phages as a therapy dates back to 1919 to treat dysentery infection. After encouraging results, two countries (USA and Brazil) have tried to use it as a therapy, however the results were not reproducible which led to decrease in their interest (Doss et al. 2017). In addition, the emergence of antibiotics has prompted Western Europe and North-American countries to stop considering phages as a therapy. However, the Soviet Union and Eastern Europe countries have not stopped their studies on phages as a treatment of infections caused by resistant bacteria (D. M. Lin et al. 2017).

4.2.1. Life Cycle of the Phage

Phages are small biological entities of about 25-200 nm lengthways, as viruses, they use bacteria as host to survive and multiply. Phages can be divided into 2 structural units, the capsid, which contains the genetic materials and the body, variable from phage to another, by which the phages interact with bacteria in order to insert their genetic materials (**Figure 17**) (Doss et al. 2017).

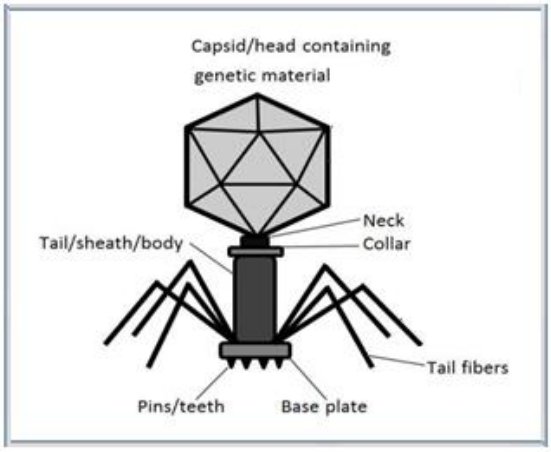

Figure 17. General structure of phage (Doss et al. 2017).

The reproduction of the phages can take place in two different cycles, lytic cycle and lysogenic cycle (**Figure 18**). The fixation process of the phages depends on the presence of specific receptors on the bacterial cells, allowing them to fix and inject their genetic materials. The integration of the genetic materials of the page inside the genome of the bacteria design the prophage step, this transformation occurs during the lysogenic cycle. The phage genome replicates during life cycle of the bacteria through typical lysogenic active cycle. When the bacteria were exposed to unfavorable environmental conditions, the lytic cycle is induced allowing the release of newly formed phages. During the lytic cycle, the phage uses directly the molecular machinery of the bacteria to reproduce. However, the production of endolysin causes the degradation of the bacterial membrane therefore the liberation of the virus, leading to lytic cycle, resulting in bacteria death (Doss et al. 2017; D. M. Lin et al.2017).

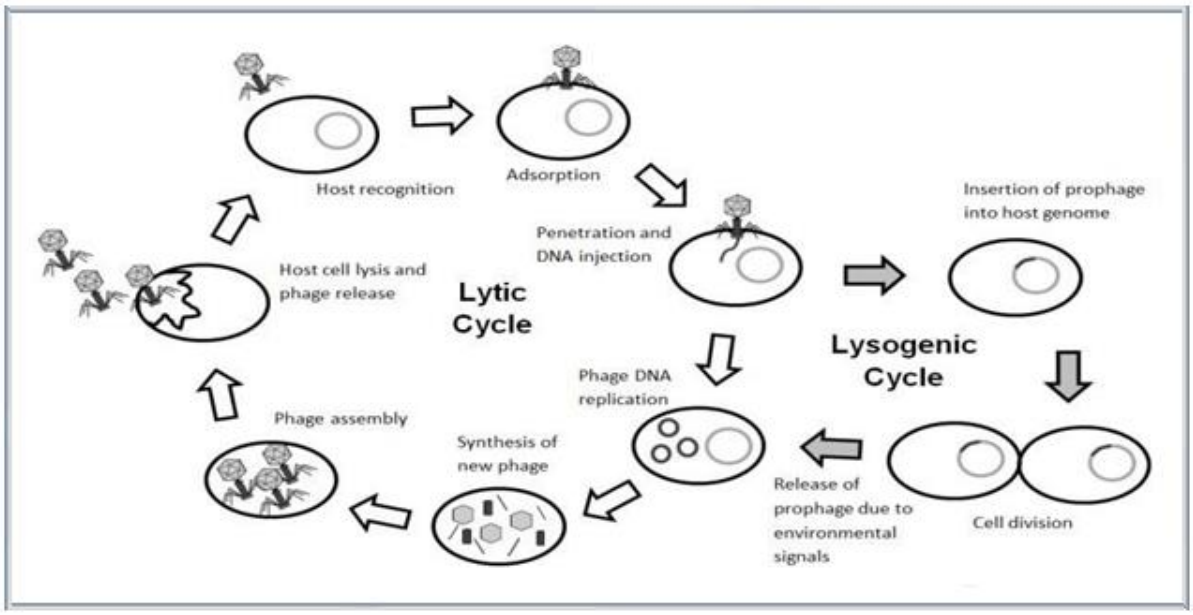

Figure 18. The two reproduction cycles of phages (Doss et al. 2017).

4.2.2. Phage Therapy Advantages over Antibiotics

The phage therapy has clear advantages compared to conventional antibiotics. Conversely to antibiotics, which are endowed with bactericidal and bacteriostatic effects, lyticphages are only bactericidal. One of the strongest points of the phage therapy is that once the phages entered the cells, they keep replicating until the targeted bacterial population is ended, which means that one administration is sufficient. In case of antibiotics, several administrations are needed, which increase the risk of overdose and ultimately emergence of resistance? In addition, phages are able to inhibit biofilms formation (Saha et al., 2019). Another advantage of phages is their specificity in hunting down only the harmful bacteria, which is not the case for the antibiotics that kill both the harmful and the beneficial bacteria (Eyre 2014).

4.2.3. Overcoming the Challenges of Phage Therapy

Despite the encouraging properties of phages, there are challenging questions which should be taken into consideration. The present issues facing the phagetherapy and its suggested

solutions are summarized in **Figure 19** (Doss et al. 2017; D. M. Lin et al. 2017; Saha et al. 2019).

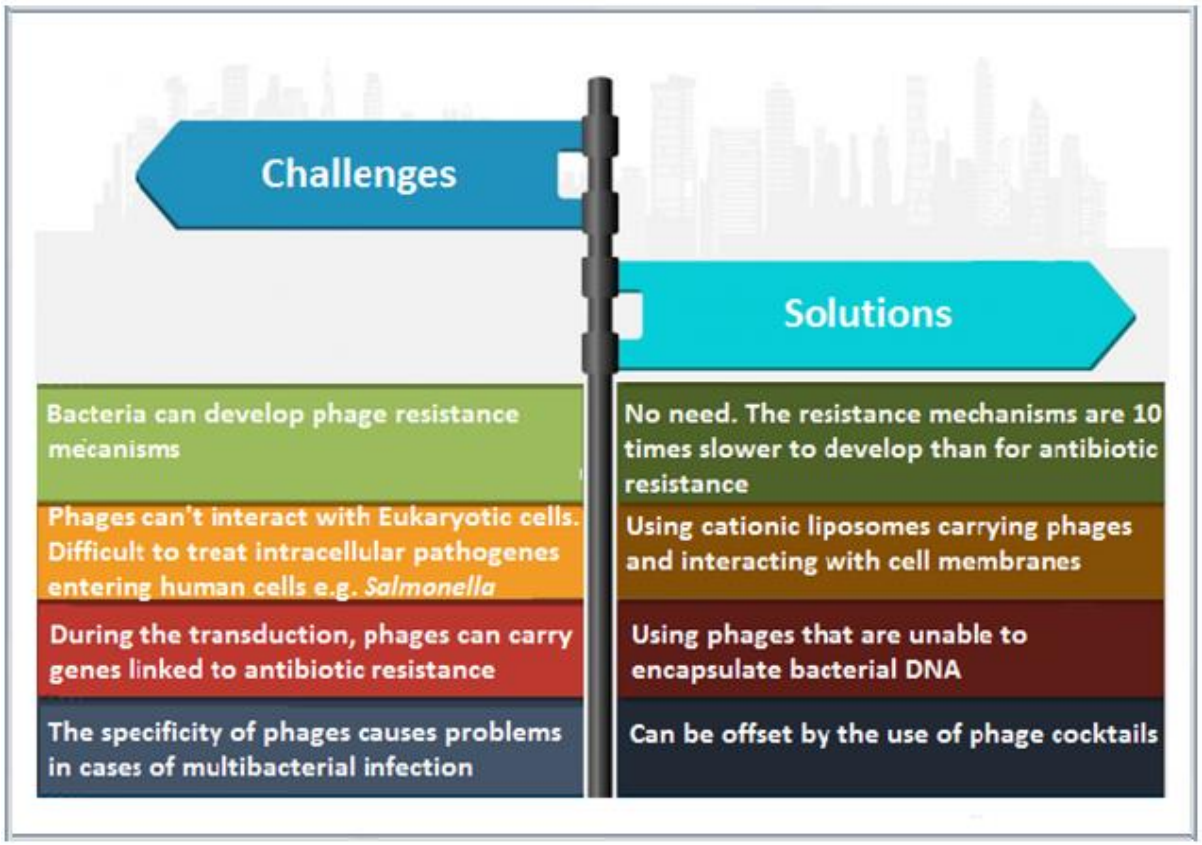

Figure 19. Challenges facing the use of phages therapy and its solutions.

4.2.4. Phage-antibiotic Combination

As mentioned, many strategies can be used to overcome antibiotic resistance. Noteworthy, the combination between phages and antibiotics could be an interesting approach in fighting the global AMR threat.

4.2.4.1. CRISPR-Molecular Tool

The lack of specificity prevailing among the conventional antibiotics, threatening both the bad and the good bacteria, initiated the scientists to look out for new strategies. The originality of

the new design was linked with the ability of re-engineering the genetics of the bacteria in our bodies to make them less pathogenic. The new research is directed towards producing a molecular "conditional-lethality device" able of hunting down the "bad" bacteria with high specificity. These novel classes of antibiotics use a system called "CRISPR", an RNA-guided nuclease, capable to destroy the virulent genes of the bacterial cells. The delivering of the DNA-based agent inside the targeted cells occurs by packaging it into a phage carrier ready to bind to bacteria (**Figure 20**) (Eyre 2014). One of the difficulties in this approach is finding the specific type of phage, which binds to a specific type of targeted bacteria. In addition, the anti-biofilms properties of phages could enhance the penetration of antibiotics inside bacteria without losing activity (Saha et al. 2019).

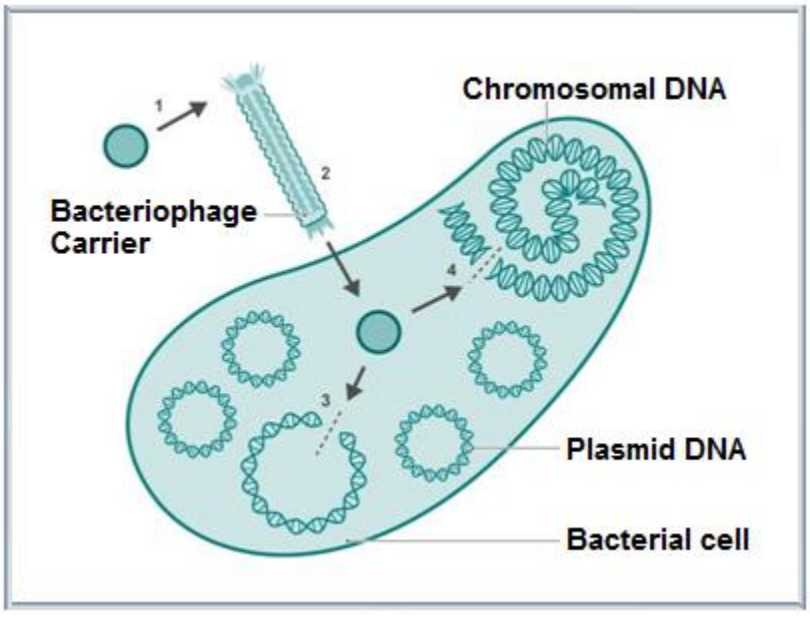

Figure 20. Illustration of the mode of action of the new CRISPR. 1- Specific CRISPR targets specific DNA sequence; 2- The CRISPR is delivered inside the cell by phage carrier; 3- The molecule starts cutting the DNA strand at the targeted location of the gene and cause damages to the plasmid- with or without cell death; 4- Bacterial cell always die after cut of chromosomal DNA (Eyre 2014).

4.3. Vaccines

Since the launch of the first immunization programs in the mid-nineteenth century, vaccination has largely changed the global landscape of infectious diseases, saving countless lives and essentially limiting disease transmissions (O'Neill 2014). Based on this, vaccines may represent an efficient way to overcome problems related to antibiotic resistance and prevent diseases (**Figure 21**) (Jansen et al. 2018).

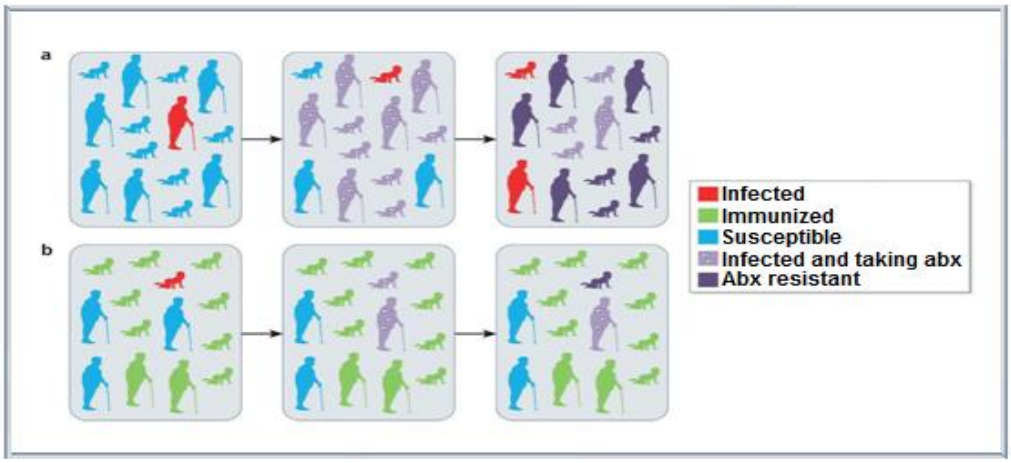

Figure 21. Effect of vaccination on the emergence of pathogens. a- Unvaccinated population, using of antibiotics increase resistance. b- immunized population, limited pathogens dissemination (Jansen et al. 2018).

Vaccines bring hope for those who do not have immunity e.g. immune-compromised people taking chemotherapy. Herd immunity (**Figure 21.1**), is a group of immunized people that have immunity against a pathogen, in this case the frequency of transfer pathogen from this group to another is weak, resulting in a decrease in antibiotics use, and therefore decrease in the emergence of resistance (Jansen et al. 2018).

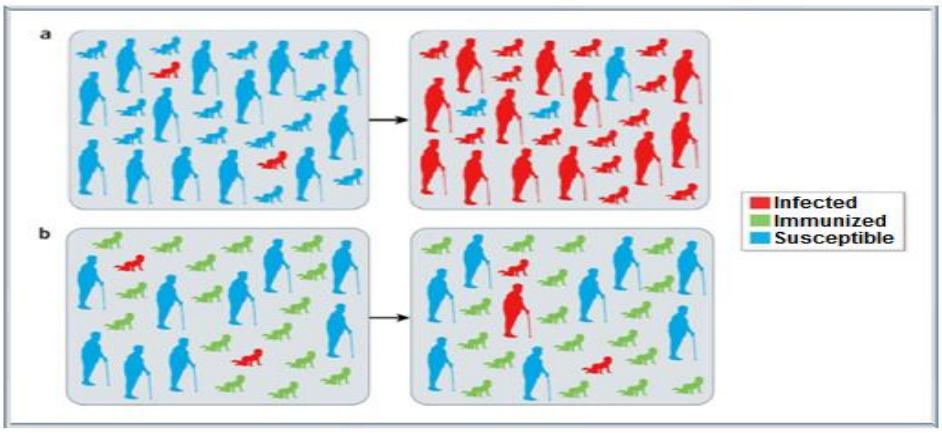

Figure 21 1. Effect of herd immunity. a- Unvaccinated population, pathogen spreads easily through the population. b- vaccinated population, pathogen spreads is limited, protecting the immune-compromised people(Jansen et al. 2018).

Vaccination has shown to be durable without creating significant resistance (**Figure 22**), which is related to the specificity of vaccines by targeting pathogens in many ways, making bacteria need more time to create multiple mutations to display resistance (Bloom et al. 2018).

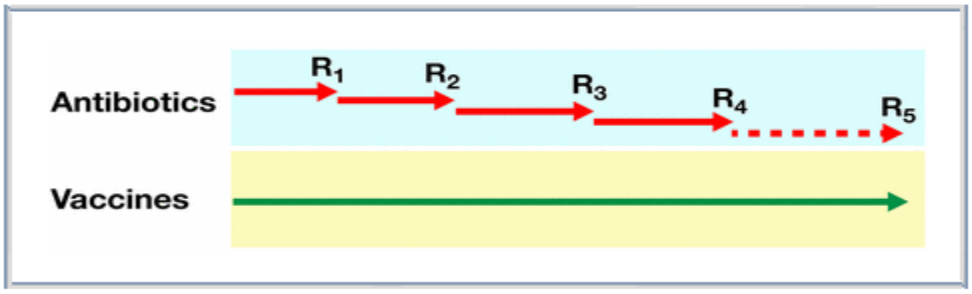

Figure 22. A comparison between antibiotics and vaccines. As shown in the representation, the antibiotics are prone to resistance (R), which limits their uses; therefore, new antibiotics are needed for treatment. However, vaccines can serve longer without causing serious resistance problems (Bloom et al. 2018).

4.3.1. Bacterial Vaccines

The first type of bacterial vaccines (Hib vaccines) was used to prevent the bacterial meningitis disease caused by *Haemophilus influenza* type b. After 20 years of using this vaccine, in the Canadian markets, the disease cases related to Hib were divided by 3. In United Kingdom, all the

children became immunized to Hib. Interestingly, a significant diminution in the resistant strains (β-lactamases strains) of Hib was also observed (Jansen et al. 2018).

4.3.2. Antiviral Vaccination's Impact on Antimicrobial Prescriptions

Viral vaccines, in addition of their role in preventing viral diseases, have shown to be useful in decreasing antibiotic resistance. It is known that the viral infections reduce the immune response therefore expose the body to secondary complications. Most of these complications are linked to bacterial infections, which require large numbers of antibiotics consumption. Antiviral vaccination not only prevents the *influenza* e.g. the seasonal flu (primary infection), but also decreases, in the same time, the frequency of a second infection e.g. pneumonia or acute otitis. In Turkey, the children have shown significant decrease (more than 50%) in acute otitis infection after being vaccinated. Viral vaccines have decreased antibiotic use, especially those used for respiratory diseases most often attributable to viral infection, and thus the development of antibiotic resistance (Jansen et al. 2018). Also antiviral vaccination decreases the case of viral infections that are sometimes misdiagnosed as a bacterial infection therefore treated with antibiotics e.g. 42% of Finnish children suffering from burden of influenza are taking antibiotics ("Vaccines Europe" 2013).

4.3.3. Vaccines of Future

Like antibiotics, the development of vaccines takes several months', even years, in order to respond to some critical criteria. Before the vaccines being commercialized, they pass by development phase (preclinical) and then by clinical phase. The preclinical phase takes place in laboratory then in animals. The human clinical trials (4 phases) start once FDA approved the

results("Sécurité et qualité des vaccins" 2017). In the present time, many vaccines, against pathogens classified as priority to fight, are in the clinical phases (**Table 8**).

Table 8. Examples of 5 Vaccines in clinical trials (Kingwell 2018).

Name	Developer	Pathogen	Status
PF-06425090	Pfizer	*Clostridium difficile*	Phase III
GSK-692342	GlaxoSmithklin/Areas	*Mycobacterium tuberculosis*	Phase II
Group B *Streptococcus* vaccine, maternal immunization	GlaxoSmithkline	Group B *Streptococcus*	Phase II
PF-06290510	Pfizer	*Staphylococcus aureus*	Phase II
JNJ-63871860	Johnson & Johnson	Extra-intestinal pathogenic *Escherichia coli*	Phase II

4.3.4. The Limits of Vaccinations

Despite the uncountable benefits of vaccines, many obstacles stand in their way (Brinth, et al., 2015; M. Dairo, et al., 2016; Yakum, et al., 2015):

- All vaccines have a very low risk of an immediate allergic reaction induced by its components. Their side effects consist of slight fever, pain at the point of inoculation, anorexia, redness and fatigue
- The process of vaccination is not economic for poor countries because of the storage temperature as well as the poor hygiene alongside the bad use of injection equipment. The optimal temperature required for vaccine conservation should be between 2°C and 8°C, which brings a problem of high cost for countries with high temperature. According to a report published by the World Health Organization, out of 16 billion injections made annually worldwide for vaccine purposes, 1,67 billion of injections (about10%) are performed without following SOPs for the safety conditions, which carries significant

risks to the population concerned (AIDS, hepatitis B C) (WHO, 2014; Pépin, et al., 2014).

Since bacteria which can present a pathogenic character are generally commensal and play other roles in the tract, for example *E. coli* (beneficial as well harmful), vaccine would not be the perfect strategy.

4.4. Antimicrobial Peptides (AMPs)

Antimicrobial peptides, also known as host defense peptides (HDPs), are found in all cells and form a part of the innate immune system. These peptides present large structural and functional diversity, displaying a broad spectrum of antimicrobial activity (Gram-positive bacteria, Gram-negative bacteria, enveloped viruses) (Mahlapuu et al. 2016). They are made up of short macromolecules containing 50 amino acids at most, positively charged with an amphiphilic nature. The classifications of AMPs are made based on their posttranslational modification and their global structures (α-helices and β-sheet) (Alvarez-Sieiro et al. 2016; Mahlapuu et al. 2016). The AMPs produced by bacteria allow them to survive in abundant microbiota and provide protection to the host by direct effect on the pathogens or by activating the host immunity (Chiu et al. 2017). The AMPs, according to their synthesis process, can be divided into two families (Hancock et al. 1999):

- Non-ribosomal peptides (NRPs), produced mostly by bacteria.
- Ribosomally and post-translationally modified peptides (RiPPs), produced by all organisms.

4.4.1. Sources of Antimicrobial Peptides and their Current Status

Due to the potent antimicrobial activity expressed by AMPs, the last two decades witnessed a significant progress in these peptides discovery, with an increase in the number of publication related to these molecules (**Fig.23**) (Mahlapuu et al. 2016). Many peptides have been brought to light; some of them were extremely strikingas the DRGN-1antimicrobial peptide extracted from the blood of the Komodo dragon by Monique van Hoek'steam (Chung et al. 2017). DRGN-1 displayed an inhibitory activity against two bacteria species, *S. aureus* and *P. aeruginosa,* and even to antibiotic resistant biofilms forming strains (Chung et al. 2017). Such achievements highlight the great potential of AMPs and stimulate the researchers to go further to reveal more AMPs in both, plants and animals. The human gut microbiota is a frequently studied ecosystem where lives cocktail of microorganisms (parasites, viruses, eukaryotic cells and bacteria). The survival of these organisms and therefore the diversity of the ecosystem are maintained by the production of antimicrobial peptides (Garcia-Gutierrez et al. 2019). The variety of the microorganisms present in the gut microbiota makes it the perfect source of novel antimicrobial peptides (Garcia-Gutierrez et al. 2019), including bacteriocins, which are potent AMP produced by bacteria, mainly lactic acid bacteria (LAB), and endowed with interesting activities against various infectious diseases and food-borne pathogens (Ahmad et al. 2017) (Drider, et al., 2011).

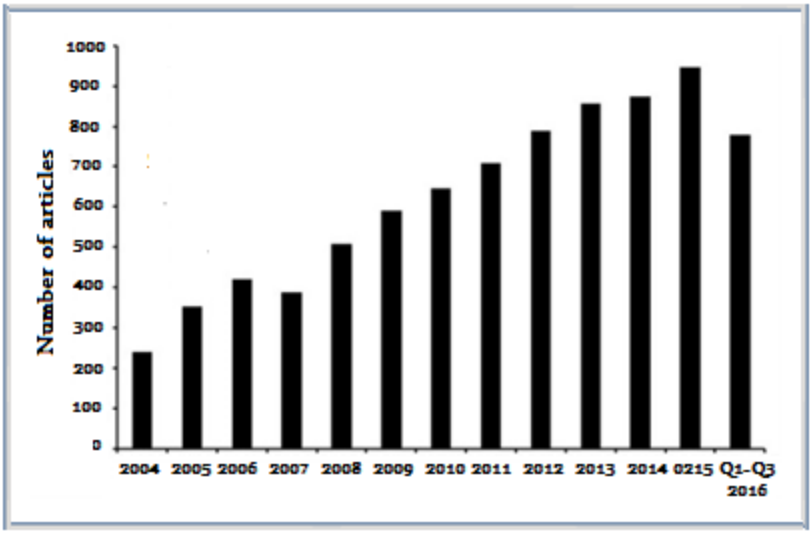

Figure 23. Number of publications on antimicrobial peptides between 2004 and September 2016 (Mahlapuu et al. 2016).

In addition, a chemical synthesis can be applied to obtain antimicrobial peptides including Solid-phase peptide synthesis (SPPS). SPPS was introduced by Bruce Merrifield in 1963, is a strategy for the synthesis of peptides which entails the repetitive coupling of the C-terminus of protected amino acids to an insoluble resin support. The attachment to a solid support via a cleavable linker enables the use of large excesses of reagents and facilitates an effective removal of byproducts to obtain peptides of high purity. Moreover, the method permits the automation of the whole process and is, nowadays, the most commonly used strategy for peptide synthesis (Münzker et al. 2016).

4.4.2. Bacteriocins

In addition to the previous cited strategies to fight against antibiotic resistance, some molecules have antibiotic properties and belong to a large family of antimicrobial peptides, the bacteriocins.

4.4.2.1. Definition

Bacteriocins are a family of proteins or antimicrobial peptides of low molecular weight, naturally synthesized and produced by immunized bacteria against their own peptides (Klaenhammer 1988). These antimicrobial peptides are ribosomally synthesized involving transcription and translation, in addition of the bacteriocin production and immunity genes which are located either on the plasmids or in the chromosomes (Drider et al. 2006; Franz et al. 2007).

These molecules play a role in the competition between bacterial species within an ecological niche. Usually, bacteriocins have narrow activity spectrum and are characterized by their diversity in terms of their biochemical properties, their molecular weight, their spectrum of inhibition and their mode of action (Klaenhammer 1988). In contrary to antibiotics, having no-specific activity, bacteriocins possess specific activity. Besides, bacteriocins, either produced by Gram-positive bacteria or Gram-negative bacteria, are synthesized during the primary growth phase while antibiotics are secondary metabolites (Zacharof et al. 2012; Jack et al. 1995). It has been suggested that most of the bacteria and *Archaea* (30 to 99%) have the ability to produce at least one bacteriocin (Klaenhammer 1988; Cotter et al. 2005).

4.4.2.2. Bacteriocins produced by Gram-positive Bacteria:

Bacteriocins from Gram-positive bacteria are mostly produced by LAB, which are non-sporulating, catalase negative, spherical (cocci) or rod–shaped (bacilli) bacteria, producing lactic acid as a major metabolic compound derived from carbohydrates fermentation (O'Sullivan et al. 2002). These bacteria are usually found in different ecological niches including milk products (Pandit et al. 2012), soil (de Melo Pereira et al. 2020) and water (Minervini et al. 2019). LAB species can be found in many food matrixes like plants (Yu et al. 2020), cherry pulp (de Melo

Pereira et al. 2020) and Napa cabbage (Miller et al. 2019). Amongst the LAB genera (**Figure 24**), the bacteriocins producer strains belong mainly to *Leuconostoc*, *Lactococcus*, *Enterococcus*, *Pediococcus*, *Lactobacillus*, *Carnobacterium*, and *Streptococcus* (Rea et al. 2011).

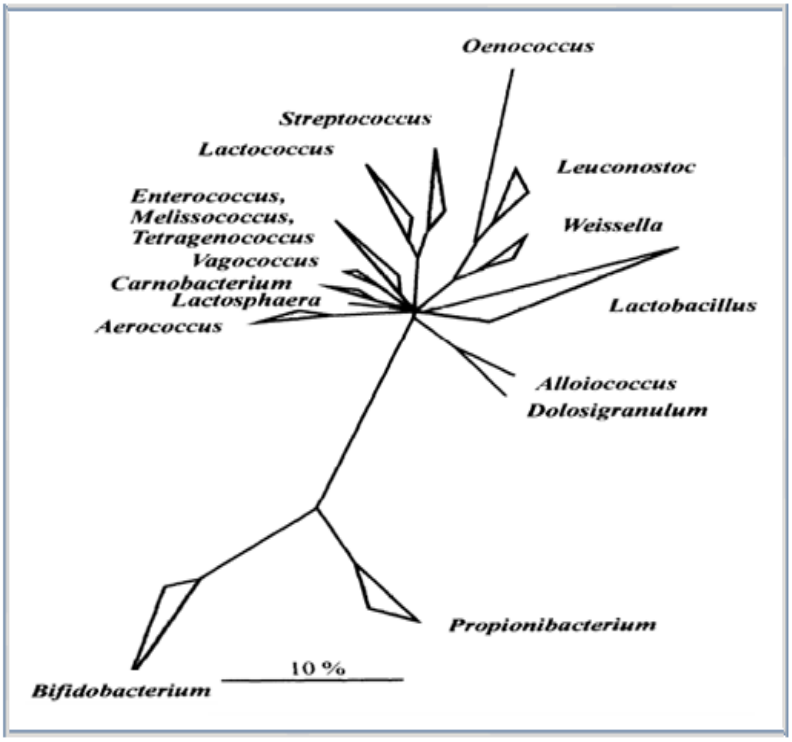

Figure 24. Major phylogenetic groups of LAB (the bar shows 10% expected sequence divergence) (Rea et al. 2011).

An old classification model of bacteriocins suggested by Klaenhammer in 1988, based on the structure and the mode of actions, includes 5 classes of antimicrobial peptides (**Table 9**) (Klaenhammer 1988). In 2005, Cotter et al. (Cotter et al. 2005) have suggested a revision of this classification. The authors proposed a classification based only on two distinct classes, which are the lanthionines (lantibiotics) and the non-lanthionines (unmodified bacteriocins). Remarkably, the large, heatlabile murein hydrolases have been separated from bacteriocins and called then

"bacteriolysins", because of their distinct mode of action, as they catalyze cell wall hydrolysis (**Table 10**) (Cotter et al. 2005). Another classificationof LAB bacteriocins, proposed by Drider et al (2006) included three classes of bacteriocins on the basis of their biochemical and genetic properties (**Table 11**) (Drider et al. 2006).

Table 9. Classification of bacteriocins according to Klaenhammer (1993).

Class	Description	Characteristics
Classe I	Contains post-translational modifications	Small peptides <5 kDa; contain specific amino acids (lanthionine and dehydrated residues).
Classe II	No post-translational modification	Small peptides <10 kDa; thermostable; pore formation in the membranes
Classe III	No post-translational modification	Proteins with molecular weight> 30 kDa; enzymatic activity
Classe IV	Complex proteins	Proteins attached to sugars or Lipids

Table 10. Classification scheme for bacteriocins suggested by Cotter et al. (2005) (Cotter et al. 2005).

Classification	Remarks/suggestions	Examples
Class I		
Lanthionine-containing bacteriocins/lantibiotics	Both single- and two-peptide lantibiotics are included; more than 11 subclasses have been proposed	Single-peptide: nisin; two-peptide: lacticin 3147, cytolysin
Class II		
Non-lanthionine-containing bacteriocins	Heterogeneous class of small peptides; contains pediocin-like (subclass a bacteriocins), two-peptide (subclass b bacteriocins), cyclic (subclass c; formerly class V), non-pediocin single linear peptides (subclass d)	Class IIa: pediocin PA1; class IIb: lactacin F; class IIc: enterocin AS48; class IId: lactococcin A
Bacreiolysins		
Non-bacteriocin lytic proteins	Large, heat-labile proteins, usually murein hydrolases	Lysostaphin

Table 11. Classification of LAB Bacteriocins according to Drider et al. (Drider et al. 2006).

Classification	Characteristics	Subcategory
Class I	Lantibiotics (containing lanthionine and -lanthionine)	Type A (elongated molecules; molecular mass, <4 kDa)

		Type B (globular molecules; molecular mass, 1.8 to 2.1 kDa)
Class II	Non modified heat-stable bacteriocins containing peptides with molecular masses of <10 kDa	Subclass IIa (antilisterialpediocin-like bacteriocins) Subclass IIb (two-peptide bacteriocins) Subclass IIc (other peptide bacteriocins)
Class III	Protein bacteriocins with molecular masses of >30 KDa	

The most known bacteriocin is "Nisin", which is used as food additive, E234 and has also potential medical applications (Shin, et al., 2016). Nisin was discovered in 1928 by Rogers and Whittier, and is produced by strains of *Lactococcus lactis subsp. Lactis* (Paul Ross, et al., 2002). This bacteriocin contains lanthionine and methyllanthionine and thus belongs to lantibiotics family (De Vuyst, et al., 1992). This group of bacteriocins is post-translationally modified, leading to unusual amino acids (Shin, et al., 2016), which might be responsible for several functional properties like heat stability, activity at low pH, acid tolerance and a unique bactericidal mode of action (De Arauz, et al., 2009; De Vuyst, et al., 1992). Nisin has more than one variant e.g. nisin A and nisin Z, these two variants which differ by the substitution of a single amino acid at position 27 (histidine in nisin A and asparagine in nisin Z) (De Vos, et al., 1993). In 1988, the use of nisin as a food preservative was approved by the FDA and it was generally regarded as safe (GRAS) peptide (Paul Ross, et al., 2002). Since then, the use of nisin was extended to human and veterinary applications. Nisin exerts a potent antimicrobial activity against wide spectrum of Gram-positive bacteria (e.g. *Pediococcus,* Enterococci, *Staphylococcus*, *Streptococcus, Lactococcus, Lactobacillus, Micrococcus, Listeria*) (Shin, et al., 2016) and Gram-negative bacteria and also the outgrowth of Gram-positive spores of *Bacilli* and *Clostridia* (De Arauz, et al., 2009). Nisin is a potent antimicrobial peptide to control the outgrowth of both vegetative cells and spores of *C. perfringens* thereby ensuring food safety (Garde, et al., 2014).

4.4.2.3. Modes of action of bacteriocins

The modes of action of bacteriocins are exerted through a pore-forming mechanism. However, some bacteriocins can act also by different ways, as lantibiotics which can interact with lipid II disturbing the cell wall of sensitive bacteria (Drider, et al., 2011). Bacteriocins exert mainly their lethal action through interaction with specific receptors, located at the surface of the targeted cell (**Figure 25**) (Hammami, et al., 2012). Based on the types of bacteriocins, several such receptors have been defined as docking molecules, e.g. undecaprenyl pyrophosphate phosphatase (UppP), lipid II, maltose ABC transporter and mannose-specific phospholipase transferase system (man-TPS) (Kumariya, et al., 2019).The binding process of the bacteriocins to the target surface (through specific or non-specific receptors) is critical for subsequent insertion in the cytoplasmic membrane to form ion selective pores. Different models of pore formation have been described, class I bacteriocins (lantibiotics) forms pores in “wedge” or “barrel-stave” model, whereas class II bacteriocins forms pores in “carpet” or “barrel stave” model (Kumariya, et al., 2019; Moll, et al., 1999).

Cationic bacteriocins can also interact directly with susceptible bacteria due to the anionic lipids properties of the cell membrane made of cardiolipins (CL) and phosphatidylglycerol (PG) and the anionic components of the bacterial cell envelope made of lipoteichoic acid (LTA) and lipopolysaccharide (LPS). Electrostatic interactions occur between the negatively charged cell membranes and the positively charged amino acids of bacteriocins, whereas the hydrophobic surfaces can easily cross the lipid bilayer, resulting in the permeabilization of the cell membrane (Kumariya, et al., 2019).

The death of sensitive bacteria induced by bacteriocins may result from the cell lysis, originating from the inhibition of cell wall synthesis, the massive release of ATP, the leakage of

K^+ and electrolytes, the dissipation of the proton motive force and inhibition of protein and nucleic acid biosynthesis (Schein, et al., 1978; Lusk, et al., 1972).

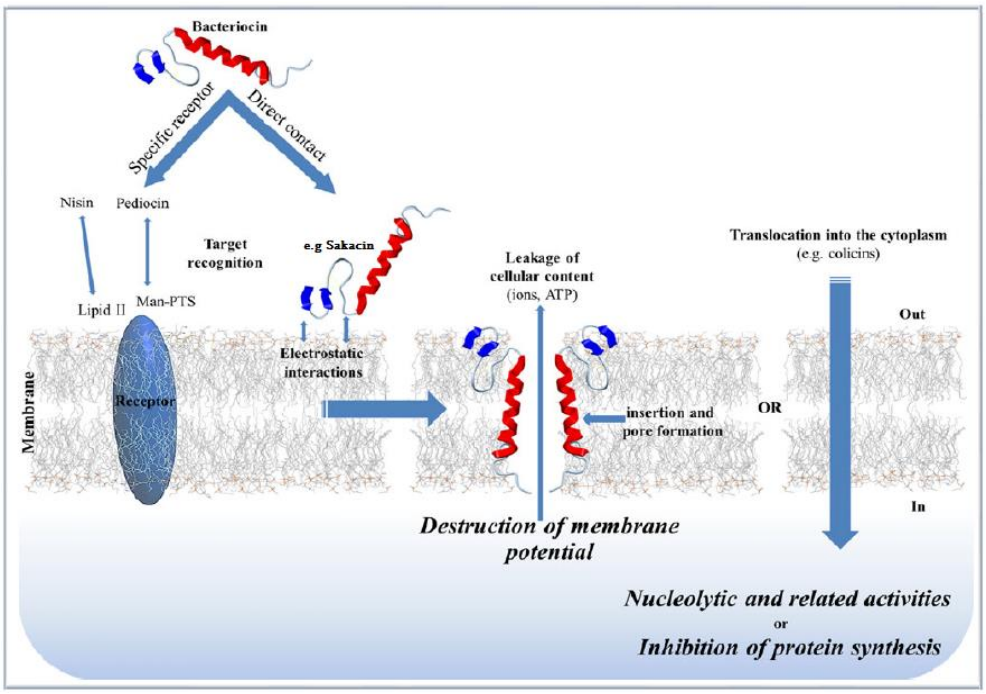

Figure 25. Mode of action of bacteriocins (Hammami, et al., 2012).

4.4.2.4. The Potent Antimicrobial Activities of Bacteriocins expand their Applications

Bacteriocins are shown to be able to prevent biofilm formation of microorganisms by inhibiting the quorum sensing of these intruders. Thus, the natural levels Sub-minimal inhibitory concentration (Sub-MIC) of bacteriocins, produced by bacteriocin-producers, lead to repel invading bacteria, thereby it was expected that the primary functions of bacteriocins is to be as signal and repel rather than kill. Further functions of bacteriocins can be revealed at higher concentrations, minimum bactericidal concentration (MBC), than the natural levels, which among the antimicrobial activity as a result of membrane disruptors, pore forming or by inhibiting cell division process (Algburi, et al., 2017; Chikindas, et al., 2018). In addition to

antimicrobial activity, some bacteriocins (subtilosin A) have anti-viral activity by perturbing the late stages of viruses'replication (Quintana, et al., 2014). Numerous studies revealed that bacteriocins can act synergistically with bacteriophages (Rubio, et al., 2015) and various food grade substances (curcumin, poly-lysine, or zinc lactate) (Amrouche, et al., 2010). Bacteriocins can be used to control post-operative infections and as a treatment for urogenital, gastrointestinal, respiratory tract and other infections (**Table 12**). Due to the selectivity of bacteriocins against cancer cells, researches are focusing now on the ability of using bacteriocins as novel anti-cancer agents (Kaur, et al., 2015). One of the most emerging beneficial effects of bacteriocins is aimed at the functional aspects of foods, where the consumption of bacteriocin-producers modulates the intestinal health, regulates the gastrointestinal microbiota and reduces the risk of some foodborne illness (Chikindas, et al., 2018). One such example is the antimicrobial potential of *E. faecalis* 14, isolated from the meconium of newborns (Al Atya, et al., 2015), which displayed a potent anti-*C. perfringens* activity associated with the production of enterocin14, EntDD14,which is a leaderless two-peptide bacteriocins.

Table 12. Spectrum of Antimicrobial Activity of Bacteriocins

Bacteriocin	**Class**	**Producer organism**	**Examples of target organsims**	***Example of anti-infective effects***	***In vitro/vivo***	**Reference**
Nisin A	Ia	*L. lactis* s-ubsp. lactis	*Enterococcus Leuconostoc ListeriaClostridium*	Multidrug-resistant strain	*In vitro*	(Piper, Draper, Cotter, & Ross RP, 2009)
Nisin F	Ia	*L. lactis* F10	*S. aureus S. carnosus L. curvatus*	Pneumonia	*In vitro*	(De Kwaadsteniet, Doeschate, & Dicks, 2009)
Nisin U	I	*S. uberis* A TCC 27958	*S.pyogenes S. uberis S. agalactiae*	Bovine mastitis	*In vitro*	(Wirawan, Klesse, Jack, & Tagg, 2006)
Nisin Z	Ia	*L. lactis* subs-p (*S. lactis*)	*Enterococcus Lactobacillus Clostridium*	Oral candidiasis	*In vitro*	(Akerey, Le-Lay, Fliss, Subirade, & Rouabhia, 2009)
DD14	IIb	*E. faecalis 14*	*C. perfringens*	Gastrointestinal disease	*In vitro*	(L Caly, et al., 2017)
E50-52	IIa	*E. faecium NRRL B-*	*Y. pseudotuberculosis Y. enterocolitica*	Tuberculosis	*In vitro/vivo*	(Sosunov, et al., 2007)

		30746	*S. epidermidis*			
Enterocin 96	II	*E. faecalis* WHE96	*E. faecalis, L. lactis, L. monocytogenes*	Cutaneous infections	*In vitro*	(Izquiedo, Wagner, Marchioni, Werner, & Ennahar, 2009)
Microcin J25	-	*E. coli*	*Enterobacteriaceae, Salmonella*	Gastrointestinal disease	*In vivo*	(Lopez, Vincent, Zenoff, Salomon, & Farias, 2007)

During the last decades more than three thousand antimicrobial peptides have been discovered (Chen and Lu 2020). Only a few of them, produced by Gram-positive soil bacteria, have been approved by Food and Drug Administration (FDA). Most of the approved ones have been used for topical medications, although some of them have been delivered inside the body to treat some diseases (Chen and Lu 2020). Yet, AMPs can display some limits as drugs, such instability and poor pharmacokinetics (Makowski et al. 2019). However, the combination of these antimicrobial peptides with carrier nano-objects (including nanoparticles) can ameliorate their properties e.g. improve their delivery, enhance their half-life and decrease their dosage therefore the eventual toxicity (Makowski et al. 2019 ; Dicks et al. 2017)

4.5. Nano-Objects

The nano-objects (also called nanostructured materials) are materials of which at least one of their 3 dimensions is less than 100 nm, which regroups nano-plates, nano-fibers, nano-particles (the 3 external dimensions are less than 100 nm) and other complex systems coupled with organic molecules used in many fields of application (Maria, 2015). Decades of research have revealed the possibility of synthesizing nano-objects using different materials such as transition metals (Ghosh et al. 2019), liposomes (Yakavets et al. 2019), oxides (Mirza-Aghayan et al. 2019) and of course biodegradable polymers like alginate (Thomas et al. 2020). Nowadays, several researches are focusing on the application of the nanosized natural polymers as a drug carrier. These are described in details in the next section.

5. Nano-antibiotics

5.1. Definition

Nanoparticle is an assembly of atoms ranging from a few dozen, for a 1 nm nanoparticle, to several million, for a 100 nm nanoparticle (Maria, 2015). The smaller is the particle, the greater the ratio of the number of atoms constitute its surface and those present in its volume. This ratio varies inversely proportional to the diameter of the particle (**Figure 26**). On the scale of nanoparticles (NPs), these particles possess unique physical and pharmacokinetic properties compared to those of micro- and macro scale particles (Zarschler et al. 2016). Due to these unique properties of NPs, many works have been done leading to a solid knowledge in this field, which appeared in the ability of synthesizing complex NPs with different shapes such as stars NPs (Minati et al. 2014) and core-shell NPs (Langlois et al. 2015).

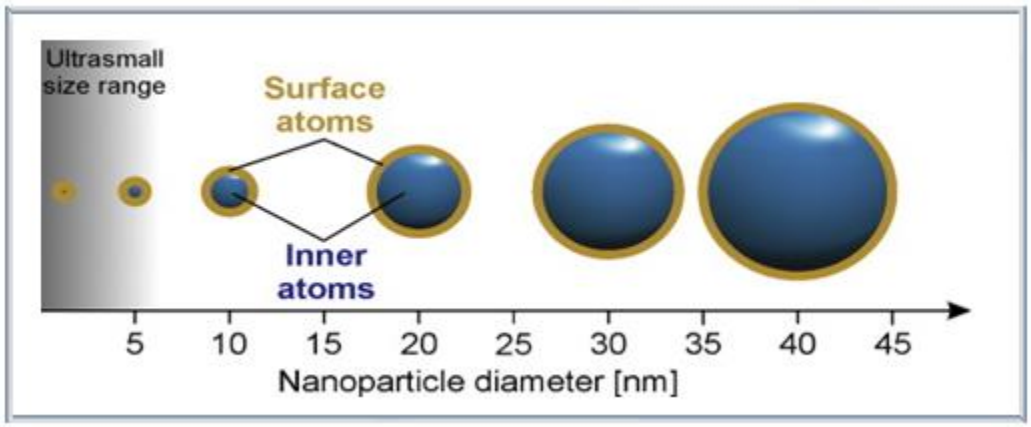

Figure 26. The number of the surface atoms is inversely proportional to the nanoparticle diameter (Zarschler et al. 2016).

5.2. A Brief History of Nano-Objects

The first empirical use of the nanoparticles by humanity dated back to Mayan times. In 800 B.C., the Mayan used a blue pigment (**Figure 27A**), called Maya Blue (MB), in the village of ChichénItzá, to decorate statues, pottery and painting which showed to be resistant to many acids (including HCl and HNO_3), organic solvents and other environmental conditions (Giustetto et al. 2005). In 1931, a group of researchers have rediscovered the presence of MB traces on the Warriors' temple (H. Morris, et al., 1931). Twenty years later, the X-ray diffraction analysis (XRD) proved the presence of traces of MB in fibrous clay (clay palygorskite) (Gettens 1962). The integration of the dye molecules inside the pores of the clay provide a stable complex capable of maintaining its color for a very long time, which is clear in the Mayan's frescoes that show a preserved blue although thousands of years have passed.

The Roman civilization also had a share in using nanoparticles in its crafts. The most famous example is the Lycurgus cup (4^{th} century AD) also known as *cage cups* or *diatreta*. Analytical transmission electron microscopy showed the presence of particles of metals ranging between 50-100 nm. X-ray analysis revealed that the glass is a mix of three types of nanoparticles i.e. gold, silver and copper (Freestone et al. 2007). What is surprising in this masterpiece (Lycurgus cup), the color of the glass can change from green to red depending on whether the light is traversing or reflected (**Figure 27B**).

Figure 27. A-Maya Blue; B- Lycurgus cup (4th century AD) lit from the front-left cup (in reflected), lit from the back-right cup (in transmitted).

Few centuries later (9th-17th centuries), the luster of glaze decorations on ceramics used in the Islamic world, and later in Europe, contained nanoparticles of silver or copper or other metallic nanoparticles (Sciau, 2012). For example, the colored glass windows of European cathedrals contain nanoparticles of metal oxides and gold nanoparticles, which were also used as photocatalytic air purifiers. This type of stained glass is visible in the cathedral of Notre-Dame in Paris (R. Allcock, 2008; Allen, 2012).

In the 10th century, another form of nanotechnology was found in steels improperly named Damascus steels. It is believed that this steel, called *wootz*, was produced in India. The particularity of these steels is their composition, made up of carbon nanotubes and cementite nanowires, giving them an exceptional quality (Reibold et al. 2006). Legend has it that during the crusader times, the blades handled by the Syrians have damaged the Crusader armor. Many attempts were unsuccessful to reforge this unique steel: too hot, it crumles and too cold, it breaks. Also, studies showed that the extraordinary abilities of these blades decrease in case

forging it for a long time. Unfortunately, the great secret behind the steel of *wootz* is now lost although the many efforts which were made by the amateurs to rediscover it (Durand-Charre 2007; Reibold et al. 2006).

In 1857, Michael Faraday, one of the Scientifics who enabled the nanotechnology in the modern area, has discovered the colloidal "ruby" gold, showing a scientific description of the colors change of the nanostructured gold exposed to certain lighting conditions (Faraday 1857; Axelle 2019).

A century later, Richard Feynman gave the first conference on technology on atomic scale "There is plenty of room at the bottom" at Caltech 1959.

In 1974, the term "nanotechnology" was invented by Tokyo University Professor Norio Taniguchi to describe the precision machining used in semiconductors (Taniguchi 1974; Axelle 2019).

In 1987, Heinrich Rohrer and Gerd Binnig of the IBM laboratory have invented the scanning tunneling microscope (STM), allowing the observation and the manipulation of nano-objects (Binning, et al., 1987). This invention helped scientists to "see" individual atoms for the first time and allowed Harold Kroto, Robert Curl and Richard Smalley to discover a sphere of 60 carbon atoms, the fullerene (Goodarzi, et al., 2017). In 1989, IBM laboratory researchers have manipulated 35 individual xenon atoms to write the three letters "IBM" on a nickel substrate. The picture (**Figure 28**), taken by STM, has entered the history (Eigler, et al., 1990).

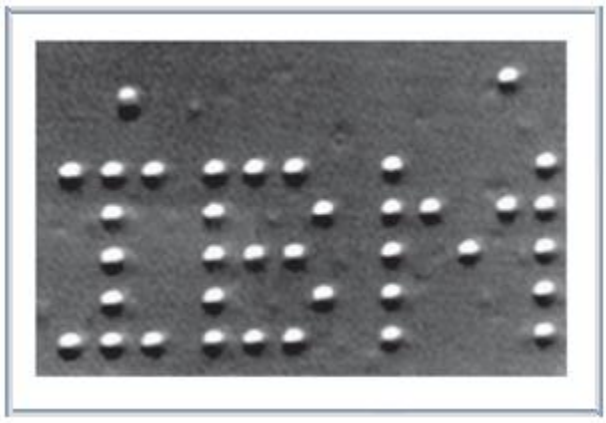

Figure 28. Letters "IBM" wrote by xenon atoms shows the capability of the IBM STM in 1989 (Eigler, et al., 1990).

In 2000, the demand of nanotechnology products started increasing to consumer market, including transparent sunscreens, antibacterial socks, consisting of nano-silver material, and therapeutic cosmetics. In the same year, the president Bill Clinton initiated the National Nanotechnology Initiative (NNI)), with a budget about 497 million dollars to promote the companies of the USA in nanotechnology field.

The widespread availability of the nanotechnology was accompanied by many questions on its environmental toxicity that pushed the WHO, in 2013, to impose precautionary data principle due to the insufficient data on its toxicity. However, labeling of the nanoparticles used in the products has been compulsory since 2013 in France (Anses, 2014).

5.3. Development of Nano-Objects and Drug Delivery

Nanoparticles represent a promising strategy in health domain with their particular properties as targeted delivery of therapeutic agent or in the field of medical imaging e.g. using the markers for diagnostic imaging tissues. The specificity of these NPs as drug delivery

prevents side effects and reduces dosage and dosage frequency by decreasing the degradation of the active ingredient (AI) and by increasing the intracellular permeability (Patra et al. 2018).

5.4. Types and Applications of Various Nanoparticles used in the Field of Veterinary Medicine

Nano materials showed their ability in solving various issues related to animal nutrition, animal breeding, animal health and common livestock production, treatment, disease diagnosis and drug delivery (Bai, et al., 2018). Different nano-systems are available, including carbon nanotubes, fullerenes, metallic nanoparticles (gold NPs, silver NPs, iron oxide NPs and zinc oxide NPs), liposomes and polymeric nanoparticles (starch NPs, chitosan NPs and alginate NPs) (**Figure 29**) (Sarei, et al., 2013; Youssef, et al., 2019; Bai, et al., 2018).

Figure 29. Types of various nanoparticles used in veterinary medicine.

5.4.1. Different Types of Nanoparticles

The previous definition of nanoparticles only integrates a notion of size and not types of nanoparticles. Although the large number of criteria by which NPs can be classified, the notion of biodegradability grouped these vectors into two main families (Fröhlich 2013):

- Degradable nanoparticles which do not have the risk of accumulation in the organs (De Fazio 2018).
- Non-degradable nanoparticles which are often used in medical imaging due to their optical properties (Nune et al. 2009).

5.4.1.1. Non-Degradable Nanoparticles

The nanometric size of these materials provides them with new properties and a wide range of applications. Various shapes of rigid NPs can be found in this group e.g. needles, tubes, ellipses and spheres.

A. Quantum Dots

These structures, known as nano-layers, have one dimension in the nano range while the other dimensions are large. These materials were discovered in colloidal solutions and in a glass matrix by Louis E Brus in 1980 (Ostiguy et al. 2010). The combination of the elements of groups III and V or groups II and IV of the Mendeleev periodic table is the origin of these materials e.g. indium / phosphorus or indium / arsenic, cadmium / tellurium or cadmium / selenium. Their number of atoms varies from 1,000 to 100,000 and their association has a diameter which can range from 2 to 10 nm (their dimensions smaller than the exciton Bohr radius). Due to their small

size and their quantum confinement effect, they display unique physicochemical properties which make them useful for many applications e.g. photo-thermal therapy, imaging, biochips, targeted surgery, drug delivery and pharmaceutical analysis (**Figure 30**). Also, they can be used as cellular probes during biological labeling, the cells are carried with quantum dots, re-injected inside patient, then traced by microscopy (Yao et al. 2018).

Figure 30. Applications of Quantum Dots (QDs) (Yao et al. 2018).

B. Fullerenes

Fullerenes are carbon structures synthesized for the first time by Harold Kroto in 1985 (Kroto 1985). These spherical cages consist of carbon atoms attached to 3 other carbon atoms in sp^2 hybridization (**Figure 31**). The different forms of fullerenes were obtained by vaporizing graphite in a helium enclosure using laser-vaporization machine and by measuring the mass spectrum of carbon. Although different structures have been synthesized e.g. a nanotube, a ring,

an ellipsoid and a sphere, this last was the most studied form. The spherical form composed of 60 carbon atoms with high stability.

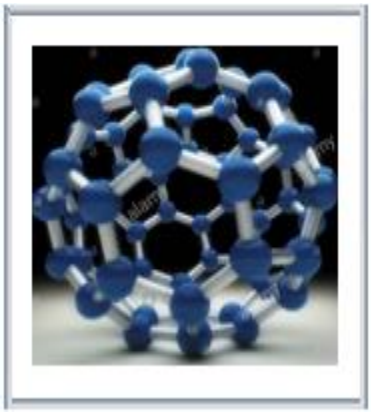

Figure 31. Schematic Representation of a Fullerene C_{60}.

Since fullerenes are being hollow atomic assemblies (**Fig.31**) with size similar to several active molecules, we can introduce different substances e.g. metallic ones (Cs, Na, K and Ni) used for medical imaging or filled them with active molecules essentials for medical applications such as therapeutic filed in case of cancer and AIDS (Kazemzadeh et al. 2019 ; Liu et al. 2018).

C. Metallic Nanoparticles

Most of metals exhibit interesting properties on the scale of nanometers compared to those of micrometric dimensions, for example, gold NPs are particularly studied as optical markers and showed a resonance spectrum in the visible range, which depends on the form and the size of the NPs (Ou, et al., 2019). Due to their several properties, they have broad spectrum of applications, especially the use of gold nano-rods (Au NRs) as photothermal therapy (PTT) of different cancers (Turcheniuk et al. 2015). Silver nanoparticles are produced in large quantities and showed to be promising antimicrobial agents mainly for inhibition of bacterial biofilm (McEvoy et al. 2020). Iron oxide nanoparticles can be used as a cell-tracking system for the detection and

distribution of intra-lesional antigens in sheep (Bai, et al., 2018). Zinc oxide nanoparticles are applied as antimicrobial agents to treat microbial infections including *Klebsiella pneumonia, S. epidermis, S. agalactiae* and *E. coli* (Bai, et al., 2018).

5.4.1.1.1. Toxicity of Non-Degradable Nanoparticles

The marriage of nano-science with biology has greatly improved the biological techniques, greatly facilitating overcoming difficulties in the biomedical fields. However, some of nanomaterials (quantum dots, metallic nanoparticles and fullerene) that are used, especially in medical filed, are toxic.

Toxicological studies revealed that quantum dots e.g. CdSe and CdSe/ZnS, pose threats to various systems (soft tissue, human fibroblast) due to the release of metal ions e.g cadmium ions (Mo et al. 2017). Quantum dots' toxicity has impact on reactive oxygen species (ROS) (Wang et al. 2018), causing ROS-related DNA damage, oxidative stress and toxicity in HeLa cell line (Mu et al. 2017). ZnS quantum dots showed significant toxicity on male reproduction system of mice (Amiri et al. 2016).

Also, the toxicological studies of metallic nanoparticles highlighted that metal oxide nanoparticles, e.g. lead oxide (PbO) and copper oxide (CuO), cause serious damage in human fibroblasts cells (Bushueva et al. 2019). Zinc oxide (ZnO), copper oxide and titanium dioxide (TiO_2) display toxic effects on cell viability of colorectal adeno-carcinoma cells (HT29) (Schneider, Westermann et al. 2017). Gold nanoparticles related with oxidative stress-related cytotoxicity causing DNA damage and cell death (Jia et al. 2017). Both gold nanoparticles and silver nanoparticles have cytotoxicity effect on HT29 viability (Schneider et al. 2017).

As for fullerene and its derivatives, limited information is available on its toxicity. However, these molecules have been shown to be toxic to benthic organisms (Ponte et al. 2019). Although fullerene has antioxidant capacity (e.g. in dermatological and cosmetic applications), it can display a wide range of activities resulting in cell dysfunction or cell death (Mousavi et al. 2017).

5.4.1.2. Degradable Nanoparticles

Degradable NPs are generally composed of sugars, lipids and biodegradable polymers (Alginate) and most of them can be dispersed in aqueous solutions. Depending on the type of polymer used and the pH of the solution, the surface charge of the NPs can be charged (positively or negatively). Optimization of these parameters make possible to control the aggregation of the particles (charged particles repelling themselves) (Cortial 2016). These polymer nanoparticles are generally used in three major domains (Pichot, Labarre, & Daniel, 2014):

- *In vitro* diagnostics and biological analysis
- Medical imaging (*in vivo* diagnosis)
- Pharmaceutical formulations

Liposomes

Liposomes are sphere-shaped vesicles consisting of lipid bilayer membrane, constituting a hydrophobic zone used to deliver hydrophobic molecules and having hydrophilic core suitable to deliver hydrophilic molecules (**Figure 32**). Most of the liposomes are formed from cholesterol and phospholipid molecules and their size ranges between 30 nm to several micrometers (Mozafari et al. 2008). In the biomedical field, these structures are able to carry active

therapeutic components (Daraee et al. 2016), the amount of the cholesterol determines their rigidity. Due to the non-toxicity and biocomptability of liposomes, they have been widely used in biology, biochemistry and medicine as a carrier of active therapeutic components. However, these particles display low encapsulation capacity, early release of the active ingredients and moderate stability (Sercombe et al. 2015; Pinto-Alphandary et al. 2000).

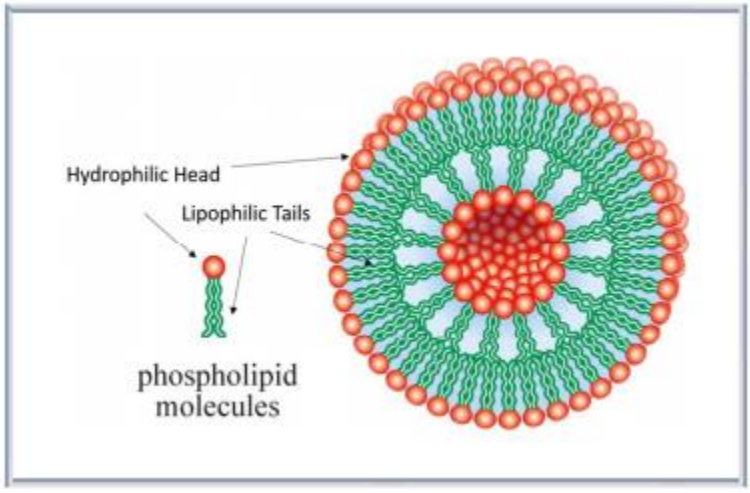

Figure 32. Schematic representation of liposome structure (Yoon Hwang et al. 2016).

Polymeric Nanoparticles

In 1979, the first polymeric NPs were developed on the basis of poly-alkyl-cyanoacrylates (Couvreur et al. 1979). Since then, research on these structures has not stopped evolving and has used new polymers, in particular naturally-derived polysaccharides (alginate based nanoparticles) used as drug delivery systems (Venkatesan et al. 2017).

These polymeric nanoparticles are biodegradable and biologically compatible, and are permitted to be used in the health sector by global authorities (European Pharmacopoeia and FDA) (Truchon 2008). Besides to not producing toxic metabolites after their degradation in

organism, their unique stability gives them particular properties for use in the biomedical field(Shariatinia 2019).

A. Starch Nanoparticles

Starch is one of the most available, abundant, biopolymer and is produced by most green plants through the process of photosynthesis and used in the form of micro-granules as energy storage. Starch micro-granules can be broken down into nano-size by both physical i.e. high-energy ball milling (H. Lin et al. 2016) and chemical methods i.e. acid hydrolysis (Bhardwaj et al. 2017). These nanoparticles have no cytotoxic effect on human health and are widely used in food and non-food industries (cosmetics, medicines). In general, nanotechnology is achieving the most important advances in biomedical application, in particular new drug delivery usage in which starch nanoparticles showed a promising performance as a drug carrier (Sandhu et al. 2017) and as enhancerof antimicrobial activity (Ismail et al. 2017).

B. Chitosan Nanoparticles

Chitosan is a linear polysaccharide obtained by deacetylation of chitin. It is a white, inelastic and hard biopolymer (Shahidi et al. 1991). Chitosan is widely used in the food and bioengineering industries to encapsulate active nutritional components, in agriculture as a plant defense, and as active biopesticieds and as a targeted drug delivery. Chitosan is a polycationic molecule with various properties such as biocompatibility, biodegradation, bioactivity, antimicrobial safety for human health. Chitosan nanoparticles can be synthesized by 5 different methods (**Figure 33**) (Divya et al. 2018).

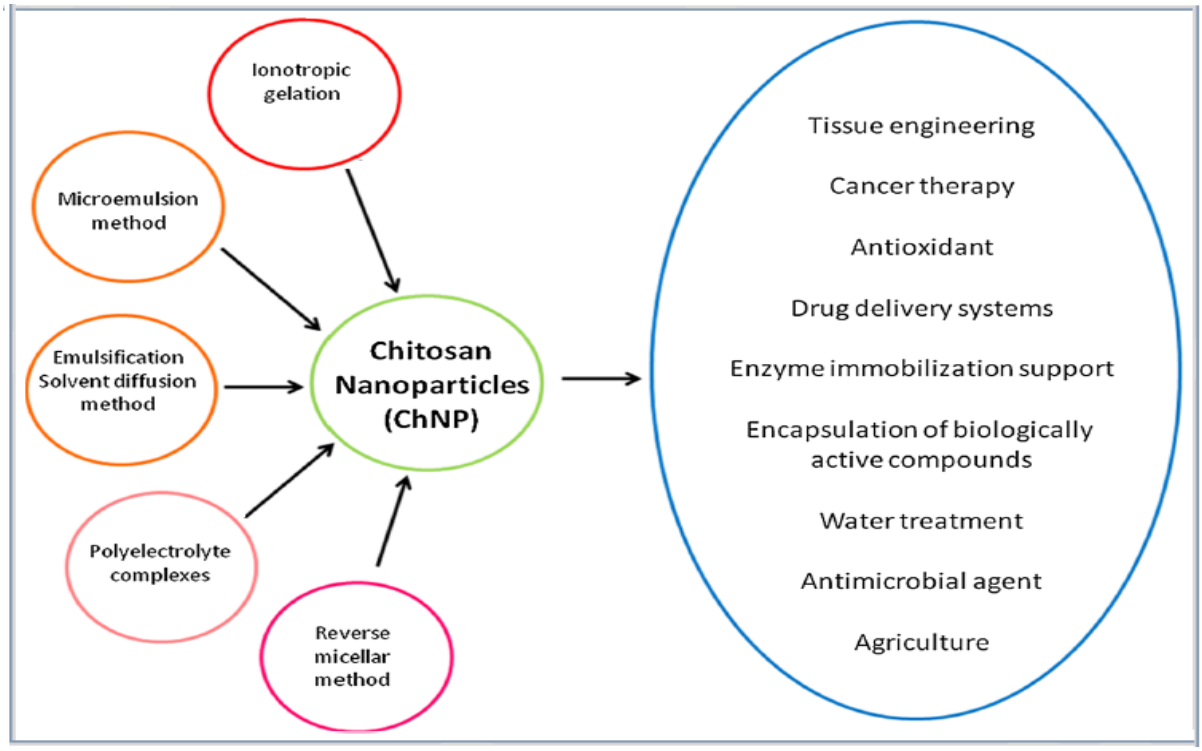

Figure 33. Schematic representation of the 5 modes of chitosan nanoparticles synthesis and their applications (Divya et al. 2018).

Chitosan nanoparticles have the particularity of a natural origin with excellent chemical, antimicrobial and biological properties, which makes them an environmentally friendly material and have safe bioactivity that does not affect human health (Malmiri 2012). Because of these unique properties, chitosan nanoparticles can be applied in a wide range of medical fields especially in tissue engineering (Rangasamy et al. 2010), cancer diagnosis (Mathew et al. 2010), enzyme immobilization support (Ghadi et al. 2015), antimicrobial agents and drug delivery (Hadidi et al. 2020; Garg et al. 2019)

C. Alginate Nanoparticles

Alginates are an ionic polysaccharide, varying in color from white to yellowish brown, found in the cell walls of all types of *Pheophyceae* (brown algae). These polysaccharides are able to

provide the hardness of algae and prevent them from drying out when they exposed to air at low tides. They compose more than 40% of the dry extract of brown algae. Alginate, considered a marine equivalent, in terms of physiological properties, of cellulose from terrestrial plants (Palluault 2010).

Primarily, alginate nanoparticles (AlgNPs) are being synthesized by polyelectrolyte complexation with chitosan and ionic gelation with calcium chloride (Venkatesan et al. 2017). In research, AlgNPs are extensively used for drug toxicity reduction as non-toxic delivery system (Spadari et al. 2019) and considered as one of the most widely recognized biomaterials used for the transport of several drugs (**Table 13**) (Severino, et al., 2019).

Table 13. Examples of alginate nanoparticles in drug delivery (Severino, et al., 2019).

Nanoparticles	Drug	Administartion Route	Applications
Polyurethane-alginate/chitosan	A model antigen	Subcutaneous	Enhance immune resposnes
Alginate-chitosan	Silver	-	Antimicrobial and anticancer
Chitosan-alginate	Crocin	-	Antioxidant and anticancer
Chitiosan-alginate	Natringenin	Oral	Diabets
Chitosan-alginate	Quecetin	-	Antioxidant
Lipid-alginate	Dexamethasone	Nasal	Anti-inflammatory
Calcium-alginate	Attenuated *Androctonus australis hector* (Aah) venom	Parenteral	Immunization against scorpion envenomation
Chitosan-alginate	Insulin	Oral	Diabets
Chitosan-alginate	Doxorubicin	-	Cancer
Polyurethan-alginate	Insulin	Oral	Diabets
Polyvinyl alcohol-alginate	Metformin	Oral	Diabets

The AlgNPs not only have unique physicochemical properties, which facilitate several modifications of the site-specific targeting, but also other properties such as mucoadhesivness, sustainability, stability, release of medication control, biodegradation and biocompatibility profiles which increase their use in drug delivery (**Figure 34**) (Severino, et al., 2019).

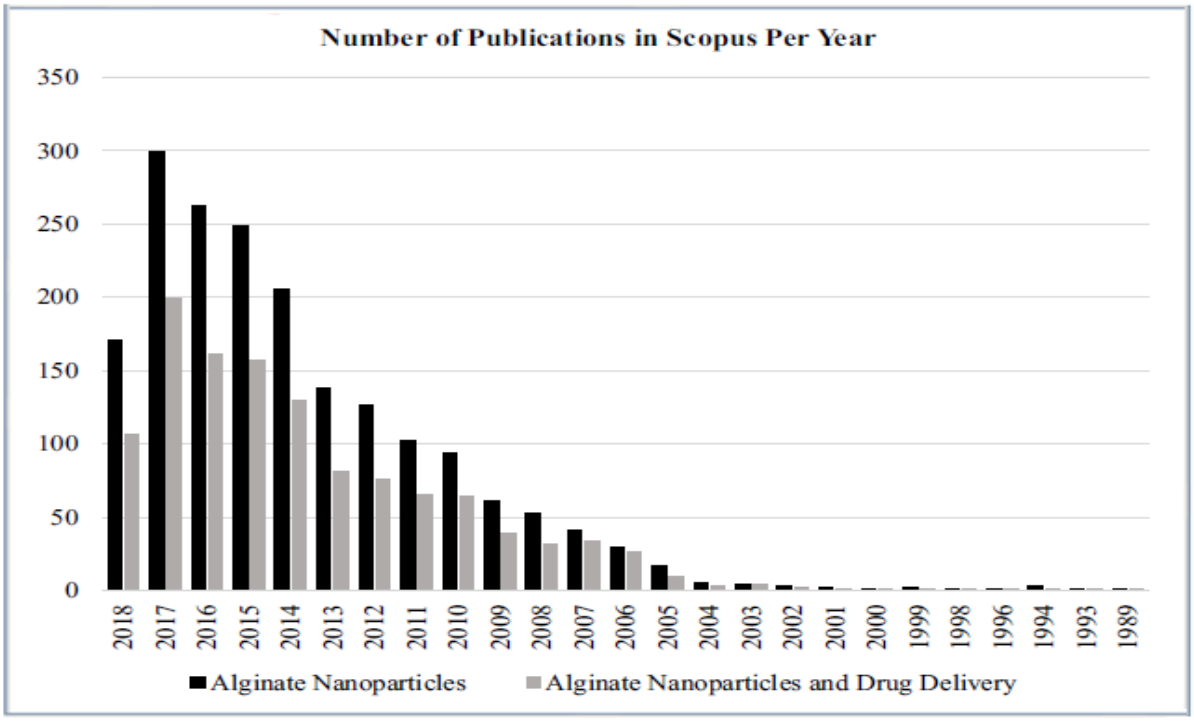

Figure 34. Number of published papers in Scopus per year (Severino, et al., 2019).

AlgNPs have received a great attention because of their targeting selectivity and specificity, by controlling the location of drug release, in chemotherapy drug delivery into hepatoma cells leading to reduction in the negative side-effects of chemotherapy drugs (Guo, et al., 2013). Alg NPs are promising agents in terms of improving the pharmacokinetics, tissue distribution, bioavailability of anti-tubercular drugs and in reducing their dosing frequency (the encapsulated drugs stayed 15 day in organs after administration, while the free drug stayed 1 day) (Ahmad, et

al., 2006). Numerous studies revealed the bioavailability of insulin loaded on Alg NPs and enabled to increase the protection of this protein from the enzymatic degradation in the gastrointestinal tract, increasing its stability at gastric pH and improving its absorption by intestinal walls (Sarmento, et al., 2007). The encapsulation of DNA, using polymers like alginate, allows protection from enzymes and other plasma proteins degradation during its passage from blood circulation to the targeted site. Thus macrophage-targeted Alg NPs are considered as a promising factor in gene therapy, for example the effective delivery of plasmid DNA leads to sustain the gene expression in macrophages (Jain, et al., 2012). Also Alg NPs can enhance the immunogenicity of vaccines due to a sustained-release profile e.g. the stability and antigenicity of diphtheria toxoid vaccine were increased after being loaded on alginate nanoparticles and thus highlighting the benefit of alginate nanoparticles as vaccine delivery vehicles (Sarei, et al., 2013).

Chapter 2. Materials and Methods

1. Mechano-chemical synthesis method of alginate nanoparticles

- Principle of ball mill method

A ball mill method is a type of grinder used to reduce the size of a material of interest (in our case, sodium alginate powder). Ball mill method is based on the top down approach (from micrometer to nanometer), in which the large molecules (macrostructures) are deconstructured to produce small materials at the nanoscale (nanostructures). The grinding balls apply a kinetic energy to the milled macromolecules inducing the break of the chemical bonds and produce smaller fresh surfaces by fracturing the initial macromolecules. The characteristics of the newly created nanosurfaces are usually more reactive than those of macromolecules (Kohane, 2007). Due to the high energy of the ball milling process, a local high temperature (over 1000°C) and high pressure (several GPa) are produced. Therefore, the ball milling process is used as a mechano-chemical synthesis method for the production of unique nanoparticles with new chemical properties.

- Preparation of alginate nanoparticles

The preparation of Alg NPs is described in the chapter 4.

2. Characterization of alginate nanoparticles

2.1. UV-visible spectrophotometry

- Principle

This technique is based on the absorption of photons by an atom or a molecule. This phenomenon allows the passage into an excited state, high in energy, by the transfer of electrons

from one orbital to another. The absorbed energy corresponds to the difference in energy between the excited state and the initial state, $\Delta E = h\nu$, where ν is the frequency of the photon ($\nu = c / \lambda$, λ the wavelength and c the speed of light). In solution, the energies of low transitions are observable and each absorption is characterized by the wavelength where the absorption reaches its maximum and its intensity is given by the molar extinction coefficient ε at this wavelength. Beer lambert's law makes it possible to relate these parameters $A = \varepsilon.l.[C]$ where A is the absorbance, L the length of the optical path and [C] the concentration of the sample (Sommer, 1989).

- Operating mode

  UV-vis analysis is described in the chapter 3.

2.2. Fourier transform infrared (FTIR) spectroscopy

FTIR analysis is described in the chapter 3.

2.3. *X-ray photoelectron spectroscopy (XPS)*

XPS analysis of Alg NPs is described in the chapter 3.

3. Biological materials

3.1. Bacterial strains

Five strains of *Clostridium perfringens* were studied, four of them were isolated from infected chickens with NE and the fifth one belongs to an infected human (**Table 14**). The

Enterococcus faecalis 14 was recently isolated from the meconium of a new born (K. Al Atya, et al., 2015)

Table 14. Strains of *C. perfringens* used as target bacteria.

Strain	Origin	Institution
***C. perfringens* DSM756**	Chicken	Leibniz-institut DSMZ
***C. perfringens* ICVB88**	Chicken	ANSES
***C. perfringens* ICVB89**	Chicken	ANSES
***C. perfringens* Clinique 1**	Chicken	ANSES
***C. perfringens* NCTC6785**	Human	NCTC

ANSES:Agence nationale de sécurité sanitaire de l'alimentation, de l'environnement et du travail(https://www.anses.fr/fr; https://www.nctc.edu)

3.2. Bacterial growth medium

Two types of culture media were used for the cultivation of the bacterial strains:

- The BHI (Brain heart infusion) broth medium (Sigma-Aldrich) for the cultivation of *C. perfringens* strains.
- The M-17 broth medium (Sigma-Aldrich) for the cultivation of *E. faecalis*14. After the autoclaving of M-17 broth medium, 5 % of sterilized glucose (concentrated at 10 %) was added to it.

3.3. Conservation of bacterial strains

All the bacterial strains were cultured in appropriate medium and incubated (aerobically in case of *E. faecalis* 14 or anaerobically in case of *C. perfringens*) for 24h at 37ºC. The anaerobic environment was established in a jar containing anaerobic reactor bag AnaeroGen^{tm} 2.5 (Thermo Fisher Inc). Then, the cultures were centrifuged 10 min at 8000 rpm at 4ºC. The

cells were transferred to fresh medium cultures containing 20% of glycerol in order to preserve them at -20ºC and -80ºC for further use.

4. Production and purification of enterocin DD14 (EntDD14)

E. faecalis 14 was grown in 100 mL of M17 broth (Sigma Aldrich), supplemented with 0.5% (w/v) of glucose and buffered with 60 mM sodium phosphate (pH 6.3), at 37°C for 24 h with 160 rpm continuous shaking. After harvesting by centrifugation (8.000 rpm, +4°C, 10 min), the cell-free supernatant was incubated 24 h at room temperature with 3g of CM Sephadex® C-25 resin (GE Healthcare Life Sciences, Sweden) previously suspended in 30 mL of distilled water and equilibrated for 30 min. The resin was then washed with 5 bed volumes (BV) of distilled water and 1 BV of 0.5 M NaCl. The resin-bound EntDD14 was eluted with 2 BV of 1.5 M NaCl. The removal of the salt from the solution containing EntDD14 was achieved by passing 1 mL of the solution into each column of PD MidiTrap G-10 columns (GE Healthcare, Chicago, IL, USA), following the manufacturer's instructions. The protocol was repeated several times to obtain a sufficient quantity of EntDD14 which was kept at 4ºC for further uses.

4.1. Matrix-Assisted Laser Desorption/Ionization Time-Of-Flight Mass Spectrometry (MALDI-TOF/MS) of EntDD14

MALDI-TOF/MS analyses of purified EntDD14 were performed using an autoflex™ speed (BrukerDaltonics, Bremen, Germany) running flex Control 3.3 (BrukerDaltonics). The instrument was calibrated in positive reflector mode using 8 proteins (MBT standard). Spectra were obtained in the range of 3,500 to 6,000 Da. Each MS spectrum was acquired in positive linear mode or positive reflectron mode by accumulating data from 5,000 laser shots distributed in random locations on the surface of the matrix spot. For analysis, a mixture of 5 µL of purified

EntDD14 and 5 µL of α-cyano-4-hydroxycinnamic acid (10 mg/mL 70:30 water/acetonitrile with 0.1% TFA) was spotted onto a MALDI-TOF MTP 384 target plate (BrukerDaltonics), according to the procedure of the dried-droplet preparation.

4.2. Quantification of EntDD14

- Principle of bicinchoninic acid

The quantification of EntDD14 was determined using the bicinchoninic acid (BCA) protein assay. The principle of BCA assay is based on the formation of a complex (Cu^{2+}-protein) under alkaline conditions, accompanied with the reduction of Cu^{2+} to Cu^{+}. This reduction is proportional to the total protein present in the sample. Studies showed that amino acids including tryptophan, tyrosine and cysteine can reduce Cu^{2+} to Cu^{1+}. Once the Cu^{1+} formed, the color of the solution turned to purple-blue. The BCA assay has several advantages such as, its color complex is stable, easy to use and can be used for broad range of protein concentrations (Wiechelman, et al., 1988).

- Dosage procedure

In the BCA assay, different samples are prepared including the blank which is the buffer without protein, the BSA protein standards of known concentrations and the unknown samples which are the solution to be assayed. Briefly, the BSA protein standards are prepared at different concentrations by making several dilutions of the BSA stock solution (e.g. 0-30 µg/mL) of total protein or according to an estimation of the concentration of the unknown samples. Also, the unknown samples should be diluted several times to make sure that the concentration of the protein is between the ranges of that of BSA standards (serve as calibration curve) (**Table 15**). After preparing all the samples, 0.5 mL of each sample is mixed with 0.5 mL of BCA working

reagent (mix of reagent A and reagent B provided with the kit). All the samples were gently vortexed and incubated 1h at 60°C. After the incubation, the solution in the tubes was transferred into cuvettes to measure the absorbance of the solution at 562 nm in a spectrophotometer.

Table 15. Example of standard assay set up table.

Tube No.	Sample volume (mL)	[BSA] Protein Standard (µg/mL)	Dilution factor	BCA Working Reagent (mL	A_{562}
1	0.5	0	x	0.5	0
2	0.5	0.5	x	0.5	0.023
3	0.5	5	x	0.5	0.134
4	0.5	10	x	0.5	0.26
5	0.5	20	x	0.5	0.60
6	0.5	30	x	0.5	0.93
7	0.5	50	x	0.5	1.36
8 to n	0.5	(unknown)	100	0.5	0.04

After obtaining the results (**Table 15**), a standard curve (A=f([BSA]) was generated to determine the protein concentration in the unknown sample by plotting the absorbance at 562 nm versus the BSA protein standard concentrations. The equation of the standard curve is as followed: $Y_n = aX_n + b$

Y_n: Represents the absorbance of n sample.

X_n: Represents the concentration of n sample (µg/mL).

"a" and "b" are parameters determined by the calculation of the standard curve.

The concentration of the protein present in the unknown sample (X_n) calculated previously is multiplied by the dilution factor.

4.3. Fixation of EntDD14 on alginate nanoparticles (EntDD14/ Alg NPs)

This was described in the following chapter (chapter 3).

5. Analyses methods of antibacterial activity

5.1. Activity of purified EntDD14 against *C. perfringens*.

Anti-*C. perfringens* activity assays of the cell-free supernatant (CFS) and purified fractions, were performed using the agar well diffusion method (Magaldi et al., 2004; Valgas et al., 2007). Tests were performed against the 5 strains of *C. perfringens*. A hundredth dilution (1/100) of a culture of *C. perfringens* (overnight, anaerobic), is spread over a BHI agar Petri dishes. After drying for 60 min at 4°C, holes of 5 mm in diameter are dug and 50 µL of samples are deposited, and plates were incubated overnight. The presence or not of halos of inhibitions around the samples is observed after anaerobic incubation at 37^0C for 24 h.

The antimicrobial activity is expressed in terms of arbitrary units (AU). It is defined as the maximum dilution produced a minimum of zone that still gives a clearly visible antagonistic zone. The reciprocal of the maximum dilution divided by the volume deposited into the hole multiplied by 1000, give the titer of antagonistic activity in AU per milliliter (L Caly, et al., 2017):

(n/v) x1000

"n" represents the reciprocal of the highest dilution showing visible inhibitory activity

"v" represents the volume deposited into the hole

5.2. Measurement of minimum inhibitory concentration (MIC)

There are many definitions for determining the MIC; in microbiology the MIC is "the lowest concentration of an antimicrobial (antibiotic, antifungal of bacteriostatic) drug able to inhibit the visible growth of a microorganism after an overnight incubation". Two methods can be used to determine the MIC (Jehl, et al., 2015).

1- Diffusion method: MIC can be determined using agar plates of solid growth medium. This method consists of depositing on the surface of the agar plate spread with desired bacteria, several diskscontaining known amount concentration of the antibiotic e.g. 30µg/mL, 60µg/mL,120µg/mL and 240µg/mL. A gradient of concentration settless down around each disk (because the antibiotic has diffused into the agar). After overnight incubation, the radius from which bacterial growth takes place determines the MIC (**Figure 35**).

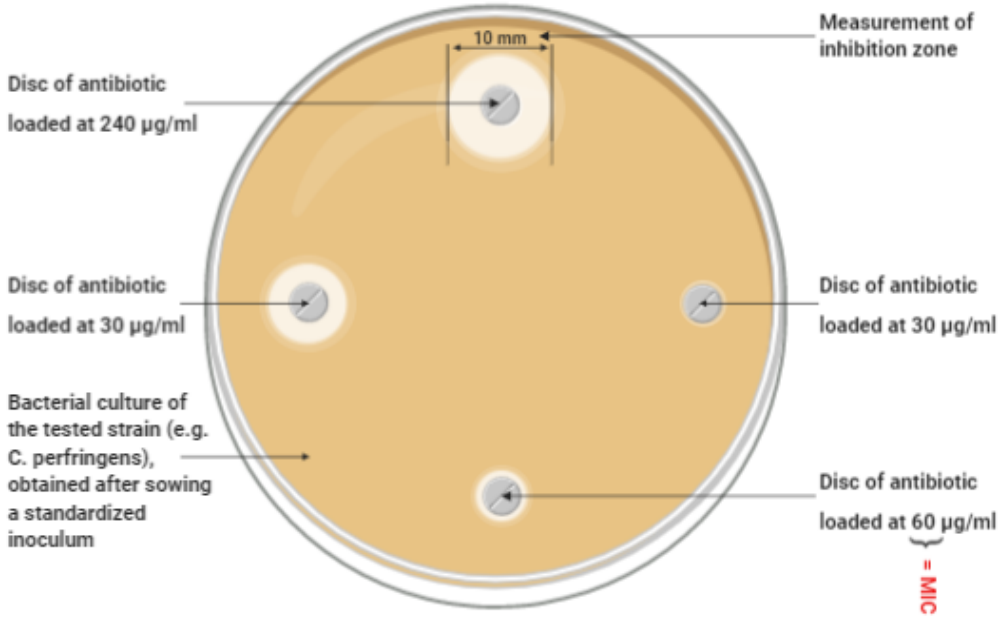

Figure 35. Determination of the MIC using diffusion method.

This method, also called Kirby–Bauer test, can be used also to identify the status of the bacteria (sensitive, intermediate or resistant).The distinction is based on the MICs and the diameter of the zones of inhibitions (**Figure 36**) (CLSI 2018; SFM 2018):

- For the sensible species “S”: The MIC of the tested antibiotic is lower or equal to the lower critical concentration “c” therefore equivalent to the higher critical diameter “D”.

The bacteria belonging to this category can be treated easily and the success of the therapeutic is high.

- For the intermediate species "I": The MIC is between the two critical concentrations (the lower critical concentration "c" and the higher critical concentration "C") and the diameter is between the two critical diameters (the lower critical diameter "d" and the higher critical diameter "D"). The species are sensible if they areexposed to high amount of concentration; however, the success of the therapeutic is not predictable.
- For the resistant species "R": The MIC of the antibiotic is greater than the higher critical concentration, which is equivalent to a diameter below the lower critical diameter. Note that the success of therapeutic is weak.

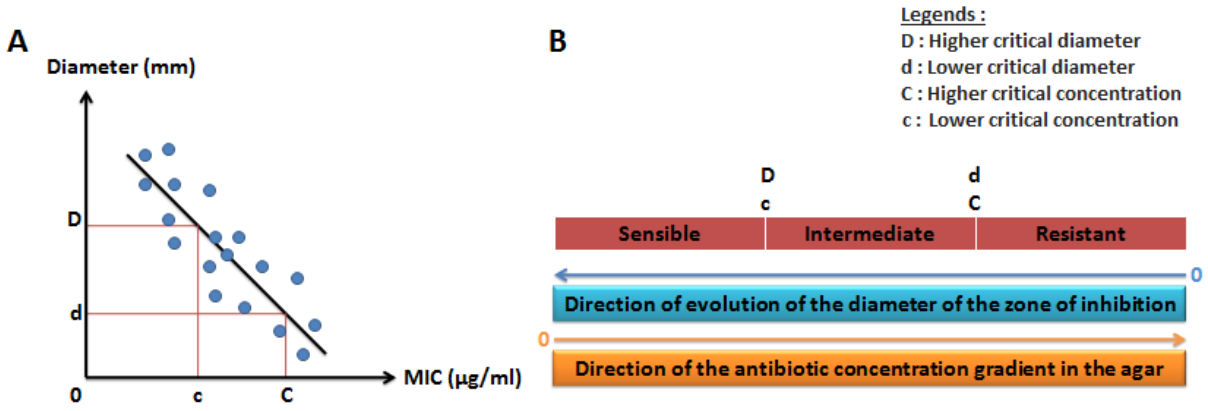

Figure 36. A, Broadcast antibiogram MIC concordance curves –diameters; B, Interpretation of the antibiogram result by diffusion in agar medium.

2- Microbroth Dilution Method

The purpose of this method is to determine the MIC of an antibiotic for a given bacteria. The principle of the technique is relatively simple (Jorgensen, et al., 2009): it involves bringing a decreasing concentration of antibiotic into contact with calibrated bacterial inoculums. The

different samples (EntDD14 and EntDD14/Alg NPs) are dissolved beforehand in sterile distilled water. Then, a series of ½ dilution is carried out in the different wells of a 96-well plate, then supplemented with a culture medium (BHI) for a final volume of 250 µL in each well. Then, a suspension of the microorganism (10^5 CFU/mL of *C. perfringens*) is introduced into the wells above the different concentrations of the antibacterial solutions. After 24 h of incubation at 37 °C, the MIC is determined according to the disorder observed in the well. If the mixture is cloudy, the bacteria have grown; if it is clear, the bacterial colonies have not grown. MIC corresponds to the lowest concentration of antibiotic in the wells where the mixture remained clear.

Reading can be visual or confirmed by spectrophotometry at a wavelength between 600 and 630 nm. The incubation methodology is as follows:

- Addition of 125 µL of sterilized distilled water in all wells (except the wells of line 2)
- Addition of 250 µL of the antibacterial solution to the wells in line 2.
- We make a dilution of ½ of each well, that is to say that from well 2 we take 125 µL of the antibacterial solution which we transfer to well 3 which already contains 125 µL of distilled water sterile. The concentration of ½ is then obtained in well 3. The operation is repeated for all the wells until the last one for which a volume of 125 µL in excess is withdrawn and eliminated.
- After adding 125 µL of BHI (containing the bacterial strain) to each well (1 to 12), then the total volume in each well will be 250 µL; in the 0, we have 125 µL of water + 125 µL of bacterial strain.
- Incubation for 24 h at 37 °C without shaking.

- After 24 h of incubation, different MICs are determined for each plate for the different antibacterial solution.

The smaller the MIC, the more sensitive the strain, and conversely the larger the MIC, the more resistant the strain.

6. Transmission electron microscopy (TEM) analysis

- Principle

TEM is an important technique widely used in many research fields including biomedical and microbiology research. This tool provides detailed analysis due to the high resolution of the accelerated electron beams, which pass through a specimen to form an image. This image is a consequence of the interaction of the electrons with the sample during the transmission of the beams through the ultrathin section placed on the grid (specimen). Then, the image is magnified and focused in the imaging device (Walther, 2017).

- Operating mode

The impacts of EntDD14, EntDD14/Alg NPs and Alg NPs alone on *C. perfringens* DSM 756/ATCC®13124TM were observed by TEM. The *C. perfringens* strain was incubated overnight at 37ºC then the Alg NPs (0.5 mg/mL) and EntDD14 (60 µg/mL), free or loaded on the Alg NPs, were added. Additionally, phosphate buffer solution (PBS) was used as a negative control. After incubation for 24h at 37°C in anaerobic conditions as described previously, treated cells of *C. perfringens* DSM756 were recovered by centrifugation and fixed using 2.5 % (v/v) glutaraldehyde solution and 0.1 M (v/v) of CaCo buffer (pH 7.4). *C. perfringens* DSM756 treated, or not, with EntDD14 alone or associated with the Alg NPs, and Alg NPs alone was prepared on a Formvar film of 300 square mesh, nickel grid (EMS FF300-Ni). The TEM images

were recorded using a JEOL JEM 2100FX TEM instrument, equipped with a GATAN CCD Orius 1000 camera and a GATAN CCD Orius 200D camera, at an acceleration voltage of 200 KV.

7. Time-kill assay

The time kill analysis reveals the effect of different concentrations of an antimicrobial over time in relation to the various phases of the bacterial growth (lag, exponential, stationary stage). The killing curves were performed on the pathogenic strain *C. perfringens* Clin1. Tubes of BHI medium containing defined concentrations of EntDD14 or EntDD14/Alg NPs (previously prepared) were inoculated with the Clin1 strain (previously prepared and incubated overnight) at a density of about 10^7 CFU/mL in a final volume of 4 mL and incubated for 0, 2, 4, and 6 h at 37°C under anaerobic condition. The killing kinetics of free EntDD14 and EntDD14/Alg NPs were assessed against *C. perfringens* Clin1 strain using standard killing over time experiments and viable bacterial counts on BHI agar plates. The final concentrations were 60 µg/mL and 30 µg/mL, respectively, for EntDD14 and EntDD14/Alg NPs (0.25 mg/mL).

Bactericidal activity was determined as $\log_{10}$ CFU/mL reduction in the colony count relative to the initial inoculum (Pankuch et al., 1994). Each culture was sampled after 0, 2, 4, and 6 h of incubation, and all were serially diluted in saline solution to determine viable cell counts. These dilutions (100 µL) were plated on BHI agar plates and colonies were counted after overnight incubation at 37°C under appropriate conditions.

8. Stability of EntDD14 and EntDD14/Alg NPs

A biochemical simulation of the internal processes occurring inside the gastrointestinal tract (GIT) of human and chicken was designed. This model lets us understand how GIT (of both

human and chicken) interacts with the purified EntDD14 alone and EntDD14/Alg NPs and therefore determine their stability at each compartment.

8.1. Simulated Chicken Gastrointestinal Tract (GIT)

The simulated chicken GIT models were designed according to Musikasang et al. (Musikasang, et al., 2012). To simulate the gastric compartment (gizzard), a solution of 1 mg/mL of pepsin (Sigma Aldrich) was prepared in PBS containing 240 µg/mL of purified EntDD14 or that of EntDD14 (120µg/mL) loaded on Alg NP (1 mg/mL). The pH was adjusted to 3.0 using 0.5 M HCl, and the obtained solution was incubated at 42°C for 90 min under agitation (160 rpm). After that, the same solutions were adjusted to pH 7 using 2 M NaOH and supplemented with 1 mg/mL pancreatin, in order to simulate the intestinal compartment prior to incubation for 3 more hours at 42°C under agitation (160 rpm). After each step, a sufficient volume was taken to test antibacterial activity against the *C. perfringens* Clin1 strain.

8.2. Simulated human Gastrointestinal Tract (GIT)

The simulated human GIT was designed as described by Grand et al. (Montgomery, et al., 1999). Briefly, the designed gastric compartment consists of 240 µg/mL of EntDD14 alone suspended in PBS adjusted at pH 3.5 with 0.5 M HCl, in which 2 mg/mL of pepsin (Sigma Aldrich) was added and incubated at 37°C with continuous agitation (160 rpm) for 90 min, followed by a simulated duodenal compartment solution by adding 2 mg/mL of pancreatin after pH adjustment to 7.0 followed by further incubation of 3 h under the same conditions. The same experiment was repeated for EntDD14 (120 µg/mL) loaded on Alg NPs. After each step, a sufficient volume was taken to test antibacterial activity against *C. perfringens* Clin1 strain.

9. Cell viability assay and *in vitro* cytotoxicity of EntDD14 and EntDD14/Alg NPs

The effects of EntDD14, EntDD14/Alg NPs, and Alg NPs alone were assessed on Caco-2 and HT-29 cell-lines.

The Caco-2 cell line obtained from Sigma Aldrich, is a continuous line of heterogeneous human epithelial colorectal adeno carcinoma cells. The cultivation of these cells, at specific conditions (detailed below), allow them to differentiate and polarize such that their phenotype, functionally, resemble the enterocytes lining the small intestine. Caco-2 cells show, microvilli, tight junctions, and a significant number of transporters and enzymes which characterize these enterocytes: peptidases, uptake transporters for amino acids, esterases, carboxylic acids and P-glycoprotein (J. Hidalgo, et al., 1989).

The HT-29 cell line, obtained from Sigma Aldrich, is originated from the human colorectaladeno carcinoma. These cells are being extensively used in cancer research; they are able to express the characteristics of the mature intestinal cells such as the mucus producer cells and the enterocytes. They represent a model *in vitro* to stimulate the intestinal epithelial response to bacterial infection. These cells, once they are mature, they can be organized in monolayer and can produce mucins (Martínez-Maqueda, et al., 2011).

- Caco-2 cell and HT-29 cell assays preparation:

The two cell lines were grown in Dulbecco's Modified Eagle's Medium (DMEM) (PAN-Biotech GmbH, Aidenbach, Germany) at 37°C in a humid atmosphere with 5% CO_2. Cells were grown for 72 h in 2 subsequent subcultures and were transferred into 96-well tissue culture plates

at a density of 8,000 cells/well, and were grown for an additional 72-96 h. During this time (7 days), the medium was changed each two days to maintain optimal conditions for cell growth.

For cytotoxicity assays, 100 µL of the appropriate concentration of each sample (purified EntDD14, EntDD14/Alg NPs and Alg NPs alone), previously re-suspended in DMEM, were added to each well of the 96-well tissue culture plates containing the Caco-2 and HT29 cell monolayers. The contact took place for 24 h at 37°C in a humid atmosphere with 5% CO_2. The viability of the Caco-2 and HT29 cells was assessed using the *Cell Counting Kit-8* assay (CCK-8) (Dojindo Molecular Technologies, Kumamoto, Japan) by adding to each well 100 µL of DMEM containing 5 µL of CCK-8 reagent. Following 2 h of incubation in the dark, the absorbance of each well was measured at 450 nm in a spectrophotometer (SAFAS Xenius; SAFAS Monaco, Monaco). Results were expressed as the percent viability compared to non-treated cells.

10. Contact of the clinical isolate *C. perfringens* Clin1 with sub-inhibitory concentrations

Real-time quantitative PCR (RT-qPCR) analysis was performed to study the impact of the different formulations associating EntDD14 and Alg NPs, prepared during this study, on the expression of the most important *C. perfringens* Clin1 toxin-encoding genes, including *plc* (toxin α), *netB* (enteritis B-like toxin), *cnaA* (collagen adhesion protein A) and *pfoA* (thiol activated cytolysin). The expression levels of these genes were assessed after treatment of the *C. perfringens* Clin1 strain with sub-inhibitory concentrations of EntDD14 (MIC/2 = 30 µg and MIC/4 =15 µg), EntDD14 loaded on Alg NP (MIC/2 = 15;125 µg and MIC/4 = 7.5;62.5 µg) and PBS as a negative control.

The clinical isolate *C. perfringens* Clin1 was grown in 10 mL of BHI broth (Sigma Aldrich) at 37°C in anaerobic conditions for 24 h. After harvesting by centrifugation (8,000 × *g*, +4°C, 10 min), the recovered cells were re-suspended in 10 mL of fresh BHI. Five samples were prepared, each containing 1 mL of bacterial culture plus 1 mL of the aforementioned formulation (including EntDD14 alone or EntDD14/Alg NPs) at MIC/2 or MIC/4 and using PBS as anegative control. The five samples were incubated overnight at 37°C in anaerobic conditions as described previously. After 12 h of incubation, 20 µLwas withdrawn from each sample to investigate the cell viability and the remaining cells were harvested (8,000 × *g*, +4°C, 10 min) for RNA extraction (as described in the section below).

10.1. RNA extraction, DNAase treatment and first strand cDNA synthesis

RNA extraction:

The *C. perfringens* Clin1 cells, treated with the different formulations of EntDD14 and Alg NPs (as described previously) were each re-suspended in 500µL of NucleoZol (Macherey-Nagel, Duren, Germany), in which 400 µL RNA-free water were added, vortexed 15 sec and incubated 15 min at room temperature (RT). The solutions were then centrifuged 15 min at 12000 g at RT (2 phases were formed: ARN in the supernatant). The supernatant was taken out and placed in new tubes, in which 500µL of Buffer Mix (v/v nucleozol) were added. Each nucleospin was charged with 700 µL of the supernatant and centrifuged 30 sec at 8000 g at RT (this step was repeated two times to allow the pass of all supernatant). The RNA was then washed two times; the first wash was achieved by charging each nucleospin with 700 µL of Buffer RA3 and centrifuged 30 sec at 8000 g at RT. The second wash was carried out by charging each nucleospin with 350µL of RA3 and centrifuged 2 min at 8000 g at RT. The nucleospins were

placed in new tubes (RNA-free) and left to dry for 3 min at RT. Finally, the RNA was collected by adding 40 µL of RNAase-free water and centrifuged 1 min at 11000 g.

DNAase treatment:

The RNA quality control and concentrations were achieved using NanoDrop Lite Spectrophotometer (Thermo Scientific) by which RNA concentrations were dosed and then diluted (with RNAase free water) to make 0.0125 % (w/v) of each sample. Then, 1 µL of DNAase and 1 µL of Buffer DNAase were added to the 8 µL of ARN. The 10 µL of each sample were incubated 30 min at 37ºC. The reaction was further inactivated with 1 µLEDTA (added to the 10µL), then the 11 µL of each sample were incubated for 10 min at 65ºC.

First strand cDNA synthesis:

In the followed protocol, we calculated the volume for one sample; the other samples were treated in the same way.

The volume of ARNs treated with DNAase is divided into 2 (5µL/tube). For a final volume of 20 µL, we added into each tube:

- Random Primer: 1µL
- Buffer: 4 µL
- Inhibitor: 0.5 µL
- dNTP Mixture (10mM): 2 µL
- RNAase-free water: 6.5 µL
- Reverse Transcriptase: 1 µL or 1 µL water for No Reverse Transcriptase control (NRT)

The PCR program was as follows: 25ºC/5 min; 42ºC/1h; 70ºC/5 min and 10ºC/10 min.

10.2. Real-time quantitative PCR (RT-PCR) measurements of *C. perfringens* Clin1 toxin-encoding genes challenged with EntDD14 and EntDD14/NPs

Real-time quantitative PCR (RT-PCR) can reliably detect and measure the products generated during each PCR (polymerase chain reaction) cycle and are directly proportional to the amount of matrix present before amplification. The products of PCR (amplicons) are quantified during the exponential phase of the reaction because it is only at this stage that it is possible to extrapolate the starting quantity of the template sequence. To measure them, it is possible to use fluorochromes which intercalate in a nonspecific way (like SYBR Green) or molecular probes containing a "quencher". The fluorescence intensity increases with each cycle which results in an increase in the amount of amplicons produced in a proportional way. The"*threshold cycle"* (Ct) is defined as the number of cycles of PCR at which the reporter fluorescence is greater than the background fluorescence. This Ct value is always reached during the exponential phase of target amplification. The Ct is inversely proportional to the relative level of original expression of the gene of interest. It is an essential element for obtaining accurate and reproducible data (Arya, et al., 2005).

The SYBR Green 1 is very popular, it is fluorescent only when it intercalates into double-stranded DNA; the fluorescence intensity therefore depends on the amount of double-stranded DNA present in the reaction

Three independent samples of total RNA were extracted from each test condition using NucleoZol (Macherey-Nagel), and were reverse transcribed into cDNA using RevertAidRT Reverse Transcription Kit (ThermoFisherInc.). For the qPCR, specific primers were designed using sequences of known *C. perfringens* toxin encoding genes retrieved from the NCBI

database (https://www.ncbi.nlm.nih.gov/). Then Primer3 software (http://frodo.wi.mit.edu/primer3/) was used to generate the adequate primers for each gene, thus generating amplicons of a normalized length of 100 bp. Additional primers allowing amplification of specific parts of 16S cDNAs were designed following the same procedure to provide internal controls (**Table 16**). The amplification (using 2 µL of a 1:10 cDNA dilution), detection (with automatic calculation of the threshold value), and real-time analysis were performed twice for each cDNA sample using the CFX Connect Real-Time PCR Detection system (Bio-Rad Laboratories, Hercules, CA, USA). Relative mRNA levels of each gene in each condition were calculated using a comparative cycle time data with analysis modules from CFX Manager™ Software Version 3.1 (Biorad). The detailed protocol of RT-qPCR is as follows:

- The pool was first achieved by mixing 5 µL of each condition followed by serial dilutions to obtain the following concentrations 1/10, 100 and 1/1000.
- To each condition, the following reagents were added: 12.5 µL Syber Mix + 1 µL forward primer + 1 µL reverse primer + 8.5 ARNase-free water + 2µLcDNA (or 2 µL water to prepare the negative control for each condition)
- For each condition, NRT was prepared (previously detailed in section 10.1) as a control of the genomic DNA contamination.

After preparing the 96-wells plate (the total volume in each hole is 25µL), the qRTt-PCR was programmed as follows: 95ºC/3.00 min; [95ºC/0.15min; 57ºC/0.30min; 72ºC/0.15min] repeated 39 fols; 95ºC/0.10min; 65ºC/0.05min; 95ºC/0.50min.

Table 16. Primers designed and used in *C. perfringens* Clin1 for RT-qPCR.

Primer	Sequences	Target gene (Function/role)
F-*plc*RT-qPCR	CTGACACAGGGGAATCACAAA	*plc* (Toxin α)
R-*plc*RT-qPCR	TAGTGCATAGCCTCTCCAAGAT	
F-*cnaA* RT-qPCR	TGATGTTGGTGGATGGGCAA	*cnaA*(Collagenadhesionprotein)
R-*cnaA* RT-qPCR	ATCTGCCCGCTTTGAACCAG	
F-*tpeL* RT-qPCR	TTTGTCGGAAATGTTGGCGC	*tpeL* (Cytotoxin produced on sporulation phase)
R-*tpeL* RT-qPCR	TAGCTGTCAACCTAAGAAGCCT	
F-*pfoA* RT-qPCR	GAGTGTACAGGCCTTGCTT	*pfoA* (Thiol activatedcytolysin)
R-*pfoA*RT-qPCR	CCAGGATATAAAGTTGTTCCCCA	
F-*netB* RT-qPCR	TGGCTTTAGCATTAACAGCACC	*netB* (Enteritis B-Like toxin)
R-*netB* RT-qPCR	TCGCCATTGAGTAGTTTCCCA	
F-*netE* RT-qPCR	GCGCCAAAAGATGCTAAAGAAT	*netE* (Leukocin/hemolysin toxin family)
R-*netE* RT-qPCR	GAACGTTTATTTGATCCCCTCCA	
F-*netF* RT-qPCR	AGGCACCAAAAGGCACAAAA	*netF* (Leukocin/hemolysin toxin family)
R-*netF* RT-qPCR	ACACGATTTTCTCCCCACCA	
F-*netG* RT-qPCR	ACTGTTGGTGGAGAAATATCAGCT	*netG* (Leukocin/hemolysin toxin family)
R-*netG* RT-qPCR	ACTGTTTTAAAGTCTGGCTGTTCA	
F-16S CpRT-qPCR	CGGTAATACGTAGGTGGCGA	16s rDNA
R-16S Cp-qPCR	AATGCAGCACCCAAGTTGAG	

N.B: Data obtained with the RT-qPCR experiments were expressed as a mean ± standard error calculated from three independent experiments. Analysis of statistical significance was performed by one-way ANOVA and the post-hoc Tukey Test ($p< 0.05$). Principal component analysis was carried out using R version 3.5.2 (r-project.org, R foundation for statistical computing).

11. Inflammation of Caco2 cells and anti-inflammatory activity of EntDD14

After the production and purification of EntDD14 (previously detailed), the enterocin was re-suspended in DMEM to study the effect on the inflammation of Caco-2 cells. The latter (prepared as described previously) were pre-stimulated with lipopolysaccharide (LPS) from *E. coli* (Sigma Aldrich) (50 µg/mL) for 36 h. Then, the EntDD14 was added, in triplicate, at two different concentrations (60 µg/mL and 240 µg/mL), dexamethasone (20 mM) (Sigma Aldrich) as positive control and DMEM containing LPS (50 µg/mL) as negative control.The plate was incubated over night at 37°C.

The next day, the supernatant of each well was removed to make dosages of interleukins by Luminex and ELISA, while Caco2 cells in the bottom of the wells were subjected to RNA extraction, DNAase treatment, first strand cDNA synthesis, Real-time quantitative PCR (RT-PCR) measurements (same protocol described in sections 10.1. & 10.2.)

NB: each condition was in triplicate.

11.1. Luminex assay

11.1.1. Introduction

Cytokines are immunomodulatory small proteins (~5–20 kDa) that play important roles in cell signaling and in both adaptive and innate immune responses (Dinarello, 2000). The generic term“cytokines” includes myokines, interferons, lymphokines and interleukins (acting as mediators between T cells) and chemokines (responsible for the migration of T-cell). Cytokines are produced by many cells, including endothelial cells as well as immune cells (B lymphocytes, T lymphocytes and macrophages). Cytokines act at the recognition, activation, or effector phases of an immune response, modulating the balance between humoral and cell-based immune responses. They also regulate the maturation, the growth and the functional activities of

particular cell populations (T cells, B cells and myeloid cells). Therefore, cytokines are involved in the multi-faceted response of the immune system to most antigens, among those responses that induce the inflammatory process (Dinarello, 2000).

Many clinical and sub-clinical diseases are accompanied with inflammation (at low level) such as diabetes, cancer and cardiovascular disease. Therefore, in such states, measuring the specific cytokines responses, are critical for better understanding diseases interactions and their pathogenesis. Luminex has the advantage of enabling quantitative detection of dozens of analytes simultaneously.

11.1.2. Principle

Analyte-specific antibodies are coated with MagPlex®-C microspheres (magnetic microparticles) fixed with fluorophores at set ratios for each unique microparticle region. Microspheres, standards and samples are pipetted into wells and the analytes are captured specifically, by the beads (each analyte has specific bead which bind to). After washing away any unbound substances, a biotinylated detection antibody cocktail is introduced to each well. After washing away any unbound biotinylated antibody, streptavidin-PE conjugate, which binds to the biotinylated antibody, is introduced to each well. After removing the unbound Streptavidin-PE, the microspheres are resuspended in buffer and read using the Luminex® MAGPIX® Analyzer. A magnet in the analyzer captures and holds the super paramagnetic microparticles in a monolayer. Two spectrally distinct Light Emitting Diodes (LEDs) illuminate the microparticles. The first LED excites the dyes inside each microsphere to identify the region and the second one excites the PE to measure the amount of analyte bound to the microparticles

11.1.3. Immunoassay procedure

Prior to beginning this assay, all the reagents were allowed to warm to room temperature (RT), then:

- The plate was first washed by introducing 200 µL of Wash Buffer (provided with the kit) into each well. The plate was sealed and mixed for 10 min at RT.
- After removing the Wash Buffer from all wells, 50 µL of each diluted Standards, the Quality Control and the Backgrounds were added to the appropriate wells (All were provided with the kit).
- After that, 25 µL of Assay Buffer (provided with the kit) were added to the sample wells, in which 25 µL of neat samples were then added.
- 25 µL of the Premixed Beads (provided with the kit) were added to each well. Then, the plate was sealed and incubated overnight, in the dark, with agitation at 4°C. Note: During the introduction of Premixed Beads, the bead bottle was intermittently shaken to avoid settling
- The incubation was followed with removing the well contents and washing plate 3 times.
- 50 µL of Detection Antibodies (provided with the kit) were added to each well and incubated 1h at RT. After that, 50 µL of Streptavidin-Phycoerythrin (provided with the kit) were added per well and incubated, in the dark, for 30 min at RT.
- All the well contents were then removed by washing the plate 3 times (each time 200 µL of Wash Buffer were added to each well).
- 150 µL of Sheath Fluid (provided with the kit) were introduced into each well. The beads were re-suspended by shaking the plate 5 min on a plate shaker.
- Finally, the plate was run on Luminex® 200™, HTS, FLEXMAP 3D® software.

  Note: The reagents were prepared and diluted according to manufacturer's instructions

11.2. High sensitive human interleukine-6 Enzyme-linked immunosorbent assay (HS human IL-6 ELISA)

11.2.1. Principle

Human IL-6 ELISA is used for quantitative measurement of human IL-6 in many medium (plasma, serum and cell culture supernatants). The principle of this assay is based on using an antibody specific for human IL-6 coated on a 96-well plate. After adding standards and samples into the wells, IL-6 present in the samples will bind to the wells by the immobilized antibody. After that, the biotinylated anti-human IL- 6 antibody is added after washing the plate. The unbound biotinylated antibody is washed away and the HRP-conjugated streptavidin is added. After removing the contents of the wells, a TMB substrate solution is added and a blue color is shown in proportion to the amount of IL-6 bound. Finally, a Stop Solution is added and a color change occurred (from blue to yellow. The intensity of the color is measured by a plate reader at 450 nm. Of note, the minimum detectable dose (MDD) of human IL-6 ranged from 0.007-0.090 pg/mL.

11.2.2. Procedure

The assay was performed as follows:

1. 100 µL of Assay Diluent RD1-75 was added to each well.
2. 100 µL of standard or sample was added per well. Then the plate was covered and incubated for 2 h at room temperature on a microplate shaker set at 500 ± 50 rpm.
3. The content was then aspirated and the wells were followed by five washes.

4. 200 µL of Human IL-6 HS Conjugate was added to each well and further incubated for 2h at RT. After the incubation the plate was washed 5.
5. 50 µL of Substrate Solution was added to each well and incubated for 60 min at room temperature.
6. After one hour, 50 µL of Amplifier Solution was added to each well, and then incubated for 30 min at room temperature. Note: The addition of Amplifier Solution initiates the color development.
7. 50 µL of Stop Solution was added to each well.
8. The optical density of each well was determined within 30 min using a microplate reader set to 490 nm, with a wavelength correction set to 650 nm or 690 nm.

11.3. Real-time quantitative PCR (RT-qPCR) measurements of *C. perfringens* Clin1 toxin-encoding genes challenged with EntDD14 and EntDD14/Alg NPs

For the RT-qPCR, after extraction of mRNA and otention of cDNA from treated Caco2 cells, as described previously, specific primers (**Table 17**) using sequences of known interleukins encoding IL6 and IL8 genes as described previously by Sugazawa et al. (2007) (Saegusa, et al., 2007). Additional primer allowing amplification of specific parts of GAPDH (house keeping gene) were designed following primer3 software to provide internal controls.

Table 17. Primers designed and used in Caco2 cells for RT-qPCR.

Primer	Sequences	Target gene (Function/role)
F-*IL6* RT-qPCR	CACTCACCTCTTCAGAACGA	*Interleukin 6*
R-*IL6* RT-qPCR	CTGTTCTGGAGGTACTCTAGG	
F-*IL8* RT-qPCR	TGGCTCTCTTGGCAGCCTTC	*Interleukin 8*
R-*IL8* RT-qPCR	TGCACCCAGTTTTCCTTGGG	
F-*GAPDH* RT-qPCR	TGAACGGGAAGCTCACTGG	*GAPDG* (house keeping gene)
R-*GAPDH*RT-qPCR	TCCACCACCCTGTTGCTGTA	

Chapter 3. Alginate nanoparticles potentiate the anti-*Clostridium perfringens* activity of enterocin DD14 (EntDD14), and down-regulate the expression of genes coding for toxins in this pathogen.

To potentiate the anti-*Clostridium perfringens* activity of the leaderless two-peptide EntDD14 (**Figure 37**), we used an orginal approach that consisted of loading this bacteriocin on the alginate nanoparticles (Alg NPs). Below is the sequence of EntDD14, which is a class IIb, consisting in two peptides.

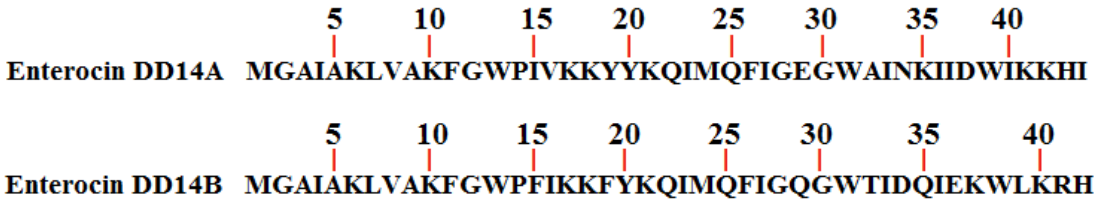

Figure 37. Amino acid sequence alignment of EntDD14A and EntDD14B (L Caly, et al., 2017).

The Alg NPs was prepared by using the ball mill method. It was then subjected to several analyses including UV-vis spectrophotometer, Fourier transform infrared spectroscopy (FTIR); X-ray photoelectron spectroscopy (XPS); X-ray photoelectron spectroscopy (XPS, dynamic light scattering (DLS) and Scanning Electron Microscopy (SEM). The results showed that alginate nanoparticles displayed similar spectral features compared with the sodium alginate precursor (a safe polymer). These results give a clear evidence that the ball mill method do not affect the structure of this polymer (**Figure 38**).

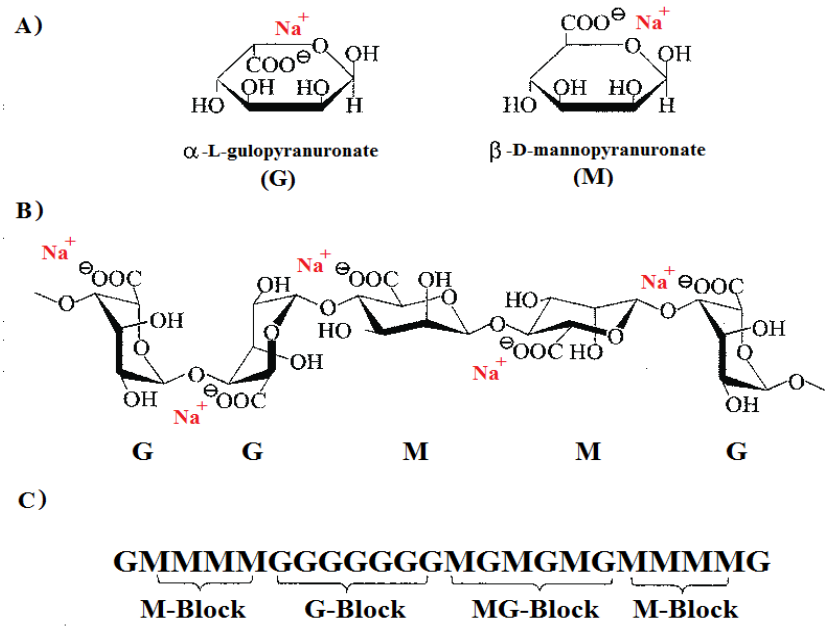

Figure 38. Sodium alginate structural data: (A) sodium alginate monomers (M versus G); (B) the macromolecular conformation of the sodium alginate polymer; (C) chain sequences (Davis, et al., 2003)

The production of EntDD14 was achieved according to the protocol described Abriouel et al. (Abriouel, et al., 2003). After ascertaining the purity of EntDD14, the latter was loaded on Alg NPs through a simple physical adsorption process.The new formulation, enterocin DD14/ alginate nanoparticles was named EntDD14/Alg NPs. Importantly, this developed formulation has reduced four folds, the minimum inhibitory concentration (MIC) of the EntDD14 when used alone (**Table 18**), dealineating a gain the antibacterial activity.

Table 18.The minimum inhibitory concentration (MIC) values of EntDD14, alone or loaded on alginate nanoparticles (EntDD14 + Alg NPs) against the various target strains.

MIC (µg/mL) *C. perfingens*strain number	Alg NP	DD14	DD14 + Alg NP	Amoxicillin
ICVB088	>1000	60	15	0.15
ICVB089	>1000	60	30	0.15
Clin1	>1000	60	30	0.15
DSM 576	>1000	60	60	0.3
NCTC6785	>1000	60	60	5

In addition to improve the activity of EntDD14, the new formulation was assessed for its safety aspects. As matter of fact, it was checked for its adverse effects against eukaryotic cells. Similarly, the stability of formulation was studied under conditions mimicking the real physiological states of human and birds. Indeed, these environments are rich en proteases, which can hamper the activity of the bacteriocin, as recently reported by Silva et al. (2018). Interestingly, the EntDD14/Alg NPs formulation appeared to defy these harsh conditions, and resulted then to be stable under both stimulated gastric tract of human and chicken, demonstrating protective effect of alginate nanoparticles. However, this protection was less efficient to protect EntDD14 from the conditions mimicking the small intestine of both human and chicken. To overcome this issue, we suggest using further systems like encapsulation in the near future.

The cytotoxicity assays of EntDD14 alone and that of the formulation were determined against two human colon adenocarcinoma cell lines (Caco-2 andHT-29). Therefore, no cytotoxic effect was registered after 24 hours of contact, advocating their safety towards eukaryotic cells.

To gain academic insights on the impact of these bacteriocins and formulation, we studied by using qPCR technology, the expression of genes coding for toxins in *C. perfringens*, after treating this malevolent strain with these antibacterial agents. The toxins studied were *netB* and α-toxin genes (*plc*) which are expected to play a determinant role in the NE infection (Keyburn, et al., 2008). Interestingly, this new formulation enabled to control expression of other genes such as *pfo*, and *cnaA* genes.

To be noted, *α*-toxin (*plc*) is a zinc metallophospholipase activated by zinc. It is a 43kDa protein encoded by *plc* gene, characterized by a phospholipase C/sphingomyelinase activity, and further lethal traits (Sakurai, et al., 569-574), while *netB* toxin produced by *C. perfringens* type A is considered as a member of the β-pore forming toxins, which is able to form pores of about 1.7 nm diameter in leghorn chicken hepatocellular carcinoma cells (LMH) (Keyburn, et al., 2008). Regarding the perfringolysine (*pfoA*), it is a pore forming toxin belonging to cholesterol-dependent cytolysine (CDCs) (Popoff, 2014), whereas c*naA* (collagen adhesion protein) encoded gene allows *C. perfringens* to bind to collagen types III, IV and V at higher levels compared to non-disease producing strains, suggesting thereof high binding levels to the extracellular matrix molecules (ECMM), which enhance the virulence of necrotic enteritis (Martin, et al., 2010).

As a conclusion, loading of EntDD14 on AlgNPs has enhanced the antibacterial activity of EntDD14 agaisnt *C. perfringens*. The MIC showed to be decreased by 4 fold upon the loading of EntDD14 on AlgNPs. Additionally, at sub-inhibitory concentration, the nanoformulation showed to be effective in terms of down regulating the expression of genes coding for virulent toxins such as toxin α, enteritis toxin B-Like, collagen adhesion protein and thiol activated cytolysin. The suggested formulation (EntDD14/AlgNPs) resulted to be stabe under conditions mimicking the human/animal GIT, and thus protected the anti-*C. perfringens* activity of

EntDD14 from acidic pH and pepsin action. Further studies need to be done for improving the stability of the new formulation EntDD14/Alg NPs under conditions mimicking intestinal/stomach compartment.

Alginate nanoparticles enhance anti-*Clostridium perfringens* activity of the leaderless two-peptide enterocin DD14 and affect expression of some virulence factors.

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Abstract

Here we report a novel approach to improve the anti-*Clostridium perfringens* activity of the leaderless two-peptide enterocin 14 (EntDD14), produced by *Enterococcus faecalis* 14. This strategy consists of loading EntDD14 onto alginate nanoparticles (Alg NPs), which are made of a safe polymer. The resulting formulation (EntDD14/Alg NPs) was able to reduce at least four times the minimum inhibitory concentration (MIC) of EntDD14 against *C. perfringens* pathogenic strains isolated from a chicken affected by necrotic enteritis (NE). Interestingly, this formulation remained active under conditions mimicking the human and chicken gastric tract. Assays conducted to establish the impact of this formulation on the intestinal epithelial cell line Caco-2 and the human colorectal adeno carcinoma cell line HT29 revealed the absence of cytotoxicity of both free-EntDD14 and EntDD14 loaded onto the alginate nanoparticles (EntDD14/Alg NPs) against the aforementioned eukaryotic cells, after 24 hours of contact. Notably, EntDD14 and EntDD14/Alg NPs, both at a sub-inhibitory concentration, affected the expression of genes coding for toxins such as toxin α, enteritis B-like toxin, collagen adhesion protein and thiol activated cytolysin. Further, expression of these genes was significantly down-regulated following the addition of EntDD14/Alg NPs, but not affected upon addition of EntDD14 alone. This study revealed that adsorption of EntDD14 onto Alg NPs leads to a safe and active formulation (EntDD14/Alg NPs) capable of affecting the pathogenicity of *C. perfringens.* This formulation could therefore be used in the poultry industry as a novel approach to tackle NE.

Keywords: *Clostridium perfringens*; necrotic enteritis; alginate nanoparticles; enterocin DD14; cytotoxicity; expression of toxin genes.

Introduction

Clostridium perfringens is a Gram-positive, anaerobic, spore-forming pathogenic bacterium, which could be found in the gastrointestinal tract (GIT) of humans and other animals [1]. The strains belonging to this species are able to produce up to 17 different toxins including α-toxin (phospholipase C) identified in *C. perfringens* type A strains, which are responsible for necrotic enteritis (NE) in chickens [2–5]. However, it has been established that α-toxin is not absolutely required to induce NE in chickens [6, 7], as other toxins and other mechanisms may be involved. For example, Enteritis B-like (Net B) toxin was also reported to play a role in the development of this infectious disease [6]. The discovery of NetB prompted the creation of the G toxinotype, including strains of *C. perfringens* carrying both α and NetB toxins [8]. Besides these major toxins, NE is also associated with a plasmidic pathogenicity NE *locus*-1 harboring numerous other virulence genes such as *cnaA*, coding a collagen-binding surface protein [9]. Furthermore, genes coding for other toxins, such as perfringolysin O (*pfoA*), classically referred to as θ toxin [10], pore-forming Leukocidin/Hemolysin family (*netE*, *netF*, and *netG*)[11], and a cytotoxin produced on sporulation phase (*tpeL*) [12], are also involved in the development of NE in chickens.

The NE caused by *C. perfringens* occurs in 2 to 5 weeks old broiler chickens, raised on litter, and in 7 to 12 weeks old turkeys. The disease remains in the flock for 5 to 10 days, causing up to 50% mortality and leading to important economic losses for the poultry industry [13, 14]. Antibiotics such as amoxicillin, bacitracin, and florfenicol can inhibit the proliferation of *C. perfringens*, but the continual use of these molecules may result in an increased prevalence of antimicrobial resistance [15, 16]. Innovative strategies to prevent this disease have been proposed and included the use of microorganisms capable of producing inhibitory compounds,

such as bacteriocins, which are ribosomally synthesized antimicrobial peptides [17]. Bacteriocins produced by lactic acid bacteria (LAB) have been classified into different groups. The criteria for these classifications include the producing strain, molecular weight, structure and post-translational modifications as proposed by Cotter et al. [18] and Mokoena [19]. Recent reviews have highlighted the capability of bacteriocins to fight against *Clostridia* [20–22]. LAB-bacteriocins possess numerous incentives, particularly their ability to act specifically against some pathogens without any deleterious effect on the commensal gut microbiota [21, 23]. Thus, they represent potential candidates for the development of a useable agent to combat NE.

The development of nanomaterials as drug carriers [24] has experienced considerable progress in recent years [25], and in this paper we report on our evaluation of a combination of a LAB bacteriocin with a polymeric nanoparticle preparation. Natural polymeric substances such as alginates offer many advantages like their stability, safety, biocompatibility, and biodegradability [26]. Studies on nanomaterials are rising worldwide for their use as drug-delivery systems. Alginate is one of the most commonly used as natural polysaccharide in drug delivery [27].

Enterococcus faecalis 14 was shown to display a broad spectrum of antimicrobial activity against Gram-positive pathogenic bacteria such as *C. perfringens* [28, 29]. *E. faecalis* 14 produces a leaderless two-peptide bacteriocin, referred as EntDD14. EntDD14 is composed of two peptides, namely EntDD14A and EntDD14B, which are potentially active in the prevention of *C. perfringens* infections and NE in broiler chickens [29].

This study aimed at establishing the safety of the EntDD14 loaded onto alginate nanoparticles (EntDD14/Alg NPs) and demonstrate the potency of this formulation against the *C. perfringens* Clin1 strain isolated from a NE infected chicken.

Materials and methods

Preparation of alginate nanoparticles

Alginate nanoparticles (Alg NPs) were obtained by a ball milling method (RETSCH- PM100 CM). Briefly, 2 g of sodium alginate (Sigma Aldrich, St Louis, MO, USA) were introduced into a 1.05 kg grinding jar for 10 h in contact with 112 g of zirconium oxide beads (3×20mm + 10×10mm). Rotation speed was fixed at 400 rpm at room temperature. To avoid aggregation of the Alg NPs, the grinding process was alternated by cycles of 10 min grinding followed by 10 min air cooling.Characterization of alginate nanoparticles

UV-vis

The UV-vis absorbance spectra were measured using a Perkin Elmer Lambda UV/vis 950 spectrophotometer (Waltham, MA, USA) with an optical path of 10 mm in a 1 cm quartz cuvette. 0.3 mg of each sodium alginate precursor and Alg NPs were dissolved in 1 mL of aqueous solution. The wavelength range was 200–600 nm.

Fourier transform infrared spectroscopy (FTIR)

FTIR analysis was performed on a Thermo Fisher Scientific, Nicolet 380 (Waltham, MA, USA). The alginate and Alg NPs were analyzed, separately, in the IR radiation in a pellet form, which is made by mixing 1 mg of the sample with 175 mg of potassium bromide (KBr). After that, the mixture was grounded properly to ensure uniform distribution in the KBr base. Finally, the grounded mixture was pressed in a hydraulic press by applying a pressure of ~7 to 9 tons.

X-ray photoelectron spectroscopy (XPS)

The chemical composition of alginate and Alg NPs was studied by XPS measurements using a Thermo Fisher Scientific ESCALAB Xi+ spectrometer with a monochromatic Al Kα X-ray source (1486.6 eV) and a spherical energy analyzer that operates in the CAE (constant analyzer

energy) mode using the electromagnetic lens mode. The CAE values for survey and high-resolution spectra are 100 and 50 eV, respectively.

Particles size distribution and zeta potential

Particles size (and zeta potential) was determined by dynamic light scattering (DLS) measurements using a Malvern Zeta sizer Nano ZS instrument (Malvern Instruments Inc., Worcestershire, UK), equipped with a 4 mV He-Ne laser emitting at 633 nm. The capillary cell was pre-rinsed with ethanol and purified water prior to loading samples. Samples were dissolved in Milli-Q water at a concentration of 0.5 mg/mL.

Scanning electron microscopy (SEM)

Scanning electron microscopy (SEM) was used to examine the particle size and morphology of the Alg NPs. A silicon wafer (1 cm^2) was washed sequentially with water, ethanol and acetone in an ultrasound bath for 10 min. Then, 20 µL of Alg NPs (at 0.1 mg/mL) was poured onto the surface of the wafer and dried overnight in a clean box. Then the samples were examined by electron microscopy (ULTRA 55 Carl Zeiss, Bordeaux, France) equipped with a thermal field emission emitter and three detectors (high-efficiency In-lens SE detector, EsB detector with filter grid, Everhart-Thornley secondary electron detector and high-efficiency In-lens SE detector).

Bacterial strains and growth conditions

E. faecalis 14, previously isolated from meconium and characterized as bacteriocinogenic strain [28] was grown in M17 broth medium (Sigma Aldrich) with 0.5 % glucose (Sigma Aldrich) at 37ºC for 18 to 24 h. Several *Clostridium* strains were used as target organisms. *C. perfringens* DSM756 /ATCC®13124TM (type A, α+), *C. perfringens* NCTC6785, an α + type A strain from human origin (EF, 1941), *C. perfringens* ICVB088 and *C. perfringens* ICVB089, which both were isolated from a chicken deceased from NE. These strains, including *C. perfringens* Clin1,

were kindly provided by ANSES (French Agency for Food, Environmental and Occupational Health & Safety, Ploufragan, France). All *Clostridium* strains were grown on brain–heart infusion (BHI) (Sigma Aldrich) at 37ºC under anaerobic conditions (AnaeroGen^{tm} 2.5, Thermo Fisher Scientific).

Purification of EntDD14

EntDD14 was purified to homogeneity, using a two–step procedure adapted from protocol described by Abriouel et al. [30]. *E. faecalis* 14 was grown in 200 mL of M17 broth (Sigma Aldrich), supplemented with glucose (0.5 %) and buffered with 60 mM sodium phosphate (pH 6.3) at 37°C for 24 h with 160 rpm continuous shaking. After harvesting by centrifugation (8.228 ×*g.*, +4°C, 10 min), the cell-free supernatant was filtrated throughout 0.2 µm filter and incubated 24 h at room temperature (20-25°C) with CM Sephadex® C-25 resin (GE Healthcare Life Sciences, Chicago, IL, USA). The resin was then washed with 100 mL of distilled water and 20 mL of 0.5 M NaCl. The resin-bound EntDD14 was eluted with 40 mL of 1.5 M NaCl. The removal of the salt from the solution containing EntDD14 was achieved by gel filtration using PD MidiTrap G-10 column (GE Healthcare Life Sciences), following the manufacturer's instructions.

Characterization of the purified fraction

The EntDD14 purity was checked using reverse-phase high performance liquid chromatography (HPLC) through a Vydac® 218TP C18 column (Grace, Columbia, MD, USA) equilibrated with 0.1% (v/v) trifluoroacetic acid (TFA). EntDD14 was eluted with a linear gradient of acetonitrile and isopropanol (2:1, v/v). The EntDD14 concentration was measured after each purification step using the QuantiPro BCA (bicinchoninic acid) assay kit (Sigma Aldrich), and its antibacterial activity was tested throughout the purification procedure.

Matrix-Assisted Laser Desorption/Ionisation Time-Of-Flight Mass Spectrometry (MALDI-TOF/MS)

MALDI-TOF/MS analyses of purified EntDD14 were performed using an autoflex^{TM} speed (Bruker Daltonics, Bremen, Germany) running flex Control 3.3 (Bruker Daltonics). The instrument positive reflector mode was calibrated using 8 proteins (MBT standard). Spectra were obtained in the range of 3,500 to 6,000 Da. MS spectrums were acquired using positive linear mode or positive reflectron mode by accumulating data from 5,000 laser shots. For analysis, a mixture of 5 µL of purified EntDD14 and 5 µL of α-cyano-4-hydroxycinnamic acid (10 mg/mL 70:30 water/acetonitrile with 0.1% TFA) was put as spot onto a MALDI-TOF MTP 384 target plate (Bruker Daltonics) and analyzed after drying.

Preparation of EntDD14 loaded on alginate nanoparticles (EntDD14/Alg NPs)

A solution containing purified EntDD14 (150 µg) was adjusted at pH 6 with 1 M sodium hydroxide prior to addition of 1 mg of Alg NPs. The solution was introduced in an ultrasound bath (FS100B, Decon Laboratories Ltd, Hove, UK) for 45 min at 50/60 Hz. The temperature of the bath was maintained below 30°C, in order to limit the aggregation of Alg NPs. The amount of EntDD14 loaded on the Alg NPs was determined after the separation by centrifugation (15,500 × *g*, +4°C, 10 min) of EntDD14/Alg NPs from the solution containing free-EntDD14. The amount of free EntDD14 in the supernatant was measured using Quantipro BCA assay kit (Sigma Aldrich). The amount of EntDD14 loaded on Alg NPs was calculated as the difference between the initial amount of EntDD14 used for the formulation and the amount of EntDD14 remaining in the supernatant. The percent of loading efficiency was calculated by the following formula:

$$\% \text{ Entrapment efficiency } = \frac{\text{Total amount of drug added} - \text{Nonbound drug}}{\text{Total amount of drug}} x\ 100$$

Activity of purified EntDD14 against *C. perfringens*

Anti-*C. perfringens* activity assays of the cell-free supernatant (CFS) and purified fractions were performed using the agar well diffusion method [31]. A uniform layer, using McFarland standard 1, of the target strains was spread on the BHI agar plates using a swab (Copan Italia, Brescia, Italy). Then, a 5 mm diameter wells were punched using a sterile pipette. Then, 50 µL of cell-free and purified fractions, and their serial dilutions, were introduced, separately, into the wells. The plates were incubated overnight at 37°C under anaerobic conditions. Each zone of inhibition was then measured and the corresponding activity was expressed in AU/mL, which is the inverse of the last active dilution multiplied by 20.

Determination of the Minimum Inhibitory Concentration (MIC)

An isolated colony of each *C. perfringens* strain used in this study was grown overnight in BHI medium at 37°C under anaerobic conditions. All *C. perfringens* strains were brought to equal density at approximately 10^7 CFU/mL by measuring the bacterial suspensions optical densities at 600nm using a spectrophotometer. Afterward, each strain was spread separately on the surfaces of the BHI (Sigma Aldrich) agar plates using a swab to form a uniform layer. Five concentrations (7.5, 15, 30, 60 and 120 µg/mL) of each EntDD14 and EntDD14 loaded on Alg NPs were prepared. Then, 50 µL of each concentration was pipetted onto the plate's surface. After overnight incubation at 37ºC, the MIC was determined according to the CLSI recommendations [32].

Killing curves

The killing curves were performed on the pathogenic strain *C. perfringens* Clin1 using the protocol described Al Atya et al. [33] with some modifications. Tubes of BHI medium containing defined concentrations of EntDD14 (60 µg/mL) or EntDD14 (30 µg/mL)/Alg NPs (0.25 mg/mL) were inoculated with the Clin1 strain at a density of about 10^7 CFU/mL in a final volume of 4 mL and incubated for 0, 2, 4, and 6 h at 37°C under anaerobic condition. The killing kinetics of free EntDD14 and EntDD14/Alg NPs were assessed against the *C. perfringens* Clin1 strain using standard killing over time experiments and viable bacterial counts on BHI agar plates. The final concentrations were 60 µg/mL and 30 µg/mL, respectively, for EntDD14 and EntDD14/Alg NPs (0.25 mg/mL). These experiments determined the effect of the studied bacteriocin-nanoparticles formulations on the number of living bacterial cells able to form colonies after treatments.

Bactericidal activity was determined regarding the reduction of colony count after each treatment, expressed in $\log_{10}$ CFU/mL, compared to the initial inoculum [34]. Each culture was sampled after 0, 2, 4, and 6 h of incubation, and all were serially diluted in saline solution to determine viable cell counts. These dilutions (100 µL) were plated on BHI agar plates and incubated overnight at 37°C under appropriate conditions, after which colonies were counted.

Transmission Electron Microscopy (TEM)

Similarly, the impacts of EntDD14, EntDD14/Alg NPs and Alg NPs alone on *C. perfringens* DSM 756/ATCC®13124TM were observed by TEM. The *C. perfringens* strain was incubated overnight at 37ºC then the Alg NPs (0.5 mg/mL) and EntDD14 (60 µg/mL), free or loaded on the Alg NPs, were added. Additionally, phosphate buffer solution (PBS) was used as a negative control. Samples were fixed using 2.5 % (v/v) glutaraldehyde solution and 0.1 M (v/v) of CaCo

buffer (pH 7.4). *C. perfringens* DSM756 treated, or not, with EntDD14 alone or associated with the Alg NPs, and Alg NPs alone was prepared on a Formvar film of 300 square mesh, nickel grid (EMS FF300-Ni). The TEM images were recorded using a JEOL JEM 2100FX TEM instrument (Tokyo, Japan), equipped with a GATAN CCD Orius 1000 camera and a GATAN CCD Orius 200D camera (Pleasanton, CA, USA), at an acceleration voltage of 200 KV.

Stability of EntDD14 and EntDD14/Alg NPs

In vitro multi-compartmental GIT simulation models were used to study the stability of purified EntDD14 alone and EntDD14/Alg NPs.

In simulated Chicken Gastrointestinal Tract (GIT)

The simulated chicken GIT model was prepared as previously reported [35, 36]. To simulate the gastric compartment (gizzard), a solution of 1 mg/mL of pepsin (Sigma Aldrich) was prepared in PBS containing 240 µg/mL of purified EntDD14 or that of EntDD14 (120 µg/mL) loaded on Alg NP (1 mg/mL). The pH was adjusted to 3.0 using 0.5 M HCl, and the obtained solution was incubated at 42°C for 90 min under agitation (160 rpm). After that, the same solutions were adjusted to pH 7 using 2 M NaOH and supplemented with1 mg/mL pancreatin (Sigma Aldrich), in order to simulate the intestinal compartment prior to incubation for 3 more hours at 42°C under agitation (160 rpm). After each step, a sufficient volume was taken to test antibacterial activity against the *C. perfringens* Clin1 strain.

In simulated human Gastrointestinal Tract (GIT)

Based on previous reports [37, 38], we used a simulated human GIT, with a gastric compartment solution containing 2 mg/mL of pepsin (Sigma Aldrich) in PBS adjusted at pH 3.5 with 0.5 M HCl, incubated at 37°C with continuous agitation (160 rpm) for 90 min, followed by a simulated duodenal compartment solution with addition of 2 mg/mL of pancreatin (Sigma Aldrich) after

pH adjustment to 7.0 and a further incubation of 3 h under the same conditions. As for the simulated chicken GIT, 240 µg/mL of EntDD14 alone or EntDD14 (120 µg/mL) loaded on Alg NPs were tested. After each step, a sufficient volume was taken to test antibacterial activity against *C. perfringens* Clin1 strain.

Cytotoxicity Assays

The effects of EntDD14, EntDD14/Alg NPs, and Alg NPs alone were assessed on Caco-2 and HT29 human colon cancer cell-lines using the protocol described by Al-Seraih et al. [39] with slight modifications. The two cell lines were grown in Dulbecco's Modified Eagle's Medium (DMEM) (PAN-Biotech GmbH, Aidenbach, Germany) at 37°C in a 5% CO_2 atmosphere. Cells were grown for 72 h in 2 subsequent subcultures and were transferred into 96-well tissue culture plates at a density of 15,000 cells/well, and were grown for an additional 72-96 h.

For cytotoxicity assays, 100 µL of the appropriate concentration of each sample (purified EntDD14, EntDD14/Alg NPs and Alg NPs alone) were added to each well of the 96-well tissue culture plates containing the Caco-2 cell and HT29 cell monolayers, and were incubated for an additional 24 h. The viability of the Caco-2 and HT29 cells was assessed using the CCK-8 assay (Dojindo Molecular Technologies, Kumamoto, Japan) by adding to each well 5 µL of CCK-8 reagent in 100 µL of supplemented DMEM. Following 2 h of incubation, the absorbance of each well was measured at 450 nm in a spectrophotometer (SAFAS Xenius; SAFAS Monaco, Monaco). Results were expressed as the percent viability compared to non-treated cells.

The Impact of EntDD14 and EntDD14/Alg NPs on the expression of genes coding for virulence factors

A reverse transcriptase quantitative PCR (RT-qPCR) analysis was carried out to study the impact of the different formulations associating EntDD14 and Alg NPs, prepared during this study, on

the expression of the most important *C. perfringens* Clin1 toxin-encoding genes, including *plc* (toxin α), *netB* (enteritis B-like toxin), *cnaA* (collagen adhesion protein A) and *pfoA* (thiol activated cytolysin). The expression levels of these genes were assessed after treatment of the *C. perfringens* Clin1 strain with sub-inhibitory concentrations of EntDD14 (MIC/2 = 30 µg and MIC/4 =15 µg), EntDD14 loaded on Alg NP (MIC/2 = 15; 125 µg and MIC/4 = 7.5; 62.5 µg) and PBS as a negative control.

The clinical isolate *C. perfringens* Clin1 was grown in 10 mL of BHI broth (Sigma Aldrich) at 37°C in anaerobic conditions for 24 h. After harvesting by centrifugation (8,000 × *g*, +4°C, 10 min), the recovered cells were re-suspended in 10 mL of fresh BHI. Five samples were prepared, each containing 1 mL of bacterial culture plus 1 mL of the aforementioned formulation (including EntDD14 alone or EntDD14/Alg NPs) at MIC/2 or MIC/4 and using PBS as a negative control. The five samples were incubated overnight at 37°C in anaerobic conditions as described previously. After 12 h of incubation, 20 µL was withdrawn from each sample to investigate the cell viability and the remaining cells were harvested (8,000 × *g*, +4°C, 10 min). Total RNAs was extracted from three independent samples of each condition tested using NucleoZol (Macherey-Nagel, Duren, Germany), and the cDNA was gathered with the RevertAid RT Reverse Transcription Kit (Thermo Fisher Scientific). For the qPCR, specific primers were designed using sequences of known *C. perfringens* toxin encoding genes retrieved from the NCBI database (https://www.ncbi.nlm.nih.gov/). Then Primer3 software (http://frodo.wi.mit.edu/primer3/) was used to generate the specific primers for each gene, thus generating amplicons of a normalized length of ~100 bp. Additional primers allowing amplification of specific parts of 16s cDNA, obtained from mRNAs, were designed following the same procedure to provide internal controls (Table 1). For each cDNA sample, the CFX

Connect Real-Time PCR Detection system (Bio-Rad Laboratories, Marne la Coquette, France) was used to perform amplification (using 2 µL of a 1:5 cDNA dilution), detection (with automatic calculation of the threshold value), and real-time analysis. Then a comparative cycle time data with analysis modules from CFX Manager™ Software Version 3.1, allowed to determine the relative mRNA levels for each gene and in each condition used.

Statistical analysis

Data obtained with the RT-qPCR experiments were expressed as a mean ± standard error calculated from three independent experiments. Analysis of statistical significance was performed by one-way ANOVA and the post-hoc Tukey Test ($p< 0.05$). Principal component analysis was carried out using R version 3.5.2 (r-project.org, R foundation for statistical computing).

Results

Characterization of alginate nanoparticles (Alg NPs)

The Alg NPs investigated in the present study were prepared from sodium alginate using a simple ball milling process at a gram-scale. The UV-vis analyses of sodium alginate precursor and Alg NPs revealed that the two forms exhibit similar spectral features with weak absorption band at approximately 265 nm (Figure S1a), which could be attributed to the carbonyl groups [40]. The results clearly indicate that the ball milling did not alter the polymer structure.

Fourier-transform infrared (FTIR) spectroscopy was performed to detect either the appearance of new chemical bonds or the modification of existing ones upon the ball milling process. FTIR spectra of sodium alginate precursor and Alg NPs display similar characteristics bands at 3 410 cm^{-1} (OH stretching), 2 928 cm^{-1} (CH_2 stretching), 1 635 cm^{-1} (asymmetric stretching vibration of

COO groups), 1 419 cm^{-1} (symmetric stretching vibration of COO groups), and 1 050 cm^{-1} (elongation of C-O groups) (Figure S1b) [41].

The chemical composition of sodium alginate before and after ball milling was assessed using X-ray photoelectron spectroscopy (XPS) measurements. This analysis showed the high-resolution XPS spectrum of the C_{1s} of the sodium alginate precursor (Figure S2a), which can be deconvoluted into three components at 284.9 eV (C-C/C-H), 286.3 eV (C-O) and 288.0 eV (COO). Similarly, the core level spectrum of the C_{1s} of Alg NPs can be curve-fitted with three bands at 284.9 eV (C-C/C-H), 286.5 eV (C-O) and 288.2 eV (COO)(Figure S2c). The O_{1s} spectra of sodium alginate precursor and Alg NPs showed the contribution of various functional groups at 531.2 eV (C=O/O-H), 532.2 eV (C-O) and 535.0 eV (COOH) (Figure S2b, d), which is in accordance with the composition of the alginate [42]. Similarly, the core level spectrum of the O_{1s} of Alg NPs can be curve-fitted with three bands at 530.0 eV (C=O/O-H), 531.5 eV (C-O) and 534.6 eV (COOH). The atomic percentages (at. %) of C_{1s} and O_{1s}, calculated from the survey spectrum of sodium alginate precursor, are 78.39 at.% and 21.61 at.%, respectively. After the ball milling process, a decrease of the atomic percentage of the C_{1s} (64.03 at. %) and an increase of the atomic percentage of the O_{1s} (35.96 at.%) were observed (Table 2). The agreement between the expected values (belongs to the sodium alginate precursor) and the measured ones (belongs compositions) revealed that the production of Alg NPs by ball mill method did not lead to a significant change in their surface structure compared to the sodium alginate precursor (control polymer). The slight change in the atomic percentage between the Alg NPs and the sodium alginate precursor could be attributed to the oxidation of samples.

The Alg NPs morphology was analyzed using scanning electron microscopy (SEM) (data not shown) and reveals that the Alg NPs have irregular form with an average size of 172 nm.

Dynamic light scattering (DLS) analyses showed the formation of Alg NPs with an average size of 172 nm, which is lower than the size of Alg NPs obtained by desolvation technique (290 nm). Also, the polydispersity index (0.466) of Alg NPs is comparable to that prepared by ionic gelation method [43]. The zeta potential of the Alg NPs was determined to be -20 mV. After loading EntDD14 of the Alg NPs, the average size increased to 203 nm.

EntDD14 is active against a wide range of *C. perfringens* strains

E. faecalis 14 CFS and EntDD14 exhibited antimicrobial activity against all of the tested strains of *C. perfringens* (data not shown). The *C. perfringens* ICVB088 strain showed the highest sensitivity to pure EntDD14, whereas the *C. perfringens* NCTC6785 strain was less affected, based on the zone diameters observed of 9 mm and 6 mm, respectively. These data indicate that the activity of EntDD14 was exerted in a strain-dependent manner.

DD14 purification, loading on Alg NP and MIC values

The method from Abriouel et al. [30] was used for the purification of EntDD14, leading to high purification yields. Indeed, this approach produced 2 mg of pure EntDD14 from 200 mL of *E. faecalis* 14 culture (Table 3). Previously, Caly et al. [29] showed that the EntDD14 is composed of two peptides bacteriocin, named EntDD14A and EntDD14B, with very close molecular masses. The specific activity of supernatant increased from 13 AU/mg to 24 AU/mg and then to 320 AU/mg after the two-steps of the purification process.

Two (2) mg of EntDD14 was collected and used for loading onto Alg NPs and subsequently to determine the MIC values of EntDD14 and EntDD14/Alg NPs against five strains of *C. perfringens* (listed in Table 4). The loading efficiency of DD14 on the Alg NPs was about 85% of the initial mass (150 µg) of EntDD14 that was loaded on 1 mg of Alg NPs. For the MIC values, all the studied strains were found to have the same MIC (60 µg/mL) for EntDD14 alone. This differs from the MIC values determined when EntDD14 was loaded on Alg NPs. While the

MIC values decreased from 60 µg/mL to 30 µg/mL for *C. perfringens* Clin1 and *C. perfringens* ICVB088, those of *C. perfringens* ICVB089 decreased from 60 µg/mL to 15 µg/mL. Nevertheless, the MIC values registered for *C. perfringens* DSM756 and *C. perfringens* NCTC6785 strains remained unchanged (Table 4). The amoxicillin used as positive control enabled us to know how the target strains are sensitive to a conventional drug that is frequently used in the veterinary medicine [44].

Killing curves kinetics confirmed the added value of Alg NP.

The killing curve experiments with *C. perfringens* Clin1 as target strain, treated with EntDD14 and EntDD14/Alg NPs, showed that the EntDD14 loaded Alg NPs have a more efficient antibacterial activity, compared to EntDD14 alone. As expected, the aforementioned enterocin have improved activity with Alg NPs. The cell-number as means of CFU/mL have decreased, comparatively, to that resulting from EntDD14 treatment alone at a concentration of 60 µg/mL. Remarkably, EntDD14 (30 µg/mL) loaded on Alg NPs (0.25 mg/mL) reduced the CFU/mL counts of *C. perfringens* Clin1 by at least 1-1.5 logs during 2–6 h of incubation, leading to the killing of high percentage of the CFU/mL of the initial bacterial populations (Figure 1).

TEM confirmed the enhancement of EntDD14 activity by Alg NPs

TEM analysis revealed that Alg NPs alone had no visible impact on the *C. perfringens* DSM 756 strain cells (Figure 2, panel B and F). Nevertheless, EntDD14 alone at a concentration of 60 µg/mL induced a slight membrane damage of the target *C. perfringens* Clin1 strain compared to the untreated sample (Figure 2, panel C and G). Remarkably, the sample treated with EntDD14/Alg NPs displayed high membrane damage, consisting in the leakage of cellular contents, whole disappearance of the cell membrane, as well as cytoplasm retraction (Figure 2, panel D and H).

Stability of EntDD14 and EntDD14 loaded on Alg NPs during their passage in the simulated chicken and human GIT

The anti-*C. perfringens* activity of EntDD14 at 240 µg/mL ceased after incubation in the simulated gastric conditions of both chicken (1 mg/mL pepsin, pH 3, 90 min at 42°C) and human (2 mg/mL pepsin, pH 3.5, 90 min at 37°C). Of note, the anti-*C. perfringens* activity was registered when the bacteriocin EntDD14 was loaded on Alg NPs at a ratio of 120/1 mg/mL. However, this activity was abolished for the bacteriocin EntDD14 alone, or EntDD14 adsorbed on Alg NPs under conditions mimicking the intestine of chicken (1 mg/mL pancreatin, pH 7, 180 min at 42°C) and human (2 mg/mL pancreatin, pH 7, 180 min at 37°C).

EntDD14, EntDD14 loaded on Alg NPs and Alg NPs are not cytotoxic.

The cytotoxicity of EntDD14, Alg NPs, and DD14/Alg NPs were tested on Caco-2 and HT29 cells for 24 h of contact. For the HT29 cells, no apparent cytotoxicity was observed after 24 h of contact between EntDD14 and HT29 cells, as shown on Figure 3a. Moreover, EntDD14 loaded on Alg NPs also revealed to be non-cytotoxic as well towards HT29 cells in the concentration range of 60 µg/mL to 120 µg/mL for EntDD14 and between 0.5 mg/mL and 1 mg/mL for Alg NPs (Figure 3a).

In the case of Caco-2 cells, Alg NPs were not cytotoxic in the concentration range between 0.5 mg/mL to 1 mg/mL. EntDD14 loaded on Alg NPs in the concentration range between 60 µg/mL and 120 µg/mL was also not cytotoxic (Figure 3b).

Impact of EntDD14 and association of EntDD14 and nanoparticles on virulence genes expression in *C. perfringens* Clin1

The counting of viable and cultivable *C. perfringens* Clin1 cells treated with sub-inhibitory concentrations of the EntDD14 and the EntDD14/Alg NPs formulation indicated a stable population of 8.3 to 9.3x10^7 CFU/mL in all tested conditions (data not shown).

Regarding the qPCR results, EntDD14 tested at MIC/2 (30 µg/mL) induced an increase of the transcripts of *plc* (toxin α), *netB* and *pfoA* (Figure 4 a,b,c) varying from 2.4 to 3.6-fold of the control. However, the *cnaA* transcript was downregulated under this condition to 0.1-fold of the level of the untreated *C. perfringens* Clin1 cells (Figure 4d). Interestingly, at MIC/4 (15 µg/mL), the effect of the EntDD14 is gene dependent, except for *cnaA* for which we observed the same behavior regardless the EntDD14 concentration (Figure 4d). In the case of *netB*, a significant increase of the transcript level was recorded, but limited to 1.5-fold the level of the untreated control (Figure 4b). Conversely, at this concentration, the EntDD14 inhibited the expression of the *plc* gene encoding α-toxin, leading to a three-fold decrease (Figure 4a). Regarding *pfoA*, at this concentration, EntDD14 did not seem to affect the transcript level of this gene (Figure 4c)

Interestingly, the association of EntDD14 and Alg NPs at MIC/2 and MIC/4 values, (15+125 µg/mL and 7.5+62.5 µg/mL) caused clearly a significant decrease in transcript levels of all toxins encoding genes (Figure 4). The mRNA levels of *plc*, *netB*, *pfoA* and *cnaA* genes were almost abolished, except for the expression of α-toxin gene (*plc*) which remain unchanged after treatment with the Alg NPs at MIC/4 (Figure 4a). Their levels of expression ranged from 0.02 to 0.19, which is 5 to 50 lower than that of the untreated control sample. Notably, no amplification was obtained for other toxins genes like *tpeL*, *netE*, *netF* and *netG* (data not shown), indicating the absence of these genes in *C. perfringens* Clin1 strain.

Discussion

Antimicrobial resistance (AMR) has become a major concern for public health in the world. In France, 125,000 infections occur yearly and cause 5,500 deaths [45]. To tackle this acknowledged problem, ambitious, collaborative and coordinated programs are needed. In the O'Neil report [46], there is a list of recommendations to face the AMR phenomenon. With respect to that, we suggest including nanomaterials as a means to fight AMR. Nanoparticles can be used as efficient delivery vectors, because of their small size and enhanced surface area [47, 48]. Dendrimer nanoparticles, liposomes, metallic, micellar, carbon nanoparticles and polymeric nanoparticles have been used for animal reproduction, nutrition, animal treatment and drug delivery [49]. Nonetheless, the use of nanoparticle compounds such as Ag NPs or ZnO NPs are limited because of their toxicity [50, 51].

Here, we have shown the appropriateness of the alginate polymer, which is a safe material that can be used as an enhancer of antibacterial activity of bacteriocins such as EntDD14. Notably, the process of loading EntDD14 on the alginate polymer does not require any chemical product as a cross-linking agent, which makes this formulation easy and straight forward. Moreover, these nanoparticles have been deeply characterized using FTIR, XPS measurements and DLS and Zeta potential to better understand their charge, size and shape following EntDD14 adsorption. As reported in the results section only very slight modifications have been observed in the size of nanoparticles and the overall charges.

Recently, we assessed the anti-*C. perfringens* activity of EntDD14 alone and established its MIC value to be 60 μg/mL for the *C. perfringens* Clin1 strain [29]. This activity, as shown here, is improved when EntDD14 is loaded on Alg NPs. In a previously published report, Zohri et al. [52] demonstrated that the anti-*Staphylococcus aureus* activity of nisin is enhanced when this

well-known bacteriocin was loaded onto chitosan/Alg NPs. Notably, nanoparticles used were composed of a mix of two different polymers and the characterizations (size, zeta potential and shape of particles) performed by SEM and DLS were related to both materials.

The approach developed here shows the incentives of interactions between EntDD14 and Alg NPs and the resulting formulation could be suggested as a means to treat the NE disease caused by *C. perfringens* in chickens. This promising *in vitro* finding will need to be strengthened by further data such as the toxicity, and the stability of its activity under conditions mimicking the GIT of birds. Therefore, we prepared a simulated GIT model representing the gastric and intestinal compartments with their associated conditions such as the pH variance, temperature and proteolytic enzymes in order to assess the stability of EntDD14 and that of EntDD14/Alg NPs.

Bacteriocins are of proteinaceous nature, and can be easily degraded by proteolytic enzymes during their passage in the GIT [53]. With regard to that, we observed that the anti-*C. perfringens* activity of EntDD14 was abolished under conditions simulating its passage through the gastric compartment. This loss of activity happened in both the human and chicken models. Importantly, the new formulation resisted these harsh conditions, demonstrating the ability of Alg NPs to protect such activity from the gastric acidity and pepsin activity. However, it should be noted that EntDD14 alone, or loaded on Alg NPs, lost its activity under the conditions simulating the intestinal compartment, under the action of the pancreatin and more particularly to the action of the proteases, which is in accordance to the data reported by Caly et al., [29]. This means that Alg NPs were not able to provide protection against the conditions mimicking the proximal small intestine of both humans and chickens. Additional experimental design is in

progress to enhance the resilience of EntDD14 loaded on Alg NPs and we hope to report this in the near future.

Another important point revealed in this study is the absence of toxicity towards human cells. Of note, the newly developed nano-antibiotic formulation developed here was safe with respect to eukaryotic cells, and resulted to be more effective than EntDD14 alone, but not than amoxicillin; which is as previously indicated as a commonly used drug for treatment of NE caused by *C. perfringens* [44]. Alg NPs offer advantages over other materials such as nanoparticles generated from NiO and Bi_2O_3, which induced more than 40% of cell death on the Caco-2 cell line and the HT29 cell line at a lower concentration (100 µg/mL) [54, 55]. On the other hand, nisin A induced 50% loss of the Caco-2 cell viability (MTT assay) at a concentration of 385 µg/mL [56]. Similarly, Ankaiah et al. [57] reported that enterocin-A, conversely to EntDD14, showed more than 56% growth inhibition of Caco-2 cells at a concentration of 120 µg/mL [57].

In this study, we have demonstrated the potency of the formulation developed by loading EntDD14 onto Alg NPs against *C. perfringens* target strains. We have also established by RT-qPCR the level of expression of several genes coding for the major toxins [7] found in pathogenic strains. The level of expression of *netB* and α-toxin genes is expected to play a determinant role in the NE infection, which is enhanced by predisposing factors such as *Eimeria* coccidial infection and a fishmeal diet [58]. These genes, which are involved in the occurrence of NE disease in chickens [6, 8], are affected by the addition of EntDD14/AlgNPs. This new formulation affected also the expression of other toxine encoding genes such as *pfo*, and *cnaA*.

Bacteriocins are mainly known for their ability to disturb the bacterial cell envelope through different mechanisms, such pore formation or interrupting cell wall synthesis [17]. Interestingly, Wang et al. [59] reported the effects of plantaricin LPL-1, a class IIa bacteriocin, on expression

of virulence factors in the malevolent *Listeria monocytogenes* strain. This bacteriocin was shown to down-regulate expression of several virulence factors genes, such as *hlyA, prfA, iap* and *actA*. The regulation of genes coding for toxins in *C. perfringens* strains are under the control of a two-component system (TCSs) including a membrane-bound sensor histidine kinase and a cytoplasmic response regulator which plays the role of a transcriptional regulator [60]. Different studies underpinned the role of the two component-system VirS/VirR in the regulation of such genes implied in the pathogenicity of *C. perfringens*. These genes include *plc*, *colA* and *pfoA* genes coding respectively for the synthesis of α, κ and θ-toxins [61]. Further genes such as *netB*, *cna* and *cpb2* genes, coding for NetB toxin, collagen-adhesin protein and beta-2 toxin, respectively, are as well related to the two component-system VirS/VirR [61, 62]. In this system, the VirS membrane sensor decrypts environment stimuli, including the presence of nutrient, other bacterial cells and even host cells [60, 63, 64]. Nonetheless the molecular mechanism by which bacteriocins could interact and influence the regulation of these genes in *C. perfringens* remain to be determined. In the present study, we report a down-regulation of genes coding for toxins in C*lostridium* following its treatment with a safe formulation based on EntDD14 loaded onto alginate nanoparticles (EntDD14/Alg NPs). Furthermore, we noticed that the relationship between concentration of bacteriocin-nanoparticles formulations and the effects on genes transcript levels are not linear and are gene specific regardless the reported MIC values which are at least two folds higher.

Conclusion

Upon loading EntDD14 on Alg NPs, we obtained a nano-formulation, named EntDD14/Alg NPs with improved anti-*C. perfringens* activity. At sub-inhibitory concentrations, we observed that

various genes coding for robust toxins, such as toxin α, enteritis toxin B-Like, collagen adhesion protein and thiol activated cytolysin were affected by this nano-formulation. Noticeably, the anti-*C. perfringens* activity of the EntDD14/Alg NPs assessed after incubation under conditions mimicking the human or animal GIT resulted to be stable, and unaffected by acidic pH and pepsin actions. Nevertheless, improvement of its activity under conditions mimicking the intestinal environment remains to be investigated.

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Compliance with ethical standards

Conflict of interest

All the authors declare no conflict interest.

Availability of data and material

All data generated or analyzed during this study are included in this published article [and its supplementary information files].

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Table 1. Primers designed and used in this study for RT-qPCR

Primer	Sequences	Target gene (Function/role)
F-*plc*RT-qPCR	CTGACACAGGGGAATCACAAA	*plc* (Toxin α)
R-*plc*RT-qPCR	TAGTGCATAGCCTCTCCAAGAT	
F-*cnaA* RT-qPCR	TGATGTTGGTGGATGGGCAA	*cnaA* (Collagen adhesion protein)
R-*cnaA* RT-qPCR	ATCTGCCCGCTTTGAACCAG	
F-*tpeL* RT-qPCR	TTTGTCGGAAATGTTGGCGC	*tpeL* (Cytotoxin produced on sporulation phase)
R-*tpeL* RT-qPCR	TAGCTGTCAACCTAAGAAGCCT	
F-*pfoA* RT-qPCR	GAGTGTACAGGCCTTGCTT	*pfoA* (Thiol activatedcytolysin)
R-*pfoA*RT-qPCR	CCAGGATATAAAGTTGTTCCCCA	
F-*netB* RT-qPCR	TGGCTTTAGCATTAACAGCACC	*netB* (Enteritis B-Like toxin)
R-*netB* RT-qPCR	TCGCCATTGAGTAGTTTCCCA	
F-*netE* RT-qPCR	GCGCCAAAAGATGCTAAAGAAT	*netE* (Leukocin/hemolysin toxin family)
R-*netE* RT-qPCR	GAACGTTTATTTGATCCCCTCCA	
F-*netF* RT-qPCR	AGGCACCAAAAGGCACAAAA	*netF* (Leukocin/hemolysin toxin family)
R-*netF* RT-qPCR	ACACGATTTTCTCCCCACCA	
F-*netG* RT-qPCR	ACTGTTGGTGGAGAAATATCAGCT	*netG* (Leukocin/hemolysin toxin family)
R-*netG* RT-qPCR	ACTGTTTTAAAGTCTGGCTGTTCA	
F-16S Cp RT-qPCR	CGGTAATACGTAGGTGGCGA	16s rDNA
R-16S Cp-qPCR	AATGCAGCACCCAAGTTGAG	

Table 2. Atomic percentages of C_{1s} and O_{1s} in sodium alginate precursor and Alg NPs.

Samples	Atomic % of C_{1s}	Atomic % of O_{1s}
Sodium alginate precursor	78.39	21.61
Alginate NPs	64.03	35.96

Table 3. Purification of EntDD14 from a 200 mL culture by cation-exchange (CEX*) and size-exclusion chromatography (SEC**).

Purification step	Total protein (mg)	Total activity (AU)	Specific activity (AU/mg)	Recovery (%)	Increased fold in specific activity
Supernatant	122	1 600	13	100	1
CEX*	50	1 200	24	75	1.8
SEC**	2	640	320	40	24.6

Table 4. The minimum inhibitory concentration (MIC) values of EntDD14, alone or loaded on alginate nanoparticles (EntDD14/Alg NPs). Alginate nanoparticles (Alg NPs) at (1 mg/mL) and amoxicillin were tested, respectively, as negative and positive control against the various target strains.

MIC (µg/mL) *C. perfingens* strain number	Alg NPs	DD14	DD14/Alg NPs	Amoxicillin
ICVB088	>1000	60	15	0.15
ICVB089	>1000	60	30	0.15
Clin1	>1000	60	30	0.15
DSM 576	>1000	60	60	0.3
NCTC6785	>1000	60	60	5

Figure captions

Figure 1. Time-killing kinetics of *C. perfringens* Clin1 at 0, 2, 4, and 6 h in the presence of EntDD14 alone at a concentration of 60 µg/mL or combined with alginate nanoparticles (EntDD14/Alg NPs) at a concentration of 30 µg/mL for EntDD14 and 0.25 mg/mL for Alg NPs.

Figure 2. Transmission electron microscopy (TEM) images of *C. perfringens* DSM 756. (A and E) corresponds to the untreated sample (control). (B and F) shows the sample treated with Alg NPs (0.5 mg/mL), (C and G) corresponds to the sample treated with EntDD14 alone (60 µg/mL) and (D and H) corresponds to the sample conditioned with EntDD14 (60 µg/mL) loaded on Alg NPs (0.5 mg/mL). Black arrows indicate the leakage of cellular contents (G) and/or whole disappearance of the cell membrane (H).

Figure 3. Cell-toxicity of Alg NPs alone, purified EntDD14 alone, or purified EntDD14 loaded Alg NPs on eukaryotic HT29 (a) and Caco-2 (b) cells. Control correspond to untreated HT29 (A) or Caco-2 cells (B).

Figure 4. The relative expressions of a: α-toxin (*plc*); b: *netB*; c: *pfoA* and d: *cnaA*; in *C. perfringens* Clin1, were calculated using the ΔΔCT method and normalized comparatively to 16S rDNA expression. Results are expressed as mean ± standard deviation n=3. Means with different letters are significantly different from the control ($p < 0.05$).

Fig. 1

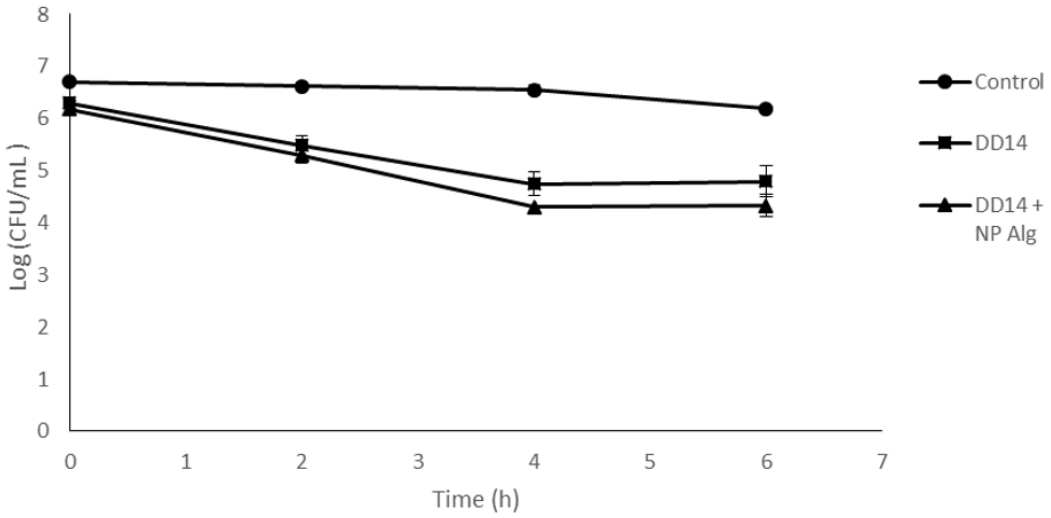


Fig. 2

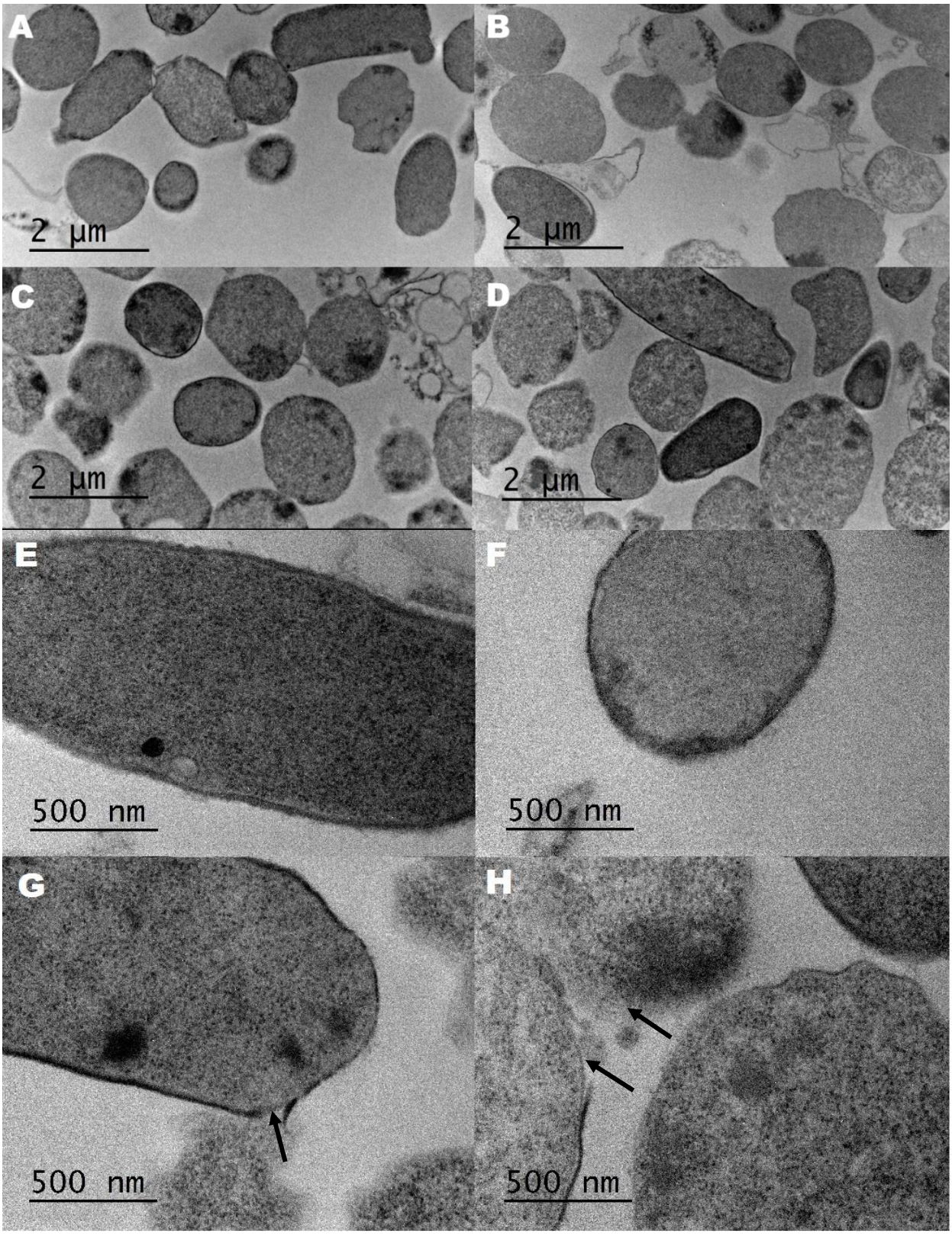


Fig.3

a.

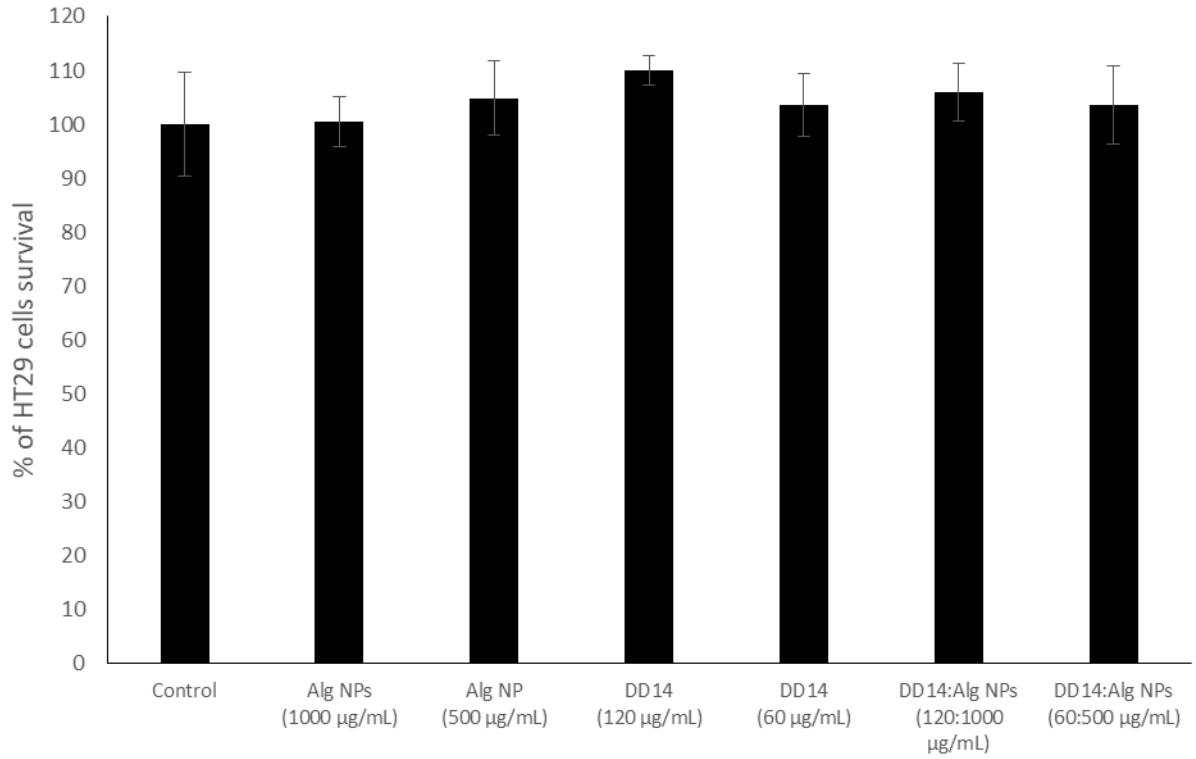


Fig. 3

b.

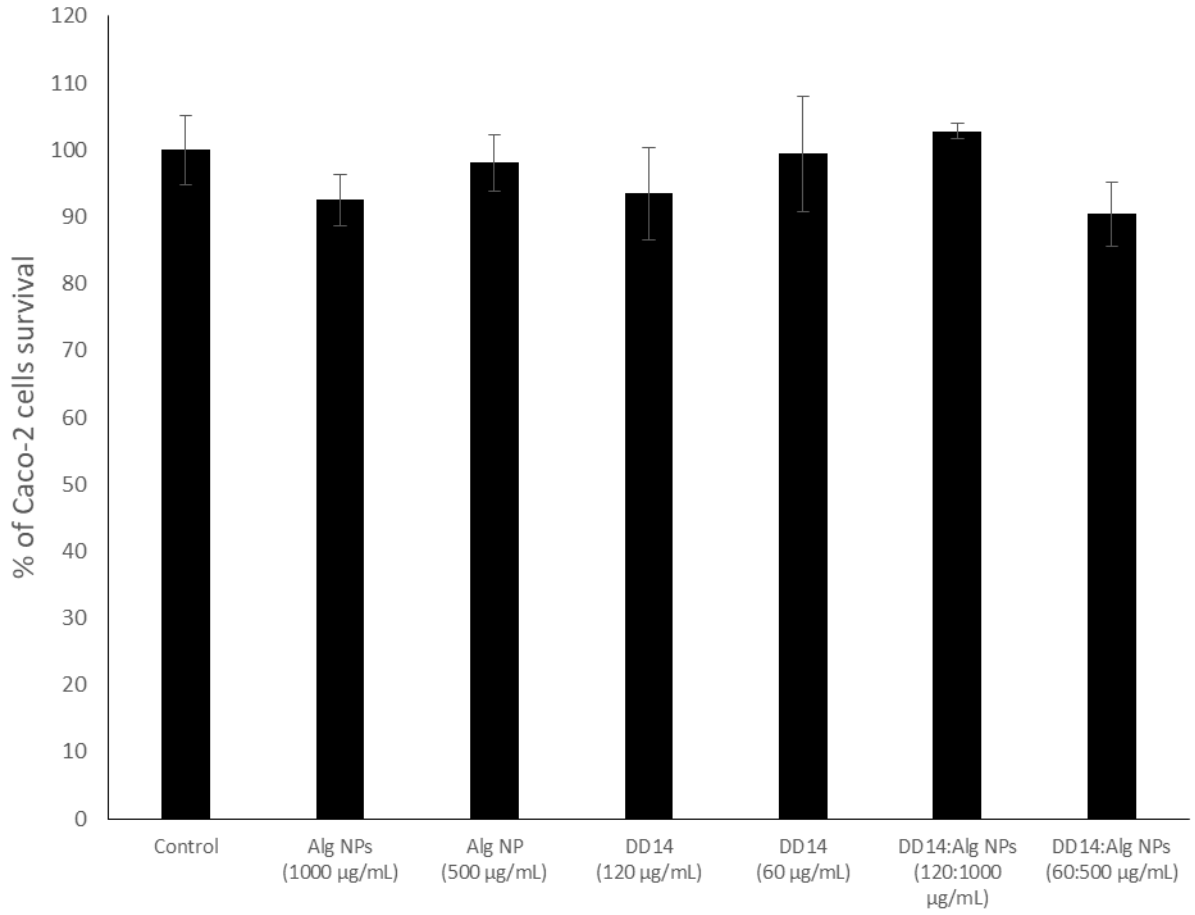
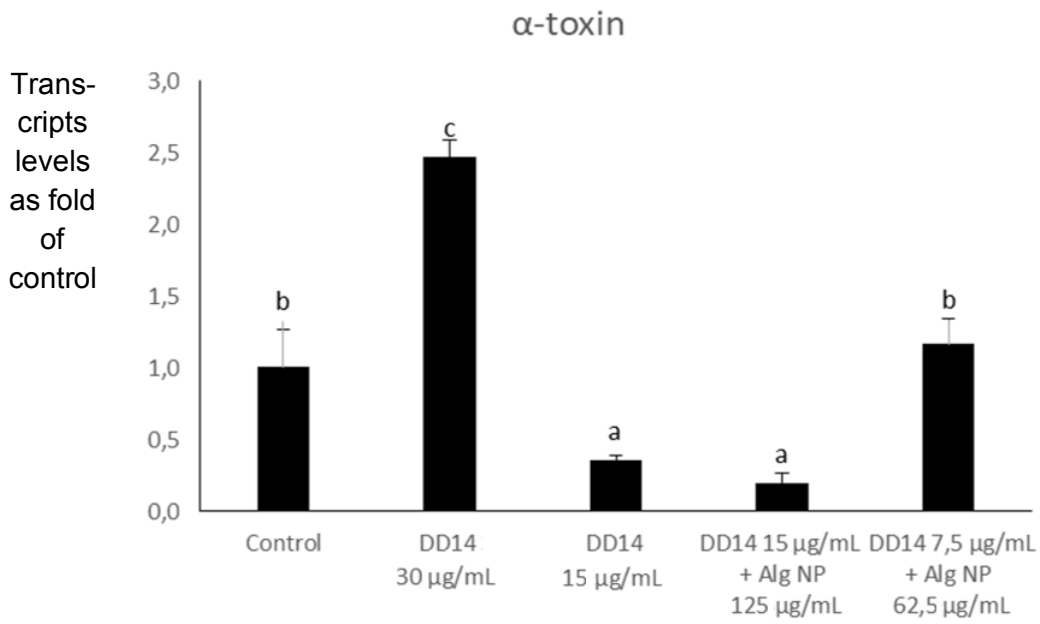
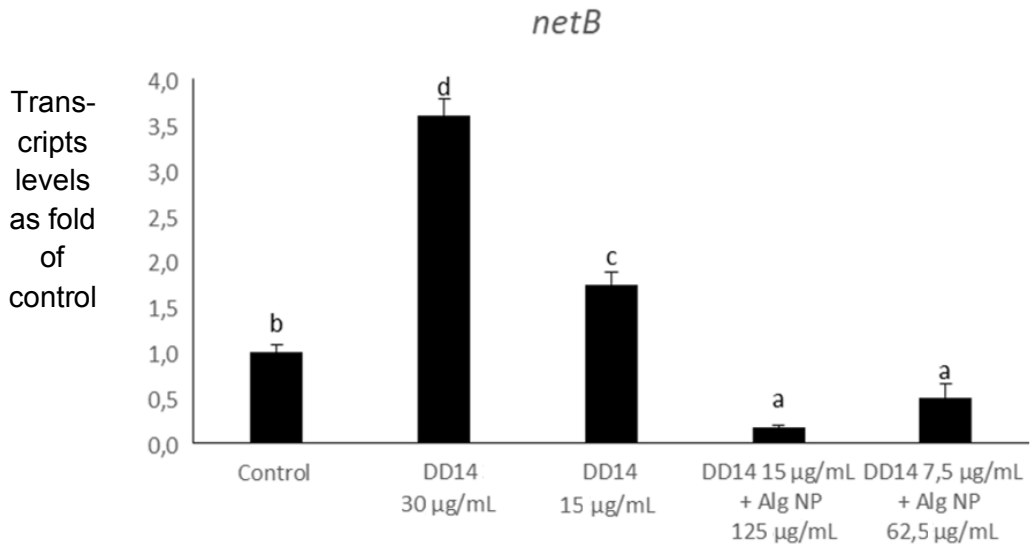


Fig. 4

a.



b.



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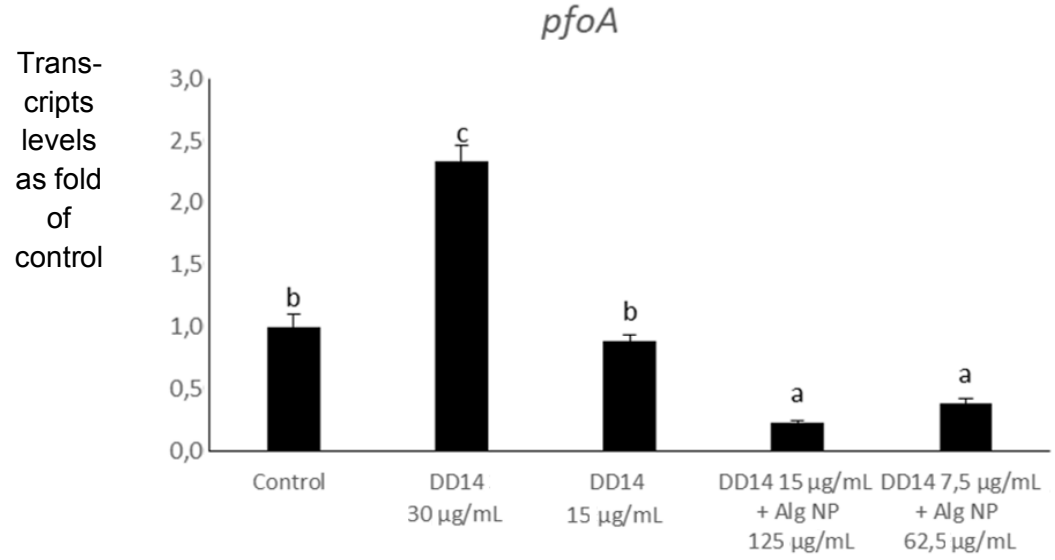

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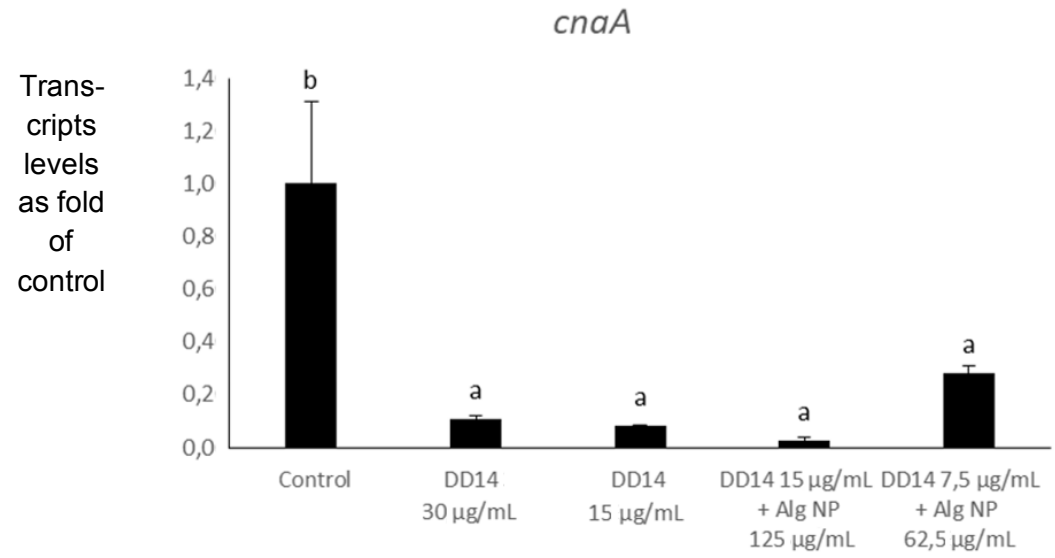

Chapitre 4: Potent anti-inflammatory activity of a leaderless enterocin DD14 (EntDD14): Down regulation of IL-6 and IL-8

Cytokines represent a diverse group of proteins that act as mediators between cells. Cytokine is a general name given for various molecules including, monokines (cytokines produced by monocytes, macrophages), lymphokines (cytokines produced by activated lymphocytes, in particular Th cells), chemokines (small cytokines responsible for leukocyte migration) and interleukins (cytokines acting as mediators between leukocytes) (Jun-Ming, et al., 2007). Cytokines can be involved in pro-inflammatory (enhance/up-regulate the inflammatory response) and anti-inflammatory reactions (control the pro-inflammatory cytokine response). However, this classification is somewhat simplistic and there are numerous examples that a given cytokine may act both as a pro- as well as an anti-inflammatory cytokine, depending on the actual cellular/molecular/immunological situation, such as interleukin 6 (IL-6) (Jun-Ming, et al., 2007). IL-6 production occurs after tissue injuries and infections, inducing host response by stimulating hematopoiesis and immune reactions. Such production is controlled by transcriptional and posttranscriptional mechanisms (Nemeth, et al., 2004). Uncontrolled production of IL-6 contributes to pathological effect on autoimmunity (Nemeth, et al., 2004). Research studies showed that IL-6 has pleiotropic effect on many biological responses including hematopoiesis, inflammation and immune response (**Figure 39**) (Kishimoto, 1985; Hirano, et al., 1986; Kishimoto, 1989).

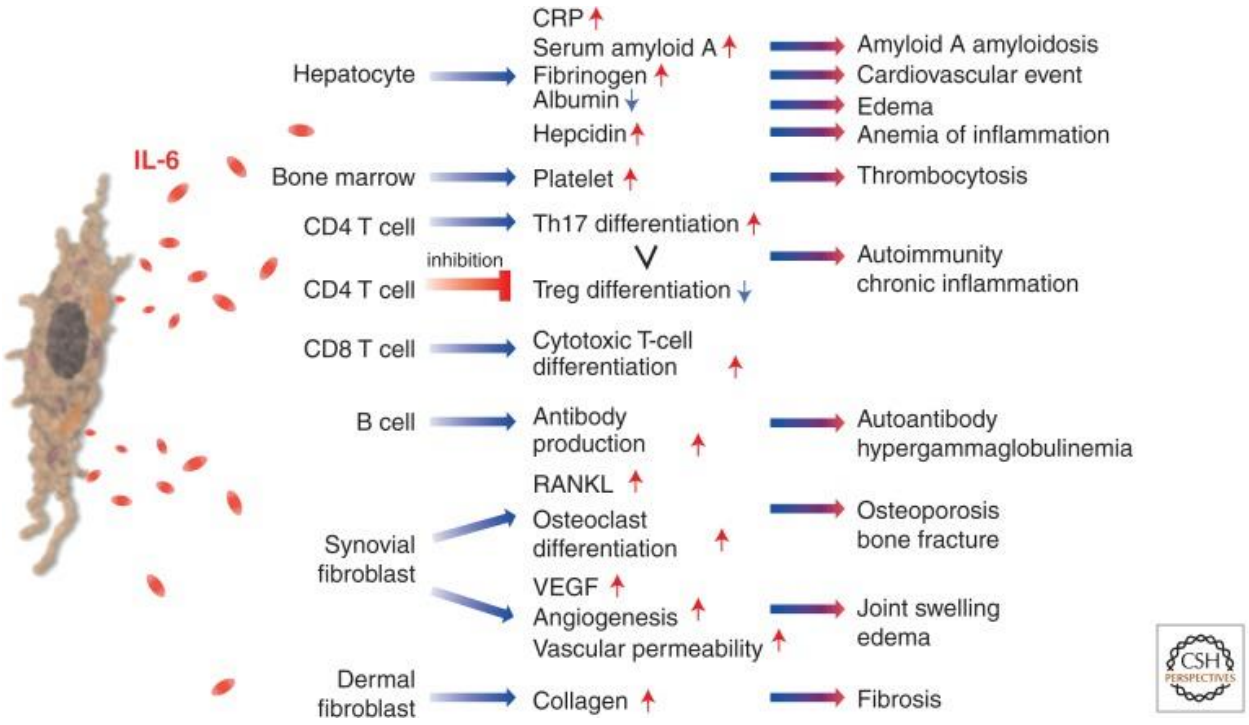

Figure 39. Pleiotropiceffect of IL-6(Tanaka, et al., 2014).

Thus, distinct functions and names have been attributed to IL-6 based on its biological activity. For example, the name interferon (IFN)-β2 (cytokines with specific anti-infectious activities particularly antiviral) was based on its interferon antiviral activity; the name hybridoma growth factor (HGF) was attributed to IL-6 due to its ability to enhance the growth of fusion cells between myeloma cells and plasma cells; the name B-cell stimulatory factor 2 (BSF-2) referred to its ability to contribute to differentiation of activated B cells to antibody (Ab)-producing cells, the name hepatocyte-stimulating factor (HSN) related to the acute phase response of hepatocytes (Kishimoto, 1985; Hirano, et al., 1986; Kishimoto, 1989). In human, IL-6 consists of 212 amino acids, including a signal peptide of 28-amino-acids. Coding gene of IL-6 was mapped to the chromosome band “7p21” (Nemeth, et al., 2004).

Furthermore, IL-6 is involved in the regulation of serum iron by inducing hepcidin production, the latter blocks the role of the iron transporter "ferroportin 1" on gut, resulting in reduction of serum iron levels (Nemeth, et al., 2004). Also, IL-6 regulates the zinc levels *via* controlling its transporter and thus IL-6 increases zinc transporter ZIP14 expression on hepatocytes, which contributes to the hypozincemia of the acute-phase response (Liuzzi, et al., 2005).

Recently, studies showed that severe acute respiratory syndrome corona virus 2 (SARS-Cov-2) selectively induces a high level of IL-6 contributing to the exhaustion of lymphocytes. Such increasing in cytokines, including IL-6, indicates that the "cytokine storm" may contribute to the mortality of COVID-19 (Tang, et al., 2020).

Another major mediator involved in the inflammatory response is IL-8, which is produced by several cell lines including macrophages, epithelial cells and endothelial cells (Bishara, 2012). It induces the chemo taxis in target cells, such as neutrophiles, inducing their migration to the sites of injury or infection, stimulates phagocytosis and acts as a promoter of angiogenesis (Bishara, 2012). Studies showed that the increased synthesis of IL-8 causes chronic inflammation disease (Kraan, et al., 2001)

Here, we report the anti-inflammatory and the antiviral potential of the leaderless enterocin DD14 (EntDD14). The obtained results showed that EntDD14 has the ability to reduce the secretion of both IL-6 and IL-8 pro-inflammatory interleukins and reduce the multiplication of HSV1 virus by a significant manner (**Figure 40**). Interestingly, the reduction of both IL-6 and IL-8 secretion by EntDD14 on Caco2 cells *in-vitro* model inflamed with lipopolysaccharide

(LPS) from *E. coli*, was more significant than that of dexamethasone (at 20mM), demonstrating the potent anti-inflammatory activity of this bacteriocin.

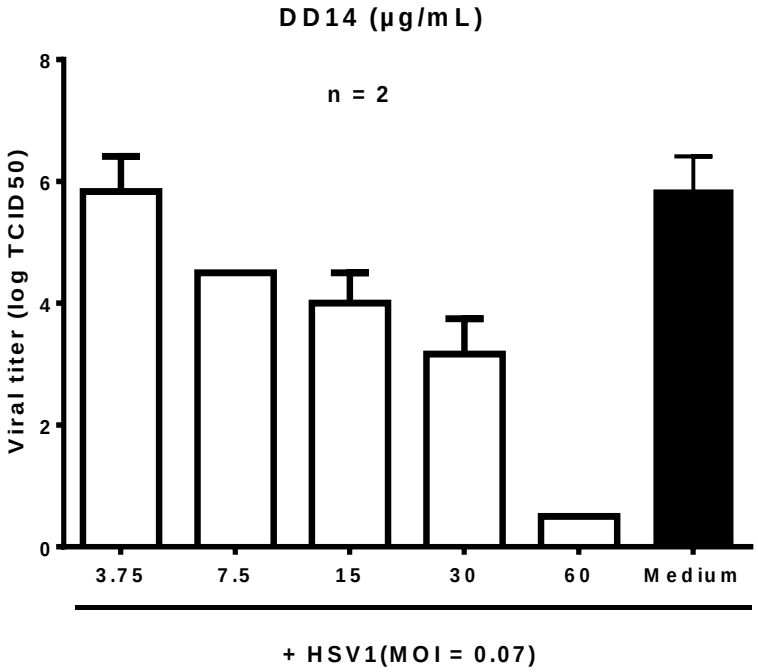

Figure 40. Anti-HSV1 activity of EntDD14 at 5 different concentrations (3.75 µg/mL ; 7.5 µg/mL ; 15 µg/mL ; 30 µg/Ml and 60µg/mL).

Anti-inflammatory and anti--viral properties of a two-peptides leaderless enterocin DD14

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Abstract

In this study we reveal further functions attributable to enterocin DD14 (EntDD14), notably anti-inflammatory and anti-viral activities. After induction of an inflammatory reaction by challenging eukaryotic Caco-2 cells with lipopolysacharide (50 µg/ml) from *Escherichia coli* the secretion of the pro-inflammatory interleukin 6 (IL-6) reached 2.5 pg/ml, whereas it was not detectable in non-inflamed Caco-2 cells. It should be noted that the decrease of secreted IL-6, induced by treatments with EntDD14 (DD14) at 60 and 240 µg/ml, is so important that it reached the detection thresholds of the luminex assay. These results were therefore confirmed by a highly sensitive ELISA method. Regarding the interleukin 8 (IL-8), which is has also a pro-inflammatory action, the effect of the different treatments with the EntDD14, was clear with a decrease in the secreted IL-8 by at least two to three folds the dose obtained with inflamed untreated control (138.85 pg/ml). Of note q-PCR analysis of the expression of IL-6 and IL-8 genes showed that DD14 (60 µg/ml) has decreased the expression of these two genes. However the analysis of the relative expression (ΔΔCt) after normalization showed that these effects on the expression of the IL6 and IL8 genes are not significant. Thus, further investigations are needed to understand the mechanism implied in the anti-inflammatory activites. Moreover, the antiviral activity of EntDD14 alone or that of the formulation was also evaluated. Thus, Vero cells, isolated from African green monkey's kidney, infected with herpes simplex virus (HSV-1) at a multiplicity of infection (MOI) of 0.07, were incubated with different concentrations of EntDD14 ranging from 7.5 to 150 µg/ml. After to 24 h of incubation, the EntDD14 showed a significant anti-HSV1 activity at higher concentrations (>30µg/ml).

Keywords: enterocin DD14; anti-inflammatory; lipopolysacharide; interleukines; anti-viral; herpes simplex virus-1.

Introduction

Inflammation is the set of reactional defense mechanisms by which the body recognizes, destroys and eliminates all foreign substances. The inflammatory reaction sometimes goes beyond its objectives and causes deleterious effects leading to immunity disorders with, sometimes, critical consequences such autoimmune diseases or anaphylaxis (Peavy and Metcalfe, 2008; Yang et al., 2020). The causes of inflammation are various including, amongst many others: infectious agents, inert foreign substances, physical agents or post-traumatic cyto-tissue injuries (Stone et al., 2020). Inflammation process generally begins with a "recognition" reaction involving monocytes, macrophages, lymphocytes or circulating proteins such antibodies, complement proteins or Hageman factor (Jukema et al., 2016; Stone et al., 2020).This recognition phase follows the sequential involvement of a whole set of cells and mediators whose order of intervention is complex and variable. Some mediators in both adaptive and innate immune responses, such as prostaglandins and cytokines, which are soluble glycoproteins (~15–20 kDa) produced by different cell types including endothelial cells as well as immune cells such as macrophages or B and T-lymphocytes (Dinarello, 2007; Rose-John, 2018). Cytokines include various molecules such myokines, interferons, lymphokines and interleukins (acting as mediators between T cells) and chemokines (responsible for the migration of T-cell) (Dinarello, 2007; Morán et al., 2013).Cytokines act at the recognition, activation, or effector phases of an immune response, modulating the balance between humoral and cell-based immune responses. They also regulate the maturation, the growth and the functional activities of particular cell populations (T cells, B cells and myeloid cells) (Morán et al., 2013; Sivakumar et al., 2004). A same cytokine can be produced by different cell types and act on a large number of different targets, reacting with specific membrane receptors present on their surface forming a kind of a cytokine network

involved in the mechanisms of inflammation and immunity (Nedoszytko et al., 2014). At least 40 cytokines have been described to date and the complexity of their actions makes them hard to study and understand their implication in immunity disorders (Binnington et al., 2020). Therefore, cytokines are involved in the multi-faceted response of the immune system to most antigens, among those responses that induce the inflammatory process (Dinarello, 2007; Stone et al., 2020)

Many clinical and sub-clinical diseases are accompanied with inflammation (at low level) such as diabetes, cancer and cardiovascular diseases (Ridker, 2017). Therefore, in such states, measuring the specific cytokines responses is critical for better understanding diseases' interactions and their pathogenesis (Aziz, 2015; Ridker, 2017). Of note, infectious pathologies due to viruses were known to induce sometime "cytokine storm", in severe cases leading to irreversible damages as described for influenza, Ebola or HCoV viruses infection complications (Liu et al., 2016; Wang et al., 2020; Younan et al., 2017) and even as a consequence of herpes viruses infections in some cases (Dulek and Thomsen, 2019).

Bacteriocins are antimicrobial peptides produced by a large number of bacteria, including Gram positive and Gram negative (Drider and Rebuffat, 2011). These small ribosomally synthesized peptides were widely studied and appeared to have multifunction abilities including anti-inflammatory properties (Chikindas et al., 2018), triggering the secretion of interleukins and mitigating inflammation (Yin et al., 2018; Yoon and Kang, 2020).

The aim of the present study is to explore the anti-inflammatory potential of the enterocin DD14 (EntDD14), a class IIb bacterioicin isolated from *Enterococcus faecalis* 14, on the secretion and genes expression of interleukins, implied in inflammation, using *in-vitro* inflamed Caco-2 cells.

Material and Methods

Production and purification of enterocin DD14 (EntDD14)

E. faecalis 14 was grown in100 mL of M17 broth (Sigma Aldrich, St Louis, MO, USA), supplemented with 5% of glucose and buffered with 60 mM sodium phosphate (pH 6.3), at 37°C for 24 h with 160 rpm continuous shaking. After harvesting by centrifugation (8.000 rpm, +4°C, 10 min), the cell-free supernatant was incubated 24 h at room temperature with 3g of CM Sephadex® C-25 resin (GE Healthcare Life Sciences, Chicago, IL, USA) previously suspended in 30 mL of distilled water and equilibrated for 30 min. The resin was then washed with 5 bed volumes (BV) of distilled water and 1 BV of 0.5 M NaCl. The resin-bound EntDD14 was eluted with 2 BV of 1.5 M NaCl. The removal of the salt from the solution containing EntDD14 was achieved by passing 1 mL of the solution into each column of PD MidiTrap G-10 columns (GE Healthcare), following the manufacturer's instructions. The protocol was repeated several times to obtain a sufficient quantity of EntDD14 which kept at 4°C for further uses.

Inflammation of Caco2 cells and anti-inflammatory activity of EntDD14

After the production and purification of EntDD14 (as previously detailed), the enterocin was re-suspended in DMEM to study the effect on the inflammation of Caco-2 cells. The Caco2 cells were grown for 72 h in 2 subsequent subcultures at 37°C under 5% CO_2 atmosphere and were transferred into 96-well tissue culture plates at a density of 8,000 cells/well, and were grown for an additional 72-96 h. During this time (7 days), the culture medium was changed each two days to maintain optimal conditions for cell growth. Then obtained Caco-2 cells monolayer were pre-stimulated with lipopolysaccharide (LPS) from *E. coli* (Sigma Aldrich) (50 µg/mL) and incubated for 36 h. Then, the EntDD14 was added, in triplicate, at two different concentrations

(60 µg/mL and 240 µg/mL), dexamethasone (20 mM) (Sigma Aldrich) as positive control and DMEM containing LPS (50 µg/mL) as negative control.The plate was then incubated over night at 37ºC under 5% CO_2 atmosphere.

The next day, the supernatant of each well was removed and a selection of thirteen interleukins and inflammation markers including: GM-CSF, IFNγ, IL-1α, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12 (p70), IL-13 and TNFα were quantified using a Milliplex® HSTCMAG-28SPMX13 kit (Merck, Darmstadt, Germany). Additional complementary dosage for IL-6 using High Sensitive ELISA kit (Bio-Techne, Minneapolis, MN, USA) was also performed. Both assays were realized according to the kit manufacturer's recommendations.

Real-time quantitative PCR (RT-PCR) measurements of interleukins secreted by Caco-2 cells.

After recovery of the supernatants from inflammation test described previously, the Caco2 cells in the bottom of the wells were subjected to RNA extraction using Trizol pruficiation kit (Macherey Nagel, Düren, Germany). Obtained RNAs were quantified and their quality was checked using RNA ScreenTape and TapeStation (Agilent technologies, Santa Clara, CA, USA). Then cDNA synthesis was performed by reverse transcription on a thermocycler using the RevertAid H Minus First Strand cDNA Synthesis Kit (ThermoFisher Scientific, Watham, MA, USA). cDNAs of interleukins were then quantified by comparative Ct experiment on a CFX Connect TM Real-Time System (Biorad, Hercules, CA, USA) using the SYBR Green PCR Master Mix (USA)and specific oligo nucleotides listed in the **table 1** and described previously by Saegusa et al. (2007) (Saegusa et al., 2007). Additional primers allowing amplification of specific parts of GAPDH (housekeeping gene) were designed following primer3 software to provide internal controls.

Table 1. Primers designed and used in Caco2 cells for RT-qPCR.

Primer	Sequences	Target gene (Function/role)
For-*IL-6* RT-qPCR	CACTCACCTCTTCAGAACGA	IL-6
Rev-*IL-6* RT-qPCR	CTGTTCTGGAGGTACTCTAGG	
For-*IL-8* RT-qPCR	TGGCTCTCTTGGCAGCCTTC	IL-8
Rev-*IL-8* RT-qPCR	TGCACCCAGTTTTCCTTGGG	
For-*GAPDH* RT-qPCR	TGAACGGGAAGCTCACTGG	GAPDG (house keeping gene)
Rev-*GAPDH*RT-qPCR	TCCACCACCCTGTTGCTGTA	

Results

Interleunkins secretion by Caco-2 cells treated with EntDD14

Among all the interleukins tested, only the secreted quantities of IL-6 and IL-8 were significantly reduced after induction of inflammation on Caco2 cells with LPS (50 µg/mL) and treatment with EntDD14. Caco-2 cells inflamed with LPS (LC control) secreted in mean 2.5 pg/mL IL-6 secretion, whereas it was not detectable in non-inflamed Caco-2 cells. It should be noted that the decrease of secreted IL-6, induced by treatments with EntDD14 (DD14) at 60 and 240µg/mL, was so important that it reaches the detection thresholds of the Milliplex assay (**Figure 1A**). Results of IL-6 quantification by High Sensitive ELISA assay confirms the previous results with a significant decrease after treatment with DD14 at both concentrations used (1x: 60 µg/mL and 4x: 240 µg/mL) from 2.4 pg/mL to less than 0.5 pg/mL (**Figure 1B**). Interestingly no dose response was observed with the two concentrations of EntDD14 used (60 and 240 µg/mL), which correspond to one and four folds the minimal inhibitory concentration (MIC) toward *Clostridium perfringens* (data not shown).

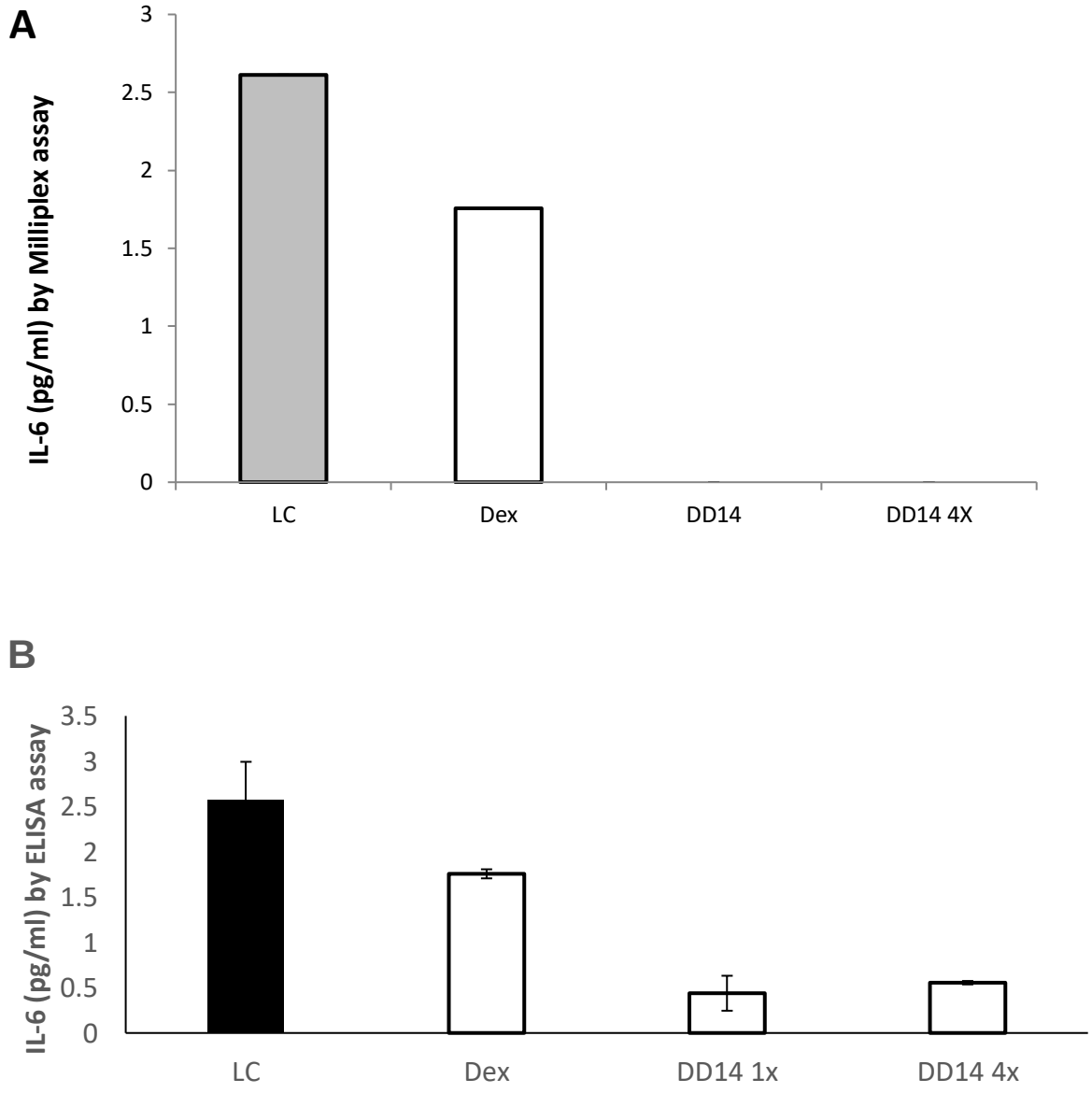

Figure 1. IL-6 quantification in the supernatant of inflamed Caco-2 cultures treated with dexamethasone, EntDD14 at 60 µg/mL (1×) and 120µg/mL (4×) by Milliplex assay (A) or High Sensitivity ELISA assay (B).

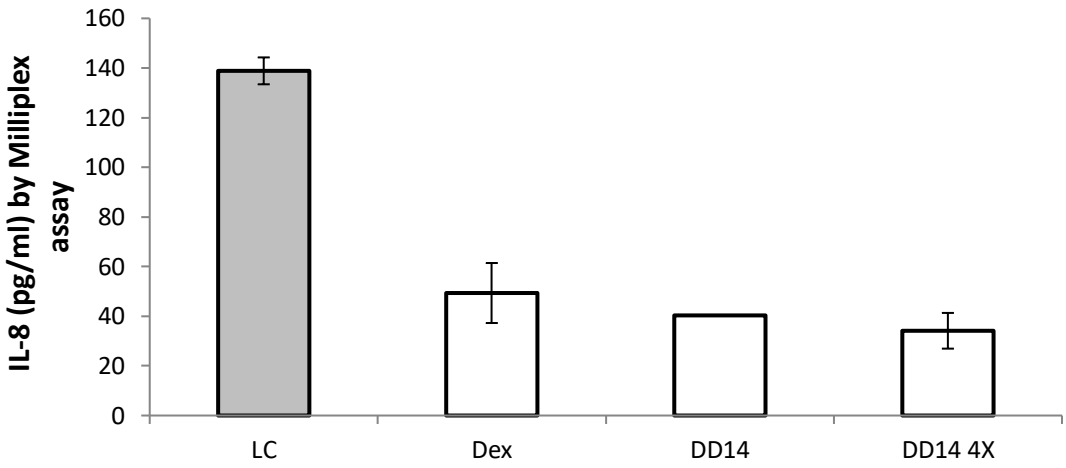

Figure 2. IL-8 quantification in the supernatant of inflamed Caco-2 cultures treated with dexamethasone, EntDD14 at 60 µg/mL(1×) and 120 µg/mL (4×) by Milliplex assay

Regarding IL-8, the effect of the different treatments is clear with a decrease of IL-8 secreted by Caco-2 cells in all cases by at least two to three-fold the dose obtained with LC control (138.85 pg/mL) (**Figure 2**).

Effect of EntDD14 on IL-6 and IL-8 genes expression

The Q-PCR analysis of the expression of IL-6 and IL-8 genes showed a decrease in the expression of these genes during treatment with EntDD14 (60 µg/mL). However, we noted variations in the expression of the GAPDH gene (House Keeping gene control). The analysis of the relative expression showed that after normalization the effects on the expression of the IL-6 and IL-8 genes are not significant as detection threshold reached after more than 38 cycles, which is too low to make interpretations.

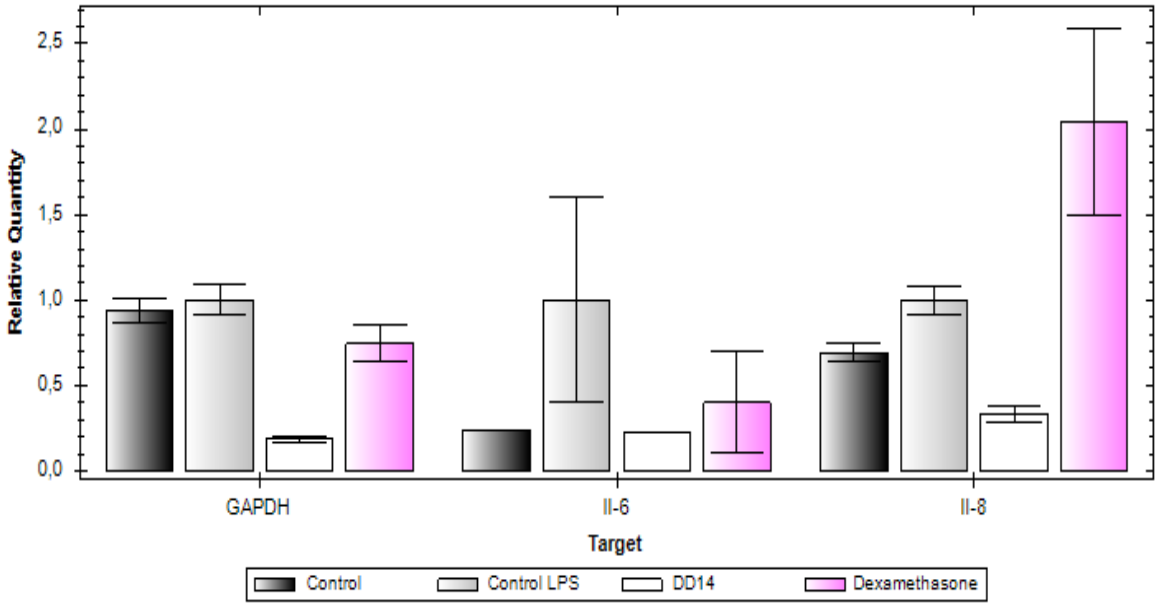

Figure 3. Relative expression of GAPDH, IL-6 and IL-8 genes quantified by specific qPCR.

Discussion

During our study, the purified EntDD14 showed the ability to reduce the secretion by inflamed Caco-2 endothelial cells with *E. coli* LPS structure, of both IL-6 and IL-8 pro-inflammatory interleukins in a significant manner. Interestingly the decrease of these interleukins induced by the bacteriocin is similar, or even higher in the case of IL-6, than the anti-inflammatory dexamethasone. Few researches interested about the anti-inflammatory effects of class IIb bacteriocins, most of the researches focusing on probiotics or bacteriocins of other classes. Of note Yin et al., (Yin et al., 2018) described the implication of plantaricin EF (PlnEF), a class IIb bacteriocin, in the reduction of colonic tumor necrosis factor alpha (TNF-α) and IL-6 levels in mice fed with a bacteriocin producer strain of *Lactobacillus plantarum* and a non bacteriocin producer mutant. Bacteriocins belonging to other classes were also demonstrated to have anti-inflammatory activities reducing the secretion of pro-inflammatory interleukins induced by

pathogens, such plantaricins (Yoon and Kang, 2020), or nisin (Małaczewska et al., 2019) under certain conditions. Among the inflammation markers, the IL-6,isproduced by most cells (monocytes, fibroblasts, synoviocytes, osteoblasts, etc.) in response to various stimuli, including certain infectious agents (bacteria, viruses, fungi…) or their components (Srirangan and Choy, 2010; West, 2019). The receptor for IL-6 is expressed on many lymphoid and non-lymphoid cells.IL-6 intervenes by stimulating the hepatocyte production of proteins of the acute phase of inflammation: CRP, SAA, haptoglobin, C3, fibrinogen, a1-antitrypsin, a2-macroglobulin (Charlie-Silva et al., 2019; Tanaka et al., 2014). IL-6 is also involved in the mechanisms of immunity by promoting the differentiation of B lymphocytes into plasma cells, by stimulating the proliferation of T lymphocytes in association with IL-2 and by promoting the generation of cytotoxic T lymphocytes (Galandrini et al., 1991; Holsti and Raulet, 1989; Tanaka et al., 2014).One other cytokine implied in acute inflammation is IL-8, called chemokine, whose main property is to attract circulating leukocytes to an inflammatory focus (David et al., 2016; Harada et al., 1994). Polymorph nuclear neutrophils are the preferred target of IL-8 *via* a specific receptor: IL-8-R. IL-8 induces chemotaxis and polymorph nuclear activation with induction of cyclooxygenase, lipooxygenase and NO-synthase (Fogh et al., 1992; Nolan et al., 2008; Rossaint et al., 2012).Recently IL-8 and particularly IL-6 were associated with cytokine storm and recently with Covid-19 disease complication (Del Valle et al., 2020; Magro, 2020)

In the present study, the decrease of available interleukins in the medium was not linked to the expression of the genes encoding them. This can be due to the anti-inflammatory mode of action of EntDD14 not necessarily affecting the regulation of interleukins genes expression. Noteworthy, that some bacteriocins were evidenced to induce an increase of interleukins genes

expression such plantaricin A or bovicin HC5 witch induce *in vitro* increase of TGF-β1, IL-8 ,TNF-α, INF-γ or IL-12 in vitro models (Paiva et al., 2013; Pinto et al., 2011).

In conclusion we established here the anti-inflammatory and the anti-viral function of the EntDD14 on *in-vitro* models. These results allow considering the new functions of bacteriocins and their perspective as new therapeutic agents for inflammation and viral infections. However, further investigations are needed on in vivo models to validate these findings before going ahead in design therapeutic protocols including this kind of bioactive compounds.

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Broadening and enhancing bacteriocins activities by association with bioactive substances

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Abstract

Bacteriocins are antimicrobial peptides some of which are endowed with anti-viral, anti-cancer and anti-biofilm properties. These properties could be improved through synergistic interactions of these bacteriocins with other bioactive molecules such as antibiotics, phages, nanoparticles and essential oils. A Number of studies are steadily reporting the effects of this combination as new and potential therapeutic strategies in the future, as they may offer incentives over existing therapies. In particular, bacteriocins can benefit from combination with nanoparticles which can improve their stability and solubility, and protect them from enzymatic degradation, reduce their interactions with other molecules and improve their bioavailability. Furthermore, the combination of bacteriocins with other antimicrobials is foreseen as a way to reduce the development of antibiotic resistance due to the involvement of several modes of action. Another relevant advantage of these synergistic combinations is that it decreases the concentration of each antimicrobial component, thereby reducing their side effects such as their toxicity. In addition, combination can extend the utility of bacteriocins as anti-viral or anti-cancer agents. Thus, in this review, we report and discuss the synergistic effects of bacteriocin combinations as medicines, and also for other diverse applications including, anti-biofilms, anti-spoilage, anti-cancer and anti-viral.

Keywords: Bacteriocin; synergism; antibiotics; nanoparticles; phages; essential oils; anti-viral; anti-cancer; anti-biofilm.

1. Bacteriocins, are sourceful antimicrobial peptides

Bacteriocins are antimicrobial peptides/proteins ribosomally synthesized by numerous bacterial strains during the primary growth phase [1]. They are secreted into the natural environment at a sub-inhibitory concentration, which is sufficient to inhibit other competitive strains. Of note, the primary function of bacteriocins was originally thought to be their signaling and repelling aptitudes, rather than the inhibition of adverse bacteria. At higher concentrations than natural levels, bacteriocins displays strong antimicrobial activity and can induce pore formation and other disturbances of the membrane, or even inhibit the cell division process [2]. Gram-positive and Gram-negative strains producing bacteriocins develops self-immunity systems to protect themselves from the toxicity of their own peptides [3,4].Moreover it was shown that DNA coding for bacteriocins their transports system and immunity genes are generally arranged in operons, which can be located either on plasmids or on chromosomes [5].

Nevertheless there is no universally accepted scheme of classification of bacteriocins. The first classification, which was proposed by Klaenhammer [1],was established on the basis of structure and modes of action of bacteriocins, and include four classes (Table 1). Thereafter, different classifications were suggested, taking into account further criteria. Thus, Drider et al. [6] considered the biochemical and genetic properties of bacteriocins. Recently Alvarez-Sieiro, et al. [7] considered the biosynthesis mechanism and biological activity of bacteriocins as key elements in their classification (Table 1).

Importantly, bacteriocins are mainly known for their antagonistic properties against both phylogenetically close or distant bacteria [8,9]. However, bacteriocins have been also associated with additional functions such as anti-viral, plant protection and anti-cancer activity [2,10].

Table 1. The main classification schemes of bacteriocins

Bacteriocins classification scheme according to :		
Klaenhammer [1]	Drider et al., [6]	Alvarez-Sieiro et al., [7]
Class I Small modified peptides <5 kDa; containing specific amino acids (lanthionine and dehydrated residues).	**Class I** Small peptides with post-translational modification (lanthionines and dehydrated residues) **Remark:** This class was subdivided in two types: **Type A** (elongated molecules with molecular mass <4 kDa) **Type B** (globular molecules with molecular mass between 1.8 to 2.1 kDa)	**Class I** Ribosomally produced and post-translationally modified peptides (RiPPs) (<10 kDa) **Remark:**Five subclasses were assigned to class I bacteriocins: **Ia:** lanthipeptides (types I, II, III, and IV) **Ib**: Cyclized peptides (Enterocin AS-48) **Ic**: Sactibiotics **Id**: linear azol(in)e-containing peptides **Ie**: Glycocins **If**: Lasso peptides
Class II Small unmodified peptides (<10 kDa); thermostables; acting by pore formation in the membranes	**Class II** Small heat-stable unmodified peptides (<10 kDa) **Remark:** Three subclasses were retained **IIa**: Pediocin-like bacteriocins **IIb**: Two-peptide bacteriocins **IIc**: Other peptide bacteriocins including cyclized ones (enterocin AS-48)	**Class II** Unmodified peptides (<10 kDa) **Remark:** Four subclasses were proposed **IIa**: Pediocin-like bacteriocins **IIb**: Two-peptides bacteriocins **IIc**: Leaderless bacteriocins **IId**: Non pediocin-like single-peptide bacteriocins (ex: laterosporulin) including cyclized ones (enterocin AS-48)
Class III Proteins with molecular weight> 30 kDa; enzymatic activity	**Class III** Large, heat-labile (>10 kDa) protein	**Class III** Thermo-labile (>10 kDa) bacteriolysins and non-lytic bacteriocins
Class IV Proteins attached to sugars or lipids	—	—

Based on their antimicrobial properties, bacteriocins are considered as potential tools to control various infections such as post-operative, urogenital, gastrointestinal, respiratory tract and others [2,11].

In addition to their antibacterial potency, few bacteriocins showed ability to inhibit the multiplication of viruses. For example, enterocin CRL35 and subtilosin, showed anti-viral activity against some viruses, like herpes simplex virus 1 (HSV-1) or poliovirus (PV-1). These bacteriocins both act by perturbing the late stages of viral replication [12,13]. Furthermore, enterocin B was shown to act against the influenza viruses H1N1 and H3N2 [14]. More recently Małaczewska et al. [15] prospected successfully the anti-viral potential of combination of nisin mixed with lysozyme and lactoferrin against the bovine viral diarrhea virus.

The multi-functionality of bacteriocins includes also their activity asa novel class of anti-cancer agents [16]. Related to that, LS10 bacteriocin, a defensin like class IId bacteriocin produced by *Brevibacillus* sp. strain SKDU10, displayed anti-cancer activity against several cancer cell-lines originated from solid tumors; including H1299 (lung carcinoma), MCF-7 (breast cancer), HeLa (cervical cancer cells) and HT1080 (human fibrosarcoma), at a concentration below 10 μM [17,18].

Furthermore, numerous studies have reported the synergistic effect of bacteriocins when combined with classic antibiotics [19], polysaccharide nanoparticles [20], phages [21], essential oils [22], organic compounds and preservatives [23]. The association of bacteriocins with such molecules was expected to have beneficial effects in various applications including anti-cancer [24], anti-biofilm [25], antimicrobial [26] and anti-fungal [22]. Of note, a major advantage of bacteriocins-bioactive substances combinations is the low amount of each molecule requested to

obtain the desired effect. This amount is expected to be significantly less important than in the case of individual use. These synergistic interactions allow as well to reduce potential toxicity or any adverse effect associated with these molecules [19].

2. Antibiotics and bacteriocins constitute an promising synergistic combinations for medical therapy

Antibiotics have been widely used, and at times misused, for decades. Their over-prescription has started to limit their effectiveness due to the emergence of antibiotic resistance. Now antibiotics are "dying" and therefore, human and animal health has started to experience untreatable infectious diseases. The combination of antibiotics and bacteriocins is increasingly being pointed out as a key strategy to confront emergent resistant pathogens [19]. This synergistic bacteriocin-antibiotic association has been reported in different circumstances [27–36]. The combinations of these molecules offers key incentives as the total amount of antibiotics administered is reduced as is their potential cytotoxicity [27,32]. Overall, the data gathered from some studies indicated the effectiveness of bacteriocins-antibiotics combinations to control resistant clinical pathogens such as methicillin-resistant *Staphylococcus aureus*(MRSA), *Enterococcus faecium* and *Clostridium difficile* [30]. Of note, the latter organism is responsible for *C. difficile*-associated diarrhea (CDAD), which is attributed to the perturbation of the gut microbiota resulting from the overuse of antibiotics [37]. Interestingly, Le Lay et al. [38] established the inhibitory activity of nisin on *C. difficile* by targeting the vegetative cells and the spore germination process. On other hand, Hanchi et al. [30] established the synergistic effect of combination of reuterin and durancin 61A on the growth of *C. difficile.* Further bacteriocins like thuricin CD [39], lacticin 3147 [40] oractagardine A [41], are active as well against *C. difficile,*

which opens a window for treatment of infections associated with this pathogens using bacteriocins.

Furthermore, Danesh et al. [42] showed that haloduracin, a lantibiotic bacteriocin, associated with chloramphenicol displayed a wide spectrum of activity against pathogens such as *Enterococcus faecalis, E.faecium, S.aureus* andeven against different strains *of Streptococcus* [42]. Recently, the combination of a bacteriocin, produced by *Lactobacillus acidophilus*, with ceftazidime, imipenem and minocycline antibioticsdecreased therequired minimal inhibitory concentrations (MICs) values of these antibiotics against a strain of *Stenotrophomonas maltophila* S19, which was previously shown to be resistant to these three aforementioned molecules [43]. Similarly, Al Atya et al. [27] demonstrated the synergetic activities of two class IIb bacteriocins, enterocins DD28 and DD93, associated with kanamycin and erythromycin against a clinical strain of MRSA. In direct line, the combinations of another bacteriocin, the lacticin 3147, with penicillin G or vancomycin and nisin z with methicillin, successfully inhibited the growth of MRSA strains [44]. Another synergistic effect of combining nisin A and cefazolin was reported against mastitis pathogensand also *S. aureus, E. faecalis, Staphylococcus intermedius, Streptococcus agalactiae, Streptococcus dysgalactiea* and *Escherichia coli* [45].

Currently, urinary tract infections (UTIs) are seen as a global public health issue caused by *E. coli* strains and other bacteria including *E. faecalis, Staphylococcus saprophyticus, Klebsiella, Citrobacter, Pseudomonas* and *Proteus mirabilis* [46,47].The high rates of occurrence of UTIs due to these pathogens and their increasing antibacterial resistance conducted to unfavourable outcomes, like complicated UTIs (cUTIs), pyelonephritis and severe urosepsis [48,49]. Thus, UTIs are infection needing the development of new therapeutic agents. The combination of garvicin KS and nisin with tetracycline or polymyxin B has revealed effective synergies against

UTIs provoked by multidrug resistant Gram-positive and Gram-negative bacteria [50,51]. Additionally, a recent study conducted by Biswas et al. [52] showed that bacteriocin-antibiotic combinations have synergistic effects against β-lactamase-producing clinical pathogens.

Bacterial living in biofilms structures are difficult to treat [53]. These aggregates of organized bacterial cells in complex structures provide efficient protection from antibiotics and detergentsaction, and therefore limit the efficacy of these antimicrobial agents [54]. The use of bacteriocins in association with classic antibiotics has shown great efficiency in terms of potent inhibition of biofilm formation and restoring antibiotic-sensitivity to the bacteria endowed in these elaborated biological structures [27]. Indeed, the combination of nisin with polymyxins reduced dramatically the requiredconcentrations of polymyxins to treat *Pseudomonas aeruginosa*, which is known forits ability to form biofilms in the lungs of patients inintensive care [29,55]. This significant reduction in the concentrations was shown to be related to the biofilm-penetrating abilities of nisin, making it a good agent for eradicating or preventing biofilm communities on medical devices and hospital equipment [56]. Furthermore, therecent study conducted by Angelopoulou et al. [57] showed a synergistic effect between nisin A and vancomycin against biofilms of multi-drug resistant *S. aureus* isolates from human milk.

The association of bacteriocins and antibiotics has provided a new life to conventional antibiotics which hadstarted to be ineffective against multidrug resistant bacteria. These combinations have extended their spectrum of activity and thus opened a new window for the treatment of infectious diseases.

Beyond their classical antimicrobial activities, bacteriocin-antibiotic associations displayed novel unexpected and very interesting activities like the anti-tumor one, which could be considered asnewanti-cancer therapy options [16]. Despite the increasing successes in some areas, the

traditional treatments for most cancers are facing to increasing limitations. In spite of the availability of various methods forcancer treatments nowadays, which includes chemotherapy, surgery and radiotherapy, the rate of mortality remains consistent with significant side effects [58].Globally, malignancy resulting from abnormal cells divisions and uncontrolled cell proliferation is one of the most difficult disease areas to treat. In addition to the non-specificity of chemotherapy, the resistance of cancer cells towards this treatmentis growing; therefore, a novel approach to anti-cancer therapy is urgently needed [16]. Recent studies demonstrated the anti-tumour activity of some bacteriocins against diverse cancer cell lines [18]. In direct line, nisin and its derivatives peptides exhibited *in vitro* and *in vivo* anti-tumor potential on mice model in head and neck squamous cell carcinoma (HNSCC) [59,60]. Interestingly, during another study the combination of nisin with doxorubicin, an anthracycline antibiotic, has effectively decreased themean skin tumour volume and tumour burden in mice during *in-vivo* assays. These observations advocate for the possible use of the nisin/doxorubicin combination to help developing alternative strategies to combat drug resistance in skin cancer cells [61].

3. Nanoparticles for carrying and improving the bacteriocin activity

Nanoparticles (NPs) are ultrafine particles with size range from 1 to 100 nanometers in diameter[62]. The surface area of the NPs can determine how they interact with their surroundings (solid, liquid or gas). As the size of the particle decreases, its surface area per unit volume increases. Due to the nanometric scale of these particles, they possess unique physical and chemical properties compared to those of solid materials at the micro- and macro-scales [63]. The increasing interest for NPs has conducted for the design of nanoparticles from different materials with different shapes [64]. NPs are distinctly different from micro- and macro-scale particles.They are influenced by physicochemical properties such as size, shape, charge among

others [65]. NPs can be divided into two families, which are non-degradable NPs (quantum dots, fullerenes and metallic NPs) displaying cytotoxic effects [66,67] and degradable NPs such as polysaccharide ones, which are generally safe [68,69]. Additionally, these functional polymers have a wide range of uses, including the pharmacological and biological ones (Fig. 1; Table 2) [70–73].

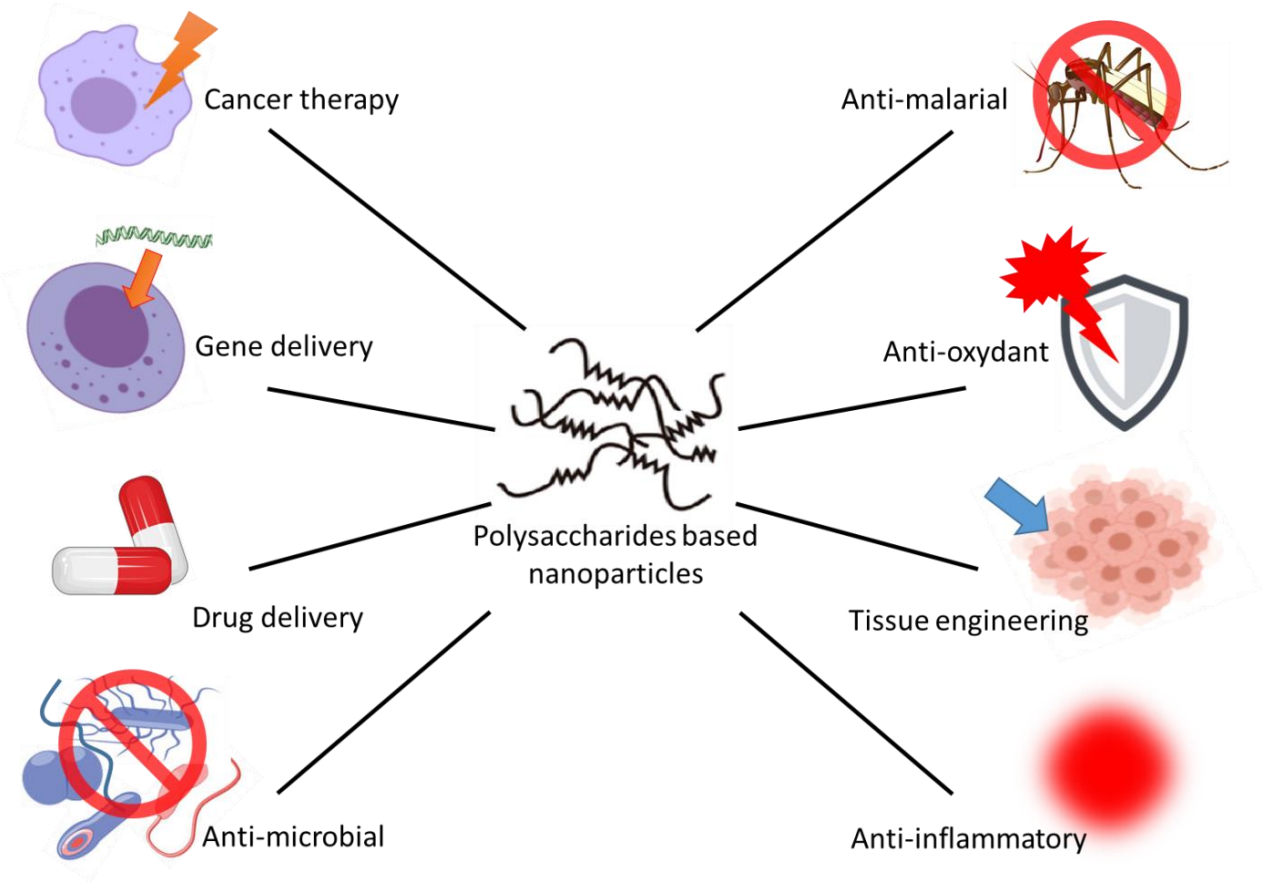

Figure 1. Schematic representation of the nano-biotechnological applications of polysaccharide-based NPs.

Table 2. Polysaccharides and the applications of their NPs in delivery systems

Polysaccharides	Origin	Applications of its NPs
Alginate	Extracted from brown algae (Phaeophyceae)	Gene and drug delivery system, tissue engineering, etc.
Cyclodextrin	Enzymatic conversion of starch	Gene and drug delivery, controlled drug release
Starch	Higher plants	Insulin controlled release & drug delivery
Pectin	Extracted from the middle lamellae of plant cells	Wound healing, delivery of amino acids and drugs
Chitosan	Extracted from the cell walls of fungi and the exoskeleton of crustaceans (crabs, shrimp)	Nanocarrier in drug delivery & antimicrobial
Cellulose	Cell wall of green plants	Nanodelivery for oral protein, for anti-cancer drugs, etc.

NPs with drug delivery properties are of major interest [71,74]. NPs can entrap drugs inside their structures or adsorb them onto their surfaces. Moreover, NPs can improve the low stability of proteins and nucleic acids in biological environments and overcome their limited passage through the biological barriers and thereby enhance their delivery to the target site [71]. Of note, the temperature, pH sensitivity and biodegradability of these particles can confer a controlled release profile. In addition NPs can also reduce adverse side effects of drugs and enhance their effectiveness [71].

Due to these interesting properties, NPs have been extensively studied for pharmaceutical, biological and medical uses. Research on this topic is currently focused on the use of NPs as protectors for the delivery of sensitive elements to the targeted sites without being degraded by pH, enzyme degradation and oxidation [75]. Several bioactive components including amino acids, fatty acids, proteins…etc. have been reported for their potential properties for inflammations, coronary heart disease (CAD) and regulation of blood pressure [76]. Nevertheless, their poor stability in the gastrointestinal tract, low solubility and bioavailability may limit their efficacy. NPs may balance these weaknesses by enhancing these molecules stability and dispersibility, protecting them thereof from digestion and increasing their absorption into the bloodstream [76].Thus, the combination between nanotechnology and biotechnology can lead to novel strategies, which can easily overcome these drawbacks especially in the biological systems[77]. Combination of bacteriocins and NPs designed as nano-antibiotics formulations offer many incentives. Indeed, the use of bacteriocins can be limited by their sensitivity to proteolytic enzymes (pancreatin, trypsin, etc). this can be resolved by adsorbing bacteriocins on NPs [78]. Furthermore, recent studies pointed out the antibacterial [79], anti-biofilm [80] and anti-cancer [81] properties of nano-antibiotics (Table3).

Table3. Synergistic effects of combining nisin and NPs against various applications.

Bacteriocin-NPs combinations	Applications	Targets	References
Nisin-alginate-chitosan NPs	Antibacterial	*L. monocytogenes*	[79]
Nisin-alginate-chitosan NPs	Antibacterial	*S. aureus* *L. monocytogenes E. coli* *S. Typhimurium*	[82]
Nisin-nano vesicles	Anti-biofilm	*S. aureus* *L. monocytogenes* *E. coli, P.aeruginosa*	[80]
Nisin-PLA-PEG-PLA NPs	Anti-cancer	Cancer cell lines: Gastrointestinal (AGS and KYSE-30) Hepatic (HepG2) Blood (K562)	[81]

4. Combined effects of bacteriocins and bacteriophages

Bacteriophages (phages) can undergo two different life cycles of reproduction: a lytic cycle or a lysogenic cycle [83]. Unlike lysogenic ones, lytic phages have a strong bactericidal effect. Once they infect their target they keep replicating until the targeted bacterial population is eliminated, which means that a low phage count, in one administration, is sufficient to eliminate the pathogens [83]. Bacteriocin-bacteriophage combinations have recently been investigated for various applications [21,26,84,85]. Recent studies highlighted the synergistic interactions of coagulin C23 (a class II bacteriocin) with two bacteriophages named FWLLm1 and FWLLm3, against the food borne pathogen *L. monocytogenes.*The combination of FWLLm1 phage and

coagulin C23 has significantly reduced the levels of *L. monocytogenes* 2000/47 (to less than 10 CFU/ml) after 96 h of food matrix storage at 4ºC [21]. However, the combination of FWLLm3 phage with the same bacteriocin failed to inhibit the growth of *L. monocytogenes*, which was attributed to a resistant mutant to the phage. Of note, the authors reported that the development of resistance was much lower when the antimicrobials were combined, explaining the synergistic effects observed in this study [21]. In direct line, Baños et al.[84], determined the ability of bacteriophages P100 and enterocin AS-48, a cyclic bacteriocin to control thegrowth of *L. monocytogenes* present in two raw fishes flesh (salmon and hake). Thus, AS-48 alone reduced the growth of *L. monocytogenes* cells counts, compared the untreated sample, by 1.9, 2.55, 2.8, and 2.8 log CFU/cm^2 (in hake) and by 1.68, 2.79, 2.9, and 3.13CFU/cm^2 (in salmon) at 1, 2, 3, and 7 days, respectively. Despite the low reductions attributed to phage P100, conversely to bacteriocin AS-48, their combination (AS-48/P100) permitted to eradicate the pathogens from both salmon and hake raws flesh after 1 to 2 days of treatment. In another study, combination of bacteriophage treatment with bacteriocins was successfully tested against *C. perfringens* strains, isolated from chicken and swine faeces [26], and against *S. aureus* KCTC 3881 reference strain [85].

5. Bacteriocins-essential oils synergy are active against pathogenic bacteria

The antibacterial activity of essential oils (EOs) and their promising properties as antimicrobial agents has been demonstrated in many studies, and their applications are anticipated to be meaningful for food industry and medicine [86]. Eos can be used in combination with other antimicrobial agents such as bacteriocins to enhance their effects, and reduce resistance as well as doses requested for such activity [87–89]. Mehdizadeh et al. [88] reported the antimicrobial effect of three EOs, extracted from basil (*Ocimum basilicum*), sage (*Salvia officinalis*) and

ajowan (*Trachys permumammi*), in combination with nisin against *E. coli* O 157 and *S. aureus*. Data obtained exhibited synergistic interactions for all these combinations against *E. coli*, and the highest onewas observed for nisin and *Salvia officinalis* EO combination. Notably, no change was observed in the antimicrobial activity of these combinations towards *S. aureus* [88]. Further, Ay and Tuncer [87], showed that when nisin is combined with carvacrol, a monoterpenoid phenol issued from oregano "*Origanum vulgare*" EO, and ethylenediaminetetra-acetic acid (EDTA), a chelating agent used in the food industry, the counts of *Salmonella typhimurium* were reduced to undetectable levels[87]. Moreover, nisin when combined with cinnamaldehyde (CA), a phenylpropanoïd isolated from cinnamon (*Cinnamo mumverum*), displayed synergistic antimicrobial activity against thirteen food-borne isolates of *S. aureus* [89]. LIVE/DEAD BacLight and scanning electron microscope (SEM) assays performed on treated bacteria cells revealed greater damage, resulting from the combination of the bacteriocin and the EO, on both cell wall and cell membrane compared with treatments bynisin or CA lonely [89].

As indicated before, biofilms allow protecting bacteria, including pathogens, from a number of antimicrobials. Indeed, when biofilms have reached the irreversible attachment stage, its extremely hard to penetrate or remove them [90]. Nonetheless, investigations requesting bacteriocins-EOs as anti-biofilms agents have been developed. Related to that, Smith et al. [91] revealed the synergism between M21A; a nisin bioengineered peptide (0.1 μg/ml), and CA (35 μg/ml) on biofilms of *L. monocytogenes* [91]. In addition, Iseppi et al. [25] revealed the effectiveness of thyme (*Thymus vulgaris*) and sage (*Salvia officinalis*) EOs, alone or in combination with the bacteriocin bacLP17 against 12 strains of *L. monocytogenes*. The authors reported that the best anti-biofilm effect was observed with the combination of EOs and bacteriocin,compared to both individual treatments and controls [25].

Of note, Issouffou et al. [22] showed the potent activity of the combination of EOs and bacteriocin against 18 spoilage microorganisms (9 strains of yeasts and 9 strains of bacteria). The 9 spoilage bacteria were *Serratiam arcescens*, *Klebsiella variicola*, *E. faecalis*, *Lactococcus lactis subsp. lactis* and *Klebsiella pneumoniae*, whereas the spoilage yeasts were *Hanseniaspora opuntiae*, *Pichia aff. fermentans*, *Candida metapsilosis*, *Pichia kundriavzevii* and *Kodamaea ohmeri* (Table 4). The results indicate that the combination of cinnamon and enterocin KT2W2G displayed a synergistic and broad action against the selected spoilage microorganisms. Notably, the use of enterocin KT2W2G alone didnot displayany activity [22].The use of EOs in food matrixes needs to be further evaluated as EOs could modify the organoleptic properties if used at concentrations exceeding a certain threshold [92]. Nevertheless, combination of bacteriocins with EOs allows diminishing the required concentrations of both compounds and therefore might circumvent this issue. As conclusion for this part, the combination of bacteriocins and EOs stands as a promising and potent protective strategy that deserves to be deeply investigated.

Table 4. Antimicrobial activities of bacteriocin-EO combinations

Bacteriocin-EO combinations	Target bacteria/Yeast
Nisin-*Salvia officinalis*	*E. coli* O 157
Nisin-Carvacrol	*S. typhimurium*
Nisin-Cinnamaldehyde	*S. aureus*
M21A- Cinnamaldehyde	*L. monocytogenes*
Bacteriocin bacLP17- *Thymus vulgaris*, *Salvia officinalis*	*L. monocytogenes*
Enterocin KT2W2G- Cinnamon	Bacteria: *Serratia marcescens*, *Klebsiella variicola*, *Enterococcus faecalis*, *Lactococcus lactis subsp. lactis*, *Klebsiella pneumonia*. Yeasts: *Hanseniaspora opuntiae*, *Pichia aff. fermentans*, *Candida metapsilosis*, *Pichia kundriavzevii* and *Kodamaea ohmeri.*

6. Conclusions

Bacteriocins have been steadily reported as alternatives to traditional antibiotics. In this review, we highlighted their multifunction and their synergistic effects once they used in combination with other molecules and antimicrobial agents. Such combinations can enhance the potency of bacteriocins and lead to novel formulations such as nano-antibiotics that can be used in the near future to fight infections, which are defying the aging and fading traditional antibiotics. Combination therapies enhance the properties of bacteriocins which become more stable, with better solubility, bioavailability and efficiency. Thus expand their spectrum of use to anti-viral, anti-biofilm and anti-cancer fields. Finally, the extent area of the multifunction of bacteriocins, along their exciting synergistic effects, could be helpful to extend the research field of their associations with other bioactive substances, once the safety and efficiency of such combinations are assessed *in vitro* and *in vivo*.

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Conflict of interest

All the authors declare no conflict interest

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Chapter 6. General conclusion and perspectives

In this Ph D project, we studied the ability of alginate nanoparticles to improve the different functions of the leaderless two-peptide bacteriocin, named enterocin DD14 (EntDD14). After preparing the alginate nanoparticles (Alg NPs) using a simple physical method involving the ball mill process which consists of grinding the sodium alginate precursor. Thus, the alginate nanoparticles were prepared without addition any chemical, such as cross-linking agents (tannic acid, ascorbic acid, citric acid, sorbitol, etc.), which gives a sustainable and green value. Numerous characterizations like UV-vis, Fourier transform infrared spectroscopy (FTIR), X-ray photoelectron spectroscopy (XPS), scanning electron microscopy (SEM) and dynamic light scattering (DLS) were performed to characterize these nanoparticles. Related to that, DLS analysis showed that the obtained alginate particles have an average size of 172 nm and zeta potential of -20 mV. Further characterizations like UV-vis, XPS, and FTIR confirmed, as expected, that the ball mill method did not affect the structure of the polymer, arguing that this method can be applied to prepare alginate nanoparticles. Besides the production of nanoparticles, we produced and successfully purified enterocin DD14 (EntDD14) with the purpose to obtain a new and active formulation. Thus, EntDD14 was prepared from *Enterococcus faecalis* 14 grown overnight in M17 medium. EntDD14 was purified by a simplified two-steps purification procedure involving cation-exchange (CEX) and size-exclusion chromatography (SEC). In a previous study, these two peptides EntDD14A and EntDD14B were characterized and their respective molecular mass were determined by MALDI-TOF/MS (5204,48 Da and 5203.90 Da). Interestingly EntDD14 was demonstrated to have a potent anti-*Clostridium perfringens* activity (L. Caly et al., 2016).

The purified bacteriocin was then successfully loaded on the alginate nanoparticles by a simple physical adsorption process for further investigations including physical properties analysis, inhibitory activities, cytotoxicity, resistance to simulated gastrointestinal harsh conditions, immunomodulating effect and anti-viral potential.

The appropriateness of using the alginate nanoparticles as an enhancer of the antibacterial activity of EntDD14 was investigated against several strains of *C. perfringens* including ICVB088, ICVB089, NCTC6785, Clin1 and DSM576. This activity was greatly improved when EntDD14 was loaded on alginate nanoparticles (EntDD14/AlgNPs) with a diminution of the minimal inhibitory concentration of the bacteriocin by four folds. The approach developed here, involving the nanoformulation (EntDD14/AlgNPs), highlights the synergistic interaction between EntDD14 and Alg NPs and therefore could be suggested as a new potential strategy to tackle diseases caused by *C. perfringens* like the NE in chickens. This promising *in vitro* finding will need to be strengthened by further data such as the toxicity and the stability of its activity under conditions mimicking the gastrointestinal tract (GIT) of birds. Therefore, we prepared simulated human and chicken GIT models representing the gastric and intestinal compartments with their associated conditions such as the pH shifting, body temperature and proteolytic enzymes in order to assess the stability of EntDD14 and that of EntDD14/Alg NPs. Bacteriocins are of proteinaceous nature, and thus can be easily degraded by proteolytic enzymes, such as pepsin and pancreatin, during their passage in the GIT. Regarding this, a disappearance of the anti-*C. perfringens* activity of EntDD14 was observed under conditions simulating its passage through the gastric compartment. The loss of activity was observed in both human and chicken GIT. Interestingly, the new formulation (EntDD14/Alg NPs) resisted against such conditions and demonstrated the ability of Alg NPs to protect the activity of EntDD14 from

the gastric acidity and pepsin action. Of note, the activity of EntDD14/Alg NPs no longer persists after its passage through the small intestinal compartment. Thus this formulation did not provide a protection against the harsh conditions mimicking the proximal small intestine of both humans and chickens. Further, the cytotoxicity assays which were performed to investigate whether or not the new formulation is toxic towards human cells have demonstrated the safety of DD14/Alg NPs towards two cell lines (Caco-2 and HT-29). Moreover, the potency of the developed formulation to down regulate the level of expression of several virulence genes coding for toxins found in pathogenic strains, such as *C. perfringens*, was assessed. The levels of expression of *netB* and α-toxin genes are expected to play a determinant role in the NE infection in chicken, and are enhanced by predisposing factors such as *Eimeria* coccidial infection and a fishmeal diet (Yang et al., 2019). The expression levels of these two genes appeared to be down regulated when the *C. perfringens* was treated with EntDD14/AlgNPs association at sub-inhibitory concentration. The expression levels of other genes coding for virulence factors shown similar behavior when exposed to the same treatment, including *pfoA* (thiol activated cytolysin) and *cnaA* (collagen adhesion protein) genes.

Another interesting feature of enterocin DD14 was revealed in this study involving its anti-inflammatory activity. This antimicrobial peptide showed its ability to reduce the secretion of both IL-6 and IL-8 pro-inflammatory interleukins by Caco-2 endothelial cells by a significant manner. Interestingly, the decrease of available interleukins in the medium was not linked to the expression of the genes encoding them. This can be due to the anti-inflammatory mode of action of EntDD14 not necessarily affecting the regulation of these interleukins' genes expression. Furthermore, in the study, the antiviral activity of enterocin DD14 was also investigated against

several types of viruses. The multiplicity of the investigated viruses was reduced after their treatment with EntDD14.

As a conclusion, EntDD14 has multifunction features, enhanced by alginate nanoparticles, in terms of antimicrobial activity. Nevertheless, in the direct line of the researches accomplished during this thesis, further studies are considered for the future including:

i. Investigating the therapeutic potential of EntDD14 and EntDD14/NPs by *in vitro* and *in vivo* experiments such as anti-cancer treatment since several experimental study have reported promising results regarding the potency of bacteriocins against several types of cancer cell lines.

ii. Exploring the possibility of enhancing the stability of EntDD14, in the intestinal compartment, towards proteolytic enzymes especially pancreatin by encapsulate the EntDD14 with alginate.

iii. Exploring possible application of EntDD14 alone or in combination with alginate nanoparticles against various pathogens including fungi and viruses.

iv. Expand the investigations of the anti-inflammatory mode of actions of EntDD14 in particular on the secretion of IL-6 and IL-8 interleukins

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