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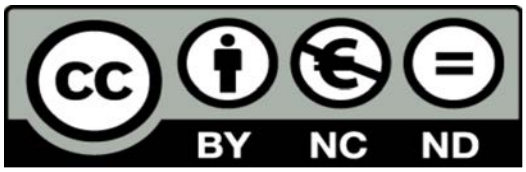

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Enterococci and its enterocins as an alternative to antibiotics in poultry affected by necrotic enteritis

Thesis in cotutel

Doctorate in Food Science – Laval University
Doctorate in Biomedical and Biotechnological Sciences – La Rioja University

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Abstract

Antibiotics have been widely used in therapeutic, prophylaxis and growth promotion in poultry farming. Due to the urgency of preventing the spread of multi-drug resistant bacteria, many governments have banned the use of antibiotics for growth promotion and are calling for a general reduction of these agents in food production, requiring alternatives to preserve human and animal health. *Clostridium perfringens* infections associated with necrotic enteritis (NE) are one of the major threats of the poultry sector. Antibiotic resistance in *C. perfringens* is emerging, but as this microorganism is not subject to surveillance programmes, more information is needed to fully understand its antibiotic resistance profile. All this underlines the need to deepen in the control of NE associated to *C. perfringens* in the poultry sector reducing the use of antibiotics. Bacteriocin-producing (BAC+) bacteria, capable of inhibiting the growth of *C. perfringens* provide a good approach. Enterococci are characterized by the production of bacteriocins (enterocins) and can therefore be used for this purpose. However, these genera often contain virulence factors and antibiotic resistance mechanisms. Hence, due to their duality as commensal and opportunistic pathogen, a deep characterisation of these BAC+ enterococci is required. For this reason, the use of their enterocins instead could be an even better and more realistic approach. This thesis attempts to address this issue through the development of three main objectives.

The first objective was to characterise at the genomic level a collection of *C. perfringens* isolates from poultry affected by NE. To this end, twenty isolates were characterised by whole genome sequencing (WGS) and data on their resistome, virulome, plasmidome, toxin genes and multilocus sequence typing were analysed. The results showed that the *tet* genes (associated with tetracycline resistance) were the most common resistance genes detected and, interestingly, two isolates carried the *erm*(T) gene associated with erythromycin resistance, which has only been reported in other Gram-positive bacteria. Twelve of the isolates were toxinotyped as type A and seven as type G. Other virulence factors encoding hyaluronases and sialidases and plasmids, were frequently detected. Identified sequence types revealed a high variability of the isolates and new allelic combinations were found. Among the isolates, *C. perfringens* MLG7307 showed unique characteristics, even lacking the housekeeping gene *colA*, suggesting that this isolate could belong to a new

species/variant. Overall, the results obtained provide insights into the genomic characteristics of *C. perfringens* and a better understanding of this pathogen.

The second objective was to screen and characterise for safety enterococcal strains of poultry origin with antimicrobial activity against *C. perfringens*. To this end, a collection of 251 enterococci from poultry was screened for antimicrobial activity against the *C. perfringens* collection and BAC+ strains were selected to perform WGS analysis in terms of the resistome, virulence, plasmidome and multilocus sequence typing. According to the results obtained, potentially harmless selected enterococci were also tested for digestive survival under poultry conditions. Among all enterococci, *E. faecium* X2893 and X2906 were the most promising candidates for further studies as protective cultures for poultry farming. Both strains belong to sequence type ST722, carry the genes encoding enterocin A and enterocin B, have no acquired resistance genes, do not carry plasmids, contain the *acm* gene involved in host colonisation and showed high survival rates under *in vitro* poultry digestive conditions. They are therefore good candidates for use as protective cultures in future studies.

The last objective was to produce and purify enterocins with activity against the *C. perfringens* collection and other relevant bacterial poultry pathogens. Enterocins A, B, P, SEK4 and L50 were obtained by microwave-assisted solid-phase peptide synthesis and their antimicrobial activity was evaluated against the *C. perfringens* collection and other relevant bacteria. Combinations of these enterocins, according to their mechanisms of action, were evaluated to achieve synergy. The results showed that the two peptides from L50 were the most active against *C. perfringens*, with L50A being more active. These peptides also showed the broadest spectrum, being active even against Gram-negative *Campylobacter coli* ATCC 33559 and *Pseudomonas aeruginosa* ATCC 27855. All combinations tested showed synergy or partial synergy. This study strengthens the idea of using enterocins alone and in synergistic combinations to inhibit the growth of *C. perfringens* and other pathogens as a promising alternative to antibiotics in the poultry sector.

In conclusion, the use of BAC+ enterococci, and especially their enterocins, represents a very attractive alternative to antibiotics in the poultry sector. Further perspectives on this topic could include the optimisation of enterocin production and more detailed studied (toxicity, enzymatic degradation, etc), to ensure that their use is safe. Also, next steps can include

in vivo infection models to assess their efficacy in preventing *C. perfringens* infection in poultry production.

Résumé

Les antibiotiques ont été largement utilisés comme promoteurs de croissance et prophylactiques pour prévenir les maladies dans l'élevage de volailles. En raison de l'urgence de la prévention de la propagation des bactéries multirésistantes, de nombreux gouvernements ont interdit leur utilisation comme facteurs de croissance. Malheureusement, ces mesures ne sont pas universellement appliquées et, comme effet secondaire, l'incidence des infections à *Clostridium perfringens* associées à l'entérite nécrotique (EN) a augmenté dans les pays où elles ont été interdites. En outre, des *C. perfringens* résistants apparaissent, mais comme ce microorganisme ne fait pas l'objet de programmes de surveillance, davantage d'informations sont nécessaires pour comprendre pleinement son profil de résistance. Tout cela souligne la nécessité de rechercher et de mettre en œuvre de nouvelles approches pour éviter l'utilisation d'antibiotiques chez les volailles. Les bactéries productrices de bactériocines (BAC+), capables d'inhiber la croissance de *C. perfringens*, constituent une bonne approche. Les entérocoques se caractérisent par la production de bactériocines (entérocines) et peuvent donc être utilisés à cette fin. Cependant, ces genres contiennent souvent des facteurs de virulence et des mécanismes de résistance aux antibiotiques. Par conséquent, en raison de leur dualité en tant que pathogènes commensaux et opportunistes, une caractérisation plus approfondie de ces bactéries est nécessaire. C'est pourquoi l'utilisation de leurs entérocines pourrait être une approche encore meilleure et plus réaliste. Cette thèse tente de répondre à cette question en développant trois objectifs principaux.

Le premier objectif était de caractériser au niveau génomique une collection d'isolats de *C. perfringens* provenant de volailles touchées par la NE. À cette fin, vingt isolats ont été caractérisés par séquençage du génome entier (WGS) et les données relatives à leur résistome, virulome, plasmidome, gènes de toxines et typage de séquences multilocus ont été analysés. Les résultats ont montré que les gènes *tet* (associés à la résistance à la tétracycline) étaient les gènes de résistance les plus fréquemment détectés et, fait intéressant, deux isolats portaient le gène *erm* (T) associé à la résistance à l'érythromycine, qui n'a été signalé que chez d'autres bactéries Gram-positifs. Douze des isolats ont été toxinotypés comme étant de type A et sept comme étant de type G. D'autres facteurs de virulence codant pour des hyaluronases et des sialidases, ainsi que

des plasmides, ont été fréquemment détectés. Les types de séquences ont révélé une grande variabilité des isolats et de nouvelles combinaisons alléliques ont été trouvées. Parmi les isolats, *C. perfringens* MLG7307 présentait des caractéristiques uniques, même en l'absence du gène conservateur *colA*, ce qui suggère que cet isolat pourrait appartenir à une nouvelle classification. Dans l'ensemble, les résultats obtenus permettent de mieux comprendre les caractéristiques génomiques de *C. perfringens* et de mieux appréhender ce pathogène.

Le deuxième objectif était de cribler et de caractériser les souches d'entérocoques d'origine avicole ayant une activité antimicrobienne contre *C. perfringens*. À cette fin, une collection de 251 entérocoques provenant de volailles a été criblée pour son activité antimicrobienne contre la collection de *C. perfringens* et des BAC+ ont été sélectionnées pour effectuer une analyse WGS en termes de profil de résistance aux antimicrobiens, de virulence, de plasmidome et de typage de séquences multilocus. Selon les résultats obtenus, des entérocoques potentiellement inoffensifs ont été sélectionnés pour tester la survie digestive dans les conditions de la volaille. Parmi tous les entérocoques, *E. faecium* X2893 et X2906 étaient les candidats les plus prometteurs pour des études ultérieures en tant que cultures protectrices pour l'élevage de volailles. Les deux souches appartiennent au type de séquence ST722, portent les gènes codant pour l'entérocine A et l'entérocine B, n'ont pas de gènes de résistance acquis, ne portent pas de plasmides, portent le gène acm impliqué dans la colonisation de l'hôte et ont montré des taux de survie élevés dans des conditions digestives in vitro chez la volaille. Ils sont donc de bons candidats pour être utilisés comme cultures protectrices dans des études à venir.

Le dernier objectif était de produire et de purifier des entérocines ayant une activité contre la collection de *C. perfringens* et d'autres bactéries pathogènes pour la volaille. Les entérocines A, B, P, SEK4 et L50 ont été obtenues par synthèse peptidique en phase solide assistée par micro-ondes et leur activité antimicrobienne a été évaluée contre la collection de *C. perfringens* et d'autres bactéries pertinentes. Des combinaisons de ces entérocines, en fonction de leurs mécanismes d'action, ont été évaluées pour obtenir une synergie. Les résultats ont montré que les deux peptides de L50 étaient les plus actifs contre *C. perfringens*, L50A étant plus actif. Ces peptides ont également montré le spectre le plus large, étant actifs même contre *Campylobacter coli*

ATCC 33559 et *Pseudomonas aeruginosa* ATCC 27855. Toutes les combinaisons testées ont montré une synergie ou une synergie partielle. Cette étude renforce l'idée d'utiliser les entérocines seules et en combinaisons synergiques pour inhiber la croissance de *C. perfringens* et d'autres agents pathogènes comme une alternative prometteuse aux antibiotiques dans le secteur de la volaille.

En conclusion, l'utilisation d'entérocoques BAC+, et en particulier de leurs entérocines, représente une alternative très intéressante aux antibiotiques dans le secteur de la volaille. D'autres perspectives sur ce sujet pourraient inclure l'optimisation de la production d'entérocines et des études plus détaillées (toxicité, dégradation enzymatique, etc.), afin de s'assurer que leur utilisation est sûre. Les prochaines étapes pourraient également inclure des modèles d'infection *in vivo* afin d'évaluer leur efficacité dans la prévention de l'infection par *C. perfringens*.

Resumen

Los antibióticos se han utilizado ampliamente en terapéutica, profilaxis y como promotores del crecimiento en producción aviar. Dada la necesidad urgente de prevenir la propagación de bacterias multirresistentes, muchos gobiernos han prohibido su uso como promotores del crecimiento en producción animal, y urgen en la necesidad de reducir de forma general el uso de antibióticos en este sector, requiriendo alternativas para preservar la salud humana y animal. Las infecciones por *Clostridium perfringens* asociadas con enteritis necrótica (EN) han aumentado en los últimos años en el sector avicola y suponen un gran problema, que exige una vigilancia estrecha. La resistencia a antibióticos esta aumentando en *C. perfringens*, pero como este microorganismo no está sujeto a programas de vigilancia, se necesita más información para conocer a fondo su perfil de resistencia. Todo esto resalta la necesidad de investigar en el control de la EN asociada a *C. perfringens*, aplicando nuevos enfoques para reducir el uso de antibióticos en producción aviar. Las bacterias productoras de bacteriocinas (BAC+), capaces de inhibir el crecimiento de *C. perfringens*, suponen un buen enfoque. Los enterococos destacan por la producción de bacteriocinas (enterocinas) y, por tanto, pueden utilizarse con este fin. Sin embargo, con frecuencia los enterococos contienen factores de virulencia y mecanismos de resistencia a los antibióticos. De ahí que, debido a su dualidad como comensales y patógenos oportunistas, sea necesario caracterizarlos exhaustivamente. Así pues, el uso de sus enterocinas podría ser un enfoque aún mejor y más realista. Esta tesis intenta abordar esta problemática mediante el desarrollo de tres objetivos principales.

El primer objetivo fue caracterizar a nivel genómico una colección de aislados de *C. perfringens* procedentes de aves de corral afectadas por NE. Para ello, se caracterizaron veinte cepas mediante secuenciación del genoma completo (WGS) y se analizaron datos sobre su resistoma, viruloma, plasmidoma, genes de toxinas y tipificación molecular. Los resultados mostraron que los genes *tet* (asociados a la resistencia a la tetraciclina) eran los más frecuentes y fue de interés la detección de dos aislados que portaban el gen *erm*(T), asociado a la resistencia a la eritromicina, que sólo se ha descrito previamente en otras bacterias Gram-positivas. Doce de los aislados fueron toxinotipados como tipo A y siete como tipo G. También se detectaron con frecuencia otros factores de virulencia que codifican hialuronasas y sialidasas,

así como plásmidos. El tipado molecular reveló una gran variabilidad de los aislados, hallándose incluso nuevas combinaciones alélicas. Entre los aislados, *C. perfringens* MLG 7307 mostró características únicas, careciendo del gen "housekeeping" *colA*, lo que sugiere que podría pertenecer a una nueva especie/variedad. En su conjunto, los resultados obtenidos permiten conocer mejor las características genómicas de *C. perfringens* y profundizar en el estudio de este patógeno.

El segundo objetivo fue caracterizar los enterococos de origen aviar con actividad antimicrobiana frente a *C. perfringens* para garantizar su seguridad. Para ello, se examinó la actividad antimicrobiana de una colección de 251 enterococos de origen aviar frente a la colección de *C. perfringens* y se seleccionaron cepas BAC+ para realizar análisis de secuenciación masiva (WGS) y determinar así su resistoma, viruloma, plasmidoma y realizar la tipificación molecular. Posteriormente, se analizó la supervivencia digestiva en condiciones aviares de los enterococos potencialmente inocuos seleccionados. De entre todos los enterococos, *E. faecium* X2893 y X2906 fueron los candidatos más prometedores para futuros estudios como cultivos protectores. Ambas cepas pertenecen al linaje ST722, son portadoras de los genes codificantes de la enterocina A y la enterocina B, no tienen genes de resistencia a antibióticos adquiridos, no portan plásmidos, poseen el gen *acm* implicado en la colonización del hospedador y mostraron elevadas tasas de supervivencia *in vitro* bajo condiciones digestivas aviares. Por lo tanto, son buenos candidatos para ser utilizados como cultivos protectores en estudios posteriores.

El último objetivo fue producir y purificar enterocinas con actividad antimicrobiana contra la colección de *C. perfringens* y otros patógenos relevantes. Las enterocinas A, B, P, SEK4 y L50 se obtuvieron mediante síntesis peptídica en fase sólida asistida por microondas y se evaluó su actividad antimicrobiana frente a la colección de *C. perfringens* y otros patógenos. Se evaluaron diferentes combinaciones de estas enterocinas, seleccionadas según sus mecanismos de acción, para lograr sinergias. Los resultados mostraron que los dos péptidos de la enterocina L50 eran los más activos contra *C. perfringens*, siendo L50A el más activo. Estos péptidos también mostraron el espectro más amplio, siendo activos incluso frente a las bacterias Gram-negativas *Campylobacter coli* ATCC 33559 y *Pseudomonas aeruginosa* ATCC 27855. Todas las combinaciones estudiadas mostraron sinergia o sinergia parcial. Este

estudio consolida la idea de emplear enterocinas solas y en combinaciones sinérgicas para inhibir el crecimiento de *C. perfringens* y otros patógenos como alternativa prometedora a los antibióticos en el sector avícola.

En conclusión, el uso de enterococos BAC+, y especialmente de sus enterocinas, representa una alternativa muy atractiva a los antibióticos en el sector avícola. Entre las perspectivas futuras sobre este tema podrían figurar la optimización de la producción de enterocinas y estudios más detallados (toxicidad, degradación enzimática, etc.), para garantizar que su uso sea seguro. Asimismo, los próximos pasos pueden incluir modelos de infección *in vivo* para evaluar su eficacia en la prevención de la infección por *C. perfringens*.

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List of abbreviations

AACs: Aminoglycoside acetyltransferases

AGP: Antimicrobial Growth Promoter

AMR: Antimicrobial Resistance

AMP: Ampicillin

AMPs: Antimicrobial peptides

ANI: Average Nucleotide Identity

ANTs: Aminoglycoside Nucleotidyltransferases

APEC: Avian Pathogenic *Escherichia coli*

APHs: Aminoglycoside Phosphotransferases

ARG: Antimicrobial Resistance Genes

Bac: Bacteriocin

BHI: Brain Heart Infusion

BP: Bacteriocin-Producing

CDC: Centers for Disease Control and Prevention

CFC: Chicken Farmers of Canada

CFU: Colony-Forming Unit

CHL: Chloramphenicol

CHUL: Hospital Center of the University of Laval

CLI: Clindamycin

CLSI: Clinical and Laboratory Standard Institute

CPE: Enterotoxin

CTX: Cefotaxime

DHFR: Dihydrofolate Reductase

DIC: Diisopropylcarbodiimide

DIEA: Diisopropylethylamine

DMF: Dimethylformamide

DNA: Deoxyribonucleic Acid

DODT: Dioxa-octanedithiol

Ent: Enterocin

EntA: Enterocin A

EntB: Enterocin B

EntCRL35: Enterocin CRL35

EntDD14: Enterocin DD14

EntL50: Enterocin L50

EntP: Enterocin P

EntSEK4: Enterocin SEK4

EntW: Enterocin W

ERY: Erythromycin

ES: Enterococcal Spondylitis

ESI: Electro-spray ionization

EU: European Union

FDA: Food and Drug Administration

GRAS: Generally Recognized as Safe

HDPs: Host-defense peptides

HFIP: Hexalfuoroisopropanol

HLR: High-Level Resistance

HPLC: High-Performance Liquid Chromatography

HPLC-MS: High-Performance Liquid Chromatography-Mass Spectometry

HS: Heated Supernatant

I: Intermediate

IMP: Imipenem

LAB: Lactic Acid Bacteria

LCMS: Liquid Chromatography Mass-Spectometry

Man-PTS: Mannose Phosphotransferase System

MDR: Multi-Drug Resistant

MIC: Minimal Inhibitory Concentration

MLST: Multi Locus Sequence Typing

MS: Mass Spectometry

MSSA: Methicillin-Susceptible *Staphylococcus aureus*

MRSA: Methicillin-Resistant *Staphylococcus aureus*

MTZ: Metronidazole

NE: Necrotic Enteritis

NHS: Non-Heated Supernatant

NRS: Non-Ribosomal peptide Synthesized

OECD: Organization for Economic Cooperation and Development

OIE: World Animal Health Oranization

PBPs: Penicillin-Binding Proteins

PCR: Polymerase Chain Reaction

QRDR: Quinolone Resistance Determinant

R: Resistant

RCM: Reinforced clostridial medium

RiPP: Ribosomally synthesized and Post-translationally modified Peptides

RNA: Ribonucleic Acid

RP-HPLC: Reverse-Phase High-Performance Liquid Chromatography

S: Susceptible

SPPS: Standard Solid-Phase Peptide Synthesis

ST: Sequence Type

TET: Tetracycline

TFA: Trifluoroacetic Acid

TIPS: Triisoproylsilyl

TSA: Tryptic Soy Agar

TSB: Tryptic Soy Broth

UppP: Undecaprenyl pyrophosphate phosphatase

VRE: Vancomycin-resistant enterococci

WHO: World Health Organization

WOAH: World Organization for Animal Health

WGS: Whole Genome Sequencing

A mi abuelo sinvergüenza,

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I would like to express my most sincere gratitude to everyone who has been part of the process of this doctoral thesis. To begin with, I would like to thank my two thesis directors, Dr. Ismail Fliss and Dr. Carmen Torres for all their teachings and support during the last three years. Carmen, muchísimas gracias por esta gran oportunidad, por abrirme las puertas del 229, y, sobre todo, por hacer que la distancia Quebec-Logroño no pareciera tal. Gracias de corazón por todo el apoyo y por estar siempre pendiente de mí. Ismail, thank you so much for welcoming me into your laboratory, for trusting me and for all the knowledge and inspiration. I'm very grateful for everything.

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Preface

This thesis is part of the AviBiocin project, the overall objective of which is to evaluate, through a multidisciplinary approach, the potential of bacteriocins produced by enterococci of poultry origin as an alternative to antibiotics in the poultry industry in Tunisia. It is a thesis in cotutel between the University of La Rioja (Logroño, Spain), under the supervision of Dr. Carmen Torres, and University of Laval (Quebec, Canada), under the supervision of Dr. Ismail Fliss.

The structure of this doctoral thesis consists of a general introduction followed by four chapters.

Chapter I provides a bibliographic review of the state of knowledge on the main topic of the document. At the end of this chapter, the main problem is summarised, the research hypothesis is established, and the general and specific objectives are defined. Each of the next three chapters (Chapter II, Chapter III and Chapter IV) corresponds to the three main objectives of the thesis.

Chapter II, "Pathogenicity and Antibiotic Resistance Diversity in *Clostridium perfringens* Isolates from Poultry Affected by Necrotic Enteritis in Canada", was conducted in the laboratories of Dr. Ismail Fliss at Laval University and Dr. Carmen Torres at La Rioja University. This work was the subject of a scientific article that was published in Pathogens in 2023 (IF: 4.531, doi: https://doi.org/10.3390/pathogens12070905), and belongs to the Special Issue: *Clostridium* Pathogenesis: Virulence, Host Responses, Microbiome, and Interventions. Results obtained from this chapter were presented in the 90 congress ACFAS (Association francophone pour le savoir), as a short communication, the 8 May 2023 in Sainte Hyacinthe, Montreal; and, as a poster participation in the INAF (Institute of Nutrition and Functional Foods) Student Symposium the 3 March 2023, Quebec, where it was awarded by the Public's favourite award.

Chapter III, "Targeting Enterococci with Antimicrobial Activity against *Clostridium perfringens* from Poultry", was conducted in the laboratories of Dr. Ismail Fliss at Laval University and Dr. Carmen Torres at La Rioja University. This work was the subject of a scientific article published in 2023 in the journal Antibiotics (IF: 5.222, doi: https://doi.org/10.3390/antibiotics12020231). An

additional experiment which was not published is also included. Results obtained from this chapter were presented in the ECCMID (European Congress of Clinical Microbiology and Infectious Diseases), on the 9 July 2021 as an E-poster and as a poster communication in the ANTIMIC (symposium international sur les antimicrobiens naturels), the 22 September 2022 in Tunisia, where it was awarder with the third's poster presentation award.

Chapter IV, "Antimicrobial Activity of Enterocin A, B, P, SEK4 and L50 against *Clostridium perfringens* is Correlated with their Respective Mechanism of Action", was conducted in the laboratories of Dr Ismail Fliss at Laval University and Dr Eric Biron, at the Hospital Center of the University of Laval (CHUL) of Quebec. This work is going to be the subject of an article that will be submitted to the journal Microbiology Spectrum (IF: 3.7). Results obtained from this chapter were presented in the McGill AMR 3rd Annual Symposium as a poster presentation.

The last section of this thesis consists on general conclusion highlighting the main strengths and weakness of the study, considering also the future perspectives of it.

Introduction

Antimicrobial resistance is a major public health concern that threatens the treatment of human and animal infections and is linked to the high use, and in many cases misuse, of antibiotics. In addition to clinical use, antibiotics are also used in veterinary medicine and animal husbandry to treat and prevent infections or, to a lesser extent, in agriculture to preserve crops (McEwen & Collignon, 2018). All these uses can contribute to the increase of antibiotic resistance, not only in pathogenic bacteria but also in commensal bacteria of the intestinal tract of humans and animals, which can contaminate food or the environment through faeces. Therefore, resistant bacteria can be transmitted to humans through food, water or contact with animals. The World Health Organisation (WHO) is proposing to address this issue from a 'One Health' perspective by developing new alternatives to the use of antibiotics in livestock and agriculture (Aslam et al., 2021).

Antibiotics have been widely employed as growth promoters and prophylactics in poultry farming. The poultry industry is one of the most promising economic sectors worldwide, including low- and middle-income countries. Currently, poultry is the first most consumed meat in the world (FAO, 2022), followed by pork and beef. Poultry farms are subject to various diseases that can lead to significant economic losses and major consequences for human health. One of the means used to prevent these diseases is the use of antibiotics, which are effective against several gram-positive bacteria such as *C. perfringens*, responsible for necrotic enteritis (NE), one of the main concerns of conventional and organic broiler producers worldwide (Alizadeh et al., 2022). Unfortunately, the overuse of antibiotics in animals, which have many similarities to those used in human medicine, has led to a worrying increase in the number of multi-resistant bacteria in this sector. This situation has resulted in the European Union imposing a total ban on the use of antibiotics as growth promoters in animal feed in 2006 under the Feed Additives Regulation 1831/2003/EC. Unfortunately, these measures are not applied worldwide and the situation remains alarming in low and middle-income countries (Góchez et al., 2019), where legislation, regulatory oversight and surveillance systems for antibiotic use and the prevention and control of multidrug-resistant bacteria are in many cases weak or inadequate.

On the other hand, in countries where the use of antibiotics as growth promoters have been banned, the incidence of *C. perfringens* associated with NE, as well as other relevant pathogens in poultry, has increased (Villagrán-de la Mora et al., 2020). The prominent feature of NE is acute death, with mortality rates that can reach 50%. Clinical signs include depression, dehydration, drowsiness, ruffled feathers, diarrhoea, and reduced feed consumption. The subclinical form of the disease causes chronic damage to the intestinal mucosa of chickens, resulting in impaired nutrient absorption, reduced weight gain and overall performance (Khalique et al., 2020), all of which lead to huge production and economic losses in broilers.

It is therefore more than necessary to do research and to implement new alternative approaches to reduce or phase out the use of antibiotics in animal husbandry. Vaccines, probiotics, bacteriophages, herbal medicines, metals, etc, are some of the proposed alternatives (Gupta & Sharma, 2022). Here, the use of bacteriocin-producing enterococci as protective cultures or their antimicrobial products, the enterocins, is proposed as an effective alternative to prevent *C. perfringens* infections and the spread of multidrug-resistant bacteria in the poultry sector.

1. Chapter I: State of Knowledge

1.1 Antibiotic resistance

Antimicrobial resistance (AMR) is an urgent global public health threat, and the World Health Organization (WHO) and many other health or scientific authorities are warning of the scale of the problem and its important clinical consequences. According to data from 2019, it is estimated that 1.27 million people worldwide died from causes directly attributable to antimicrobial resistance and 4.5 million died from causes associated with AMR (Lancet, 2022). In the United States, more than 2.8 million antibiotic-resistant infections occur each year and more than 35,000 people die as a result (CDC, 2019). This problem has been growing since the discovery of penicillin. **Figure 1. 1** summarises the main events on antimicrobial resistance through history.

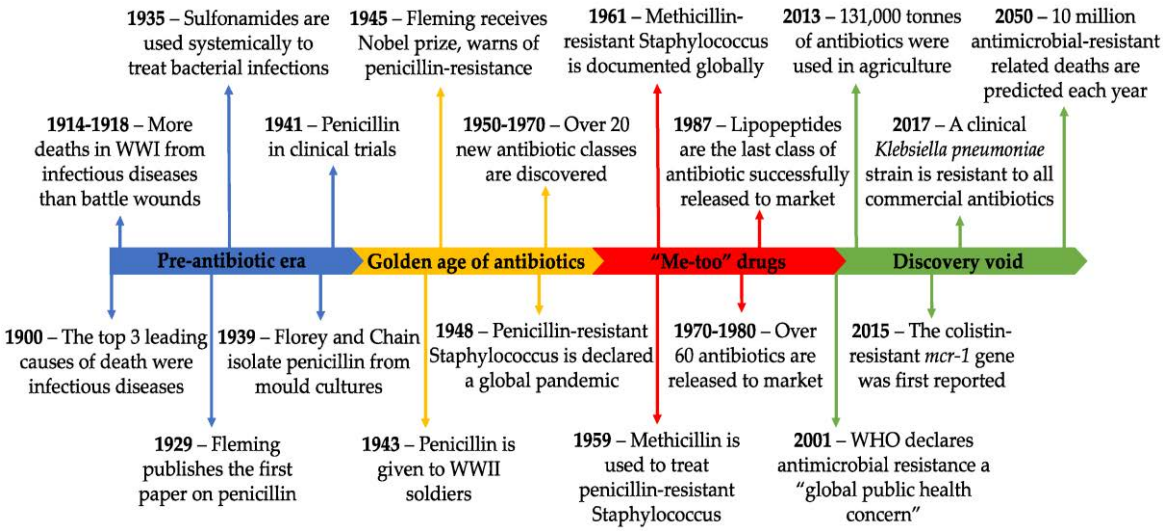


Figure 1. 1. Main event in antimicrobial use and antimicrobial resistance through history. Obtained from Browne et al., 2020.

Resistance is defined as the capability of a microorganism to resist the action of an antimicrobial agent in the presence of concentrations of an antibiotic that would normally inhibit or kill susceptible strains of the same species. Microorganisms can acquire resistance by two mechanisms: by mutation of their genes (less likely) or by acquisition of resistance genes by horizontal transfer through mobile genetic elements (Miller et al., 2014; Munita & Arias., 2016). Resistance is a tool that allows microorganisms to survive, adapt to the environment and compete with other microorganisms. Today it is

known that some of these resistance genes have their origin in saprophytic bacteria and fungi that, for thousands of years, have been synthesizing chemical products (bacitracin, penicillin, polymyxin, etc.) to avoid the aggressions of other microorganisms in their environment. Consequently, and in order to survive, microorganisms develop mechanisms to counteract the toxicity of the antibiotics they synthesize (Raphael & Riley., 2017).

The two types of antibiotic resistance mechanisms displayed by bacteria are intrinsic and acquired resistance:

- Intrinsic resistance: refers to the natural or inherent ability of certain bacterial species or strains to withstand the effects of certain antibiotics due to their inherent biological characteristics. Intrinsic resistance is not acquired through genetic changes or exposure to antibiotics but is a result of the bacteria's inherent genetic composition. It varies between bacterial species and can limit the effectiveness of certain antibiotics against specific pathogens (Livermore et al., 2003).
- Acquired resistance: it occurs when bacteria that were previously susceptible to an antibiotic develop mechanisms to resist its effects. This type of resistance occurs through genetic mutations or the acquisition of resistance genes from other bacteria. It is often the result of selective pressure exerted by the use of antibiotics, leading to the survival and proliferation of resistant bacteria. Acquired resistance can spread within bacterial populations, making it a major concern in healthcare settings and the community (Nikaido et al., 2009).

Intrinsic and acquired resistance both contribute to the global challenge of antimicrobial resistance, making it essential for healthcare professionals and researchers to understand these mechanisms in order to develop effective strategies to combat bacterial infections.

Molecular mechanisms that bacteria develop in order to became resistant to the antibiotics, and thus, survive and proliferate, depends on the type of antibiotics as they act on different targets. Generally, antibiotics target the wall cell synthesis, cell membrane synthesis, deoxyribonucleic acid (DNA)/ ribonucleic acid (RNA) synthesis, protein synthesis and different metabolic pathaways (Wright, 2010; Blair et al., 2015). **Figure 1. 2** shows different molecular mechanisms of antimicrobial resistance.

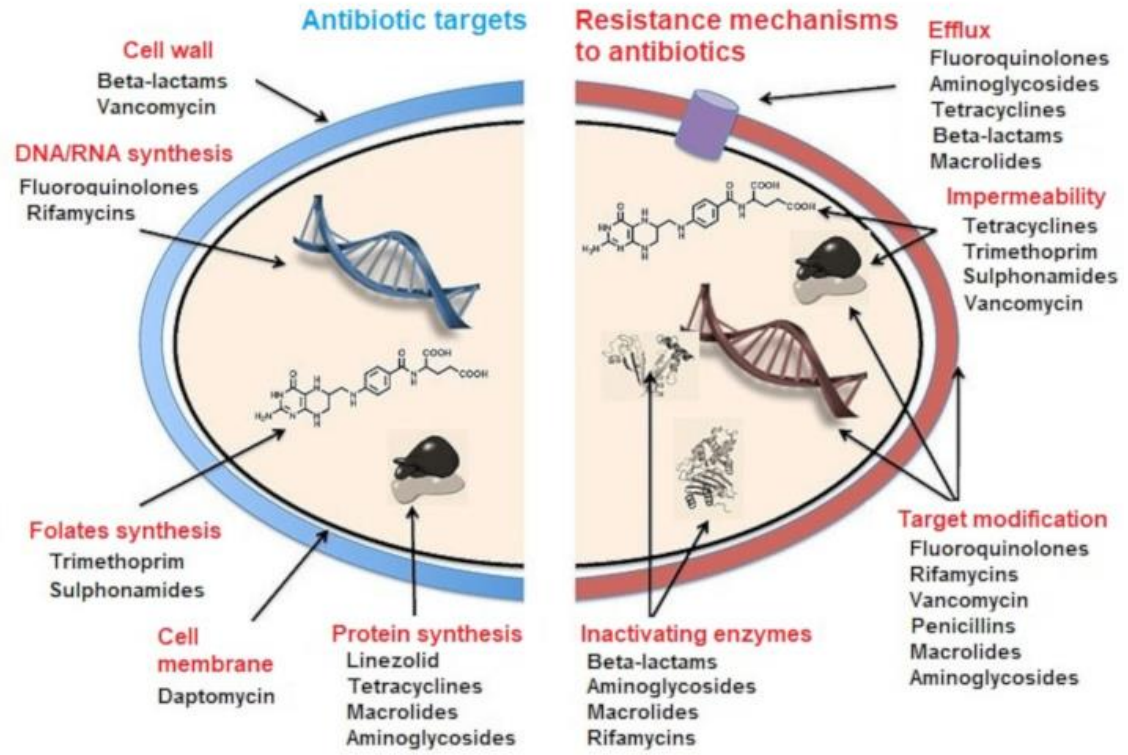


Figure 1. 2. Different molecular antibiotic resistance mechanisms. In the left part there is a representation of different antibiotic targets and, in the right part the mechanisms of resistance that bacteria develop to became resistant to the antibiotics (Wright, 2010).

Antibiotic resistance can be transmitted between bacteria by several mechanisms, throghout mobile genetic elements (Partridge et al., 2018). Plasmids are small, circular DNA molecules that are separated from the bacterial chromosome and can carry antibiotic resistance genes. They can be transferred horizontally between bacteria, allowing the rapid spread of resistance genes within a bacterial population or even between different bacterial species (Munita & Arias., 2016). This transfer can occur by processes such as conjugation, where plasmids are transferred directly from one bacterium to another, or transformation, where bacteria acquire free DNA from the environment. Transposons, on the other hand, are DNA sequences that can 'jump' from one location in the bacterial genome to another, and often carry antibiotic resistance genes within their structure. When a transposon inserts itself into a new location in a bacterium's genome, it can confer resistance to antibiotics. Some transposons can also be found within plasmids, increasing the potential for rapid spread of resistance genes (Partridge et al., 2018; Lerminiaux & Cameron., 2019). These mechanisms of horizontal gene transfer

play an important role in the spread of antibiotic resistance in bacterial populations.

Antibiotic-resistant bacteria occur not only in clinical human isolates but are also widespread in animals and in environmental settings. In fact, in addition to their clinical use in humans, antibiotics are also used in veterinary medicine and in animal husbandry. Antibiotics have also been used extensively as growth promoters in food-producing animals, but although this practice has been banned in Europe since 2006 (Wang et al., 2020) and in several other countries, it is still allowed in some others (Gochez et al., 2021). This contributes to the increase and spread of antibiotic resistance, not only among pathogenic bacteria, but also among commensal bacteria in the human and animal intestinal tract, which can lead to contamination via faeces. Resistant bacteria can therefore be transmitted to humans through the food chain and water, or through contact with animals. For this reason, the WHO has proposed to address this issue from a "One Health" perspective and to establish new alternatives to the use of antibiotics in livestock and agriculture (McEwen & Collignon., 2018).

1.1.1 Alternatives to antibiotics

As infections caused by antibiotic-resistant bacteria increase, new approaches to treatment and prevention are needed. Therefore, the development and number of alternatives to antibiotics has increased in recent decades. Phytochemicals, vaccines, antibodies, probiotics, bacteriophages and antimicrobial peptides are some of the most promising current alternatives (Ghosh et al., 2019; Deb Adhikari et al., 2022).

Phytochemicals from medicinal plants are of growing interest as potential sources of new therapeutic agents. To date, many phytochemicals with diverse biological activities, such as antibacterial, antifungal and anticarcinogenic, have been reported with low toxicity and adverse effects. In addition, anti-quorum sensing, a promising strategy for cell-cell communication that plays an important role in the regulation of various bacterial physiological functions such as pathogenicity, luminescence, mobility, sporulation, etc., has been reported in a variety of novel plant-based compounds. Thus, the use of phytochemicals represents a promising alternative to antibiotics (Nag et al., 2022).

Antibodies (proteins with the ability to recognise and neutralise specific components of a pathogen, produced naturally by the immune system) represent useful alternatives for the treatment of challenging bacterial infections, although their use has several drawbacks, such as the cost of antibody production or allergic reactions in recipients. Antibodies can treat bacterial infections either directly by attacking the bacterial surface or indirectly by neutralizing bacterial toxins and virulence factors. Numerous antibodies against staphylococci, *P. aeruginosa*, *Bacillus anthracis* and *Clostridium difficile* are in various stages of clinical development and some have already been approved by the Food Drug Administration (FDA) (Ghosh et al., 2019; Tasin et al., 2022).

Bacteriophages are unique organisms that replicate at the expense of specific bacteria. Phage therapy was first used almost a century ago, although it was undermined and neglected after the effective introduction of antibiotics. Phage therapy relies primarily on obligately lytic phages to kill their bacterial hosts, leaving human cells unharmed and reducing the shock to commensal bacteria that often results from antibiotic use. Phage therapy offers several advantages, but concerns have been raised about its use, such as bacterial resistance to phages. To date, several phage therapies have been approved or are in clinical trials (Ghosh et al., 2019; Jaiswal., 2022).

Probiotics, prebiotics and synbiotics offer one of the most sustainable and non-alarming alternatives to antibiotics. The probiotic species, such as *Lactobacillus, Bifidobacterium*, etc., produce a wide range of antimicrobial compounds that can act on bacteria that are resistant to antibiotics and limit their growth. Prebiotics such as inulin, on the other hand, act directly in the gut and help to enrich beneficial microorganisms. Synbiotics are the combined use of probiotics and prebiotics to achieve similar effects through the growth of healthy microbiota. As well as controlling the growth of antimicrobial resistant / multi-resistant pathogens, they also provide other immunomodulatory functions that would be even more beneficial to health (Ranjan., 2022).

Vaccines have been used for years to protect against viral or bacterial infections and are proving to be an important alternative to antibiotics in some situations, leading to a reduction in antibiotic use. Vaccines induce a protective immune response similar to that of a natural infection, but without the adverse consequences of the clinical course of the disease. Although they have great

potential for disease prevention, they are not easy to implement. For example, most vaccines are administered by injection, which increases labour costs, and some vaccines are only effective against a small number of bacterial or viral strains, while others may have unexpected side effects. Many of these issues are still under investigation. All in all, however, in certain circumstances vaccination may be a better alternative to antibiotics in the near future (Islam et al., 2022).

Antimicrobial peptides (AMPs) and host-defense peptides (HDPs) are produced by many organisms against invading pathogens. They have a wide variety of activities varying from antibacterial, antifungal, antiviral, anticancer, antiplasmodial, antiprotistal, insecticidal and spermicidal to immunomodulatory. There have been reports of bacteria developing resistance to AMPs. However, disruption of the bacterial cell membrane is too energetically unfavourable for bacteria to easily develop resistance. The wide range of properties and their advantages have led the scientific world to consider AMPs as the antibiotics of the future, and many of them are in clinical trials (Ghosh et al., 2019).

Bacteriocins are a type of AMP that some bacteria produce to prevent competition and increase survival. Bacteriocins are ribosomally synthesized peptides that are often active against drug-resistant pathogens of clinical importance, making them a promising alternative to antibiotic use that will be further developed in the next sections of this document.

1.2 Bacteriocins

Bacteriocins are ribosomal peptides with antimicrobial activity against bacteria closely related to the producing strain, but to which the producing strain is resistant. They have become the center of attention as a promising substitute for antibiotics because of their narrow spectrum of activity, nontoxicity, biodegradability, and non-immunogenicity (Yang et al. 2014; Soltani et al., 2021; Choi et al., 2022). Bacteriocin production is an important trait for bacterial fitness, allowing them to compete with other microorganisms within a niche. In fact, target and producer strains typically share an ecological niche (Ness et al., 2014).

1.2.1 Classification

The classification of bacteriocins is in undergoing constant changes as new developments appear regarding their structure, amino acid sequence, and the recognized mechanism of their action. One method of classification is based on the cell wall type of the producer bacteria (Gram-negative or Gram-positive). Their antimicrobial activities depend on their structure and mechanisms of action, thus, bacteriocins from the same group generally presents similar mechanisms of action.

Bacteriocins produced by Gram-negative bacteria are classified into four groups: colicins, colicin-like, phage-tail-like bacteriocins, and microcins (Zimina et al., 2020). Colicins are proteins produced by *Escherichia coli* strains containing specific plasmids. They are large proteins (30–80 kDa) that are sensitive to proteases and heat. Colicin synthesis occurs under stress and is lethal to the producing cells due to co-expression with lysis protein. They can be categorized into three groups based on their mechanisms: pore-forming, nuclease, and peptidoglycan-degrading. Colicin-like bacteriocins are those bacteriocins that share structural and functional characteristics with colicins but are produced by bacteria other than *E. coli*. Phage-tail-like bacteriocins or tailocins are larger protein structures (20–100 kDa) which consist of multiple polypeptide subunits and are similar in structure to bacteriophage tail modules. They can be classified into two groups: R-type and F-type. Their mechanism of action involves penetrating the cell wall to create channels or pores that disrupt the target cell's membrane potential. Lastly, microcins are small, low-molecular-weight peptides (<10 kDa) produced by members of the Enterobacteriaceae family. They are highly stable and resistant to proteases, extreme pH, and temperature. There are two classes of microcins: Class I, which have complex post-translational modifications and inhibit essential bacterial and Class II, which create pores in the target cell's membrane.

About the classification of bacteriocins produced by the Gram-positive bacteria, **Table 1. 1** represents one of the most recent updates, including information on their receptors and mechanisms of action. Most of the bacteriocins studied to date belong to this Gram-positive group.

Table 1. 1. Gram-positive bacteriocin's classification with their main targets and distinctive structural characteristics. Modified from Zimina et al., 2020.

Class	Group	Distinctive Characteristics	Target	Bacteriocins
Class I	Lantibiotics	Residues (methyl)lanthionine	Lipid II	Nisin, microbisporicin, bovicin HJ50, mersacidin, lacticin 3147, haloduracin
			Phosphatidyl ethanolamine	Cinnamycin
	Lipolantins	N-terminal fatty acid and avionin fragment	-	-
	Thiopeptides	6-membered nitrogen heterocycle, azole ring	50S ribosomal subunit	Thiostrepton, nosiheptide, micopoccine
	Botromycins	Macrocyclic amidine, decarboxylated C-terminal thiazole, β-methylated residues	50S ribosomal subunit	Botromycin A2
	Linear azole-containing peptides	thiazole and (methyl)oxazole rings, linear back bone	-	-
	Sactibiotics (sactipeptides)	Disulfide α-carbon bridges	-	-
	Lasso peptides	Cyclization of an N-terminal amine into a γ-acid	ClpC1 ATPase	Lassomycin
			Regulator of cellular response WalR	Streptomonomicin
	Cyclic bacteriocins with a "head-to-tail" connection	Cycling from N-terminus to C-terminus	Maltose ABC-transporter	Garvicin ML
	Glycocins	Glycosylated residues	Glucose-phosphotransferase system	Sublancin 168, glycocin F
			MscL mechanosensitive channel	Sublancin 168
Class II	YGNG-motif containing bacteriocins	Consensus YGNG-motif, at least one disulfide bridge	Mannose-phosphotransferase system	Pediocin PA-1, leucocin A, carnobacteriocin B2, sacacin P, curvacin A, enterocin HF

	Linear two-peptide bacteriocins	Synergy of two peptides	Undecaprenyl-pyrophosphate-phosphatase Amino acid-polyamine-organocation transporter	Lactococcin G, enterocin 1071 Plantaricin JK
	Leaderless bacteriocins	Lack of a leader peptide	Zn-dependent membrane-bound metallopeptidase	LsbB, enterocin K1
	Other linear bacteriocins	Non-YGNG-like linear peptides	Mannose-phosphotransferase system Lipid II	Lactococcin A, garviacin Q Lactococcin 972
Class III	Bacteriolysins	Large lytic polypeptides	Peptidoglycan	Lysostaphin, zoocin A, millericin B, enterolisin A
	Non-lytic bacteriocins	Large non-lytic polypeptides	-	-
	Tailocins	Multiprotein complex, a structure similar to a phage tail	Lipopolysaccharides	Diffocin, monocin

1.2.2 Bacteriocin production

In general, bacteriocins are most commonly produced by fermentation. It involves the cultivation of bacteriocin-producing bacteria in bioreactors under controlled conditions. The producer strain is typically grown in a nutrient-rich medium, and bacteriocin production is induced through specific growth conditions or triggers. After fermentation, the culture is harvested, and bacteriocins are isolated and purified from the culture broth (Garsa et al., 2014). However, in several cases, low isolated yields and difficulties associated with their purification severely limit their use on a large scale. Chemical synthesis has been proposed for their production and recent advances in peptide synthesis methods have allowed the production of several bacteriocins. The main advantage of this approach is the increasing in the speed production. In addition, the significant reduction in the cost of peptide synthesis reagents and building blocks has made the chemical synthesis of bacteriocins more attractive and competitive (Bédard & Biron., 2018).

1.2.3. Bacteriocin's main applications

Some of the most well-known bacteriocins from Gram-positive bacteria are nisin and pediocin PA-1. Nisin, which has been successfully used in food as a preservative, mainly used in dairy and meat products, inhibits pathogenic food borne bacteria such as *Listeria monocytogenes* (a foodborne pathogen of special concern among the food industries) and many other Gram-positive food spoilage microorganisms (Gharsallaoui et al., 2016). Pediocin PA-1 is a broad-spectrum bacteriocin produced by lactic acid bacteria that shows a particularly strong activity against *L. monocytogenes*. This antimicrobial peptide is the most extensively studied class Ila (or pediocin family) bacteriocin, and it has been sufficiently well characterized to be used as a food biopreservative (Rodríguez et al., 2022).

While food preservation has always been the main application of bacteriocins, the emergency of antimicrobial resistance provides new opportunities to explore the use of bacteriocins in a variety of healthcare products where unwanted and potentially resistant microorganisms need to be controlled. Potential areas of interest include oral and skin care, respiratory, gastrointestinal, urogenital and other infections, and post-surgical control of infectious bacteria. In addition to antibacterial activity, bacteriocins have shown anti-viral and anti-cancer activity, one of the most intriguing new areas of investigation (Chikindas et al., 2018).

1.3. Poultry sector

The poultry industry holds immense economic potential globally, especially in low- and middle-income countries. Presently, poultry stands as the most popularly consumed meat across the world (FAO, 2022), surpassing both pork and beef (**Figure 1. 3**). However, poultry farms face the challenge of contending with various diseases that can result in substantial financial setbacks and pose significant risks to health.

The consumption of animal protein is the foundation of the human diet. Production of poultry meat, which accounts for about one-third of total meat production worldwide (Ritchie et al. 2017; FAO, 2022), has increased rapidly over the past 50 years. The United States, Brazil, the European Union and China remain the world's leading producers of poultry meat (OECD-FAO, 2020). Of the total global meat trade (37.6 million tonnes), 14.1 million tonnes (37.5%) are poultry meat exported by the leading producing countries, making chicken

the most exported meat. Consumer preference for poultry is driven by the increasing affordability of poultry meat compared to beef and pork. The poultry industry benefits from economies of scale and cost efficiency, leading to advances in nutrition, breeding, management and health. This all highlights the major economic importance of the poultry industry as a global commodity, leveraging global economic activity in the industrial, commercial and service sectors. Proper management of poultry litter is essential to prevent the spread of contaminants and antimicrobial resistance into the environment (De Mesquita Souza Saraiva et al., 2022).

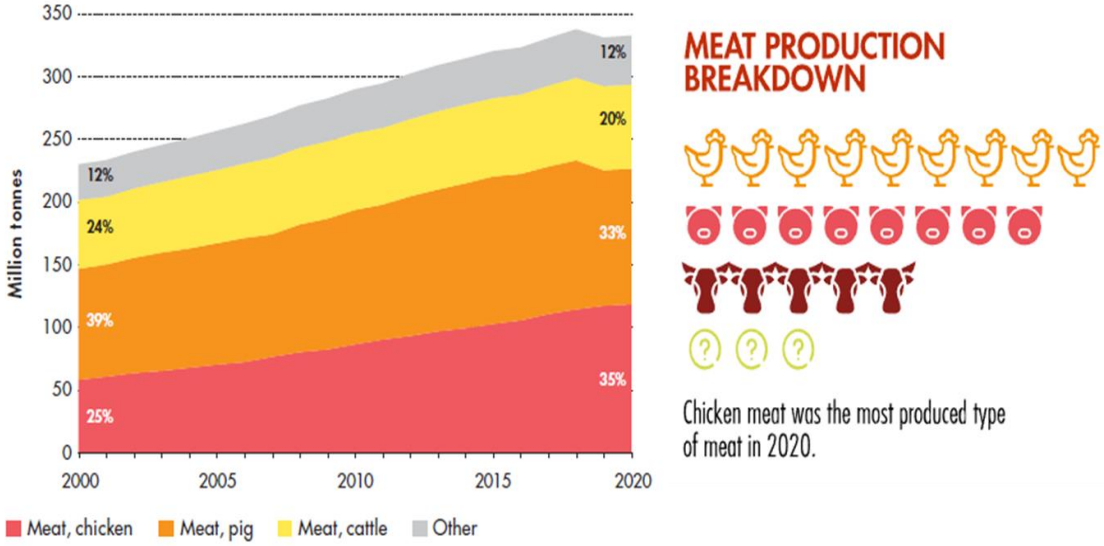


Figure 1. 3. Meat production from 2000 to 2020. Obtained from FAO, 2022.

1.3.1 Antibiotic growth promotion and antibiotic resistance in poultry

Antimicrobial growth promotion involves the administration of antibiotics to animals in order to increase the rate of weight gain or feed conversion efficiency. Elucidation of the mechanism of action of antimicrobial growth promoters (AGPs) is complicated because of the complexity of the mammalian gastrointestinal tract. Modulation of the gut microbiota is the primary mode of action of AGPs and it is hypothesised that AGPs induce changes in bacterial communities that lead to improved growth by modulating the microbiota to create a more efficient system. This may involve altering competition for nutrients, preventing pathogen colonisation and/or selecting for bacteria that are able to extract more energy from the diet (Brown et al., 2017).

During the 2016 World Organization for Animal Health (WOAH) General Session, WOAH Members adopted Resolution No. 36, 'Combating Antimicrobial Resistance through a One Health Approach: Actions and OIE (World Organization for Animal Health) Strategy' agreeing to the recommendation that: 'OIE Member Countries fulfil their commitment under the Global Action Plan to implement policies on the use of antimicrobials in terrestrial and aquatic animals, respecting OIE intergovernmental standards and guidelines on the use of critically important antimicrobial agents, and the phasing out of the use of antibiotics for growth promotion in the absence of risk analysis'. However, even if regulatory polices are taken place, 26% of the WOAH participants in 2021 (41 out of 157, 26.1%), still use antibiotics as growth promoters. The Americas have the highest percentage of respondents using antimicrobials as a growth promoter, while Europe is one of the regions with the lowest percentage (WOAH, 2023).

The WOAH performed a *List of Antimicrobial Agents of Veterinary Importance* and recommends an urgent ban on the use of colistin, fluoroquinolones and third and fourth generation cephalosporins as growth promoters (WOAH, 2023). **Figure 1. 4** shows the antimicrobials used for growth promotion in animals in 2021 in the countries they used them.

Most antibiotics used for disease prevention and growth promotion in broiler production are tetracycline and penicillin, as well as avilamycin, bambermycin, bacitracin and monocarboxylic polyether ionophores such as salinomycin, narasin, and monensin (de Mesquita Souza Saraiva et al., 2021; Mak et al. 2022).

In 1951, the United States Food and Drug Administration (FDA) authorised the use of antimicrobials in feed without veterinary prescription. The use of antimicrobials in feed has varied from country to country. In 2006, the European Union Regulation (No. 1831/2003) restricted the use of antimicrobials in animal nutrition. Then in 2013, the FDA restricted the use of AGPs (antimicrobial growth promoters). In some countries, based on FDA restrictions, AGPs are banned, but the non-OECD (Organisation for Economic Cooperation and Development) countries such as Brazil, India, South Africa etc., which are the leading poultry producers, still use AGPs (Sagar et al., 2023). In Canada, the Public Health Agency of Canada classify antibiotics in four categories (I, II, III, and IV) based on their importance in medicine, where agents

in category I are “very high importance” and those in category IV have “low importance” in human medicine. Te Chicken Farmers of Canada (CFC) progressively eliminated the preventive use of Category I to III antibiotics by 2020 (Mak et al., 2022). Overall, the trend is to remove antibiotics from poultry production slowly but concerns about bird’s health and cost-eficiency remain to be issues in the development of antibiotic replacements.

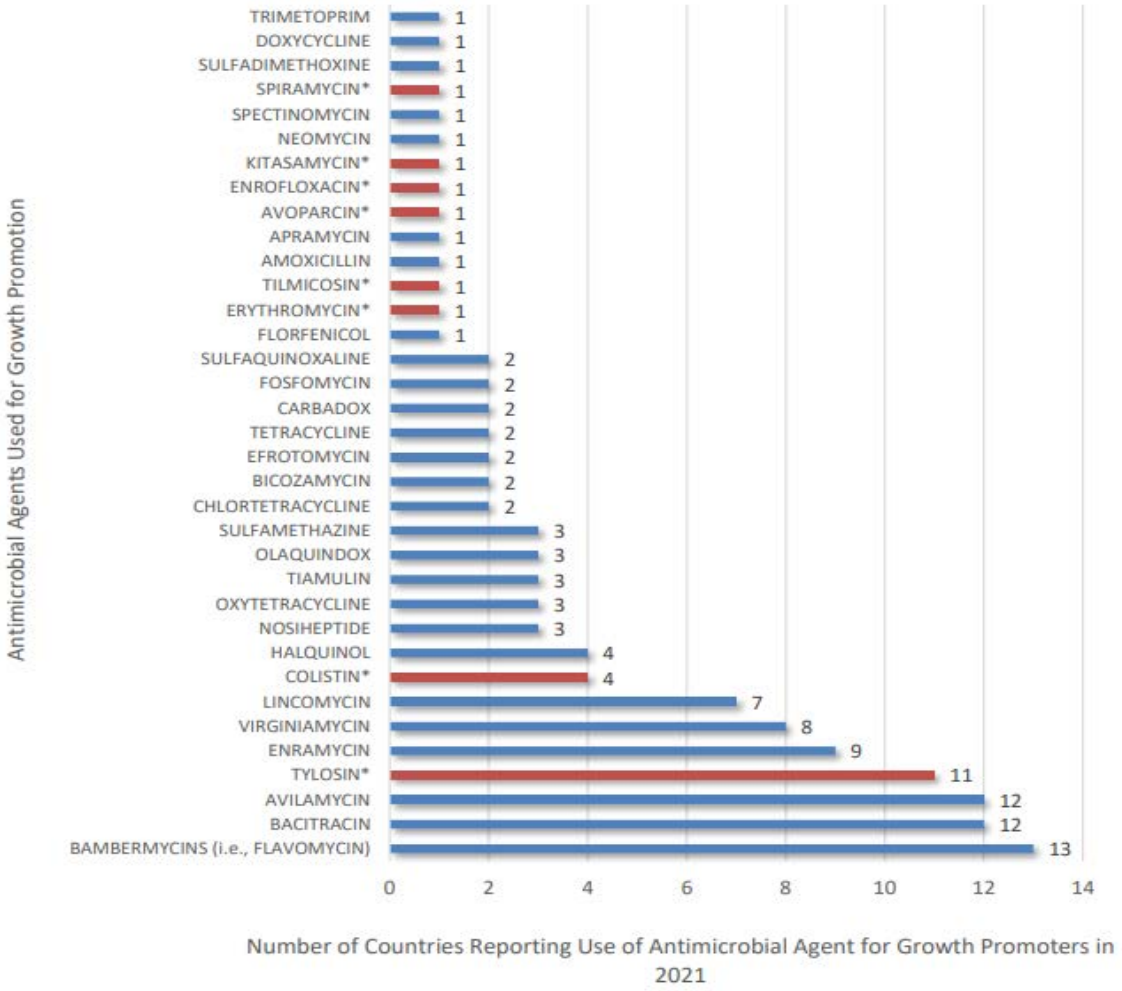


Figure 1. 4. Antimicrobial agents used for growth promotion in animals in the 2021. Data collected from 24 WOAH participants that still use antimicrobials for growth promotion. The symbol “*” indicates the classes in the WHO category of Highest Priority Critically Important Antimicrobials. Obtained from the WHOA 7th report (WOAH, 2023).

As in other sectors, the use of antibiotics in poultry production eliminates susceptible bacteria and favours the selection of resistant isolates. These persistent isolates become dominant and can transfer their resistance genes both to clonal progeny and to other isolates of the same species or even to other

species by horizontal transfer. In addition, the resistant isolates can be transmitted between sectors other than poultry (de Mesquita Souza Saraiva et al., 2021). **Figure 1. 5** shows the potential transfer pathways within the poultry industry.

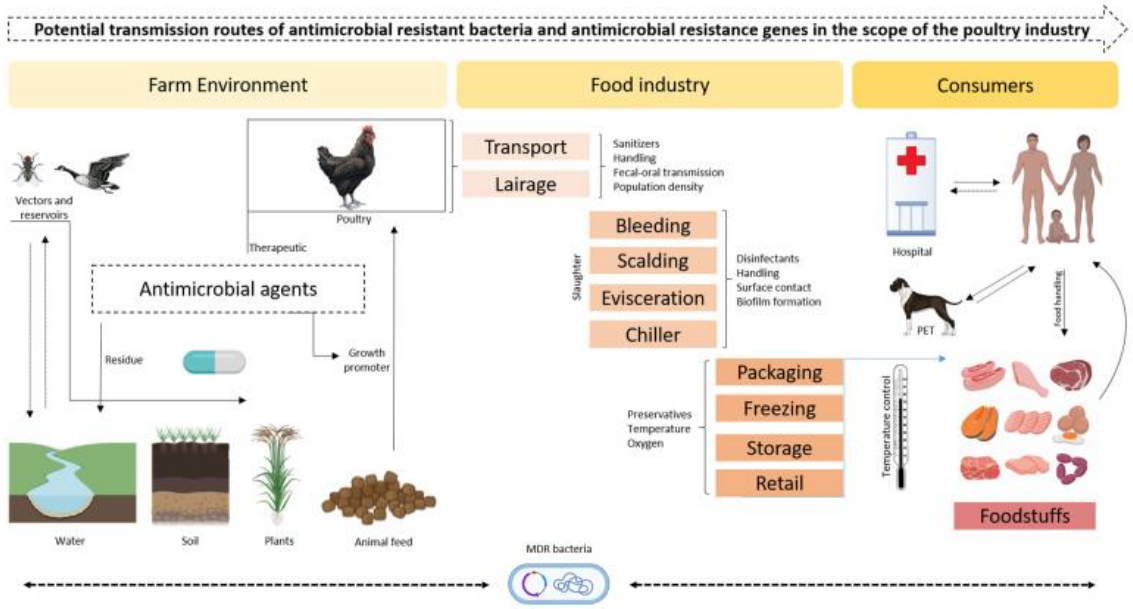


Figure 5. Potential transmission routes of antimicrobial-resistant bacteria and antimicrobial resistance genes in the scope of the poultry industry (de Mesquita Souza Saraiva et al., 2022).

1.3.2 Main pathogenic bacteria in the poultry sector

The poultry sector is facing many challenges. Among them, the emergence of bacterial infections threatens the sector in terms of health problems, economic losses and biosecurity concerns. Here, it is highlighted some of the most relevant infectious diseases in poultry caused by Gram-negative microorganisms such as colibacillosis, salmonellosis, campylobacteriosis and Gram-positive bacteria as is the case of enterococcal spondylitis (ES) and necrotic enteritis (NE).

Avian colibacillosis is caused by a group of pathogens known as avian pathogenic *Escherichia coli* (APEC). Although known for over a century, avian colibacillosis remains one of the most important endemic diseases affecting the poultry industry worldwide, responsible for severe respiratory and systemic disease in broilers, threatening food security and bird welfare worldwide. APEC can be transmitted to the human host, where it is thought to cause urinary tract

infections and meningitis (Dziva & Stevens., 2008, Guabiraba & Schouler., 2015).

Salmonellosis is considered a serious problem in the poultry sector worldwide, usually spread by contamination of chicken meat and eggshells with chicken gut contents, which can also affect human hosts. In broilers it causes diarrhoea, lethargy, loss of appetite, among other syntomps, reaching high mortalities. Several species of *Salmonella* cause salmonellosis. *S. pullorum* and *S. gallinarum* are the species considered to be specific for avian pathogens. However, infections with other serovars of low avian specificity such as *S. typhimurium*, *S. heidelberg*, *S. enteritidis*, *S. infantisa* and others also occur (El-Saandony et al., 2022).

Avian campylobacteriosis is an infection threatening human health caused by *Campylobacter spp. Campylobacter* is usually found in the intestines of birds, which exhibit mild signs in infected birds and can usually contamitante chicken carcasses. Consequently, contaminated poultry products are the main source of human infection and result in severe clinical symptoms (El-Saadony et al., 2023).

Enterococcal spondylitis (ES) is emerging as one of the most important new threats to the poultry sector, the main feature of which is paralysis due to an inflammatory mass that develops in the spinal column of broilers infected with *Enterococcus cecorum*. *E. cecorum* has been identified as a harmless commensal in the gastrointestinal tract of chickens. However, there are pathogenic strains of *E. cecorum* that become a significant cause of morbidity and mortality in broiler breeders and, recurrent outbreaks occur although an environmental reservoir for pathogenic *E. cecorum* has not yet been identified. In addition, pathogenic *E. cecorum* carry increased antimicrobial resistance compared to commensal strains (Jung et al., 2018). There is a strong need for research in this area to fully understand this emerging pathogen.

Necrotic enteritis (NE) is one of the most serious problems facing the poultry industry, which leads to around 6$ billion annual losses (Emami & Dalloul., 2021), and is the main subject of this thesis. It is discussed in more detail in the following sections.

There are many other infections affecting the poultry sector, such as those caused by *Pseudomonas aeruginosa*, wich leads to septicemia, respiratory

and enteric infections, and high mortality (Abd El-Ghany., 2021), *Staphyloccoccus spp.*, causing mainly skeletal disorders (Szafraniec et al., 2022), *Mycoplasma gallisepticum* and *M. synoviae*, causing respiratory problems (Chaidez-Ibarra et al., 2022).

1.4. Necrotic enteritis by *Clostridium perfringens*

Necrotic enteritis (NE), which is caused by *C. perfringens*, stands as one of the most prevalent infections affecting poultry and leads to significant financial losses within the industry (Alizadeh et al., 2021). A distinguish characteristic of NE is its sudden and severe fatality, with mortality rates reaching as high as 50%. Clinical manifestations include depression, dehydration, ruffled feathers, diarrhea, and reduced feed intake (Van Immerseel et al., 2004). The subclinical manifestation of this disease consists on chronical damages the intestinal mucosa in chickens, resulting in compromised nutrient absorption, reduced weight gain, and overall decline in performance. While *C. perfringens* is typically present in low quantities (<10^5 CFU/g) in the intestines of healthy chickens, an increase in its prevalence can render poultry susceptible to NE (Alizadeh et al., 2021). The pathogenesis of *C. perfringens* is resumed in **Figure 1. 6**.

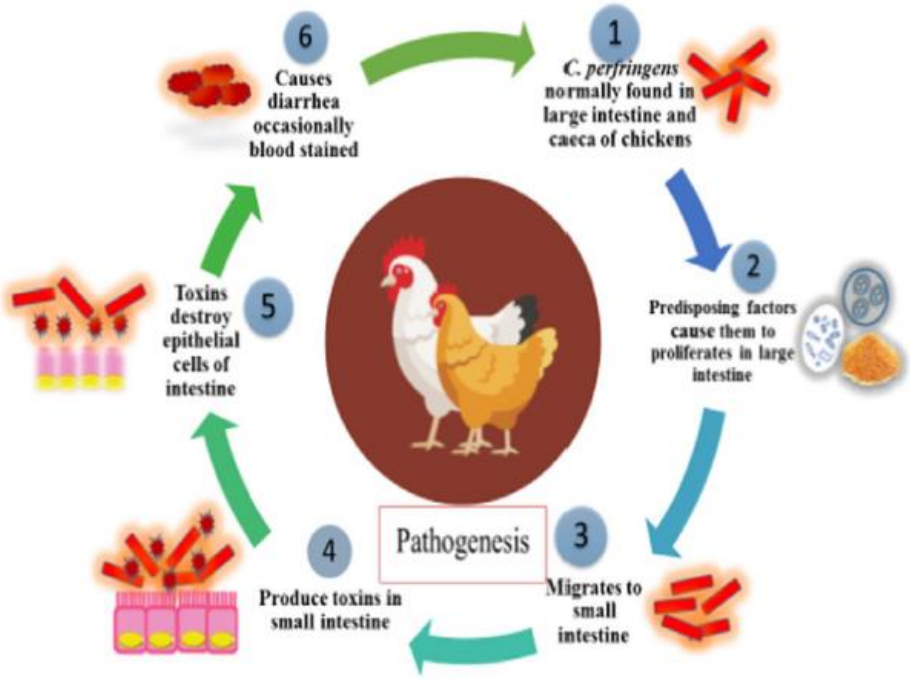


Figure 6. Pathogenesis of necrotic enteritis in broiler chickens, causing destruction of epithelial cells of intestine that leads to blood-stained diarrhea. Obtained from Rajput et al., 2020.

The pathogen responsible of the developing of this disease is *C. perfringens*. It is a Gram-positive, spore-forming, strictly anaerobic bacterium that can be found in a variety of environments, including food, soil, and in the gastrointestinal tracts of both diseased and healthy animals and humans (Kiu & Hall., 2018). It is a widespread pathogen that can be classified as toxin types A–G (**Figure 1. 7**), depending on the combination of the following toxins: α-toxin, β-toxin, ε-toxin, ι-toxin, enterotoxin (CPE), and NetB. This microorganism also produces other toxins which are not considered for typing. These include β2-toxin, λ-toxin, and θ-toxin (Shrestha et al., 2018; Villagrán-de la Mora et al., 2020). Hence, they produce a diversity of diseases in both animal and human hosts (Kiu & Hall., 2018; Shrestha et al., 2018; Villagrán-de la Mora et al., 2020), which are represented in **Figure 1. 7**.

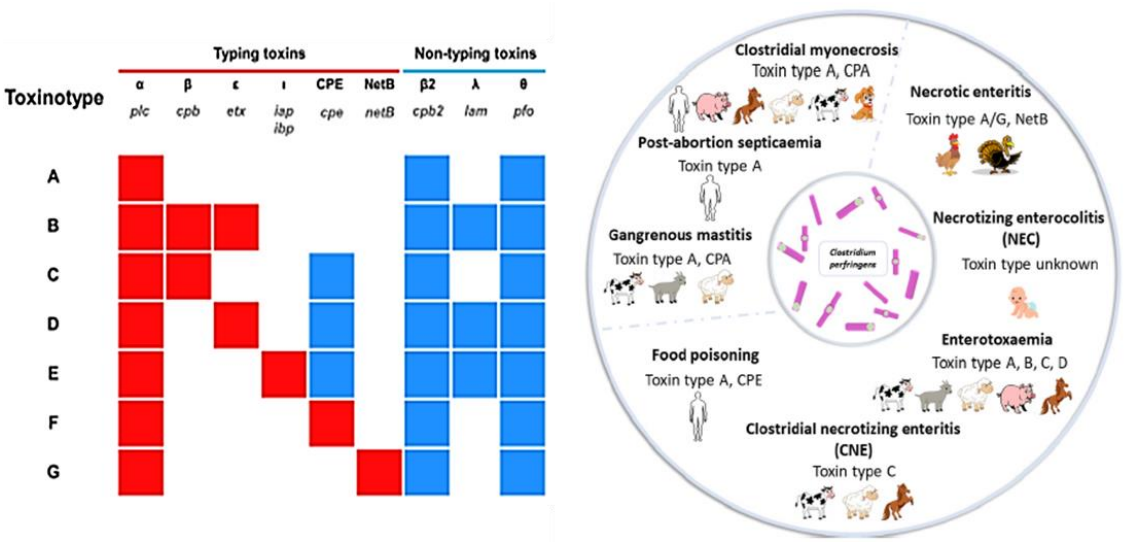


Figure 7. *Clostridium perfringens* current toxinotyping system and the *C. perfringens* induded diseases according to toxinotypes. The names of toxin genes are printed in grey italics. Red cells indicate toxin production and blue cells indicate potential toxin production. Image combined from Kiu & Hall., 2018 and Fu et al., 2022.

Toxinotype G is a proven cause of NE in chickens (Rood et al., 2018), in which NetB plays an important role. It is a plasmid-encoded, pore-forming toxin exclusive for *C. perfringens* coming from poultry affected by NE. It is a key virulence factor in the pathogenesis and is similar to *Staphylococcus aureus* alpha-hemolysin. It forms heptameric pores on its target cell membranes (Keyburn et al., 2008; Yan et al., 2013). Sequences of *netB* genes from isolates from around the world show that the coding sequence is highly conserved across all strains (Lacey et al., 2013). Other toxins present in *C. perfringens* from poultry with NE are α-toxin, β2-toxin, and θ-toxin. The α-toxin is a secreted zinc-metalloenzyme with lethal, hemolytic, and dermonecrotic activities, as well

as phospholipase C and sphingomyelinase activities, and it is a major pathogenic factor in the development of gas gangrene. At low doses, it causes limited phospholipid hydrolysis, which in turn activates diacylglycerol- and ceramide-mediated signaling pathways, leading to cell apoptosis (Naylor et al., 1998; Sakurai et al., 2004; Urbina et al., 2011). The β2-toxin has no significant homology with the sequence of β-toxin or any other known protein sequence, and its mechanism is still unknown (Gibert et al., 1997; Van Asten et al., 2010). The θ-toxin is a cholesterol-dependent cytolysin and is a member of the β pore-forming family of toxins (Popoff., 2014).

C. perfringens also carries other virulence genes such as those encoding sialidases, exoenzymes, and adhesion proteins. The most common degradative enzymes are proteases (e.g., clostripain), hyaluronidase (μ-toxin), collagenase, endoglycosidases, and the sialidases NanJ, NanI, and NanH (neuraminidases), which generate free sialic acids (Mehdizadeh Gohari et al., 2021).

The production of antimicrobial peptides, such as bacteriocins, by *C. perfringens* has also been reported. This trait is sometimes considered virulence factors, as they could inhibit the growth of not only pathogenic bacteria, but also commensals for competition with the ecological niche in the host gut. Bacteriocin BCN5 and perforin are the well-known plasmid-encoded bacteriocins produced by *C. perfringens* (Villagrán-de la Mora et al., 2020; Mehdizadeh Gohari et al., 2021). Recently, the structural gene of Lactococcin A has been detected in a *C. perfringens* strain from poultry (Elnar & Kim., 2021). More in-depth studies are necessary for the study of bacteriocin production in *C. perfringens* and the possible link with the virulence of this pathogen.

1.4.1 Antibiotic resistance in *Clostridium perfringens*

Antimicrobials have been used successfully as treatment for NE. The antimicrobials of choice are beta-lactams (benzylpenicillin or aminopenicillins) followed by tylosin. Coccidiostats are also used to control this disease due to their activity against coccidian parasites, such as *Eimeria spp.*, an important predisposing factor for *C. perfringens* infections (Agunos et al., 2012). However, the continued increase in the emergence of multidrug-resistant bacteria has led to a scenario where resistant *C. perfringens* isolates are also becoming a concern.

Isolates of *C. perfringens* resistant to bacitracin, penicillin, streptomycin, tetracyclines and gentamicin have been reported in poultry. Despite the importance of *C. perfringens* on poultry health, there are a lack of studies that investigated the prevalence of antimicrobial resistance (AMR) in *C. perfringens* of poultry production (Mak et al., 2022).

Antimicrobial resistance, together with a gradual decrease in the susceptibility of some strains of *Eimeria* spp. to anticoccidial agents (a predisposing factor for NE), can lead to an increase in the occurrence of *C. perfringens* strains (Agunos et al., 2020). Acquired antimicrobial resistance genes are commonly plasmid-associated. Plasmid-carrying tetracycline resistance genes (*tet*) are frequent in *C. perfringens* (Adams et al., 2018), as well as those related to macrolide and lincosamide resistance (mainly erythromycin and lincomycin) (Kiu & Hall., 2018). Multidrug resistance among *C. perfringens* isolates has been described in different studies. Resistance to tetracycline, lincomycin, enrofloxacin, cefoxitin/ampicillin, and erythromycin by the carriage of *tet*, *Inu*, *qnr*, *bla*, and *erm*(B) genes, respectively, has been identified in *C. perfringens* of foodborne infections by polymerase chain reaction (PCR) in Egypt (Bendary et al., 2022). This phenomenon is also frequent in *C. perfringens* coming from birds, as well as in those coming from other sources. However, many studies only include the phenotypic detection of antimicrobial resistance (Haider et al., 2022; Yadav et al., 2022). Thus, further studies are needed to determine the current status of resistance genetic profile in *C. perfringens*.

Whole genome sequencing (WGS) could be a good tool for this purpose. In this regard, the *C. perfringens* genomes of isolates (n= 372) from different locations and sources (including strains from cattle, dogs and horses) were analysed by WGS to assess their genetic diversity and phylogenetic relatedness (Camargo et al., 2022); this study found that the genetic diversity of *C. perfringens* is based on a large number of virulence factors, which vary between phylogroups, and antibiotic resistance markers. They found that a high percentage of genomes (72.8%, 270/372) carried some type of AMR gene, with a large number of isolates carrying genes putatively associated with resistance to tetracycline, macrolides and aminoglycosides. The AMR genes detected in the study were: *tet*(A), *tet*(B), *tet*(M), *tet*(44), associated with tetracycline resistance *erm*(A), *erm*(B), *erm*(Q), *mef*(A), associated with macrolide resistance, *ant(6)-Ib*,

aadE, associated with aminoglycoside resistance; *Inu*(D), associated with lincosamide resistance; *fex*(A), associated with chloramphenicol resistance; and, *optr*(A), associated with linezolid resistance. Thus, WGS approaches may contribute to a better understanding of this pathogen in terms of its resistance profile.

1.4.2. Alternatives to antibiotics in poultry

Some of the ongoing studies on alternatives to antibiotics in NE include improvements in feed quality, anticoccidial strategies, vaccination and improvements in broiler gut health through the use probiotics and bacteriocins (Abd El-Hack et al., 2022).

Diet strongly influences the incidence of NE in broilers. Diets with high levels of indigestible, water-soluble non-starch polysaccharides, known to increase the viscosity of the intestinal contents, predispose to NE. This is the case for diets rich in rye, wheat and barley relative to diets rich in corn. Also, diets rich in fish meal predispose to NE (Van Immerseel et al., 2004). Therefore, the use of more adequate diets are one of the main preventions to NE.

Coccidia is a protozoan parasite that multiplies in the intestine of its host and causes enteric disease. *Eimeria* species, such as *E. acervulina* and *E. maxima*, are the best known predisposing factors for NE in chickens. It is not clear how coccidia induce NE, but possible causes include damage to the intestinal lumen, usually during coccidial multiplication, leading to haemorrhage; the leaked plasma proteins become a source of growth substrate for *C. perfringens*. In addition, coccidiosis induces mucus production and provides a suitable environment for *C. perfringens* growth. Therefore, the prevention of coccidiosis by the use of coccidiostats is one of the proposed alternatives to antibiotics for the prevention of NE in broilers (Abd El-Hack et al., 2022).

Other preventive measure against *C. perfringens* infections is vaccination against clostridial toxins (Van Immerseel et al., 2004; Khalique et al., 2020). However, difficulties with production costs and implementation make this alternative less feasible than others (Abd El-Hack et al., 2022).

Supplementation with a single bacterial strain or a mixture of different bacterial strains or yeasts prevents the growth of pathogens in the gut. Probiotics reduce the risk of NE by enhancing host immunity, improving the

balance of the gut microbiota and stimulating metabolism. These probiotics also produce antimicrobial substances that inhibit the growth of pathogenic bacteria. They also compete with pathogenic bacteria in the chicken gut for growth and nutrients, a process known as competitive exclusion (Van Immerseel et al., 2004; Khalique et al., 2020; Kulkarni et al., 2022). Possible modes of action of probiotics include competitive exclusion, increased digestive enzyme activity, production of substances that can inhibit pathogen growth or neutralise enterotoxins, modulation of host immune development, and alteration of gut microbial activity (Khalique et al., 2020). Thus, many studies have focused on the use of probiotics to prevent NE (Kulkarni et al., 2022).

A realistic alternative to conventional antibiotics is the use of purified bacteriocins or the producing strains as feed additives. One of the main advantages of using bacteriocins is that some of them have highly specific antimicrobial activity, so that they can be used to treat specific infections without altering the commensal gut microbiota. Indeed, secretion of bacteriocins is the mechanism of action of many probiotic strains. Several examples of well-described bacteriocins with beneficial effects for broilers can be found, including pediocin A, divercin and nisin (Caly et al., 2015).

Here, the use of bacteriocin-producing enterococci and its enterocins are further developed in the document as an alternative to antibiotics to prevent *C. perfrinengs* infections.

1.5. Enterococci

1.5.1 General characteristics of *Enterococcus spp.*

The genus *Enterococcus* belongs to the family Enterococcaceae, which also includes the genera *Bavariicoccus*, *Catellicoccus*, *Melissococcus*, *Pilibacter*, *Tetragenococcus* and *Vagococcus*. They are ubiquitous Gram-positive cocci found in the gastrointestinal tract of healthy humans and animals, as well as in water, soil, plants and food. An important key to their widespread distribution in nature is that they are remarkably resilient organisms, able to withstand a wide range of pH and temperature, as well as hypotonic and hypertonic conditions (Lebreton et al., 2014). There are many species described in the genus *Enterococcus*. **Figure 1. 8** shows the phylogenetic relationship between them and their distribution in nature.

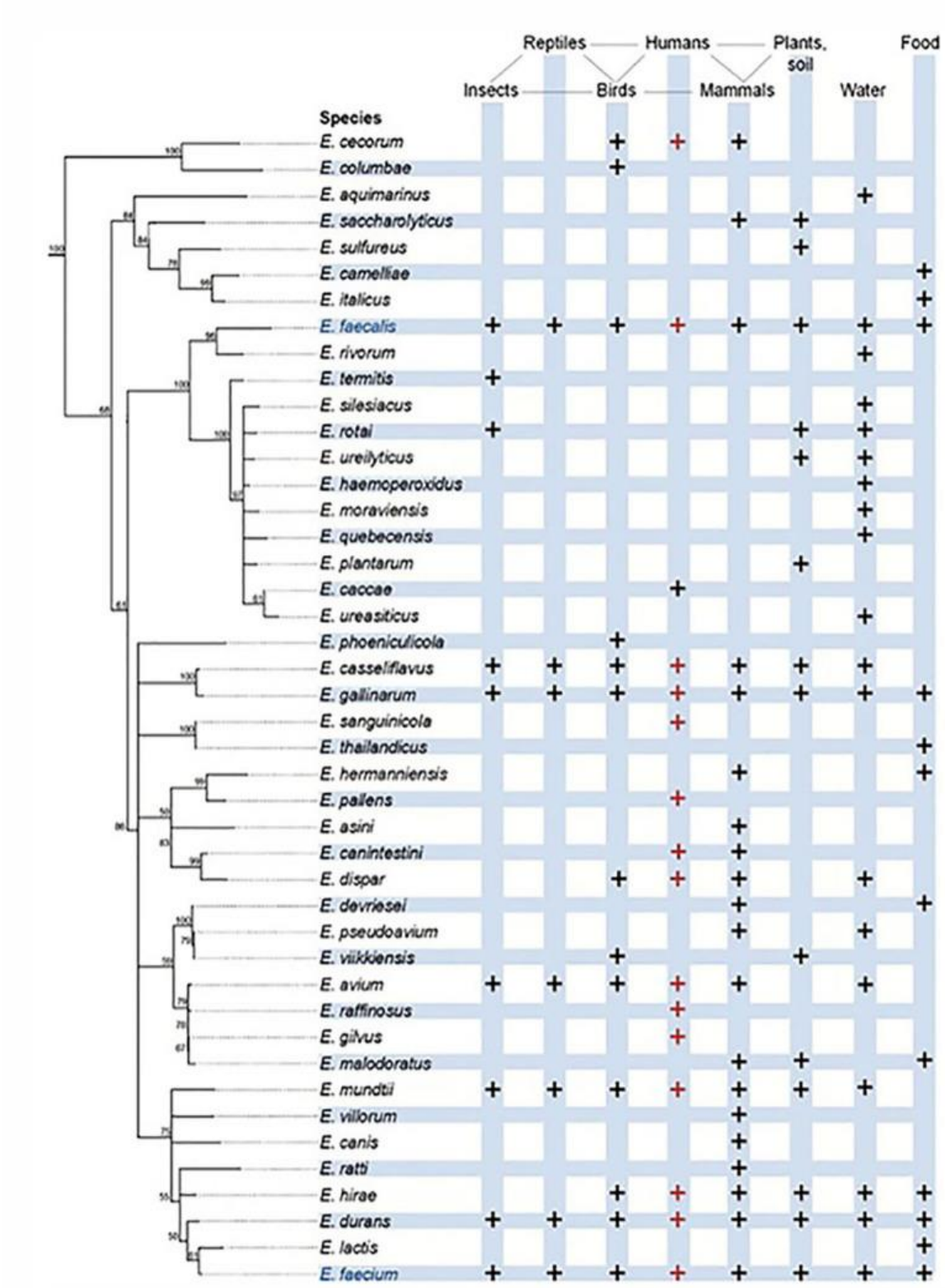


Figure 1. 8. Distribution of species of the genus *Enterococcus* in nature. Phylogenetic relationships are shown in the dendrogram. The sources of isolation are given for each species. A simplified food chain is shown. Red and black symbols indicate species described in human infections and colonisation, respectively. Figure taken from Leberton et al., 2014.

The main characteristics of all enterococci include being Gram-positive, spherical or ovoid cells arranged in pairs or chains. They are non-spore-forming facultative anaerobes and obligate fermentative chemoorganotrophs. They have a growth range of 10-45 °C and an optimum growth temperature of 35 °C. They are catalase-negative, although some species produce a catalase and appear catalase-positive with weak effervescence. Genomically, they present a low G+C content, ranging from 34.29 to 44.75%. They are generally homofermentative and produce lactic acid as the end product from glucose fermentation without gas production. Among the species, some are motile, such as *E. gallinarum* and *E. casseliflavus* (Lebreton et al., 2014, Ramsey et al., 2014).

Generally, *E. faecium*, *E. faecalis*, *E. hirae* and *E. durans* are the most prevalent enterococcal species in human and other mammalian gastrointestinal tracts. *E. cecorum* is also an important member of the normal enterococcal community in the intestines of livestock, pets (bovine, porcine, canine, feline) and avian species (poultry and pigeon). In chickens, however, a significant age-related increase in the colonisation of the gut has been reported in this species. In addition, *E. cecorum* was found to be a dominant part of the enterococcal gastrointestinal microbiota in mature chickens, and currently, even if first described as commensal, pathogenic *E. cecorum* strains are one of the main concerns in this sector (Torres et al., 2018).

1.5.2 Bacteriocin-producing *Enterococcus spp.*

Protective cultures are essentially bacteria that have been specifically selected for their ability to inhibit the growth of other pathogenic organisms or microbiological spoilage agents and have GRAS status (Generally Recognized as Safe), being most of them producers of bacteriocins. They therefore can provide a useful "green" benefit to food labelling (Young & O'Sullivan, 2011). Bacteriocin-producing strains have gained considerable interest in recent years. They are considered to be one of the most promising alternatives to antibiotics.

Enterococci play an important role in biotechnology because of their potential relevance as protective cultures. The ability of enterococci to produce bacteriocins is of interest for their use in food to control and prevent undesirable microorganisms such as *L. monocytogenes*, *Bacillus spp.* or *S. aureus,* among others (Giraffa, 1995, Hanchi et al. 2018). Bacteriocins are defined as ribosomal peptides with antimicrobial activity against bacteria closely related to the producing strain, but to which the producing strain is resistant (Yang et al.

2014; Soltani et al., 2021). Bacteriocin production is an important trait for bacterial fitness, allowing them to compete with other microorganisms within a niche. Indeed, target and producer strains typically share an ecological niche, and specific resistance mechanisms contribute to the survival of the producer strain (Ness et al., 2014).

Importantly, safe enterococcal strains should not carry virulence factors in their genome, as well as antibiotic resistance genes and mobile genetic elements, without receptors for bacteriophages, but with appropriate receptors for adherence to epithelial cells (Krawczyk et al., 2021). Among potential advantages of the use of enterococci as probiotic and commensal strains it can be highlighted their rol in bacteriocin production against pathogens, capacity to block the spread of putrefactive bacteria, bio-preservation, starter cultures in dairy products, dietary supplementation for animals, etc (Krawczyk et al., 2021).

However, in recent years, the use of enterococci in the food industry has been debated because of their implications for opportunistic infections and their potential acquisition of antimicrobial resistance and virulence genes (Ben Braïek & Smaoui., 2019). Thus, they still don't have the GRAS category by the FDA. Therefore, developing new enterococcal probiotics requires a strict safety assessment to select the truly harmless enterococcal strains for safe applications (Hanchi et al., 2018).

1.5.2.1 Enterocins

Enterocins are the bacteriocins produced by *Enterococcus spp*. In general, enterocins have activity against phylogenetic species close to the producing bacteria, but also in some of them we can find a broad-spectrum activity, including gram-positive microorganisms (*L. monocytogenes*, *Bacillus cereus*, *Staphylococcus spp*, ampng others) Gram-negative microorganisms (less frequently as *Pseudomonas aeruginosa*, *Escherichia coli* or *Vibrio cholera*) and even have been reported some antimicrobial activity against fungi and viruses (Ermolenko et al., 2019; Soltani et al., 2021; Wu et al., 2022).

Descriptions of enterococci producing new enterocins, which are active against different bacterial species, are common and have increased in recent years. **Figure 1. 9** shows a timeline of key events for enterocins discovery.

There are several systems for classifying enterocins according to their structure, modification, mode of action, etc. (Franz et al., 2007; Ness et al.,

2014; Ben Braïek & Smaoui, 2019). They can be included in the general bacteriocin classification system, which is still controversial as it's an ongoing field of research, or they can be classified alone. **Table 1. 2** shows a simplified classification for enterocins.

Lantibiotics are small peptides produced by Gram-positive bacteria and active against Gram-positive bacteria. They are ribosomally synthesized as prepeptides. Their genes are highly organized in operons containing all the genes required for maturation, transport, immunity and synthesis. The best characterized lantibiotic is nisin from *Lactococcus lactis* (Bierbaun & Sahl, 2009; Alkhatib et al., 2012). There are only two enterocins described which can be classified as Class I. Lantibiotics; Cytolisin and Enterocin W.

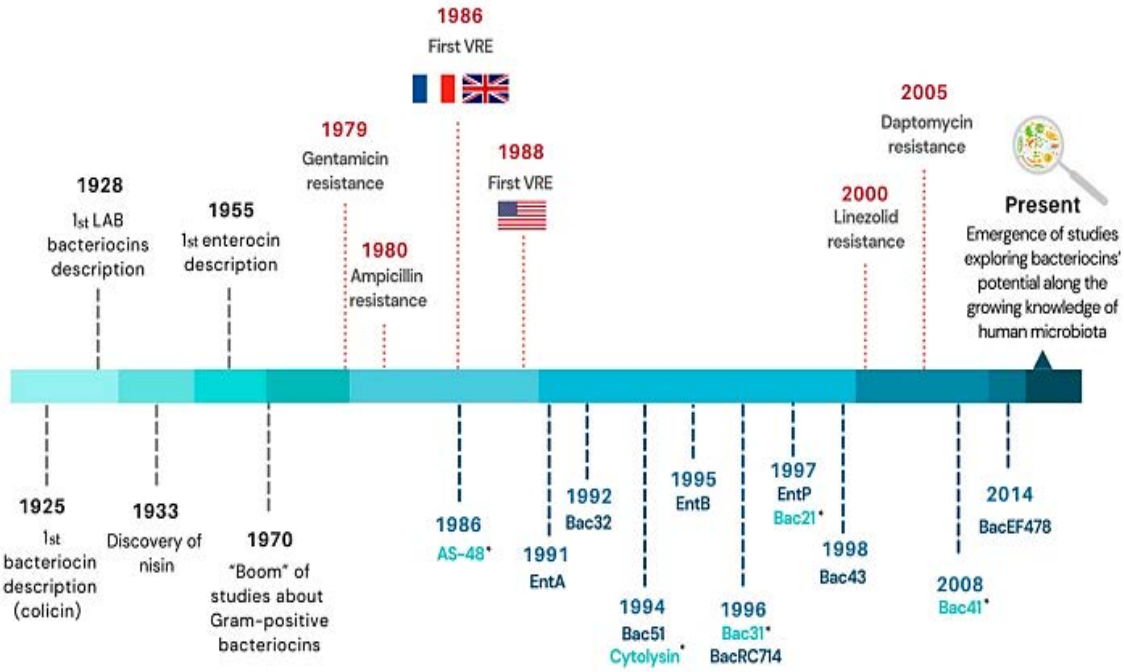


Figure 1. 9. Timeline of important events in the history of bacteriocins. Enterocins specifically described in clinical enterococci isolates to date are shown as blue lines: dark blue indicates enterocins produced by *E. faecium* and light blue by *E. faecalis*; the years given correspond to the first mention of a given enterocin, either the year of isolation or, if unknown, the year of bacteriocin description/publication (marked with * in the latter case). The years corresponding to the emergence of the most clinically relevant antibiotic resistance in enterococci are shown as red lines. Abbreviations: LAB, lactic acid bacteria; VRE, vancomycin-resistant enterococci; Bac, bacteriocin; Ent, enterocin. Obtained from Almeida-Santos et al., 2021.

Table 1. 2. Proposed simplified classification for enterocins.

Class	Subclass	Examples
I. Lantibiotics		Cytolysin, Enterocin W
II. Small non-lantibiotic enterocins	II.a Pediocin-like enterocins	Enterocin A, Enterocin P
	II.b Two component enterocins	L50A/B, MR10A/B
	II.c Circular enterocins	AS-48, NKR5-3
	II.d Others	Enterocin B, Bacteriocin 32
III. Large enterocins		Enterolysin A, Enterocin 7

Cytolysin is an enterocin produced by *E. faecalis*. It was first characterized in 1934 (Todd, 1934). As mentioned before, it shows activity against eukaryotic cells, particularly erythrocytes, as it exhibits haemolytic activity, and against gram-positive bacteria (Booth et al., 1996, Franz et al., 2007). The haemolytic phenotype has been found to be more prevalent in clinical isolates of *Enterococcus spp*. and is considered to contribute to bacterial virulence (Franz et al., 2007). Thus, it is considered to be a virulence factor. Cytolysin consists of two peptides: CylLL and CylLS, both of which are required for cytolysin to exhibit its bacteriocidal and haemolytic activities. The operon responsible for cytolysin production is encoded both chromosomally and in a pheromone-sensitive conjugative plasmid, pAD1 (Van Tyne et al., 2013).

Enterocin W (encoded by *ent*W gene) is an enterocin initially produced by *Enterococcus faecalis* NKR-4-1 isolated from pla-ra, type of traditional Japanese fish ferment (Sawa et al., 2012). It shows activity against gram-positive bacteria, even at concentrations below 1 μM (Ness et al., 2014). It shows activity against *Bacillus coagulans*, *Pediococcus pentosaceus*, *E. faecalis*, *Lactobacillus lactis* and *L. sakei*. EntW consists of two peptides, Wα and Wβ, which act synergistically to exert antibacterial activity (Sawa et al., 2012). It has strong homology to plantaricin W (bacteriocin produced by *L. plantarum*).

Class II enterocins comprise the majority of enterocins. The classification of bacteriocins in this group is controversial. In general, class II enterocins are unmodified and heat-stable proteins, although some do have some modifications (Ness et al., 2014).

The pediocin-like enterocins are the largest subgroup of class II enterocins. They all contain a hydrophilic cationic region with the conserved

YGNGVXC "pediocin box" motif and two cysteine residues connected by a disulfide bridge, which establishes the β-sheet structure (Franz et al., 2007), and is also a prerequisite for the antimicrobial activity of the protein (Ness et al., 2014).

Class II pediocin-like enterocins target the sugar transporter man-PTS and destroy its functionality by irreversibly opening it (Kumariya et al., 2019). They use two different secretion systems. Some are secreted by an ABC transporter that recognises the N-terminus of prebacteriocin, others are synthesised by a *sec*-dependent leader and thus secreted by the *sec* system (Ness et al., 2014). Among the enterocins described in this group, we can highlight Enterocin A, Enterocin B and Enterocin P as the most studied. Others are Enterocin SEK4 or Enterocin M, among others.

Enterocin A (EntA) is produced by several strains of *E. faecium*: CTC492, T136 and P21 from Spanish sausages, BFE900 from black olives, DPC 1146, WHE 81 and EFM01 from dairy products and by the N5 strain from "nuka", a Japanese rice paste (Aymerich et al., 1996). Enterocin A consists of 47 amino acids with a theoretical molecular weight of 4829 Da. EntA is produced as a prepeptide with a leading 18 amino acid peptide (Aymerich et al., 1996). The genetic determinants of this enterocin are chromosomally encoded. This enterocin shows activity against *Enterococcus spp*, *Lactobacillus spp*, *Pediococcus spp* and *Listeria spp* including the pathogen *L. monocytogenes* (Franz et al, 2007). This enterocin is usually coproduced with enterocin B (EntB) and together they form a heterodimer. Studies demonstrate the potential antibacterial and anti-biofilm of EntA-EntB heterodimer, which is active against *S. aureus*, *Acinetobacter baumanni*, *L. monocytogenes* and *E. coli* (Ankaiah et al., 2018). Studies on different applications of this enterocin show that EntA has an anti-listerial effect in different types of meat products alone (Aymerich et al., 2000; Liu et al., 2008) as well as in combination with essential oils (Ghrairi & Hani., 2015). New studies also highlight its possible application in preventing or reducing the incidence of *Campylobacter spp.* in poultry farming (Ščerbová & Lauková., 2016). In addition, EntA has shown anticancer activity when combined with EntB (Ankaiah et al., 2018) and when combined with colicin E1 (Fathizadeh et al., 2021).

Enterocin P (EntP) is produced by *E. faecium* P13 isolated from Spanish fermented sausages (Cintas et al., 1997). The spectrum of activity of EntP

includes *Lactobacillus spp.*, *Pediococcus spp., Propinobacterium spp., Enterococcus spp.*, and the pathogens *L. monocytogenes, S. aureus, C. perfringens* and *C. botulinum* (Cintas et al., 1997). EntP is processed and secreted by the *sec*-dependent pathway. EntP acts by forming specific, potassium ion-conducting pores in the cytoplasmic membrane of target cells (Herranz et al., 2001). Its antimicrobial activity has been studied against beer-spoilage lactic acid bacteria in broth, wort (hopped and unhopped), and alcoholic and non-alcoholic lager beers (Basanta et al., 2008).

The two-peptide class II bacteriocins consist of two different unmodified peptides, typically referred to as "component A" and "component B", both of which must be present for these bacteriocins to exert optimal antimicrobial activity (Oppegård et al., 2007). Nevertheless, some two component enterocins have shown antimicrobial activity on each component by their own (Cintas et al., 1998). These bacteriocins render the membrane of target cells permeable to various small molecules (Oppegård et al., 2007). Some of the well-known two component enterocins are enterocin L50, and the recently discovered enterocin DD14; but there are others described such as enterocin MR10 and enterocin 1071A (Balla et al., 2000; Ruiz-Rodríguez et al., 2013).

Enterocin L50A/B was first detected in an *E. faecium* L50 strain isolated from Spanish fermented sausage (Cintas et al., 1998). Enterocin L50A/B consists of two peptides, L50A and L50B, which synergistically promote their antimicrobial activity. The strain *E. faecium* L50 has also been shown to produce enterocins Q and P at different temperatures (Cintas et al., 2000; Criado et al., 2006). Enterocin L50 A/B exhibits a broad spectrum of antimicrobial activities, including inhibition of *Enterococcus spp., Lactobacillus spp.*, *Lactococcus lactis*, *Pediococcus pentosaceus*, *L. monocytogenes*, *S. aureus*, *B. cereus*, *C. botulinum*, *Streptococcus pneumoniae*, *S. mitis*, *S. oralis*, *S. parasanguis*, *S. agalactiae*, and *C. perfringens*. Other enterocins, such as enterocins 7A/7B and MR10A/10B, share a strong homology with enterocin L50 A/B (Ness et al., 2014). This enterocin has been used to control *L. monocytogenes* from goat's milk using an enterococcal strain that produces it as a probiotic (Achemchem et al., 2006). However, no further applications of this promising enterocin have been investigated.

Enterocin DD14 (EntDD14) is a two-peptide leaderless bacteriocin produced by *Enterococcus faecalis* 14, a human strain isolated from meconium.

Research on EntDD14 has demonstrated its activity against Gram-positive bacteria such as L. *monocytogene*s, *C. perfringens*, *E. faecalis* and *S. aureus*. EntDD14 has also been shown to potentiate the activity of several antibiotics, including erythromycin, kanamycin and methicillin, when tested against methicillin-resistant *S. aureus* (MRSA) *in vitro* and *in vivo* mouse models. In addition, EntDD14 has antiviral activity and reduced the secretion of pro-inflammatory IL-6 and IL-8 in inflamed human intestinal Caco-2 cells (Ladjouzi et al., 2023).

Circular class IIc enterocins are a unique class of these biomolecules distinguished by a seamless circular topology and are widely assumed to be ultra-stable based on this constraining structural feature. Thus, they are known for their resistance against proteolytic degradation, making them potentially more resistant to enzymes that might break down linear peptides. They often exhibit potent antimicrobial activity against various bacteria, including both Gram-positive and Gram-negative species (Masuda et al., 2012). Enterocin AS48 is the most studied circular bacteriocin. Apart from AS48, only a few circular enterocins have been described, these being enterocin NKR-5-3B and enterocin LNS18 (Himeno et al., 2015; Al-Madboly et al., 2020).

Enterocin AS-48 is a bacteriocin that forms a circular structure through a head-to-tail peptide bond (Samyn et al., 1994; Cobos et al., 2001). It exhibits a broad inhibitory effect against Gram-positive bacteria. When used alongside treatments that increase outer-membrane permeability, its effectiveness extends to some Gram-negative bacteria. Notably, eukaryotic cells are resistant to this bacteriocin. Its mechanism involves inserting into bacterial membranes, causing membrane permeability that leads to cell death. The potential of enterocin AS-48 as a food biopreservative has been demonstrated against various foodborne pathogens (*L. monocytogenes*, *B. cereus*, *S. aureus*, *E. coli*, *S. enterica*) and spoilage bacteria (*Alicyclobacillus acidoterrestris*, *Bacillus spp.*, *Paenibacillus spp.*, *Geobacillus stearothermophilus*, *Brochothrix thermosphacta*, *S. carnosus*, *L. sakei*,) (Abriouel et al., 2010). Its efficacy is significantly enhanced when used in combination with chemical preservatives, essential oils, phenolic compounds, and various physico-chemical treatments (Grande-Burgos et al., 2014). Interestingly, it also shows potential as leishmanicidal agent (Abengózar et al., 2017). Advances in preclinical characterization have been performed (Cebrian et al., 2023).

Class IId enterocins are enterocins that cannot be subgrouped elsewhere because of their unique structural characteristics.

Enterocin B (EntB), which always appears in coproduction with EntA, belongs to class IId enterocins. EntB was first produced by *E. faecium* T136 isolated from Spanish fermented sausages (Casaus et al., 1997). Enterocin B shows antimicrobial activity against gram-positive bacteria, such as *L. monocytogenes*, *Propionibacterium spp.*, *C. sporogens*, and *C. tyrobutyricum* (Casaus et al., 1997). When EntA and EntB are co-produced, they form a heterodimer, and studies have demonstrated its potential anti-bacterial and anti-biofilm activities against *S. aureus*, *Acinetobacter baumannii, L. monocytogenes*, and *E. coli* (Ankaiah et al., 2018).

Class III enterocins are large heatlabile proteins with a high molecular weight (>30 kDa). Enterolysin A was the only and most well-known class III enterocin. However, recently a novel class III enterocin has being reported; enterocin 7 (Vasilvhenko et al., 2018).

Enterolysin A, produced by E*. faecalis LMG* 2333 inhibits growth of enterococci, pediococci, lactococci, and lactobacilli. Mature enterolysin A consists of 316 amino acids (Nilsen et al., 2003). It cleaves the peptide bonds within the stem peptide as well as in the interpeptide bridge of Gram-positive bacterial cell walls (Khan et al., 2013). Differently, Enterocin-7 displayed a broader spectrum of activity, being active against some Gram-positive and Gram-negative microorganisms (Vasilvhenko et al., 2018).

As mentioned above, enterocins exhibit antimicrobial activity against several relevant pathogens. Thus, they could be used as an alternative to antibiotics to prevent infections by *C. perfringens*, responsible of NE in poultry. There are different enterocins described in the literature with activity against *C. perfringens*, shown in the **Table 1. 3.**

Table 1. 3. Enterocins with anti-*C. perfringens* antimicrobial activity.

Enterocin	Class	Length	Producer strain	Origin	Activity spectrum	References
Munditicin KS	IIa	58 amino acids	*E. mundtii* AT06	Processed vegetables	E*nterococcus spp., Leuconostoc spp., Pediococcus spp., L. monocytogenes, C. perfringens and C. botulinum*	Bennik et al., 1998; Kawamoto et al., 2002; Saavedra et al., 2004; Franz et al., 2007
Enterocin P	IIa	44 amino acids	*E. faecium* P13	Spanish fermented sausage	*Lactobacillus spp., Pediococcus spp., Emterococcus spp., L. monocytogenes, S. aureus, C. perfringens, C. botulinum*	Cintas et al., 1997; Franz et al., 2006; Marekova et al., 2007
Enterocin SEK4	IIa	43 amino acids	*E. faecalis* K4	Grass silage	*Enterococcus spp., Bacillus subtilis, C. beijerinckii, C. perfringens, L. monocytogenes*	Eguci et al., 2001; Doi et al., 2002
Enterocin L50	IIb	44 amino acids (L50A), 43 amino acids (L50B)	*E. faecium* L50	Spanish fermented sausage	*Enterococcus spp., Lactobacillus spp., Lactococcus spp., Pediococcus spp., Streptococcus spp., L. monocytogenes, S. aureus, B. cereus, C. perfringens, C. botulinum*	Cintas et al., 1998; Cintas et al., 200; Ness et al., 2014
Enterocin 1071	IIb	57 amino acids (1071A), 59 amino acids (1071B)	*E. faecalis* BFE1071	Faeces of minipigs	*Propionibacterium spp., Lactobacillus spp., Streptococcus spp., Micrococcus spp., Listeria spp., Clostridium spp.*	Balla et al., 2000; Balla & Dicks 2005

Enterocin AS48	IIc	105 amino acids	*E. faecalis* AS48	Clinical sample	*Enterococcus spp., Bacillus spp., S. pneumonie, Corynebacterium bovis, C. glutamicum, Mycobacterium phlei, M. smegmatins, Nocaria caranilla, L. monocytogenes, C. perfringens, C. sporogenes, C. tetani, E. coli, Shigella sonnei, Myzococcus xantus, Leishmania spp.*	Galvez et al., 1986; Maqueda et al., 2004; Abengózar et al., 2017

In summary, there are several enterocins that seems to have activity against the avian pathogen *C. perfrignens*. In addition, some of them have a broader spectrum, being active against other relevant pathogens such as *L. monocytogenes*, *Streptococcus spp.* or even Gram-negative bacteria, such as *E. coli*. They therefore might represent a promising alternative to the use of antibiotics in the poultry sector, aspect that should be carefully evaluated.

1.5.3 Enterococci as opportunistic pathogens

Enterococci infections happens when these bacteria overwhelm the host defenses and replicate at rates that exceed clearance and when pathologic changes result. *E. faecalis* and *E. faecium* are the two enterococcal species that are more frequently implicated in human infections, and they are found in relative abundance in human feces, but typically represent less than 1% of the total microbial population (Fiore et al., 2019). Enterococci must compete with other microbes in the gut for space, binding sites, and nutrients. Enterococal overgrowth in the colon increases the chance, by simple numeric probability, of dissemination into the bloodstream and contamination of other body sites and could result in infections (Fiore et al., 2019). *E. cecorum*, on the other hand, is a commensal present in the gastrointestinal tract of poultry that can migrate to spinal cord becoming pathogenic and generating the SE pathology, previously mentioned (Jung et al., 2018).

1.5.3.1 Virulence in *Enterococcus spp.*

The genetic determinants that some enterococcal species have acquired increase the capacity of this genus to generate infection. Different virulence factors are involved in attachment to host cells and extracellular matrix proteins (AS, Esp, Hyl, EfaA), in macrophage resistance (AS), and in cell and tissue damage (Cyl, GelE). Enterococcal virulence factors can be classified into externally secreted virulence factors, surface proteins, extracellular surface proteins and others (Fisher & Philips, 2009; Gilmore et al., 2002; Leberton et al., 2014). Thus, although enterococci are commensal bacteria found in the intestine, they can cause infection. Therefore, the FDA has not yet given them the GRAS category. Their major virulence factors are represented in **Table 1. 4.**

Table 1. 4. Virulence factors in enterococci.

Adherence	Ace (Adhesin to collagen of *E. faecalis*) Acm - *E. faecium* AS (Aggregation substance) Ebp pili (Endocarditis- and biofilm-associated pilus) EcbA - *E. faecium* EfaA Esp (Enterococcal surface protein) Scm (Second collagen adhesin of *E. faecium*) - *E. faecium* SgrA (Serine glutamate repeat A) - *E. faecium*
Exotoxin	Cytolysin
Exoenzyme	Gelatinase Hyaluronidase SprE
Immune modulation	Capsule
Biofilm	BopD Fsr (*E. faecalis* regulator)

The genes encoding virulence factors are located in conjugative plasmids (*agg*, *cyl* or *hyl*), in the chromosome (*gelE* or *fsr*), or in regions of the chromosome called pathogenic islands (*esp*, *cyl*) (Nakayama et al., 2002; Shankar et al., 2002). However, the presence of these genes is not always related to the capacity of virulence, since sometimes they are silent genes and the associated phenotype is not detected. Some of the most common genes encoding virulence factors are *esp*, *hyl* and *gelE*.

The *esp* gene that encodes the enterococcal surface protein, was described in 1999, being mostly found in strains of *E. faecalis* causing infection (Shankar et al., 1999). It plays an important role in adherence, facilitating colonization and persistence (Shankar et al., 2001), as well as in the capacity of biofilm formation (Tendolkar et al., 2004). In addition, this gene has been found in association with a pathogenicity island in *E. faecium,* being transferable by conjugation (Van Schaik et al., 2010).

The virulence gene *hyl* in enterococci encodes a glycosyl-hydrolase, enzyme that in many microorganisms facilitates intestinal colonization (Freitas et al., 2010). This gene has been found mostly in multiresistant *E. faecium* strains causing infection (Rice et al., 2003). It has also been found in plasmids, megaplasmids of more than 150 kb, which are associated with, or co-transferred with, other plasmids carrying the *vanA* gene for vancomycin resistance (Arias-Moliz et al., 2009).

Gelatinase is a secretory protease encoded by the gene *gelE*. In general, the proteases of bacteria have the function of providing peptide nutrients to these microorganisms. However, directly or indirectly, these enzymes cause damage to the host by degrading connective tissues, deregulating some important processes that facilitate the entry and survival of the bacteria in the host, etc, so that they can be considered as virulence factors. This enzyme is capable of hydrolyzing gelatin, collagen, casein, lactoglobulin and other small biologically active peptides. The gene is included in the *fsr* locus complex, with several genes of this locus being involved in the expression of the *gelE* gene. Thus, the presence of the complete locus is necessary for the expression of gelatinase activity. This virulence factor has been found in higher percentage among *E. faecalis* strains. In addition, it can be related to the pathogenicity of enterococci because of its possible role in biofilm formation, especially in strains lacking *esp* gene (Creti et al., 2004).

On another hand, there is a bacteriocin produced by *Enterococcus spp.* that is considered as a virulence factor, Cytolysin, that has been previously described, and was firstly characterized in 1934 (Todd, 1934). This enterocin, composed by two peptides CylLL and CylLS, presents activity against eukaryotic cells, specifically, against erythrocytes due to its hemolytic activity, and it presents activity against Gram-positive bacteria (Booth et al., 1996, Franz et al., 2006). The haemolytic phenotype has been seen to be present to a greater extent in clinical isolates of *Enterococcus spp.*, contributing to bacterial virulence (Franz et al., 2007).

In addition to secreted proteins, enterococci have also been shown to produce toxic oxygen metabolites that can cause cell or organ damage, being more frequent in the case of *E. faecalis* than in *E. faecium* (Fiore et al., 2019).

1.5.3.2 Antibiotic resistance in *Enterococcus spp.*

Enterococci are well known to present a variety of antibiotic resistance mechanisms, both of intrinsic and acquired character.

As mentioned before, resistance to antibiotics can be intrinsic, when it is naturally present in the cell and acquired. The genus *Enterococcus* is characterized by its intrinsic resistance to a large number of antibiotics and its ability to acquire new resistance mechanisms.

Enterococci are resistant, by nature, to semisynthetic penicillins (reduced susceptibility), to aminoglycosides (in low level), to vancomycin (low level and only the species *E. gallinarum* and *E. casseliflavus*/*E. flavescens*, carriers of *vanC* genes), to lincosamides (mostly) and to polymyxins and streptogramins (the specie *E. faecalis*) (Fontana et al., 1996; Gholizadeh & Courvalin, 2000; Torres et al., 2018).

Antibiotic resistance acquisition can occur by mutations in the DNA (mostly in antibiotic targets), or by acquisition of genetic elements containing the resistance genes, which is favored when there is a selective antibiotic pressure. Acquired resistance in enterococci generally takes place by the exchange of pheromone-responsive genes or plasmids, or transposons. Most of the enterococcal strains hold multiple plasmids and transposons which can contain antibiotic resistant or virulence genes (Sarathy et al., 2020). The wide range of antibiotic-resistant mechanisms observed in enterococci is depicted in **Figure 1. 10**.

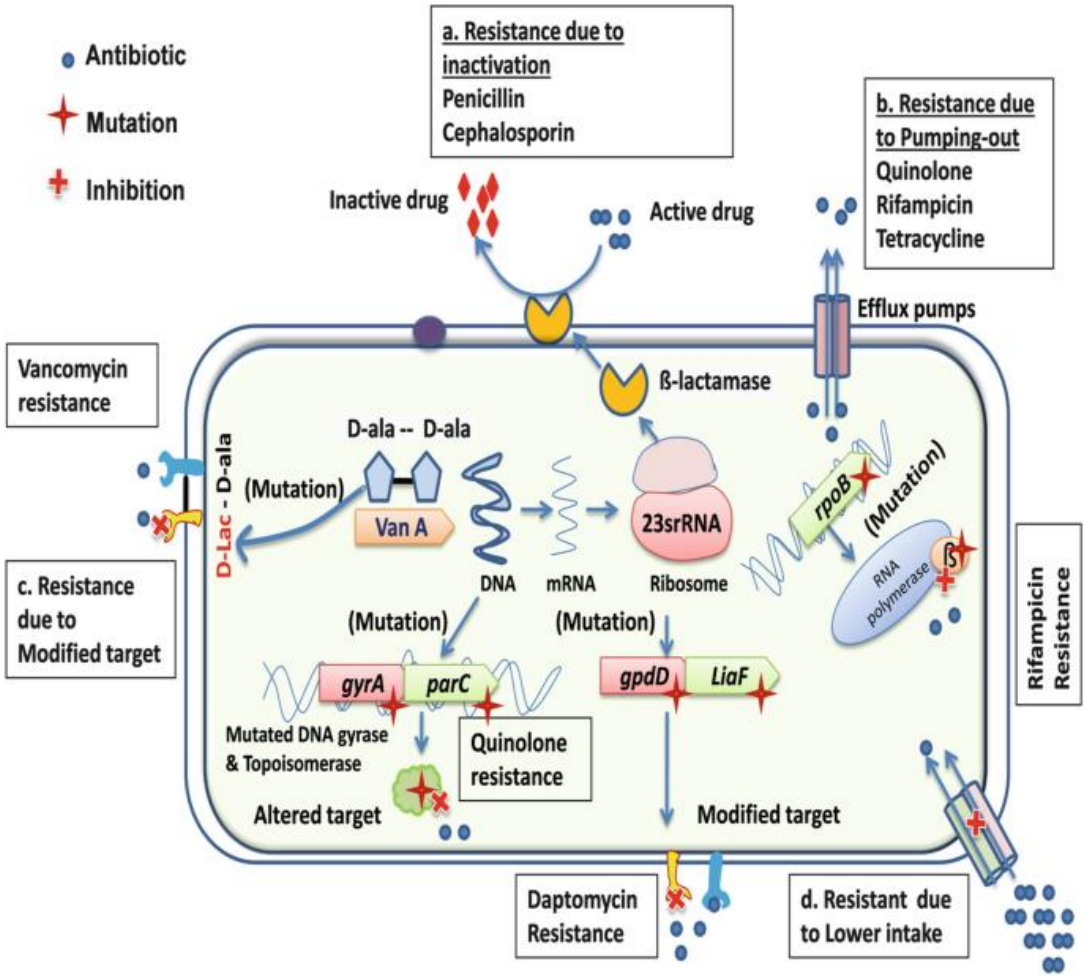


Figure 1. 10. Antibiotic resistance mechanisms in enterococci (Sarathy et al., 2020).

Enterococci have a reduced susceptibility to β- lactams. This susceptibility is due to the low affinity of their penicillin-binding proteins (PBPs) (Cetinkaya et al., 2000; Gilmore et al., 2002). Additionally, they can also acquire high levels of resistance to β-lactams, due to the production of a high amount of an alternative penicillin-binding protein (PBP5), which presents specific amino acid changes that contribute to the low affinity of this protein (Fontana et al. 1996; Jureen et al. 2003; Klibi et al., 2008; Zorzi et al. 1996). This has been frequently detected among clinical *E. faecium* isolates, being rare in *E. faecalis* (Torres et al., 2018). The amino acid substitutions near the Ser-Thr-Phe-Lys, Ser-Asp-Ala, and Lys-Thr-Gly motifs, which are part of the active-site cavity, seem to be the most significant ones.

Mechanisms for the production of β-lactamases have also been identified. However, this mechanism has been detected on few occasions, being the isolates of *E. faecalis* producers of β-lactamases very infrequently (Murray, 1992; Rice

& Carias, 1998; Shepard & Gilmore, 2002), and exceptionally in *E. faecium* (Coudron et al.,1992).

Aminoglycosides act by interfering in protein synthesis by binding to the 16S rRNA of the 30S subunit of the ribosome. Enterococci have an intrinsic low level of resistance to aminoglycosides due to a deficient transport of the antibiotic to the interior of the bacteria. However, when the use of aminoglycosides is combined with an active antibiotic against the cell wall, such as β-lactams or glycopeptides, the entry of aminoglycosides into the bacterium increases, producing a synergistic effect between the two (Moellering et al., 1971). Enterococci present acquired resistance to aminoglycosides by mutation in the target or by the acquisition of genes that confer high level resistance (HLR), encoding enzymes that modify aminoglycosides: phosphotransferases (APHs), acetyltransferases (AACs) and nucleotidyltransferases (ANTs). The most common are those encoded by the genes *ant(6)-Ia* and *ant(3'')-Ia* which allow the expression of the enzymes ANT(6)-Ia and ANT(3'')-Ia, respectively, and generate HLR to streptomycin*; aph(3')-IIIa* which encodes the enzyme APH(3')-III which confers HLR to kanamycin; *aac(6')-Ie-aph(2'')-Ia* encodes the bifunctional enzyme AAC(6')-APH(2'') which has both acetyl and phosphotransferase activities conferring resistance to a broad spectrum of aminoglycosides including gentamicin and tobramycin but not to streptomycin (Torres & Cercenado, 2010; Torres et al., 2018). There are other species-specific genes such as *aac(6')-Ii*, *aac(6')-Iih* and *aac(6')-Iid* that produce resistance to the aminoglycosides kanamycin and tobramycin and are found in the chromosome of the species *E. faecium*, *E. durans* and *E. hirae*, respectively, but not in other enterococcal species. In addition, other less frequent genes that also confer HLR to aminoglycosides have been described as *ant(4')-Ia, aph(2'')-Ib*, *aph(2'')-Ic*, and *aph(2'')-Id* (Del Campo et al., 2005; Chen et al., 2018; Torres et al., 2018).

Regarding the resistance to macrolides, lincosamides and streptogramins, the most common resistance phenotype detected in *Enterococcus* is MLS_B, that affect to macrolides, lincosamides and streptogramis of goup B. It is mediated by the presence of the *erm*(B) gene, and in lesser frequency by *erm*(A), which encodes methylases that modify the target of the antibiotic (Portillo et al., 2000; Ben Said et al., 2016). Other relevant genes are the efflux genes *mef(A)*, which confers resistance to macrolides, *vgb(A)*, which confers resistance to virginiamycin, *lnu(B)*, which confers resistance to

lincosamide, and *vat(D)* and *vat(E)*, which confers resistance to streptogramins. Chromosomal intrinsic resistance to macrolides by *msr*(A) and to lincosamides by *linB* in *E. faecium* has also been described (Torres et al., 2018).

Resistance to chloramphenicol, antibiotic that acts inhibiting bacterial protein synthesis, specifically by binding to the bacterial ribosome and interfering with the peptidyl transferase activity, which is necessary for protein synthesis (Oong & Tadi., 2022), is produced by acetyltransferases encoded by *cat* genes (Torres et al., 2018).Resistance to chloramphenicol associate to the presence of the *fexA* and *fexB* genes has also been reported (Ruiz-Ripa et al., 2020), that frequently is associated to genes of resistance to linezolid

Tetracycline inhibits bacterial protein synthesis, specifically by binding to the bacterial ribosome and interfering with the attachment of aminoacyl-tRNA to the ribosome. This action prevents the addition of new amino acids to the growing peptide chain, ultimately inhibiting bacterial growth and replication (Shutter & Akhondi., 2022). Resistance to this antibiotic is produced by different *tet* genes that produce the reduction of affinity for the target or the efflux of the antibiotic. In enterococci the most frequent ones are those implicated in ribosomal protection [*tet*(M), *tet*(O), *tet*(S)], efflux, or enzymatic inactivation [*tet*(K), *tet*(L)] (Bentorcha et al., 1991; Torres et al., 2018).

The antimicrobial activity of quinolones is based on the inhibition of DNA gyrase, formed by the subunits GyrA and GyrB, and DNA topoisomerase IV, composed by the subunits ParC and ParE, both essential for DNA replication. Quinolone resistance in Gram-positive microorganisms is generally due to mutations in the quinolone resistance determinant (QRDR) regions of the *gyrA* and *parC* genes and/or to decreased intracellular accumulation (Oyamada et al., 2006; Petersen & Jensen, 2004).

Trimethoprim is a bacteriostatic compound that inhibits the activity of the enzyme dihydrofolate reductase (DHFR), leaving the bacteria deficient in di- and tetrahydrofolic acid, an essential cofactor in the biosynthesis of purines, and, therefore, of DNA (Wisell et al., 2008). The most frequent resistance mechanisms against this antibiotic are the presence of antimicrobial efflux or reduced permeability, natural mutations in the DHFR coding genes, overproduction of DHFR enzymes and production of additional DHFR enzymes resistant to trimethoprim (Huovinen, 2001). This last mechanism is the most

frequently detected, and is due to the presence of *dfr* genes, which encode these enzymes, and which are usually found in mobile genetic elements. There are more than 30 *dfr* genes that confer high level resistance to trimethoprim, most of which have been found in Gram-negative bacteria. Only a few *dfr* genes have been described in Gram-positive bacteria. For the genus *Enterococcus*, the genes *dfrG* and *dfrF* are mostly reported (Cattoir et al., 2009).

Vancomycin and teicoplanin are used in humans, while avoparcin has been widely used as a growth promoter in animals, until its ban in many countries, but still allowed in others. The mechanism of action of glycopeptide antibiotics is based on the inhibition of cell wall synthesis. The mechanisms of resistance to glycopeptides in enterococci are based on the modification of the target, generating a pentapeptide with a modified end formed by DAla-D-Lac (D-Lac) or D-Ala-D-Ser (D-Ser). Thus, the modified pentapeptides have a much lower affinity for vancomycin: up to 1000 times lower in the case of D-Lac and about seven times lower in the case of D-Ser (Arthur et al., 1996; Billot-Klein et al., 1994). This modification is produced by the presence of a cluster of genes, organized in the structure of an operon, called *van* operons (there are different ones), which encode enzymes needed for this modification (Courvalin, 2006). To date, nine types of resistance to glycopeptides have been described, one of them being of intrinsic character (*vanC*) for three species (*E. gallinarum*, *E. casseliflavus*, and *E. flavescens*) and eight of acquired character (*vanA, vanB*, *vanD*, *vanE, vanG*, *vanL*, *vanN* and *vanM*) (Cattoir & Leclercq, 2010; Torres et al., 2018). The *vanA* and *vanB* are the most frequent genotypes among vancomycin resistant enterococci (VRE) with acquired resistance mechanisms of humans and animals, mostly among *E. faecalis* and *E. faecium*. The genotypes *vanD*, *vanE*, *vanG*, *vanL*, *vanM*, and *vanN* are very unusual in VRE isolates (Torres et al., 2018).

The prevalence of VRE in many countries makes it necessary to seek other therapeutic options, and linezolid plays a role. Linezolid resistance is still uncommon among enterococci but has emerged in human and animal isolates in recent years. Mutations in the central loop of domain V of the 23S rDNA are the most common mechanism of resistance in enterococci. However, the emergence of transferable resistance to linezolid associated with the acquisition of the *cfr* gene or the recently described *optrA* gene has raised concerns in recent years (Torres et al., 2018; Ruiz-Ripa et al., 2020; Abdullahi et al., 2023).

1.5.4 Link between pathogenicity and enterocin production

Little is known about the correlation between the presence of virulence factors and the occurrence of bacteriocin production in enterococci. Most of the studies just focus on the characterization of virulence factors and antimicrobial resistance determinants in pathogenic enterococci (Say Coskun, 2019) or they characterize enterococci with probiotic potential, and thus, these enterococci do not carry virulence factors or antimicrobial resistance mechanisms. However, this doesn´t mean that enterococci with bacteriocin genetic determinants doesn´t carry undesirable genes. In fact, many bacteriocins have been first described from enterococci coming from clinical samples (Gálvez et al., 1986; Tomita et al., 1996) or/and from resistant strains (Del Campo et al., 2001; Inoue et al., 2006).

Poeta et al., 2006 detected structural genes of bacteriocins and virulence factors from enterococci obtained from faecal samples of poultry: *gelE*, *cpd*, *ace*, *agg*, *fsr*, *esp* and *cyl* were detected in *E. faecalis* strains; *gelE* was also found in *E. faecium* and *E. durans* strains. Structural genes for Enterocin A and Enterocin B were found in *E. faecium* strains. This is a common combination. Enterocin A determinants are considered as housekeeping genes in *E. faecium* (Ness et al., 2014), and Enterocin B, might not be present in *E. faecium*, but in the case it is being produced, it is always coproduced with Enterocin A.

Lengliz et al., 2021 studied bacteriocinetic enterococci from rabbits in search for strains with probiotical potential. Among the 50 enterococci, 47 presented at least one structural enterocin gene (*entA, entB* and/or *entP*), and only four of them didn´t present any virulence factor studied (*hyl*, *esp*, *gelE*, *agg*, *ace*, *efa*, *CylLL/s*, *cob*, *cpd* and *ccf*), belonging to the species *E. durans*, *E. gallinarum*, and *E. avium*. The other enterococcal species were more virulent (*E. faecalis* and *E. faecium*). Regarding the antimicrobial resistance phenotype, nine of them were susceptible to antibiotics, the rest were resistant to at least one antibiotic, noting that resistance to tetracycline and ampicillin were the most frequent resistance profiles, detected in 20 of the 50 isolates studied, and vancomycin resistance in 15 of the 50 enterococci (twelve *E. faecalis*, two *E. avium* and one *E. faecium*; resistance was of acquired character in all these isolates). In addition, linezolid resistance was also detected in three *E. faecalis* strains. Of all the strains studied, one was susceptible to antimicrobials, non-virulent and with bacteriocin determinants, from the species *E. durans*.

Moraes et al., 2019 characterized and analysed the fingerprints of 43 enterococci from raw milk and soft cheese by RAPD-PCR. Most isolates harboured at least one lantibiotic or enterocin gene and were positive for several tested virulence genes, mainly *asa1*, *gelE* and *efaA*. The authors concluded that the isolates presented an interesting potential application for food preservation but however, virulence-related genes were identified in all isolates.

Avci & Tuncer., 2016 determined the antimicrobial activity and occurrence of bacteriocin structural genes in *Enterococcus spp.* isolated from different cheeses as well as the presence of virulence factors. Structural enterocin genes *entA*, *entB, entP* and *entX* were detected in some isolates. Six strains showed multiple antibiotic resistance patterns (including strains carrying the vancomycin resistance gene *vanA*) and in addition, several virulence genes were detected in many strains.

Sánchez Valenzuela et al., 2012 screened *E. faecalis* and *E. faecium* from wildflowers, which were clustered in well-defined groups by ERIC-PCR fingerprinting. A high incidence of antibiotic resistance was detected among the enterococcal isolates. Most isolates (especially *E. faecalis*) carried the gelatinase gene *gelE* as well as other potential virulence factors (*ace*, *efaA, ccf*, *cpd*, *cylL* and *hyl)*. Many isolates produced bacteriocins and carried genes for enterocins, predominating *ent*A, *ent*B, and *ent*L50.

Dahlén et al., 2012 evaluated the presence of virulence factors and antibiotic susceptibility among enterococcal isolates (sixty *E. faecalis* strains and one *E. faecium*) from oral mucosal and deep infections. Virulence genes were detected in most of the *E. faecalis* strains, and forty-six strains produced bacteriocins.

Concluding, bacteriocin-producing enterococci frequently carry genes encoding virulence factors, and also could carry antimicrobial resistance genes of clinical importance, what it is a relevant issue. The expression of bactericins could facilitate the colonization process of enterococci and if they contain virulence or antibiotic resistance genes could increase the pathogenicity of the strain and could worsen the therapeutical options in case of infection. However, little is known about the connection of pathogenicity and production of enterocins. Thus, deeply characterization of virulence factors and antimicrobial resistance mechanisms are needed for determining safety in bacteriocin-

producer enterococci. Whole genome sequencing (WGS) of enterococci could give a global view of the content in bacteriocin, virulence and antibiotiuc resistance genes, relevant for safety determination of the strains intended for potential use as protective cultures.

1.6 Problematic, hypothesis and objectives

1.6.1 Problematic

The spread of antibiotic-resistant bacteria, together with the increase in infections caused by *C. perfringens* in poultry, the etiological cause of NE in poultry and responsible for many economic losses, leads us to a situation where new alternatives to antibiotics are more than necessary. In addition, resistant *C. perfringens* occurs, but since this microorganism is not under surveillance programs, more information is needed to fully understand its resistance profile, both phenotypically and genomically.

New and effective approaches need to be implemented in the poultry sector to avoid excessive use of antimicrobials for prophylaxis, treatment and growth promotion. Bacteriocin-producing bacteria are one possibility, and safe enterococci could be used for this purpose. However, these bacteria contain genetic determinants that encode virulence factors and very often carry antibiotic resistance mechanisms that can be transferred to other bacteria. Therefore, due to their duality as commensal and opportunistic pathogens, a very in-depth characterisation of these bacteria is required.

Another promising alternative to antibiotics in this area is the use of bacteriocins. Some has already been used as food preservatives. However, more studies are needed to fully characterize them and describe new ones. Finding bacteriocins that are active against *C. perfringens* could be a good option, and the enterocins produced by *Enterococcus spp.* could be good candidates for this purpose. Some of them have already been described as active against this pathogen. However, their applications will be enhanced by the identification of others that may also inhibit the growth of other important pathogens in the sector. Another problem associated with the use of bacteriocins is the difficulty of purification methods, as they are usually produced by fermentation. It is therefore necessary to find new approaches to their production and purification to make their production more feasible.

Therefore, this thesis will address several points to overcome this issue. Firstly, NE-associated *C. perfringens* isolates will be characterized at the genomic level, with special attention to resistance mechanisms, in order to increase the information on this topic and to better understand the pathogen. Secondly, an in-depth characterization of bacteriocin producing enterococci with activity against *C. perfringens* will be carried out to ensure safety and to select potential strains for further use in the poultry sector. Finally, enterocins will be produced and their potential to inhibit the growth of *C. perfringens* and other relevant pathogens in the sector will be evaluated.

1.6.2 Hypothesis

Enterococcus spp. of poultry origin producers of bacteriocins and/or its antimicrobial peptides could represent an effective alternative to antibiotics in poultry farming, preventing infections by *C. perfringens* responsible for necrotic enteritis as well as the spread of multidrug resistant bacteria.

1.6.3 Objectives

1st Objective: To characterize at the genomic level a collection of *C. perfringens* isolates coming from poultry affected by necrotic enteritis:

1- To analyse their antibiotic resistant phenotype.
2- To perform WGS analysis of isolates, and to determine:
    - Resistome
    - Multilocus-sequence-typing
    - Toxin genes and toxinotypes
    - Other virulence factors as well as plasmids
    - Phylogenetic relationships among the *C. perfringens* isolates.
3- To detect bacteriocin-genes and to determine the bacteriocinetic activity of the *C. perfringens* isolates.

2nd Objective: To screen and characterize in terms of safety enterococcal strains of poultry origin with antimicrobial activity against *C. perfringens*.

1- To isolate enterococci from poultry carcasses and carry out their identification.
2- To check the collection of enterococci for antimicrobial activity against *C. perfringens* using different methods.
3- To evaluate the antimicrobial activity of the enterococci against other relevant bacteria.
4- To carry out a phenotypic and genotypic analysis of selected bacteriocin-producer enterococci in terms of:
    a. Antimicrobial resistance
    b. Virulence
    c. Plasmid content
    d. Molecular typing (MLST)
5- To select potential harmless enterococci to analyze their digestive survival under poultry conditions.

3rd Objective: To produce and purify enterocins with activity against the *C. perfringens* collection and other relevant bacterial poultry pathogens.

1- To obtain by chemical synthesis enterocins A, B, P, SEK4 and L50 (L50A and L50B).
2- To evaluate their antimicrobial activity against the collection of *C. perfringens* isolates.
3- To study differences in the enterocin receptors from selected isolates of the *C. perfringens* collection according to differences in their enterocin susceptibility profiles.
4- To evaluate the antimicrobial activity of the enterocins produced against other relevant poultry pathogens.
5- To combine different enterocins according to their mechanisms of action and elucidate if those combinations achieve synergy against *C. perfringens*.

2. Chapter II: Pathogenicity and Antibiotic Resistance Diversity in *Clostridium perfringens* Isolates from Poultry Affected by Necrotic Enteritis in Canada

Abstract

Necrotic enteritis (NE) caused by *C. perfringens* is one of the most common diseases of poultry and results in a huge economic loss to the poultry industry, with resistant clostridial strains being a serious concern and making the treatment difficult. Whole-genome sequencing (WGS) approaches represent a good tool to determine resistance profiles and also shed light for a better understanding of the pathogen. The aim of this study was to characterize, at the genomic level, a collection of 20 *C. perfringens* isolates from poultry affected by NE, giving special emphasis to resistance mechanisms and production of bacteriocins. Antimicrobial resistance genes were found, with the *tet* genes (associated with tetracycline resistance) being the most prevalent. Interestingly, two isolates carried the *erm*(T) gene associated with erythromycin resistance, which has only been reported in other Gram-positive bacteria. Twelve of the isolates were toxinotyped as type A and seven as type G. Other virulence factors encoding hyaluronases and sialidases were frequently detected, as well as different plasmids. Sequence types (ST) revealed a high variability of the isolates, finding new allelic combinations. Among the isolates, *C. perfringens* MLG7307 showed unique characteristics; it presented a toxin combination that made it impossible to toxinotype, and, despite being identified as *C. perfringens*, it lacked the housekeeping gene *colA*. Genes encoding bacteriocin BCN5 were found in five isolates even though no antimicrobial activity could be detected in those isolates. The *bcn5* gene of three of our isolates was similar to one previously reported, showing two polymorphisms. Concluding, this study provides insights into the genomic characteristics of *C. perfringens* and a better understanding of this avian pathogen.

Resumé

L'entérite nécrotique (EN) causée par *C. perfringens* est une des maladies les plus courantes chez les volailles et entraîne une perte économique considérable pour l'industrie avicole, avec des souches clostridiennes résistantes qui constituent une préoccupation sérieuse et rendent le traitement difficile. Les approches de séquençage du génome entier représentent un bon instrument pour déterminer les profils de résistance et permettent également de mieux comprendre l'agent pathogène. L'objectif de cette étude était de caractériser, au niveau génomique, une collection de 20 isolats de *C. perfringens* provenant de volailles touchées par la NE, en mettant l'accent sur les mécanismes de résistance et la production de bactériocines. Des gènes de résistance aux antimicrobiens ont été trouvés, les gènes *tet* (associés à la résistance à la tétracycline) étant les plus répandus. Il est intéressant de noter que deux isolats portaient le gène *erm* (T) associé à la résistance à l'érythromycine, qui n'a été signalé que chez d'autres bactéries Gram-positifs. Douze des isolats ont été toxinotypés comme type A et sept comme type G. D'autres facteurs de virulence codant pour des hyaluronases et des sialidases ont été fréquemment détectés, ainsi que différents plasmides. Les types de séquences (ST) ont révélé une grande variabilité des isolats, avec la découverte de nouvelles combinaisons alléliques. Parmi les isolats, *C. perfringens* MLG7307 présentait des caractéristiques uniques ; il présentait une combinaison de toxines qui le rendait impossible à toxinotyper et, bien qu'il ait été identifié comme *C. perfringens*, il était dépourvu du gène hautement conservé *colA*. Des gènes codant pour la bactériocine BCN5 ont été trouvés dans cinq isolats, bien qu'aucune activité antimicrobienne n'ait pu être détectée dans ces isolats. Le gène *bcn5* de trois de nos isolats était similaire au gène précédemment rapporté, présentant deux polymorphismes. En conclusion, cette étude permet de mieux comprendre les caractéristiques génomiques de *C. perfringens* et de mieux appréhender ce pathogène aviaire.

Resumen

La enteritis necrótica (EN) causada por *C. perfringens* es una de las enfermedades más comunes de las aves de corral y ocasiona enormes pérdidas económicas a la industria avícola. Las cepas clostridiales resistentes constituyen un serio motivo de preocupación y dificultan el tratamiento de la EN. Los métodos de secuenciación del genoma completo representan una buena herramienta para determinar los perfiles de resistencia a la vez que aportan conocimientos para una mejor comprensión de este patógeno. El objetivo de este estudio es caracterizar, a nivel genómico, una colección de 20 aislados de *C. perfringens* procedentes de aves de corral afectadas por EN, haciendo hincapié en los mecanismos de resistencia y la producción de bacteriocinas. Se encontraron genes de resistencia a antibióticos, siendo los genes *tet* (asociados a la resistencia a la tetraciclina) los más prevalentes. Es de interés señalar que dos cepas eran portadoras del gen *erm*(T), asociado a la resistencia a la eritromicina, que sólo se había descrito en otras especies de bacterias Gram positivas. Doce de los aislados fueron toxinotipados como tipo A y siete como tipo G. También se detectaron con frecuencia otros factores de virulencia que codifican hialuronidasas y sialidasas, así como diferentes plásmidos. Las Secuencias Tipo (ST) detectadas revelaron una gran variabilidad de los aislados, encontrándose nuevas combinaciones alélicas. Entre los aislados, *C. perfringens* MLG7307 mostró características únicas; presentaba una combinación de toxinas que hacía imposible su toxinotipado y, a pesar de ser identificado como *C. perfringens*, carecía del gen housekeeping *colA*. Se encontraron genes que codifican la bacteriocina BCN5 en cinco aislados, si bien no se pudo detectar ninguna actividad antimicrobiana en ellos. El gen *bcn5* de tres de nuestros aislados era similar a uno descrito con anterioridad, mostrando dos polimorfismos. En conclusión, este estudio permite conocer mejor las características genómicas de *C. perfringens* y ayuda a una mejor comprensión de este patógeno aviar.

2.1 Introduction

Necrotic enteritis (NE) caused by *Clostridium perfringens* is one of the most common diseases of poultry and results in a huge economic loss to the poultry industry (Alizadeh et al., 2021). A distinguishing feature of NE is acute death, with mortality rates as high as 50%. Clinical symptoms include depression, dehydration, drowsiness, ruffled feathers, diarrhea, and reduced feed consumption (Van Immerseel et al., 2004). The subclinical form of the disease causes chronic damage to the intestinal mucosa in chickens, resulting in poor absorption of nutrients, reduced weight gain, and a decrease in overall performance. In healthy chickens, *Clostridium perfringens* can be found at low levels in the intestines ($<10^5$ CFU/g), but this level may increase, and poultry become prone to NE (Alizadeh et al., 2021).

C. perfringens is a Gram-positive, spore-forming, strictly anaerobic bacterium that can be found in a variety of environments, including food, soil, and in the gastrointestinal tracts of both diseased and healthy animals and humans (Kiu & Hall., 2018). It is a widespread pathogen that can be classified as toxin types A–G, depending on the combination of the following toxins: α-toxin, β-toxin, ε-toxin, ι-toxin, enterotoxin (CPE), and NetB. This microorganism also produces other toxins which are not considered for typing. These include β2-toxin, λ-toxin, and θ-toxin (Shrestha et al., 2018; Villagrán-de la Mora et al., 2020). Hence, they produce a diversity of diseases in both animal and human hosts (Kiu & Hall., 2018; Shrestha et al., 2018; Villagrán-de la Mora et al., 2020).

Toxinotype G is a proven cause of NE in chickens (Rood et al., 2018), in which NetB plays an important role. It is a plasmid-encoded, pore-forming toxin exclusive for *C. perfringens* coming from poultry affected by NE. It is a key virulence factor in the pathogenesis and is similar to *S. aureus* alpha-hemolysin. It forms heptameric pores on its target cell membranes (Keyburn et al., 2008; Yan et al., 2013). Sequences of netB genes from isolates from around the world show that the coding sequence is highly conserved across all strains (Lacey et al., 2013). Other toxins present in *C. perfringens* from poultry with NE are α-toxin, β2-toxin, and θ-toxin. The α-toxin is a secreted zinc-metalloenzyme with lethal, hemolytic, and dermonecrotic activities, as well as phospholipase C and sphingomyelinase activities, and it is a major pathogenic factor in the development of gas gangrene. At low doses, it causes limited phospholipid

hydrolysis, which in turn activates diacylglycerol- and ceramide-mediated signaling pathways, leading to cell apoptosis (Naylor et al., 1998; Sakurai et al., 2004; Urbina et al., 2011). The β2-toxin has no significant homology with the sequence of β-toxin or any other known protein sequence, and its mechanism is still unknown (Gibert et al., 1997; Van Asten et al., 2010). The θ-toxin is a cholesterol-dependent cytolysin and is a member of the β pore-forming family of toxins (Popoff., 2014).

C. perfringens also carries other virulence genes such as those encoding sialidases, exoenzymes, and adhesion proteins. The most common degradative enzymes are proteases (e.g., clostripain), hyaluronidase (μ-toxin), collagenase, endoglycosidases, and the sialidases NanJ, NanI, and NanH (neuraminidases), which generate free sialic acids (Mehdizadeh Gohari et al., 2021).

Antimicrobial resistance is also a concern in infections caused by *C. perfringens*. The continued widespread use of antibiotics in poultry during the last years has led to changes in the bacterial environment, eliminating susceptible strains and allowing antimicrobial-resistant bacteria to persist and predominate. Antibiotics have been used as growth promoters for decades, although this practice is now banned in many countries (Góchez et al., 2019). Antimicrobial resistance, together with a gradual decrease in the susceptibility of some strains of *Eimeria* spp. to anticoccidial agents (a predisposing factor for NE), can lead to an increase in the occurrence of *C. perfringens* strains (Agunos et al., 2020). Acquired antimicrobial resistance genes are commonly plasmid-associated. Plasmid-carrying tetracycline resistance genes (*tet*) are frequent (Adams et al., 2018), as well as those related to macrolide and lincosamide resistance (mainly erythromycin and lincomycin) (Kiu & Hall., 2018). Multidrug resistance among *C. perfringens* isolates has been described in different studies. Resistance to tetracycline, lincomycin, enrofloxacin, cefoxitin/ampicillin, and erythromycin via the detection of *tet*, *Inu*, *qnr*, *bla*, and *erm*(B) genes, respectively, has been identified in *C. perfringens* of foodborne infections by PCR in Egypt (Bendary et al., 2022). This phenomenon is also frequent in *C. perfringens* coming from birds, as well as in those coming from other sources. However, many studies only include the phenotypic detection of antimicrobial resistance (Haider et al., 2022; Yadav et al., 2022). Thus, further studies are needed to determine the current status of resistance genetic profile in *C. perfringens*.

The whole-genome sequencing (WGS) approach could be a good tool for this purpose. In this respect, the *C. perfringens* genomes of isolates of different locations and sources (including strains from cattle, dogs, and horses) were previously analyzed by WGS to assess their genetic diversity and phylogenetic relatedness (Camargo et al., 2022); this study established that the genetic diversity of *C. perfringens* is based on a large number of virulence factors that vary among phylogroups and antibiotic resistance markers. These methods may help to develop future strategies to prevent disease caused by this emerging and poorly understood pathogen.

The production of antimicrobial peptides, such as bacteriocins, by *C. perfringens* has also been reported. This trait is sometimes considered virulence factors, as they could inhibit the growth of not only pathogenic bacteria, but also commensals for competition with the ecological niche in the host gut. Bacteriocin BCN5 and perforin are the well-known plasmid-encoded bacteriocins produced by *C. perfringens* (Villagrán-de la Mora et al., 2020; Mehdizadeh Gohari et al., 2021). Recently, the structural gene of Lactococcin A has been detected in a *C. perfringens* strain from poultry (Elnar & Kim., 2021). More in-depth studies are necessary for the study of bacteriocin production in *C. perfringens* and the possible link with the virulence of this pathogen.

2.2 Objective

The main objective of this study was to characterize via WGS a collection of *C. perfringens* isolates of poultry affected by NE, giving special emphasis to the characterization of antimicrobial resistance determinants, as well as to the presence of virulence and bacteriocin genes and its correlation with the expression of antimicrobial activities by the isolates. Moreover, molecular typing of isolates and phylogenetic relationships among them was also addressed.

2.3 Methodology

2.3.1 Strain collection

A collection of 20 *C. perfringens* isolates, previously recovered from poultry affected by NE, and belonging to the University of Laval collection (Quebec, QC, Canada), was included in this study. *C. perfringens* ATCC 13124

was used as a control strain. The isolates were preserved in glycerol 40% at −80 °C. A reinforced medium for clostridia (HiMedia, Kelton, PA, USA) was used for the propagation of the isolates (incubation at 37 °C, 24 h, under strict anaerobic conditions).

2.3.2 Antibiotic susceptibility testing

Antibiotic susceptibility testing was performed by calculating the minimal inhibitory concentration (MIC) with the 20 *C. perfringens* isolates following the recommendations of the Clinical and Laboratory Standard Institute (CLSI, 2022). The following antibiotics were tested: ampicillin, cefotaxime, imipenem, tetracycline, chloramphenicol, clindamycin, metronidazole, and erythromycin. The strains were then identified as susceptible (S), resistant (R), or intermediate (I) in accordance with the protocol interpretation guidelines (CLSI, 2022).

2.3.3 Whole genome sequencing analysis

DNA was extracted using the DNeasy Blood and Tissue Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions for Gram-positive bacteria. The DNA was subjected to WGS using the Illumina technique at the Hospital Center of the University of Laval (CHUL), Quebec, Canada.

Raw sequencing data were processed using fastp 0.20.0 for trimming and quality control of trimmed reads (Chen et al., 2018). De novo assembly, without alignment to a reference genome, was performed with SPAdes 5.0.2 (Bankevich et al., 2012), using QUAST 1.14.6 for checking the assembled quality (Gurevich et al., 2013). Prokka 1.14.6 (Seemann et al., 2014), which uses Prodigal for prediction of coding sequences (Hyatt et al., 2010), was used for gene prediction and annotation.

For detection of genes associated with antibiotic resistance, ResFinder 4.1 was used (Camacho et al., 2009; Zankari et al., 2017; Bortolaia et al., 2020). For plasmid detection, the program PlasmidID 1.6.4 (Carattoli & Hasman., 2020) was used. Genes encoding virulence factors were detected using the ABRicate 1.0.1 program with the VFDB database (Liu et al., 2022). Toxinoytpe assignment was performed using TOXIper v1.1 (Kiu et al., 2017).

Multi-locus sequence typing (MLST) was tested in the genome data using MLST 2.0 (Lemee et al., 2004; Bartual et al., 2005; Wirth et al., 2006; Jaureguy et al., 2008; Griffiths et al., 2010; Larsen et al., 2012). The representation of phylogenetic relationships in a tree was performed using R version 4.2.1 (R Core

Team., 2013), and phylogenetic distances were calculated using the average nucleotide identity (ANI) method, calculated with pyANI, a program that uses the ANI for whole-genome comparisons, and renders graphical summary output (Pritchard., 2016). The graphic was generated using R version 4.2.1 (R Development Core Team., 2013) with ape 5.0 (Paradis & Schliep., 2019).

For the detection of bacteriocin genes, antiSMASH 7 beta (Blin et al., 2021) and BAGEL4 (Van Heel et al., 2018) were used. Blastp of the secondary metabolites detected was performed for identification of the peptides produced. Jalview 2.11.2.5. (Waterhouse et al., 2009) and Clinker (Gilchrist & Chooi., 2021) were used to align bacteriocin genes detected among the *C. perfringens* species and to compare their genetic environments, respectively. GenBank database was used to obtain genes and plasmids of reference.

Multiple sequence alignment and visualization of the *erm*(T) gene products and the *bcn5* products, as well as generation of phylogenetic relationships between the *erm*(T) and *bcn5* products of our *C. perfringens* isolates and those of other bacterial species was established with the program Jalview 2.11.2.5 (Waterhouse et al., 2009). The representation of the genetic environment of *erm*(T) and *bcn5* genes in comparison with other genetic environments of *erm*(T) present in different bacterial species and a reference plasmid carrying the *bcn5* gene, respectively, was performed using the program Clinker (Gilchrist & Chooi., 2021). The *erm*(T) genes from other bacterial species and the *bcn5* genes and genetic environments from other *C. perfringens* isolates were obtained from the GenBank databases.

2.3.4 Screening for antimicrobial activity

The antimicrobial activity of the collection of *Clostridium perfringens* isolates was studied using the *spot-on the lawn* method and agar well diffusion, as previously described (García-Vela et al., 2023). In the case of *spot-on the lawn* method, the following indicator bacteria were used: *Clostridium tyrobutyricum* ATCC25755, *Pediococcus acidilactici* UL5, and *Enterococcus faecalis* ATCC29212. The same indicator bacteria were used for the well diffusion method, but *Micrococcus luteus* ATCC10240 was also included. For the *spot-on the lawn* method, tryptic soy agar plates were used with a thin layer of tryptic soy broth supplemented with 8% agar and 3% yeast extract that was inoculated with the indicator bacteria. The medium used for agar well diffusion was “reinforced medium for clostridium” supplemented with

8% agar for the indicator strain *Clostridium tyrobutyricum* ATCC25755, Tryptic Soy Agar for *Enterococcus faecalis* ATCC29212, MRS supplemented 8% agar for *Pediococcus acidilactici* UL5, and Nutrient Broth supplemented 8% agar for *Micrococcus luteus* ATCC10240.

Briefly, for the *spot-on the lawn* method a fresh culture of the indicator strain was suspended in brain–heart infusion broth (BHI) (turbidity 0.5 MacFarland). Subsequently, 10 µL of this indicator microorganism solution was added to tubes containing 5 mL of semi-solid melted tryptic soy broth (TSB) and supplemented with 0.7% agar and 0.3% yeast extract. Finally, the semi-solid TSB medium with the indicator microorganism was poured onto tryptic soy agar plates (TSA). Once the plates were dried, the *C. perfringens* isolates were sting-seeded, and the plates were incubated at 37 °C for 24 h under strict, anaerobic conditions.

For agar well diffusion, as a first step, supernatants of the *C. perfrignens* isolates were prepared. To that end, the isolates were inoculated in 10 mL of reinforced clostridial medium (RCM) in sterile tubes and were incubated overnight at 37 °C. Then, the culture medium was centrifuged at 5000× *g* rpm for 5 min and filtrated using 0.20 µm filters. Secondly, the agar plate containing the indicator strains were prepared. Different media according to indicator strains were used: reinforced medium for clostridium supplemented with 8% agar for the indicator strain *Clostridium tyrobutyricum* ATCC25755, Tryptic Soy Agar for *Enterococcus faecalis* ATCC29212, MRS supplemented 8% agar for *Pediococcus acidilactici* UL5, and Nutrient Broth supplemented 8% agar for *Micrococcus luteus* ATCC10240. Once dried, wells with a 10 mL pipete were performed and 80 µL of the supernatants were poured into them. The plates were incubated overnight at 37 °C.

2.4 Results

2.4.1 Resistance phenotype

The rates of antibiotic resistance in the collection of 20 *C. perfringens* isolates were as follows (**Table 2. 1**): tetracycline (50%; MIC ≥ 16 µg/mL), clindamycin (40%; MIC ≥ 8 µg/mL), and cefotaxime (5%; MIC ≥ 64 µg/mL); no resistant isolates were detected for metronidazole, chloramphenicol, ampicillin, and imipenem. Isolates in the intermediate susceptibility category

were identified for tetracycline (15%; MIC 8 µg/mL), clindamycin (25%; MIC 4 µg/mL), and ampicillin (5%, MIC 1 µg/mL). In the case of erythromycin, there are no breakpoints in CLSI to classify isolates as resistant, intermediate, or susceptible for this agent. However, most of the isolates showed a MIC for erythromycin of 1–16 µg/mL, and only three isolates showed a very high MIC value (>128 µg/mL). According to these data, we consider these last three isolates as erythromycin-resistant (15%).

Table 2. 1. MICs values (in µg/mL) for the collection of 20 *C. perfringens* isolates.

***C. perfringens* Isolate**	**Resistance Phenotype**	**TET**	**CLI**	**AMP**	**CTX**	**CHL**	**MTZ**	**IPM**	**ERY**
MLG0418	Susceptible	2	<0.25	<0.25	2	4	4	<0.25	16
MLG2203	Susceptible	<0.25	<0.25	<0.25	<0.25	4	2	<0.25	8
MLG4201	TET	16	2	<0.25	1	4	2	<0.25	16
MLG5719	TET	8	2	<0.25	2	4	4	<0.25	8
MLG5806	TET	16	2	<0.25	<0.25	4	8	<0.25	16
MLG7814	TET	32	2	<0.25	1	4	2	<0.25	8
MLG1819	CLI	<0.25	4	<0.25	1	4	8	<0.25	16
MLG1619	CLI	<0.25	8	<0.25	4	4	8	<0.25	16
MLG6907	CLI	4	8	<0.25	<0.25	4	4	<0.25	8
MLG4206	CLI	4	4	<0.25	1	4	4	<0.25	1
MLG0618	TET, CLI	32	4	<0.25	2	4	2	<0.25	8
MLG0712	TET, CLI	16	8	<0.25	2	4	4	<0.25	16
MLG2314	TET, CLI	16	>128	<0.25	4	8	4	1	16
MLG2919	TET, CLI	64	>128	<0.25	2	8	8	0.5	8
MLG3406	TET, CLI	8	4	<0.25	1	4	4	<0.25	16
MLG7309	TET, CLI	16	4	<0.25	1	4	1	<0.25	4
MLG3111	TET, ERY	16	1	<0.25	0.5	4	1	<0.25	>128
MLG1108	TET, CLI, ERY	8	>128	<0.25	1	4	4	<0.25	>128
MLG7009	TET, CLI, ERY	16	>128	0.5	2	4	8	<0.25	>128
MLG7307	CLI, AMP, CTX	2	32	1	64	4	8	2	4

TET: tetracycline; CLI: clindamycin; AMP: ampicillin; CTX: cefotaxime; CHL: chloramphenicol; MTZ: metronidazole; IMP: imipenem; ERY: erythromycin. Red cells indicate resistance values (R); orange cells indicate intermediate values (I) according to the CLSI standards. White cells indicate the susceptible category. Note: For ERY, there are no breakpoints to establish susceptibility by CLSI. We consider as resistant the isolates with an MIC higher than 128µg/mL.

2.4.2 Whole genome sequencing analysis

2.4.2.1 Resistome

Antimicrobial resistance genes (ARG) were found in 16 of the 20 isolates analyzed (**Table 2. 2**). Those isolates in which no resistance genes were detected were *C. perfringens* MLG2203, susceptible to all antibiotics tested, *C. perfringens* MLG1819 and MLG1819, resistant to clindamycin, and *C. perfringens* MLG7307, which was resistant to clindamycin and cefotaxime and presented intermediate susceptibility to ampicillin. Different tetracycline

resistance genes (*tetA*, *tetB*, and *tet44*) were found in 16 of the isolates. The *InuP* gene was found in three isolates from our collection, two of them resistant to clindamycin.

Table 2. 2. Resistance phenotype and genotype of the *C. perfringens* collection.

***C. perfringens* Isolate**	**Resistance Phenotype[a]**	**Resistance Genotype Detected**		
		Resistance Genes	**Identity**	**Accession Number[c]**
MLG0418	Susceptible	*tetA*	99.17	L20800
MLG2203	Susceptible	No genes detected		
MLG4201	TET	*tetA*	99.84	AB001076
		tetB	99.74	NC_010937
		InuP	99.8	FJ589781
MLG5719	TET	*tetA*	100	AB001076
		tetB	99.74	NC_010937
MLG5806	TET	*tetA*	100	AB001076
		tetB	99.74	NC_010937
MLG7814	TET	*tetA*	99.84	AB001076
		tetB	99.74	NC_010937
		lnuP	99.8	FJ589781
MLG1819	CLI	No genes detected		
MLG1619	CLI	No genes detected		
MLG6907	CLI	*tetA*	99.26	AB001076
MLG4206	TET, CLI	*tetA*	100	AB001076
		tetB	99.74	NC_010937
MLG0618	TET, CLI	*tetA*	99.84	AB001076
		tetB	99.74	NC_010937
		lnuP	99.8	FJ589781
MLG0712	TET, CLI	*tetA*	99.84	AB001076
		tetB	99.74	NC_010937
MLG2314	TET, CLI	*tetA*	99.18	AB001076
		tet(44)	98.75	NZ_ABDU01000081
		lnuP	99.8	FJ589781
		ant(6)-Ib	100	FN594949
MLG2919	TET, CLI	*tetA*	100	AB001076

		tetB	99.67	NC_010937
MLG3406	TET, CLI	*tetA*	100	AB001076
		tetB	99.74	NC_010937
MLG7309	TET, CLI	*tetA*	99.84	AB001076
		tetB	99.74	NC_010937
MLG3111	TET, ERY	*tetA*	99.84	AB001076
		tetB	99.74	NC_010937
		lnuP	99.8	FJ589781
MLG1108	TET, CLI, ERY	*tetA*	99.84	AB001076
		tetB	99.74	NC_010937
		erm(T)	99.86	AY894138
MLG7009	TET, CLI, ERY	*tetA*	99.84	AB001076
		tetB	99.74	NC_010937
		erm(T)	99.86	AY894138
MLG7307	CLI, AMP[b], CTX	No genes detected		

[a]TET: tetracycline; CLI: clindamycin; AMP: ampicillin; CTX: cefotaxime; ERY: erythromycin; [b] intermediate susceptibility; [c] accession number of the resistance gene used in the comparison.

The *erm*(T) gene was detected in two of our strains (*C. perfringens* MLG1108 and MLG 7009), and both strains showed very high MIC values for erythromycin (>128 µg/mL). Another additional *C. perfringens* isolate (MLG3111) showed resistance to this antimicrobial agent (MIC > 128 µg/mL), but it lacked known erythromycin resistance genes (**Table 2. 2** and **Table 2. 3**).

Figure 2. 1 shows phylogenetic relationships and alignments of the *erm*(T) product (metyltransferase) found in *Clostridium perfringens* MLG1108 and MLG7009 (which were identical) with the metyltransferases from other Gram-positive bacteria. When comparing the methylases encoded by the *erm*(T) gene of our *C. perfringens* strains with those of others strains such *as Streptococcus suis* CP061278, *Erysipelothix rhusiopathiae* KM576795, *Staphylococcus* spp. CP068248, *Staphylococcus aureus* FN390947, *Enterococcus faecalis* CP089585, and *Bacillus paranthracis* KC991136, we spotted only one difference in the amino-acid sequences. Indeed, the lysine at position 30 of the *C. perfringens* methylase was replaced by a threonine (Lys30Thr) in the ErmT sequence of the other Gram-

positive bacteria. A comparison with the ErmT sequence of *Lactobacillus reuteri* AF310974 revealed three substitutions: Lys30Thr, Arg204Ile, and Leu476Phe. With respect to the *Streptococcus dysgalactiae* HE862394 methylase, we detected the Lys30Thr substitution plus two deletions present at positions 74 and 75 in the *Streptococcus dysgalactiae* methylase. Lastly, 16 amino-acid substitutions were identified in the *Haemophilus parasius* KC405064 methylase respect to that of *Clostridium perfringens* (**Figure 2. 2**).

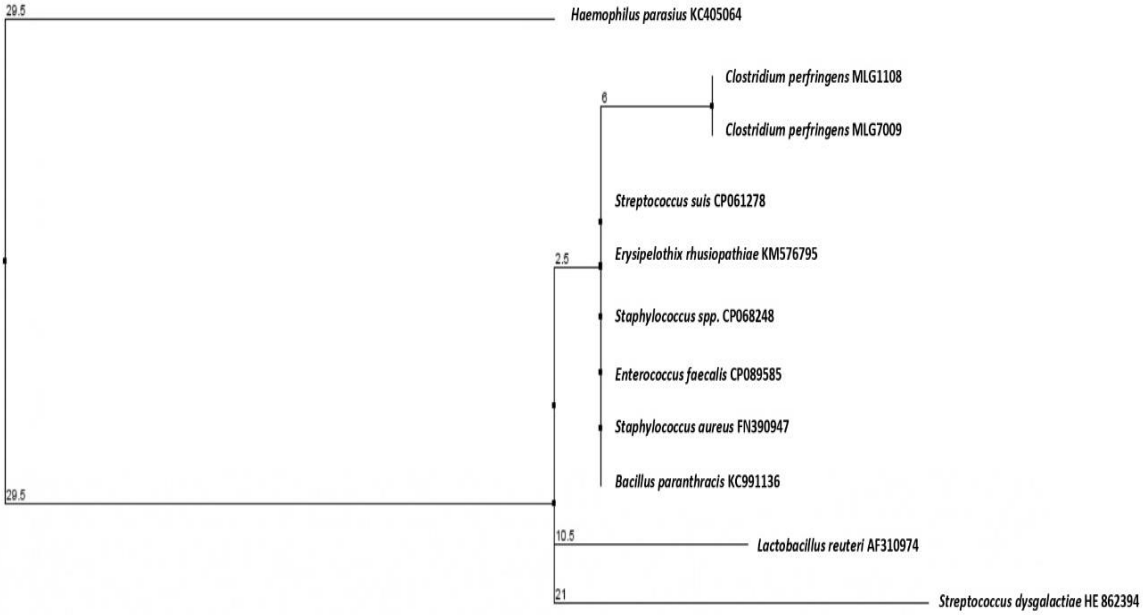


Figure 2. 1. Phylogenetic relationships of the *erm*(T) gene present in *C. perfringens* MLG1108 and MLG7009 strains and in those of other different Gram-positive species. Sequences of the *erm*(T) gene from different strains were obtained from GenBank database and were grouped in a tree according to their average phylogenetic distances with the program Jalview 2.11.2.5. Numbers indicate phylogenetic distances.

Figure 2. 3 shows a comparison of the genetic environments of the *erm*(T) gene from other Gram-positive bacteria with those of our *C. perfringens* isolates. As we can see, the genetic environments of our *C. perfringens* isolates MLG1108 and MLG7009 were identical to each other and had few similarities with other genetic environments previously described. Only *mob* and *moba* genes were found to be 36% identical to those in *Staphylococcus spp.* CP068248 and *E. faecalis* CP089585. The remainder of the predicted genes in the genetic environment on the *C. perfringens erm*(T) gene showed no similarities with those contemplated for other genetic environments. Overall, we can see that the *erm*(T)

gene is highly preserved among different species, but its genetic environments are very different from one species to another.

The aminoglycoside resistance gene *ant(6)-Ib* was found in *C. perfringens* MLG2314, being the first report in which *ant(6)-Ib* gene is reported in a toxitype A *C. perfringens* isolate. No resistance genes were detected by WGS in *C. perfringens* MLG7307.

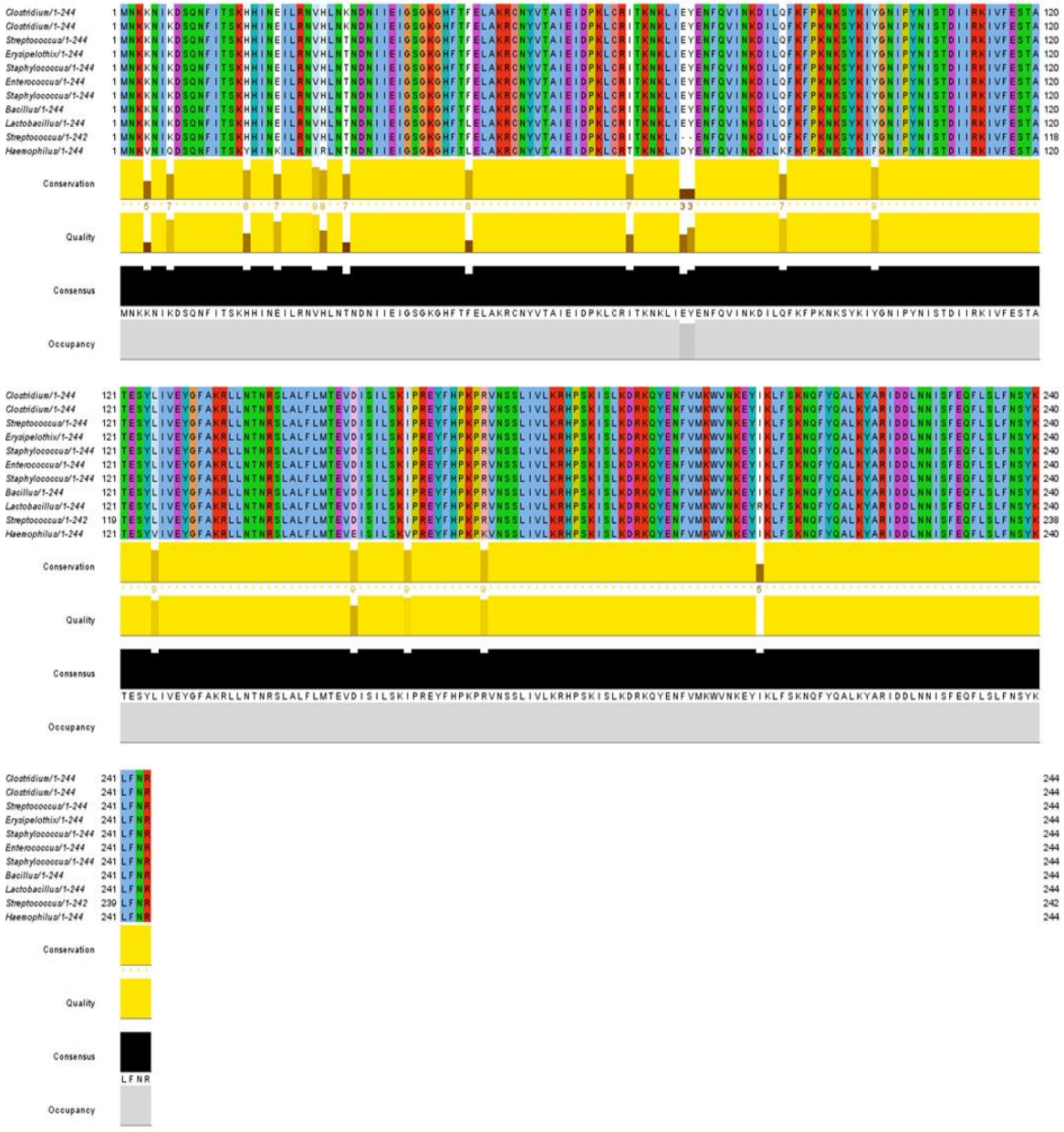


Figure 2. 2. Genetic alignments of the Erm(T) methylases of *C. perfringens* MLG1108 and MLG7009 with Erm(T) methylases from other species.

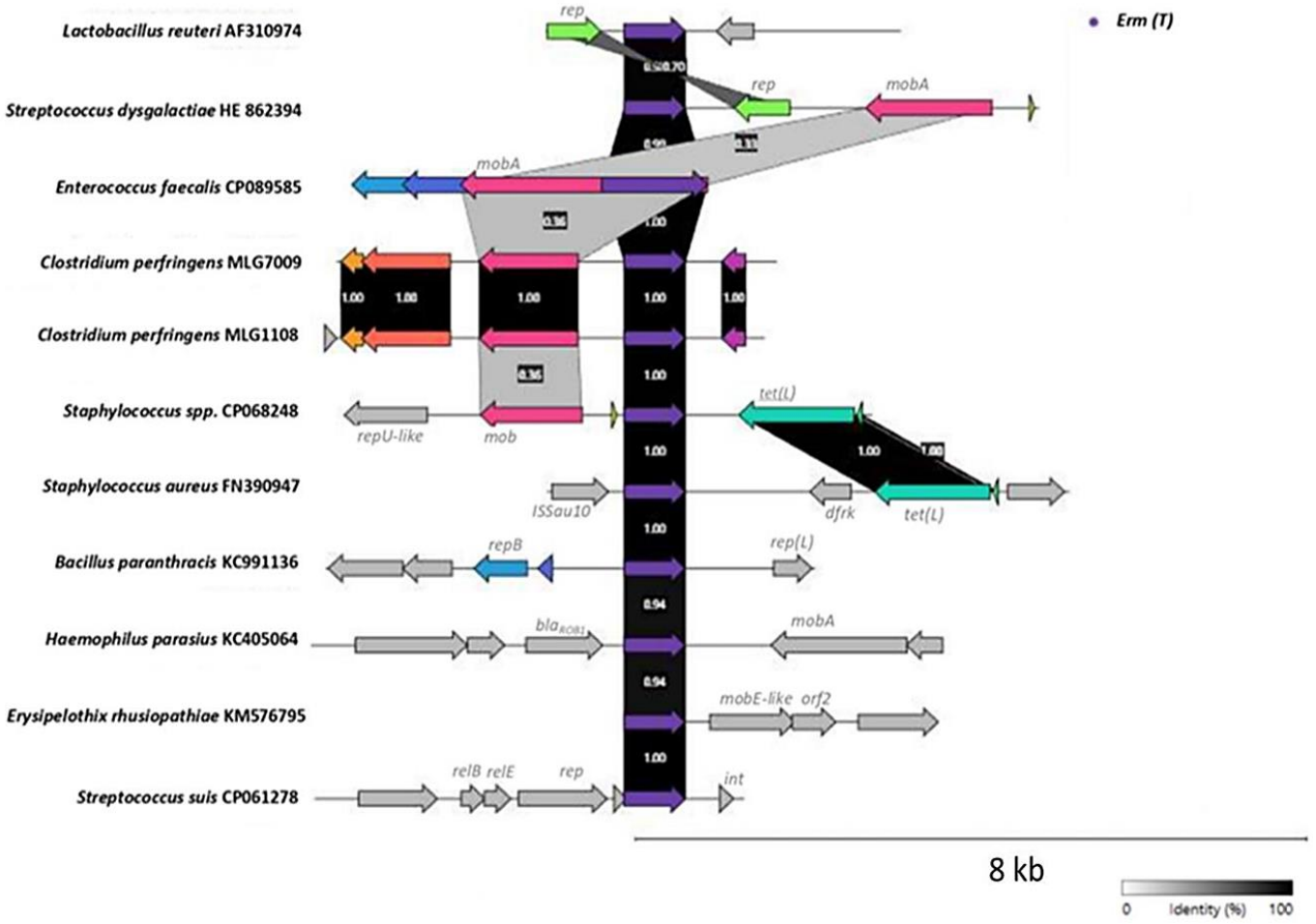


Figure 2. 3. Comparison of genetic environments on *erm*(T) gene from *C. perfringens* strains MLG1108 and MLG7009 with the genetic environments of *erm*(T) from other Gram-positive bacteria. Different genes are indicated with arrows. Colors in the arrows represent the genes which shows similarities, and identities between them are indicated with numbers and a scale of gray. The *erm*(T) gene from different bacteria is represented with the purple arrows.

4.4.2.2 Toxinotyping, virulence factors and plasmidome

Twelve of the *C. perfringens* isolates were toxinotyped as type A, carrying the *plc* gene encoding α-toxin. Seven of the strains were toxinotyped as type G, carrying *netB* in addition to *plc*. The gene of the non-typing toxin PFO (*pfoA*) was detected in both A and G toxinotypes. **Figure 2. 4** shows the phylogenetic relationships, toxinotyping, plasmids, antimicrobial resistance genes (ARG), and main virulence factors detected in our collection of *C. perfringens* isolates. *C. perfringens* MLG7307 could not be toxinotyped since it did not carry the *plc* gene, present in all toxinotypes. Instead, it carried the genetic determinants for the non-typing β2-toxin (*cpb2*). Other virulence factors such as *cloSI* and *colA* were present in the majority of the isolates, except for *C. perfringens* MLG7307. Other virulence factors detected in our collection include the genes of mu-toxin and of the three sialidases NanH, NanI, and NanJ. Thirteen of the isolates carried at least one plasmid. Plasmids detected are represented in **Figure 2. 4**.

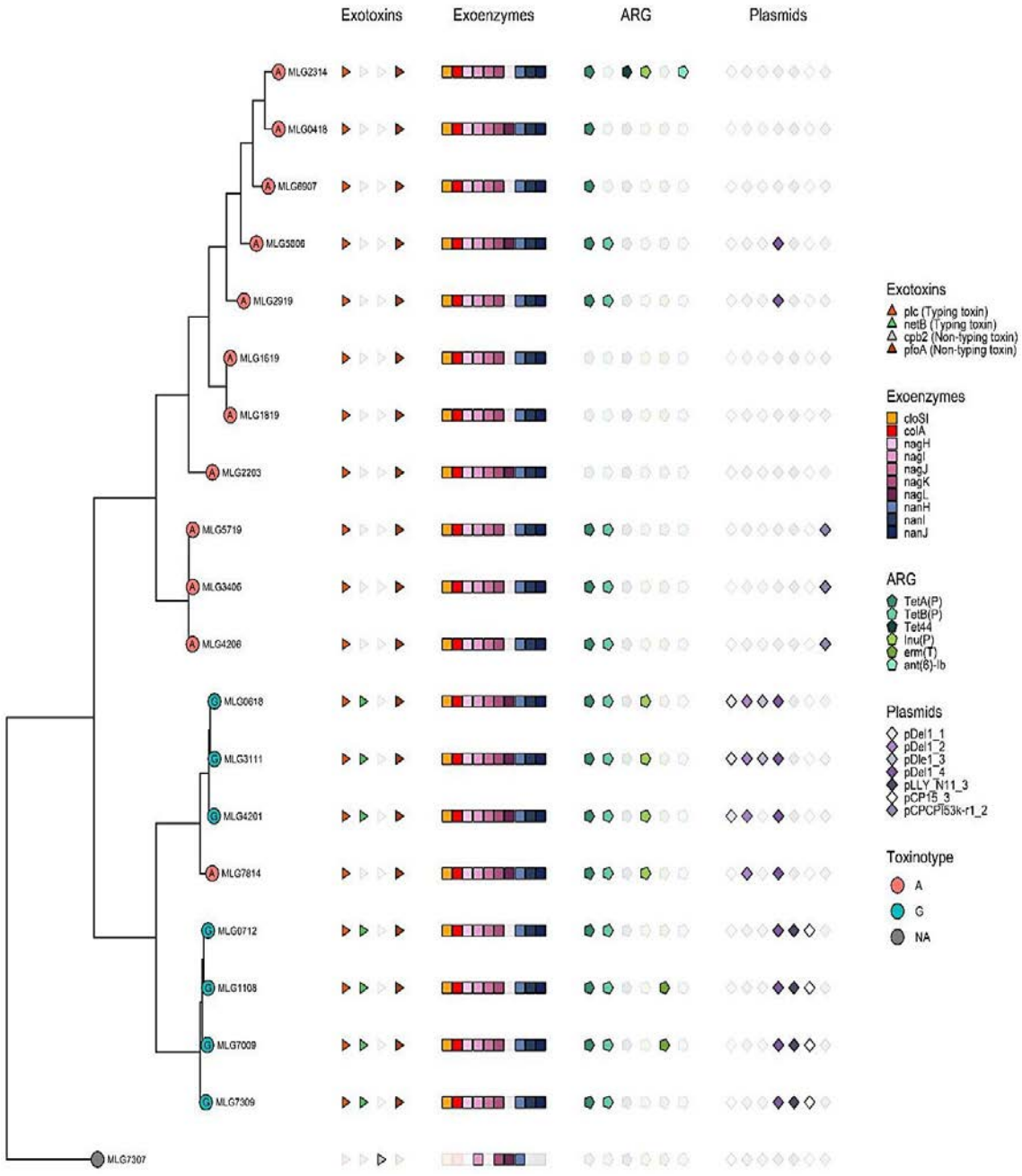


Figure 2. 4. Phylogenetic relationships and main features of the *20 C. perfringens* isolates; toxinotypes, exotoxins produced, exoenzymes, antimicrobial resistance genes (ARG), and plasmids detected. Letters A and G indicate toxinotype A and G, respectively. Different shapes of the symbols colored or uncolored indicate different genes detected. Triangles indicate exotoxins used for toxinotyping, Squares indicate presence of genes encoding exoenzymes: in yellow, *cloSI*, encoding the alpha-clostripain; in red, *colA* encoding the kappa-toxin; in purple, *nagH*, *nagI*, *nagJ*, *nagK*, and *nagL*, encoding the mu-toxin; in blue, *nanH*, *nanI*, and *nanK*, encoding the sialidases. Pentagons correspond to ARG, and purple diamonds indicate the presence of different plasmids.

4.4.2.3 Sequence types (ST) of the strains

Table 2. 3 includes the sequence type (ST) of the isolates, new allelic combinations, and alleles with <100% identity or coverage of the isolates. A sequence type could be established for nine of the isolates, with ST73 (n = 4) and ST21 (n = 4) being the most prevalent, followed by ST279 (n = 1). Three additional isolates showed two new allelic combinations. Moreover, another six isolates showed alleles with <100% identity, suggesting the existence of new alleles and, as a consequence, of new STs. Another isolate showed an allele (*sigk*) with <100% of coverage, and ST could not be assigned. Lastly, *C. perfringens* MG7307 could not be typed because it lacked the housekeeping *colA* gen.

Table 2. 3. Sequence type and new allelic combinations of the 20 *C. perfringens* isolates.

Sequence Types (ST)									
Strain	ST	Housekeeping Genes							
		colA	*groEL*	*gyrB*	*nadA*	*pgk*	*plc*	*sigk*	*sodA*
C. perfringens MLG0712	21	3	1	3	1	1	4	2	3
C. perfringens MLG1108	21	3	1	3	1	1	4	2	3
C. perfringens MLG7009	21	3	1	3	1	1	4	2	3
C. perfringens MLG7309	21	3	1	3	1	1	4	2	3
C. perfringens MLG0618	73	39	19	3	1	1	4	5	1
C. perfringens MLG3111	73	39	19	3	1	1	4	5	1
C. perfringens MLG4201	73	39	19	3	1	1	4	5	1
C. perfringens MLG7814	73	39	19	3	1	1	4	5	1
C. perfringens MLG2314	279	77	41	8	1	4	1	19	1
New or Unknown ST[a]									
Strain	ST[b] (Nearest ST)	Housekeeping Genes							
		colA	*groEL*	*gyrB*	*nadA*	*pgk*	*plc*	*sigk*	*sodA*
C. perfringens MLG0418	NAC (53)	37	22	17	28	1	27	18	19
C. perfringens MLG1619	NAC (629)	6	1	3	13	1	4	2	3
C. perfringens MLG1819	NAC (629)	6	1	3	13	1	4	2	3
C. perfringens MLG2203	New (131)	41	44	37[c]	47[c]	18	101[c]	25[c]	38[c]
C. perfringens MLG2919	New (625)	6[c]	5	24	1	7	33	4	1
C. perfringens MLG3406	New (340, 613)	3	6	1	1	4	43	5	71[c]
C. perfringens MLG4206	New (340, 613)	3	6	1	1	4	43	5	71[c]
C. perfringens MLG5719	New (340, 613)	3	6	1	1	4	43	5	71[c]
C. perfringens MLG5806	New	3[c]	56[c]	29[c]	49	8	88[c]	5	79[c]
C. perfringens MLG6907	unknown (200)	4	1	3	13	1	109	80[d]	20
C. perfringens MLG7307	Unknown	No hit[e]	121	83	135	63	163	87	125

[a]In this section is recorded (a) new allelic combinations (NACs), (b) potential new STs, because some of the gene sequences showed differences with those registered in MLST database (the closest allele is included), and (c) unknown STs, because incomplete coverage or lack of some alleles occurred. [b] The type of ST is recorded as NAC (new allelic combination), new ST (new sequence for any of the intrinsic genes), or unknown ST (not complete sequence of any of the intrinsic genes or lack of any of the genes). The closest ST/STs are also included, when possible. [c] Alleles with <100% identity. [d] Alleles with <100% coverage. [e] No hit: this strain lacked the housekeeping gene and ST could not be defined.

4.4.2.4 Secondary metabolites

Genes encoding secondary metabolites were detected in all *C. perfringens* isolates (**Table 2. 4**). Among them, genes encoding the bacteriocin BCN5, sactipeptides, lassopeptides, RiPP-like (ribosomally synthesized and post-translationally modified peptides), and NRS-like (non-ribosomal peptide synthesized) were identified. A bacteriocin-like peptide was also detected in one isolate, *C. perfringens* MLG4206.

Table 2. 4. Secondary metabolites detected in the *C. perfringens* isolates.

C. perfringens **Isolate**	**Secondary Metabolites**
MLG0418	Sactipeptides
MLG0618	Sactipeptides
MLG0712	Sactipeptides
MLG1108	Sactipeptides
MLG1619	Sactipeptides
MLG1819	Sactipeptides
MLG2203	Sactipeptides
MLG2314	Sactipeptides
MLG2919	Sactipeptides, lasso-peptides, bacteriocin BCN5
MLG3111	Sactipeptides, RiPP-like
MLG3406	Sactipeptides, RiPP-like, bacteriocin BCN5
MLG4201	Sactipeptides, NRPS-like
MLG4206	Sactipeptides, bacteriocin-like, bacteriocin BCN5
MLG5719	Sactipeptides, bacteriocin BCN5, NRPS-like
MLG5806	Sactipeptides, NRPS-like
MLG 6907	Sactipeptides, lasso-peptides
MLG7009	Sactipeptides
MLG7307	Sactipeptides, lasso-peptides, bacteriocin BCN5
MLG 7309	Sactipeptides
MLG7814	Sactipeptides

BCN5 was detected in five of our isolates. **Figure 2. 5** represents the phylogenetic relationships and distances among the bacteriocin BCN5 present in the five *C. perfringens* isolates and two bacteriocin BC5 of reference. Alignments of the BCN5 at the amino-acid level can be seen in **Figure 2. 6**. Bacteriocin BCN5 from MLG3406, MLG4206, and MLG5719 presented a length of 890 amino acids; they were identical among themselves and to bacteriocin BCN5 P08696 from the GenBank database. They showed a 65.2% of identity with bacteriocin BCN5 from MLG2919, which presents a length of 910 amino acids. Comparing the BCN P08696 with the bacteriocin BCN5 of *C. perfringens* MLG7307, whose length is 577 amino acids, the identity was 93.89%. The other bacteriocin of reference considered from GenBank databases (accession number BAD90628) was phylogenetically closer to BCN5 from *C.*

perfringens MLG7307 and MLG2919, and it presented just 50.3% identity with BCN P08696.

The genetic environment of the gene encoding the bacteriocin BC5, *bcn5*, was compared among our isolates and with the reference strain, as presented in **Figure 2. 7**.

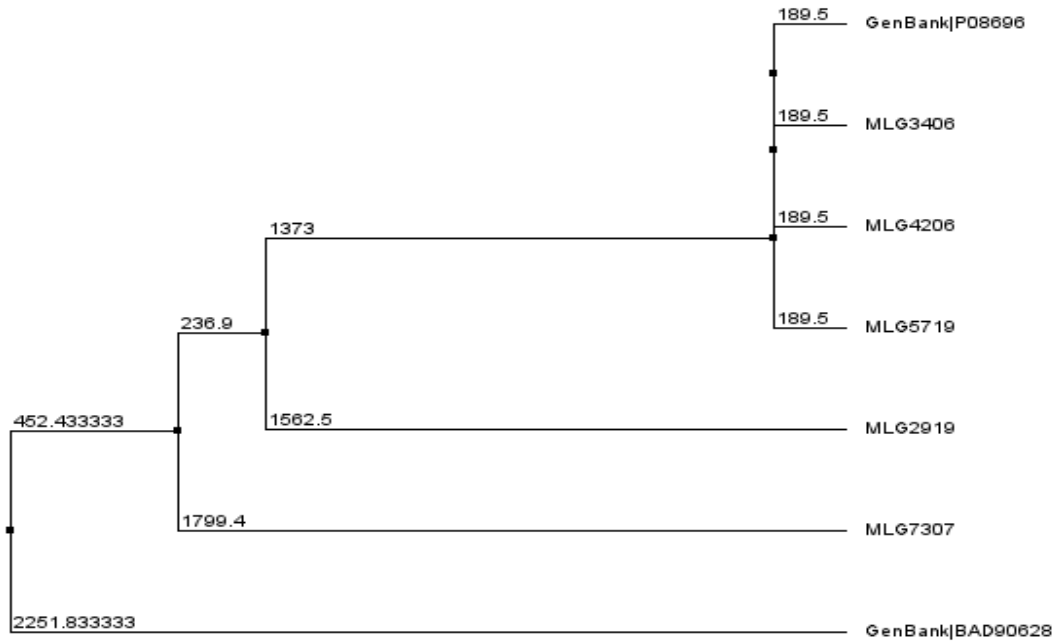


Figure 2. 5. Phylogenetic relationships of the bacteriocin BCN5 from the five isolates of our collection (*C. perfringens* MLG3406, MLG4206, MLG5719, MLG2919, and MLG7307) and two bacteriocin BCN5 from GenBank database (P08696 and BAD90628). Numbers indicate phylogenetic distances. Tree generated with Jalview 2.11.2.5.

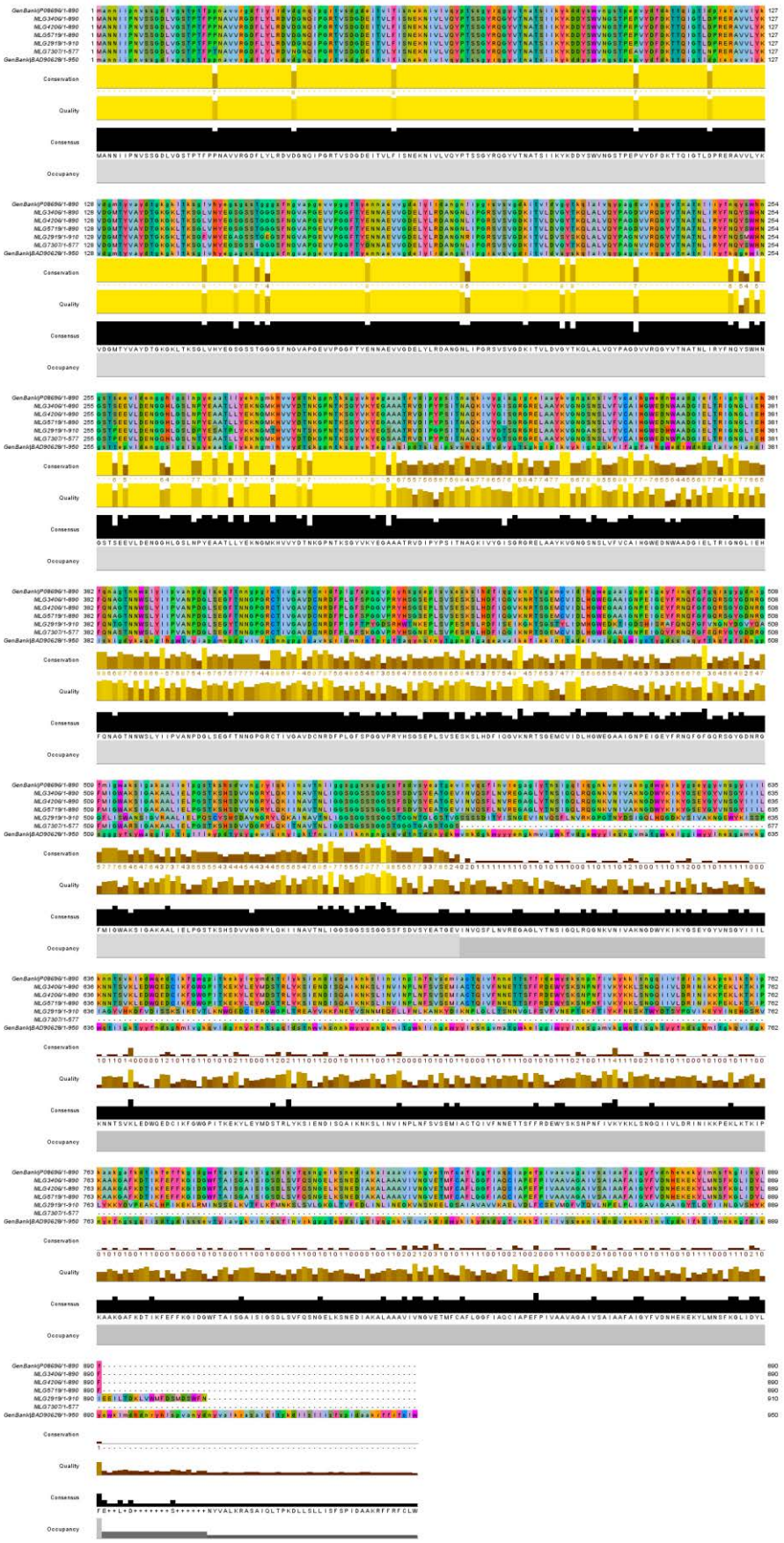


Figure 2. 6. Alingment of the bacteriocin BCN5 from the five isolates of our collection and two bacteriocin BCN5 from GenBank database.

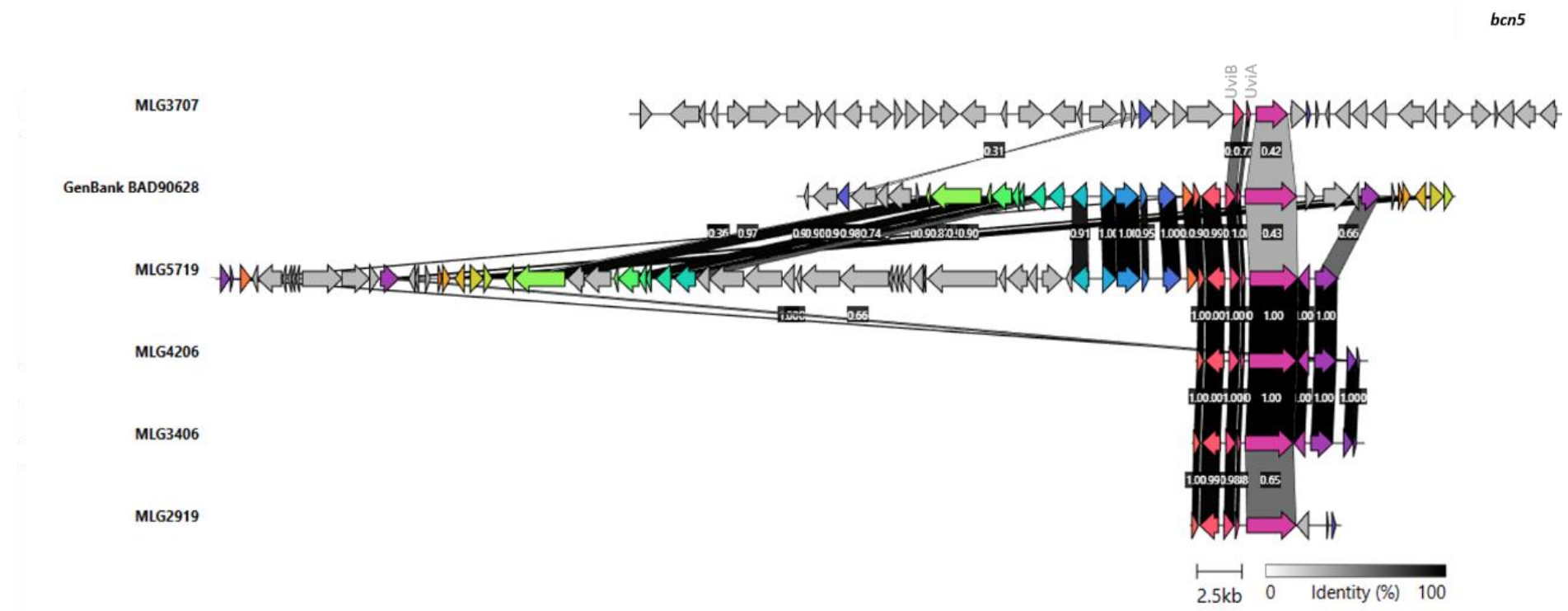


Figure 2. 7. Genetic environments of the *bcn5* gene present in the five *C. perfringens* isolates from our collection and the environment of *bcn5* from the plasmid of reference (accession number AD90628) from GenBank database. Different genes are indicated with arrows. Colors in the arrows represent the genes, which show similarities, and identities between them are indicated with numbers and a scale of gray. The *bcn5* gene from different bacteria is represented with the purple arrow. The *uviA* and *uviB* genes were located next to the *bcn5* in all the isolates.

4.4.3 Bacteriocinetic activity of the strains

Both methods used to detect antimicrobial activity by the *C. perfringens* strains (spot-on-the-lawn and agar well diffusion methods) gave negative results with the conditions and indicator bacteria used. No antimicrobial activity was detected in any of the *C. perfringens* isolates.

2.5 Discussion

Resistance to antibiotics happens frequently among *C. perfringens* isolates. Tetracycline resistance is very common, both among our isolates and in other studies (Bendary et al., 2022; Haider et al., 2022). Ampicillin resistance is not as frequent but has been previously reported (Nhung et al., 2017).

Different tetracycline resistance genes were found in 16 of our isolates. The presence of *tet* genes has been reported in *C. perfringens* isolates in other studies using WGS and appear to be commonly involved in isolates implicated in all types of infections (Kiu & Hall., 2018). The *InuP* gene, which encodes a lincosamide nucleotidyltransferase associated with lincomycin resistance (Nhung et al., 2017) was found in three isolates from our collection, two of them resistant to clindamycin.

Macrolide resistance has been previously detected in *C. perfringens*, carrying the gene *erm*(B) (Bendary et al., 2022). There are three pathways for the acquisition of macrolide resistance: target site modification, efflux pump, and drug inactivation. Target site modification is mediated by 23S rRNA methylation enzymes encoded by *erm* genes, conferring resistance to macrolides (Dinos., 2017; Torres et al., 2018). Interestingly, in our study, the *erm*(T) gene was detected in two of our strains (*C. perfringens* MLG1108 and MLG 7009), being the first report to find *erm*(T) in *C. perfringens* isolates. Both strains were phenotypically resistant to erythromycin (MIC > 128 µg/mL). Nevertheless, another additional *C. perfringens* isolate (MLG3111) showed resistance to this antimicrobial agent (MIC > 128 µg/mL), although no genes associated with erythromycin resistance were detected by WGS. The mechanisms of macrolide resistance in this isolate should be analyzed in the future to see if it could carry a new mechanism of resistance.

The gene *erm*(T) has been previously detected in other Gram-positive bacteria, such as *Enterococcus spp.*, *Streptococcus spp.*, or *Staphylococcus spp.* (DiPersio & DiPersio., 2007; DiPersio et al., 2008; Gómez-Sanz et al., 2013; Li et al. 2022) but never before in *C. perfringens*. Genetic environments of the *erm*(T) gene have been described in other species (Li et al., 2022). Overall, we can see that the *erm*(T) gene is highly preserved among different species, but its genetic environments are very different from one species to another.

Anaerobic bacteria such as *C. perfringens* usually present low susceptibility to aminoglycosides as they present intracellular reduced transport of the antibiotic (Kiu & Hall., 2018). *C. perfringens* MLG2314 harbors the *ant(6)-Ib* gene, which is associated with streptomycin resistance. This gene has previously been reported in toxinotype C (Kiu & Hall., 2018). This is the first report in which *ant(6)-Ib* gene is reported in a toxinotype A *C. perfringens* isolate.

Toxinotyping revealed toxinotypes A and G among our isolates, carrying different toxins. In addition to toxin production, *C. perfringens* is known to produce a variety of other virulence factors. The *cloSI* and *colA* genes, present in most of our isolates, except in *C. perfringens* MLG7307, encode alpha-clostripain and kappa-toxin respectively. Alpha-clostripain is a cysteine endopeptidase. It has been shown to not be essential for disease development (Labrou & Rigden., 2004; Chakravorty et al., 2011). Kappa-toxin is a clostridial collagenase that actively degrades host tissues to support growth, survival, and dissemination in infected hosts, or to potentiate other toxins by facilitating their diffusion (Matsushita & Okabe., 2001). The gene *colA* is considered a housekeeping gene for MLST. It is interesting to note that *C. perfringens* MLG7307 did not carry it, which, together with other characteristics of the isolate, made it unique in the collection.

Other virulence factors were frequently present, e.g., mu-toxin encoded by the genes *nagH*, *nagI*, *nagJ*, *nagK*, and *nagL*, and sialidases, encoded by *nanH*, *nanI*, and *nanJ*. Mu-toxin consists of hyaluronidases that facilitate the degradation of polysaccharides, such as hyaluronic acid and chondroitin sulfate, thus helping the microorganism to spread into deeper tissues (Rood., 1998; Wang., 2020). In our isolates, we frequently detect *nagH*, *nagI*, *nagJ*, *nagK*, and, with lesser frequency, *nagL*. The three sialidases NanH, NanI, and NanJ were also highly abundant in our isolates. They are considered to be important virulence factors that promote the pathogenesis of *C. perfringens*;

among them, NanI promotes colonization in the intestinal tract and enhances cytotoxic activity (Wang., 2020).

Among the isolates from our study, we detected pDel1, pDel2, pDel3, and pDel4 plasmids, previously described in *C. perfringens* Del1, a strain from a chicken affected by NE, in which the *netB* gene was included within pDel1 (Li et al., 2017). However, in our study, we found pDel1 in three of our type G isolates, but not in all isolates that carried *netB* gene, belonging to type G toxinotypes. Instead, all *C. perfringens* type G isolates carrying *netB* harbored the pDel4 plasmid, suggesting that, in our isolates, *netB* may reside in this plasmid. Our isolates also harbored pLLY_N11_3, a plasmid previously detected by (Li et al., 2017), pCP15_3, which has been frequently detected in *C. perfringens* in other studies, and pCPCPI53k-r1_2, which has not yet been studied (Feng et al., 2020).

Among poultry, the most common STs previously found were ST143 and ST215(*Camargo et al., 2020*). Among our isolates, ST21, ST73, and ST279 were detected. The other three isolates presented two new allelic combinations (*C. perfringens* MLG1619 and MLG1819 with the same allelic combination, and MLG0418 with a different one). In addition, six isolates presented four new STs, showing new housekeeping alleles. These results reveal a high level of diversity among the *C. perfringens* isolates in our collection. In contrast, studies on MLST of *C. perfringens* from poultry indicate that *C. perfringens* isolates from NE diseased birds and healthy birds within outbreaks tend to be closely related. Even though *C. perfringens* is very diverse, there are subpopulations of *C. perfringens* types commonly found in NE birds that are not as variable as those found in healthy chickens (Feng et al., 2020). Among the STs of our isolates, only ST21 has previously been reported in NE chickens (Hibberd et al., 2011). In addition, we found new allelic combinations and STs, highlighting that most of our strains are from undescribed STs, thus adding more variability to *C. perfringens* from chickens affected by NE.

All isolates carried genes of sactipeptides (sulfur-alpha carbon thioether crosslinked peptides), a novel type of lantibiotic that presents various biological activities such as antibacterial, spermicidal, and hemolytic properties. However, their function is still being studied (Chen et al., 2021). Genes encoding lassopeptides were also found in three of the isolates. They belong to a specific family of RiPPs with an unusual lasso structure. Lasso peptides possess

remarkable thermal and proteolytic stability and diverse biological activities such as antimicrobial activity, enzyme inhibition, receptor blocking, anticancer properties, and HIV antagonism. They have promising potential therapeutic effects in gastrointestinal diseases, tuberculosis, Alzheimer's disease, cardiovascular diseases, fungal infections, and cancer (Sosunov et al., 2007; Cheng & Hua., 2020).

The gene encoding bacteriocin BCN5 was detected in five of our *C. perfringens* isolates. It is a plasmid-encoded bacteriocin with promising activity against *Mycobacterium tuberculosis* (Garnier & Cole., 1986). Its activation has been shown to depend in vivo and in vitro on the activity of the UviA and UviB proteins (Dupuy et al., 2005), and it is inducible by UV irradiation (Miyamoto et al., 2015). The phylogenetic relationships of bacteriocin BNC5 of our stains and those included in Genbank revealed that BNC5 of three of our *C. perfringens* isolates (MLG3406, MLG4206, and MLG5719) were identical among themselves, as well as to bacteriocin BCN5 P08696 from the GenBank database, which corresponds to the bacteriocin first described and characterized (Miyamoto et al., 2015). Moreover, the BCN5 sequence of the remaining two *C. perfringens* isolates of our collection and the bacteriocin BNC5 BAD90628 from the GenBank database (Miyamoto et al., 2015) clustered separately. With regard to the alignments of bacteriocin BCN5, the low level of identity between them may be evidence that they are different bacteriocins. Overall, bacteriocin BCN5 was identical to and well conserved within three of the *C. perfringens* isolates from our collection and the bacteriocin BCN5 P08696 of reference, whereas it presented polymorphisms with the other isolates of our collection and with the other BCN5 of reference (BAD90628). The genetic environments of the contigs containing the *bcn5* gene of our isolates and the reference plasmid BAD90628 showed many differences among them, whereas the genetic environments of *C. perfringens* MLG2919, MLG3406, and MLG4206 were similar. *C. perfringens* MLG5719 showed similarities with the plasmid of reference BAD90628, with two insertions. The *C. perfringens* MLG7307 showed no similarity to the others. As no plasmids were identified in strain MLG7307, the *bcn5* gene in this strain could be located at the chromosome.

Surprisingly, although many genes encoding for antimicrobial peptide production have been detected, no antimicrobial activity was observed in *C. perfringens* isolates against the indicator strains used. This is probably because

the presence of encoding genes detected by WGS does not necessary lead to gene expression and to synthesis of the antimicrobial protein, perhaps requiring specific conditions for gene expression. This phenomenon has already been described for others bacteriocins, such as nisin and microcins (Moreno et al., 2002; Zhou et al., 2006). Future studies should investigate this issue.

To sum up, *C. perfringens* infection of avian species is a serious animal health concern. Among all the characteristics studied in the 20 isolates from NE poultry of toxinotypes A and G, we could highlight the presence of the *erm*(T) gene, reported only in other Gram-positive bacteria, including ARG, as well as the presence of multiple toxins and virulence factors, and the existence of variability among the ST of the isolates. In addition, of great interest is the detection of genes encoding different bacteriocins, with BCN5 being of relevance.

Moreover, it should also be noted that the *C. perfringens* MLG7307 isolate was clearly distinct from the other strains. This strain was phenotypically resistant to clindamycin and cefotaxime, and it had intermediate susceptibility to ampicillin, although no resistance genes were observed by WGS. It also had a combination of toxins that made it impossible to toxinotype. Interestingly, even though it was identified as *C. perfringens*, it lacked the housekeeping gene *colA*. Further studies should be carried out on this strain to determine its characteristics and possible classification.

WGS analysis provides insights into the genomic characteristics of bacteria and is a promising tool for the study and better understanding of the avian pathogen *C. perfringens*.

2.6 Conclusions

I. Antibiotic resistance is frequent in *C. perfringens* isolates implicated in NE of poultry, with tetracycline resistance being the most common.
II. Macrolide resistance, via the *erm*(T) gene, was reported for the first time in two *C. perfringens* from our collection.
III. The *erm*(T) gene is highly conserved among different Gram-positive bacterial species, but its genetic environment varies from species to species.

IV. The ERY-resistant *C. perfringens* MLG3111 may carry novel resistance mechanisms that should be further investigated.

V. Isolate *C. perfringens* MLG7307 showed unique characteristics in terms of toxinotyping and resistance profile, and also lacked the housekeeping gene *colA*, which might indicate that this isolate may represent a new species or subspecies of *C. perfringens* that should be further analyzed and classified.

VI. The *C. perfringens* collection shows a wide variety of ST in which new possible alleles as well as new allelic combinations are detected.

VII. Secondary metabolites, including structural genes for bacteriocins, were detected among *C. perfringens* isolates although none of them showed antimicrobial activity.

VIII. WGS analysis provides insight into the genomic characteristics of *C. perfringens*.

3. Chapter III: Targeting Enterococci with Antimicrobial Activity against *Clostridium perfringens* from Poultry

Abstract

Necrotic enteritis (NE), caused by *Clostridium perfringens*, is an emerging issue in poultry farming. New approaches, other than antibiotics, are necessary to prevent NE development and the emergence of multidrug-resistant bacteria. Enterococci are commensal microorganisms that can produce enterocins, antimicrobial peptides with activities against pathogens, and could be excellent candidates for protective cultures. This study aimed to screen and characterize *Enterococcus* strains of poultry origin for their inhibitory activity against *C. perfringens*. In total, 251 *Enterococcus* strains of poultry origin plus five bacteriocin-producing (BP+) *E. durans* strains of other origins were screened for antimicrobial activity against the indicator *C. perfringens* X2967 strain using the "spot on the lawn" method. We detected thirty-two BP+ strains (eleven *Enterococcus faecium*, nine *E. gallinarum*, eight *E. faecalis*, three *E. durans*, and one *E. casseliflavus*). We further studied the antimicrobial activity of the supernatants of these 32 BP+ strains using agar well diffusion and microtitration against a collection of 20 *C. perfringens* strains. Twelve BP+ enterococci that were found to exhibit antimicrobial activity *against C. perfringens* were characterized using whole genome sequencing. Among these, *E. faecium* X2893 and X2906 were the most promising candidates for further studies as protective cultures for poultry farming. Both strains belong to the sequence type ST722, harbor the genes encoding for enterocin A and enterocin B, do not possess acquired resistance genes, do not carry plasmids, and present the *acm* gene, which is implicated in host colonization. Further research is needed to determine the utility of these strains as protective cultures.

Résumé

L'entérite nécrotique (EN), causée par *Clostridium perfringens*, est un problème émergent dans l'élevage de volailles. De nouvelles approches, autres que les antibiotiques, sont nécessaires pour prévenir le développement de l'EN et l'émergence de bactéries multirésistantes. Les entérocoques sont des microorganismes commensaux qui peuvent produire des entérocines, des peptides antimicrobiens ayant des activités contre les pathogènes, et pourraient être d'excellents candidats pour les cultures protectrices. Cette étude visait à cribler et à caractériser les souches d'*Enterococcus* d'origine avicole pour leur activité inhibitrice contre *C. perfringens*. Au total, 251 souches d'*Enterococcus* d'origine avicole et cinq souches d'*E. durans* produisant des bactériocines (BP+) d'autres origines ont été criblées pour leur activité antimicrobienne contre la souche indicatrice *C. perfringens* X2967 en utilisant la méthode du "spot on the lawn". Nous avons détecté trente-deux souches BP+ (onze *Enterococcus faecium*, neuf *E. gallinarum*, huit *E. faecalis*, trois *E. durans* et un *E. casseliflavus*). Nous avons ensuite étudié l'activité antimicrobienne des surnageants de ces 32 souches BP+ en utilisant la diffusion sur puits de gélose et la microtitration contre une collection de 20 souches de *C. perfringens*. Douze entérocoques BP+ présentant une activité antimicrobienne contre *C. perfringens* ont été caractérisés par séquençage du génome entier. Parmi celles-ci, *E. faecium* X2893 et X2906 étaient les candidates les plus prometteuses pour des études ultérieures en tant que cultures protectrices pour l'élevage de volailles. Les deux souches appartiennent au type de séquence ST722, abritent les gènes codant pour l'entérocine A et l'entérocine B, ne possèdent pas de gènes de résistance acquise, ne portent pas de plasmides et présentent le gène *acm*, qui est impliqué dans la colonisation de l'hôte. Des recherches supplémentaires sont nécessaires pour déterminer l'utilité de ces souches en tant que cultures protectrices.

Resumen

La enteritis necrótica (EN), causada por *Clostridium perfringens*, es un problema emergente en avicultura. Se necesitan nuevos enfoques, distintos de los antibióticos, para prevenir el desarrollo de la EN y la aparición de bacterias multirresistentes. Los enterococos son microorganismos comensales que pueden producir enterocinas, péptidos antimicrobianos con actividad inhibitoria contra patógenos, que podrían ser excelentes candidatos para su utilización como cultivos protectores. El objetivo de este estudio es realizar un cribado y caracterización de cepas de *Enterococcus* de origen avícola que presenten actividad inhibidora contra *C. perfringens*. En total, se analizó la actividad antimicrobiana de 251 cepas de *Enterococcus* de origen avícola y cinco cepas de *E. durans* productoras de bacteriocinas (BP+) de otros orígenes frente a la cepa indicadora *C. perfringens* X2967 mediante el método de "spot on the lawn". Se detectaron treinta y dos cepas BP+ (once *Enterococcus faecium*, nueve *E. gallinarum*, ocho *E. faecalis*, tres *E. durans* y un *E. casseliflavus*). Además, se estudió la actividad antimicrobiana de los sobrenadantes de estas 32 cepas BP+ mediante difusión en pocillos de agar y microtitulación frente a una colección de 20 cepas de *C. perfringens*. Doce enterococos BP+ que mostraron actividad antimicrobiana contra *C. perfringens* se caracterizaron mediante secuenciación del genoma completo. Entre ellas, destacan *E. faecium* X2893 y X2906 por ser las candidatas más prometedoras para futuros estudios como cultivos protectores en ganadería avícola. Ambas cepas pertenecen al linaje genético ST722, albergan los genes que codifican la enterocina A y la enterocina B, no poseen genes de resistencia adquirida, no son portadoras de plásmidos y presentan el gen *acm*, implicado en la colonización del hospedador. Se necesitan más investigaciones para determinar la utilidad de estas cepas como cultivos protectores.

3.1 Introduction

Antibiotic resistance is a serious public health concern that compromises the treatment of infections in humans and animals and is associated with the unnecessary prescription and/or misuse of antibiotics. Besides their clinical use in humans, antibiotics are also used in veterinary and animal farming. Antibiotics have also been extensively used as growth promoters in food-producing animals; however, even though this practice has been banned in Europe since 2006 (Wang et al., 2020) and also in several other countries, it is still allowed in some others (Gochez et al., 2021). This contributes to the increase and spread of antibiotic resistance, not only among pathogenic bacteria but also among commensal bacteria of the intestinal tract of humans and animals, which can lead to contamination via feces. Therefore, resistant bacteria can reach humans via the food chain and water or by contact with animals. For this reason, the World Health Organization (WHO) proposed to address this issue from a "One Health" perspective, establishing new alternatives to the use of antibiotics in livestock and agriculture (McEwen & Collignon., 2018).

Clostridium perfringens is associated with necrotic enteritis (NE) in poultry, and its prevalence has been increasing in countries that no longer use antibiotic growth promoters, which suggests that the same trend could also originate among other relevant pathogens (Villagrán-de la Mora et al., 2020). NE caused by *C. perfringens* is one of the most common poultry diseases that cause substantial economic losses to the industry (Alizadeh et al., 2021). A prominent characteristic of NE is acute death, with mortality rates reaching 50%. Clinical signs include depression, dehydration, somnolence, ruffled feathers, diarrhea, and decreased feed consumption (Van Imersel et al., 2004). The subclinical form of this disease causes chronic damage to the intestinal mucosa of the chickens, leading to impaired nutrient absorption, reduced weight gain, and decreased overall performance. *Clostridium perfringens* is present in the intestines of healthy chickens but in a small proportion (less than 10^5 CFU/g of the intestinal content); when its count increases, hen birds become susceptible to NE (Agunos et al., 2016).

Antibiotic-resistant bacteria are prevalent in different environments and can be introduced into the food chain at various points. Poultry is a reservoir for antibiotic-resistant bacteria that can be transmitted to humans. The continuous and widespread use of antibiotics in farm animals may lead to

changes in the bacterial environment, eliminating susceptible strains and allowing antimicrobial-resistant bacteria to survive and predominate. Furthermore, the continuous administration of antibiotics in feed may cause cross-resistance to therapeutic antimicrobial agents. Antimicrobial resistance and a gradual decrease in antibiotic sensitivity to anticoccidials in some strains of *Eimeria spp.* (a predisposing factor for NE) can exacerbate the presence of *C. perfringens* strains (Agunos et al., 2016).

Protective cultures essentially consist of bacteria specifically selected for their ability to inhibit the growth of other pathogenic organisms or microbiological spoilage agents, having the status of GRAS (Generally Recognized as Safe). These bacterial species are entirely natural. Therefore, they provide a useful "green" benefit to food product labeling (Young & O'Sullivan., 2011). Bacteriocin-producing strains have gained considerable interest in recent years. They are considered one of the most promising alternatives to antibiotics for use as protective cultures.

Enterococci are ubiquitous microorganisms found in the gastrointestinal tracts of humans and animals and in water, soil, plants, and food. These microorganisms produce bacteriocins known as enterocins (Ben Braïek & Smaoui., 2019), which exhibit an inhibition spectrum against taxonomically close bacteria and even those with a broad spectrum of action, inhibiting a wide range of bacteria, including the emergent *C. perfringens* (Franz et al., 2007; Silva et al., 2018). Using enterococci as potential probiotic strains or protective cultures can be an excellent alternative to antibiotic use in poultry farming (Hamanami et al., 2019).

However, in recent years, the use of enterococci in the food industry has been debated because of their implications for opportunistic infections and their potential acquisition of antimicrobial resistance and virulence genes (Ben Braïek & Smaoui., 2019). Therefore, developing new enterococcal probiotics requires a strict safety assessment to select the truly harmless enterococcal strains for safe applications (Hanchi et al., 2018).

3.2 Objective

This study aimed to isolate and characterize *Enterococcus* strains of poultry origin showing antimicrobial activity against the collection of *C.*

perfringens implicated in NE of poultry of the previous study and other relevant microorganisms. Phenotypic and genotypic analysis of the bacteriocin producing enterococci will be performed as well as other characteristics, as digestive survival under poultry conditions.

3.3 Methodology

3.3.1 *Enterococcus* sampling and identification

In total, 251 enterococcal isolates of poultry origin were used in this study: (a) 60 isolates were collected during this study from poultry carcass samples obtained from different supermarkets and butchers in the La Rioja Region (Spain), the isolates recovered in the Slanetz–Bartley agar (OXOID); (b) 166 isolates were previously obtained from poultry carcasses at the slaughterhouses' level in Tunisia; (c) 25 poultry isolates were obtained from the University of La Rioja's collection (Spain). Additionally, five bacteriocin producing enterococci of other origins (2 isolates from cow milk and 3 from camel milk) were obtained from the University of LAVAL's strain collection (Canada).

3.3.2 Screening for anti-*C. perfringens* activity using the "spot on the lawn" method

The antimicrobial activity of the 256 *Enterococcus* isolates against the indicator strain *C. perfringens* X2967 (a clinical strain obtained from the Hospital San Pedro, Logroño, Spain), was analyzed using the "spot-on-the-lawn" method (Poeta et al., 2007). The active isolates were identified as bacteriocin producing (BP+) isolates. Briefly, a fresh culture of *C. perfringens* strain X2967 was suspended in brain–heart infusion broth (BHI) (turbidity 0.5 MacFarland). Subsequently, 10 µL of this indicator microorganism solution was added to tubes containing 5 mL of semi-solid melted tryptic soy broth (TSB) and supplemented with 0.7% agar and 0.3% yeast extract. Finally, the semi-solid TSB medium with the indicator microorganism was poured onto tryptic soy agar plates (TSA). Once the plates were dried, the enterococcal microorganisms were sting-seeded, and the plates were incubated at 37 °C for 24 h under strict, anaerobic conditions.

Strains that showed inhibitory activity against *C. perfringens* strain X2967 were tested against other relevant pathogens and multidrug-resistant

(MDR) bacteria using the same test. This panel included *E. casseliflavus* C1232, *E. gallinarum* C2310, *E. faecium* C2321, *E. faecalis* C410, *E. durans* C1433, *E. hirae* C1436, MSSA C411, MRSA C1570, *M. luteus* C157, *L. monocytogenes* C137, *S. suis* C2058, *E. coli* C408, *S. enterica* C660, *Y. enterocolitica* X3080, and *P. aeruginosa* X3282. A blood agar plate was used for *S. suis* testing. All strains used as indicator bacteria came from the University of La Rioja's collection.

3.3.3 Screening for Anti-*C. perfringens* activity using the agar diffusion method

Non-heated supernatant (NHS) and heated supernatant (HS) extracts were prepared from *Enterococcus* isolates showing inhibitory activity in the spot-on-the-lawn assay. These supernatants were tested against a collection of 20 *C. perfringens* isolates using the previously described agar diffusion method (Bennett et al. 2021), with nisin as a positive control. The *C. perfringens* isolates were collected from the NE of poultry origin (University of Laval, Quebec, QC, Canada).

To prepare the NHS, enterococci were inoculated in 10 mL of TSB in sterile tubes and were incubated overnight at 37 °C. Then, the culture medium was centrifuged at 5000× *g* rpm for 5 min and filtrated using 0.20 µm filters. Next, a fraction of this supernatant was heated at 100 °C for 15 min and used as the HS. For the concentrated supernatants, the culture cell media were concentrated 10 times using a Speed Vac (Thermo Scientific Savant, Asheville, NC, United States) after centrifugation.

For the agar well-diffusion method, *C. perfringens* was cultured in a reinforced clostridial medium (RCM) (Himedia, Kennett Square, PA, USA) supplemented with 10% agar. The plates were incubated overnight at 37 ºC under strict, anaerobic conditions.

3.3.4 Anti-*C. perfringens* activity determination using microtitration assay

A microtitration assay was performed to determine the total activity (AU/mL) of the active supernatant of BP+ enterococci against the *C. perfringens* ATCC 13124 strain, as described previously (Lo Verso et al., 2018; Soltani et al., 2022). The BHI was used as the growth medium for *C. perfringens* and was added to the wells, with a final bacterial concentration of

~10^5 CFU/well. The microplate was incubated for 24 h at 37 °C under strict, anaerobic conditions. After incubation, the optical density was measured at 595 nm using a microplate reader (Infinite M200, Tecan, Männedorf, Switzerland) to determine the number of wells in which inhibition occurred.

The following formula was used to calculate the total arbitrary activity:

$$Activity\ \left(\frac{AU}{mL}\right) = 2^{n*\left(\frac{1000}{25}\right)} = 2^{n+3}$$

where 2 is the dilution factor, n is the number of inhibition wells, 1000 is the factor for reporting the result per mL, and 125 is the volume of the solution tested in microliters.

3.3.5 Characterization of BP+ enterococci

Twelve BP+ enterococci were chosen for further characterization based on their antimicrobial activity against *C. perfringens* strains.

3.3.5.1 Susceptibility to antibiotics

The susceptibility of BP+ enterococci to nine antibiotics was tested using the disk diffusion method according to the Clinical and Laboratory Standard Institute (CLSI) guidelines (2021) (CLSI., 2021). The antibiotics tested were as follows (disk charge): penicillin (10 units), tetracycline (30 µg), erythromycin (15 µg), chloramphenicol (30 µg), linezolid (30 µg), high-level gentamicin (120 µg), high-level streptomycin (300 µg), vancomycin (30 µg), and ciprofloxacin (5 µg). Strains were then identified as susceptible (S), resistant (R), or intermediate (I) using the protocol interpretation guidelines (CLSI, 2021).

3.3.5.2 Gelatinase activity and hemolysis

The gelatinase activity and hemolytic capacity of BP+ enterococci strains were determined as reported previously (Poeta et al., 2006), using TSA supplemented with 3% skim milk and blood agar, respectively.

3.3.5.3 Whole Genome Sequencing analysis

DNA from BP+ enterococci was extracted using a DNeasy Blood & Tissue Kit (QIAGEN, Hilden, Germany), following the manufacturer's instructions for gram-positive bacteria. The DNA was subjected to WGS using an Illumina sequencing system at the Hospital Center of University Laval (CHUL). Data were analyzed using the following programs; fastp for trimming and quality check of the trimming (Chen et al., 2018), SPAdes for the assembly (Bankevich et al.,

2012), QUAS for checking the assembled quality (Gurevich et al., 2013), and prokka for annotation (Seemann., 2014). Anti-SMASH 6.0 (Blin et al., 2021) and BAGEL4 (Van Heel et al., 2018) were used to detect genes encoding bacteriocins. ResFinder 4.1 (Camacho et al., 2009; Zankan et al., 2017; Bortolaia et al., 2020) was used to detect genes associated with antibiotic resistance and mutations in the *pbps*, *parC*, and *gyrA* genes. VirulenceFinder 2.0 was used to detect virulence factors (Camacho et al., 2009; Joensen et al., 2014; Malberg Tetzschner et al., 2020) and PlasmidFinder 2.1 for plasmid detection (Camacho et al. 2009; Carattoli et al., 2014). Multi-locus sequence typing (MLST) was performed using MLST 2.0 (Lemee et al., 2004; Bartual et al., 2005; Jauneguy et al., 2008; Wirth et al., 2010; Larsen et al., 2012). Representation in the phylogenetic tree was performed using R version 4.2.1 (R Development Core Team., 2013), and the phylogenetic distances were calculated using the average nucleotide identity (ANI) method.

3.3.6. Digestive Survival of Selected BP+ enterococcal isolates

An *in vitro* digestive survival assay with the isolates *E. faecium* X2893 and X2906, which showed potential as candidates for protective cultures, was performed following the INFOGEST protocol (Brodkorb et al., 2019). This protocol simulates the gastrointestinal conditions in an *in vitro* model. Adaptations were made in order to simulate the poultry conditions: 42 °C temperature and pH adjustments for saliva (6.75), stomach (3.5) and intestine (6.4). 100 µL of each bacterial culture were recovered just after each step of the digestion (initial, salivary, gastric and intestinal) and plaqued into BHI agar to evaluate their growth by counting CFU/mL.

3.4 Results

3.4.1 *Enterococcus* Sampling and Identification

Sixty *Enterococcus* strains were isolated from poultry meat samples collected from local markets in La Rioja, Spain. These strains were identified using MALDI-TOF mass spectrometry as *E. faecium* (n = 33), *E. faecalis* (n = 19), *E. gallinarum* (n = 5), *E. casseliflavus* (n = 1), *E. durans* (n = 1), and *E. avium* (n = 1). These isolates were combined with another 191 *Enterococcus*, previously obtained from poultry (in Spain and Tunisia), and with five bacteriocin-producing (BP+) *Enterococcus* from other origins, to develop the

entire collection of 256 *Enterococcus* used to detect and characterize the BP+ isolates.

3.4.2 Screening of enterococci for antimicrobial, specifically Anti-*C. perfringens* activity

In total, 32 of the 256 enterococci tested (12.84%) demonstrated antimicrobial activity against *C. perfringens* X2967 using the "spot on the lawn" method. These strains belonged to the species *E. faecium* (*n* = 11), *E. gallinarum* (*n* = 9), *E. faecalis* (*n* = 8), *E. durans* (*n* = 3), and *E. casseliflavus* (*n* = 1). Among them, 27 (84,37%) were active against *Listeria monocytogenes, Micrococcus luteus*, and *Streptococcus suis* (**Table 3. 1**). One *Enterococcus* strain alone showed antimicrobial activity against methicillin-susceptible *Staphylococcus aureus* (MSSA) and methicillin-resistant *S. aureus* (MRSA). None of the tested strains showed inhibitory activity against gram-negative bacteria (*Escherichia coli, Salmonella enterica, Yersinia enterocolitica*, and *Pseudomonas aeruginosa*). **Figure 3. 1** shows the inhibition halo against *C. perfringens* X2967 produced by two of the thirty-two BP+ strains.

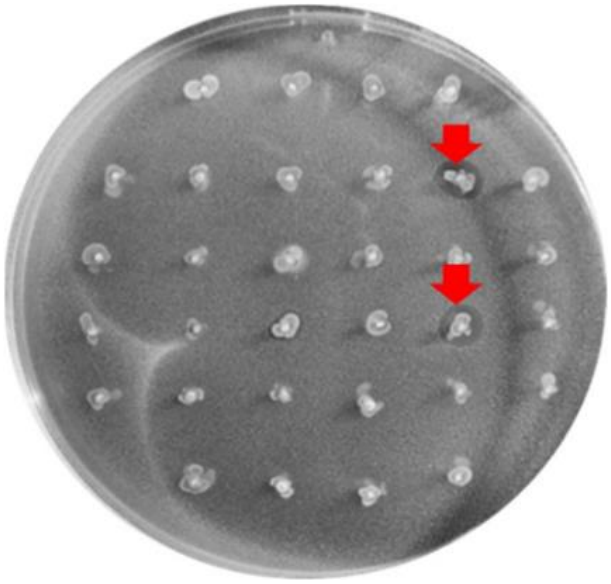


Figure 3.1. Inhibition halos (marked with the red arrow) produced by 2 of the BP+ enterococci tested against the *C. perfringens* X2967 indicator strain.

Table 3. 1. Antimicrobial activity of the 32 bacteriocin producer (BP+) enterococci against *C. perfringens* X2967 and other relevant indicator bacteriaa, as detected by the "spot on the lawn" as-say.

		Number of BP+ strains with activity against the indicator strain					
		***E. faecium* (*n*=11)**	***E. gallinarum* (*n*=9)**	***E. faecalis* (*n*=8)**	***E. durans* (*n*=3)**	***E. casseliflavus* (*n*=1)**	**Total**
Indicator strains[a]	*C. perfringens* (X2967)	11	9	8	3	1	32
	E. hirae (C1436)	11	1	7	3	1	23
	E. durans (C1433)	11	5	2	3	1	22
	E. casseliflavus (C1232)	8	3	6	3	-	20
	E. faecium (C2321)	10	3	3	3	-	19
	E. faecalis (C410)	11	1	3	3	-	18
	E. gallinarum (C2310)	9	3	3	3	-	18
	L. monocytogenes (C137)	8	4	4	3	-	19
	M. luteus (C157)	1	1	7	3	1	13
	S. suis (C2058)	2	-	6	3	1	7
	MRSA[b] (C411)	-	1	-	-	-	1
	MSSA[b] (C1570)	-	1	-	-	-	1

[a]None of the isolates showed antimicrobial activity against *E. coli* (C408), *S. enterica* (C660), *Y. enterocolitica* (X3080) or *P. aeruginosa* (X3282).
[b] Abbreviation: MRSA: methicillin-resistant *S. aureus*; MSSA: methicillin-susceptible *S. aureus*

3.4.3 Effects of the supernatants of BP+ enterococci on *C. perfringens* Isolates

The supernatants of the 32 BP+ enterococci were tested against a collection of 20 *C. perfringens* isolates of poultry origin. The antimicrobial activity was detected in 18 concentrated supernatants against at least one of the *C. perfringens* strains. Nevertheless, antimicrobial activity was observed in six of the heated supernatants (HS) and non-heated supernatants (NHS) (**Figure 3. 2, Table 3. 2**), corresponding to four *E. faecium* and two *E. durans* isolates. In general, the inhibitory activities of the HS and NHS were similar; both inhibited the growth of 2–8 strains of the 20 *C. perfringens* tested. The

concentrated supernatants showed a broad spectrum of inhibition against 2–20 *C. perfringens* isolates (**Table 3. 2**). The remaining 14 supernatants, either HS, NHS, or concentrated supernatants, did not show any inhibitory activity.

Figure 3. 2. Inhibition halo of the *E. faecium* strain, X3179, against one of the 20 *C. perfringens* isolates. The bigger halo corresponds to the activity of nisin, used as a control.

Table 3. 2. The number of *C. perfringens* isolates to which the supernatants of 18 BP+ enterococci present antimicrobial activity in their supernatants.

Strain	**Origin**	**Specie**	**Number of *C. perfringens* (of 20 Tested) Inhibited by the Antimicrobial Activity of the Extracts of BP+ Strains**		
			Non Heated Supernatant	**Heated Supernatant**	**Concentrated supernatant**
C1446	Poultry	*E. gallinarum*	-a	-	11/20
X2829	Poultry	*E. faecium*	2/20	2/20	12/20
X3036	Poultry	*E. gallinarum*	-	-	2/20
X3179	Poultry	*E. faecium*	4/20	4/20	18/20
X2903	Poultry	*E. faecium*	-	-	8/20
X2947	Poultry	*E. faecium*	-	-	4/20
X2956	Poultry	*E. faecium*	-	-	3/20
X2960	Poultry	*E. faecium*	-	-	4/20

X3187	Poultry	*E. faecalis*	-	-	1/20
X3220	Poultry	*E. faecium*	-	-	1/20
X3198	Poultry	*E. faecalis*	-	-	1/20
X3204	Poultry	*E. faecium*	2/20	2/20	16/20
X2906	Poultry	*E. faecium*	2/20	2/20	8/20
61A	Cow milk	*E. durans*	4/20	3/20	20/20
42G	Cow milk	*E. durans*	8/20	5/20	18/20
LCW03	Camel milk	*E. durans*	-	-	18/20
LCW44	Camel milk	*E. durans*	-	-	16/20
LCW06	Camel milk	*E. durans*	-	-	16/20

[a] The symbol -: no antimicrobial activity was detected against any of the *20 C. perfringens* isolates tested as the indicator bacteria.

Supernatant activity could only be quantified for the *E. faecalis* X3198 and *E. faecium* X3179 strains (16 AU/mL).

3.4.4 Phenotypic and genotypic characterization of the selected BP+ enterococci

For a complete genome analysis, 12 BP+ enterococci were selected based on their antimicrobial activity detected using the previously described methods. Five *E. faecium* and two *E. faecalis* of poultry origin were selected, as well as five *E. durans* of milk and camel milk origin, chosen as the BP+ controls.

3.4.4.1 Bacteriocinome

Structural genes encoding for bacteriocins were detected in 12 BP+ strains (**Table 3. 3**). The structural genes for enterocin P and Enterocin L50 A/B were detected in all five *E. durans* isolates, and the genes for bac 32 were also observed in three of them. Genes encoding enterocin A and enterocin B were detected in all the *E. faecium* strains; two of these strains carried the genes encoding enterocin NKR-5-3-A/D/Z. Moreover, the genes encoding enterocin SE-K4 and staphylococcin C55a/b were identified in two *E. faecalis* strains.

Table 3. 3. Putative enterocins detected by WGS in the 12 selected BP+ enterococci.

Strain	Specie	Putative enterocins
42G	*E. durans*	Enterocin P, Enterocin L50 A/B
61A	*E. durans*	Enterocin P, Enterocin L50 A/B
LCW03	*E. durans*	Enterocin P, Enterocin L50 A/B, Bacteriocin 32

LCW06	*E. durans*	Enterocin P, Enterocin L50 A/B, Bacteriocin 32
LCW44	*E. durans*	Enterocin P, Enterocin L50 A/B, Bacteriocin 32
X2893	*E. faecium*	Enterocin A, Enterocin B
X2903	*E. faecium*	Enterocins NKR-5-3A; Enterocin NKR-5-3D, Enterocin NKR-3-5-3-Z
X2906	*E. faecium*	Enterocin A, Enterocin B
X3179	*E. faecium*	Enterocin A, Enterocin B, Enterocin NKR-5-3A, Enterocin NKR-5-3D, Enterocin NKR-5-3-Z
X3204	*E. faecium*	Enterocin A, Enterocin B
X3198	*E. faecalis*	Ent SE-K4, Staphylococcin C55a/b
X3187	*E. faecalis*	Ent SE-K4, Staphylococcin C55a/b

3.4.4.2 Antibiotic Resistance phenotype and resistome

Five of the twelve selected BP+ enterococci (41.7%) were susceptible to the nine antibiotics tested, all of them from the species *E. durans*. The remaining strains were resistant to at least one of the antibiotics tested. The most frequent resistance was against ciprofloxacin (58.3%), followed by tetracycline (25.0%), erythromycin (25.0%), penicillin (16.7%), chloramphenicol (8.3%), high-level streptomycin (8.3%), and high-level gentamicin (8.3%). In addition, all the isolates showed susceptibility to vancomycin and linezolid.

Genes encoding antibiotic resistance were detected in all 12 BP+ strains (**Table 3. 4**), although only five (three *E. faecium* and two *E. faecalis* isolates) had genes for acquired-type resistance. The mutations associated with resistance phenotypes for beta-lactams (*pbp5*) and fluoroquinolones (*gyrA* and *parC*) were detected only in *E. faecium* isolates (**Table 3. 5**).

Table 3. 4. Antibiotic resistance phenotype and genotype of the BP+ enterococci.

				Resistance genotype	
Strain	**Specie**	**Origin**	**Resistance phenotype**	**Intrinsic mechanisms**	**Adquired mechanisms**
X2893	*E. faecium*	Poultry	CIP	*msr(C), aac(6')-Ii*	-
X3179	*E. faecium*		CIP, E	*msr(C), aac(6')-Ii*	*erm(B)*
X2903	*E. faecium*		CIP, E, P, C	*msr(C), aac(6')-Ii*	*erm(B), fexB, poxtA*
X3187	*E. faecalis*		CIP, E, P, TE, CN, S	*Isa(A)*	*erm(B), aac(6')-aph(2''), tet(M)*
X3198	*E. faecalis*		CIP, TE	*Isa(A)*	*erm(B), aac(6')-aph(2''), tet(M)*
X3204	*E. faecium*		CIP, TE	*msr(C), aac(6')-Ii*	str, tet(M), tet(L), cat
X2906	*E. faecium*		CIP	*msr(C), aac(6')-Ii*	-
61A	*E. durans*	Other	Susceptible	*aac(6')-Iih*	-
42G	*E. durans*		Susceptible	*aac(6')-Iih*	-
LCW03	*E. durans*		Susceptible	*aac(6')-Iih*	-
LCW44	*E. durans*		Susceptible	*aac(6')-Iih*	-
LCW06	*E. durans*		Susceptible	*aac(6')-Iih*	-

Table 3. 5. Mutations detected in the *pbp5*, *gyrA* and *parC* genes of the BP+ enterococci.

Strain	Mutations associated with Beta-lactam resistance				No class defined mutations			
	Mutation	Nucleotide change	Amino acid change	Phenotype	Mutation	Nucleotide change	Amino acid change	Phenotype
X2893	pbp5:p.E100Q	gag -> cag	e -> q	ampicillin[a]	gyrA:p.N708D	aat -> gat	n -> d	Unknown[b]
	pbp5:p.N496K	aat -> aaa	n -> k	ampicillin	pbp5:p.D644N	gac -> aac	d -> n	Unknown
	pbp5:p.A216S	gca -> tcc	a -> s	ampicillin	parC:p.E138A	gaa -> gca	e -> a	Unknown
	pbp5:p.E525D	gag -> gat	e -> d	ampicillin	pbp5:p.T25A	act -> gct	t -> a	Unknown
	pbp5:p.T324A	aca -> gca	t -> a	ampicillin	pbp5:p.S39T	agc -> acc	s -> t	Unknown
	pbp5:p.G66E	gga -> gaa	g -> e	ampicillin				
	pbp5:p.L177I	tta -> ata	l -> i	ampicillin				
	pbp5:p.T172A	aca -> gca	t -> a	ampicillin				
	pbp5:p.R34Q	cgg -> cag	r -> q	ampicillin				
	pbp5:p.V24A	gta -> gca	v -> a	ampicillin				
	pbp5:p.A499T	gca -> aca	a -> t	ampicillin				
	pbp5:p.S27G	agt -> ggt	s -> g	ampicillin				
	pbp5:p.K144Q	aaa -> caa	k -> q	ampicillin				
X3179	pbp5:p.E100Q	gag -> cag	e -> q	ampicillin	pbp5:p.T25A	act -> gct	t -> a	Unknown
	pbp5:p.E85D	gaa -> gat	e -> d	ampicillin	gyrA:p.N708D	aat -> gat	n -> d	Unknown
	pbp5:p.T324A	aca -> gca	t -> a	ampicillin	pbp5:p.P406A	cct -> gct	p -> a	Unknown
	pbp5:p.A68T	gca -> aca	a -> t	ampicillin	pbp5:p.S39T	agc -> acc	s -> t	Unknown
	pbp5:p.N496K	aat -> aaa	n -> k	ampicillin	pbp5:p.D644N	gac -> aac	d -> n	Unknown
	pbp5:p.A216S	gca -> tcc	a -> s	ampicillin				
	pbp5:p.S27G	agt -> ggt	s -> g	ampicillin				
	pbp5:p.R34Q	cgg -> cag	r -> q	ampicillin				
	pbp5:p.E525D	gag -> gat	e -> d	ampicillin				
	pbp5:p.L177I	tta -> ata	l -> i	ampicillin				
	pbp5:p.V24A	gta -> gca	v -> a	ampicillin				
	pbp5:p.G66E	gga -> gaa	g -> e	ampicillin				
	pbp5:p.T172A	aca -> gca	t -> a	ampicillin				
	pbp5:p.A499T	gca -> aca	a -> t	ampicillin				
	pbp5:p.K144Q	aaa -> caa	k -> q	ampicillin				
X2903	pbp5:p.S27G	agt -> ggt	s -> g	ampicillin	pbp5:p.Q461K	caa -> aaa	q -> k	Unknown
	pbp5:p.L177I	tta -> ata	l -> i	ampicillin	pbp5:p.D472L	gac -> ctt	d -> l	Unknown
	pbp5:p.D204G	gac -> ggc	d -> g	ampicillin	pbp5:v468	g-tg -> gat	v -> d	Unknown
	pbp5:p.K144Q	aaa -> caa	k -> q	ampicillin	pbp5:p.L473K	tta -> aaa	l -> k	Unknown
	pbp5:p.E100Q	gag -> cag	e -> q	ampicillin	pbp5:p.Q470S	caa -> agt	q -> s	Unknown
	pbp5:p.E85D	gaa -> gat	e -> d	ampicillin	pbp5:p.T475F	act -> ttc	t -> f	Unknown
	pbp5:p.A216S	gca -> tcc	a -> s	ampicillin	pbp5:p.D481*	gat -> taa	d -> *	Unknown
	pbp5:p.V24A	gta -> gca	v -> a	ampicillin	pbp5:p.L477D	ttg -> gat	l -> d	Unknown
	pbp5:p.G66E	gga -> gaa	g -> e	ampicillin	pbp5:p.V471R	gta -> aga	v -> r	Unknown
	pbp5:p.A68T	gca -> aca	a -> t	ampicillin	pbp5:p.K474N	aaa -> aac	k -> n	Unknown
	pbp5:p.T172A	aca -> gca	t -> a	ampicillin	pbp5:p.I478L	att -> cta	i -> l	Unknown
	pbp5:p.R34Q	cgg -> cag	r -> q	ampicillin	pbp5:p.S39T	agc -> acc	s -> t	Unknown
	pbp5:p.T324A	aca -> gca	t -> a	ampicillin	pbp5:p.A476F	gct -> ttt	a -> f	Unknown
					pbp5:p.Y479F	tat -> ttc	y -> f	Unknown
					pbp5:s469	--tca -> gtatcaca	s -> vs?	Unknown
					pbp5:p.T25A	act -> gct	t -> a	Unknown
					pbp5:p.S480R	tcc -> cga	s -> r	Unknown
X3204	pbp5:p.E525D	gag -> gat	e -> d	ampicillin	parC:p.I45M	ata -> atg	i -> m	Unknown
	pbp5:p.S27G	agt -> ggt	s -> g	ampicillin	pbp5:p.D644N	gac -> aac	d -> n	Unknown
	pbp5:p.T324A	aca -> gca	t -> a	ampicillin	parC:p.T740M	acg -> atg	t -> m	Unknown
	pbp5:p.A499T	gca -> aca	a -> t	ampicillin	parC:p.S442N	agc -> aac	s -> n	Unknown
	pbp5:p.G66E	gga -> gaa	g -> e	ampicillin	pbp5:p.S39T	agc -> acc	s -> t	Unknown
	pbp5:p.V24A	gta -> gca	v -> a	ampicillin	pbp5:p.T25A	act -> gct	t -> a	Unknown
	pbp5:p.R34Q	cgg -> cag	r -> q	ampicillin	parC:p.E484Q	gaa -> caa	e -> q	Unknown
	pbp5:p.T172A	aca -> gca	t -> a	ampicillin				
	pbp5:p.A216S	gca -> tcc	a -> s	ampicillin				
	pbp5:p.D204G	gac -> ggc	d -> g	ampicillin				
	pbp5:p.K144Q	aaa -> caa	k -> q	ampicillin				
	pbp5:p.N496K	aat -> aaa	n -> k	ampicillin				
	pbp5:p.E100Q	gag -> cag	e -> q	ampicillin				
	pbp5:p.L177I	tta -> ata	l -> i	ampicillin				

X2906	pbp5:p.N496K	aat -> aaa	n -> k	ampicillin	pbp5:p.T25A	act -> gct	t -> a	Unknown
	pbp5:p.T324A	aca -> gca	t -> a	ampicillin	gyrA:p.N708D	aat -> gat	n -> d	Unknown
	pbp5:p.K144Q	aaa -> caa	k -> q	ampicillin	pbp5:p.S39T	agc -> acc	s -> t	Unknown
	pbp5:p.A499T	gca -> aca	a -> t	ampicillin	pbp5:p.D644N	gac -> aac	d -> n	Unknown
	pbp5:p.E100Q	gag -> cag	e -> q	ampicillin	parC:p.E138A	gaa -> gca	e -> a	Unknown
	pbp5:p.S27G	agt -> ggt	s -> g	ampicillin				
	pbp5:p.V24A	gta -> gca	v -> a	ampicillin				
	pbp5:p.A216S	gca -> tcc	a -> s	ampicillin				
	pbp5:p.T172A	aca -> gca	t -> a	ampicillin				
	pbp5:p.E525D	gag -> gat	e -> d	ampicillin				
	pbp5:p.L177I	tta -> ata	l -> i	ampicillin				
	pbp5:p.R34Q	cgg -> cag	r -> q	ampicillin				
	pbp5:p.G66E	gga -> gaa	g -> e	ampicillin				

[a]The nineteen pbp5 mutations must be present simultaneously for resistance phenotype

[b]Phenotype not included in databases

3.4.4.3 Virulence

Among the 12 selected BP+ enterococci, only *E. faecalis* X3198 was positive for gelatinase activity, and all the strains showed gamma hemolysis.

Among the 12 BP+ enterococcal strains, virulence genes were detected *in E. faecium* and *E. faecalis* but not in *E. durans* (**Table 3. 6**).

Table 3. 6. Virulence genes and sequence types detected in the BP+ *E. faecalis* and *E. faecium* isolates of poultry origin by WGS.

Strain	Specie	Virulence genes	Sequence type
X3187	*E. faecalis*	*ElrA, SrtA, ace, cCF10, cOB1, cad, camE, ebpC, efaAfs, fsrB, gelE, hylA, hylB & tpx*	ST397
X3198	*E. faecalis*	*ElrA, SrtA, ace, cCF10, cOB1, cad, camE, ebpC, efaAfs, fsrB, gelE, hylA, hylB & tpx*	ST397
X2893	*E. faecium*	*Acm*	ST722
X2903	*E. faecium*	*Acm*	New allelic combination: *adk_1, atpA_2, ddl_7, gdh_57, gyd_1, pstS_80, purK_6*
X2906	*E. faecium*	*Acm*	ST722
X3179	*E. faecium*	*Acm*	New allelic combination: *adk_1, atpA_2, ddl_7, gdh_76, gdh_2. gyd_1, pstS_1, purK_3*
X3204	*E. faecium*	*Acm*	ST784

3.4.4.4 Plasmidome

The replicon plasmids identified in the selected enterococci are listed in **Table 3. 7**. All of the *E. durans* strains carried *RepA_N, Inc18*, and *Rep3* or *Rep1* plasmidic replicons. Both *E. faecalis* strains carried the type *Rep trans*. Moreover, most of the *faecium* strains carried at least three different types of plasmidic replicons.

Table 3. 7. Plasmidome of the 12 BP+ enterococci detected by WGS.

Strain	Species	Type	Replicon Plasmid
X2893	*E. faecium*	-	-
X3179	*E. faecium*	Rep3	*rep29*
		Inc18	*rep1, rep2*
		RepA_N	*repUS15*
X2903	*E. faecium*	Rep3	*rep29*
		Rep1	*repUS58*
		Inc18	*rep1*
		Rep_trans	*rep14a*
		RepA_N	*repUS15*
X3187	*E. faecalis*	Rep_trans	*repUS43*
X3198	*E. faecalis*	Rep_trans	*repUS43*
X3204	*E. faecium*	RepA_N	*repUS15*
		Inc18	*rep1*
		Rep1	*rep22*
X2906	*E. faecium*	-	-
61A	*E. durans*	RepA_N	*repUS15*
		Rep1	*repUS64*
		Inc18	*rep1, rep2*
42G	*E. durans*	RepA_N	*repUS15*
		Rep1	*repUS64*
		Inc18	*rep1, rep2*
LCW03	*E. durans*	Rep3	*rep18a*
		Inc18	*rep1, rep2*
		RepA_N	*repUS15*

		Inc18	*rep1, rep2*
LCW44	*E. durans*	Rep3	*rep18a*
		RepA_N	*repUS15*
		Inc18	*rep1, rep2*
LCW06	*E. durans*	Rep3	*rep18a*
		RepA_N	*repUS15*

3.4.4.5 Genetic lineages

Multi-locus sequence typing (MLST) of the two *E. faecalis* and five *E. faecium* strains yielded the following results: (a) the two *E. faecalis* strains were typed as ST397; (b) the five *E. faecium* strains showed four different sequence types, with two isolates typed as ST722, one isolate typed as ST784, and the remaining two with an unknown ST (**Table 3. 6**, **Figure 3. 3**).

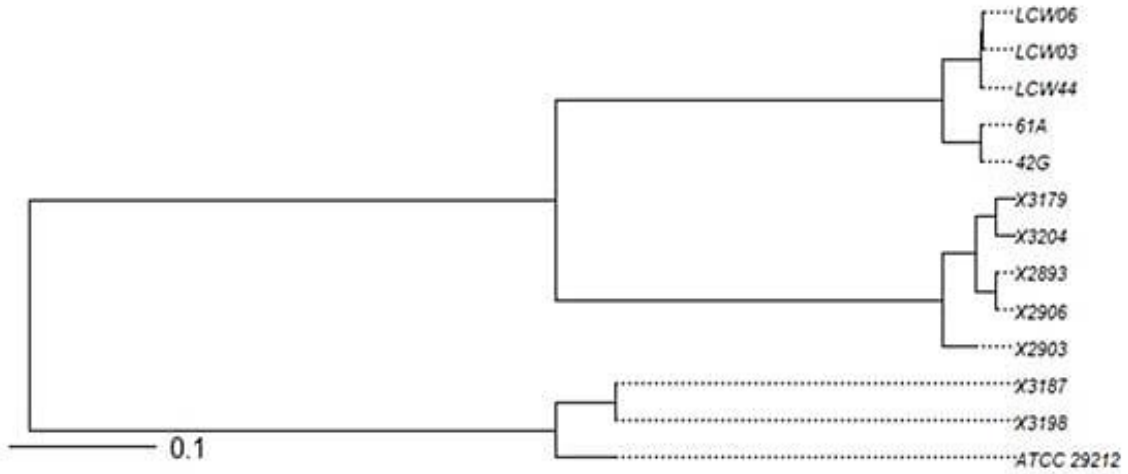


Figure 3. 3. Phylogenetic tree based on the average nucleotide identity (ANI) of the 12 BP+ enterococci. The reference strain, ATCC 29212, was also included.

3.4.5 Digestive survival of selected *E. faecium* X2893 and X2906

Isolates *E. faecium* X2893 and X2906 were selected for the digestive survival assay because of their characteristics; non-carriers of acquired resistance mechanisms, non-carriers of virulence factors and plasmids, and bacteriocin producers. **Table 3. 8** shows the bacterial growth (CFU/mL) of each of the two selected isolates.

Table 3. 8. Bacterial growth in CPU/mL of *E. faecium* X2893 and X2906 after each step of the digestive *in vitro* digestion.

	X2893	X2906
Initial	1.4 x 10^8 CFU/mL	2.2 x 10^8 CFU/mL
After salivary digestion	2.7 x 10^8 CFU/mL	3.7 x 10^8 CFU/mL
After gastric digestion	8.7 x 10^7 CFU/mL	5.1 x 10^6 CFU/mL
After intestinal digestion	7.5 x 10^7 CFU/mL	4.2 x 10^6 CFU/mL

3.5 Discussion

A total of 32 of the 256 enterococci tested (12.84%) showed antimicrobial activity against the *C. perfringens* X2967 strain, as determined using the "spot on the lawn" method; however, among these, only 18 supernatants of the BP+ strains were active against the collection of 20 *C. perfringens* isolates used as indicators. The inhibitory activities of these supernatants were attributed to the *Enterococcus*-derived enterocins (Gilmore et al., 2014). The absence of inhibitory activity in the supernatants obtained from the strains showing inhibition using the spot-on-the-lawn method may be explained by the fact that bacteriocins sometimes remain attached to the cell wall and are not released in the supernatant. Furthermore, the production of bacteriocins is commonly mediated by quorum sensing (Perez et al., 2022); hence, we detected 14 strains as BP+ via the spot-on-the-lawn method (in which the producer and the indicator strains are confronted) but without activity in their supernatants (the extract produced without previous exposure to the indicator bacteria) (Simons et al., 2020).

According to their antimicrobial activity, 12 BP+ enterococci were selected for further characterization.

The structural genes for enterocins P and Enterocin L50A/B were detected in all five *E. durans* isolates. Enterocin P (entP) was first detected in an *E. faecium* strain isolated from a dry-fermented sausage (Cintas et al., 1997), showing activity against gram-positive pathogenic bacteria such *as C. perfringens*, *L. monocytogenes*, and *S. aureus*. Enterocin P is chromosomally encoded (Cintas et al., 2000; Criado et al., 2008); however, other studies have detected entP genes in the plasmid location (Abriouel et al., 2006). Enterocin P

and L50A/B have been detected in different enterococcal species (Ness et al., 2014). This study is the first study to detect Enterocin P in *E. durans*.

Enterocin L50A/B was first detected in an *E. faecium* L50 strain isolated from Spanish fermented sausage (Cintas et al., 1998). Enterocin L50A/B consists of two peptides, L50A and L50B, which synergistically promote their antimicrobial activity. The strain *E. faecium* L50 has also been shown to produce enterocins Q and P at different temperatures (Cintas et al., 2000; Criado et al., 2006). Enterocin L50 A/B exhibits a broad spectrum of antimicrobial activities, including inhibition of *Enterococcus spp.*, *Lactobacillus spp.*, *Lactococcus lactis*, *Pediococcus pentosaceus*, *L. monocytogenes*, *S. aureus*, *B. cereus*, *C. botulinum*, *Streptococcus pneumoniae*, *S. mitis*, *S. oralis*, *S. parasanguis*, *S. agalactiae*, and *C. perfringens*. Other enterocins, such as enterocins 7A/7B and MR10A/10B, share a strong homology with enterocin L50 A/B (Ness et al., 2014).

Enterocin Bac32 was identified in three of our five *E. durans* strains. This peptide was firstly detected in a vancomycin-resistant clinical *E. faecium* VRE200 strain, exhibiting activity against *Enterococcus spp* (Inoue et al., 2006). Although this bacteriocin has not been extensively studied, it seems to be identical to enterocin IT (Izquierdo et al., 2009).

The strain *E. durans* 61A has been previously described, and durancin 61A and enterocins L50A and L50B were identified using mass spectrometry (Hanchi et al., 2016; Hanchi et al., 2017). However, the genetic determinants for these bacteriocins were not detected in strain 61A using whole genome sequencing (WGS) in our study; instead, enterocin P was detected. Duracin 61A is not in the anti-SMASH and BAGEL4 databases (which uses data from the NCBI and NCBI plus UniProt, respectively), whose genetic determinants have yet to be described. In contrast, enterocin P might not have been detected in other studies, as it is a temperature-regulated bacteriocin that is synthesized optimally at 37–47 °C (Criado et al., 2006).

Genes encoding enterocin A and enterocin B were detected in all of our five *E. faecium* strains, two of which also carried the genes encoding Enterocin NKR-5-3-A/D/Z.

Enterocin A was first identified in 1996 (Aymerich et al., 1996) and is produced by several strains of *E. faecium*—CTC492, T136, and P21—isolated from Spanish sausage; BFE900 from black olives; DPC 1146, WHE 81, and

EFM01 from dairy products; and the N5 strain of "nuka", a Japanese rice paste. Enterocin A shows activity against *Enterococcus spp.*, *Lactobacillus spp.*, *Pediococcus spp.*, and *L. monocytogenes* (Franz et al., 2007). However, its activity has not been tested against clostridial species. Enterocin A is usually co-produced with enterocin B, which is produced by *E. faecium* T136 isolated from Spanish fermented sausages (Casaus et al., 1997). Enterocin B shows antimicrobial activity against gram-positive bacteria, such as *L. monocytogenes*, *Propionibacterium spp.*, *C. sporogens*, and *C. tyrobutyricum* (Casaus et al., 1997). When enterocin A and enterocin B are co-produced, they form a heterodimer, and studies have demonstrated its potential anti-bacterial and anti-biofilm activities against *S. aureus*, *Acinetobacter baumannii, L. monocytogenes*, and *E. coli* (Ankaiah et al., 2018).

The genetic determinants for enterocin NKR-5-3-A/B/C/D/Z were detected in two of our *E. faecium* strains. These enterocins have been purified and studied previously (Ishibashi et al., 2012). NKR-5-3-A (identical to brochocin A) and NKR-5-3-Z are class IIb bacteriocins and exhibit synergistic antimicrobial activity. NKR-3-5-B is a novel circular bacteriocin belonging to class IIc bacteriocins with a broad spectrum of antimicrobial activities against *Bacillus spp.*, *Enterococcus spp.*, and gram-negative bacteria (*E. coli* and *Salmonella*). NKR-5-3-C is a class IIa bacteriocin with strong antimicrobial activity against *L. monocytogenes*. NKR-5-3-D, a class IId bacteriocin, has a weak antimicrobial activity but can be produced even under unfavorable conditions (Himeno et al., 2015; Ishibashi et al., 2021). NKR-5-3-A, D, and Z variant genes were detected in the two *E. faecium* strains. The genetic determinants of enterocins NKR-5-3-A/C/D/Z are closely located in a gene cluster (13 kb long) and include specific bacteriocin biosynthetic genes, such as an ABC transporter gene (*enkT*), two immunity-related genes (*enkIaz* and *enkIc*), a response regulator (*enkR*), and a histidine protein kinase (*enkK*). This gene cluster is essential for the biosynthesis and regulation of NKR-5-3 enterocins (Ishibashi et al., 2014).

Genes encoding enterocin SE-K4 and staphylococcin C55a/b were identified in the two *E. faecalis* strains in this study. Enterocin SE-K4 was first identified in *E. faecalis* K-4 isolated from grass silage (Eguchi et al., 2001); it grows at 43–45 °C and exhibits antimicrobial activities against *E. faecium*, *E. faecalis*, *B. subtilis*, *C. beijerinckii*, and *L. monocytogenes*. This enterocin has a

high degree of homology to bacteriocin 31 and T8/43 (Franz et al., 2007). Staphylococcin C55a/b was originally found to be produced by *S. aureus* C55 (Navaratna et al., 1998), consisting of three distinct peptide components termed staphylococcins C55a, C55b, and C55g. Staphylococcins C55a and C55b (lantibiotic components) acted synergistically against *S. aureus* and *M. luteus* (Navaratna et al., 1998). It is a plasmid-encoded bacteriocin (Kawada-Matsuo et al., 2016); thus, the plasmid transfer between the producer, *Staphylococcus*, and the *E. faecalis* strains could account for the presence of the genetic determinants of this bacteriocin.

Five of the twelve BP+ enterococci, all from *E. durans* isolates, were susceptible to the nine antibiotics tested. The remaining strains showed resistance to at least one of the antibiotics. Generally, the enterococci of poultry origin have more resistance genes than those of other origins (camel and camel milk). The only gene discovered in *the E. durans* strains of milk origin was *aac(6')-Iih*, which is intrinsically present in *E durans* (Portillo et al., 2000; Del Campo et al., 2005). Antibiotics are commonly used in poultry farming, leading to the development of acquired resistance mechanisms in poultry-derived strains.

The genus *Enterococcus* is characterized by its intrinsic resistance to several antibiotics and ability to acquire new resistance mechanisms (Miller et al., 2014). Enterococci are naturally resistant to semisynthetic penicillins (a reduced susceptibility), aminoglycosides (in low levels), vancomycin (at a low level and only in the species *E. gallinarum* and *E. casseliflavus/E. flavescens*, which are carriers of *vanC* genes), to lincosamides, polymyxins, and streptogramins (the species *E. faecalis*) (Fontana et al., 1996). In addition, *E. faecium* carries some intrinsic genes, such as *msrC* and *aac(6')-Ii,* whereas *E. durans* harbors the gene *aac(6')-Iih* (Portillo et al., 2000; Del Campo et al., 2005). Antibiotic resistance can occur either through the acquisition of genetic elements containing the resistance genes or via DNA mutations (mostly in genes encoding antibiotic targets), which are favored when there is a selective antibiotic pressure (Miller et al., 2014).

Among the acquired resistance genes detected in the *E. faecium* and *E. faecalis* strains, the genes associated with erythromycin [*erm(B)*], chloramphenicol [*fexB* and *cat*], tetracycline [*tet(M)* and *tet(L)*], streptomycin [*str*], gentamicin and tobramycin [*aac(6')-aph(2")*], and linezolid resistance

(*poxtA)* have been reported. Vancomycin resistance genes have not been reported (Hollenbeck & Rice., 2012).

E. faecium strains X2893 and X2906 carry only chromosomal and intrinsic resistance genes (*msr(C)* and *aac(6')-Ii)*, which are non-transferable; therefore, these strains are excellent candidates for use as potential protective cultures.

Specific mutations in the *pbp5* and *gyrA*/*parC* genes are associated with resistance to beta-lactams and fluoroquinolones, respectively (Poeta et al., 2007; Cercenado., 2011; López et al., 2011). Different mutations in the *pbp5*, *gyrA,* and *par*C genes have been detected in our strains, although, in most cases, with an unknown resistance phenotype associated.

Different virulence factors are involved in the attachment to host cells and extracellular matrix proteins (AS, Esp, Hyl, and EfaA), macrophage resistance (AS), and cell and tissue damage (Cyl and GelE) (Gilmore et al., 2002; Fisher & Phillips., 2009). Thus, although enterococci are commensal bacteria found in the intestine, they can still cause infections. Therefore, the Food and Drug Administration (FDA) has not yet assigned them to the GRAS category. Genes encoding these virulence factors are located in conjugative plasmids (*agg*, *cyl*, or *hyl*), in the chromosome (*gelE* or *fsr*), or in regions of the chromosome called pathogenic islands (*esp* and *cyl*) (Nakayama et al., 2002; Shankar et al., 2002).

In the 12 enterococcal strains, virulence genes were detected in *E. faecium* and *E. faecalis* but not in *E. durans*. *E. faecalis* has already been described as more virulent than other species (Eaton & Gasson., 2001). Fifteen virulence genes were detected in both *E. faecalis* strains. However, the presence of these genes is not always related to the virulence potential, as they are sometimes silenced and not associated with the phenotype (Shankar et al., 2002). Both strains carried the *gelE* gene, which is associated with gelatinase activity, but only strain X3198 was positive for gelatinase activity.

All the *E. faecium* strains carried the functional collagen adhesin gene, *acm*, which plays an essential role in colonization by binding to collagen type I, with less affinity to collagen type IV (Nallapareddy et al., 2008). As these *E. faecium* strains did not carry other virulence factors, the presence of *acm* might be positive, as it could facilitate the colonization of this beneficial strain.

Nevertheless, as mentioned before, the presence of a virulence gene does not always indicate that it is being expressed (Shankar et al., 2002). Therefore, further studies must uncover whether *acm* is, in fact, expressed as a virulence factor.

Ten of the BP+ enterococci harbored at least one plasmid. Interestingly, strains X2893 and X2906 did not present any mobile genetic elements, which, along with the other characteristics, makes them good candidates for potential protective cultures (Krawczyk et al., 2021).

Among the 12 enterococci that showed inhibitory activity against *C. perfringens*, the strains *E. faecium* X2893 and X2906 seem to be the most promising candidates for use as protective cultures in poultry farming. Both strains belong to the sequence type ST722 and harbor enterocin A and Enterocin B genetic determinants. These strains also do not have acquired resistance genes, do not carry plasmids, and only carry the *acm* gene, which is implicated in host colonization and might be a desirable feature for protective strains. Both are gelatinase-negative and gamma-hemolytic.

The strains derived from other origins (milk and camel milk) and belonging to the species *E. durans* might be also good candidates as protective cultures, as they do not harbor any virulence factors or resistance genes, and they produce bacteriocins. However, these strains carry more than one plasmid and have not been isolated from poultry.

E. faecium X2893 and X2906 showed potential to be considered in further studies as protective cultures in poultry farming, a promising alternative to antibiotic use in this sector. Digestive survival of this promising BP+ strains revealed a high survival rate, reducing bacterial counts in only 10 times in the case of *E. faecium* X2893 and 100 times in the case of *E. faecium* X2906. Further studies must be completed to evaluate their safety and potential use on *in vivo* models.

3.6 Conclusions

I. Strains with antimicrobial activity against *C. perfringens* are commonly found among enterococcal isolates from poultry origin.

II. In addition to the antimicrobial activity against *C. perfringens*, these enterococci show activity against other enterococcal isolates, as *L. monocytogenes* and, to a lesser extent, *M. luteus* and methicillin-susceptible and -resistant *S. aureus*.

III. The antimicrobial activity of the isolates is mediated by production of enterocins, which are thermostable.

IV. The genes of Staphylococcin C55a/b have been detected among our enterococci, what may suggest potential transfer from staphylococci.

V. The genes encoding Enterocin A, B, P and L50 are frequently detected in enterococci of poultry origin.

VI. Antibiotic resistance is very common among enterococci of poultry origin, what could indicate high antibiotic selective pressure.

VII. Virulence factors are frequently detected in enterococci of poultry origin, especially in the *E. faecalis* species.

VIII. The bacteriocin–producing *E. durans* isolates (L50A/B) lacking antibiotic resistant and virulence genes could be good candidates for protective cultures, although relevance of their non-poultry origin needs to be further evaluated.

IX. The bacteriocin producing *E. faecium* X2893 and X2906 isolates (Enterocoin A/B) showed potential to be considered in further studies as protective cultures in the poultry sector.

X. *E. faecium* X2893 and X2906 showed high survival rates in the *in vitro* poultry digestion model, reinforzing their promising potential to be considered as protective cultures in future research.

4. Chapter IV: Antimicrobial activity of Enterocin A, B, P, SEK4 and L50 against *Clostridium perfringens* is correlated with their respectice mechanism of action

Abstract

Multidrug resistant *Clostridium perfringens* infections in poultry are a major threat to the industry. Effective alternatives to antibiotics are urgently needed to prevent these infections and limit the spread of multi-drug resistant bacteria. The aim of the study was to produce by chemical synthesis a range of enterocins with different mechanism of action and to compare their spectrum of inhibition activity, either alone or in combination, against a large panel of *C. perfringens*. Enterocins A, B, P, SEK4 (class IIa bacteriocins) and L50 (class IIb bacteriocin) were produced by microwave-assisted solid-phase peptide synthesis. Their antimicrobial activity against *C. perfringens* was determined by agar well diffusion and microtitration methods against twenty *Clostridium* isolates. The FIC_{INDEX} of different combinations of the selected enterocins was calculated in order to identify combinations with synergistic effect. The results showed that L50A and L50B were the most active against *C. perfringens*. These peptides also showed the broadest spectrum of activity when tested against other non-clostridial indicator strains, being also active against the Gram-negative *Campylobacter coli* ATCC 33559 and *Pseudomonas aeruginosa* ATCC 27855. The selected enterocins were combined on the basis of their different mechanisms of action and all combinations tested showed synergy or partial synergy. In conclusion, because of their high activity against *C. perfringens* and other pathogens, the use of enterocins alone or as a consortium can be a good alternative to the use of antibiotics in the poultry sector.

Résumé

Les infections à *Clostridium perfringens* multirésistantes chez les volailles constituent une menace majeure pour l'industrie. Des alternatives efficaces aux antibiotiques sont nécessaires de toute urgence pour prévenir ces infections et limiter la propagation des bactéries multirésistantes. L'objectif de cette étude était de produire par synthèse chimique différentes entérocines ayant des mécanismes d'action différents et de comparer leur spectre d'activité d'inhibition obtenu, seul ou en combinaison, contre un large panel *de C. perfringens*. Les entérocines A, B, P, SEK4 (bactériocines de classe IIa) et L50 (bactériocine de classe IIb) ont été produites par synthèse peptidique en phase solide assistée par micro-ondes. Leur activité antimicrobienne contre *C. perfringens* a été réalisée par des méthodes de diffusion sur puits d'agar et de microtitration contre vingt isolats de *Clostridium*. Le FIC_{INDEX} de différentes combinaisons des entérocines sélectionnées a été calculé afin d'identifier les combinaisons ayant un effet synergique. Les résultats ont montré que les peptides L50A et L50B étaient les plus actifs contre *C. perfringens*. Ces peptides ont également montré le spectre d'activité le plus large lorsqu'ils ont été testés contre d'autres souches indicatrices non clostridiennes, étant également actifs contre les Gram-négatifs *Campylobacter coli* ATCC 33559 et *Pseudomonas aeruginosa* ATCC 27855. Toutes les combinaisons d'entérocines testées ont montré une synergie ou une synergie partielle, tout en combinant différents mécanismes d'action. En conclusion, en raison de leur forte activité contre *C. perfringens* et d'autres pathogènes, l'utilisation d'entérocines seules ou en consortium peut constituer une bonne alternative à l'utilisation d'antibiotiques dans le secteur de la volaille.

Resumen

Las infecciones por *Clostridium perfringens* multirresistentes en aves de corral constituyen una grave amenaza para el sector avívola. Se necesitan urgentemente alternativas eficaces a los antibióticos para prevenir estas infecciones y limitar la propagación de bacterias multirresistentes. El objetivo de este estudio fue producir mediante síntesis química diferentes enterocinas con distintos mecanismos de acción y comparar su espectro de actividad inhibitoria frente a un amplio panel de cepas de *C. perfringens*, ya sea solas o en combinación. Las enterocinas A, B, P, SEK4 (bacteriocinas de clase IIa) y L50 (bacteriocina de clase IIb) se produjeron mediante síntesis peptídica en fase sólida asistida por microondas. Su actividad antimicrobiana frente a *C. perfringens* se evaluó mediante métodos de difusión en pocillos de agar y microtitulación frente a veinte aislados de *Clostridium*. Con el fin de identificar combinaciones con efecto sinérgico, se calculó el FIC_{INDEX} de distintas combinaciones de las enterocinas seleccionadas. Los resultados mostraron que los péptidos L50A y L50B eran los más activos frente a *C. perfringens*. Estos péptidos también mostraron el espectro de actividad más amplio cuando se estudiaron frente a otras cepas indicadoras no clostridiales, siendo también activos frente a las bacterias Gram-negativas *Campylobacter coli* ATCC 33559 y *Pseudomonas aeruginosa* ATCC 27855. Todas las combinaciones de enterocinas analizadas mostraron sinergia o sinergia parcial, al combinar diferentes mecanismos de acción. En conclusión, por su elevada actividad contra *C. perfringens* y otros patógenos, el uso de enterocinas solas o en consorcio puede ser una buena alternativa al uso de antibióticos en el sector avícola.

4.1 Introduction

Antimicrobial resistance is a serious public health problem that compromises the treatment of infections in both humans and animals. This problem is linked to the unnecessary prescription and/or misuse of antibiotics. In addition to the clinical use of antibiotics in humans, they are also used in veterinary medicine and animal husbandry to treat and prevent infections, and even in agriculture to preserve crops, even if at low levels. Antibiotics have also been used extensively as growth promoters in food-producing animals, but although this practice has been banned in Europe since 2006 and in other countries, it is still allowed in some others ((McEwen & Collignon., 2018; Gochez et al., 2021). In countries where antibiotic growth promoters are no longer used, infections such as poultry-associated necrotic enteritis (NE) induced by *Clostridium perfringens* have increased. Several of these *Clostridium* strains are multidrug-resistant (García-Vela et al., 2023). This suggests that this may also be the case for other relevant pathogens (Villagrán de la Mora et al., 2020). Necrotic enteritis caused by *C. perfringens* is one of the most common poultry diseases and causes huge economic losses in the industry (Alizadeh et al., 2021). Effective alternatives to antibiotics are needed to prevent the spread of multi-drug resistant bacteria and the development of emergent infections in the poultry industry.

Among the most promising alternatives, bacteriocins show very attractive properties (Rahman et al., 2022). Bacteriocins are ribosomally synthesized peptides that have antimicrobial activity against bacteria closely related to the producing strain. Enterococci are ubiquitous microorganisms that can be found everywhere; in water, plants, soil, food and in the gastrointestinal tract of humans and animals (Gilmore et al., 2014). They have traditionally been used as starters in food fermentation or as protective cultures in food biopreservation or as probiotics, as they produce bacteriocins called enterocins (Silva et al., 2018; Hanchi et al., 2018). In recent years, the direct use of enterococci as a starter or as a probiotic has generated an important debate due to presence of virulence and antibiotic resistance genes and the high risk associated with genetic transfer mechanisms (Ben Braïek & Smaoui., 2019). Thus, the use of their antimicrobial products instead of the isolates could be a promising alternative to the use of antibiotics.

Enterocins are short cationic peptides (20-60 amino acids) with hydrophobic sections, which are highly stable to heat and over a wide range of pH (Franz et al., 2007). In general, they have activity against phylogenetic species close to the producing bacteria, but some of them exhibit a broad-spectrum of activity, including Gram-positive microorganisms such as *Listeria monocytogenes*, *Bacillus cereus*, *Staphylococcus spp.*, *Clostridium spp.*; and Gram-negative microorganisms such as *Pseudomonas aeruginosa*, *Escherichia coli* or *Vibrio cholera*; and even against fungi and viruses (Hammami et al., 2019; Simons et al., 2020). Enterocins have several advantages when used as an alternative to antibiotics. First, their narrow spectrum of action causes less destabilization of the microbiota. Moreover, their high potency makes them very effective and their sensitivity to proteases ensures biosafety (Soltani et al., 2021). Plus, they can be modified by bioengineering, which makes them easy to handle.

In general, bacteriocins are most commonly produced by bacterial fermentation using the producing strains. However, the low production yields combined to difficulties associated with their purification severely limit their potential for large scale use. Chemical synthesis has been proposed as an alternative to produce several bacteriocins such as pediocin (Bédard et al., 2018) and bactofencin (Bédard et al., 2019). The main advantage of this approach is the increase in the speed at which large quantities of pure bacteriocins can be produced. In addition, the significant reduction in the cost of peptide synthesis reagents and building blocks has made the chemical synthesis of bacteriocins more attractive and competitive (Bérard & Biron., 2018).

For this study, enterocins with different mechanisms of action namely enterocin A (EntA), enterocin B (EntB), enterocin P (EntP), enterocin SEK4 (EntSEK4) and enterocin L50 (L50) were selected and produced by chemical synthesis. These class II bacteriocins, are unmodified low molecular weight (<10 kDa) and thermostable bacteriocins, which do not involve the use of non-proteogenic amino acids. Therefore, no special enzymes other than signal peptides or transporters are required to complete the maturation and activation of such bacteriocins. Enterocin A, enterocin P and enterocin SEK4 are class IIa bacteriocins (pediocin-like bacteriocins) containing the consensus YGNGV sequence and a disulfide bond formed by two cysteines in the N-terminal section, both being signatures of this class. Enterocin L50 is composed of the

two peptides L50A and L50B and belongs to class IIb bacteriocins, but do not have a consensus sequence and are synthesized without a leader peptide nor the ABC transporter secretion system. Enterocin B is a non subgrouped class II linear bacteriocin (Ben Braïek & Smaoui., 2019; Wu et al., 2022).

In general, pediocin-like class IIa bacteriocins act by forming pores in the membrane of Gram-positive bacteria via their interaction with the mannose phosphotransferase system (Man-PTS), as is the case for enterocin A, P and SEK4 (Ríos-Colombo et al., 2018). The Man-PTS system consists of 4 subunits: IIA, IIB, IIC and IID. The phosphotransfer subunits IIA and IIB are not required for the interaction, but it is the subunits IIC and IID that are involved in the mechanism of action of these bacteriocins. Nevertheless, it is controversial, whether IIC or IID is involved in bateriocin pore formation, or whether it simply assists in membrane penetration and pore assembly. Bacteriocin sensitivity is correlated with the expression level of the receptor/target protein but also mutations of the target (subunit IID) can attenuate bacteriocin sensitivity (Jeckelmann et al., 2020). On the other hand, the undecaprenyl pyrophosphate phosphatase (UppP), a membrane-spanning protein involved in cell wall synthesis, has been identified as the receptor for two-peptide bacteriocins, as is the case for enterocin 1071 (Kjos et al., 2014; Cotter., 2014; Ekblad et al., 2016). However, there is no evidence that UppP is the receptor for enterocin L50. The mechanism of action of enterocin B remains unknown.

The enterocins used in this study have been reported to have antimicrobial activity against several pathogenic bacteria. Enterocin A was first identified in 1996 and is produced by several *Enterococcus faecium* strains (Aymerich et al., 1996) and was produced by several *Enterococcus faecium* strains. Enterocin A shows activity against *Enterococcus spp.*, *Lactobacillus spp.*, *Pediococcus spp.* and *Listeria monocytogenes* (Aymerich et al., 1996). Enterocin A is usually co-produced with enterocin B, which is initially produced by *E. faecium* T136 isolated from Spanish fermented sausages (Casaus et al., 1997). Enterocin B shows antimicrobial activity against Gram-positive bacteria such as *L. monocytogenes*, *Propionibacterium spp.*, *C. sporogens* and *C. tyrobutyricum*. When enterocin A and enterocin B are co-produced, they form a heterodimer and studies have demonstrated its potential antibacterial and anti-biofilm activities against *S. aureus*, *Acinetobacter baumannii*, *L. monocytogenes* and *E. coli* (Ankaiah et al., 2018). Enterocin P is produced by *E. faecium* P13

isolated from Spanish fermented sausages. The spectrum of activity of Enterocin P includes *Lactobacillus spp.*, *Pediococcus spp.*, *Propinobacterium spp.*, *Enterococcus spp.* and the pathogens *L. monocytogenes*, *S. aureus*, *C. perfringens* and *C. botulinum* (Cintas et al., 1997). Enterocin SEK4 was first identified in *E. faecalis* K-4 isolated from grass silage growing at 43-45 °C and has antimicrobial activity against *Enterococcus spp.*, *B. subtilis*, *C. beijerinckii* and *L. monocytogenes* (Eguchi et al., 2001). Enterocin L50 was first detected in an *E. faecium* L50 strain isolated from Spanish fermented sausage (Cintas et al., 1998). It consists of two peptides, L50A and L50B, which synergistically promote their antimicrobial activity. Enterocin L50 A/B has a broad spectrum of antimicrobial activity, including *Enterococcus spp.*, *Lactobacillus spp.*, *Lactococcus lactis*, *Pediococcus pentosaceus*, *L. monocytogenes*, *S. aureus*, *B. cereus*, *C. botulinum*, *Streptococcus spp.* and *C. perfringens* (Ness et al., 2014).

Although there is a great amount of information available on the inhibitory activity of several enterocins, this information has been obtained using disparate isolates of various origins and using different *in vitro* methods. To our knowledge, no study has compared the inhibition spectrum of enterocins with different mechanisms of action against a large panel of bacteria using the same method at the same time. Furthermore, an in-depth analysis of the correlation between the mechanism of action and the extent of the inhibition spectrum has never been performed.

4.2 Objective

The aim of this study is to produce by chemical synthesis different enterocins exhibiting different mechanism of action and to compare their spectrum of inhibitory activity, either alone or in combination against a large panel of *C. perfringens* and other relevant bacteria.

4.3 Materials and methods

4.3.1 Srain collection, manteinance and propagation

A collection of 20 *C. perfringens* isolates previously obtained from poultry affected by NE and belonging to Laval University (Canada) collection was included in this study, which were previously characterized (García-Vela et al.,

2023). Other isolates used in the study belong to strain type collections: *Enterococcus faecalis* ATCC 23212, *Micrococcus luteus* ATCC 10240, *Staphylococcus aureus* ATCC 6538, *Listeria monocytogenes* ATCC 1911, *Streptococcus pyogenes* ATCC 19615, *Salmonella enterica* ATCC 69162, *Escherichia coli* ATCC 24922, *Pseudomonas aeruginosa* ATCC 27855 and *Campylobacter coli* ATCC 33559. Additionally, *Enterococcus cecorum* CECO0009 from Laval University collection, *Streptococcus suis* C2058 and methicillin-resistant *Staphylococcus aureus* C411 from La Rioja University collection were also used.

All isolates were preserved in 40% glycerol at -80°C. Reinforced medium for clostridia (HiMedia, Pensylvania, United States) was used for the propagation of *C. perfringens* isolates (incubation at 37°C, 24 h, under strict anaerobic conditions). Brain Heart Infusion (Becton, Dickinson and Company, Helidelberg, Germany) was used for the propagation of non-clostridial aerobic isolates (incubation at 37°C, 24h, under aerobic conditions) and BD BBL supplemented with 5% blood (Becton, Dickinson and Company, Helidelberg, Germany) for *C. coli* (incubation at 42°C, under microaerophilic conditions).

4.3.2 Genome analysis of *C. perfringens* isolates

Whole genome sequencing of the *C. perfringens* collection was performed previously (García-Vela et al., 2023) using the Illumina technique at the Hospital Center of the University of Laval (CHUL), Quebec, Canada. Some of the sequences were further analyzed in this study. Briefly, raw sequencing data were processed using fastp 0.20.0 for trimming and quality control of trimmed reads (Chen et al., 2018). De novo assembly, without alignment to a reference genome, was performed with SPAdes 5.0.2 (Bankevich et al., 2012), using QUAST 1.14.6 for checking the assembled quality (Gurevich et al., 2013). Prokka 1.14.6 (Seemann., 2014) was used for gene prediction and annotation, using Prodigal for coding sequence prediction (Hyatt et al., 2010).

Pairwise alignments and visualization of the products of the genes encoding the Man-PTS subunit IID *(manZ_1*, *manZ_2* and *manZ_3*) and UppP (*uppP*) receptors from selected isolates were performed with the program Jalview 2.11.2.5 (Waterhouse et al., 2009) in order to detect mutations and explain differences in enterocin susceptibility between strains. The isolates C. *perfringens* MLG 0418, 2919 and 3707 (which previously showed unique characteristics (García-Vela et al., 2023)) were chosen for analysis of the Man-

PTS receptor. Sequences from *C. perfringens* ATCC13124 were added as a reference.

4.3.3 Production of enterocins

On the basis of their different mechanisms of action, five enterocins were selected for this study and their amino acid sequences are shown in **Table 4.1**.

Table 4.1. Enterocins synthetized in the study.

Enterocin	Lenght	Amino acid sequence
Enterocin L50A	44 AA	MGAIAKLVAKFGWPIVKKYYKQIMQFIGEGWAINKIIIEWIKKHI
Enterocin L50B	43 AA	MGAIAKLVTKEGWPLIKKFYKQIMQFIGQGWTIFQIEKWLKRH
Enterocin A	47 AA	TTHSGKYYGNGVYCTKNKCTVDWAKATTCIAGMSIGGFLGGAIPG KC
Enterocin B	53 AA	ENDHRMPNELNRPNNLSKGGAKCGAAIAGGLFGIPKGPLAWAAGL ANVYSKCN
Enterocin P	44 AA	ATRSYGNGVYCNNSKCWVNWGEAKENIAGIVISGWASGLAGMGH
Enterocin SEK4	43 AA	ATYYGNGVYCNKOKCWVDWSRARSEIIDRGVKAYVNGFTKVLG

All reagents and solvents were purchased from commercial suppliers and used without additional purification. Fmoc-protected amino acids, 2-chlorotrityl chloride resin and DIC were purchased from Matrix Innovations (Québec, QC) and the Oxyma Pure acquired from CEM (Matthews, NC, USA). Other reagents and solvents were purchased from Sigma-Aldrich (St-Louis, MO, USA) or Fisher Scientific (Hampton, NH, USA).

Chemical synthesis of the six enterocins (enterocin A, B, P, SEK4, L50A and L50B) was performed on a microwave-assisted peptide synthesizer (CEM Liberty Blue 2.0, Matthews, NC, USA). The peptides were prepared by standard solid-phase peptide synthesis (SPPS) on a 0.05 mmol scale using Fmoc/tBu strategy on a preloaded 2-CTC polystyrene resin (typically 0.3 mmol/g). Briefly, the Fmoc protecting group was removed from the resin by treating with a solution of 10% piperidine in DMF (v/v) for 5 min at 60°C and amino acid couplings were performed with Fmoc-Xaa-OH (5 equiv), Oxyma pure (5 equiv), DIEA (0.1 equiv), and DIC (10 equiv) in DMF for 20 min at 50°C. After the

synthesis, the resin was washed successively with DMF (5 × 5 ml) and CH_2Cl_2 (5 × 5 ml).

The peptides were cleaved from the resin by treating with 10 ml of a solution of 20% HFIP in CH_2Cl_2 (2 × 20 min) and the amino acid side chains were deprotected by treating with 10 ml of a deprotection cocktail containing TFA/TIPS/H_2O/Phenol/DODT (90:2.5:2.5:2.5:2.5) for 3 h. The resulting peptide was precipitated in cold diethyl ether and the solid was washed twice with diethyl ether before drying under vacuum overnight.

The peptides were purified by semi-preparative RP-HPLC with a Shimadzu Prominence system on a Phenomenex Kinetex EVO C18 column (250×21.2 mm, 300Å, 5 µm) using H_2O (0.1% TFA) (A) and CH_3CN (0.1% TFA) (B), with a linear gradient of 10-50% of 20 min at a rate of 12 mL/min and detection at 220 and 254 nm. The collected fractions were lyophilized to afford the desired peptide as a white fluffy pouder. Peptide purity and composition were confirmed by HPLC and mass spectrometry on a Shimadzu Prominence LCMS-2020 system equipped with an electrospray ionization (ESI) probe using a Phenomenex Kinetex EVO C18 column (100 mm× 4.6 mm, 100 Å, 2.6 µm) with a 10.5 min gradient from water (0.1% HCOOH) and CH_3CN (0.1%HCCOH) (10 to 100% CH_3CN) and detection at 220 and 254 nm.

4.3.4 Antimicrobial activity assay

Antimicrobial activity of the peptides was first studied by agar-well diffusion against the strain collection. After, minimal inhibitory concentration (MIC) of the strains was calculated for those enterocins showing antimicrobial activity against indicator strains, as in (García-Vela et al., 2023).

For agar-well diffusion, 25 µL of a bacterial suspension 0.5 McFarland of each indicator strain was diluted in 25 mL of Mueller-Hinton soft agar (Oxoid) and placed in a petri dish. Once dried, wells were performed by using a 10 mL pipette. 80 µL of each enterocin at a concentration of 200 µg/mL was placed in each well. Nisin, at a concentration of 100 µg/mL was added as positive control. Incubation of 24 h at 37ºC. For the *C. perfringens* and *C. coli* isolates, instead, brucella soft agar (HiMedia) media was used and the incubation was under strict anaerobic or microaerophilic conditions, respectively.

A microtitration assay was performed to determine MIC of the enterocins against the collection of indicator bacteria. Mueller Hinton broth (Oxoid) was used as the growth medium for the aerobic isolates. For clostridial species and

C. coli, brucella media for anaerobes (HiMedia) was used. In a 96 well plaque, 175 µL of the culture medium was added to the wells of column 1 (= negative control) and 125 µL to the wells of columns 2-12. Then 125 µL of each enterocin (stock concentration of 200 µg/mL) and nisin as positive control (stock concentration of 100 µg/mL) were added to the wells in column 3 and mixed by pipetting up and down 10 times. 125 µL from column 3 was removed and placed in column 4. The process was repeated until the column 12 was reached. After mixing, 125 µL from column 12 was discarded. Later, 50 µL of the indicator strains suspension were then inoculated in all wells, except for the column 1, to achieve a final bacterial concentration ≈ 105 CFU/well. The microplate was incubated for 24 h at 37 °C in aerobic conditions for all isolates of the collection, except for clostridial species and *C. coli* which were incubated under strict anaerobic and microaerophilic conditions, respectively. After incubation the number of wells where inhibition was occurring was recorded to calculate the MICs.

4.3.5 Checkboard assay

The activity of different enterocins combined was evaluated by calculating the FIC_{INDEX} of eight different combinations, using the microdilution checkerboard method following the CLSI guidelines (CLSI., 2022), as in (Telhig et al., 2022). The FIC index was calculated as follows:

$$FIC_{INDEX} = FIC_A + FIC_B, \text{ where:}$$

$$FIC_A = CMI_{SYNERGY}/CMI_A \; ; \; FIC_B = CMI_{SYNERGY}/CMI_B$$

The effect of the different combinations was interpreted as: synergetic effect FIC ≤ 0.5, partial synergy 0.5 < FIC ≤ 0.75, additivity 0.75 < FIC < 1, neutral 1 ≤ FIC ≤ 4, and antagonism FIC > 4.

The enterocin combinations were selected according to their mechanisms of actions: L50A-L50B, EntA-L50A, EntA-L50B, EntA-EntB, EntB-L50A, EntB-L50B, EntP-L50A and EntP-L50B. The strain *C. perfringens* MLG3111 was selected as indicator strain for this assay due to its high susceptibility to the enterocins tested.

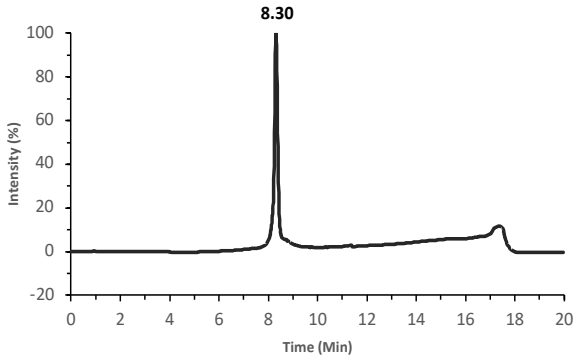
4.4 Results

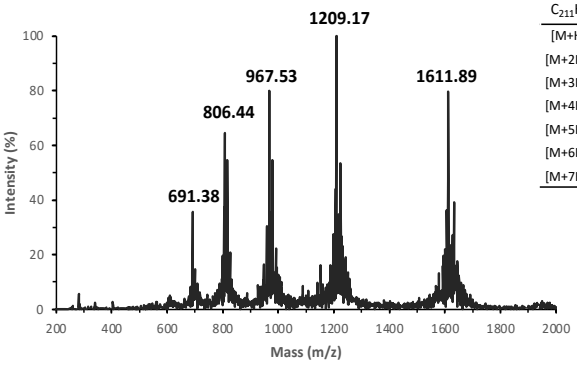
4.4.1 Production of enterocins

Enterocins A, B, P, SEK4, L50A and L50B were successfully produced by microwave-assisted solid-phase peptide synthesis. After release from the solid support and deprotection of the side chains, each enterocin has been purified by preparative HPLC and characterized by mass spectrometry (MS) (**Figure 4.1**). The synthesized enterocins were obtained in purities greater than 95% in 3-10% overall yields.

Enterocin A

NH2-TTHSGKYYGNGVYCTKNKCTVDWAKATTCIAGMSIGGFLGGAIPGKC-COOH

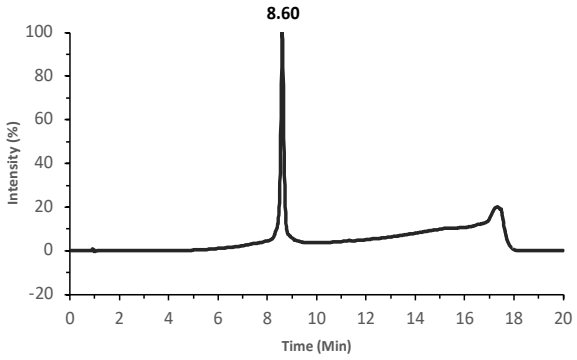


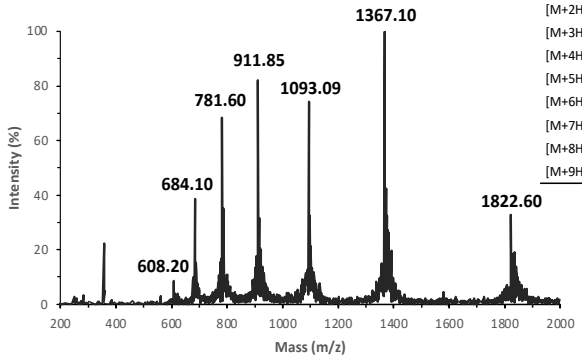


Calculated for $C_{211}H_{330}N_{57}O_{63}S_5$	
$[M+H]^+$	4833.64
$[M+2H]^{2+}$	2417.32
$[M+3H]^{3+}$	1611.65
$[M+4H]^{4+}$	1209.00
$[M+5H]^{5+}$	967.35
$[M+6H]^{6+}$	806.40
$[M+7H]^{7+}$	691.45

Enterocin B

NH2-ENDHRMPNELNRPNNLSKGGAKCGAAIAGGLFGIPKGPLAWAAGLANVYSKCN-COOH

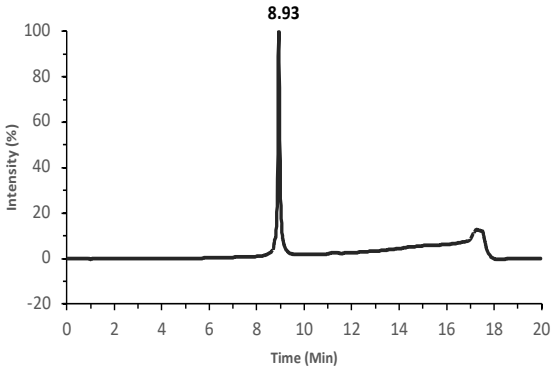


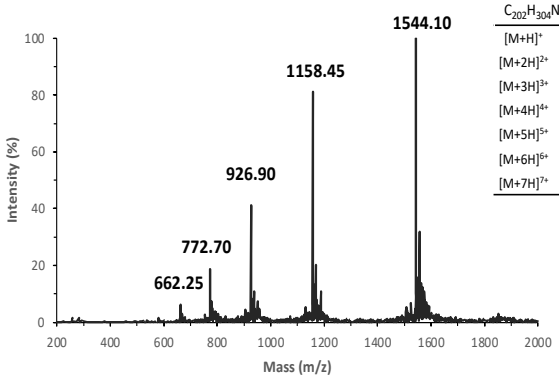


Calculated for $C_{237}H_{378}N_{73}O_{70}S_3$	
$[M+H]^+$	5466.29
$[M+2H]^{2+}$	2733.65
$[M+3H]^{3+}$	1822.77
$[M+4H]^{4+}$	1367.33
$[M+5H]^{5+}$	1094.06
$[M+6H]^{6+}$	911.89
$[M+7H]^{7+}$	781.76
$[M+8H]^{8+}$	684.17
$[M+9H]^{9+}$	608.26

Enterocin P

NH2-ATRSYGNGVYCNNSKCWVNWGEAKENIAGIVISGWASGLAGMGH-COOH

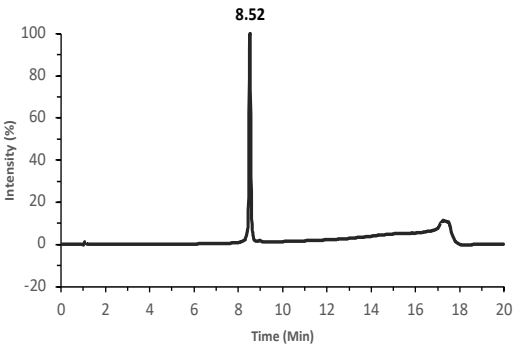


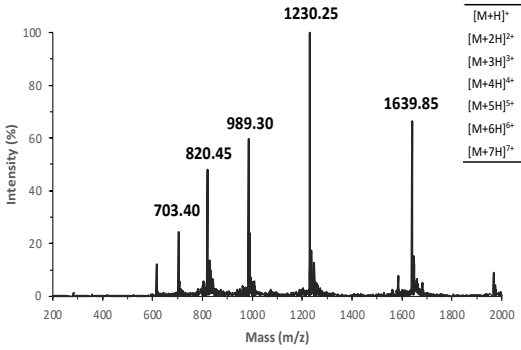


Calculated for $C_{202}H_{304}N_{59}O_{61}S_3$	
$[M+H]^+$	4631.22
$[M+2H]^{2+}$	2316.11
$[M+3H]^{3+}$	1544.41
$[M+4H]^{4+}$	1158.56
$[M+5H]^{5+}$	927.05
$[M+6H]^{6+}$	772.71
$[M+7H]^{7+}$	662.47

Enterocin SEK4

NH2-ATYYGNGVYCNKQKCWVDWSRARSEIIDRGVKAYVNGFTKVLG-COOH

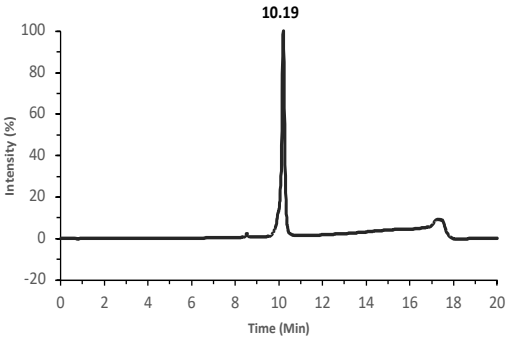


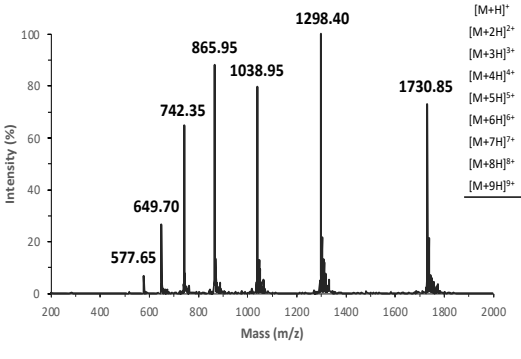


Calculated for $C_{221}H_{337}N_{62}O_{62}S_2$	
$[M+H]^+$	4918.65
$[M+2H]^{2+}$	2459.83
$[M+3H]^{3+}$	1640.22
$[M+4H]^{4+}$	1230.42
$[M+5H]^{5+}$	984.54
$[M+6H]^{6+}$	820.61
$[M+7H]^{7+}$	703.53

Enterocin L50A

NH2-MGAIAKLVAKFGWPIVKKYYKQIMQFIGEGWAINKIIEWIKKHI-COOH





Calculated for $C_{252}H_{393}N_{66}O_{54}S_2$	
$[M+H]^+$	5191.43
$[M+2H]^{2+}$	2596.22
$[M+3H]^{3+}$	1731.15
$[M+4H]^{4+}$	1298.61
$[M+5H]^{5+}$	1039.09
$[M+6H]^{6+}$	866.08
$[M+7H]^{7+}$	742.50
$[M+8H]^{8+}$	649.81
$[M+9H]^{9+}$	577.72

Enterocin L50B

NH2-MGAIAKLVTKFGWPLIKKFYKQIMQFIGQGWTIDQIEKWLKRH-COOH

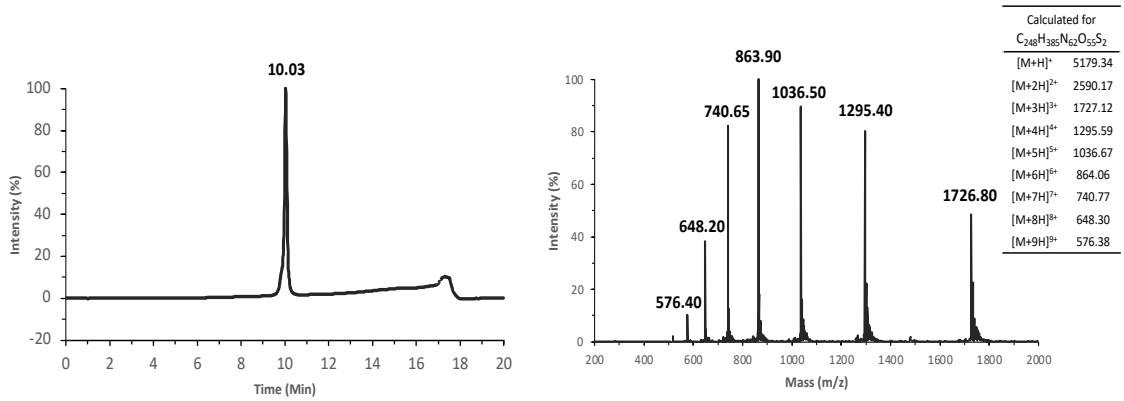


Figure 4.1. HPLC profiles (λ = 220 nm) and ESI-MS spectra of synthetized enterocins.

4.4.2 Antimicrobial activity of the enterocins against *C. perfringens* strains

The zones of inhibition obtained from agar well diffusion assays are included in **Table 4.2**. **Figure 4.2** shows inhibition halos for the enterocins against the susceptible strain *C. perfringens* MLG3111. For these results, only clear inhibition halos were considered as positive. Enterocin A, enterocin B, enterocin P and the two peptides of enterocin L50 inhibited the growth of the entire *C. perfringens* collection, whereas enterocin SEK4 only showed activity against five *C. perfringens* isolates. The most active enterocin tested against *C. perfringens* was enterocin A, with an average diameter of the inhibition halos of 20.3 mm.

Table 4.2. Inhibition halos (in mm) of the different enterocins against the *C. perfringens* collection. Nisin was added as control.

C. perfringens isolate	Enterocin A	Enterocin B	Enterocin P	Enterocin SEK4	Enterocin L50A	Enterocin L50B	Nisin
MLG 0418	18	16	15	-	16	14	23
MLG0618	23	20	22	11	21	19	14
MLG0712	21	12	19	-	16	14	17
MLG1108	22	15	19	-	16	15	16
MLG1619	18	12	18	-	17	16	20
MLG1819	21	12	18	-	16	15	19
MLG2203	21	16	20	-	15	14	24
MLG2314	21	16	17	-	16	14	20
MLG2919	20	10	18	-	16	13	21
MLG3111	18	15	19	-	15	14	27
MLG3406	20	12	20	-	16	15	19
MLG4201	21	11	19	-	16	15	18
MLG4206	22	12	19	8	16	14	25
MLG5719	20	11	17	9	15	14	19
MLG5806	19	9	17	8	14	14	19
MLG6907	19	9	17	-	16	13	23
MLG7009	21	12	19	-	16	14	20
MLG7307	22	11	22	-	17	15	20
MLG7309	21	12	18	-	15	14	17
MLG7814	19	11	19	11	15	13	23

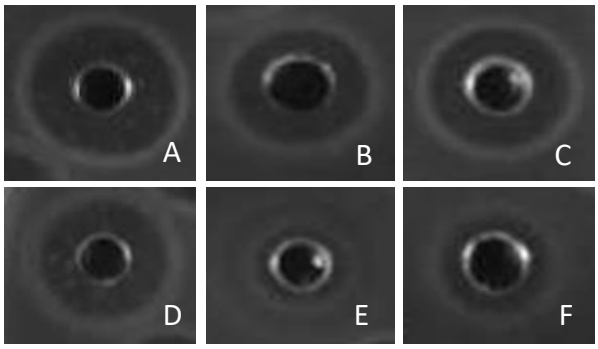


Figure 4.2. Inhibition halos with enterocin A (A), enterocin P (B), enterocin L50A (C), enterocin L50B (D), enterocin SEK4 (E) and enterocin B (F), against *C. perfringens* MLG3111.

The minimal inhibitory concentration (MIC) values of the different enterocins against the collection of *C. perfringens* isolates are shown in **Table 4.3.** Enterocin L50A and L50B showed the lowest MICs, being the most active, followed by enterocin B and enterocin A. Enterocin SEK4 and enterocin P showed very high MICs. A heat map representing those values is shown in **Figure 4.3.**

Table 4.3. MIC (μg/mL) of the enterocins against the collection of *C. perfringens* isolates.

C. perfringens isolate	L50A	L50B	Enterocin A	Enterocin B	Enterocin P	SEK4	Nisin
MLG 0418	6.25	12.5	25	50	50	-	1.56
MLG0618	6.25	12.5	>100	100	>100	>100	0.39
MLG0712	1.56	25	>100	100	>100	-	0.39
MLG1108	3.12	25	>100	25	>100	-	0.78
MLG1619	12.5	50	>100	100	>100	-	0.19
MLG1819	6.25	12.5	>100	25	50	-	0.39
MLG2203	12.5	25	>100	50	>100	-	<0.09
MLG2314	3.12	12.5	>100	50	100	-	1.56
MLG2919	6.25	12.5	>100	>100	>100	-	1.56
MLG3111	3.12	12.5	25	50	50	-	0.39
MLG3406	6.25	12.5	>100	50	>100	-	3.12
MLG4201	6.25	25	50	100	100	-	0.78
MLG4206	6.25	12.5	50	50	>100	>100	1.56
MLG5719	6.25	25	>100	>100	>100	>100	1.56
MLG5806	6.25	25	>100	100	>100	>100	1.56
MLG6907	12.5	50	>100	>100	>100	-	1.56
MLG7009	6.25	25	>100	50	>100	-	1.56
MLG7307	1.56	6.25	3.12	6.25	1.56	-	0.78
MLG7309	6.25	50	>100	100	100	-	0.78
MLG7814	12.5	50	>100	>100	>100	>100	3.12

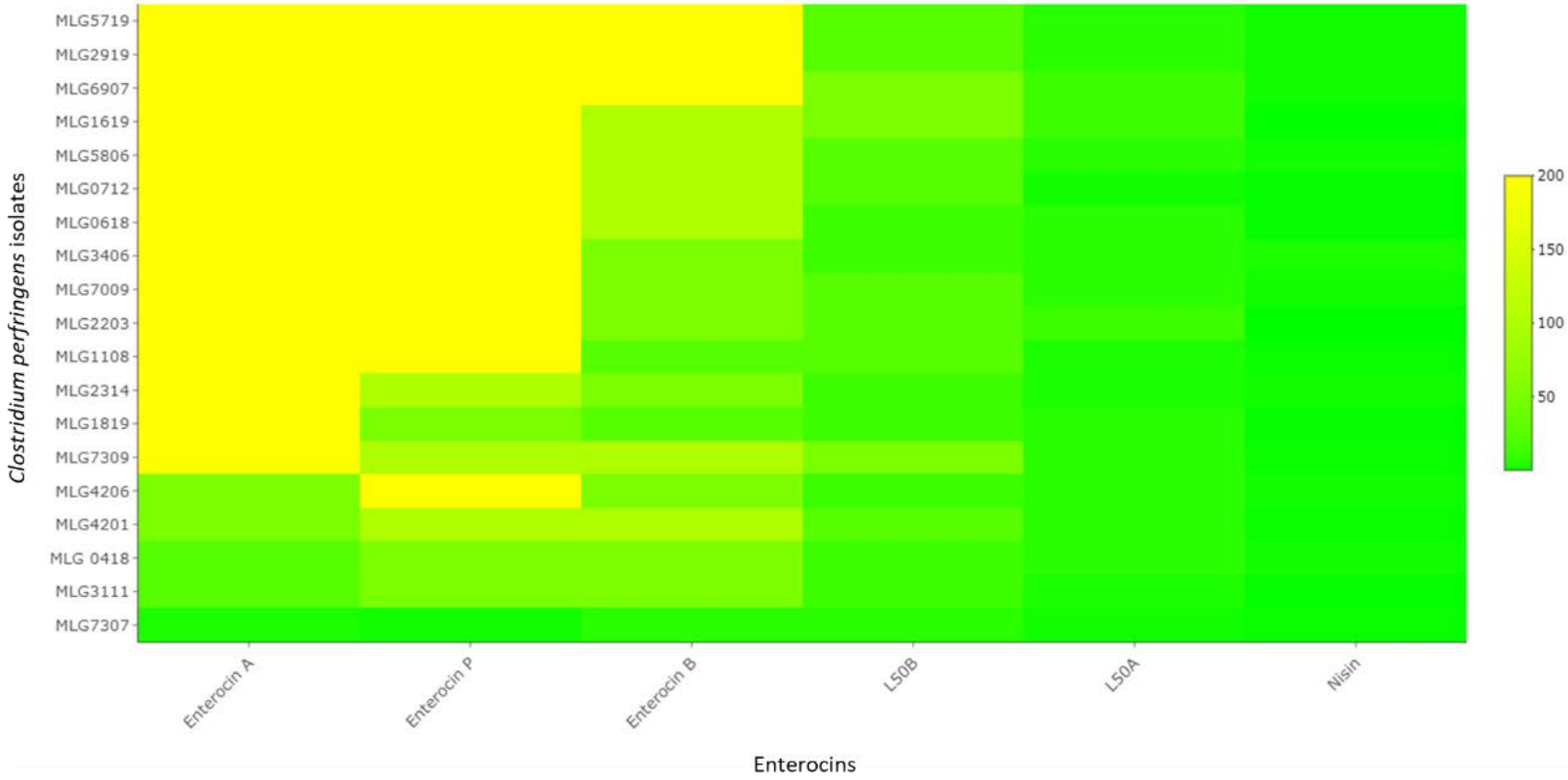


Figure 4.4. Heatmap with the MIC values of the studied enterocins against the *C. perfringens* collection. Heatmap generated with R (Galili et al., 2018).

4.4.3. Whole genome sequencing analysis

Pairwise alignments of the product of the genes encoding the IID subunit of the Man-PTS (*manZ_1*, *manZ_2* and *manZ_3*) revealed differences between the *C. perfringens* ATCC13124 strain and the isolates from our collection (**Figures 4.4**, **4.5** and **4.6**). Regarding the product of *manZ_1*, similarities can be observed between the three *C. perfringens* isolates from U. Laval collection, being *C. perfringens* 7307 clearly different from the other two of them. The alignments of the four isolates can be visualized in **Figure 4.4**. *C. perfringens* ATCC 13124 *man_Z1* product presented a 31.80% of similarity with *C. perfringens* MLG2919. Among many mutations observed, we can highlight the presence of two deletions: one deletion of 11 amino acids from positions p207 to p218; and other of 17 amino acids from position p232 to p249. About the similarities and differences in the *man_Z1* product of the *C. perfringens* isolates from the collection; *C. perfringens* MLG0418 (one of the most susceptible to the class II enterocins) and MLG2319 (one of the less susceptible) showed a 99.96% of identity, finding one substitution in p294, in which an Ala is replaced by a Val in MLG2919. Comparing them with the *manZ_1* product of MLG3707, which had the lowest MICs of the study, with MLG0418 and MLG2919, we detected a 93.40 and 93.73% of identity, respectively. MLG7307 *manZ_1* product presented 13 substitutions in compared with the *manZ_1* product of MLG0418 with the *manZ_1* product of MLG0418 and 14 substitutions the *manZ_1* product of MLG2919.

In regard with the similarities of the product of *manZ_2*, only two of the isolates of the collection (MLG0418 and MLG2919) were identical to one another (100% of identity). They presented a 30.29% of identity with *C. perfringens* ATCC 13124 and a 29.39% of identity with the *manZ_2* products of the MLG7307 isolate. The alignments can be visualized in **Figure 4.5**.

For the product *manZ_3*, the strain MLG3707 did not present that gene in its genome. Comparing its products from the other two isolates from *the C. perfringens* collection, a 99.63% of identity was detected between the MLG2919 and MLG0418, with a substitution in Asn55Lys. The alignments can be visualized in **Figure 4.6**.

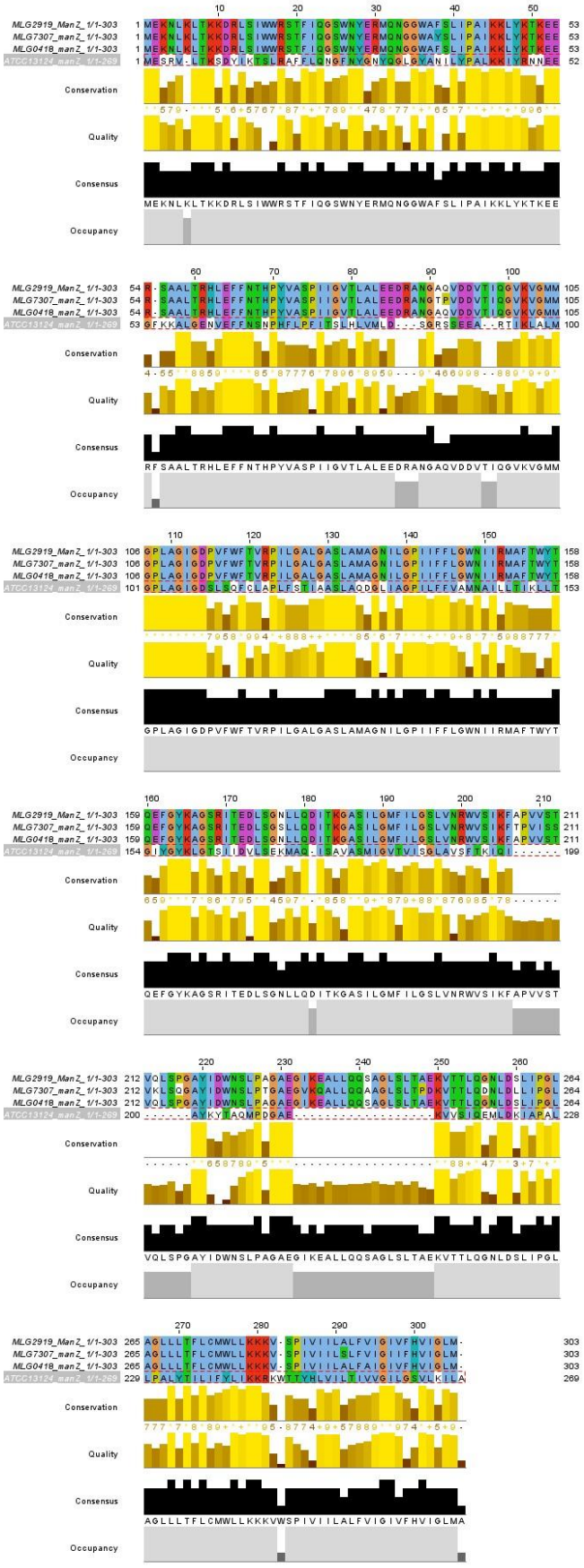


Figure 4.5. Alingments of *manZ_1* product of *C. perfringens* MLG 0418, 2919 and 7307. The product from the *C. perfringens* ATCC 13124 strain was also alinged.

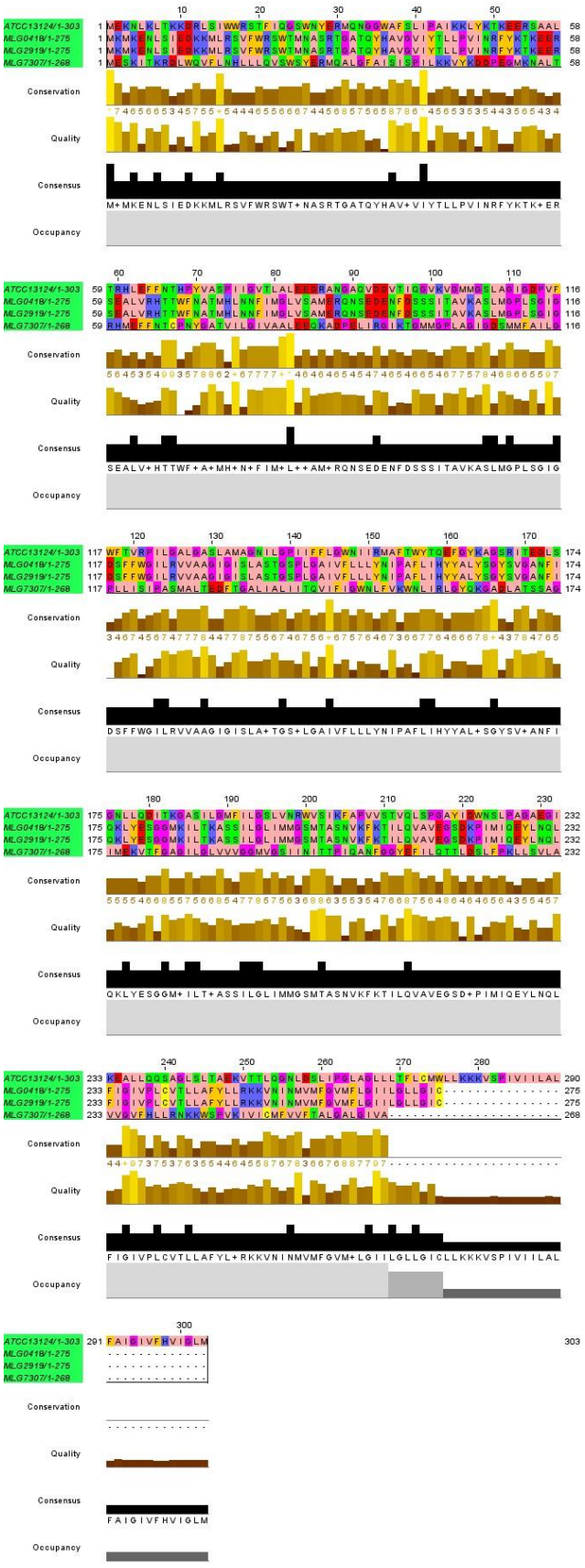


Figure 4.6. Alingments of *manZ_2* product of *C. perfringens* MLG 0418, 2919 and 7307. The product from the *C. perfringens* ATCC 13124 strain was also alinged.

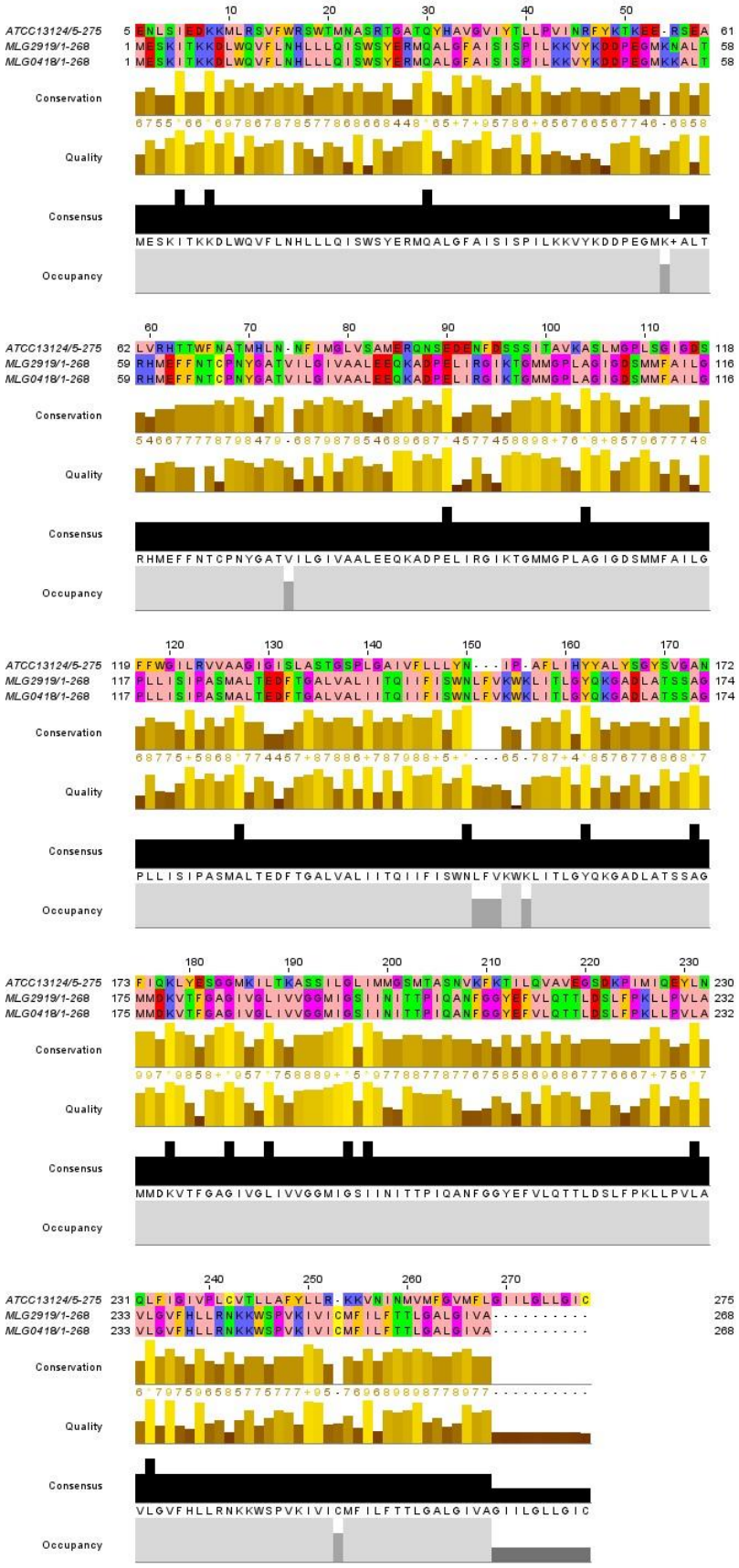


Figure 4.7. Alingments of *manZ_3* product of *C. perfringens* MLG 0418 and 2919. The product from the *C. perfringens* ATCC 13124 strain was also alinged.

4.4.4 Antimicrobial activity of the enterocins against other relevant pathogens

All synthesized enterocins showed strong activity against *L. monocytogenes*. Enterocin L50A and L50B showed the broadest spectrum of activity and were even active against the Gram-negative *P. aeruginosa* ATCC 27855 (**Figure 4.7**) and *C. coli* ATCC 33559. Inhibition diameters (in mm) from the enterocins against the other relevant bacteria used as indicators are shown in **Table 4.4**. MIC values obtained against these pathogens are represented in **Table 4.5**. While the results showed that *L. monocytogenes* was the most sensitive strain to the tested enterocins, enterocin L50A and L50B yielded the widest spectrum of activity with L50A exhibiting the lowest MICs.

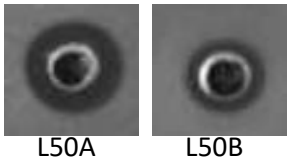


Figure 4.8. Inhibition halos of the two peptides of enterocin L50 (L50A, L50B), against the Gram-negative *P. aeruginosa* ATCC 27855.

Table 4.4. Inhibition halos (in mm) of the enterocins against relevant bacteria.

	Enterocins					
Pathogens	L50A	L50B	EntA	EntB	EntP	EntSEK4
L. monocytogenes ATCC1911	15	17	25	16	21	13
E. faecalis ATCC29212	15	14	18	16	20	12
E. cecorum C0009	28	26	-[a]	17	-	16
S. suis C2058	15	14	12	17	13	-
S. pyogenes ATCC19615	26	26	-	11	-	-
M. luteus ATCC10240	20	16	-	-	-	-
S. aureus ATCC6538	15	15	-	-	-	-
S. aureus C411	13	11	-	-	-	-
P. aeruginosa ATCC27855	14	13	-	-	-	-
C. coli ATCC33559	10	10	-	-	-	-

a: Not active
Note: No activity was detected against *E. coli* ATCC 24922 and *S. enterica* ATCC 69162

Table 4.5. MIC (μg/mL) of the enterocins against different pathogens.

	L50A	L50B	Enterocin A	Enterocin B	Enterocin P	Enterocin SEK4
Listeria monocytogenes ATCC1911	<0.19	<0.19	<0.19	3.12	<0.19	1.56
Enterococcus faecalis ATCC29212	3.12	6.25	1.5S6	1.56	-	-
Enterococcus cecorum CECO0009	1.56	1.56	-[a]	-	-	-
Streptococcus suis C2058	1.56	1.56	50	1.56	-	-
Streptococcus pyogenes ATCC19615	0.78	0.78	-	<0.19	-	-
Micrococcus luteus ATCC10240	1.56	3.12	-	-	-	-
Staphylococcus aureus ATCC6538	6.25	6.25	-	-	-	-
Staphylococcus aureus C411	12.5	25	-	-	-	-
Pseudomonas aeruginosa ATCC27855	12.5	25	-	-	-	-
Campylobacter coli ATCC33559	25	50	-	-	-	-

a: Not active.

4.4.5 Synergistic effects of different enterocin's combinations

FIC index to evaluate synergistic, additive and antagonism was calculated using the following combinations of enterocins: L50A-L50B, EntA-EntB, EntA-L50A, EntA-L50B, EntB-L50A, EntB-L50B, EntP-L50A and EntP-L50B. *C. perfringens* MLG 3111 was chosen as indicator strain due to its high susceptibility to the enterocins. Results are shown in **Table 4.6.** Four combinations resulted synergistic and other four resulted additive or indifferent effect.

Table 4.6. FIC values of different combinations of enterocins against *C. perfringens* MLG3111. MICs values in µg/mL.

COMBINATION		MICA	MICB	MIC of synergy A	MIC of synergy B	FIC_A	FIC_B	FIC INDEX	EFFECT
Comp A	Comp B								
L50A	L50B	3.12	50	1.56	3.12	0.5	0.06	0.56	PARTIAL SYNERGY
EntA	L50A	100	6.25	25	0.78	0.25	0.12	0.37	SYNERGY
EntA	L50B	100	100	25	25	0.25	0.25	0.5	SYNERGY
EntA	EntB	50	100	25	12.5	0.5	0.12	0.56	PARTIAL SYNERGY
EntB	L50A	400	12.5	200	0.78	0.5	0.06	0.56	PARTIAL SYNERGY
EntB	L50B	400	50	50	25	0.5	0.12	0.62	PARTIAL SYNERGY
EntP	L50A	400	6.25	12.5	0.39	0.03	0.024	0.05	SYNERGY
EntP	L50B	400	25	12.5	3.12	0.03	0.12	0.15	SYNERGY

Note: MICs in µg/mL

4.5 Discussion

Enterocins A, B, P, SEK4, L50A and L50B were successfully obtained by microwave-assisted solid-phase peptide synthesis, highlighting the potential of chemical synthesis to produce long peptides (i.e >40 AA) such as bacteriocins. Because the *in situ* formation of a disulfide bond in biological media has been recently demonstrated with linear and cyclic pediocin PA-1 and bactofencin A showing the same activity (Bédard et al.,2018; Bédard et al., 2019), no disulfide bond formation was performed during the synthesis and linear enterocins A, B, P, and SEK4 were used as is in the antimicrobial assays. Further studies are currently underway to optimize the synthesis steps and increase yields. Access to these enterocins by chemical synthesis allows modifications to be made to optimize their physicochemical and pharmacological properties as well as further studies for their use in the food and animal production industry.

All the enterocins produced showed antimicrobial activity against the *C. perfringens* collection, with the exception of enterocin SEK4, which showed antimicrobial activity against only five isolates, even though it has shown activity against clostridial isolates in other studies (Eguchi et al., 2001). MIC values of class IIa enterocin A and enterocin P were around 100 µg/mL, although they showed large inhibition halos in the agar well diffusion assay. The situation was different with enterocin B. Even when the halo was small, the

MICs were lower than those of class IIa enterocins. This could be attributed to the fact that enterocin B is a large peptide and may not diffuse properly through the agar pores. Both peptides of enterocin L50 showed promising results in terms of antimicrobial activity against the whole *C. perfringens* collection, with the L50A peptide being more active than the L50B peptide. The L50A peptide even showed MIC values very close to those produced by nisin, which is known to be very active (Cintas et al., 1998). Considering that enterocins selected for this study have different modes of action, enterocins that bind to the manPTS receptor showed similar MICs against *C. perfringens* isolates. The same phenomenon occurred with enterocin L50, which probably binds to the UppP receptor, and nisin, which binds to the lipid II receptor (Bierbaum & Sahl., 2009; Islam et al., 2012). Enterocin B, whose receptor has not yet been identified, showed MIC values similar to those of enterocins A and P.

The products of the different genes encoding subunit II of the Man-PTS system showed different substitutions and deletions when compared with isolates of different susceptibility levels and with the reference strain *C. perfringens* ATCC 13124. However, as resistance to pediocin-like IIa bacteriocins is not only due to mutations on the Man-PTS system but also to overexpression of the genes (Jeckelmann & Erni., 2020), it cannot be concluded that these differences in the amino acid sequences of sub IID of the Man-PTS system are the only ones responsible for the differences in enterocin susceptibility. Further studies, such as qPCRs analyzing the level of expression of the genes, are required to complete the explanation. Likewise, the differences in susceptibility to the pediocin-like class IIa enterocins were not very remarkable.

In terms of the spectra of activity, all enterocins produced were active against *L. monocytogenes* ATCC 1911 with very low MICs. Pediocin-like antimicrobial peptides have previously been used to control foodborne pathogens such as *Listeria* (Kaur et al., 2011). This study highlights the fact that they can be used for this purpose. It also shows that they can be produced by chemical synthesis, which facilitates their purification and further uses (Bédard & Biron., 2018). In addition to *Listeria*, class IIa enterocins were also active against other relevant bacteria. These included *E. faecalis* ATCC 29212, *S. suis* C2058 and *S. pyogenes* ATCC 19615. Typically, bacteriocins act against closely related bacteria because they are produced to compete for the ecological niche (Ness et al., 2014). That is the case for enterocin A and enterocin B, which

are active against *E. faecalis* ATCC 29212. In this study, enterocin P and enterocin SEK4 were only active against *L. monocytogenes* ATCC 1911, representing a very narrow spectrum of activity, and this may be positive for applications targeting only this pathogen. In contrast, enterocin L50 showed a broader spectrum of activity, with both peptides also active against Gram-negative bacteria, which is not common for bacteriocins produced by Gram-positive bacteria (Ness et al., 2014; Balandin et al., 2019). Both L50A and L50B peptides are promising antimicrobial peptides for further studies, not only because of their broader spectrum of action, but also because of their high activity at low concentrations. Therefore, they can be effective not only against Gram-positive poultry pathogens such as *C. perfringens* and *E. cecorum*, which cause huge damage in the poultry sector (Jung et al., 2018; Abd El-Hack et al., 2022), but also against Gram-negative pathogens such as *C. coli* and *P. aeruginosa*. In addition, L50A and L50B are active against S. aureus ATCC 6538 and also against the methicillin-resistant *S. aureus* C411. This underlines the idea that they can be used as an alternative to antibiotics in the case of multi-drug resistant bacteria.

The FIC_{INDEX} was calculated to evaluate the activity of different enterocin combinations. All combinations tested had partial or synergistic effects, supporting the idea that the combination of enterocins with different modes of action can be used to enhance antimicrobial activity. The different mechanisms of action of the chosen enterocins are represented in **Figure 4.8**. The combinations EntA-L50A, EntA-L50B, EntP-L50A and EntP-L50B, combining the ManPTS and probably the UppP receptor, were synergistic. The combination of the two peptides of enterocin L50, L50A-L50B showed partial synergy. This combination had shown synergy in previous studies (Cintas et al., 1998). However, the methods used to interpret this can vary and the FIC_{INDEX} for L50A-L50B was 0.56, which is very close to synergy. Given that these two peptides are very active at low concentrations and that they have a broad spectrum of activity, this combination is very promising for further applications. For the combinations of enterocin B, whose mechanism of action is still unknown, with other enterocins, the FIC_{INDEX} value showed a partial synergy. This can be explained by the idea that enterocin B do not bind to the ManPTs system and has a different mechanism of action, which needs to be further studied by other methods. FIC_{INDEX} was very low for other synergistic combinations such as EntA-L50A and EntP-L50A. However, as the MIC values for enterocin P are high, the

combination EntA-L50A may be a better candidate as lower concentrations of enterocin A than enterocin P are required to achieve inhibitory activity.

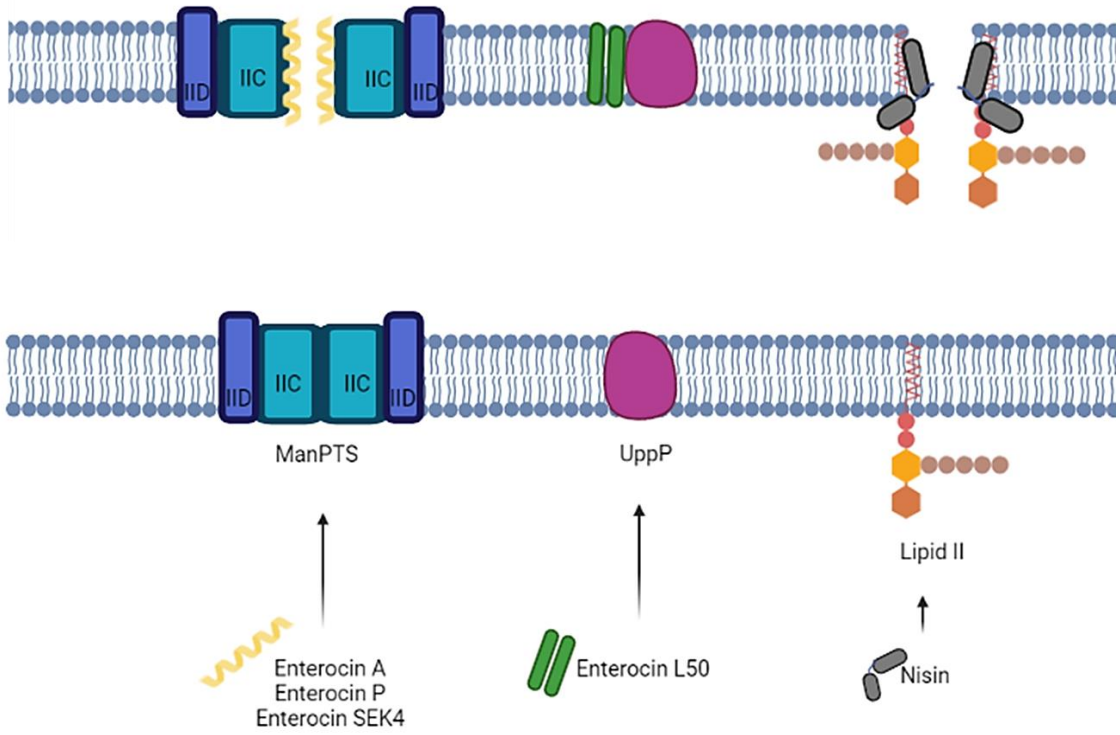


Figure 4. 8. Mechanisms of action of the enterocin A, P, SEK4, L50 and nisin. Enterocin A, P and SEK4, belonging to class IIa bacteriocins binds to the subunit IIC of the Man-PTS system to allow pore formation. The two peptides of enterocin L50, belonging to class IIb bacteriocins, binds to the UppP receptor in the membrane and facilitates pore formation. Nisin, a lantibiotic belonging to class I, binds to the lipid II receptor facilitating the pore formation. Created with BioRender.com.

In summary, the enterocins produced in this study are active against, among others, the poultry pathogen *C. perfringens*, *L. monocytogenes and E. cecorum*. Their activity depends on their mode of action and enterocins using the ManPTS as a receptor showed a similar spectrum of activity while enterocin L50, which has a different mode of action, showed a broader spectrum with inhibitory activity even against Gram-negative *P. aeruginosa* and *C. coli*. Combining enterocins with different modes of action resulted in increased antimicrobial activity against *C. perfringens* as they appeared to be synergistic or at least partially synergistic.

This study reinforces the idea of using enterocins as a promising alternative to antibiotics in the poultry sector, since they exhibit antimicrobial activity against relevant and problematic bacterial pathogens and can be easily produced by chemical synthesis. Moreover, our study also demonstrated that

combinations of enterocins based on their mode of action can significantly enhance antimicrobial activity and efficacy.

4.6. Conclusions

I. Microwave-assisted solid phase peptide synthesis is a suitable and effective tool for the production of enterocins.

II. Enterocins A, B, P and L50A/B are active against *C. perfrignens* isolates from poultry affected by NE, with L50 being the most active and thus the most competitive candidate as an alternative to antibiotics in the sector.

III. Enterocins A, B, P, SEK4 and L50 were active against *L. monocytogenes* and others, demonstrating potential use not only against *Clostridium*.

IV. Enterocin L50 is the most promising of the enterocins produced, as it exhibits the higher activity against the collection of *C. perfrignens* (lower MICs) and showed a broader spectrum of activity, inhibiting the growth of even gram-negative *P. aeruginosa* and *C. coli*.

V. The combinations EntA-L50A, EntA-L50B, EntP-L50A and EntP-L50B combining the ManPTS and probably the UppP receptor are synergistic.

VI. Even if resulted in partial synergy, combination of the two peptides of L50A represents a promising combination due to their elevate activity at very low concentrations.

VII. Because of their elevated activity at low concentrations, combinations of L50A or L50B peptides with pediocin-like enterocins such as EntA represent the most efficient combination to enhance antimicrobial activity against *C. perfrignens*.

Conclusions and future perspectives

In recent years, research into alternatives to the use of antibiotics has increased as a result of the spread of multi-resistant bacteria. As the poultry sector is currently the most consumed meat, new alternatives need to be implemented in this industry to avoid this problem. As many governments have banned the use of antibiotics as feed additives in livestock, some infections that were previously prevented by these drugs have increased. This is the case of *C. perfringens* infections, which are responsible for many economic losses. The present thesis, through the development of its objectives, brings an innovative and original vision to the use of enterococci and their enterocins as an effective alternative to those antibiotics used in the poultry sector to prevent infections by *C. perfringens*.

As the first objective of this thesis "to characterise at the genomic level a collection of *C. perfringens* isolates coming from poultry affected by necrotic enteritis", the results obtained contribute to a better understanding of this avian pathogen through the use of WGS, which provided new information, such as the presence of the *erm*(T) gene, associated with macrolide resistance and detected for the first time in *C. perfringens*. In addition, the study found new STs and even new possible alleles, indicating the presence of genetic variability in this species, providing more information than previously described by other authors. In this study, the twenty *C. perfringens* isolates analysed came from chickens affected by NE, making the information obtained very targeted. However, the population sample may be small, and the results may be limited. Expanding the collection, isolating from different farms and characterising new *C. perfringens* strains in the same way could be a good idea for future studies. Furthermore, within this study we characterised the strain *C. perfringens* MLG 7307, which presented unique characteristics (such as a combination of toxins never found before and the absence of one of the housekeeping genes), indicating that it could be a subspecies or even another species within the genus *Clostridium*. Nevertheless, finding new species was not one of the specific objectives of this chapter, and the means used are not sufficient to determine whether it is indeed a new species or subspecies. Further experiments should be carried out to better determine the characteristics of this strain and its classification.

With regard to the results of the second objective "to screen and characterise in terms of safety enterococcal strains of poultry origin with antimicrobial activity against *C. perfringens*", the results obtained were very promising. The sample of enterococci analysed was very large and a significant number of enterocin-producing enterococci were found. A large percentage of the enterococci tested showed antibiotic resistance mechanisms, which is a cause for concern, including resistance mechanisms to linezolid (an antimicrobial reserved for human use). Enterocin-producing enterococci with fewer antibiotic resistance mechanisms, although not ideal candidates for further use as a protective culture in broilers, could have been found if enterococci of other origins had been considered. The strains selected for future studies showed very promising characteristics, being very active against *C. perfringens*. As a future perspective, a search for strains that are also active against other emerging pathogens in the sector, particularly *E. cecorum*, which has become more important in recent years, could be considered.

As enterococci do not have GRAS status, the use of their enterocins rather than the strains as such could be a more feasible alternative to the use of antibiotics. Therefore, the third objective was "to produce and purify enterocins with activity against the *C. perfringens* collection and other relevant bacterial poultry pathogens". Five enterocins were successfully produced by chemical synthesis, representing the first time that enterocins have been produced by this method, which offers an innovative perspective. Furthermore, by comparing this method with the most common production method, fermentation, we found advantages such as the easy purification of enterocins. However, further studies are needed to optimise the production process and to be able to produce them on a large scale. The enterocins produced showed antimicrobial activity not only against *C. perfringens* but also against other important pathogens such as *E. cecorum* and *L. monocytogenes*, and one of them, L50, showed activity against gram-negative *Campylobacter coli* and *Pseudomonas aeruginosa*, making this enterocin one of the most promising for use in this sector. Therefore, future studies should focus on this enterocin. With regard to the combination of bacteriocins to achieve synergistic effects, the results were also very promising, especially those involving the use of L50 with other bacteriocins that have different mechanisms of action. However, the combinations chosen were only enterocin-enterocin. Perhaps combining enterocins with other types of bacteriocins, or even with other compounds of

antimicrobial nature produced by other microorganisms, would have been a good idea to achieve a broader spectrum of action.

Overall, the use of bacteriocin-producing enterococci, and in particular their enterocins, represents a very attractive alternative to antibiotics in the poultry sector. Further studies on this topic could include the optimisation of enterocin production and their combination with other antimicrobial compounds. In addition, these compounds should be studied in detail to ensure that their use is safe; studies on toxicity, enzymatic degradation, etc. should be carried out. These compounds should then be tested in *in vivo* infection models to assess their efficacy in preventing *C. perfringens* infection and their potential as novel growth factors. On the other hand, it is not only *C. perfringens* that plays havoc in the sector, so the efficacy of these compounds should also be tested against other relevant pathogens, such as the aforementioned *E. cecorum*.

Conclusions et perspectives d'avenir

Ces dernières années, la recherche sur les alternatives à l'utilisation d'antibiotiques a augmenté en raison de la propagation de bactéries multirésistantes. Étant donné que le secteur avicole représente actuellement le type de viande le plus consommée au monde, il est nécessaire de mettre en place de nouvelles alternatives dans ce secteur pour éviter ce problème. À mesure que de nombreux pays ont interdit l'utilisation d'antibiotiques comme additifs alimentaires dans l'élevage, certaines infections précédemment évitées avec ces médicaments ont augmenté. C'est le cas des infections à *C. perfringens*, responsables de nombreuses pertes économiques. Cette thèse, par le biais de la réalisation de ses objectifs, apporte une vision novatrice et originale dans laquelle il est proposé d'utiliser des entérocoques et leurs entérocoques en tant qu'alternative efficace aux antibiotiques pour prévenir les infections à *C. perfringens* dans le secteur avicole.

En ce qui concerne le premier objectif de cette thèse, "caractériser au niveau génomique une collection d'isolats de *C. perfringens* provenant de volailles atteintes de l'entérite nécrotique", les résultats obtenus contribuent à une meilleure compréhension de ce pathogène grâce à l'utilisation du séquençage complet du génome (WGS), apportant de nouvelles informations, comme la présence du gène *erm* (T), associé à la résistance aux macrolides et détecté pour la première fois chez *C. perfringens*. De plus, l'étude a trouvé de nouveaux ST (séquence types) et même de nouveaux allèles possibles, ce qui indique la présence de variabilité génétique dans ce genre, fournissant plus d'informations que ce qui avait été décrit précédemment par d'autres auteurs. Dans cette étude, les vingt isolats de *C. perfringens* analysés provenaient de poulets atteints d'entérite nécrotique, de sorte que les informations obtenues sont très spécifiques. Cependant, la taille de l'échantillon peut être réduite et les résultats limités. Élargir la collection en isolant des souches de différentes fermes pourrait être une bonne idée pour les prochaines études. De plus, au sein de cette étude, la souche *C. perfringens* MLG 7307 a été caractérisée, présentant des caractéristiques uniques (comme une combinaison de toxines jamais auparavant trouvée et l'absence de l'un des gènes de maintenance), ce qui suggère qu'il pourrait s'agir d'une sous-espèce ou même d'une autre espèce au sein du genre *Clostridium*. Cependant, la recherche de nouvelles espèces n'était pas l'un des objectifs spécifiques de ce chapitre, et les moyens utilisés ne

sont pas suffisants pour déterminer s'il s'agit effectivement d'une nouvelle espèce ou sous-espèce. Des expériences supplémentaires devraient être réalisées pour mieux définir les caractéristiques de cette souche et ainsi la classer.

Par rapport aux résultats du deuxième objectif « sélectionner et caractériser des entérocoques d'origine aviaire en termes d'innocuité avec une activité antimicrobienne contre *C. perfringens* », les résultats obtenus étaient très prometteurs. L'échantillon d'entérocoques analysé était très vaste, et un nombre significatif d'entérocoques producteurs d'enterocins a été trouvé. Cependant, un pourcentage élevé des entérocoques analysés présentaient des mécanismes de résistance aux antibiotiques, ce qui est préoccupant, car cela inclut des mécanismes de résistance à la linézolide (un antimicrobien réservé à un usage humain). Bien que les entérocoques d'origine autre que la volaille ne soient pas considérés comme idéaux pour une utilisation en tant que cultures protectrices chez les volailles, la collection d'entérocoques pourrait être étendue à d'autres origines, augmentant ainsi la probabilité de trouver des souches plus sûres en relation à la résistance et de virulence. Dans tous les cas, les deux souches sélectionnées comme candidates à la culture présentaient des caractéristiques très prometteuses, étant très actives contre *C. perfringens*. En tant que perspective, la recherche de souches actives contre d'autres pathogènes émergents du secteur, en particulier *E. cecorum*, qui a pris de l'importance ces dernières années, pourrait être envisagée.

Étant donné que les entérocoques n'ont pas de statut GRAS, l'utilisation de leurs entérocoques, plutôt que des souches elles-mêmes pourrait être une alternative plus réalisable à l'utilisation d'antibiotiques. Par conséquent, le troisième objectif était « produire et purifier des entérocoques avec une activité antimicrobienne contre la collection de *C. perfringens* et d'autres pathogènes aviaires ». En résultat, cinq entérocines ont été produites avec succès par synthèse chimique, ce qui représente la première fois qu'ils sont produits par cette méthode, offrant une perspective novatrice. De plus, en comparant cette méthode avec la méthode de production la plus courante, la fermentation, nous avons trouvé des avantages tels que leur purification facile. Cependant, davantage d'études sont nécessaires pour optimiser le processus de production et les produire à grande échelle. Les entérocines produits ont montré une activité antimicrobienne non seulement contre *C. perfringens*, mais aussi contre

d'autres pathogènes importants tels qu'*E. cecorum* et *L. monocytogenes*, et l'une d'entre elles, l'entérocine L50, a montré une activité contre les bactéries à Gram négatif *Campylobacter coli* et *Pseudomonas aeruginosa*, ce qui fait de cette entérocine l'une des plus prometteuses pour une utilisation dans le secteur avicole. Par conséquent, les futures études devraient se concentrer sur cette entérocine. En ce qui concerne la combinaison de bactériocines pour obtenir des effets synergiques, les résultats étaient également très prometteurs, en particulier ceux qui impliquaient l'utilisation de L50 avec d'autres bactériocines ayant des mécanismes d'action différents. Cependant, les combinaisons choisies étaient uniquement entre entérocine-entérocine. Il est possible que la combinaison d'entérocoques avec d'autres types de bactériocines, voire avec d'autres composés antimicrobiens, ait pu être une bonne idée pour élargir le spectre d'action.

En général, l'utilisation d'entérocoques producteurs de bactériocines, et en particulier de leurs entérocines, représente une alternative très attractive aux antibiotiques dans le secteur avicole. D'autres études sur ce sujet pourraient inclure l'optimisation de la production d'entérocines et leur combinaison avec d'autres composés antimicrobiens. De plus, les entérocines devraient être étudiées en détail pour garantir leur sécurité d'utilisation ; des études sur la toxicité, la dégradation enzymatique, etc., devraient être menées. D'autre part, ces composés devraient être testés dans des modèles d'infection *in vivo* pour évaluer leur efficacité dans la prévention de l'infection à *C. perfringens* et leur potentiel en tant que nouveaux facteurs de croissance. Enfin, étant donné que ce n'est pas seulement *C. perfringens* qui cause des ravages dans le secteur, l'efficacité de ces composés devrait également être étudiée contre d'autres pathogènes pertinents, comme l'*E. cecorum* mentionné précédemment.

Conclusiones y perspectivas

En los últimos años ha aumentado la investigación sobre alternativas al uso de antibióticos como consecuencia de la propagación de bacterias multirresistentes. Puesto que el sector avícola representa actualmente el tipo de carne más consumida a nivel mundial, es necesario implantar nuevas alternativas en este sector para evitar dicha problemática. A medida que muchos países han ido prohibiendo el uso de antibióticos como aditivos alimentarios en el ganado, han aumentado algunas infecciones que antes se evitaban con estos fármacos. Este es el caso de las infecciones por *C. perfringens*, responsables de muchas pérdidas económicas. La presente tesis, a través del desarrollo de sus objetivos, aporta una visión innovadora y original en la cual se propone el uso de enterococos y sus enterocinas como alternativa eficaz a los antibióticos para prevenir las infecciones por *C. perfringens* en el sector avícola.

Como primer objetivo de esta tesis, "caracterizar a nivel genómico una colección de aislados de *C. perfringens* procedentes de aves de corral afectadas por enteritis necrótica", los resultados obtenidos contribuyen a un mejor conocimiento de este patógeno mediante el uso de WGS, aportando nueva información, como la presencia del gen *erm*(T), asociado a la resistencia a macrólidos y detectado por primera vez en *C. perfringens*. Además, el estudio encontró nuevos ST e incluso nuevos posibles alelos, lo que indica la presencia de variabilidad genética en este género, aportando más información que la descrita anteriormente por otros autores. En este estudio, los veinte aislados de *C. perfringens* analizados procedían de pollos afectados por NE, por lo que la información obtenida es muy específica. Sin embargo, la muestra poblacional puede ser pequeña y los resultados limitados. Ampliar la colección, aislando cepas de distintas granjas podría ser una buena idea para los próximos estudios. Además, dentro de este estudio se caracterizó la cepa *C. perfringens* MLG 7307, que presentaba características únicas (como una combinación de toxinas nunca antes encontrada y la ausencia de uno de los genes "*housekeeping*"), lo que indica que podría tratarse de una subespecie o incluso de otra especie dentro del género *Clostridium*. No obstante, la búsqueda de nuevas especies no era uno de los objetivos específicos de este capítulo, y los medios utilizados no son suficientes para determinar si se trata efectivamente

de una nueva especie o subespecie. Deberían realizarse más experimentos para determinar mejor las características de esta cepa y así poderla clasificar.

En cuanto a los resultados del segundo objetivo "seleccionar y caracterizar en términos de inocuidad enterococos de origen aviar con actividad antimicrobiana contra *C. perfringens*", los resultados obtenidos fueron muy prometedores. La muestra de enterococos analizada fue muy amplia y se encontró un número significativo de enterococos productores de enterocinas. Sin embargo, un gran porcentaje de los enterococos analizados presentaron mecanismos de resistencia a los antibióticos, lo cual es motivo de preocupación, ya que estos se incluyen mecanismos de resistencia al linezolid (un antimicrobiano reservado para uso humano). Si bien los enterococos de origen diferente al aviar no se consideran ideales para el uso como cultivos protectores en aves, la colección de enterococos se podría ampliar a otros orígenes, aumentando así la probabilidad de encontrar cepas más seguras en cuanto a resistencia y virulencia. En cualquier caso, las dos cepas seleccionadas como candidatos a cultivos protectores mostraron características muy prometedoras, siendo muy activas frente a *C. perfringens*. Como perspectiva futura, podría considerarse la búsqueda de cepas que también fueran activas frente a otros patógenos emergentes en el sector, en particular *E. cecorum*, que ha cobrado mayor importancia en los últimos años.

Debido a que los enterococos no tienen estatus GRASS, el uso de sus enterocinas en lugar de las cepas como tal podría ser una alternativa más factible al uso de antibióticos. Por lo tanto, el tercer objetivo fue "producir y purificar enterocinas con actividad antimicrobiana contra la colección de *C. perfringens* y otros patógenos avícolas". Como resultado, se produjeron con éxito cinco enterocinas por síntesis química, siendo la primera vez en la que se producen por este método, ofreciendo una perspectiva innovadora. Además, al comparar este método con el método de producción más común, la fermentación, encontramos ventajas como su fácil purificación. Sin embargo, son necesarios más estudios para optimizar el proceso de producción y poder producirlas a gran escala. Las enterocinas producidas mostraron actividad antimicrobiana no sólo contra *C. perfringens*, sino también contra otros patógenos importantes como *E. cecorum* y *L. monocytogenes*, y, una de ellas, la enterocina L50, mostró actividad contra los Gram-negativos *Campylobacteri coli* y *Pseudomonas aeruginosa*, lo que hace de esta enterocina una de las más

prometedoras para su uso en el sector avícola. Por lo tanto, los estudios futuros deberían centrarse en esta enterocina. En cuanto a la combinación de bacteriocinas para lograr efectos sinérgicos, los resultados también fueron muy prometedores, especialmente los que implicaban el uso de L50 con otras bacteriocinas que tienen mecanismos de acción diferentes. Sin embargo, las combinaciones elegidas fueron únicamente enterocina-enterocina. Tal vez la combinación de enterocinas con otros tipos de bacteriocinas, o incluso con otros compuestos de naturaleza antimicrobiana, habría sido una buena idea para lograr un espectro de acción más amplio.

En general, el uso de enterococos productores de bacteriocinas, y en particular de sus enterocinas, representa una alternativa muy atractiva a los antibióticos en el sector avícola. Otros estudios sobre este tema podrían incluir la optimización de la producción de enterocinas y su combinación con otros compuestos antimicrobianos. Además, las enterocinas deberían estudiarse en detalle para garantizar que su uso sea seguro; deberían llevarse a cabo estudios sobre toxicidad, degradación enzimática, etc. Por otro lado, estos compuestos deberían probarse en modelos de infección *in vivo* para evaluar su eficacia en la prevención de la infección por *C. perfringens* y su potencial como nuevos factores de crecimiento. Por último, ya que no sólo *C. perfringens* causa estragos en el sector, la eficacia de estos compuestos también debería ser estudiada contra otros patógenos relevantes, como el ya mencionado *E. cecorum*.

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