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À la mémoire de mes chers grands-parents
Lamia et Sadok

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Avant-propos

Cette thèse de doctorat comporte une introduction générale suivie de quatre chapitres et d'une conclusion générale.

L'introduction générale présente la problématique, l'hypothèse de recherche, l'objectif général ainsi que les objectifs spécifiques de cette étude.

Le chapitre 1 correspond à une revue de littérature récente qui met en évidence les connaissances actuelles sur l'utilisation des bactériocines, et leur potentiel comme alternative fort prometteuse aux antibiotiques chez le porc.

Le chapitre 2 intitulé « Fate and biological activity of the antimicrobial lasso peptide microcin J25 under gastrointestinal tract conditions » a été réalisé en partie dans le laboratoire du Dr Ismail Fliss ainsi qu'à l'institut sur la nutrition et les aliments fonctionnels (INAF) affilié à l'université Laval à l'aide de la plateforme de digestion *in vitro* qui dispose du modèle de digestion gastro-intestinale (TIM-1). La deuxième partie de ces travaux a été réalisée au laboratoire Molécules de Communication et Adaptation des Microorganismes (MCAM) au Muséum National d'Histoire Naturelle (MNHN) à Paris à l'aide de la plateforme de spectrométrie de masse Bio-organique. J'ai réalisé l'ensemble des expériences qui portent sur la digestion de la MccJ25 au niveau du TIM-1 et en présence des protéases ainsi que l'évaluation de son activité inhibitrice. L'analyse des résultats ainsi que la plus grande partie de la rédaction ont été également réalisés par moi-même. Les expériences relatives à l'analyse des produits de dégradation de la MccJ25 par LC-MS/MS et par l'approche des réseaux moléculaires ont été réalisées en grande partie sous la supervision du Dre Séverine Zirah. Ce premier article dont je suis le premier auteur a été soumis en avril 2018 au journal *Frontiers in Microbiology*.

Le chapitre 3 intitulé « The antimicrobial lasso peptide microcin J25 exhibits inhibitory activity against *Salmonella* in swine colonic conditions » a été entièrement réalisé dans le laboratoire du Dr Ismail Fliss et dont l'ensemble des expériences, le traitement des résultats ainsi que la rédaction ont été réalisés par moi-même. Je suis le premier auteur de cet article qui a été soumis au journal *International Journal of Antimicrobial Agents*.

Le chapitre 4 intitulé « Bioavailability and biological activity of microcin J25: metagenomic and metabolomic analysis of its impact on the porcine microbiome in a continuous culture model » a été

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réalisé en grande partie à l'aide de la plateforme de digestion *in vitro* de l'INAF qui dispose du modèle *in vitro* de fermentation en continu (PolyFermS) permettant de simuler les conditions de la partie proximal du côlon porcin. L'ensemble des expériences relatives à la fermentation colique et au traitement des échantillons prélevés du fermenteur ont été réalisées par moi-même sous la supervision du Dr Ismail Fliss. J'ai également effectué l'analyse des résultats ainsi que la plus grande partie de la rédaction. Une partie de l'analyse métagénomique incluant le séquençage du gène bactérien ARNr 16S a été réalisée à l'Institut de biologie intégrative et des systèmes (IBIS) de l'Université Laval à l'aide de la technologie Illumina MiSeq. Toute la partie relative à l'analyse et le traitement des données LC-MS pour l'étude du métabolome du microbiote colique a été supervisée par les Dres Séverine Zirah et Sylvie Rebuffat au MNHN à l'aide de la plateforme de spectrométrie de masse Bio-organique. Cet article, dont je suis premier auteur sera soumis au journal *Gut*.

La thèse se termine par une conclusion générale incluant les perspectives soulevées par ces travaux.

Introduction générale

Au cours des dernières années, la production porcine est parmi les secteurs économiques qui ont connu la plus forte expansion surtout dans les pays industrialisés (Steinfeld et al., 2006). En effet, le porc est l'une des viandes les plus consommées au monde. Près de 100 millions de tonnes de viande de porc sont produites par année dominant ainsi le marché mondial de la viande avec un part du marché d'environ 36% (Atlas, 2014). Malgré cette expansion, la production porcine a connu d'importantes pertes de revenus en raison de l'augmentation de l'incidence des infections entériques chez les porcs. Des pathogènes bactériens tels que *Escherichia coli*, *Salmonella spp.*, et *Clostridium spp.* sont les principaux responsables de ces maladies (Heo et al., 2013).

Les infections à *Salmonella* sont très répandues chez le porc et sont connues pour causer la salmonellose, une maladie entérique aiguë qui entraine des symptômes de diarrhée et de fièvres engendrant une perte rapide du poids des animaux, un taux de mortalité élevé et une baisse considérable de la productivité des exploitations porcines (Yuan et al., 2018). Les animaux infectés par cette bactérie pathogène peuvent transmettre la maladie à l'humain notamment à travers l'alimentation ce qui entraîne un problème majeur de santé publique poussant l'industrie porcine en collaboration avec les autorités réglementaires à prendre toutes les mesures nécessaires afin de prévenir de telles maladies. L'utilisation des antibiotiques est parmi les mesures qui ont été mises en œuvre depuis des décennies dans plusieurs pays industrialisés (Cromwell, 2002).

Depuis leur découverte au milieu du XXe siècle, les antibiotiques ont été largement utilisés à grande échelle à des fins thérapeutique, prophylactique et/ou comme facteurs de croissance dans le secteur vétérinaire (Viola and DeVincent, 2006). Plus particulièrement, l'utilisation des antibiotiques comme facteurs de croissance est largement répandue dans l'élevage porcin en raison de leur contribution à l'amélioration de la productivité des porcs et ainsi à la réduction des coûts de production. Cependant, l'utilisation massive des antibiotiques a entrainé l'apparition du phénomène de résistance chez les bactéries commensales et pathogènes qui peuvent par la suite se transmettre à l'homme via l'alimentation et causer des problèmes de santé graves (Gill et al., 2015). Conscients des dangers liés à ce phénomène d'antibiorésistance, plusieurs pays ont décidé de bannir cette pratique. Depuis 2006, l'utilisation des antibiotiques comme facteurs de croissance est interdite par l'Union Européenne (Mathew et al., 2007). Au Canada, l'utilisation des antibiotiques appartenant à la catégorie I et II a été récemment interdite pour l'industrie avicole (Santé.Canada, 2017). Il est donc clair qu'il y'a un besoin

urgent de développer de nouvelles alternatives aux antibiotiques conventionnels pour le secteur de l'élevage. Parmi les alternatives proposées, les peptides antimicrobiens naturels (AMPs) et plus particulièrement les bactériocines, représentent une des solutions les plus prometteuses compte tenu de leur caractère naturel et leur différence par rapport aux antibiotiques.

Les bactériocines sont des peptides de faibles poids moléculaires qui sont produits par des bactéries de différentes origines et qui sont dotés d'une activité inhibitrice dirigée contre des microorganismes phylogénétiquement proches de la souche productrice (Riley and Wertz, 2002). Contrairement aux antibiotiques, les bactériocines sont synthétisées par voie ribosomale et sont actives à des concentrations du l'ordre du nanomolaire. Depuis leur découverte, près de 200 bactériocines se caractérisant par la diversité de leurs structures et leurs mécanismes d'action ont été identifiées, classées en plusieurs catégories et répertoriées dans une base de données en ligne appelée BACTIBASE (Hammami et al., 2010). La majorité des bactériocines étudiées sont produites par des bactéries à Gram-positif essentiellement des bactéries lactiques (BL). Les bactériocines produites par les bactéries à Gram-négatif sont beaucoup moins nombreuses et moins diversifiées. Les bactériocines couvrent un champ d'application très large tel que le secteur agro-alimentaire ainsi que le domaines biomédical et vétérinaire (Hammami et al., 2013a). En effet, de nombreuses études ont démontré le fort potentiel des bactériocines à contrôler la prolifération des souches pathogènes et à prévenir les contaminations des produits alimentaires, notamment la nisine qui est la seule bactériocine autorisée et commercialisée comme additif alimentaire depuis la fin des années 80. D'autres études ont pu montrer le potentiel des bactériocines en tant qu'agents thérapeutiques permettant de prévenir et de traiter de diverses maladies infectieuses humaines et animales causées par des bactéries pathogènes. Malgré l'abondance des études qui mettent en évidence le fort potentiel des bactériocines, très peu d'études se sont intéressées aux bactériocines produites par les bactéries à Gram-négatif et à leur activité antibactérienne qui cible des bactéries pathogènes appartenant à cette catégorie.

La microcine J25 (MccJ25) est l'une des bactériocines de bactéries à Gram-négatif les plus étudiées. Cette bactériocine est connue pour son activité inhibitrice contre les bactéries pathogènes appartenant à la famille des *Enterobacteriaceae* telles que *Escherichia coli*, *Shigella* et *Salmonella* (Salomon and Farías, 1992b; Blond et al., 1999b). La MccJ25 est un peptide de 2,1 kDa qui possède une structure très particulière appelée structure en lasso lui conférant des caractéristiques très intéressantes

notamment une stabilité remarquable aux températures et pH extrêmes ainsi qu'une forte résistance à de nombreuses protéases (Rosengren et al., 2004). L'ensemble de ces caractéristiques suggère un fort potentiel de la MccJ25 pour son utilisation comme alternative aux antibiotiques non seulement comme agent thérapeutique contre des pathogènes animaux telle que *Salmonella*, mais également comme agent pour promouvoir la croissance des animaux dès leur jeune âge en remplacement aux antibiotiques.

Dans cette étude, nous avons adopté des approches très originales de microbiologie, de métagénomique et de métabolomique pour évaluer le potentiel de la MccJ25 comme alternative aux antibiotiques en production porcine. Ainsi, sa stabilité gastro-intestinale, son activité inhibitrice contre *Salmonella* Newport et son impact sur le profil métagénomique et métabolomique du microbiote colique du porc ont été étudiés à l'aide de modèles *in vitro* simulant les conditions physiologiques et microbiologiques du tube digestif chez les porcelets en post-sevrage.

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Chapitre 1. Revue de littérature

1.1. Production porcine au Canada

La production porcine est devenue l'un des secteurs économiques les plus dynamiques dans les pays industrialisés (Kanis et al., 2003; Steinfeld et al., 2006).

À l'échelle mondiale, le porc domine le marché mondial de la viande avec une production d'environ 100 millions de tonnes, correspondant à 36,3% de la production totale. Au Canada, la production de porc représente près de 30 % des productions animales du secteur agricole ce qui a entrainé des exportations d'une valeur de plus de 3,8 milliards de dollars en 2016. (Agriculture et Agroalimentaire Canada, 2018). En 2013, le porc représente la deuxième viande la plus consommée au Canada après le bœuf (Statistique Canada, 2015).

1.2. Appareil digestif et microbiote intestinal du porc

Le porc représente l'espèce animale majeure pour la production alimentaire mondiale. Il est aussi largement utilisé comme animal modèle en biomédecine. Contrairement aux ruminants (bovins, ovins, caprins…), le porc est un animal monogastrique qui se nourrit d'aliments d'origine animale et végétale. Son appareil digestif est constitué du tube digestif (estomac, intestin grêle et côlon) (Figure- 1) et des glandes annexes (glandes salivaires, foie et pancréas). L'estomac représente environ 30% du volume total du tube digestif et assure le rôle de la digestion des aliments grâce à l'acidité et les sécrétions. Quant au gros intestin, il représente le lieu de la fermentation des fibres alimentaires contrairement aux polygastriques qui possèdent un rumen assurant cette fonction.

Le microbiote du tube digestif porcin représente un écosystème complexe et diversifié comme chez tous les mammifères. Il est composé de bactéries, d'archées, ainsi que d'eucaryotes (champignons, levures) et de virus. Cependant, la plus grande population de microorganismes est majoritairement constituée de bactéries. Selon plusieurs études, la diversité bactérienne globale au niveau GI porcin est dominée par trois groupes majeurs : les *Firmicutes*, *Bacteroidetes* et *Proteobacteria* (Allen et al. 2011; Lamendella et al. 2011; Looft et al. 2014). Le jéjunum et l'iléon abritent majoritairement le

phylum des *Firmicutes* et des *Proteobacteria*, tandis qu'au niveau du caecum et du côlon on retrouve plus les groupes *Firmicutes* suivis des *Proteobacteria*, et des *Bacteroidetes* (Figure -2).

Il a été démontré que la plus grande diversité bactérienne se trouve au niveau du côlon. En effet, plusieurs genres bactériens qui composent le microbiote colique ont été identifiés notamment le genre *Prevotella* qui représente le genre le plus abondant au niveau colique suivi par les genres *Lactobacillus* et *Clostridium.* L'abondance du genre *Prevotella* chez le porc est associée à la production de certains acides gras à courtes chaînes (SCFA) au niveau intestinal tel que l'acétate et le butyrate qui sont bénéfiques pour la santé de l'hôte (Flint et al. 2012; Looft et al. 2014).

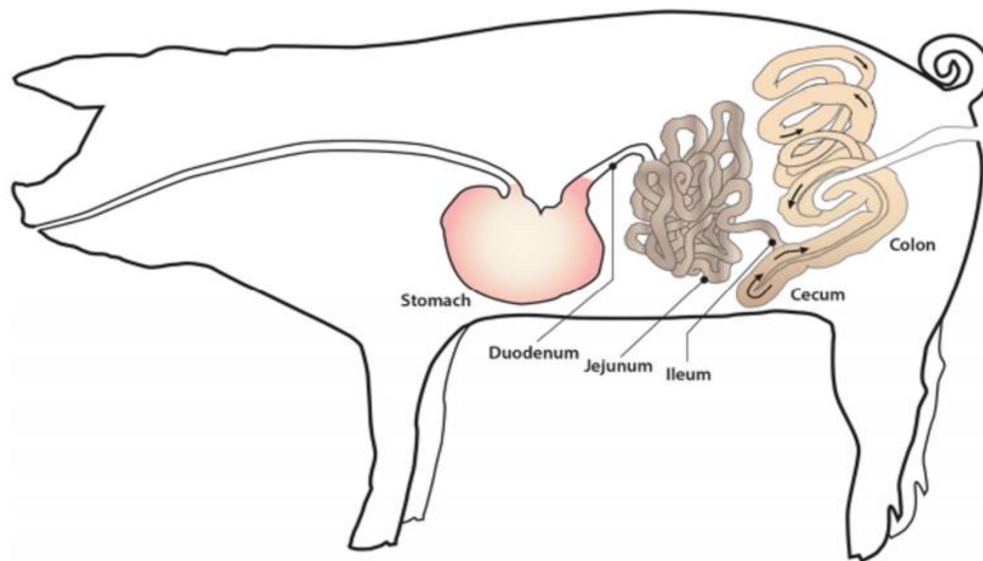

Figure- 1. Schéma du tube digestif du porc (Holman et al., 2017)

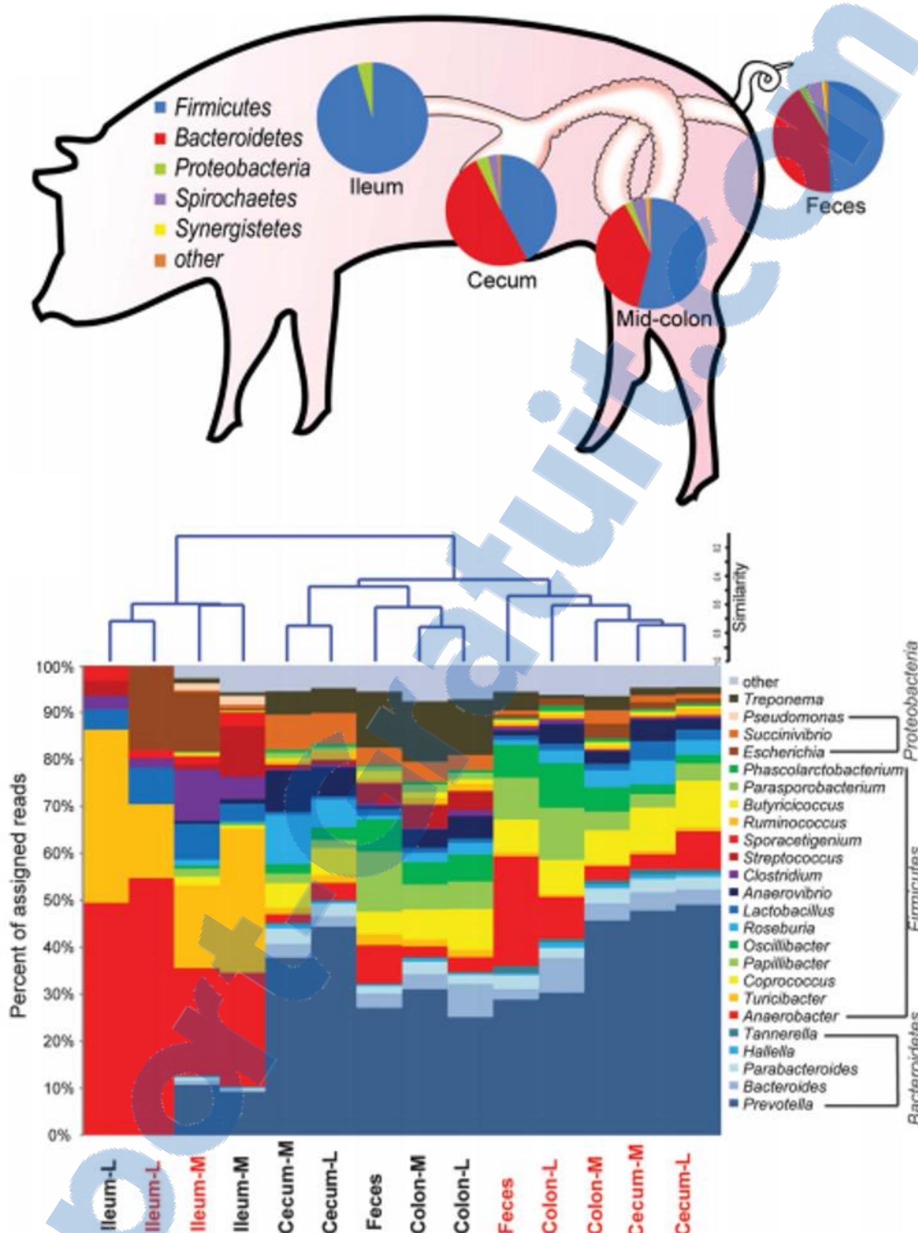

Figure- 2. Distribution des principaux phyla bactériens au niveau du tractus intestinal du porc (Looft et al., 2014a)

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1.3. Usage des antibiotiques en production porcine

Depuis leur découverte au milieu des années 1950, les antibiotiques ont été largement utilisés en médecine humaine et vétérinaire pour traiter et/ou prévenir certaines infections causées par les bactéries pathogènes ainsi que pour améliorer la croissance des animaux d'élevage (Viola and DeVincent, 2006). Les antibiotiques sont connus comme étant des molécules généralement produites par un organisme vivant et sont administrées chez l'hôte dans le but de tuer des bactéries pathogènes. Ces molécules sont largement utilisées aussi bien chez l'homme que chez l'animal.

Les antibiotiques occupent une place importante dans le secteur vétérinaire. L'utilisation des antibiotiques en production porcine ne cesse de croître en raison de leur contribution de manière significative à l'amélioration de la productivité des porcs et à la réduction des coûts de production (Holman and Chénier, 2014). Les antibiotiques utilisés en tant que facteurs de croissance en production animale possèdent un mécanisme d'action qui repose principalement sur la stimulation de la croissance de la microflore intestinale des animaux (Figure-3). Ils peuvent agir de deux façons. Une façon directe qui consiste à réduire la compétition avec l'hôte pour les nutriments et ainsi améliorer la croissance et l'efficacité alimentaire, ou bien une façon indirecte tel que la réduction de l'inflammation et l'amélioration de la santé intestinale.

L'utilisation d'antibiotiques dans l'alimentation porcine présente un moyen efficace pour l'amélioration de la qualité de la viande et le rendement de l'élevage. Par contre, les antibiotiques sont connus pour leur large spectre d'activité, ciblant aussi bien les bactéries pathogènes mais aussi les bactéries bénéfiques pour l'hôte. L'utilisation des antibiotiques peut également fragiliser la flore commensale et favoriser le développement des microorganismes pathogènes (Leser et al. 2000).

Il a été démontré que l'utilisation accrue des antibiotiques a conduit à l'émergence du phénomène de la résistance des bactéries multirésistante, qui peut par la suite se transmettre à l'homme principalement par l'alimentation ainsi que par le contact direct avec les animaux (Barza, 2002). Ceci a amené certains pays à bannir cette pratique, notamment l'Union Européenne qui a interdit totalement en 2006 l'utilisation des antibiotiques comme facteurs de croissance chez les animaux. Au Canada, de nouvelles mesures et modifications réglementaires ont été proposées au cours des dernières années afin de réduire l'utilisation des antibiotiques. Tout récemment, l'utilisation des antibiotiques

appartenant à la catégorie I et II a été interdite dans l'industrie avicole (Santé.Canada, 2017). Ces deux catégories sont considérées de haute importance en médecine humaine selon Santé Canada.

Catégorie	Exemples
Catégorie I : Très haute importance	Carbapénèmes Céphalosporines - de troisième et quatrième générations Fluoroquinolones Glycopeptides Glycylcyclines Cétolides Lipopeptides Monobactams Nitroimidazoles (métronidazole) Oxazolidinones Pénicillines résistantes aux β-lactamases (Associations) Polymyxines (colistin) Agents thérapeutiques antituberculeux (p. ex., éthambutol, isoniazide, pyrazinamide et rifampicine)
Catégorie II : Haute importance	Aminoglycosides (sauf agents topiques) Céphalosporines - première et deuxième générations (et céphamycines) Acide fusidique Lincosamides Macrolides Pénicillines Quinolones (sauf fluoroquinolones) Streptogramines Triméthoprime/sulfaméthoxazole
Catégorie III : Moyenne importance	Aminocyclitols Aminoglycosides (agents topiques) Bacitracines Fosfomycine Nitrofuranes Phénicols Sulphonamides Tétracyclines Triméthoprime
Catégorie IV : Faible importance	Flavophospholipols Ionophores

Tableau. 1. Catégorisation des antibiotiques par Santé Canada en se basant sur leur importance en médecine humaine Adapté de (Santé.Canada, 2009)

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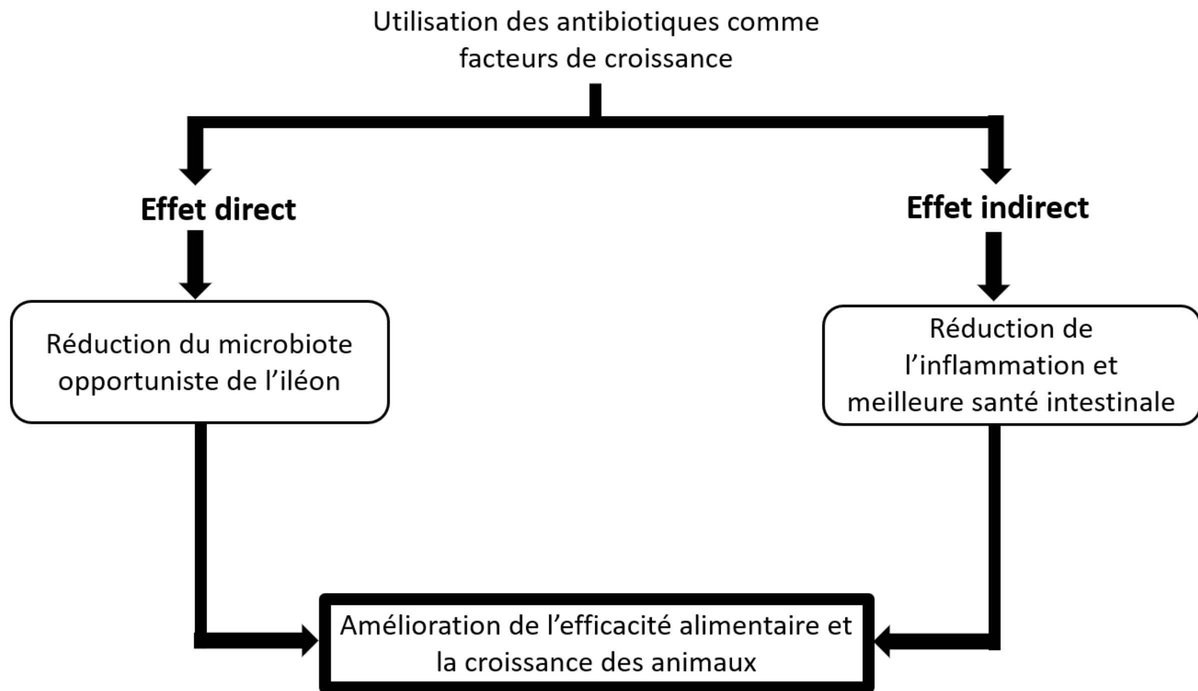

Figure- 3. Mécanismes d'action des antibiotiques utilisés comme facteur de croissance en production animale adapté de (Gaskins et al., 2002).

1.4. Alternatives aux antibiotiques

Face à l'émergence du phénomène d'antibiorésistance, plusieurs travaux réalisés depuis les dernières années laissent entrevoir des perspectives prometteuses permettant de remplacer l'usage des antibiotiques en production animale.

1.4.1. Les probiotiques

Le concept des probiotiques est bien loin d'être nouveau. Il est né à partir des observations faites en 1907 par le biologiste russe Elie Metchnikoff, lauréat du prix Nobel de médecine et physiologie (Gordon 2008). Il a établi un lien entre la longévité « inhabituelle » de certaines populations rurales en Bulgarie et leur grande consommation de produits laitiers fermentés (Anukam and Reid 2007). Il a pu alors identifier deux souches bactériennes nécessaires à la fermentation : *Lactobacillus bulgaricus* (bacille bulgare) et *Streptococcus thermophilus*. La présence de ces souches dans le tube digestif crée un environnement défavorable à l'implantation de pathogènes entériques. Metchnikoff fut ainsi le premier à suggérer que la consommation des bactéries lactiques présentes dans ces laits fermentés pourrait avoir un effet bénéfique sur la santé.

Plusieurs définitions ont été proposées depuis la théorie de Metchnikoff pour définir le terme probiotique (Salminen, Ouwehand et al. 1999; Schrezenmeir and de Vrese 2001; Sanders 2008). La définition la plus utilisée est celle proposée par un comité d'experts, qui définit les probiotiques comme étant des microorganismes vivants qui, lorsqu'ils sont ingérés en quantité adéquate, produisent des bienfaits sur la santé de l'hôte (WHO/FAO 2001). Il s'agit le plus souvent de bactéries, principalement des bactéries lactiques telles que les espèces de *Lactobacillus* et de *Bifidobacterium*, ou bien de levures (Tableau-2) qui peuvent être intégrés dans différents types de produits, y compris les aliments (notamment les produits laitiers fermentés), les substances médicamenteuses et les suppléments alimentaires.

Plusieurs études ont démontré l'efficacité de plusieurs souches probiotiques le traitement de diverses maladies infectieuses animales causées par certaines bactéries pathogènes (Hammami et al., 2013b). En effet, il a été démontré que l'utilisation d'une souche de *Lactobacillus plantarum* comme supplément alimentaire entraine la réduction des comptes de *Salmonella* au niveau du microbiote intestinal porcin via un phénomène de compétition (van Winsen et al., 2001).

Lactobacillus species	*Bifidobacterium* species	Other lactic acid bacteria	Nonlactic acid bacteria
L. acidophilus	*B. adolescentis*	*Enterococcus faecalis*[2]	*Bacillus cereus var.* toyoi[2,3]
L. amylovorus	*B. animalis*	*Enterococcus faecium*	*Escherichia coli* strain nissle
L. casei	*B. bifidum*	*Lactococcus lactis*[4]	*Propionibacterium freudenreichii*[2,3]
L. crispatus	*B. breve*	*Leuconstoc mesenteroides*	*Saccharomyces cerevisiae*[3]
L. delbrueckii subsp. *bulgaricus*[4]	*B. infantis*	*Pediococcus acidilactici*[4]	*Saccharomyces boulardii*[3]
L. gallinarum[2]	*B. lactis*[5]	*Sporolactobacillus inulinus*[2]	
L. gasseri	*B. longum*	*Streptococcus thermophilus*[4]	
L. johnsonii			
L. paracasei			
L. plantarum			
L. reuteri			
L. rhamnosus			

[1] Data from reference
[2] Main application for animals.
[3] Applied mainly as pharmaceutical preparations.
[4] There is either little known about the probiotic properties or the microorganism is nonprobiotic.
[5] Probably synonymous with *B. animalis.*

Tableau. 2. Microorganismes considérés comme des probiotiques (Holzapfel et al., 2001)

1.4.2. Les prébiotiques

Les prébiotiques sont connus comme étant des ingrédients, dont la fermentation de façon sélective entraîne des changements spécifiques de la composition et/ou de l'activité du microbiote gastrointestinal, dont en résultent des bénéfices pour la santé de l'hôte (Gibson et al., 2010). Les prébiotiques regroupent principalement les oligosaccharides solubles non-digestibles composés de sucres simples notamment le glucose, le fructose, ainsi que le mannose (Chambers and Gong, 2011). Il a été démontré que certains prébiotiques, notamment les fructo-oligosaccharides inhibent la croissance de *Salmonella* chez la volaille (Chambers and Gong, 2011). En production animale, il est possible d'administrer des synbiotiques, c'est-à-dire la combinaison des probiotiques et prébiotiques afin d'améliorer l'efficacité du traitement.

1.4.3. Les enzymes

Les enzymes sont connues comme étant des protéines impliquées dans la catalyse de nombreuses réactions chimiques ainsi que la libération de nombreux produits. Plusieurs études ont démontré l'effet bénéfique des enzymes chez les animaux notamment l'effet d'enzymes lipolytiques sur la flore colique des porcelets (Dierick et al 2008).

1.4.4. Les huiles essentielles

Ce sont des huiles aromatiques produites principalement par les végétaux et sont constituées généralement de composés phénoliques tels que le thymol ainsi que d'autres composés comme les aldéhydes et les polypeptides, connus pour avoir une activité antimicrobienne (Chambers and Gong, 2011). Une étude précédente réalisée chez le porc, a démontré une amélioration significative du poids et de la performance des porcs suite à l'administration du thymol et de cinnamaldéhyde (Li et al., 2012).

1.4.5. Les bactériocines

1.5.5.1. Définition

Les bactériocines furent découvertes en 1925 par André Gratia qui observa l'inhibition de la croissance de certaines souches d'*Escherichia coli* en présence d'un composé antibactérien, auquel il a donné le nom de colicine V (De Vuyst and Vandamme, 1994). Depuis leur découverte, près de 200 bactériocines se caractérisant par la diversité de leurs structures et leurs mécanismes d'action ont été identifiées. Elles sont répertoriées dans la littérature selon la base de données BACTIBASE (Hammami et al. 2010). Les bactériocines sont définies comme étant des peptides antimicrobiens synthétisés par des bactéries qui permettent l'inhibition de la croissance d'espèces proches de la souche productrice et parfois d'espèces plus éloignées (Nishie et al. 2012). Contrairement aux antibiotiques, les bactériocines sont caractérisées par une synthèse par voie ribosomale, par un spectre d'activité restreint et par une activité inhibitrice à des faibles concentrations (Nes, 2011).

La majorité des bactériocines sont produites par des bactéries à Gram-positif. Les bactériocines produites par les bactéries à Gram négatif sont beaucoup moins nombreuses et moins diversifiées (Hammami et al., 2013b). Ceci pourrait être dû au fait que les bactéries à Gram positive productrices de bactériocines regroupent en majorité les bactéries lactiques (BL). Ces dernières ont été très étudiées au cours des dernières années et utilisées dans l'industrie alimentaire (Arthur et al. 2014).

Figure- 4. Utilisation de la base de données BACTIBASE disponible sur le site Web http://bactibase.hammamilab.org/main.php pour la recherche des applications thérapeutiques des bactériocines(Hammami et al., 2013b)

1.4.5.2. Applications

Les bactériocines sont utilisées dans plusieurs domaines notamment le secteur agroalimentaire. La nisine est la bactériocine la plus étudiée et la seule approuvée pour une utilisation commerciale en tant qu'additif alimentaire. La nisine est utilisée pour le contrôle de la prolifération de souches pathogènes telles que *Clostridium botulinum* et *Listeria monocytogenes* dans les produits laitiers (Davies et Delves-Broughton, 1999 ; Wirjantoro et al. 2001). Elle est également utilisée pour l'inhibition des souches pathogènes dans les viandes crues et le saumon fumé (Thomas et al. 2000 ; Neetoo et al. 2008). La pédiocine PA-1 a aussi démontré des effets antimicrobiens dans les crèmes fromagères et les produits carnés contaminés par *L. monocytogenes* (Rodríguez et al. 2002). Il a été aussi démontré que d'autres bactériocines, notamment la Lacticine 3147 et l'entérocine AS-48, permettent de prévenir la contamination des produits laitiers (Morgan et al. 2001 ; Ananou et al. 2010).

Les bactériocines sont aussi utilisées dans le domaine médical et vétérinaire comme alternatives aux antibiotiques pour la prévention et le traitement des infections dues à des bactéries devenues résistantes aux traitements conventionnels. Il a été démontré que la mersacidine, une bactériocine produite par la souche *Bacillus sp.* HIL Y-85,54728, est utilisée pour le traitement d'infections cutanées causées par de nombreuses bactéries pathogènes telles que *Listeria*, *Staphylococcus* ou *Streptococcus* (Sass et al. 2008). La nisine A, une bactériocine produite par *Lactococcus lactis* subsp. *lactis,* a été officiellement commercialisée à la fin des années 1980 en tant qu'additif alimentaire, notamment dans les produits laitiers (Gharsallaoui et al., 2016). Une étude précédente a démontré le fort potentiel inhibiteur de la nisine de la bactérie pathogène du porc *Streptococcus suis* (LeBel et al., 2013) et de plusieurs bactéries pathogènes dans les viandes crues et le saumon fumé (Pawar et al., 2000; Neetoo et al., 2008). La pédiocine PA-1, une bactériocine de la classe IIa, a démontré une activité inhibitrice contre *L. monocytogenes* dans certains produits laitiers et carnés (Rodríguez et al., 2002). D'autres bactériocines, notamment la lacticine 3147 et l'entérocine AS-48, inhibent la propagation de contaminants bactériens dans les produits laitiers (Morgan et al., 2001, Ananou et al., 2010). Il a été également démontré que la colicine E1 inhibe la croissance des souches pathogènes d'*Escherichia coli* causant plusieurs maladies chez les porcs en post-sevrage (Stahl et al., 2004a). L'administration de la colicine E1 a également entrainé l'amélioration de la croissance des porcelets ainsi que la réduction de l'incidence de la diarrhée par *E. coli* entérotoxinogène (Cutler et al., 2007a).

1.4.5.3. Classification

Plusieurs classifications ont été proposées pour répertorier les bactériocines en raison de la constante évolution des travaux de recherche portant sur ce domaine. Elles peuvent être classées en deux catégories : les bactériocines synthétisées par les bactéries à Gram négatif et les bactériocines synthétisées par les bactéries à Gram positif. La classification présentée ci-dessous repose sur celle proposée par Cotter *et al.* (Cotter, 2005a) (bactériocines de Gram-positif) et sur Rebuffat *et al.* (bactériocines de Gram-négatif) (Rebuffat, 2011).

La catégorie des bactériocines produites par les bactéries à Gram positif est plus diversifiée et plus étudiée puisqu'elles incluent les bactériocines issues des bactéries lactiques. Ces dernières sont très utilisées dans le domaine alimentaire. Dans cette catégorie, on retrouve trois grandes classes : la classe I, la classe II et les bactériolysines (Cotter et al. 2005). La classe I regroupe les bactériocines subissant des modifications post-traductionnelles et subdivisée en trois sous-classes : les lantibiotiques (peptides de petite taille contenant de la lanthionine et/ou de la β-méthyllanthionine en plus d'autres acides aminés), les labyrinthopeptines (caractérisées par la présence de l'acide aminé la labionine) et les sactibiotiques (peptides cycliques caractérisés par la présence d'un soufre lié au carbone α). La classe II regroupe les bactériocines ne subissant aucune modification post-traductionnelle. Elle est subdivisée en quatre sous-classes : la classe II a (bactériocines ressemblant à la pédiocine et actives contre les bactéries appartenant au genre *Listeria*), la classe II b (bactériocines à deux composantes non-modifiées), la classe II c (bactériocines circulaires) et la classe II d (autres bactériocines linéaires non-modifiés ne ressemblant pas à la pédiocine). Les bactériolysines constituent la troisième classe des bactériocines produites par les bactéries à Gram positif et sont caractérisées par leur mécanisme d'action qui consiste à inhiber les bactéries sensibles en hydrolysant leurs parois bactériennes.

Dans la catégorie des bactériocines produites par les bactéries à Gram négatif, on retrouve deux classes : les colicines qui correspondent aux peptides de haut poids moléculaire (compris entre 30 et 80 kDa) et les microcines qui regroupent les peptides de faible poids moléculaire (taille comprise entre 1 et 10 kDa) (Rebuffat, 2011). Les colicines sont divisées en deux groupes : les colicines du groupe A utilisent un récepteur relié au système membranaire Tol, sont codées sur de petits plasmides et sont excrétées dans le milieu de culture (Cascales et al. 2007; Fernandez, 2014). Quant aux colicines du

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groupe B, elles utilisent un récepteur relié au système membranaire Ton, sont codées sur de grands plasmides et ne sont pas excrétées dans le milieu de culture. Les microcines sont aussi divisées en deux groupes : les microcines de classe I qui regroupent les peptides de taille comprise entre 1 et 3 kDa et qui ont subi de nombreuses modifications post-traductionnelles (Duquesne et al. 2007). Les microcines de classe II, quant à elles, regroupent les peptides de taille comprise entre 5 et 9 kDa et sont subdivisées en deux sous-classes : la classe II a qui incluent les microcines caractérisées par l'absence de modification post-traductionnelle et la classe II b qui incluent les microcines caractérisées par la présence de gènes codant pour des enzymes impliquées dans les modifications post-traductionnelles.

<table>
<tr><th>Groupe</th><th>Classe</th><th>Sous-classe</th><th>Exemple</th></tr>
<tr><td rowspan="8">Gram-positif</td><td rowspan="3">I</td><td>Lantibiotiques</td><td>Nisine; Epidermine</td></tr>
<tr><td>Labyrinthopeptines</td><td>Peptide A2</td></tr>
<tr><td>Sactibiotiques</td><td>Thuricine CD, Subtilolisine</td></tr>
<tr><td rowspan="4">II</td><td>a) « pediocin-like »</td><td>Pédiocine PA-1</td></tr>
<tr><td>b) à 2 composantesnon-modifiées</td><td>Lactococcine G</td></tr>
<tr><td>c) circulaires</td><td>Entérocine AS-48</td></tr>
<tr><td>d) linéaires « non pediocin-like » à une composante</td><td>Lactococcine A, lacticine Q</td></tr>
<tr><td>Bactériolysines</td><td>-</td><td>Lysostaphine, entérolysine A</td></tr>
<tr><td rowspan="4">Gram-négatif</td><td rowspan="2">Colicines</td><td>A</td><td>Colicine E1, colicine A</td></tr>
<tr><td>B</td><td>Colicine B, colicine M</td></tr>
<tr><td rowspan="2">Microcines</td><td>I</td><td>Microcines J25, Microcines B17</td></tr>
<tr><td>II</td><td>Microcines 492, Microcines M</td></tr>
</table>

Tableau. 3. Classification des bactériocines selon (Cotter, 2005a) et (Rebuffat, 2011)

1.4.5.4. Exemple de bactériocine de bactérie à Gram négatif : la microcine J25

En raison de son unique structure et mécanisme d'action, la microcine J25 (MccJ25) a été choisie parmi toutes les bactériocines étudiées de bactérie à Gram négatif pour faire l'objet de ces travaux de thèse. La MccJ25 fait partie de la classe I des microcines possédant un faible poids moléculaire d'environ 2,1 KDa (Duquesne et al., 2007b). Elle est produite naturellement par la souche *Escherichia coli* AY25 isolée à partir de matières fécales de nouveau-né (Salomon and Farías, 1992b; Rebuffat, 2011). La MccJ25 fait partie des RiPPs, peptides synthétisés par la voie ribosomale et qui subissent des modifications post-traductionnelles, aboutissant à la formation d'une structure très particulière connue par la structure en lasso (Duquesne et al., 2007b). Cette structure lui confère des caractéristiques très intéressantes telles que la résistante à de nombreuses protéases, aux pH extrêmes et à la température (Rebuffat, 2011). La MccJ25 est aussi caractérisée par son activité inhibitrice contre certaines bactéries pathogènes appartenant à la famille des *Enterobacteriacea* telles que *Escherichia coli*, *Salmonella* et *Shigella* (Salomon and Farías, 1992b; Sable et al., 2000; Rintoul et al., 2001b; Vincent and Morero, 2009).

1.4.5.4.1. Biosynthèse et maturation de la MccJ25

Le processus de biosynthèse de la MccJ25 est semblable à celui d'autres RiPPs, notamment les peptides lasso. En effet, la MccJ25 est d'abord synthétisée sous la forme d'un peptide précurseur linéaires qui sera soumis à des modifications post-traductionnelles le transformant en peptide mature (Arnison et al., 2013). La MccJ25 représente un bon modèle pour l'étude de la biosynthèse des peptides lasso.

La synthèse et la maturation de la MccJ25 sont réalisées chez la cellule productrice, *Escherichia coli*, grâce au plasmide pTUC100 de 4,8 kb (Rosengren et al., 2004). Trois gènes, soient *mcjA*, *mcjB*, *mcjC* se trouvant sur le plasmide pTUC100 sont impliqués dans le mécanisme de biosynthèse de la MccJ25 (Clarke and Campopiano, 2007; Severinov et al., 2007; Cheung et al., 2010). Le gène *mcjA* code pour le peptide précurseur de la MccJ25, le McjA qui comporte 58 acides aminés. Les gènes *mcjB* et *mcjC* codent pour les enzymes McjB et McjC qui sont indispensables à la maturation de la MccJ25 (Figure-5). McjB est une protéase qui nécessite la présence de l'ATP pour le clivage et le pré-repliement de McjA, tandis que McjC est une lactame-synthétase impliquée dans la catalyse de la cyclisation du

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cycle macrolactame de la MccJ25 en N-terminal. Ces deux enzymes, dont chacune nécessite la présence de l'autre pour être active, semblent former un complexe McjB / McjC appelé lasso-synthétase (Yan et al., 2012). Un quatrième gène, *mcjD* faisant partie du système génétique de la MccJ25 code pour un transporteur membranaire McjD responsable de l'export la MccJ25 mature à l'extérieur de la cellule et également impliqué dans défense immunitaire de la bactérie productrice (Solbiati et al., 1999; Duquesne et al., 2007c). Les quatre gènes *mcjA*, *mcjB*, *mcjC* et *mcjD* sont organisés en deux opérons transcrits en sens contraire, dont le premier est sous le contrôle du promoteur P_{mcjA} impliqué dans l'expression du gène *mcjA*, tandis que le deuxième opéron est sous le contrôle du promoteur P_{mcjBCD} et impliqué dans l'expression des trois gènes *mcjB*, *mcjC* et *mcjD* (Figure-6).

L'activation du système génétique de la MccJ25 et l'expression des gènes impliqués dans la MccJ25 synthétase sont induites dans les conditions de stress environnemental, plus particulièrement de stress nutritif que subit la bactérie productrice. Comme dans le cas du gène *mcjA* qui est exprimé lors d'une carence en carbone et en phosphate inorganique (Duquesne et al., 2007b; Vincent and Morero, 2009).

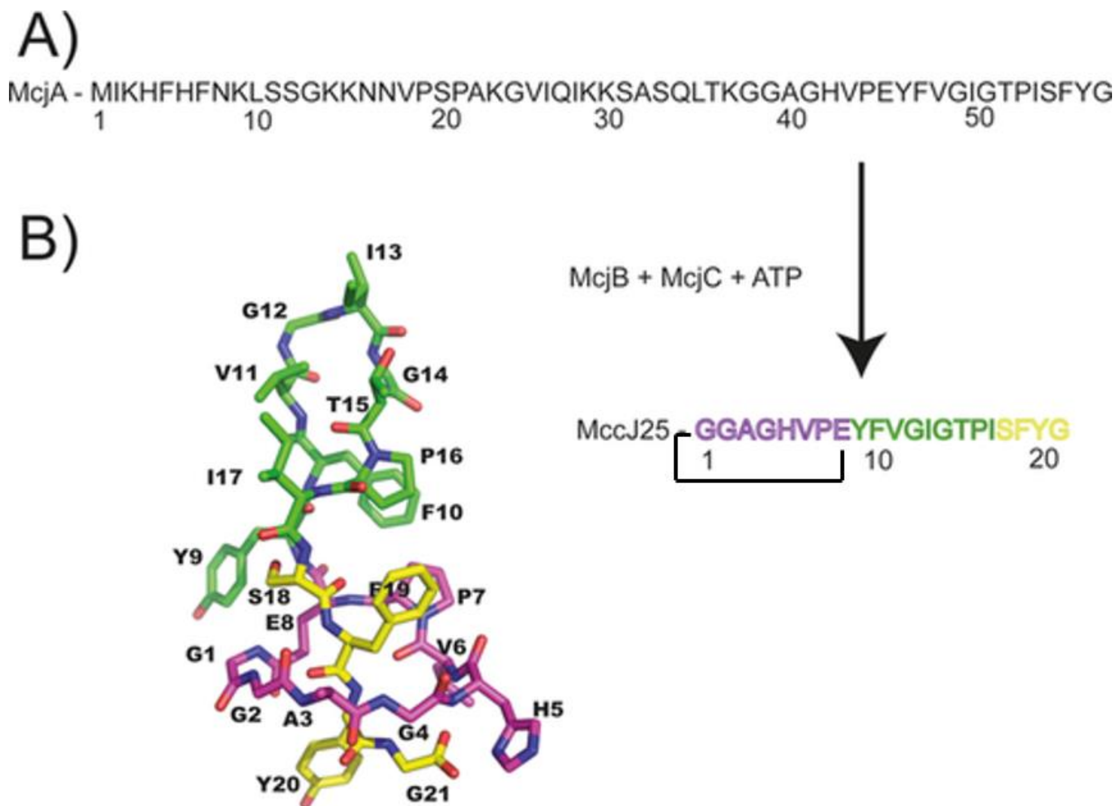


Figure- 5. Représentation schématique du processus de maturation de la MccJ25. A) Structure primaire du peptide précurseur McjA (58 acides aminés) impliqué dans synthèse de la MccJ25 mature (21 acides aminés) grâce au complexe McjB/McjC et la présence d'ATP. B) Structure tridimensionnelle de la MccJ25 mature déterminée par RMN (PDB ID: 1Q71) D'après (Assrir et al., 2016).

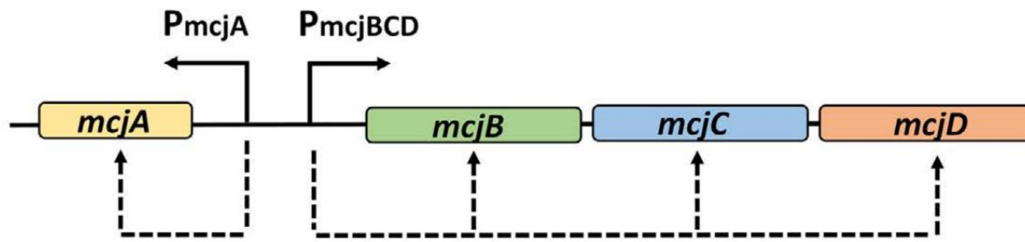


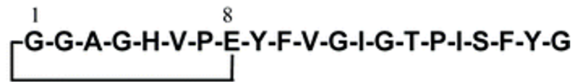
Figure- 6. Illustration schématique du système génétique de la MccJ25 montrant les promoteurs et les gènes responsables du processus de la biosynthèse de la MccJ25 mature. Schéma modifié et adapté de (Forkus et al., 2017).

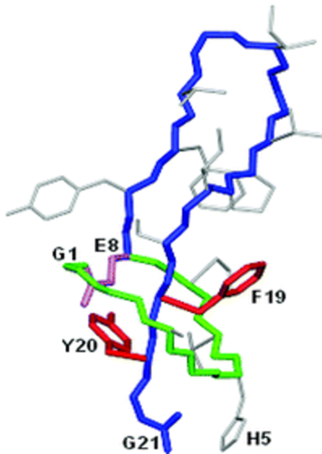
1.4.5.4.2. Structure de la MccJ25

La MccJ25 se distingue des autres microcines par son unique topologie mécaniquement verrouillée, incluant une structure entrelacée qui rappelle la forme d'un lasso de cow-boy donnant ainsi le nom de structure lasso (Bayro et al., 2003; Rosengren et al., 2003; Wilson et al., 2003). Comme chez tous les peptides lasso, la topologie de la MccJ25 est indispensable à son activité biologique. La MccJ25 présente une unique topologie appelée [1]rotaxane qui est caractérisée par la présence d'un seul macrocycle lié d'une manière covalente à un fragment linéaire qui le traverse de part et d'autre (Maksimov et al., 2012).

La MccJ25 est constituée de 21 résidus présentant la chaîne d'acides aminés suivante : GGAGHVPEYFVGIGTPISFYG. Les huit premiers acides aminés, Gly^{1}–Glu^{8} forment un cycle macrolactame ou une boucle dans la région N-terminale, tandis que les treize acides aminés de la région C-terminale Tyr^{9}–Gly^{21} forment une queue qui est insérée à l'intérieur de la boucle par des interactions non-covalentes et maintenue étroitement grâce à un encombrement stérique créés par les deux résidus aromatiques Phe^{19} et Tyr^{20} (Hammami et al., 2015), ce qui donne la forme lasso de la structure tridimensionnelle de la MccJ25 (Figure-7).

Cette structure particulière est très rigide et confère à la MccJ25 une stabilité remarquable pouvant supporter des niveaux de température et de pH extrêmes (Salomon and Farías, 1992b; Blond et al., 2001b) et une grande résistance à la lyse enzymatique par de nombreuses protéases (Blond et al., 1999a; Rosengren et al., 2004).





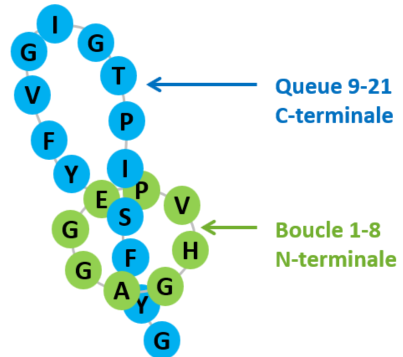


Figure- 5. Représentation schématique de la structure en lasso de la MccJ25. Structures primaires et tridimensionnelles de la MccJ25 (figure de droite) adapté de (Duquesne et al., 2007a). Structure secondaire de la MccJ25 (figure de gauche).

1.4.5.4.3. Mécanisme d'action de la MccJ25

La MccJ25 cible principalement les bactéries pathogènes appartenant à la famille des *Enterobacteriaceae* notamment *Escherichia coli* ainsi que des espèces de *Salmonella* et *Shigella* en inhibant leurs croissances à une concentration minimale inhibitrice (CMI) comprise entre 2 et 5 nM (Duquesne et al., 2007b; Rebuffat, 2012). Cette activité inhibitrice s'effectue plusieurs modes d'action (Rebuffat et al., 2004; Galván et al., 2018).

Le premier représente le mode d'action le plus prédominant de la MccJ25. Il repose sur son action sur l'ARN polymérase de la cellule cible (Bellomio et al., 2007; Duquesne et al., 2007b). En effet, la MccJ25 pénètre au travers la membrane de la cellule cible pour inhiber l'ARN polymérase bloquant ainsi la transcription et empêchant la synthèse des protéines (Figure-8). L'import de la MccJ25 dans la cellule cible implique le récepteur transmembranaire FhuA localisé à la membrane externe (Salomón and Farías, 1993), ainsi que le complexe TonB-ExbB-ExbD (Braun, 1995) et la protéine transmembranaire SbmA de la membrane interne (Salomón and Farias, 1995). La MccJ25 est d'abord reconnue sur la membrane externe de la cellule cible par le récepteur FhuA, utilisé généralement par les entérobactéries pour l'import du fer (Destoumieux-Garzón et al., 2005). Sa structure en lasso lui permet de prendre la place du ligand naturel de FhuA. Le complexe TonB/ExbB/ExbD est alors activé permettant ainsi la translocation de la MccJ25 au travers de la membrane externe et l'entrée dans l'espace périplasmique (Duquesne et al., 2007b). La MccJ25 pénètre ensuite dans le cytoplasme au travers la membrane interne grâce à la protéine de transport SbmA pour ensuite bloquer la synthèse de l'ARN par obstruction du canal secondaire de l'ARN polymérase.

La MccJ25 agit aussi selon un deuxième mode d'action qui consiste à cibler la mitochondrie et à bloquer la chaîne respiratoire en inhibant la consommation d'oxygène nécessaire à la viabilité de la cellule cible (Galván et al., 2018). Ce mécanisme d'action été observé chez *Salmonella* Typhimurium et *Escherichia coli* (Vincent et al., 2004; Vincent and Morero, 2009). Un autre mode d'action a été observé chez *Salmonella* Newport qui consiste en une perméabilisation par la MccJ25 de la membrane cytoplasmique provoquant ainsi une dissipation du potentiel de membrane (Rintoul et al., 2001b).

Le lien entre ces différents modes d'action par lesquelles la MccJ25 inhibent la croissance de plusieurs bactéries cibles demeure inconnu jusqu'à présent (Rebuffat, 2012).

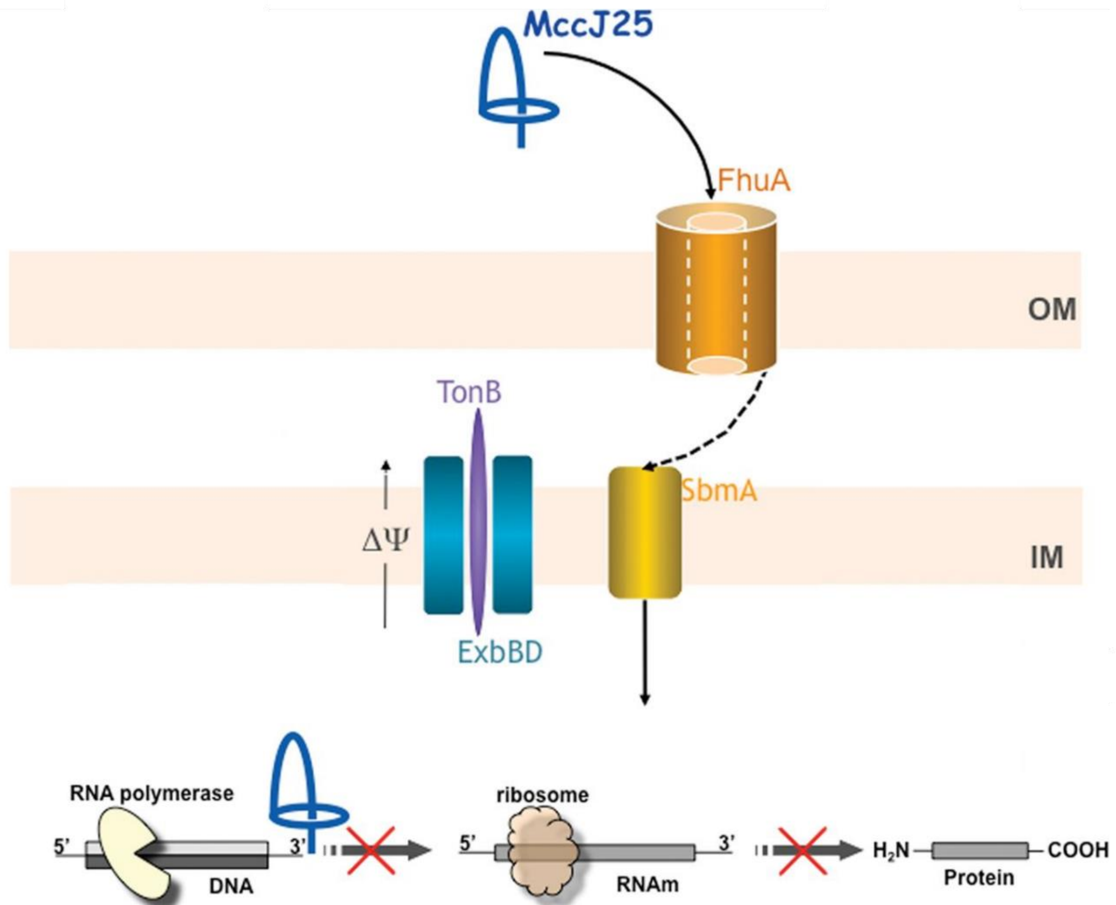


Figure- 6. Illustration schématique du principal mécanisme d'action de la MccJ25. Schéma modifié et adapté de (Rebuffat, 2012). FhuA (récepteur transmembranaire); SbmA (protéine transmembranaire); TonB et ExbBD (complexe TonB-ExbB-ExbD); OM (membrane externe), IM (membrane interne).

1.5. Travaux antérieurs et problématique

Contrairement à la diversité des études portant sur la structure, la biosynthèse et les mécanismes d'action de la MccJ25 citées auparavant, celles qui évaluent son activité inhibitrice dans des modèles *in vivo* et *in vitro* demeurent plus rares.

Une étude publiée en 2000 par Sablé *et al.* a évalué *in vitro* l'activité antibactérienne de la MccJ25 contre une collection de différentes souches d'*Escherichia coli* entéropathogènes (Sable et al., 2000). Il a été démontré que la MccJ25 inhibe significativement la croissance de la souche *E. coli* O157:H7 dans trois produits alimentaires testés, soient du lait écrémé stérile, du jaune d'œuf dilué dans de l'eau stérile et dans un extrait de viande. Ces résultats suggèrent la possibilité d'utiliser la MccJ25 pour le contrôle des aliments contaminés par les souches d'*E. coli* incluant le sérotype O157:H7.

En 2007, une étude *in vivo* publiée par Lopez *et al.* et utilisant un modèle murin a démontré qu'un traitement intra-péritonéal à la MccJ25 diminue significativement les comptes de *Salmonella* Newport chez la souris (Lopez et al., 2007a). Dans cette étude, il a été également démontré *ex vivo* la stabilité et l'efficacité anti-infectieuse de la MccJ25 dans le sang humain, le plasma et le sérum. De plus, aucune activité hémolytique de la MccJ25 n'a été observée.

Une étude récente publiée en 2017 par Yu *et al.* et menée chez les porcelets au sevrage a évalué l'impact *in vivo* de la MccJ25 sur les performances de croissance, la digestibilité des nutriments, le microbiote intestinal et la fonction de barrière intestinale des porcelets sevrés (Yu et al., 2017). Dans cette étude, il a été démontré que contrairement à un antibiotique conventionnel, la supplémentation alimentaire en MccJ25 à des concentrations égales à 1 et à 2 mg/kg améliore la performance de croissance des porcelets sevrés et réduit efficacement l'incidence de la diarrhée post-sevrage porcine. L'administration de la MccJ25 comme supplément alimentaire a également amélioré la fonction de barrière intestinale et la composition du microbiote intestinal et a activé la réponse immunitaire. Cette étude suggère que la MccJ25 pourrait être potentiellement utilisé comme une alternative aux antibiotiques traditionnels chez les porcelets sevrés.

La stabilité et l'activité inhibitrice de la MccJ25 dans les conditions gastro-intestinales n'a cependant jamais été étudiée jusqu'à présent. De plus, son impact sur l'équilibre et la diversité microbienne ainsi que l'activité métabolique du microbiote intestinal n'a jamais été étudié.

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1.6. Hypothèse et objectifs

1.6.1. Hypothèse de recherche

Compte tenu de sa structure particulière en lasso et son spectre d'activité restreint, la MccJ25 serait capable de résister aux différentes barrières rencontrées dans le tractus gastro-intestinal et de conserver son activité biologique sans pour autant affecter la composition et l'activité métabolique du microbiote colique du porc.

1.6.2. Objectif général

L'objectif général de cette thèse consiste à évaluer le potentiel de la MccJ25 comme alternative aux antibiotiques en production porcine à l'aide de modèles dynamiques *in vitro* du tube digestif.

1.6.3. Objectifs spécifiques

Objectif 1 : Évaluer la stabilité et l'activité biologique de la MccJ25 durant son transit dans le tractus gastro-intestinal en utilisant le simulateur dynamique *in vitro* du tube digestif (TIM1).

Objectif 2 : Étudier *in vitro* l'activité inhibitrice de la MccJ25 dans les conditions physiologiques du côlon porcin.

Objectif 3 : Démontrer l'activité inhibitrice de la MccJ25 dans les conditions coliques du porc et élucider son impact sur le métagénome du microbiote colique porcin à l'aide du modèle *in vitro* de fermentation en continu (PolyFermS).

Objectif 4 : Étudier l'impact de la MccJ25 sur le métabolome du microbiote colique du porc.

Chapitre 2. Fate and biological activity of the antimicrobial lasso peptide microcin J25 under gastrointestinal tract conditions

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Keywords: microcin J25, lasso peptide, GI TIM-1 model, degradome, duodenum, pancreatic enzymes

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2.1. Résumé

La microcine J25 (MccJ25) est une bactériocine qui possède une activité inhibitrice contre des bactéries pathogènes à Gram-négatif telles que *Salmonella*, *Shigella*, et *Escherichia coli*. Grâce à sa structure en lasso, ce peptide de 21 acides aminés est remarquablement stable en présence de la chaleur et à des valeurs extrêmes de pH et résistance à de nombreuses protéases. Dans cette étude, nous avons utilisé le simulateur dynamique *in vitro* du tube digestif TIM-1 afin d'évaluer la stabilité et l'activité antibactérienne de la MccJ25 lors de son passage dans la partie proximale du tractus gastro-intestinal humain. La concentration de la MccJ25 a été mesurée par HPLC dans les différents compartiments du TIM-1, et l'inhibition de *Salmonella enterica* serotype Enteritidis a été évaluée en utilisant les tests qualitatifs et quantitatifs de l'activité antibactérienne. Les analyses par LC-MS/MS ainsi qu'une analyse subséquente par l'approche des réseaux moléculaires utilisant la plateforme GNPS (Global Natural Product Social Molecular Networking) et l'analyse de la dégradation peptidique en présence d'enzymes protéolytiques mimant les conditions gastro-intestinales ont permis de déterminer le comportement de la MccJ25 par l'identification de ces principaux produits de dégradation. La MccJ25 s'est montrée relativement stable dans les conditions gastriques, mais se dégrade rapidement dans le compartiment duodénal, notamment en présence de la pancréatine. Parmi les composants de la pancréatine, l'élastase I est apparue principalement responsable de la dégradation de la MccJ25, alors que l'α-chymotrypsine était moins efficace.

2.2. Abstract

The bacteriocin microcin J25 (MccJ25) inhibits the growth of Gram-negative pathogens including *Salmonella* and *Shigella* species, and *Escherichia coli*. This 21-amino acid peptide has remarkable stability to heat and extreme pH values and resistance to many proteases, thanks to a characteristic lasso structure. In this study, we used the dynamic simulator TIM-1 as gastro-intestinal tract model to evaluate the stability and antibacterial activity of MccJ25 during passage through the proximal portion of the human gastrointestinal tract. MccJ25 concentration was measured in the different simulator sections by HPLC, and inhibition of *Salmonella enterica* serotype Enteritidis was evaluated using qualitative and quantitative assays. LC-MS/MS analysis and subsequent molecular networking analysis on the Global Natural Product Social Molecular Networking platform (GNPS) and analysis of the peptide degradation in the presence of proteolytic enzymes mimicking the gastro-intestinal conditions permitted to delineate the fate of MccJ25 through identification of the main degradation products. MccJ25 was relatively stable under gastric conditions, but degraded rapidly in the compartment mimicking the duodenum, notably in the presence of pancreatin. Among pancreatin components, elastase I appeared primarily responsible for MccJ25 breakdown, while α-chymotrypsin was less efficient.

2.3. Introduction

Antibiotics have been used for nearly a century in human and veterinary medicine and for therapeutic or growth-promoting purposes in livestock production (Gustafson and Bowen, 1997; Gyles, 2008). They remain the most widely used chemicals for treating infections. Their use in animal feed began to grow steadily in the early 1950s, when it was observed that keeping animals free of bacterial infections increased livestock productivity considerably and thus reduced production costs (Viola and DeVincent, 2006). However, overuse of antibiotics with structural features similar to those used in human medicine led to the emergence of pathogens with resistance to at least one antibiotic used to treat infections (Reardon, 2014). This worrying development led the European Union to declare a total ban on antibiotics as growth promoters in animal feeds since 2006 (Castanon, 2007a). The first industries affected by the ban were hog and poultry production, the principal users of antibiotics as feed additives. It is clear that a satisfactory alternative to antibiotics would be welcome in these industries. Among the alternatives being proposed are natural antimicrobial peptides produced by bacteria, essentially lactic acid bacteria, termed bacteriocins (Ennahar et al., 2000). The production of bacteriocins by bacterial strains is well documented in the literature (Hammami et al., 2013b). However, very few bacteriocins have been approved by regulatory agencies for use in food, medical or veterinary products, despite the increasing urgency of the demand for such compounds.

A bacteriocin is most often defined as a peptide of molecular mass mostly < 10,000 Da that is inhibitory to species closely related to the producer species. Bacteriocins differ from antibiotics in being synthesized by translation of an mRNA transcript, having generally a narrow spectrum of activity and being effective at concentrations as low as nanomolar (Nes, 2011). Some bacteriocins may undergo post-translational modifications and as such belong to the family of ribosomally-synthesized and post-translationally-modified peptides (RiPPs) (Arnison et al., 2013) Their potential as antimicrobials, especially in food matrices such as dairy, meat and plant products, has been evaluated repeatedly (Cleveland et al., 2001). However, few studies have focused on their potential as inhibitors of enteric pathogens in animals or humans. The stability of bacteriocins in the gastrointestinal tract is uncertain, and their resistance to acid, bile and gastric and pancreatic enzymes is a current research topic. In a previous study, we showed that the class IIa bacteriocin pediocin PA-1 was sensitive to gastrointestinal conditions, losing completely its antibacterial activity in the small intestine (Kheadr et al., 2010).

In this work, we examined the potential of microcin J25 (MccJ25), a well-studied bacteriocin produced by the Gram-negative species *Escherichia coli* and of high interest as a potential alternative to conventional antibiotics, because of its potent inhibitory activity against pathogens belonging to the genera *Escherichia, Salmonella* and *Shigella* (Salomon and Farías, 1992b; Sable et al., 2000; Rintoul et al., 2001b; Vincent and Morero, 2009). Interestingly its activity is maintained in complex matrices and in a mouse model of infection (Lopez et al., 2007a). MccJ25 is produced by *Escherichia coli* AY25 as a translated peptide precursor, which undergoes posttranslational modification ensured by two dedicated enzymes (Duquesne et al., 2007c; Yan et al., 2012). The process leads to the 21 amino-acid mature peptide (Figure 1), which encompasses an N-terminal macrolactam ring of 8 residues, through which the C-terminal tail threads and is tightly maintained by steric hindrance of two aromatic bulky side chains (Phe19 and Tyr20) (Bayro et al., 2003; Rosengren et al., 2003; Wilson et al., 2003). This confers to the peptide a unique [1]rotaxane topology known as the lasso structure. This peculiar highly rigid structure has been shown to confer to MccJ25 remarkable stability to high temperatures and extreme pHs (Salomon and Farías, 1992b; Blond et al., 2001b) and to make the molecule highly resistant to many proteases (Blond et al., 1999a; Rosengren et al., 2004). Such features are important if this bacteriocin is to be considered for use as a natural alternative to antibiotics. However, although its properties hold promise, there are no reports on its stability and inhibitory activity under the human GI tract conditions.

The goal of the present study was therefore to evaluate the stability and the inhibitory activity of MccJ25 against *Salmonella enterica* serotype Enteritidis in the upper portion of the human GI tract using a dynamic simulator (TIM-1), as gastrointestinal (GI) tract model.

2.4. Material & methods

2.4.1. Bacterial strains and growth conditions

MccJ25 was produced by a clone of *E. coli* MC4100 carrying a mutated pTUC202 plasmid that confers chloramphenicol resistance (Solbiati et al., 1999). *S. enterica* subsp. *enterica* ser. Enteritidis (Ducasse et al., 2012) was used as a sensitive strain for antibacterial activity assays. Both bacterial strains were obtained from Prof. Sylvie Rebuffat (Muséum national d'Histoire naturelle, MCAM laboratory, Paris, France). As described by (Ducasse et al., 2012), *E. coli* MC4100 harbouring the pTUC202 plasmid was cultured overnight at 37°C in Luria–Bertani (LB) broth (Difco, Sparks, MD, USA) supplemented

with 34 mg/mL chloramphenicol under aerobic condition while *S. enterica* ser. Enteritidis was cultured overnight at 37°C in LB broth under aerobic condition.

2.4.2. Enzymes and chemicals

Pancreatin from porcine pancreas, pepsin from porcine gastric mucosa (EC 3.4.23.1), trypsin (EC 3.4.21.4), elastase type IIA from porcine pancreas (EC 3.4.21.36) and α-chymotrypsin from bovine pancreas (EC 3.4.21.1) were purchased from Sigma-Aldrich. Lipase from *Rhizopus oryzae* (EC 3.1.1.3) was from Amano Enzymes Inc. (Nagoya, Japan). All chemicals (HPLC solvents and salts for bacteriological media) used in the study were of high analytical grade.

2.4.3. Production and purification of MccJ25

Production and purification of MccJ25 was adapted from Blond *et al.* (Blond et al., 1999a; Rebuffat et al., 2004). Briefly, 3 L of minimal medium (M63) containing KH_2PO_4 (3 g/L), K_2HPO_4 (7 g/L), $(NH_4)_2HPO_4$ (2 g/L) and casamino acids (1 g/L) were supplemented after autoclaving with $MgSO_4$ 1 mL/L, glucose 10 mL/L and thiamine 1 mL/L, added as 0.2 µm-filtered solutions (respectively 20%, 20% and 1 g/L) and inoculated with *E. coli* MC4100 pTUC202 culture (2% v/v) grown in LB broth. Overnight culture grown at 37°C with rotary shaking at 250 rpm was centrifugated at 8000 *g* for 20 min at 4°C. MccJ25 was isolated from the culture supernatant by solid-phase extraction using a Sep-Pak C18 35 cc cartridge (Waters) washed previously with 200 mL of methanol and 200 mL of ultra-pure water. The bacteriocin was eluted with acetonitrile/water (30% v/v) containing 0.1% HCl. Acetonitrile was eliminated using a rotary evaporator R-215 (Büchi Labortechnik AG, Flawil, Switzerland) before MccJ25 was purified by reverse-phase high-performance liquid chromatography (RP-HPLC, Beckman Coulter System Gold Preparative HPLC system, Mississauga, ON, Canada) on a preparative C18 column (Luna 10 µm, 250 mm × 21.10 mm, Phenomenex, CA, USA) at a flow rate of 10 mL/min using a 25–100% linear gradient of filtered acetonitrile/5 mM HCl with absorbance measurement at 214 nm. The purified MccJ25 eluted as a single peak at 47.69 % acetonitrile (Amp: 1.8 AU, 18 min) was lyophilized and stored at -20°C.

2.4.4. Dynamic simulator of the GI tract (TIM-1)

A TIM-1 apparatus (TNO Nutrition and Food Research Institute, Zeist, The Netherlands, described previously by Minekus *et al.,* (Minekus et al., 1995)) was used to study the behavior of MccJ25 during passage through the stomach and small intestine. This apparatus is composed of four compartments

that simulate the stomach, duodenum, jejunum and ileum. These compartments are interconnected in series by computer-controlled peristaltic valve pumps (Fernandez et al., 2014). Hollow-fiber membrane dialysis units connected to the jejunal and ileal compartments provide simulation of nutrient absorption without loss of MccJ25. The gastric pH initially set at 4.5 was decreased gradually to 2.7 after 40 min, 2.0 after 60 min and 1.8 after 90 min of digestion by injection of HCl (1 mol/L), and subsequently to 1.7, where it was maintained from 120 to 300 min. The pH was adjusted to 6.3, 6.5 and 7.4 in the duodenal, jejunal and ileal compartments, respectively by injection of $NaHCO_3$ solution (1 mol/L). Simulated gastric secretions containing pepsin (0.19 mg/mL) or lipase (0.25 mg/mL), both in an electrolyte solution (NaCl 6.2 g/L, KCl 2.2 g/L, $CaCl_2$ 0.3 g/L, $NaHCO_3$ 1.5 g/L), were delivered into the gastric compartment at a flow rate of 0.25 mL/min. Simulated duodenal secretions consisting of solutions of 8% pancreatin (pancreatin from porcine pancreas 4 x USP, Sigma-Aldrich), 4% porcine bile (porcine bile extract, Sigma-Aldrich) and an electrolyte solution (NaCl 5.0 g/L, KCl 0.60 g/L, $CaCl_2$ 0.30 g/L, pH 7.0) were injected at 0.25, 0.5 and 0.25 mL/min respectively. The total injected volumes were logged. A 2 mg/mL trypsin solution (EC 3.4.21.4 from bovine pancreas, Sigma-Aldrich) was also injected into the duodenal compartment. The initial jejunal and ileal contents consisted of the electrolyte solution at pH 7.0. The fast transit protocol, digestion of a liquid, low-calorie feed condition and adult conditions were applied for all experiments.

The simulated digestion was performed in duplicate. 310 mL of a sterile 0.1 mg/mL MccJ25 solution were injected into the gastric compartment, and aliquots of 7 mL were collected therefrom in duplicate at 0, 30 and 60 min, from the duodenal compartment at 30, 60 and 120 min and from the jejunal and ileal compartments at 60, 120 and 240 min. Each sample was heat-treated at 70°C for 10 min to inactivate all enzymes and then centrifugated at 8300 *g* for 10 min at 4°C. The supernatant was loaded onto a Sep-Pak C18 35 cc cartridge (Waters) washed beforehand with 200 mL of methanol, 200 mL of pure acetonitrile and 200 mL of 0.1% formic acid in ultra-pure water. MccJ25 was eluted with 100 mL of 0.1% formic acid solution, and a 1/1 (v/v.) blend of filtered acetonitrile and 0.1% formic acid. The eluted fraction containing MccJ25 was concentrated using a rotary evaporator R-215 (Büchi Labortechnik AG, Flawil, Switzerland), lyophilized, re-dissolved in 600 µL of ultra-pure water supplemented with 0.1 % acetonitrile.

2.4.5. Quantification of MccJ25 by reverse-phase HPLC

MccJ25 was quantified by reverse-phase HPLC using an analytical C18 column (Aeris™ 3.6 µm, PEPTIDE XB-C18, 250 mm × 4.6 mm, Phenomenex, CA, USA). A linear standard curve was generated by injecting stock solutions of MccJ25 known quantities (100, 50, 20, 10, 5, 2, 1, 0.5, 0.25, 0.1, 0.05 and 0.025 µg) into the HPLC column at a flow rate of 1 mL/min and a gradient of 100% solvent A (ultra-pure water/ 0.1% trifluoroacetic acid) and 0% solvent B (acetonitrile/ 0.1% TFA) to 50% solvent A and 50% solvent B and measuring the absorbance at 230 and 280 nm. The area of the peak corresponding to MccJ25 was calculated by integration using the HPLC software and the standard curve was obtained from the area for each known concentration. Samples collected from TIM-1 compartments and processed as described above were then injected on the HPLC column to monitor the MccJ25 concentrations.

2.4.6. Static models simulating gastric and duodenal digestions

A 50 mL volume of the gastric solution described above was adjusted to 1.7 using 1 mol/L HCl. Duodenal solution (35 mL) consisted of 8% pancreatin, 4% porcine bile solutions and 2 mg/mL trypsin solution in intestinal electrolyte solution adjusted to pH 6.3 using 1 mol/L $NaHCO_3$. To both solutions, MccJ25 was added to obtain a final concentration of 0.1 mg/mL. They were then shaken at 150 rpm at 37°C. Aliquots of 5 mL were collected in duplicate at 0, 30 and 60 min from the gastric mixture and at 30, 60 and 120 min from the duodenal mixture for MccJ25 quantification and antibacterial activity assays. Each sample was heated at 70°C for 10 min and centrifugated at 8300 g for 10 min at 4°C, and the supernatant was mixed with pure acetone (1:1) and centrifugated at 12,000 g for 10 min at 4°C. The pellet was washed twice with acetone and finally dissolved in ultra-pure water and concentrated using a Speed-Vac concentrator (Model SC110A, Savant Instruments Inc., Farmingdale, NY) to a final volume of 1 mL for MccJ25 determination using the reverse-phase HPLC method described above.

The duodenal experiment was repeated with pancreatin, trypsin, elastase and α-chymotrypsin, each used alone and at a final concentration of 8 mg/mL in the intestinal electrolyte solution. Aliquots of 3 mL were collected in duplicate and analyzed as described above.

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2.4.7. Antibacterial activity assays

Inhibitory activity of MccJ25 against *S. enterica* ser. Enteritidis (Ducasse et al., 2012) was tested first using the critical dilution micro-method adapted from Turcotte et al. (Turcotte et al., 2004). In a sterile flat-bottom 96-well polystyrene micro-plate (Falcon; Becton Dickinson), 125 µL of sample were diluted in twofold series in LB medium, and 50 µL of an overnight LB culture of *S. enterica* ser. Enteritidis diluted 1000-fold in fresh LB medium were then added to each well. The micro-plate was incubated at 37°C for 18 h. MccJ25 activity was expressed as arbitrary units (AU) /mL using the formula 2^n (1000/125) where 2 is the dilution factor, n is the number of wells showing inhibition of *S. enterica* ser. Enteritidis (absorbance < 0.1) and 125 is the volume in µL of the tested fraction.

The inhibitory activity was evaluated qualitatively using the agar well diffusion method described previously by Tagg et al. (Tagg et al., 1976b). Sterile LB (25 mL) containing 0.75% (w/v) agar was seeded with 150 µL of an overnight culture of *S. enterica* ser. Enteritidis and poured into a sterile Petri dish. Wells (7 mm diameter) were cut in the solidified agar using the open end of a sterile 5 mL pipette and filled with 80 µL of tested sample. Plates were incubated at 37°C for 18 h, and the diameter of the zone of inhibition was measured.

2.4.8. Analysis of MccJ25 degradation products by LC-MS/MS

Samples collected from all experiments were analyzed by LC-MS/MS on an ultra-high-performance LC system (Ultimate 3000 RSLC, Thermo Scientific) connected to a high-resolution electrospray ionization – quadrupole – time of flight (ESI-Q-TOF) mass spectrometer (Maxis II ETD, Bruker Daltonics). Separation was achieved on an Acclaim RSLC Polar Advantage II column (2.2 µm, 2.1 × 100 mm, Thermo Scientific) at a flow rate of 300 µL/min, using the following gradient of solvent A (ultra-pure water / 0.1% formic acid) and solvent B (HPLC-MS grade acetonitrile / 0.08% formic acid) over a total run time of 17.5 min: linear increase from 10% B to 60% B for 12 min, linear increase to 100% B for 0.2 min, decrease to 10% B for 0.5 min. The ESI-Q-TOF instrument was externally calibrated before each run using a sodium formate solution consisting of 10 mM sodium hydroxide in isopropanol / 0.2% formic acid (1:1, v/v). The MS spectra were acquired in positive ion mode in the mass range *m/z* 60 – 2000. The source parameters were as follows: nebulizer gas 35 psi, dry gas 8 L/min, capillary voltage 3500 V, end plate offset 500 V, temperature 200 °C. Automatic MS/MS was carried out using the following set-up: absolute threshold 500 counts, preferred charge states: 1–3, unknown charge states excluded, cycle time 3 s, MS spectra rate: 2 Hz, MS/MS spectra rate: 3 Hz at

5000 counts increasing to 6 Hz at 50 000 counts or above. MS/MS active exclusion was set after 1 spectrum unless intensity increased fivefold. Collision energy was automatically calculated from *m/z* and charge states. The LC-MS/MS data were treated with Data Analysis 4.3 (Bruker Daltonics).

2.4.9. Molecular Networking

The high resolution LC-MS/MS data were converted into mgf files using Data Analysis 4.3 (Bruker Daltonics) and subjected to the online GNPS workflow (http://gnps.ucsd.edu) (Yang et al., 2013; Wang et al., 2016), using the following set-up: parent ion mass tolerance of 0.05 Da, fragment ion mass tolerance 0.05 Da, cluster minimal size 3, minimum matched peaks 4 and minimum cosine similarity score 0.4. Resulting networks were visualized using Cytoscape 3.5.1.

2.4.10. Nomenclature

To avoid any ambiguity, the rotaxane species generated by hydrolysis of MccJ25 were denoted using the nomenclature previously proposed by our group (Ducasse et al., 2012). They are written {Ax-By/Cz-Dt} where A, B, C, D represent the one-letter amino acid of the extremities of the two associated peptides and x, y, z, t indicate their respective positions in the sequence. The [2]rotaxane product ions generated by MS/MS were termed using the $[(b_r)^*(y_s)]$ nomenclature previously introduced (Zirah et al., 2011), where r and s indicate the number of amino acids in the non-covalently associated b- and y-type product ions.

2.5. Results

2.5.1. Stability and antibacterial activity of MccJ25 in the upper portion of the human GI tract

The stability and antibacterial activity of MccJ25 were first evaluated using a dynamic simulator of the upper GI tract (TIM-1 system). MccJ25 stability was determined using reverse-phase HPLC. Figure 2 shows the theoretical and actual distributions of MccJ25 in the three TIM-1 compartments over time. In the stomach, the two profiles did not differ markedly, indicating that MccJ25 tolerated gastric conditions. In the duodenum compartment however, the experimental concentration was significantly lower than the theoretical level, suggesting that the microcin was degraded to a considerable extent at this stage. Samples collected from the TIM-1 model were also tested for their antimicrobial activity against *S. enterica* ser. Enteritidis using the micro-titration assay. As shown in Table 1, 1406.2 AU of MccJ25 inhibitory activity were detected per mL of stomach contents at 0 min. This activity decreased

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after 30 and 60 min, which is likely due to the dynamic nature and function of the TIM-1 system (Minekus et al., 1995). However, the drop to 58.1 AU/mL observed at 30 min in the duodenum and later in this compartment, as well as in the jejunum confirmed the concentration measurements. These results were also confirmed by LC-MS/MS as shown in Figure 3. MccJ25 was eluted at 8.7 min and showed doubly charged $[M+2H]^{2+}$ and triply charged $[M+3H]^{3+}$ ions at *m/z* 1054.02 and 703.01, respectively. Its MS/MS spectrum showed the typical rotaxane fragment ions diagnostic of the lasso structure (Zirah et al., 2011; Jeanne Dit Fouque et al., 2018). These fragment ions result from multiple cleavages within the Y9-I18 loop region, which generate b- and y-type product ions associated through steric hindrance provided by the side chains of F19 and Y20. The MccJ25 species was detected as an intense peak in the stomach compartment but was absent from the early stages of the duodenum section.

In order to further delineate the nature of MccJ25 degradation products, a molecular network was generated from high-resolution LC-MS/MS data using the GNPS workflow. In the TIM-1 stomach compartment, a single degradation product named DP1 was revealed at 2124 Da (Figure S1, Table 2, Table S1). The + 18 u increment from the molecular mass of MccJ25 indicated a hydrolysis in the Y9-I18 loop of the peptide, yielding a [2]rotaxane entity, as already reported (Ducasse et al., 2012). The amino acid carrying the + 18 u increment, determined from the MS/MS spectra, permitted to locate the hydrolysis site at G14-T15 (Figure S2). The dynamic nature of the TIM-1 model hampered the detection of MccJ25 degradation products formed in the duodenum section, due to the dilution effect. Therefore, the stability and inhibitory activity of MccJ25 were further evaluated in a static model that simulates individually gastric and duodenal physicochemical conditions with a fixed total volume.

A molecular network constructed from the LC-MS/MS data of the duodenal incubations permitted to determine the degradation pattern of MccJ25 in duodenal conditions (Figure 4). Analysis of the a, b and c clusters revealed nine degradation products named DP2 to DP10 (Table 2, Table S1), which hydrolysis sites were determined from the MS/MS spectra (Figures S3-S11), as described above. These degradation products, which differ from the degraded form DP1 identified in the stomach, result from either single or multiple hydrolyses in the Y9-G14 region. The degraded forms DP2 to DP4, detected from t_0, correspond to hydrolysis of MccJ25 at Y9-F10 (Figure S3), F10-V11 (Figure S4), and I13-G14 (Figure S5) peptide bonds, respectively. The six other products (DP5 to DP10) formed at 60

or 120 min correspond to hydrolysis of MccJ25 at two cleavage sites, leading to the release of a single amino acid or of two to four amino acid peptide segments (Figures S6-S11).

Measurement of the inhibitory activity of MccJ25 against *S. enterica* ser. Enteritidis in the static model simulating gastric and duodenal conditions confirmed these results (Table 3). The antibacterial activity measured at t_0 and after 30 and 60 min under gastric conditions was 16384 arbitrary units per mL. This activity decreased significantly (to 4096 AU/mL) as soon as the beginning of exposure to duodenal conditions, confirming that MccJ25 is degraded instantly in the duodenum. MccJ25 activity increased unexpectedly at 60 min (to 8192 AU/mL) from its level measured at 30 min, then decreased at 120 min to the initial value.

2.5.2. Stability and antibacterial activity of MccJ25 in the presence of proteolytic enzymes

In order to determine which duodenal component is responsible for the breakdown of MccJ25, the effects of pancreatin, trypsin, elastase and chymotrypsin were examined separately in the duodenal solution. The degradation of MccJ25 in these conditions was first monitored by LC-MS/MS (Figure 5, Table 2). MccJ25 appeared only slightly degraded in the presence of pancreatin, highly impacted by elastase and almost unaffected in the presence of chymotrypsin (Figure 5A). The degradation products previously identified in the static model of duodenum digestion were searched in priority and assigned from their retention times and MS/MS spectra. Degradation products with a single hydrolysis in the Y9-I18 loop (*m/z* 709) were detected in the three conditions (Figure 5B). Pancreatin hydrolysis yielded mainly DP2, while elastase generated DP3 and DP4 and chymotrypsin yielded DP2 and DP3. Finally, the degradation products DP7 to DP10, corresponding to multiple hydrolysis in the loop region, were formed in the presence of pancreatin as well as in the presence of elastase (Figure 5C-F).

The action of pancreatic enzymes on MccJ25 was also evaluated using the antibacterial activity assay. The inhibitory activity of MccJ25 measured after each experiment by the microdilution assay is reported in Table 3. In the presence of trypsin, no significant decrease in Mcc25 activity was observed even after 120 min of reaction (data not shown). By contrast, pancreatin decreased the inhibitory activity of MccJ25 instantly by four-fold relative to the positive control. A further eight-fold decrease then occurred over the 120 min of reaction. In the presence of elastase, the antibacterial activity was reduced instantly (since t_0) by a factor of 256 (128 AU/mL compared to 32768 AU/mL for the positive control, and decreased by an additional four-fold (8 AU/mL) over the next 2 hours. Chymotrypsin also

affected the antibacterial activity but to a lower extent, with a reduction to 2048 and 1024 AU/mL, respectively, at 0 and 120 min time points. These results were confirmed qualitatively by using the agar diffusion assay (Figure 5G-H). An inhibitory activity was detected in the presence of pancreatin at 0 min of digestion, showing an inhibition zone of 1.5 cm compared with the 1.8 cm inhibition zone of the positive control (data not shown). This inhibitory activity decreased after 120 min of digestion by pancreatin, showing an inhibition zone of 1.2 cm. Contrary to pancreatin, trypsin did not induce any reduction of the inhibitory activity of MccJ25 over digestion (data not shown). This could be explained by the absence of trypsin cleavage site in the MccJ25 sequence. The inhibitory activity of MccJ25 in presence of elastase was markedly reduced at t_0 showing an inhibition zone of 1.1 cm compared with the positive control (1.8 cm). After 120 min of digestion, MccJ25 was shown to have totally lost its antibacterial activity as evidenced by the absence of inhibition zone. Therefore, LC-MS/MS data and antibacterial assays both indicate that MccJ25 is partially degraded in the presence of pancreatin and completely degraded in the presence of elastase after 120 min, while chymotrypsin has no significant effect on the stability and antibacterial activity of MccJ25.

2.6. Discussion

The identification of peptide and protein degradation products in complex samples has emerged in the past years thanks to the advance in analytical techniques. Such approaches provide key information on the degradome, defined as the sum of the products of various degradation processes, which include substrates of proteases and their generated cleavage products (Strlič et al., 2009). They rely generally on bottom-up proteomic approaches (*i.e.*, hydrolysis followed by identification of the generated peptides through database searching) (Lai et al., 2015), and have benefited from biochemical methods such as N-terminal enrichment strategies (Sabino et al., 2017), gel electrophoresis-based methods: protein topography and migration analysis platform (PROTOMAP) (Dix et al., 2014; Kaisar et al., 2016) and in-gel degradomics (Vidmar et al., 2017) as well as from the development of targeted analytical workflows (Savickas and auf dem Keller, 2017) and specific algorithms (El-Assaad et al., 2017). The [1]rotaxane lasso structure of MccJ25, yielding [2]rotaxane species upon hydrolysis, precludes the use of database search to identify degradation products. Nevertheless, the use of MS/MS-based molecular networking revealed very efficient to reveal the degradome. This study thus constitutes to our knowledge the first application of molecular networking to degradomics. In addition, the + 18 u increment permitted to assign unambiguously the hydrolysis site of the [2]rotaxane degradation products.

The present study has provided evidence of the degradation of MccJ25 in both dynamic and static models of digestion. MccJ25 was stable in the stomach compartment but underwent multiple hydrolysis as soon as it entered the duodenal compartment, resulting in a drastic decrease in its antibacterial activity against *Salmonella enterica*. Interestingly, a slight increase of the inhibitory activity of MccJ25 was observed after 30 min of duodenal digestion followed by an additional decrease reaching the initial arbitrary activity value. This fluctuation might be due to the presence of bile salts in the duodenal medium. Bile is produced continuously by the liver and enters the duodenum via the bile duct during digestion in order to facilitate digestion by emulsifying dietary lipids (Redinger and Small, 1972). The microscopic micelles thus formed are hydrophilic at the external surface and hydrophobic inside. MccJ25 contains several hydrophobic amino acids (Rebuffat et al., 2004), which may bind to micelles formed in the duodenum and be released later, as is reported to occur in microencapsulation processes (Corona-Hernandez et al., 2013). Altogether, these data indicate that MccJ25 is degraded by proteases upon entering the duodenum. Thus, the compact and stable structure of MccJ25 permits to tolerate acidic conditions of stomach but does not provide protection towards duodenal proteases, which limit its application as orally administrated antimicrobial.

The stability of MccJ25 were later evaluated in the presence of pancreatic enzymes separately in order to determine which among the duodenal components are responsible for the MccJ25 degradation. Pancreatin is known as a mixture of five proteases, namely trypsin, chymotrypsin, elastase, carboxypeptidase A and carboxypeptidase B (Whitcomb and Lowe, 2007), the first of these having no cleavage site in the MccJ25 amino acid sequence. Carboxypeptidases A and B are exopeptidases that cleave peptide bonds at the carboxyl terminus, next to amino acids having aromatic, neutral or acidic side chains for the first one and to basic amino acids, primarily arginine and lysine, for the second (Whitcomb and Lowe, 2007). MccJ25 does not contain any cleavage site for either of these two exopeptidases. Elastase and chymotrypsin are endopeptidases that hydrolyze peptide bonds next to uncharged (Ala, Gly, Ser) and aromatic (Phe, Tyr, Trp) amino acids, respectively (Walker, 2004). Thus, MccJ25 does have cleavage sites for elastase and chymotrypsin, some of which are located in the more exposed loop region of the compact lasso structure. The action of elastase and chymotrypsin was further evaluated by LC-MS/MS. Elastase yielded the same degradation products previously detected in the presence of pancreatin (DP7 to DP10) involving the F10-V11 cleavage as well as the Y9-F10 cleavage in addition to the loss of one to four residues. The action of elastase also revealed a novel degradation form DP4, not observed in the presence of

pancreatin, which is located at the I13-G14 position. However, only two degraded forms with no cleaved segments resulting from the action of chymotrypsin were observed. The first degraded form is located at the Y9-F10 position was observed at the beginning of treatment with chymotrypsin but not with elastase, while the second degraded form located at the F10-V11 position was detected in the presence of both chymotrypsin and elastase. MccJ25 thus appears to have two major cleavage sites for the two pancreatic proteases: the first is located at the Y9-F10 position corresponding to the chymotrypsin cleavage site, and the second is located at the I13-G14 position which correspond to the elastase cleavage site.

The effects of pancreatic enzymes on MccJ25 were confirmed using antimicrobial activity assays. The results showed a significant decrease of the inhibitory activity of MccJ25 in the presence of pancreatin, contrary to trypsin which did not induce any significant reduction of the antibacterial activity of MccJ25 over the reaction time (data not shown). These results are consistent with the mechanism of action of trypsin, which is an endopeptidase known to participate in the digestion of proteins in the small intestine by catalyzing the hydrolysis of peptide bonds located on the carboxyl side of lysine or arginine residues (Whitcomb and Lowe, 2007), and the absence of such residues in MccJ25. A remarkable reduction of the antibacterial activity of MccJ25 was observed since the addition of elastase. This reduction was continued during the reaction time until a total loss of the activity detected at 120 min. Chymotrypsin was not shown to induce a significant effect as shown with elastase. Nevertheless, the chymotrypsin cleavage pattern was observed in samples treated with elastase. This suggests that cleavage by elastase is activated by chymotrypsin. Indeed, it has been reported that elastase is secreted by the pancreas in an inactive pro-enzyme form called proelastase and then activated in the duodenum by trypsin (Ghelis et al., 1978; Whitcomb and Lowe, 2007). This appears to explain the different degradation patterns obtained with the same enzyme according to the model.

2.7. Conclusion

The present study has provided compelling evidence that despite its highly stable structure in various harsh conditions, MccJ25 is partly degraded in the human gastrointestinal tract, practically as soon as it enters the duodenum, thus losing its antibacterial activity, and that the pancreatic protease elastase is largely responsible for this degradation (Figure 6). This finding suggests that MccJ25 is unlikely to affect the human GI tract and may therefore be considered as a safe alternative to antibiotics and for food applications. However, this also means that in order to be considered as an antibacterial agent to

be potentially used in the GI tract in man or animals, MccJ25 would have to be encapsulated or bioengineered to stabilize the loop region towards hydrolysis while maintaining the antibacterial activity.

2.8. Authors contributions

SN participated in the experimental design, was responsible for the laboratory analysis, data analysis, and writing. RH participated in the experimental design, laboratory analysis and data analysis. SZ participated in the experimental design, laboratory analysis, data analysis, and writing. BF participated in the experimental design and contributed to data analysis. SR participated in the experimental, design data analysis, and writing. IF participated in the experimental design, data analysis, and writing.

2.9. Funding

Financial support for this work was received from the National Science and Engineering Research Council of Canada (NSERC). The Fond de Recherche du Québec-Nature et Technologies (FQRNT) is gratefully acknowledged for awarding a scholarship to Sabrine Naimi.

2.10. Conflict of interest statement

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

2.11. Acknowledgments

We would like to thank the Analytical Platform of the Muséum national d'Histoire naturelle (Paris, France) for access to the mass spectrometry facilities and the MNHN staffs of the mass spectrometry (Arul Marie, Lionel Dubost) and bacteriology (Manon Vandervennet) platforms for their technical assistance. We also would like to thank Steven Davis for reading the manuscript and providing language help.

2.12. Tables & figures

2.12.1. Tables

Table 1. Inhibitory activity of MccJ25 against *S. enterica* ser. Enteritidis in the TIM-1 compartments.

TIM-1 compartment	Reaction time (min)	Antibacterial activity (AU/mL)
Stomach	0	1406.2
	30	751.6
	60	848.4
Duodenum	30	58.1
	60	67.7
	120	3.8
Jejunum	60	22.3
	120	13.4

Table 2. Degradation pattern of MccJ25 in the *in vitro* digestive models. Incubation time of first detection (min), molecular weight (Da) and retention times (RT, min) of MccJ25 degradation products.

Incubation time of first detection (min)	Degradation product	Mw (Da)	RT (min)	Assignment	Segment cleaved off
0	-	2106.02	8.6	MccJ25	-
Stomach dynamic model (TIM-1)					
60	DP1	2124.03	7.2	{G1-G14/T15-G21}	-
Duodenum static model					
0	DP2	2124.03	7.0	{G1-Y9/F10-G21}	-
0	DP3	2124.03	7.3	{G1-F10/V11-G21}	-
0	DP4	2124.03	7.4	{G1-I13/G14-G21}	-
60	DP5	2010.95	6.6	{G1-G12/G14-G21}	{I}
60	DP6	1953.93	6.9	{G1-V11/G14-G21}	{GI}
60	DP7	1854.86	6.6	{G1-F10/G14-G21}	{VGI}
60	DP8	1976.96	6.5	{G1-Y9/V11-G21}	{F}
60	DP9	1707.79	5.7	{G1-Y9/G14-G21}	{FVGI}
120	DP10	1877.89	6.2	{G1-Y9/G12-G21}	{FV}
Pancreatin					
0	DP2	2124.03	7.1	{G1-Y9/F10-G21}	-
0	DP3	2124.03	7.3	{G1-F10/V11-G21}	-
0	DP7	1854.86	6.7	{G1-F10/G14-G21}	{VGI}
0	DP8	1976.96	6.5	{G1-Y9/V11-G21}	{F}
0	DP9	1707.79	5.8	{G1-Y9/G14-G21}	{FVGI}
0	DP10	1877.89	6.3	{G1-Y9/G12-G21}	{FV}
Elastase					
0	DP3	2124.03	7.3	{G1-F10/V11-G21}	-
0	DP4	2124.03	7.4	{G1-I13/G14-G21}	-
0	DP7	1854.86	6.7	{G1-F10/G14-G21}	{VGI}
0	DP8	1976.96	6.5	{G1-Y9/V11-G21}	{F}
0	DP9	1707.79	5.8	{G1-Y9/G14-G21}	{FVGI}
0	DP10	1877.89	6.3	{G1-Y9/G12-G21}	{FV}
Chymotrysin					
0	DP2	2124.03	7.1	{G1-Y9/F10-G21}	-
0	DP3	2124.03	7.3	{G1-F10/V11-G21}	-

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Table 3. Inhibitory activity of MccJ25 against *S. enterica* ser. Enteritidis in the stomach and duodenal static model and in the presence of pancreatin, elastase or chymotrypsin, based on the micro-dilution assay.

Condition	Reaction time (min)	Antibacterial activity (AU/mL)
Stomach static model	0	16384
	30	16384
	60	16384
Duodenum static model	0	4096
	60	8192
	120	4096
Positive control	-	32768
Pancreatin	0	4096
	120	512
Elastase	0	128
	120	8
Chymotrypsin	0	2048
	120	1024

2.12.2. Figures

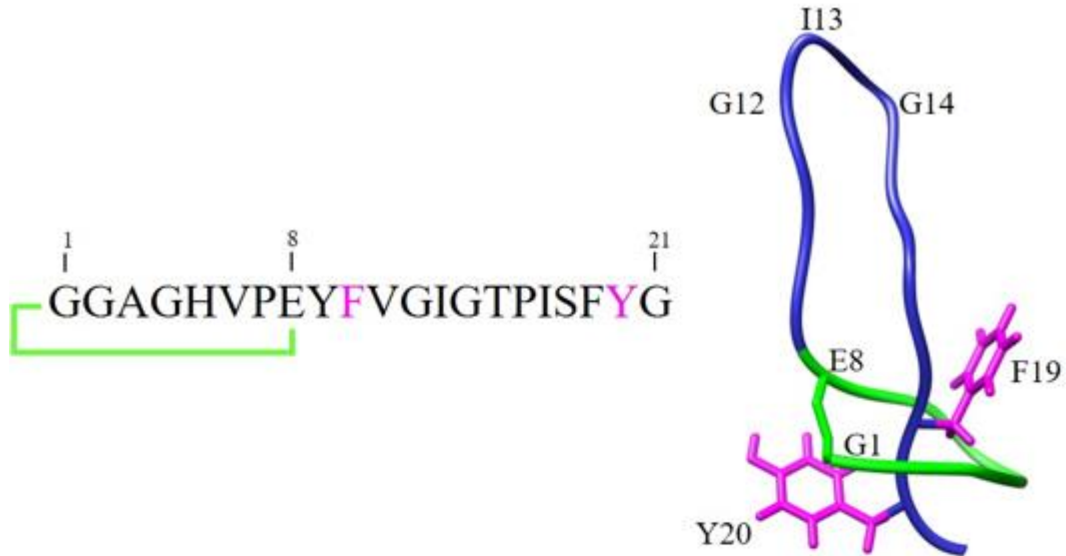

Figure 1. Schematic representation of the primary and three-dimensional structures of MccJ25.

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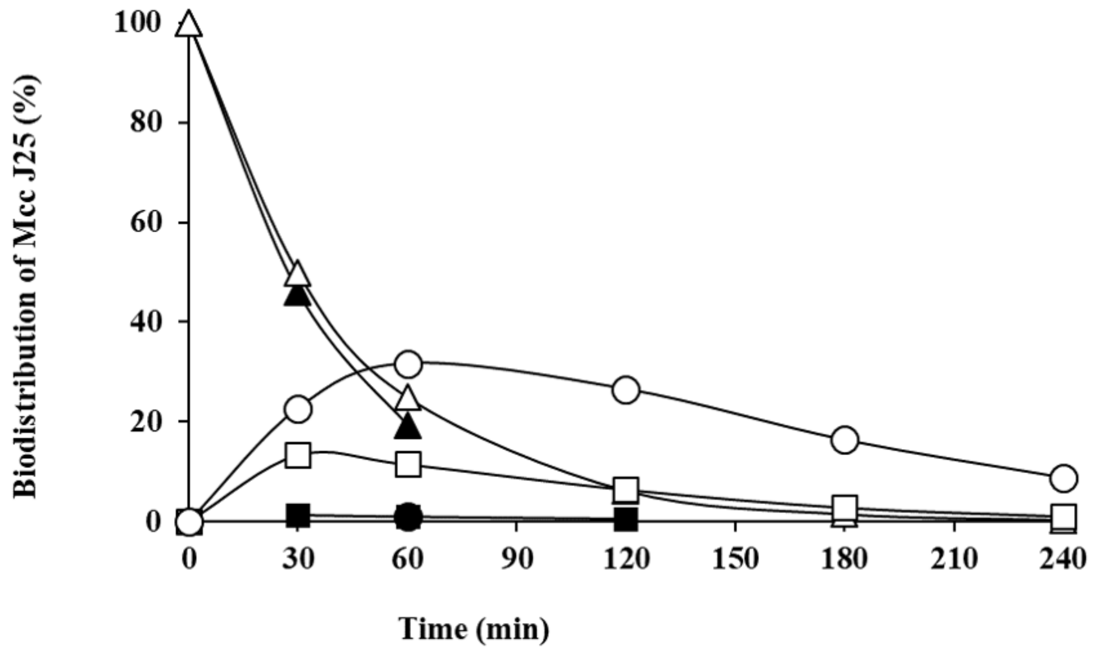


Figure 2. Theoretical and actual distributions of MccJ25 (black: measured values; white: software-calculated theoretical values) in the three compartments of the TIM-1 simulator of the human GI tract over time (triangles: gastric compartment; squares: duodenum; circles: jejunum). Data are means of two independent repetition experiments.

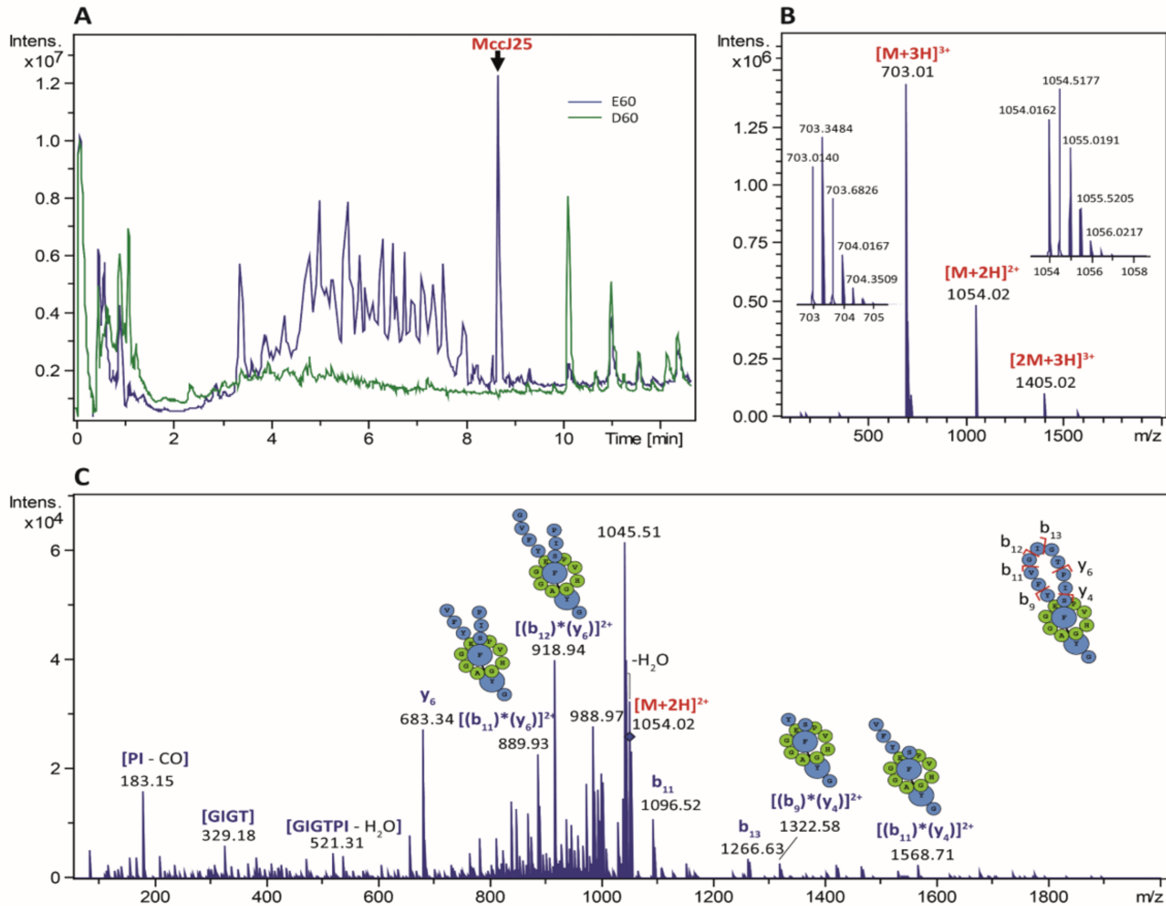
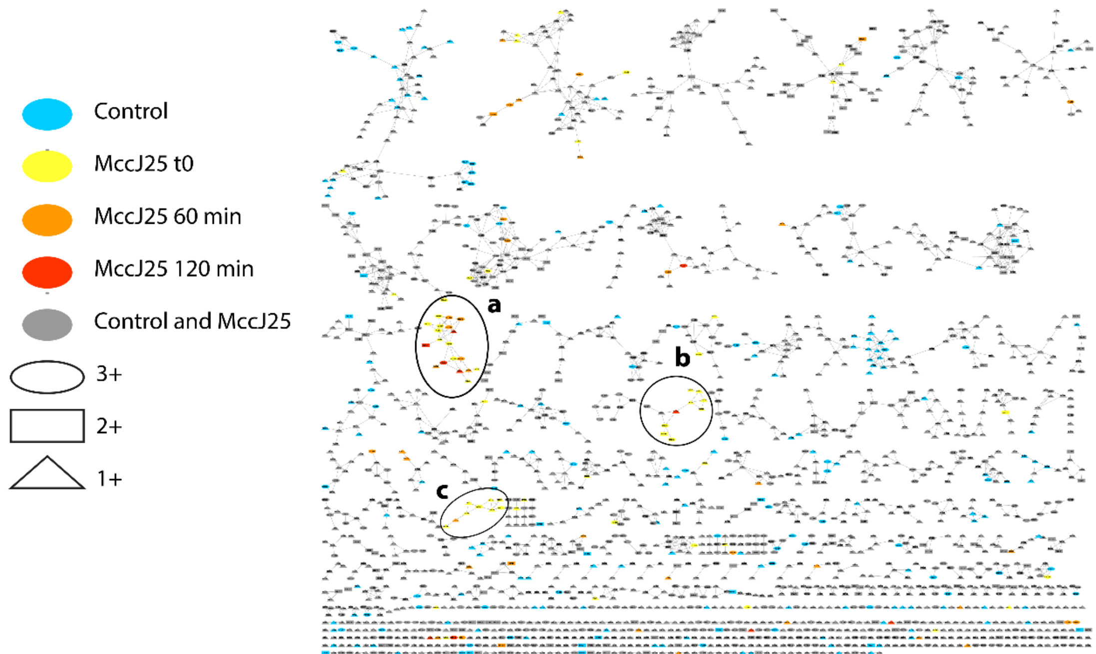


Figure 3. Digestion of MccJ25 in the TIM-1 model. (A) Total ion chromatograms generated by LC-MS/MS after 60 min digestion of MccJ25 in the TIM-1 stomach (E60, in blue) and duodenum (D60, in green) sections. (B) Positive ion ESI-MS spectrum of MccJ25 detected after digestion in the TIM-1 stomach section (retention time 8.7 min). (C) MS/MS spectrum of the $[M+2H]^{2+}$ ion of MccJ25 (*m/z* 1054.02, collision voltage 40 V) showing the typical rotaxane fragment ions diagnostic of the lasso structure.



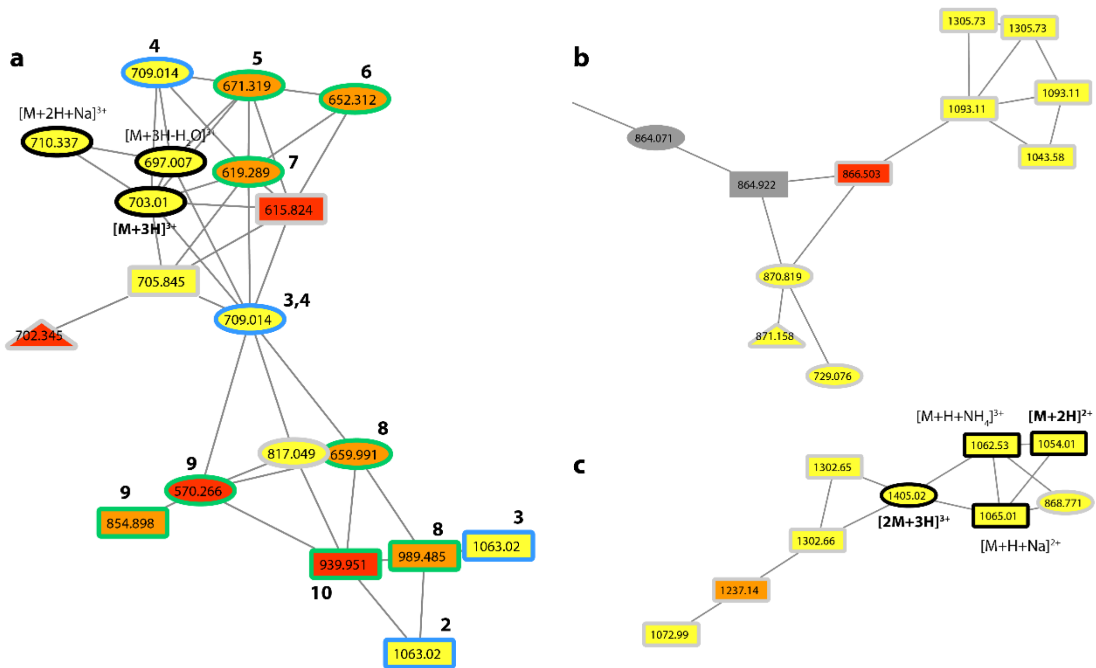


Figure 4. Molecular network showing MccJ25 degradome in the static model of duodenum digestion. The three clusters (a, b, c) corresponding to the degradation compounds derived from MccJ25 are enlarged. The nodes assigned to MccJ25 are bordered in black and annotated. The nodes corresponding to degradation products with one and two hydrolysis are bordered in blue and green, respectively and annotated with numbers referring to Table 2. The nodes, which were not considered to delineate the degradome are bordered in grey. They correspond to compounds with *m/z* values higher than those of MccJ25 or to mixed precursor selection in MS/MS experiments.

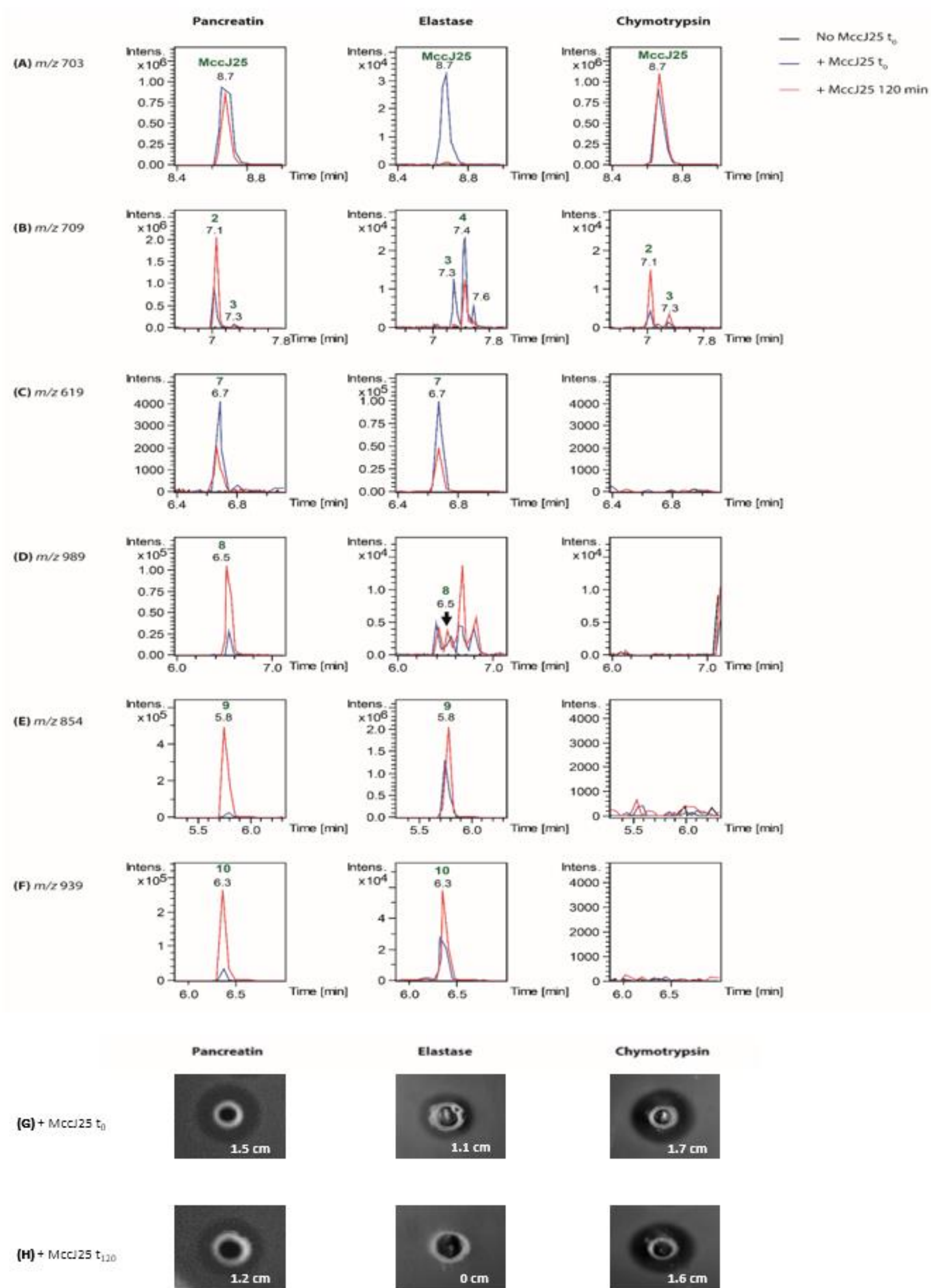


Figure 5. Detection of MccJ25 and selected degradation products formed in the presence of duodenal proteases. (left: pancreatin, middle: elastase, right: chymotrypsin). Extracted ion chromatograms (*m/z* $\pm$ 0.01) of (A) *m/z* 703.01 corresponding to the $[M+3H]^{3+}$ species of MccJ25, (B) *m/z* 709.02 corresponding to the $[M+3H]^{3+}$ ion of degradation products with a single hydrolysis in the loop region of MccJ25, (C) *m/z* 619.29 corresponding to the $[M+3H]^{3+}$ species of compound DP7 {G1-F10/G14-G21}, (D) *m/z* 989.49 corresponding to the $[M+2H]^{2+}$ species of compound DP8 {G1-Y9/V11-G21}, (E) *m/z*

854.90 corresponding to the $[M+2H]^{2+}$ species of compound DP9 {G1-Y9/G14-G21}, (F) *m/z* 939.95 corresponding to the $[M+2H]^{2+}$ species of compound DP10 {G1-Y9/G12-G21}. The peaks are annotated with the compound numbers defined in Table 2. (G) Inhibitory activity of MccJ25 against *S.* enterica ser. Enteritidis in the presence of pancreatin, elastase and chymotrypsin either upon initial contact (t_0) or (H) after 120 min of contact (t_{120}), based on the agar diffusion assay.

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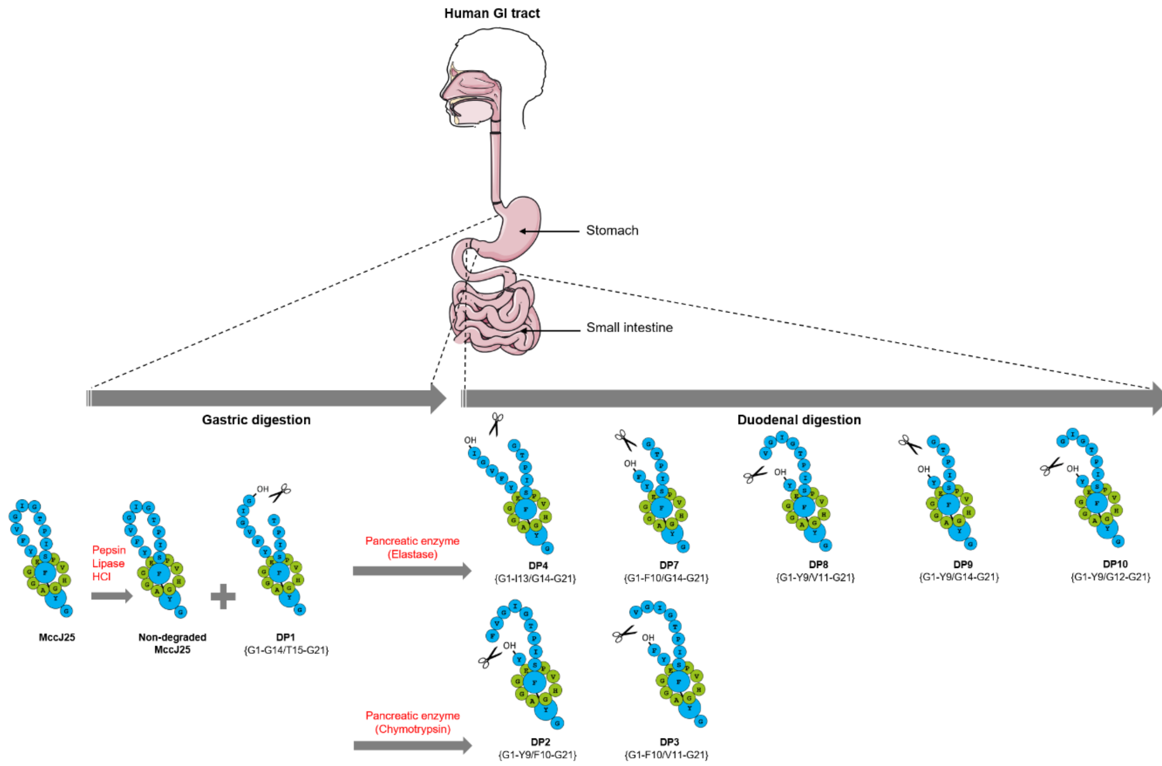

Figure 6. Schematic representation of MccJ25 breakdown in the human stomach and duodenum.

2.13. Supplementary data

2.13.1. Tables

Table S 1. Degradation pattern of MccJ25 in the *in vitro* digestive models. Incubation time of first detection (min), experimental and calculated monoisotopic *m/z*, corresponding error (ppm), molecular mass and retention times (RT) of the species detected on the molecular network for MccJ25 and its degradation products.

Inc. time (min)	Ion species	Exp. *m/z*	Calc. *m/z*	Error (ppm)	M (Da)	RT (min)	Assignment	Degradation product
0	$[M+3H]^{3+}$	703.0145	703.0143	-0.57	2106.02	8.6	MccJ25	-
	$[M+2H]^{2+}$	1054.0172	1054.0178	-0.28				
	$[2M+3H]^{3+}$	1405.0187	1405.0213	-1.85				
	$[M+2H+Na]^{3+}$	710.3404	710.3416	-1.69				
	$[M+3H-H_2O]^{3+}$	697.0103	697.0108	-0.72				
	$[M+H+NH_4]^{2+}$	1062.5296	1062.5311	-1.41				
	$[M+H+Na]^{2+}$	1065.0087	1065.0088	-0.09				
Stomach dynamic model (TIM-1)								
60	$[M+3H]^{3+}$	709.0184	709.0178	0.85	2124.03	7.2	{G1-G14/T15-G21}	DP1
	$[M+2H]^{2+}$	1063.0231	1063.0231	0.00				
Duodenum static model								
0	$[M+3H]^{3+}$	709.0182	709.0178	0.56	2124.03	7.0	{G1-Y9/F10-G21}	DP2
	$[M+2H]^{2+}$	1063.0229	1063.0231	-0.19				
0	$[M+3H]^{3+}$	709.0186	709.0178	1.13	2124.03	7.3	{G1-F10/V11-G21}	DP3
	$[M+2H]^{2+}$	1063.0236	1063.0231	0.47				
0	$[M+3H]^{3+}$	709.0184	709.0178	0.85	2124.03	7.4	{G1-I13/G14-G21}	DP4
	$[M+2H]^{2+}$	1063.0234	1063.0231	0.28				
60	$[M+3H]^{3+}$	671.3238	671.3231	1.04	2010.95	6.6	{G1-G12/G14-G21}	DP5
60	$[M+3H]^{3+}$	652.3175	652.3160	2.30	1953.93	6.9	{G1-V11/G14-G21}	DP6
60	$[M+3H]^{3+}$	619.2935	619.2932	0.48	1854.86	6.6	{G1-F10/G14-G21}	DP7
60	$[M+3H]^{3+}$	659.9955	659.9950	0.76	1976.96	6.5	{G1-Y9/V11-G21}	DP8
	$[M+2H]^{2+}$	989.4889	989.4889	0.00				
60	$[M+3H]^{3+}$	570.2706	570.2704	0.35	1707.79	5.7	{G1-Y9/G14-G21}	DP9
	$[M+2H]^{2+}$	854.9018	854.9019	-0.12				
120	$[M+2H]^{2+}$	939.9551	939.9547	0.43	1877.89	6.2	{G1-Y9/G12-G21}	DP10

2.13.2. Figures

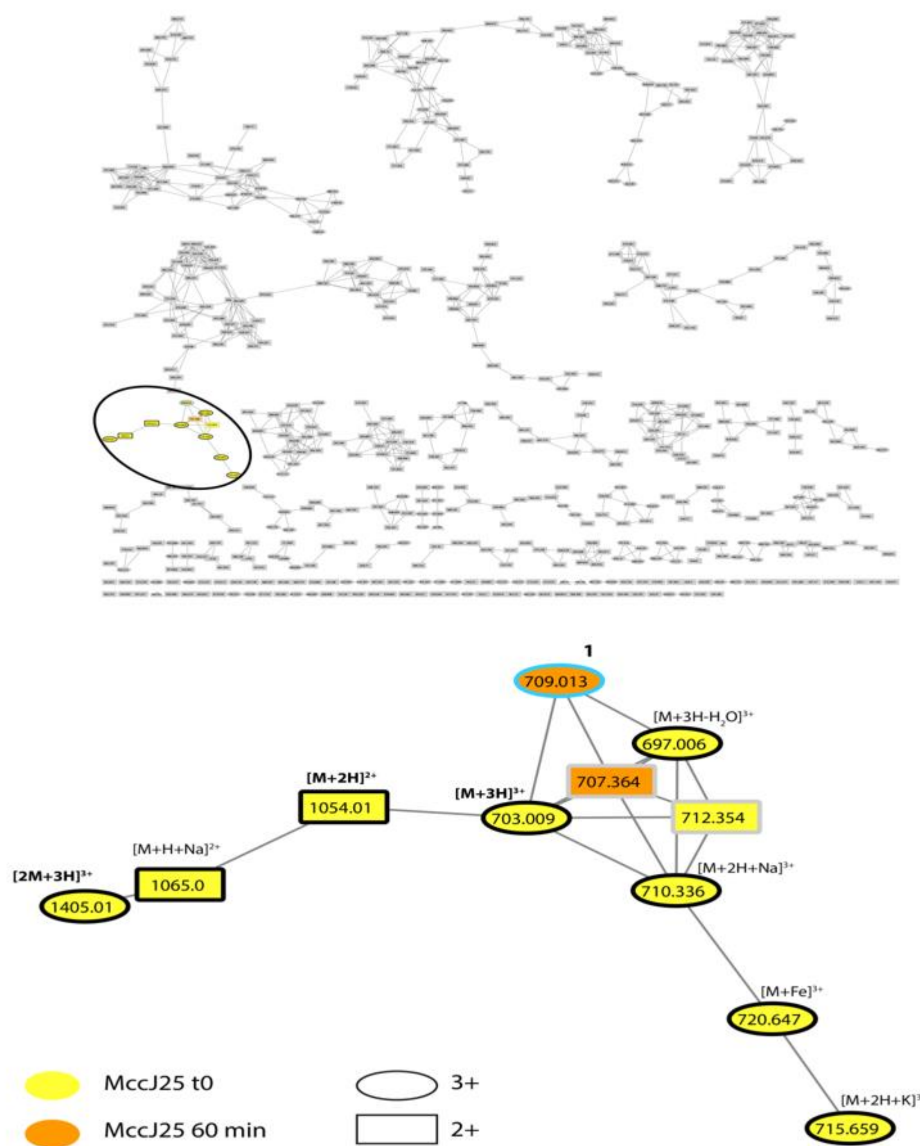

Figure S 1. Molecular network showing MccJ25 degradome in the stomach compartment of the TIM-1 dynamic model of digestion. The cluster corresponding to MccJ25 is enlarged. The nodes assigned to MccJ25 are bordered in black and annotated. The node corresponding to hydrolysed MccJ25 is bordered in blue and numbered as in Table 2. The nodes not considered to delineate the degradome are bordered in grey. They correspond to mixed precursor selection in MS/MS experiments.

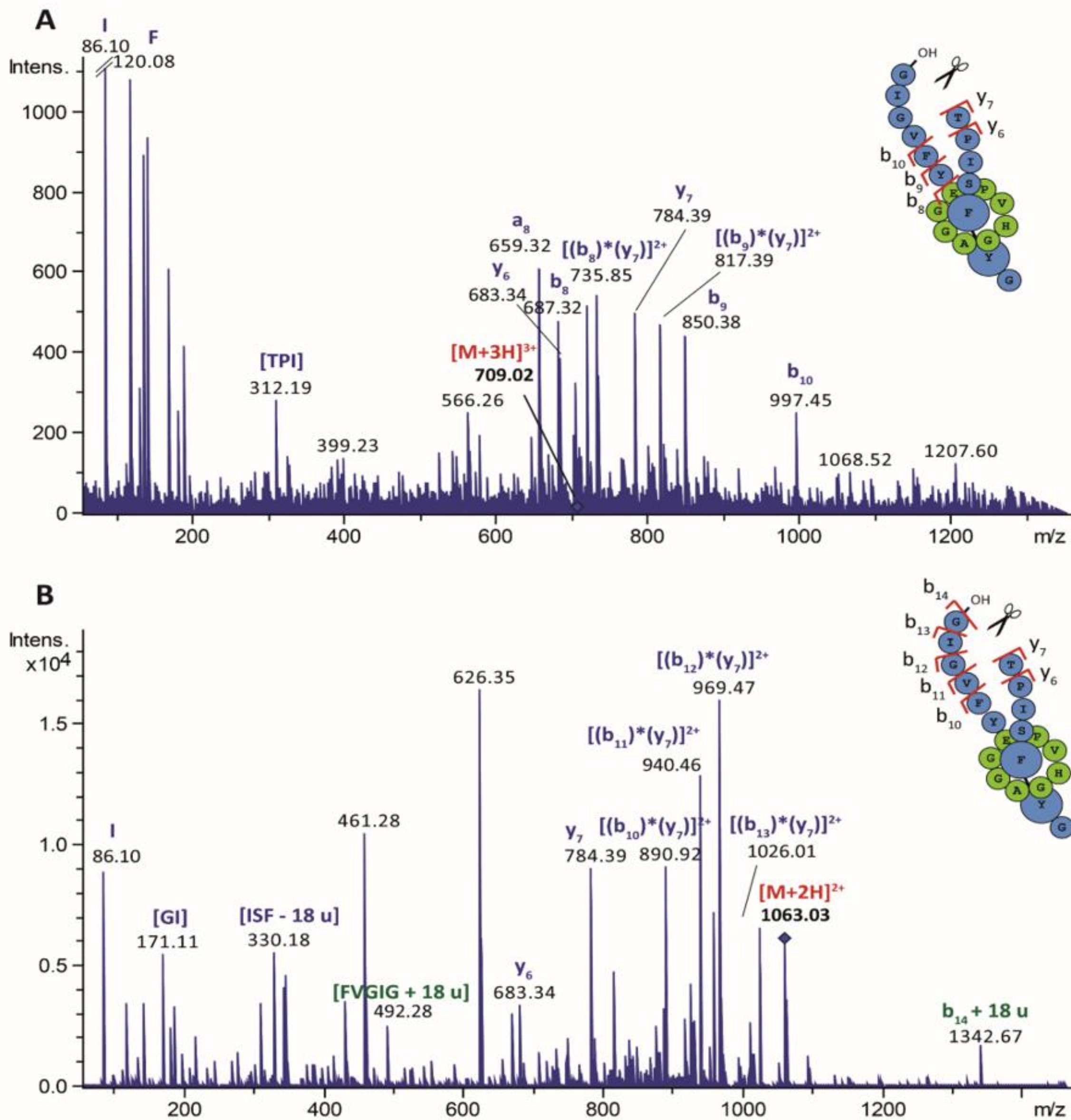

Figure S 2. MS/MS spectra of MccJ25 hydrolyzed at G14-T15 formed in the stomach compartment of the TIM-1 dynamic model, DP1 {G1-G14/T15-G21}. A: [M+3H]3+ (m/z 709.02, CE 32.6 eV), B: [M+2H]2+ (m/z 1063.03, CE 40 eV). The hydrolysis site was determined from the + 18 u increment product ions (in green).

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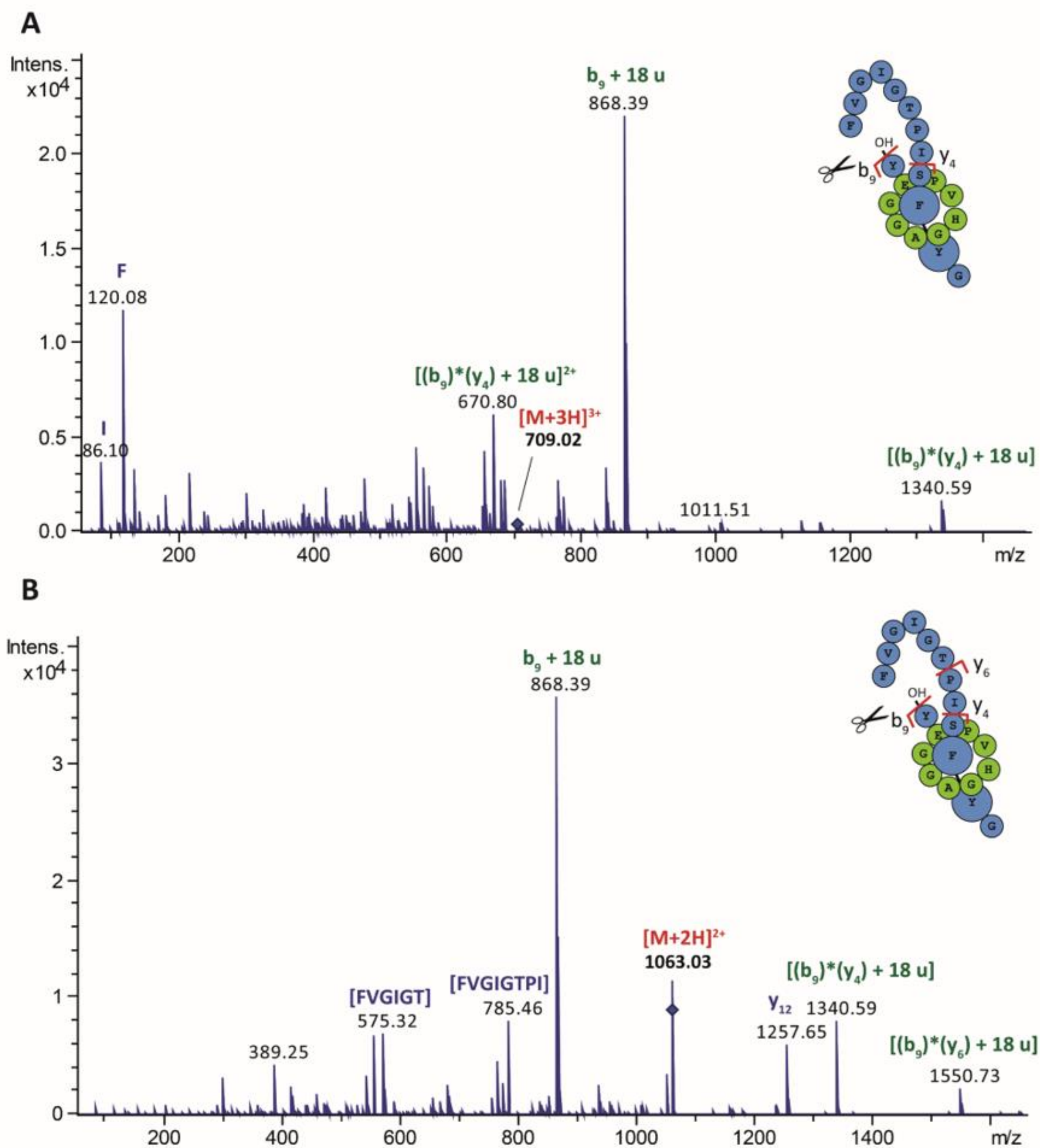

Figure S 3. MS/MS spectra of MccJ25 hydrolyzed at Y9-F10 formed in the static model of duodenum, DP2 {G1-Y9/F10-G21}. A: [M+3H]3+ (m/z 709.02, CE 32.6 eV), B: [M+2H]2+ (m/z 1063.03, CE 40 eV). The hydrolysis site was determined from the + 18 u increment product ions (in green).

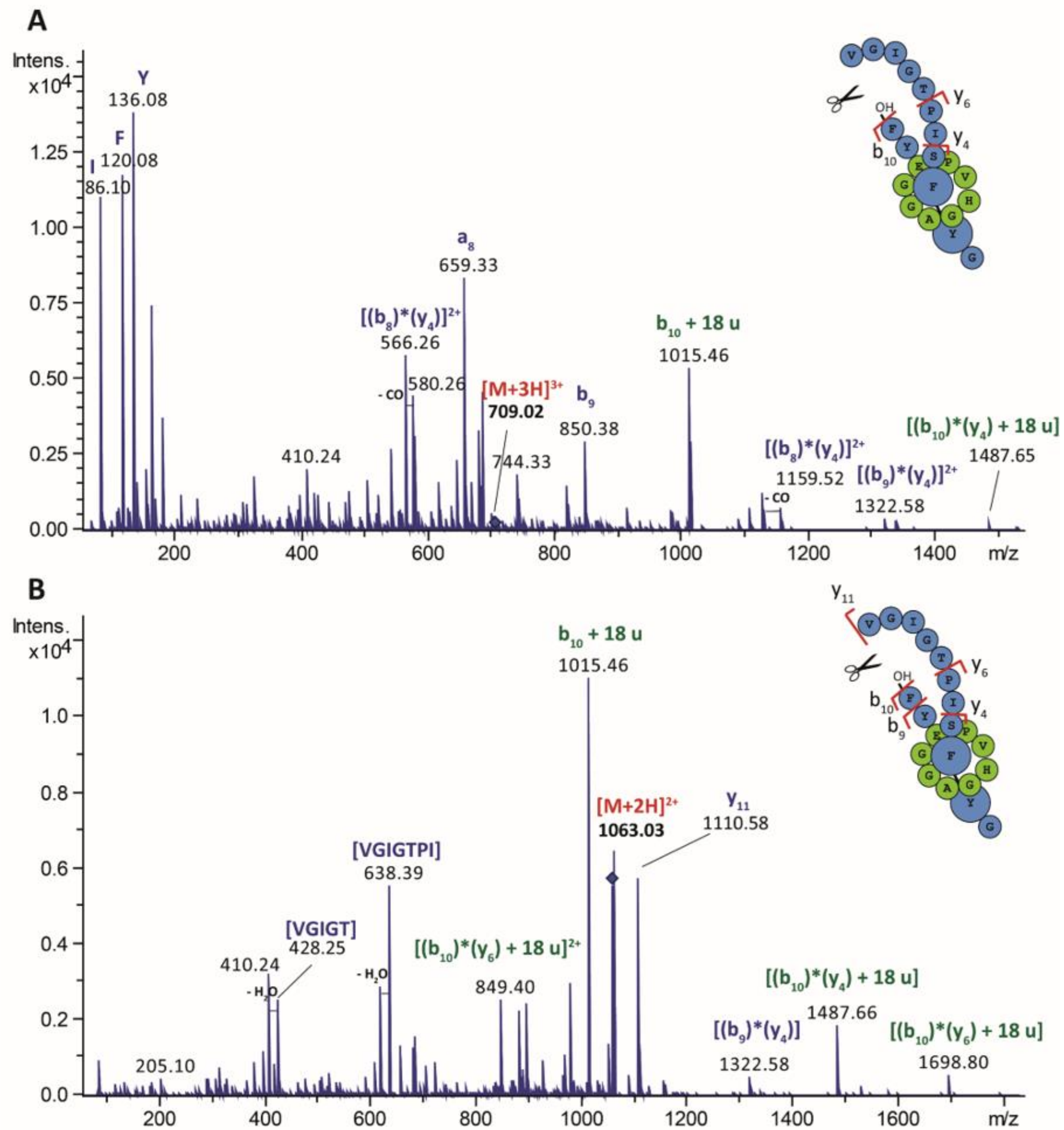

Figure S 4. MS/MS spectra of MccJ25 hydrolyzed at F10-V11 formed in the static model of duodenum, DP3 {G1-F10/V11-G21}. A: [M+3H]3+ (m/z 709.02, CE 32.6 eV), B: [M+2H]2+ (m/z 1063.03, CE 40 eV). The hydrolysis site was determined from the + 18 u increment product ions (in green).

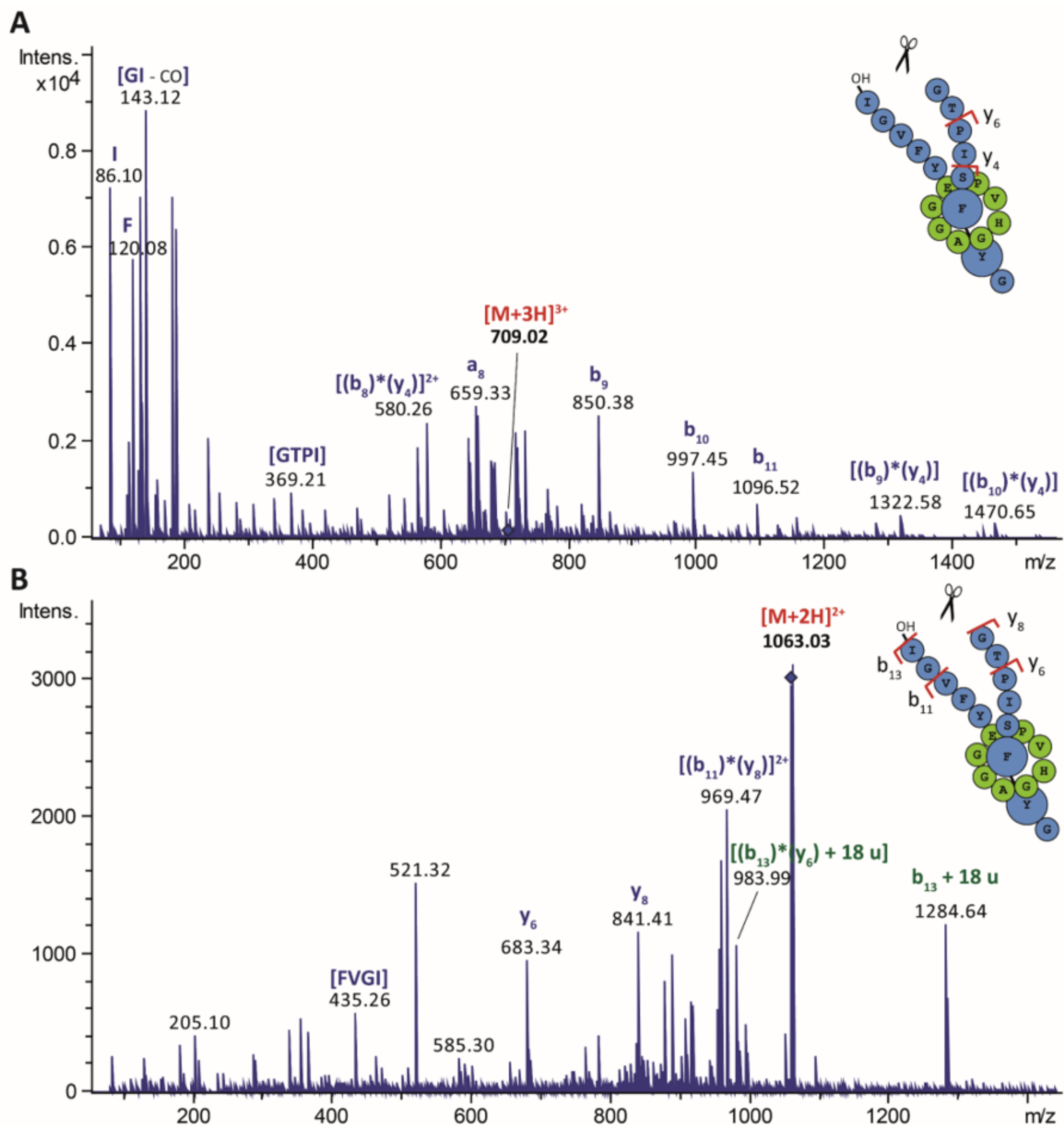

Figure S 5. MS/MS spectra of MccJ25 hydrolyzed at I13-G14 formed in the static model of duodenum, DP4 {G1-I13/G14-G21}. A: [M+3H]3+ (m/z 709.02, CE 32.6 eV), B: [M+2H]2+ (m/z 1063.03, CE 40 eV). The hydrolysis site was determined from the + 18 u increment product ions (in green).

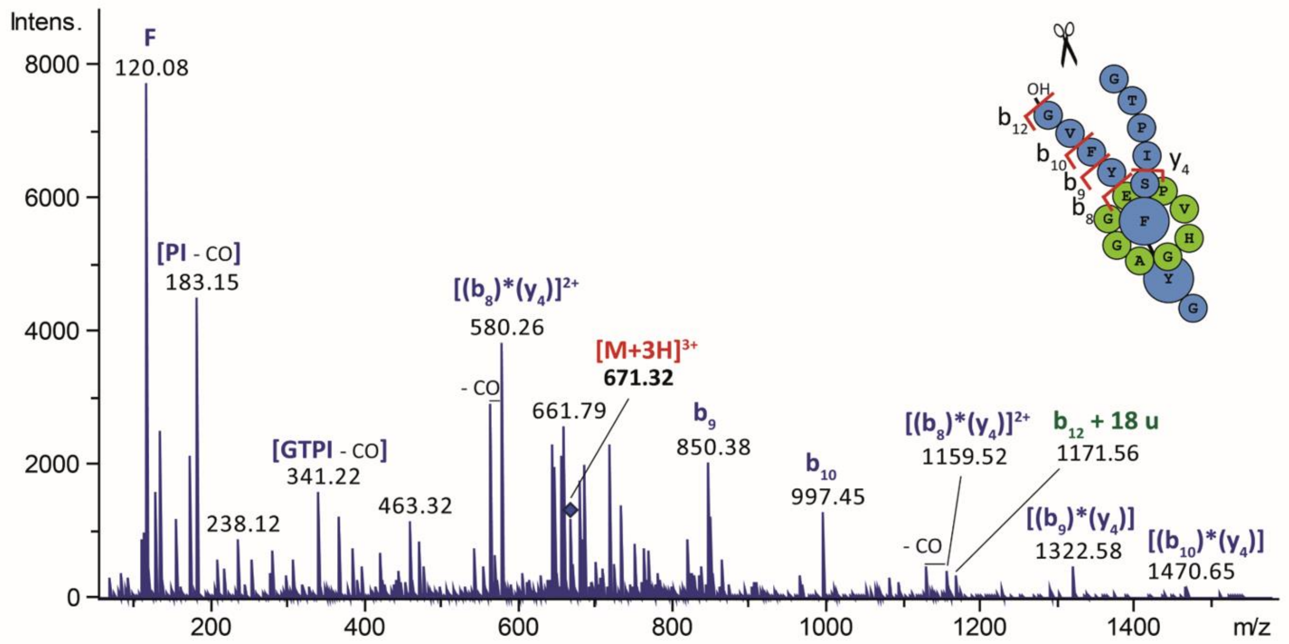

Figure S 6. MS/MS spectra of McсJ25 hydrolyzed both at G12-I13 and I13-G14 formed in the static model of duodenum, DP5 {G1-G12/G14-G21}: [M+3H]3+ (m/z 671.32, CE 30.3 eV). The hydrolysis site was determined from the + 18 u increment product ions (in green).

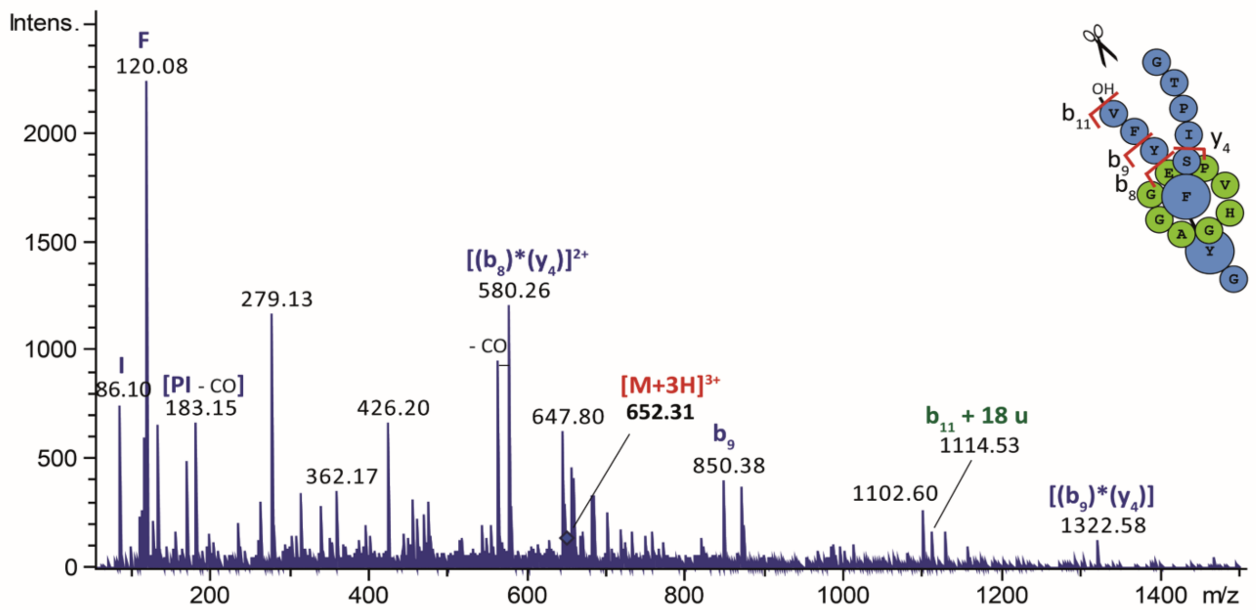

Figure S 7. MS/MS spectra of MccJ25 hydrolyzed both at V11-G12 and I13-G14 formed in the static model of duodenum, DP6 {G1-V11/G14-G21}: [M+3H]3+ (m/z 652.31, CE 29.2 eV). The hydrolysis site was determined from the + 18 u increment product ions (in green).

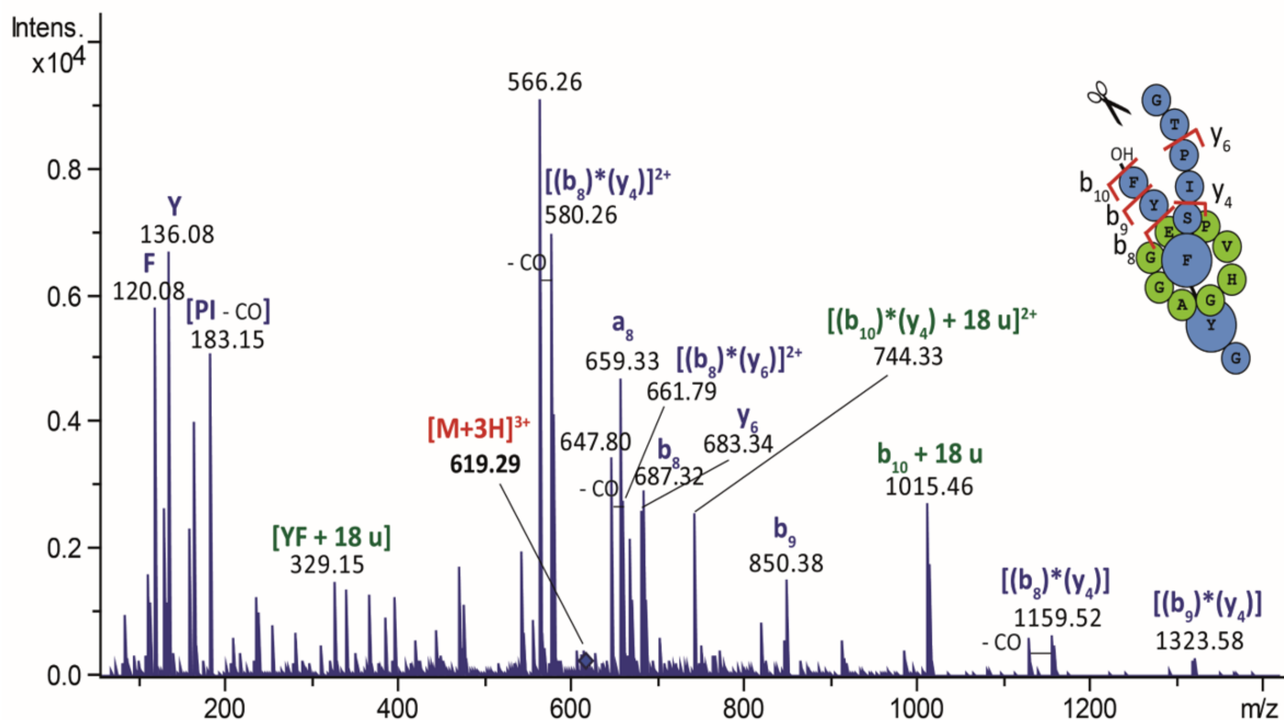

Figure S 8. MS/MS spectra of MccJ25 hydrolyzed both at F10-V11 and I13-G14 formed in the static model of duodenum, DP7 {G1-F10/G14-G21}: [M+3H]3+ (m/z 619.29, CE 27.2 eV). The hydrolysis site was determined from the + 18 u increment product ions (in green).

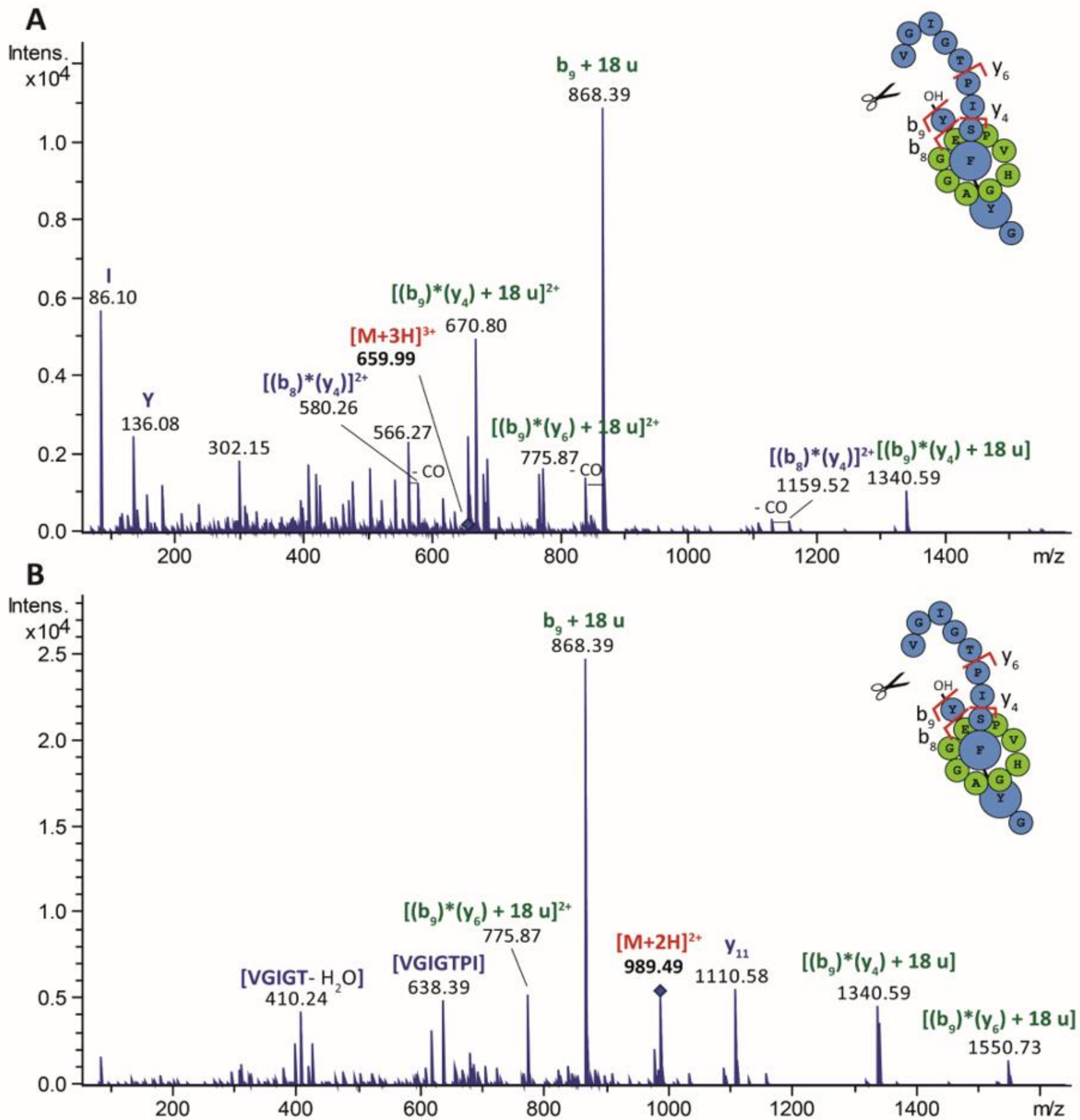

Figure S 9. MS/MS spectra of MccJ25 hydrolyzed after at Y9-F10 and F10-V11 formed in the static model of duodenum, DP8 {G1-Y9/V11-G21}. A: [M+3H]3+ (m/z 659.99, CE 29.6 eV), B: [M+2H]2+ (m/z 989.49, CE 39.8 eV). The hydrolysis site was determined from the + 18 u increment product ions (in green).

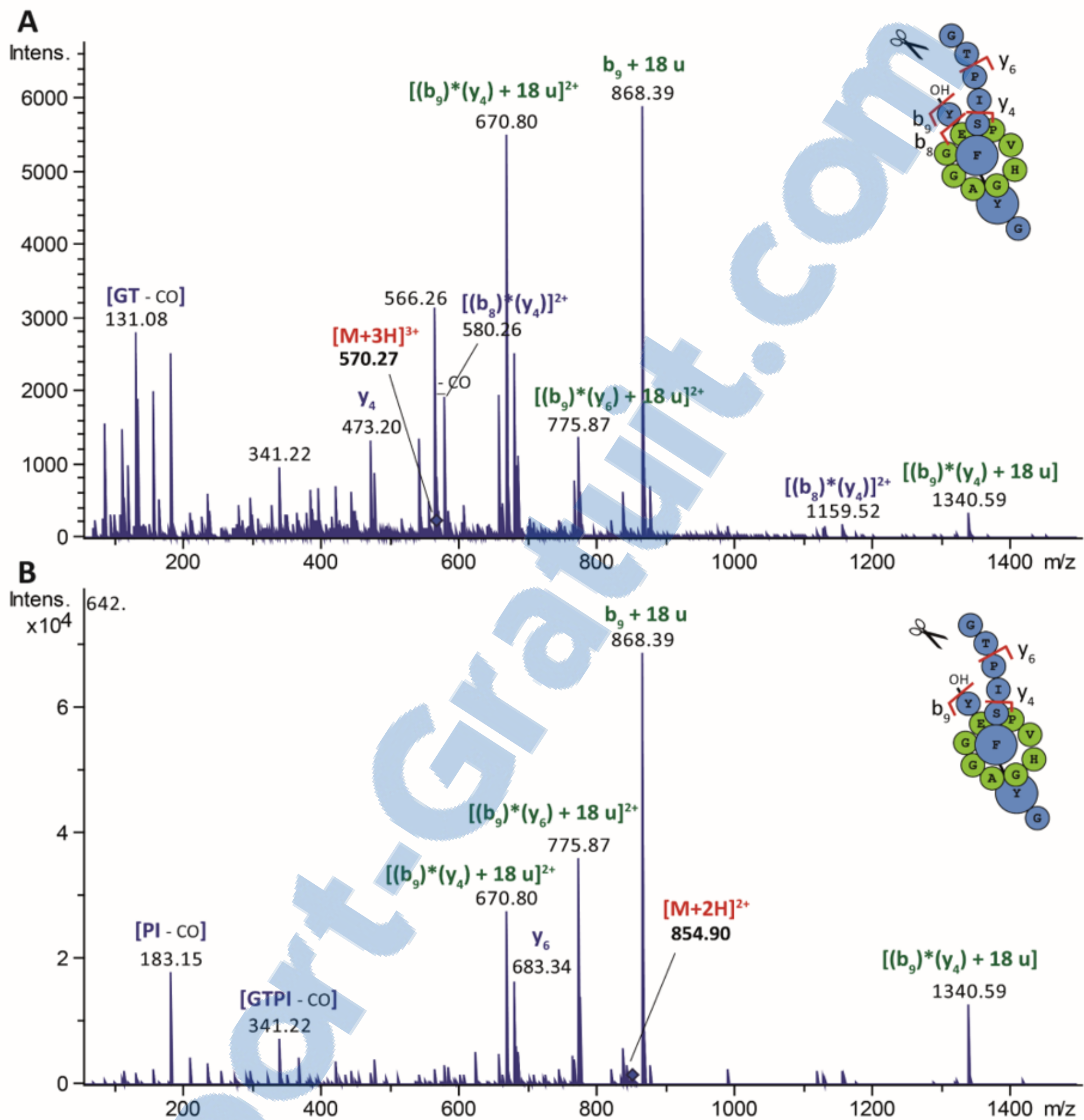

Figure S 10. MS/MS spectra of MccJ25 hydrolyzed both at Y9-F10 and I13-G14 formed in the static model of duodenum, DP9 {G1-Y9/G14-G21}. A: [M+3H]3+ (m/z 570.27, CE 24.2 eV), B: [M+2H]2+ (m/z 854.90, CE 37.1 eV). The hydrolysis site was determined from the + 18 u increment product ions (in green).

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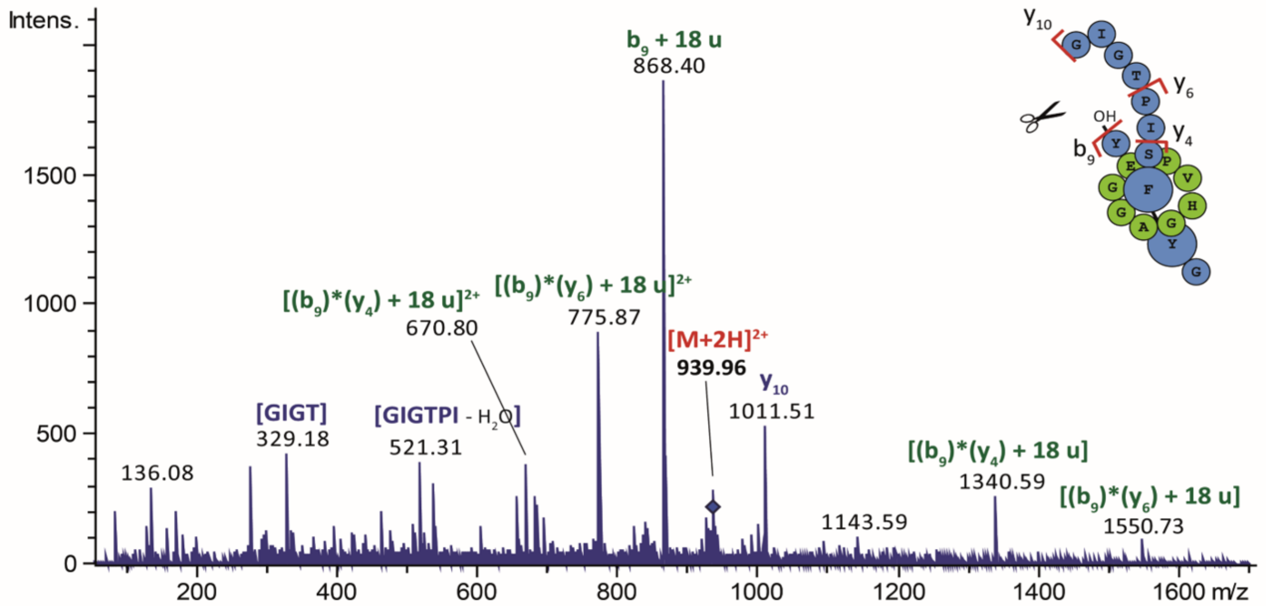

Figure S 11. MS/MS spectra of MccJ25 hydrolyzed both at Y9-F10 and V11-G12 formed in the static model of duodenum, DP10 {G1-Y9/G12-G21}: [M+2H]2+ (m/z 939.96, CE 38.8 eV). The hydrolysis site was determined from the + 18 u increment product ions (in green).

Chapitre 3. The antimicrobial lasso peptide microcin J25 exhibits inhibitory activity against *Salmonella* in swine colonic conditions

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Keywords: antimicrobial peptides, microcin J25, reuterin, rifampicin, *Salmonella*, Macfarlane medium, pig colon

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3.1. Résumé

La microcine J25 (MccJ25), une bactériocine de 21 acides aminés produite par *Escherichia coli*, est un puissant inhibiteur des entérobactéries, y compris les bactéries pathogènes d'*E. coli*, *Salmonella* et *Shigella*. Sa structure en lasso lui confère une grande stabilité qui la rend intéressante pour une utilisation possible en tant qu'agent antimicrobien dans les aliments ou comme alternative aux antibiotiques en production animale. Dans cette étude, l'activité inhibitrice de la MccJ25 contre *Salmonella enterica* subsp. *enterica* serovar Newport ATCC 6962 a été évaluée dans le milieu LB ainsi que dans le milieu Macfarlane non fermenté et fermenté simulant les conditions coliques du porc. L'activité inhibitrice de la MccJ25 a été comparée à celle de deux agents antimicrobiens, la réutérine et la rifampicine. La concentration minimale inhibitrice de la MccJ25 a été estimée à 0,03 µM dans le milieu LB, comparativement à 1079 µM et 38 µM pour la réutérine et la rifampicine, respectivement. Une inhibition totale de *Salmonella* a été observée dans le milieu LB pendant 24 h d'incubation à des concentrations correspondant à la CMI ou plus. Une inhibition significative a également été observée dans les milieux Macfarlane non fermentés et fermentés, mais des concentrations plus élevées de MccJ25 étaient nécessaires. Cette étude suggère un potentiel intéressant de la MccJ25 comme alternative aux antibiotiques chez le bétail.

3.2. Abstract

Microcin J25 (MccJ25), a 21-amino acid bacteriocin produced by *Escherichia coli*, a potent inhibitor of some *Enterobacteriaciae* including some strains of *E. coli*, *Shigella*, and *Salmonella*. Its lasso structure makes it highly stable and therefore of interest as a possible antimicrobial agent in foods or as an alternative to antibiotics in livestock production. In this study, the growth inhibition activity of MccJ25 against *Salmonella enterica* subsp. *enterica* serovar Newport ATCC 6962 was evaluated in LB medium as well as in unfermented and fermented Macfarlane medium simulating the swine colonic conditions. The inhibition activity of MccJ25 was compared to those of two antimicrobial agents, reuterin and rifampicin. The minimal inhibitory concentration of MccJ25 was estimated at 0.03 μM in LB medium, compared to 1079 μM and 38 μM for reuterin and rifampicin, respectively. Total inhibition of *Salmonella* was observed in LB medium during 24 h of incubation at concentrations corresponding to the MIC or higher. Significant inhibition was also observed in unfermented and fermented Macfarlane media, but higher concentrations of MccJ25 were needed. This study suggests an interesting potential of MccJ25 as an alternative to antibiotics in livestock.

3.3. Introduction

Currently generating billions of dollars in export income, pork production is one of the most promising sectors of the world economy. Unfortunately, as is the case with other livestock, pigs are sensitive to various infectious diseases, which cause major losses of revenue. Indeed, foodborne gastrointestinal (GI) tract infections in animals have become a major health problem that has reached epidemic proportions, especially in industrialized countries (Scallan et al., 2011; Crim et al., 2015). The increased incidence of these infections is due mainly to bacterial pathogens such as *Salmonella* spp., which are known to be responsible for one of the most widespread enteric diseases in swine. Swine is a major reservoir for *Salmonella enterica,* the causative agent for salmonellosis, a disease that reduces animal weight gain and increases mortality, thus decreasing productivity (Kim and Isaacson, 2017).

Antibiotics have long been used widely as feed additives to enhance livestock growth or to treat bacterial infections including salmonellosis (McEwen and Fedorka-Cray, 2002). In commercial pork production, piglets are weaned early, followed by abrupt introduction to a solid diet. This combination of stress factors often leads to diarrhea and slow growth. Antibiotics are therefore often used at this stage to suppress the activity of gut pathogens and thereby ensure growth. However, this practice has led to the emergence of antibiotic resistance in human commensal and pathogenic bacteria, which has raised public concern and intensified the search for alternative feeding strategies as well as alternatives to antibiotics in livestock production. Given the potential danger to humans, this line of research has become urgent. To complicate matters, several pharmaceutical companies have abandoned their searches for new families of antibiotics because of the high costs and risks associated with the development of such products. Meanwhile, the spread of resistance to antibiotics has led to a total ban on their use in animal feeds in several countries, such as those of the European Union (Castanon, 2007b). Therefore, there is an acute need for developing alternative strategies to the use of conventional antibiotics. One interesting track for preventing bacterial infections and reducing the incidence of enteric diseases in livestock is the use of natural antimicrobial peptides (AMPs), of which several are produced by bacteria and are called bacteriocins (Ageitos et al., 2017).

Many bacterial AMPs, especially bacteriocins, have been well characterized over the years. Bacteriocins are defined as RNA-transcribed antimicrobial peptides with molecular masses generally less than 10 kDa (Rea et al., 2011; Hammami et al., 2013a). Unlike antibiotics, they are typically active

at nano-molar concentrations and have narrow spectra of activity, targeting species closely related to the producer species (Nes, 2011). The use of bacteriocins as natural antimicrobial agents has already been considered in several foodstuffs including dairy products (Benech et al., 2002), meats (Abee et al., 1995; Siragusa et al., 1999) and plant products (Allende et al., 2007). Nisin A has been approved as a food additive in more than 55 countries, and Nisaplin™, a commercialized 2.5% solution of nisin A, has been used effectively in several foods. The use of bacteriocins as a growth factor in the livestock sector is becoming more and more frequent. Significant inhibition of enterotoxigenic *Escherichia coli* responsible for severe diarrhea in piglets may be obtained using colicins E1 and N (Stahl et al., 2004b) and colicin E1 added to feed has been used to decrease the incidence of post-weaning diarrhea and improve piglet growth (Cutler et al., 2007c). *Bacteroides* and *Enterobacteriacae* counts in the ileum of broiler chickens have been decreased and mean daily weight gain improved using nisin added to the feed (Józefiak et al., 2013). It should be noted that nisin-based veterinary preparations namely Wipe-Out® Dairy Wipes and Mast Out ® and developed by ImmuCell Corporation have been available commercially for several years for the prevention of mastitis in dairy cows (Cotter, 2005b; Pieterse and Todorov, 2010).

Although the chemical structure and biological activities of bacteriocins suggest great potential for application as alternatives to antibiotics in livestock feeds, very few products authorized by veterinary regulatory agencies are available for commercial use. Potential suppliers appear to be waiting for additional research before investing in the production of these natural compounds. Therefore, to overcome barriers that make suppliers reluctant to develop bacteriocins and other antimicrobial peptides as novel treatments in animal husbandry it is required to provide data on the stability and activity of the compounds under gastrointestinal conditions.

Moreover, it should be noted that most of the studies reported so far focus on bacteriocins produced by Gram-positive bacteria such as lactic acid bacteria. Very few concern bacteriocins from Gram-negative bacteria, which are called microcins (Duquesne et al., 2007b). One of the most studied Gram-negative bacteriocins is microcin J25 (MccJ25). This 21-amino acid peptide produced by *Escherichia coli* has been shown long ago to inhibit pathogenic *Enterobacteriaceae* including *Salmonella* (Salomon and Farías, 1992a; Rintoul et al., 2001a). This activity has been demonstrated to be maintained in a murine model of *Salmonella* infection (Lopez et al., 2007b). However, no study of

MccJ25 stability and biological activity under animal or human gastrointestinal conditions has been published.

The present study aims at providing data on MccJ25 activity and stability under colonic conditions. The antibacterial activity of MccJ25 was evaluated *in vitro* against *Salmonella enterica* subsp. *enterica* serovar Newport ATCC 6962 under conditions simulating the swine proximal colon using a modified Macfarlane medium. It was compared to the properties exhibited in similar conditions by two other antimicrobial compounds, namely the antibiotic rifampicin and reuterin, a broad spectrum antimicrobial produced by the probiotic bacterium *Lactobacillus reuteri* (Cleusix et al., 2007b; Schaefer et al., 2010).

3.4. Material & methods

3.4.1. Bacterial strains

E. coli MC4100 harboring the plasmid pTUC202 was used for the production of MccJ25. *Lactobacillus reuteri* ATCC 53608 was used for the production of reuterin. *Salmonella enterica* subsp. *enterica* serovar Newport ATCC 6962 (hereinafter called *Salmonella* Newport), the test strain for antibacterial activity assays, was purchased from Microbiologics Inc. (St. Cloud, Minnesota, USA).

3.4.2. Antimicrobial compounds

MccJ25 was produced from a culture of *E. coli* MC4100 harboring the plasmid pTUC202, as described previously (Ducasse et al., 2012). Briefly, minimal medium (M63) composed of KH_2PO_4 (3 g/L), K_2HPO_4 (7 g/L), $(NH_4)_2HPO_4$ (2 g/L) and casamino acids (1 g/L) supplemented with $MgSO_4$ (0.25 g/L), glucose (10 g/L) and thiamine (1 mg/L), was inoculated with a preculture of the bacteria in LB broth (2% v/v), and the culture was incubated overnight at 37°C. The culture was centrifuged (8000 *g*, 20 min, 4°C) and the supernatant was submitted to solid-phase extraction on a Sep-Pak C18 35 cc cartridge (Waters, Milford, USA). MccJ25 was eluted with acetonitrile/water (30% v/v) containing 0.1% HCl. The peptide was further purified by RP-HPLC (Beckman Coulter System Gold, Mississauga, ON, Canada) on a preparative C18 column (Luna 10 µm, 100 Å, 21.10 × 250 mm, Phenomenex, CA, USA) at a flow rate of 10 mL/min using a 25–100% linear gradient of filtered acetonitrile/5 mM HCl in ultra-pure water (PureLab Ultra, ELGA, USA) with absorbance measurement at 214 nm for peptide detection. The purified MccJ25 was lyophilized and stored at -20°C until use.

Reuterin (3-HPA) was produced from a culture of *Lactobacillus reuteri* ATCC 53608, as described previously (Doleyres et al., 2005). Briefly, MRS medium supplemented with 20 mM glycerol was inoculated (1% v/v) and incubated anaerobically at 37°C for 16 h. The culture was centrifuged (1500 *g*, 10 min, 20°C) and the cells were washed with 0.1 M potassium phosphate buffer (pH 7.0), centrifuged again, re-suspended in 300 mM glycerol solution and incubated anaerobically at room temperature for 45 min. This suspension was centrifuged (15,000 *g*, 5 min, 4°C), filtered (0.22 µm, Millipore) and lyophilized. Reuterin was then purified on a silica gel 60 (0.060-0.2 mm, 70-230 mesh; Alfa Aesar) preparative chromatography column (2.8 × 35 cm, Bio-Rad Econo-Column), with acetone:ethyl acetate (2:1) as eluent (Cleusix et al., 2007b) at a flow rate of 5 mL/min. Reuterin was quantified using a colorimetric method described previously (Lüthi-Peng et al., 2002a). Acrolein was used for calibration and generating a linear standard curve with known initial concentrations (0.001–1.0 M). The filtrate containing reuterin (1 mL) was added to 750 µL of 10 mM tryptophan dissolved in 0.05 N HCl solution and the resulting mixture was added to 3 mL of 37% HCl and maintained for 20 min at 37°C. Absorbance at 560 nm was recorded on an Infinite R F200 Pro spectrophotometer (Tecan Inc., NC, USA).

Rifampicin (purity ≥ 97%, HPLC) was purchased from Sigma-Aldrich (St. Louis, MO, USA) and stored at -20°C until use.

3.4.3. Modified Macfarlane medium

A nutrient medium described previously (Macfarlane et al., 1998a) was modified to obtain carbohydrate and protein concentrations simulating the conditions in the pig proximal colon, as described previously (Tanner et al., 2014a). The medium was autoclaved at 121°C for 20 min and then stored at 4°C until use. Vitamins and L-cysteine HCl were added separately in filter-sterilized (0.22 µm, Millipore) solutions (Michel et al., 1998a) for *in vitro* antimicrobial activity assays.

3.4.4. Preparation of fermented Macfarlane medium

A fecal sample (from a healthy pig raised under farm conditions and being not given any antibiotic during the previous three months) was collected in a sterile 50 mL Falcon tube. Anaerobiosis was maintained in a Gas-Pak anaerobic jar (Oxoid, Thermo Fisher Scientific) during transport to the laboratory (Tanner et al., 2014a). Fermented modified Macfarlane medium was obtained by inoculating 95 mL with 5 g of fecal sample and further incubating for 24 h at 37°C under anaerobic

conditions. The fermented Macfarlane medium was then centrifuged (1000 *g*, 5 min), filtered (0.45 µm) and divided into aliquots in sterile 15 mL Falcon tubes that were kept at -20°C until use.

3.4.5. Survival of *Salmonella* Newport in fermented Macfarlane medium

The survival of *Salmonella* Newport in fermented Macfarlane medium was studied in a sterile 24-well micro-plate (EMD Millipore, MA, USA) using additions of fresh medium at 1%, 5%, 10%, 15% and 20% to mimic the influx of nutrients to the pig colon. Growth in LB broth and in non-fermented Macfarlane broth was observed for comparison. Each tested medium (1.5 mL) was inoculated with 600 µL of an overnight *Salmonella* Newport culture diluted in the same medium to a final concentration of 5×10^5 cfu/mL. The micro-plate was then incubated for 24 h at 37°C and 50 µL of culture were collected from each well (containing 2.1 mL). *Salmonella* Newport was enumerated by counting on LB agar using sterile 0.1% peptone water for serial 10-fold dilution. Plates were incubated aerobically at 37°C for 12 h. Viable cell count was expressed as colony-forming units (cfu) per mL of tested medium. Since *Salmonella* Newport survived better in fermented Macfarlane medium supplemented with 20% fresh medium, this blend was used for subsequent inhibitory activity assays.

3.4.6. *In vitro* inhibition assays

3.4.6.1. Antibacterial activity measurement based on optical density

A micro-dilution method described in the Clinical and Laboratory Standards Institute (CLSI) guidelines was used first. Twofold serial dilutions of MccJ25 (0.475 mM), reuterin (138.1 mM) or rifampicin (1.215 mM) were made in sterile flat-bottom 96-well polystyrene micro-plates (BD Labware, Franklin, NJ, USA) starting with 125 µL of antimicrobial solution in 125 µL of LB broth. These were inoculated with 50 µL of an overnight culture of *Salmonella* Newport diluted to 5×10^5 cfu/mL. After incubation for 24 h at 37°C, the optical density at 595 nm was recorded using an Infinite R F200 Pro spectrophotometer (Tecan Inc., NC, USA).

Antimicrobial activity was then evaluated using 24-well plates (EMD Millipore, MA, USA) as described in the CLSI guidelines. The solutions mentioned above were diluted in LB broth in twofold series starting with 500 µL of each and inoculated with 500 µL of an overnight culture of *Salmonella* Newport diluted in LB broth to 5×10^5 cfu/mL. Plates were incubated for 24 h at 37°C and optical density was recorded at 595 nm using the same spectrophotometer.

3.4.6.2. Measurement of MccJ25 antibacterial activity based on viable count

Inhibition of *Salmonella* Newport by MccJ25 was further evaluated using LB broth, unfermented Macfarlane broth and fermented Macfarlane broth (supplemented with 20% fresh medium) in 24-well micro-plates. Twofold serial dilutions of MccJ25 solution (0.949 mM) in the tested medium (1.5 mL each) were inoculated with 600 µL of an overnight culture of *Salmonella* Newport diluted in LB broth to 5×10^5 cfu/mL. Plates were incubated for 24 h at 37°C. Cultures were sampled (50 µL) every 2 h for the first 14 h and at 24 h and dilutions in sterile 0.1% peptone water were plated on LB agar. Plates were incubated aerobically at 37°C for 12 h. Viable counts were expressed as cfu/mL of tested medium.

3.4.7. Stability of MccJ25 and development of MccJ25 resistance

Cultures in LB broth, unfermented or fermented Macfarlane broth (500 µL, 18 h or 24 h, in 24-well plates) were centrifuged (5,000 *g*, 10 min, 4°C). The inhibitory activity of the residual MccJ25 in the filtered supernatant (0.22 µm, Millipore) was evaluated using the agar well diffusion method (Tagg et al., 1976a). *Salmonella* Newport centrifugal pellet was washed twice in sterile 0.1% peptone water, re-suspended in LB broth and incubated at 37°C for 18 h. An overnight culture of *Salmonella* Newport never exposed to MccJ25 was prepared in LB broth and used as a positive control. In both cases, 150 µL of culture were suspended in 25 mL of LB 0.8% agar. Previously purified MccJ25 (0.475 mM), reuterin (138.1 mM) and rifampicin (1.215 mM) were used as positive controls. LB broth was used as a negative control. Inhibition zone diameters were measured after 18 h of incubation at 37°C.

3.5. Results

3.5.1. Purification of MccJ25 and reuterin

The bacteriocin MccJ25 was produced from a 1.5 L of *E. coli* MC4100 pTUC202 culture (Ducasse et al., 2012). The culture supernatant was first vacuum-concentrated to about 117 mL of active fraction containing MccJ25 (fraction of 30% acetonitrile) (Table 1). MccJ25 eluted as a single peak at 45 % acetonitrile (Amp: 2.7 AU, 27.283 min), as shown in the HPLC chromatogram (Fig. 1). HPLC allowed recovery of about 45 mg of pure MccJ25, corresponding to a yield of 58%.

Reuterin was produced by *Lactobacillus reuteri* ATCC 53608 according to the two-step procedure developed previously (Doleyres et al., 2005). The concentration of reuterin from 300 mM of glycerol was about 138 mM. Pure reuterin free of the accompanying contaminants, essentially glycerol and 1,3

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propanediol, was recovered with a yield of 46 % using HPLC as a peak eluting at 26.2 min (Fig. 2) and exhibiting an antibacterial activity of 2048 AU/mL.

3.5.2. Determination of MIC and MBC values of MccJ25, reuterin and rifampicin against *Salmonella* Newport

The minimal inhibitory and minimal bactericidal concentrations of the antimicrobial compounds tested on *Salmonella* Newport are presented in Table 2. *Salmonella* Newport was highly sensitive to MccJ25 (MIC and MBC at 0.03 and 3.71 µM), which was a much more potent efficiency than that of reuterin (MIC and MBC at 1078.91 and 4315.63 µM) or rifampicin (MIC and MBC at 37.97 and 1215.15 µM). The MBC/MIC ratio of MccJ25 was about 123, which is in favour of a bacteriostatic-like mechanism of action according to the interpretation suggested in the CLSI guidelines (Vollenweider and Lacroix, 2004).

3.5.3. Inhibition of *Salmonella* Newport by MccJ25, reuterin and rifampicin in LB broth

Fig. 3 shows the growth curves of *Salmonella* Newport in LB broth in the presence of MccJ25, reuterin or rifampicin during 24 h of incubation in micro-assay plates. MccJ25 inhibited *Salmonella* Newport completely at 29.6, 3.7 and 0.03 µM, which represent around 8 x MBC, 1 x MBC and 1 x MIC values respectively. At 0.004 µM (black triangles), a growth curve close to that of the positive control (black squares) was obtained, indicating only a very slight inhibition at this nanomolar concentration. In the same conditions, *Salmonella* Newport was fully inhibited by reuterin at 138.1 µM and 4315.6 µM (Fig. 3.B) and by rifampicin at 37.9 µM and 1215.1 µM (Fig. 3.C).

In order to examine the effect of the assays volume on the *Salmonella* Newport growth inhibition, 24-well micro-assay plates were used. Fig. 4.A shows the growth of *Salmonella* Newport during 24 h in the presence of MccJ25 at four concentrations. Growth was unexpectedly not inhibited completely throughout the incubation period even at a concentration of 237 µM, which corresponds to 64 times the MBC (white triangles). The inhibition lasted for only 8 hours regardless of the tested concentration. Different results were observed with reuterin (Fig. 4.B), which inhibited *Salmonella* Newport growth throughout the 24 h at concentrations of 69, 11 and 5 mM. Rifampicin was also inhibitory throughout the 24 h period when tested at a concentration of 607 µM, as shown in Fig. 4.C.

The inhibition of *Salmonella* Newport growth in LB broth by MccJ25 was further confirmed by plating on LB agar. Again, MccJ25 at concentrations corresponding to ~128 and ~64 times the MBC inhibited growth only for 8 h (Fig. 5.A, 5.B), while no inhibition was observed at concentrations corresponding to 8 times the MBC or the MBC (Fig. 5.C, 5.D).

3.5.4. Inhibition of *Salmonella* Newport by MccJ25 in unfermented and fermented Macfarlane broth

The activity of MccJ25 was evaluated first in unfermented Macfarlane broth (Fig. 6). MccJ25 was inhibitory only for the first 4 h at a concentration about 128 times the MBC (Fig. 6.A). It did not inhibit *Salmonella* Newport growth at 64 times or 8 times the MBC or at the MBC (Fig. 6.B, 6.C, 6.D). By contrast, when tested in fermented MacFarlane broth, the growth of *Salmonella* Newport was inhibited completely for 14 h at MccJ25 concentrations 128 times and 64 times greater than the MBC (Fig. 7.A, 7.B). This inhibition was not observed at 8 times the MBC or at the MBC.

3.5.5. Stability of MccJ25 during inhibitory activity assays

In order to determine whether or not MccJ25 remains stable and active against *Salmonella* Newport after incubation in LB broth or in unfermented or fermented Macfarlane broth, the residual activity was tested using the agar diffusion assay. As shown in Fig. 8.A, MccJ25 at a concentration corresponding to the MBC remained active even after 18 h of incubation in any of the three media. It was also shown to be active after 24 h of incubation in fermented Macfarlane broth in micro-assay wells. As shown in Fig. 8.B, the fermented Macfarlane supplemented with MccJ25 at concentrations of ~128, 64 and 8 times the MBC, as well as at the MBC, retained a good inhibitory activity against *Salmonella* Newport after 24 hours cultivation, although it decreased gradually as the tested concentration of MccJ25 was progressively reduced.

3.5.6. Development of MccJ25 resistance in *Salmonella*

Single colonies were isolated from *Salmonella* Newport culture exposed or not to MccJ25 for 24 h in micro-assay wells. These colonies were tested for sensitivity to MccJ25 using the agar diffusion assay. Sensitivity to reuterin and rifampicin was tested for comparison. Fig. 9.A shows high sensitivity of non-exposed *Salmonella* Newport to MccJ25, reuterin and rifampicin, with inhibition zones of 2.2, 2.4 and 1.9 cm respectively. However, *Salmonella* Newport derived from a culture exposed to MccJ25 at 128 times the MBC became completely resistant (Fig. 9.B). No resistance to reuterin or rifampicin was

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noted, which produced inhibition zones of 2.4 and 1.9 cm respectively in similar assay conditions. Similar results were obtained when *Salmonella* Newport was exposed to MccJ25 at lower concentrations corresponding to 64 times (Fig. 9.C) or 8 times the MBC (Fig. 9.D). No inhibition zone was observed in such conditions, unlike for reuterin and rifampicin, which produced inhibition zone diameters similar to those obtained without exposition of *Salmonella* Newport to MccJ25.

3.6. Discussion

The abusive use of antibiotics in livestock has raised a lot of questions in recent years. This more and more controversial practice is pointed out in the emergence of the phenomenon of resistance and multi-resistance to antibiotics. The use of antibiotics as a growth promotor is no longer allowed in several countries, which makes the search for alternatives more and more urgent. Among the alternative approaches proposed, the use of natural antimicrobials such as bacteriocins has attracted a lot of interest.

The main objectives of the present work were to evaluate the stability and inhibition activity of MccJ25 under the physiological conditions encountered in the swine colon. MccJ25 is a 21-amino acid bacteriocin produced by *E. coli* which has a narrow spectrum of inhibitory activity directed against various pathogenic *Enterobacteriaceae* including *Salmonella* (Salomon and Farías, 1992a; Blond et al., 2001a; Rintoul et al., 2001a). MccJ25 is probably one of most studied Gram-negative bacteriocins because of its compact lasso structure, which confers it high stability and activity.

In the present study, we unambiguously showed that MccJ25 exhibits a potent inhibitory activity against *Salmonella* Newport under simulated swine colonic conditions. MccJ25 was purified from the supernatant of a large-scale culture of the *E. coli* producer and the purification protocol applied led to a single major peak corresponding to MccJ25, which was recovered with a total yield of 58%. An increase of about 3-fold in the specific activity of MccJ25 against *Salmonella* Newport was obtained during the purification procedure. For comparison of MccJ25 activity in the swine colonic conditions, we selected reuterin, a potent multi-component antimicrobial naturally produced by several *Lactobacillus reuteri* strains used as probiotics (Schaefer et al., 2010). Reuterin constitutes a good reference model of antimicrobial for our study, as it has the particularity to have a limited impact on the gut microbiota despite a broad spectrum antimicrobial activity (Shornikova et al., 1997; Casas and Dobrogosz, 2000). Reuterin results from microbial degradation of glycerol (Cleusix et al., 2007b) and

consists of a mixture of 3-hydroxypropionaldehyde (HPA), its hydrate, its dehydration product (acrolein) and its dimer, which are in a dynamic equilibrium depending on the global concentration. In this study, reuterin was produced and purified according to the two-step protocol described by Cleusix which involves production of *L. reuteri* cells followed by degradation of glycerol by the harvested cells (Cleusix et al., 2007b).

Evaluation of MccJ25 activity in LB broth allowed us to confirm MIC and MBC values (0.03 µM and 3.7 µM) for *Salmonella* Newport. A similar MIC value (0.05 µM) has been already determined in an early study using a clinical isolate of *Salmonella* Newport (Rintoul et al., 2001a). The MBC value for the same sensitive strain was also determined and was shown to be 123 times higher than the MIC under the same conditions, which means that the antimicrobial activity of MccJ25 is bacteriostatic and not bactericidal (Peterson and Shanholtzer, 1992; Pankey and Sabath, 2004).

Salmonella Newport was significantly more sensitive to MccJ25 than to reuterin or rifampicin, for which MIC and MBC values were much higher. This is consistent with results published previously (Duquesne et al., 2007b). We showed here that the inhibition activity of MccJ25 seems to be influenced by the final reaction volume. In a microplate format (test volume of 125 µL), all three compounds completely inhibited *Salmonella* Newport growth throughout the incubation period. However, when we performed the same experiment using larger volumes in 24-well micro-titer plates (test volume of 2.1 mL), only reuterin and rifampicin were bactericidal. However, MccJ25 allowed a moderate growth of *Salmonella* Newport after 8 h of incubation even at the highest concentration tested (64 times the MBC). This loss of inhibitory activity after 8 h could be due to degradation of the molecule or to the appearance of resistant mutants.

To answer the first hypothesis, agar diffusion assays were used to evaluate the stability of MccJ25 by monitoring the residual antibacterial activity of MccJ25 in samples taken from micro-plate tests at the MBC and 8 and 64 times the MBC. The obtained results showed a strong inhibition activity against *Salmonella* Newport even after 24 h of incubation. This result is in agreement with the high stability known for MccJ25, which is due to its highly rigid and compact lasso structure, making it remarkably stable and resistant to various severe conditions (Blond et al., 2001a; Rosengren et al., 2004). To answer the second hypothesis, agar diffusion assays were performed to compare the sensitivity of *Salmonella* Newport to MccJ25 before and after an initial exposure to the bacteriocin. Based on the obtained sensitivity profiles, it is quite clear that *Salmonella* Newport developed resistance to MccJ25

during the first incubation period in LB. This very likely explains the apparent loss of sensitivity noted in assays with larger volumes of culture. These results corroborate earlier reports indicating that continuous exposure of bacteria to bacteriocins may select resistant cells, as it is well known to occur with conventional antibiotics (Cotter et al., 2013; de Freire Bastos et al., 2015; Egan et al., 2017). This phenomenon has been observed notably with lantibiotic bacteriocins produced by Gram-positive bacteria, the resistance mechanism in this case being associated with alteration of the cell wall and membrane (Draper et al., 2015). Resistance to bacteriocins can also occur, according to their mechanisms of action, from mutations in genes encoding either the receptors they use for internalization into the sensible cells, or the intracellular targets. Indeed, mutations in genes encoding the iron siderophore receptor FhuA hijacked by MccJ25 to penetrate target bacteria, and in the RNA polymerase subunit that is obstructed by MccJ25, have been shown to be responsible for resistance of *E. coli* strains to MccJ25 (Salomón and Farías, 1993; Yuzenkova et al., 2002; Duquesne et al., 2007b). Although bacteriocin resistance has not been defined clearly in the literature, the potential emergence of pathogens resistant to these compounds is a concern that must be addressed. The risk of selecting resistant variants as a result of prolonged exposure to any antimicrobial agent under investigation with a view to clinical application must therefore be evaluated (Cotter et al., 2013).

One of the ultimate objectives of this study was to evaluate the potential of using MccJ25 as an alternative to antibiotics in livestock. We have therefore evaluated the inhibition activity of MccJ25 in a more complex medium, which simulate the physiological conditions encountered in the large intestine of swine. When unfermented Macfarlane broth was used, a significant inhibition of *Salmonella* Newport was observed during the first 4 h at a concentration corresponding to 128 times the MBC only. Surprisingly, in fermented Macfarlane broth, a complete inhibition of *Salmonella* Newport was observed for 14 h at MccJ25 concentrations 128 times and 64 times greater than the MBC. Preliminary experiments have showed that higher MccJ25 concentrations are needed for significant inhibition of *Salmonella* Newport in Macfarlane medium than in LB medium (data not shown). The lower activity observed in unfermented Macfarlane is probably due to the non-specific interaction of MccJ25 with different compounds encountered in the medium which will affect the bioavailability of the MccJ25 and thus its activity. Similar results have been reported previously by Gänzle (Gänzle et al., 1999) for the bacteriocin-producing *Lactobacillus curvatus* LTH 1174 and by Nascimento (Nascimento et al., 2010) for the bacteriocin-producing *Enterococcus faecium* FAIR-E. The greatest inhibitory activity observed in the fermented medium is most likely due to the limited growth of *Salmonella*

Newport for which the bacterial count never exceeds 10^6 ufc/ml in fermented Macfarlane (20% of fresh unfermented medium), compared to 1.23 x 10^9 ufc/ml in 100% unfermented Macfarlane and 6.5 x 10^8 ufc/ml in LB medium. The lowest level of basal growth of *Salmonella* Newport in the fermented Macfarlane medium could be explained by the presence of organic acids biosynthesized by the faecal microflora (Fernandez et al., 2013).

In this study we have clearly shown that addition of MccJ25 in a medium simulating swine colonic condition allows significant inhibition of *Salmonella* Newport suggesting great promise for use as novel alternatives to current antibiotics. Even though higher bacteriocin concentrations are needed, this approach offers several advantages compared to the use of a bacteriocin producing strains. Indeed, the implication of bacteriocins in inhibition of enteric infections by producing strains remains not clearly demonstrated, as many reported results were contradictory due to lack of sensitive and specific methods of detection and quantification of the bacteriocins produced in the gastrointestinal tract and/or to the high diversity of bacteriocin producing strains and their bacteriocins (Hegarty et al., 2016). Bacteriocin-producing strains must survive to the stressful conditions in the stomach and the small intestine. It must then implement, survive and multiply in the colon to produce their bacteriocins. The use of purified bacteriocins allows avoiding all these limitations, especially when the structure of the molecule confers a high stability over the gastrointestinal transit, as it is the case of MccJ25 whose lasso structure gives it a high resistance to both gastric pH and proteases. In the case where the bacteriocin is less stable at the gastrointestinal level, the use of galenic forms allowing the protection and controlled release of the molecule is required.

3.7. Conclusion

In summary, the bacteriocin MccJ25 was shown to be a potent inhibitor of *Salmonella* Newport when tested under growth conditions simulating the pig colon environment. This bacteriocin might be considered as a good alternative to antibiotics for animal feed applications, although further in vivo studies are still needed to evaluate its activity in farmed animals and validate the potential of this bacteriocin from a Gram-negative bacterium.

3.8. Acknowledgements

We would like to thank Steven Davis for reading the manuscript and providing language help. Funding: This work was supported by the Natural Sciences and Engineering Research Council of Canada (NSERC) and the NSERC-METABIOLAC industrial research chair.

3.9. Competing interest

The authors declare that they have no conflicts of interest with the contents of this article.

3.10. Authors contributions

I. F. and S. N. designed the project. I.F., S. R. and S. Z. managed the overall project. S. N. produced and purified the compounds for microbiology experiments. B. F. contributed to production and purification of reuterin. S. N. performed inhibitory activity measurements and all associated experiments. S. N. and I. F. analyzed the data. S. N. wrote the manuscript with help from all other authors.

3.11. Tables & figures

3.11.1. Tables

Table. 1. Purification of MccJ25 produced by *E. coli* MC4100 pTUC202

	Volume (mL)	Protein concentration* (mg/mL)	Total protein (mg)	Antibacterial activity (AU/mL)	Specific activity (AU/mg)	Recovery† (%)
Culture supernatant	1500	0.1598	239.69	16384	102533	100
Active fraction (30% acetonitrile)	117.8	0.4188	49.33	131072	313007	62.8
Purified MccJ25	13.6	3.3203	45.16	1048576	315811	58.0

*Lowry assay †Remaining protein concentration as % of the initial concentration

Table. 2. Minimal inhibitory concentrations (MIC) and minimal bactericidal concentrations (MBC) of MccJ25, reuterin and rifampicin in LB broth using *Salmonella enterica* subsp. *enterica* serovar Newport ATCC 6962 (*Salmonella* Newport) in 96-well micro-assay plates. MIC is defined as the lowest concentration that inhibited *Salmonella* Newport growth completely based on optical density measurement. MBC is the lowest concentration at which no colony appeared on agar after 72 h of incubation.

Sensitive strain	MccJ25			Reuterin			Rifampicin		
	MIC (µM)	MBC (µM)	MBC/MIC ratio	MIC (µM)	MBC (µM)	MBC/MIC ratio	MIC (µM)	MBC (µM)	MBC/MIC ratio
Salmonella Newport ATCC 6962	0.03	3.71	123.66	1078.91	4315.63	3.99	37.97	1215.15	32.00

3.11.2. Figures

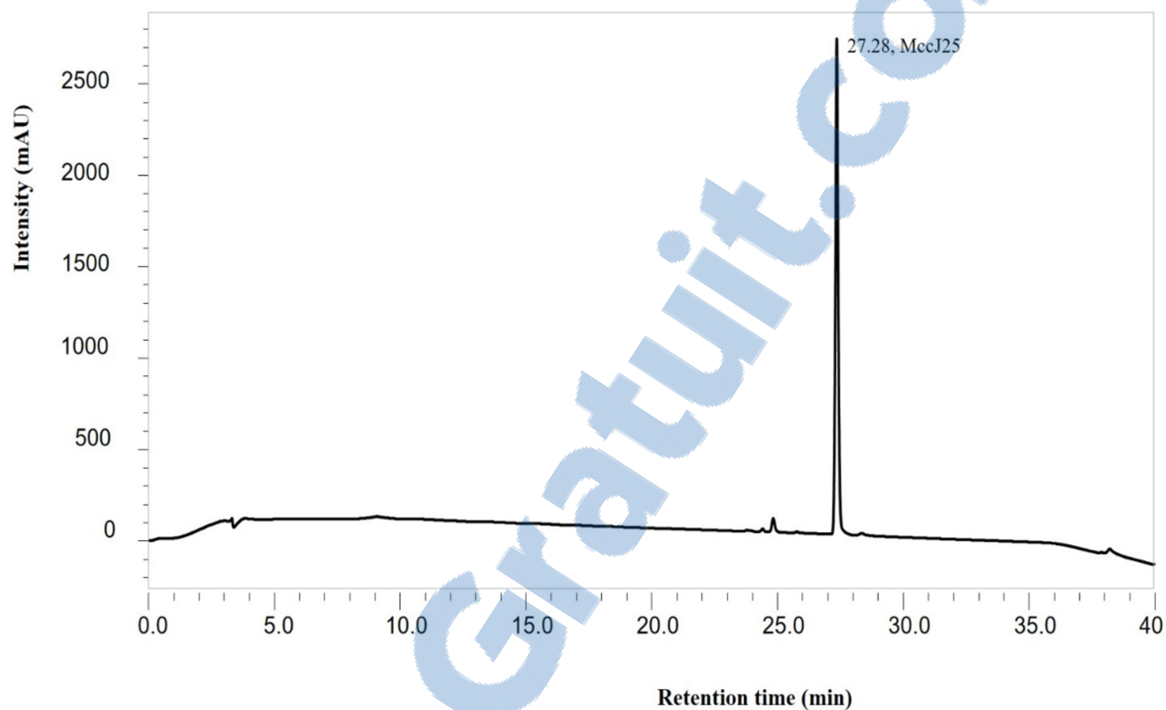

Figure. 1. RP-HPLC chromatogram of purified MccJ25 obtained using a preparative C18 column (2.1 × 25 cm) eluted with a 25–100% linear gradient of acetonitrile in 5 mM HCl in ultra-pure water at a flow rate of 10 mL/min (detection at 214 nm).

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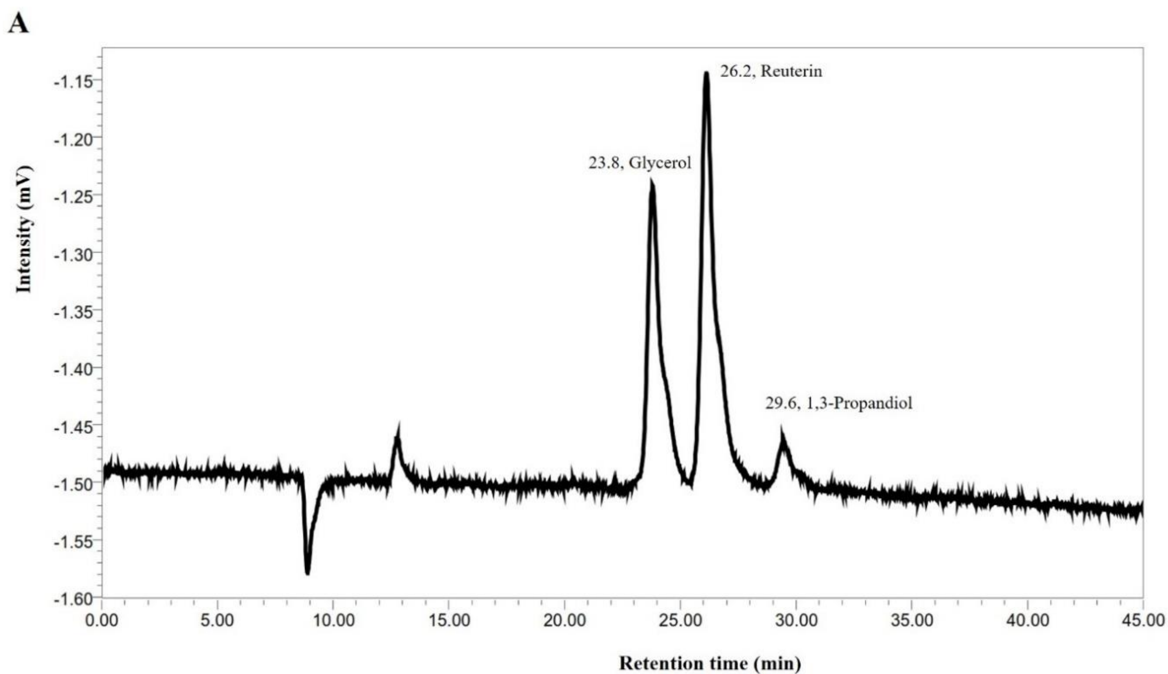

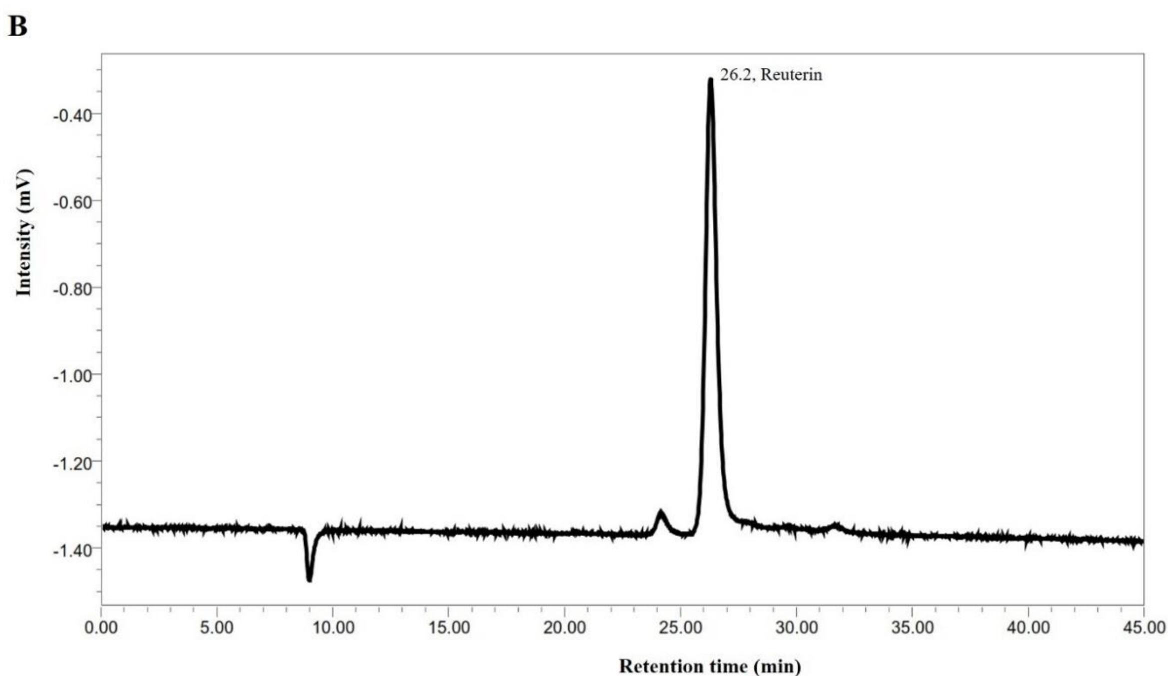

Figure. 2. HPLC chromatograms of reuterin obtained along the purification procedure using a silica gel chromatography column (2.8 × 35 cm) eluted with acetone: ethyl acetate (2:1) as eluent; (A) supernatant analyzed before purification and exhibiting a mixture of reuterin, glycerol and 1,3-propanediol; (B) reuterin after purification.

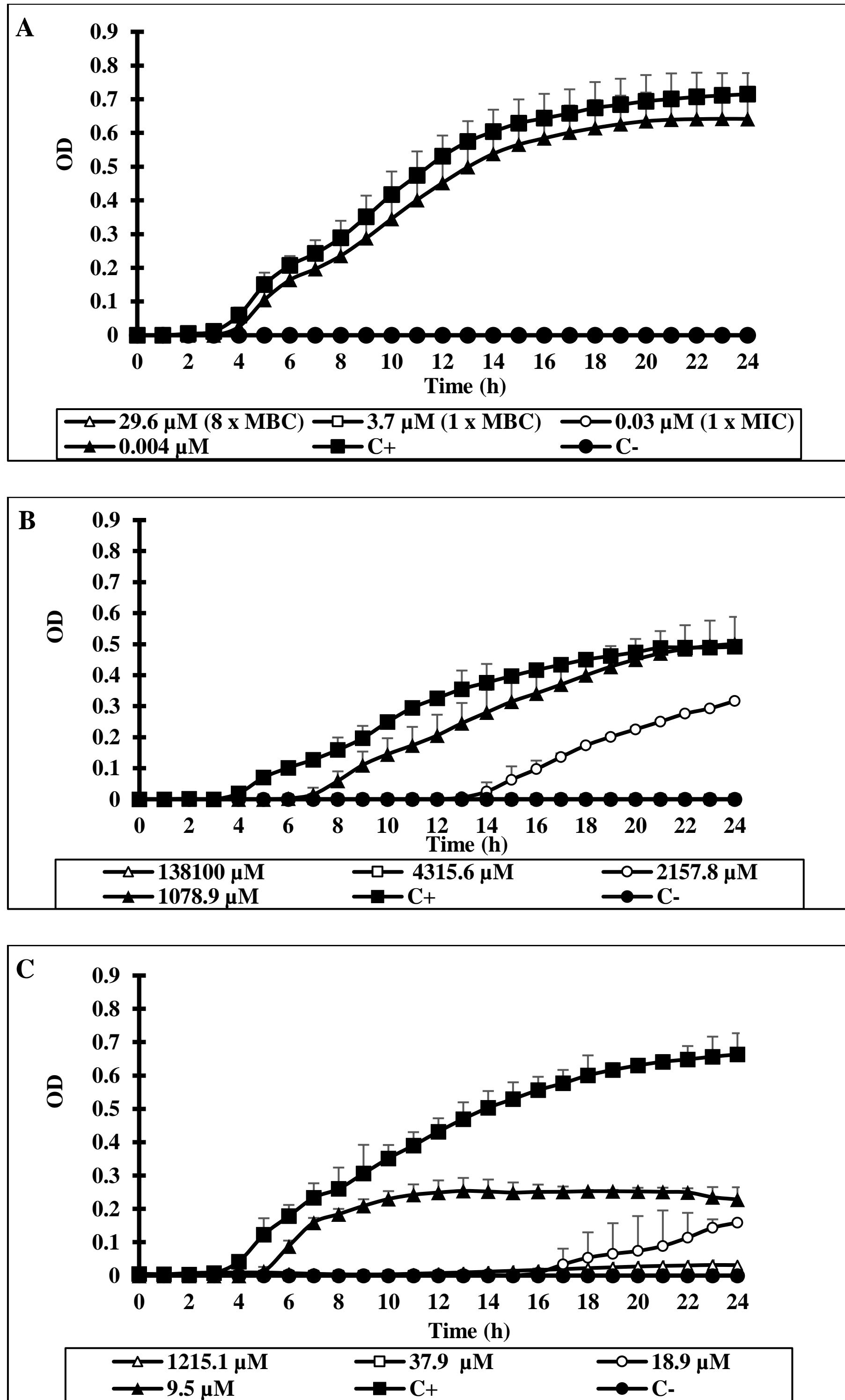

Figure. 3. Growth of *Salmonella enterica* subsp. *enterica* serovar Newport ATCC 6962 in LB broth supplemented with MccJ25 (A), reuterin (B) or rifampicin (C) at four different concentrations. Optical density in 96-well micro-plates was measured at 595 nm. C- is the negative control (LB broth only) and C+ the positive control (LB broth + MccJ25 at 0.475 mM). Data are means of two independent experimental repetitions.

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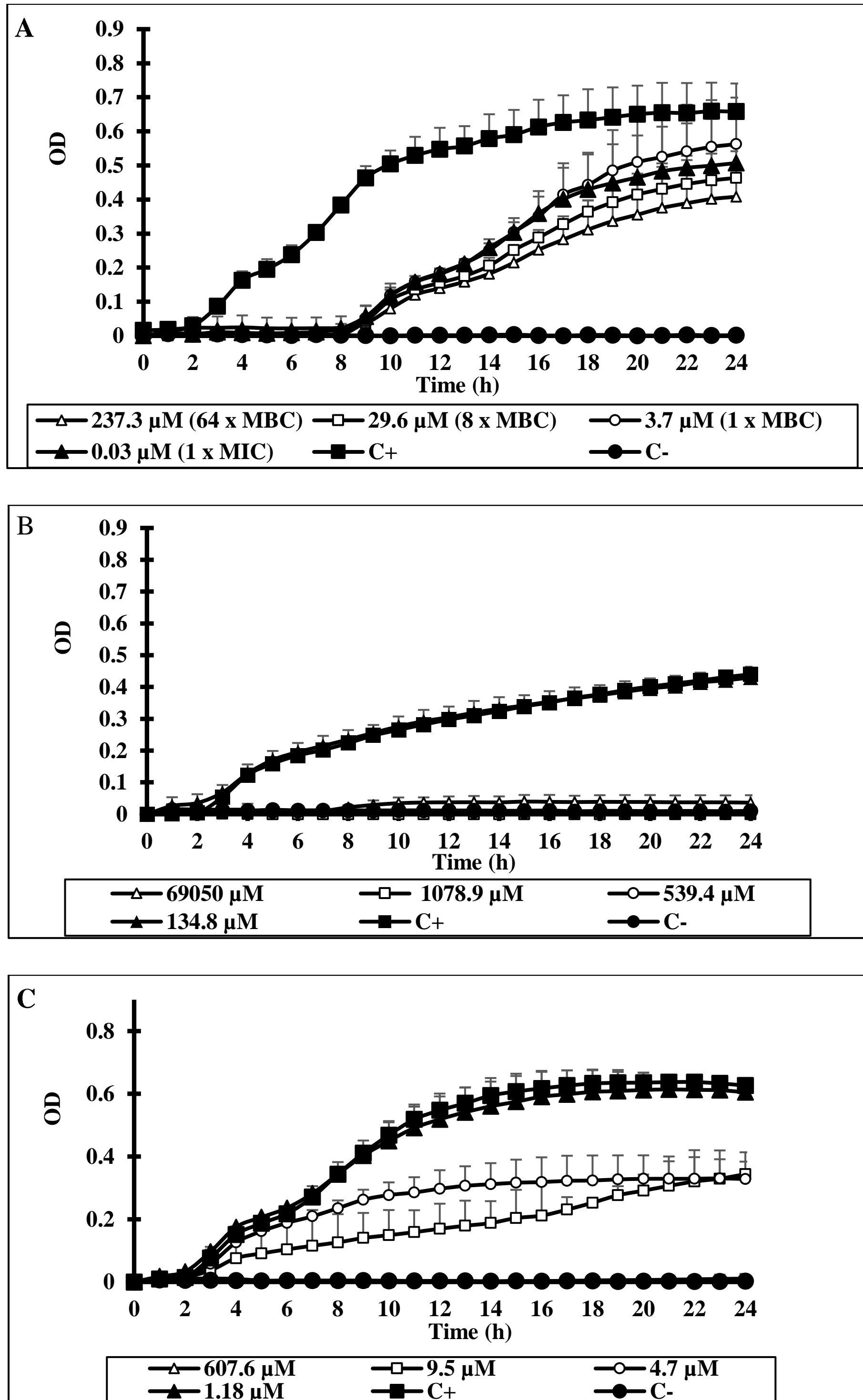

Figure. 4. Growth of *Salmonella enterica* subsp. *enterica* serovar Newport ATCC 6962 in LB broth containing MccJ25 (A), reuterin (B) or rifampicin (C) at four different concentration. Optical density in 24-well micro-plates was measured at 595 nm. C- is the negative control (LB broth only) and C+ is the positive control (LB broth + MccJ25 at 0.475 mM). Data are means of two independent experimental repetitions.

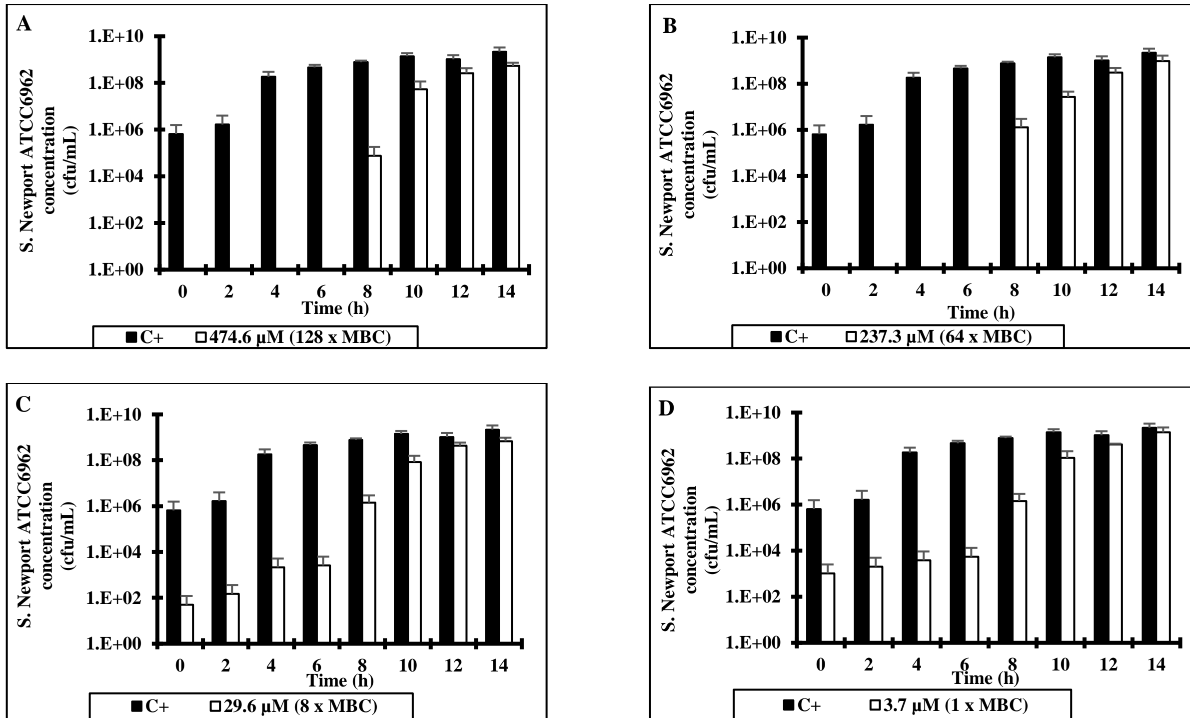

Figure. 5. Growth of *Salmonella enterica* subsp. *enterica* serovar Newport ATCC 6962 in LB broth containing MccJ25 at 128 times (A), 64 times (B), 8 times (C) and equal to (D) the MBC determined in 96-well micro-assay plates. Viable counts (cfu/mL) in 24-well micro-plates were determined by plating on LB agar. C+ is the positive control (*Salmonella* Newport at an initial concentration of 5×10^5 cfu/mL without MccJ25) Data are means of two independent experimental repetitions.

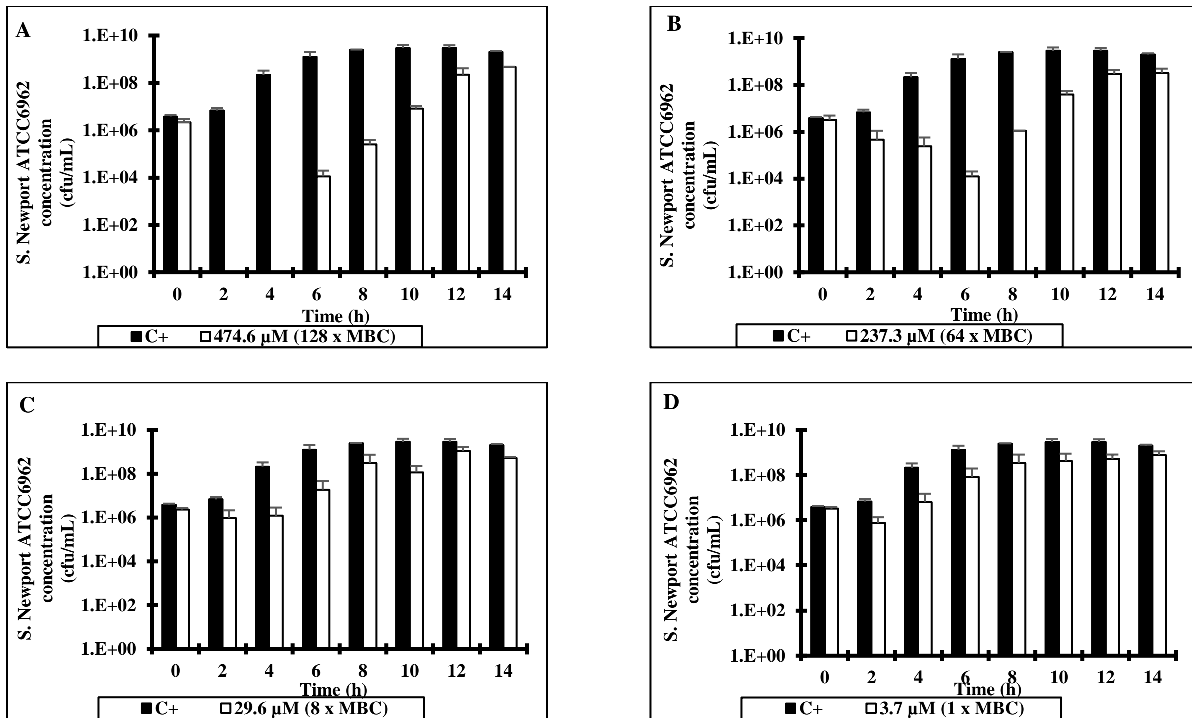

Figure. 6. Growth of *Salmonella enterica* subsp. enterica serovar Newport ATCC 6962 in unfermented Macfarlane broth containing MccJ25 at 128 times (A), 64 times (B), 8 times (C) and equal to (D) the MBC determined in 96-well micro-assay plates; viable counts (cfu/mL) in 24-well micro-plates were determined by plating on LB agar. C+ is the positive control (*Salmonella* Newport at an initial concentration of 5×10^5 cfu/mL without MccJ25). Data are means of two independent experimental repetitions.

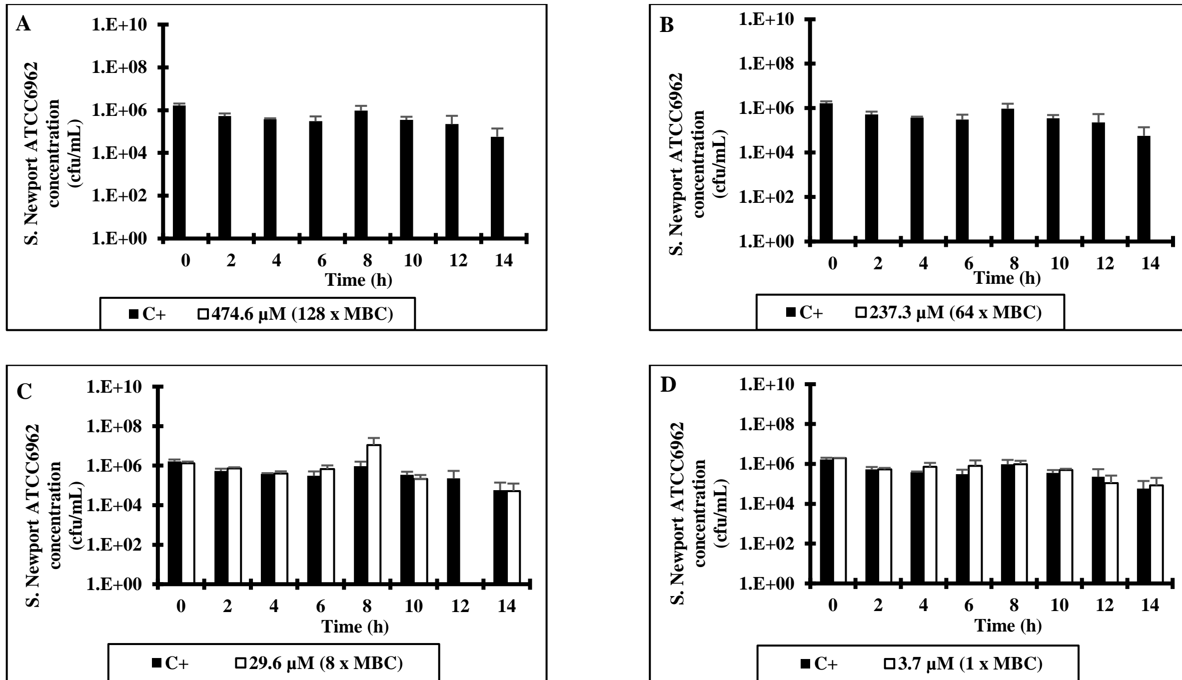

Figure. 7. Growth of *Salmonella enterica* subsp. enterica serovar Newport ATCC 6962 in fermented Macfarlane broth (+ 20% fresh medium) containing MccJ25 at 128 times (A), 64 times (B), 8 times (C) and equal to (D) the MBC determined in 96-well micro-assay plates; viable counts (cfu/mL) in 24-well micro-plates were determined by plating on LB agar. C+ is the positive control (*Salmonella* Newport at an initial concentration of 5×10^5 cfu/mL without MccJ25). Data are means of two independent experimental repetitions.

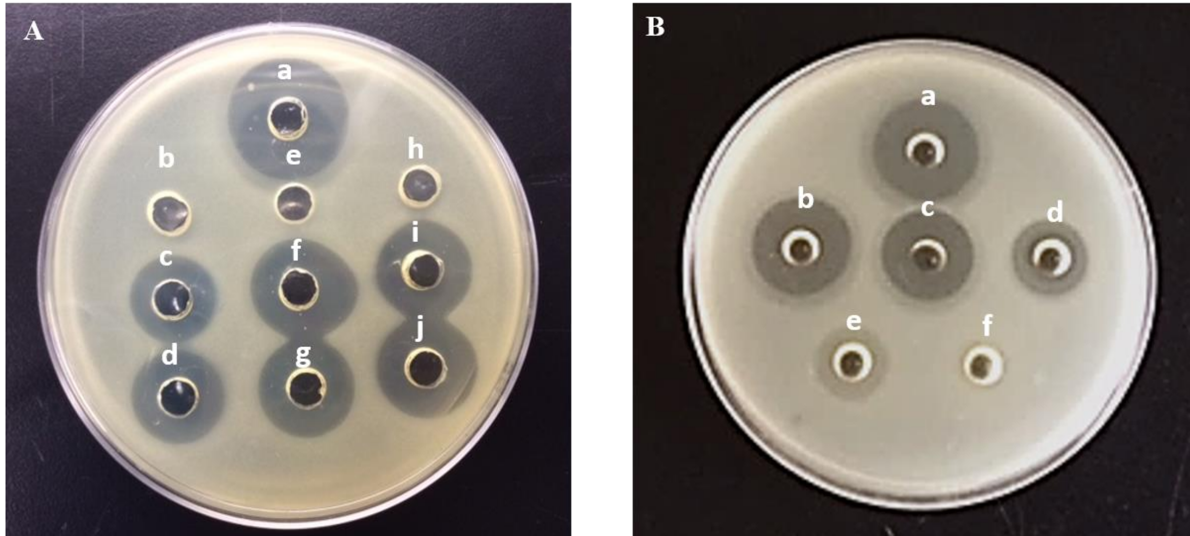

Figure. 8. (A) Bioavailability of MccJ25 (tested at a concentration corresponding to MBC) as shown by the inhibition activity against *Salmonella enterica* subsp. *enterica* serovar Newport ATCC 6962 in different media. (a) MccJ25 at a concentration of 0.5 mM, (b) LB broth without MccJ25, (c) activity of MccJ25 in LB broth before a 18 h incubation period, (d) activity of MccJ25 in LB broth after 18 h incubation, (e) unfermented Macfarlane medium without MccJ25, (f) activity of MccJ25 in unfermented Macfarlane medium before 18 h incubation, (g) activity of MccJ25 in unfermented Macfarlane medium after 18 h incubation, (h) fermented Macfarlane medium without MccJ25, (i) activity of MccJ25 in fermented MacFarlane before 18 h incubation, (j) activity of MccJ25 in fermented Macfarlane medium after 18 h incubation, (B) Bioavailability of MccJ25 as shown by the inhibition activity after 24-hour cultures in fermented Macfarlane medium. (a) MccJ25 at a concentration of 0.5 mM as a positive control, (b), (c), (d) and (e) show the activities of MccJ25 at 128 times, 64 times, 8 times and equal to the MBC respectively, (f) fermented Macfarlane medium used as negative control.

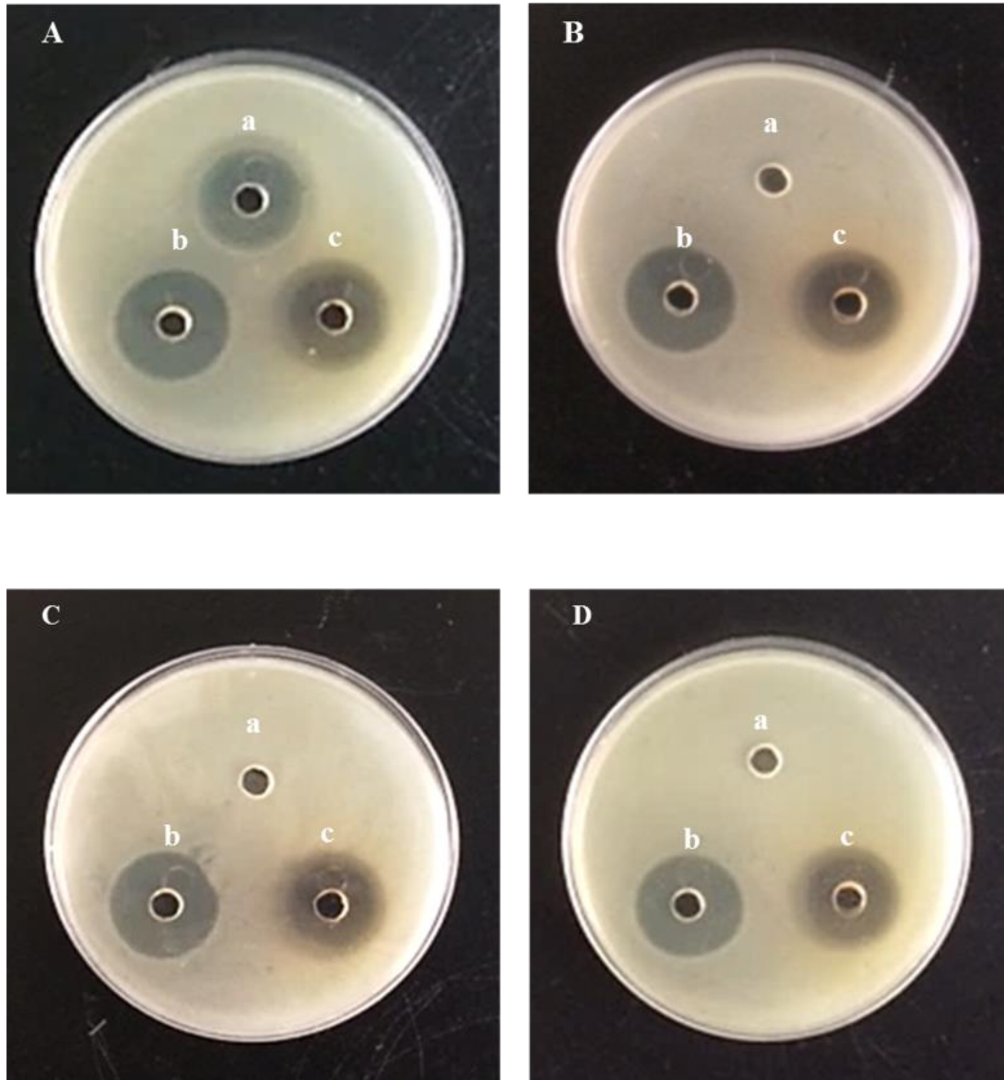

Figure. 9. Sensitivity of *Salmonella enterica* subsp. *enterica* serovar Newport ATCC 6962 to MccJ25 (a), reuterin (b) and rifampicin (c) before (A) or after an initial exposure to concentrations of MccJ25 of 128 times (B), 64 times (C) and 8 times (D) the MBC, as shown by the agar diffusion assay.

Chapitre 4. Bioavailability and biological activity of microcin J25: metagenomic and metabolomic analysis of its impact on the porcine microbiome in a continuous culture model

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Keywords: Swine colonic microbiota, PolyFermS model, *Salmonella enterica* subsp. *enterica* serovar Newport, bacteriocin, microcin J25, reuterin, rifampicin

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4.1. Résumé

La recrudescence alarmante du phénomène de résistance aux antibiotiques chez *Salmonella spp.* ainsi que chez d'autres bactéries pathogènes causant des maladies entériques chez le porc incite la communauté scientifique à rechercher de nouvelles alternatives. Parmi celles-ci, les antimicrobiens naturels, plus particulièrement les bactériocines, semblent représenter l'une des alternatives les plus prometteuses. La microcine J25 (MccJ25), une bactériocine produite par *Escherichia coli*, est un puissant inhibiteur de plusieurs bactéries pathogènes, y compris *Salmonella enterica*. Dans cette étude, nous avons évalué *in vitro* l'activité inhibitrice de la MccJ25 contre la souche *Salmonella enterica* subsp. *enterica* serovar Newport ATCC 6962 (*Salmonella* Newport) et son impact sur la composition et l'activité métabolique du microbiote colique porcin. Le modèle *in vitro* de fermentation en continu (PolyFermS) et le milieu nutritive Macfarlane ont été utilisés dans cette étude pour simuler les conditions du côlon proximal porcin. Pendant 35 jours de fermentation colique, un bioréacteur contenant du microbiote fécal de porc immobilisé a été utilisé pour inoculer en permanence deux bioréacteurs qui ont été alimentés avec le milieu nutritive Macfarlane. Ces deux bioréacteurs ont été utilisés pour étudier l'activité anti-*Salmonella* de la MccJ25 et son impact sur le métagénome et le métabolome du microbiote colique du porc. Deux autres composés antimicrobiens, soient la réutérine et la rifampicine ont été utilisés à des fins de comparaison. Les effluents des bioréacteurs ont été prélevés après l'ajout de chaque antimicrobien testé, puis analysés pour évaluer l'activité inhibitrice contre *Salmonella* Newport en utilisant la méthode de qPCR-PMA et le test de diffusion sur gélose. Les effluents collectés ont été également analysés pour étudier la composition et la diversité bactérienne par qPCR-PMA et par le séquençage de l'ARNr 16S à l'aide de l'approche Illumina MiSeq. En parallèle, l'impact des antimicrobiens testés sur le métabolome extracellulaire et intracellulaire du microbiote colique a été analysé par chromatographie liquide couplée à la spectrométrie de masse (LC-MS) suivie d'analyses multivariées des données générées. Les résultats ont démontré une activité inhibitrice remarquable de la MccJ25 contre *Salmonella* Newport contrairement à celle de la réutérine et la rifampicine. Par contre, aucun effet significatif sur la composition du microbiote colique n'a été démontré suite à l'ajout de la MccJ25 tandis que des variations au niveau du profil métabolomique ont été observées. Bien que des essaies *in vivo* soient toujours nécessaires pour valider ces résultats, notre étude suggère fortement la MccJ25 en tant qu'un candidat intéressant pour une utilisation comme alternative aux antibiotiques destinés aux applications vétérinaires.

4.2. Abstract

Recrudescence of resistance among *Salmonella spp.* and other pathogens increasingly prevalent in swine is spurring the search for alternatives to antibiotics added to pig feed. Natural antimicrobials, particularly bacteriocins, appear to hold promise. Produced by *Escherichia coli*, microcin J25 (MccJ25) is a potent inhibitor of several pathogenic bacteria including *Salmonella enterica*. We aimed in the present study to evaluate *in vitro* the inhibitory activity of MccJ25 against *Salmonella enterica* subsp. *enterica* serovar Newport ATCC 6962 (*Salmonella* Newport) and its impact on the composition and metabolic activity of the colonic microbiota of pigs. The PolyFermS *in vitro* continuous fermentation model was used with modified Macfarlane medium to simulate the porcine proximal colon. During 35 days of fermentation, a first-stage reactor containing immobilized fecal bacteria fed two second-stage test reactors in which the anti-*Salmonella* activity of MccJ25 and its impact on the microbiota were monitored. Reuterin and rifampicin were tested for comparaison. Propidium-monoazide-coupled quantitative PCR (PMA-qPCR) was performed to evaluate inhibition of *Salmonella* Newport and the effluent bacterial metagenome. Agar diffusion assays were also performed. Sequencing of 16S rRNA was performed using the Illumina MiSeq platform to evaluate microbial diversity. Liquid chromatography coupled to mass spectrometry (LC-MS) followed by multivariate analysis of the generated data. MccJ25 was a strong inhibitor of *Salmonella* Newport growth compared to reuterin or rifampicin. However, MccJ25 did not show any significant effect on the microbiota composition while it induced variations in the metabolic activity. Although this finding needs to be validated *in vivo*, our results suggest that MccJ25 could be an interesting alternative to antibiotics for veterinary applications.

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4.3. Introduction

Due to continued expansion of international markets, swine production has become one of the fastest growing economic sectors in developing countries (Kanis et al., 2003; Steinfeld et al., 2006) In 2012, pork dominated the world market for meat, at nearly 100 million tons or 36.3% of total production (Lagha et al., 2017) In spite of this, the swine industry has suffered major losses in recent years, due largely to the increasing incidence of enteric bacterial infections by pathogens such as *Salmonella spp., Escherichia coli* and *Campylobacter* (Davies, 2011). Salmonellosis is particularly prevalent in swine (Funk and Gebreyes, 2004; Foley et al., 2008), in which it causes acute disease resulting in increased mortality and reduced productivity (Boyen et al., 2008; Yuan et al., 2018). The transmission of *Salmonella* to humans via food processing and distribution is a major public health problem and pork producers strive to reduce sources of contamination. In industrialized countries, this has been achieved in large part through the use of antibiotics (Cromwell, 2002).

Ever since their commercialisation began in the 1950s, antibiotics have been used widely in human and veterinary medicine as well as in livestock production in order to treat bacterial diseases and also to promote animal growth (Viola and DeVincent, 2006). Their use as growth promoters in swine production is commonplace and has contributed substantially to improved livestock productivity and reduced production costs (Holman and Chénier, 2014). However, their overuse has led to the emergence of multiresistant bacteria, both commensal and pathogenic, which can be transmitted to humans through food consumption, contact with animals or the release of excreta into the environment (Barza, 2002). This has raised public concern and has led several countries to ban the use of antibiotics as growth promoters in animal feeds, as the European Union did in 2006 (Mathew et al., 2007). Effective replacements for conventional antibiotics are now urgently needed in order to maintain swineherd health and productivity. Among the most promising alternatives proposed so far are natural antimicrobial peptides (AMPs) called bacteriocins (Cavera et al., 2015).

Bacteriocins are defined as bacterially produced peptides of molecular mass usually less than 10 kDa (Riley and Wertz, 2002; Cotter, 2005a) Unlike antibiotics, bacteriocins are translated from mRNA and have a narrow spectrum of activity, inhibiting strains of the same or closely related species at nanomolar concentrations (Cleveland et al., 2001). Bacteriocins have been classified in categories and are listed in an open-access database called BACTIBASE (Hammami et al., 2010). The majority of bacteriocins studied so far are produced by Gram-positive bacteria and often lactic acid bacteria,

which are used widely in the food industry (Arthur et al., 2014). A few bacteriocins from Gram-negative bacteria are known but are less diverse (Hammami et al., 2013b). Bacteriocins find applications in a wide range of sectors including agri-food, the biomedical field and veterinary medicine. Nisin A, a bacteriocin produced by *Lactococcus lactis* subsp. *lactis*, is the most studied bacteriocin, having been approved in the late 1980s and commercialized for use as a food preservative, notably in dairy products (Gharsallaoui et al., 2016). Nisin is known as an effective inhibitor of the swine pathogen *Streptococcus suis* (LeBel et al., 2013) and of several pathogenic bacteria in raw meats and smoked salmon (Pawar et al., 2000; Neetoo et al., 2008). Pediocin PA-1 displays antimicrobial effects in cream cheeses and meat products contaminated with *Listeria monocytogenes* (Rodríguez et al., 2002). Other bacteriocins, including lacticin 3147 and enterocin AS-48, have been shown to prevent bacterial contaminants from propagating in dairy products (Morgan et al., 2001; Ananou et al., 2010). Potential applications in the treatment of various human and animal infectious diseases (Hammami et al., 2013b) and their use as inhibitors of enteric pathogens in animals (swine in particular) and as alternatives to antibiotics have been studied. Colicin E1, a bacteriocin produced by a Gram-negative bacterium, has been shown in vitro to inhibit strains of *Escherichia coli* that cause post-weaning diarrhea and edema in pigs (Stahl et al., 2004a) and as a feed additive to improve piglet growth by reducing the incidence of experimental post-weaning diarrhea induced by an enterotoxigenic *E. coli* (Cutler et al., 2007a). Bacteriocin-producing species such as *Lactobacillus plantarum* added to pig feed have been shown to reduce *Salmonella* counts in the intestinal microflora by out-competing the pathogen (van Winsen et al., 2001).

Although fragmentary, the studies mentioned above show that the inhibitory activity of bacteriocins and their impact on the colon microbiota are real albeit not yet well understood. Moreover, there are no published data on the real impact of bacteriocins on the overall metabolic profile of the colon microbiota. And very few studies have focused on bacteriocins of Gram-negative bacteria and their potential as inhibitors of *Salmonella* in swine.

Microcin J25 (MccJ25) is one of the most studied Gram-negative bacteriocins, having been shown to be a potent inhibitor of *Enterobacteriaceae* including *Salmonella* (Rintoul et al., 2001b). This 21-amino-acid peptide produced by *Escherichia coli* is characterized by a unique lasso structure that confers to it remarkable stability under denaturing conditions and in the presence of some proteases (Blond et al., 2001c; Rebuffat et al., 2004; Vincent and Morero, 2009). In a recent study, dietary supplemented

MccJ25 was shown to effectively improved performance, attenuated diarrhea and systematic inflammation, enhanced intestinal barrier function, and improved fecal microbiota composition of weaned pigs (Yu et al., 2017). Interesting features such as these make MccJ25 appealing for possible use as a natural alternative to antibiotics in pig feed.

In this study, the antibacterial activity of MccJ25 against *Salmonella enterica* subsp. *enterica* serovar Newport ATCC 6962 and its impact on the colonic microbiota were evaluated using the PolyFermS *in vitro* continuous fermentation model designed previously to simulate the pig proximal colon (Tanner et al., 2014a). MccJ25 was compared to the broad-spectrum antibiotic rifampicin and to reuterin, an antimicrobial aldehyde produced by the probiotic bacterium *Lactobacillus reuteri* (Cleusix et al., 2007a).

4.4. Material & methods

4.4.1. Bacterial strains and growth conditions

The MccJ25-producing strain was obtained from Prof. Sylvie Rebuffat (Muséum national d'Histoire naturelle, MCAM laboratory, Paris, France). It is a clone of *Escherichia coli* MC4100 carrying a mutated pTUC202 plasmid conferring chloramphenicol resistance (Solbiati et al., 1999). It was cultured overnight at 37°C in Luria–Bertani (LB) broth (Difco, Sparks, MD, USA) containing 34 µg/mL chloramphenicol under aerobic conditions as described by Ducasse *et al.* (Ducasse et al., 2012). Reuterin producer *Lactobacillus reuteri* ATCC 53608 was cultured overnight at 37°C in MRS broth (Oxoid Ltd., Basingstoke, Hampshire, UK) under anaerobic conditions (Doleyres et al., 2005). The swine pathogen model and sensitive strain for antibacterial activity assays in this study, namely *Salmonella enterica* subsp. *enterica* serovar Newport ATCC 6962 (obtained from Microbiologics Inc. St. Cloud, Minnesota, USA), hereinafter called *Salmonella* Newport, was cultured overnight at 37°C in LB broth under aerobic conditions.

Standard curves for enumerating bacterial groups representing the colonic microbiota were produced using ATCC strains (American Type Culture Collection, Rockville, MD, USA). *Bacteroides thetaiotaomicron* ATCC 29741 was cultured for 48 h at 37°C in brain-heart infusion (BHI) broth (Difco Laboratories) containing 0.05 % L-cysteine-HCl (Sigma, Oakville, ON, Canada) under anaerobic conditions (chamber model 1025, Forma Scientific, Marietta, OH, USA). *Lactobacillus acidophilus* ATCC 4356 was cultured for 24 h under aerobic conditions at 37°C in De Man-Rogosa-Sharpe (MRS)

broth (Oxoid, Nepean, ON, Canada) containing 0.05 % L-cysteine-HCl. *Escherichia coli* ATCC 25922 was cultured for 24 h at 37°C under aerobic conditions in LB medium. All of these strains were kept frozen at -80°C before culturing and were sub-cultured at least three times before use.

4.4.2. Antimicrobial compounds

MccJ25 was produced from a culture supernatant of *E. coli* MC4100 that harbors the plasmid pTUC202 following the protocol previously described by Ducasse *et al*. (Ducasse et al., 2012) In this study, 40 liters of broth culture of MccJ25-producing strain were used. Briefly, Minimal medium (M63) containing KH_2PO_4 (3 g/L), K_2HPO_4 (7 g/L), $(NH_4)_2HPO_4$ (2 g/L) and casamino acids (1 g/L) plus $MgSO_4$ (0.25 g/L), glucose (10 g/L) and thiamine (1 mg/L) inoculated with a preculture of strain MC4100 in LB broth (2% volume transfer) was incubated overnight at 37°C in a 30 L pilot fermenter (BIOSTAT® C plus, Göttingen, Germany). The culture was centrifuged (8,000 x g, 20 min, 4°C) and the supernatant was run through a Sep-Pak C18 35 cc cartridge (Waters, Milford, USA). MccJ25 was eluted with acetonitrile/water (30% v/v) containing 0.1% HCl then further purified by reverse-phase HPLC (Beckman Coulter System Gold, Mississauga, ON, Canada) on a preparative C18 column (Luna 10 µm, 100 Å, 21.10 × 250 mm, Phenomenex, CA, USA) at a flow rate of 10 mL/min using a 25–100% linear gradient of filtered acetonitrile/5 mM HCl in ultra-pure water (PureLab Ultra, ELGA, USA) with absorbance measurement at 214 nm for peptide detection. The purified MccJ25 was quantified by RP-HPLC using an analytical C18 column (AerisTM 3.6 µm, PEPTIDE XB-C18, 250 mm × 4.6 mm, Phenomenex, CA, USA). Nearly 418 mg of pure MccJ25 were obtained from 40 L of culture containing about 10.5 mg/mL. The purified MccJ25 was stored at 4°C until use.

Reuterin (3-hydroxypropanal) was produced using a culture of *Lactobacillus reuteri* ATCC 53608 in MRS medium containing 20 mM glycerol as described previously by Doleyres *et al*. (Doleyres et al., 2005). The inoculation volume was 1% and the temperature was 37°C (anaerobic conditions). The 16 h culture was centrifuged (1,500 x g, 10 min, 20°C) and the cells were washed with 0.1 M potassium phosphate buffer (pH 7.0), centrifuged again, re-suspended in 300 mM glycerol solution, held at room temperature for 45 min under anaerobic conditions and centrifuged (15,000 x g, 5 min, 4°C). The filtrate (0.22 µm, Millipore) was lyophilized. Reuterin was later purified on a silica gel 60 (0.060-0.2 mm, 70-230 mesh; Alfa Aesar) preparative chromatography column (2.8 × 35 cm, Bio-Rad Econo-Column) with acetone:ethyl acetate (2:1) as eluent (Cleusix et al., 2007a) at a flow rate of 5 mL/min.

Reuterin was quantified using a colorimetric method described previously by Lüthi-Peng *et al*. (Lüthi-Peng et al., 2002b). Acrolein was used for calibration and generating a linear standard curve with known initial concentrations (0.001–1.0 M). The eluate containing reuterin (1 mL) was added to 750 µL of 10 mM tryptophan dissolved in 0.05 N HCl solution and the resulting mixture was added to 3 mL of 37% HCl and maintained for 20 min at 37°C. Absorbance at 560 nm was recorded on an Infinite R F200 Pro spectrophotometer (Tecan Inc., NC, USA).

Rifampicin (purity ≥ 97%, HPLC) was purchased from Sigma-Aldrich (St. Louis, MO, USA) and stored at -20°C until use.

4.4.3. PolyFermS fermentation model of the pig colon

4.4.3.1. Immobilization of fecal bacteria

A fecal sample from a healthy adult pig raised under farm conditions and not given any antibiotic during the previous three months was collected in a sterile 50 mL Falcon tube. Anaerobiosis was maintained in a Gas-Pak anaerobic jar (Oxoid, Thermo Fisher Scientific) during transport to the laboratory and until the immobilization procedure (Tanner et al., 2014a). About 20 g were suspended in 80 mL of 0.1% peptone water (Difco Laboratories) containing 0.05% L-cysteine-HCl (Sigma-Aldrich, St. Louis, MO, USA) at 37°C and homogenized for 5 min at 200 rpm using a Stomacher (Seward model 400, Norfolk, UK). The liquid portion was recovered using a serological pipette and centrifuged for 1 min at 700 x g to remove large particles. The immobilization procedure was begun in an anaerobic chamber (model 1025, Forma Scientific, Marietta, OH, USA) by mixing 20 mL of supernatant into 1 L of a solution of 2.5 % w/v gellan, 0.25 % w/v xanthan and 0.2 % w/v sodium citrate (Cinquin et al., 2004). Beads 1-2 mm in diameter were then formed by pouring this mixture into rapidly stirred canola oil in a flask placed on ice and allowing them to settle for 5 min before removing the canola oil. A 0.1 M $CaCl_2$ solution was then added to harden the beads. After stirring for 30 min, the beads were washed with a 0.27 M KCl / 0.03 M $CaCl_2$ solution and sieved. Gel Beads were recovered and about 75 g were transferred to the inoculum reactor (BIOSTAT® Q plus, Sartorius AG, 79 Göttingen, Germany) containing 250 mL of fresh fermentation medium.

4.4.3.2. Fermentation medium

Macfarlane broth (Macfarlane et al., 1998b) with modified carbohydrate and protein concentrations was fed to the reactors in order to simulate the conditions in the pig proximal colon, as described by

Tanner *et al.* (Tanner et al., 2014a). The medium was autoclaved at 121°C for 20 min and then stored at 4°C until use. Solutions of L-cysteine-HCl monohydrate (5%) and vitamins as described previously by Michel *et al.* (Michel et al., 1998b) were filter-sterilized (0.22 μm, Millipore) and added separately to the sterilized medium (16 mL/L and 0.5 mL/L respectively). All components of the nutrient medium were purchased from Sigma-Aldrich (St. Louis, MO, USA).

4.4.3.3. Fermentation procedure

The fermentation-based *in vitro* model of the pig colon has been described elsewhere in detail (Tanner et al., 2014a). Briefly, the continuous culture was carried out for 35 days using the experimental set-up shown in Figure 1. The PolyFermS model is a two-stage system comprising a total of three reactors (BIOSTAT® Q plus, Sartorius AG, 79 Göttingen, Germany) controlled at 38°C and pH 6.0 with anaerobic conditions maintained by continuous flow of pure CO_2 through the medium. Seeded with 30% (v/v) swine fecal beads, the inoculum reactor IR (250 mL) was fed fresh nutrient medium continuously and fed test reactors TR1 and TR2, which also received fresh medium such that their continuous feed was 10% inoculum broth and 90% fresh medium. This required half of the the IR effluent; the other half of which was discarded. The mean retention time in the test reactors was 9 h. The feed rate of the three reactors was controlled using peristaltic pumps (model 120U, Watson-Marlow, Falmouth, Cornwall, UK). For the first 3 days, the IR was run in fed-batch mode, the medium replaced every 12 h with fresh medium, in order to stabilize fecal bead colonization. Continuous feeding was then started, followed by 12 days of stabilization before connection to TR1 and TR2. Continuous culture was split into three periods: stabilization, treatment and wash (Figure 1). Conditions were kept constant in IR to assess the temporal stability of the system without any manipulation during the entire fermentation, while TR1 and TR2 were subjected to treatments for various periods. Between each period, culture was flushed from TR1 and TR2 as described previously (Tanner et al., 2014a). After each flush, they were stabilized for 3 days until a steady state was reached before starting the next treatment period.

Performed simultaneously in TR1 and TR2, the four treatments consisted of adding *Salmonella* Newport alone at an initial concentration of 10^7 cfu/mL to each reactor, followed by adding *Salmonella* Newport at this concentration along with each tested antimicrobial: 0.397 mM MccJ25, 4 mM reuterin or 0.608 mM rifampicin. The antimicrobial test concentrations were determined in preliminary experiments (data not shown).

For each treatment, samples of test reactor effluent were collected at 0, 2, 4, 6, 8, 10, 12, 24, 48 and 72 h for analysis and stored at -80°C.

4.4.4. PMA-qPCR

4.4.4.1. Treatment with propidium monoazide

The inhibitory activity of MccJ25, reuterin and rifampicin against *Salmonella* Newport and the major bacterial species constituting the swine colonic microbiota were quantified using propidium-monoazide-coupled quantitative polymerase chain reaction (PMA-qPCR). Samples collected from the reactors were treated with propidium monoazide (PMA dye, Biotium, Inc., Hayward, CA, USA) for viable bacteria counts as described previously by Fernandez *et al.* (Fernandez et al., 2016). Briefly, 2.5 µL of 20 mM PMA solution (1 mg of PMA dissolved in 91 µL of 20% dimethylsulfoxide, stored at -20°C) was added to each 1 mL sample collected from the reactors to obtain a final concentration of 50 µM. Samples were kept for 5 min at room temperature in the dark with occasional vortex mixing, then placed for 5 min on ice 20 cm below a 500 W halogen lamp. Samples were then frozen and stored at -80°C until DNA extraction.

4.4.4.2. DNA extraction

DNA was extracted from samples of fermentation broth using the PowerSoil™ DNA Isolation Kit (MO BIO Laboratories, CA, USA). Samples were thawed and centrifuged for 10 min at 12,000 x g and the pellets were washed twice in Tris-EDTA buffer (10 mM Tris-HCl, 1 mM EDTA). Subsequent steps were performed following the PowerSoil kit instructions.

4.4.4.3. Real-time PCR analysis

The quantitative polymerase chain reactions were performed in MicroAmp® Fast Optical 96-Well Reaction Plates with Barcode (Life Technologies Inc., Burlington, ON, Canada) on an ABI 7500 Real-Time PCR System (Applied Biosystems, Streetsville, ON, Canada). Each sample was analyzed in duplicate. Extracted DNA was diluted 1/10 (v/v) in DNase-free water (Invitrogen) and placed in the microplate wells (5 µL of diluted extract per well). Each well also contained 12.5 µL of Fast SYBR® Green Master Mix (Applied Biosystems, Burlington, ON, Canada), 1 µL of each primer, forward and reverse, at a final concentration of 5 µM (Sigma-Aldrich, St. Louis, MO, USA) and 5.5 µL of DNase-free water. The primers used in this study are shown in Table 1. An amplification program described previously was used (Fernandez et al., 2016). Standard curves were produced for the quantified bacterial species following the protocol established previously (Fernandez et al., 2016).

4.4.5. Agar diffusion assay for the evaluation of antibacterial activity

The inhibitory activity of MccJ25, reuterin and rifampicin against *Salmonella* Newport in the pig colon model was evaluated using the agar well diffusion method as described previously (Wolf and Gibbons, 1996). Fermentation broth (500 µL samples) collected from test reactor TR1 after treatment with MccJ25, reuterin and rifampicin at 0, 4, 8, 12 and 24 h was centrifuged at 10,000 x g for 10 min. The supernatant was filtered through a 0.2 µm syringe filter (VWR, Mississauga, ON, Canada) then tested for inhibitory activity in LB medium (0.75% agar w/v) seeded with 150 µL of an overnight culture of *Salmonella* Newport. Wells 7 mm in diameter were cut in the solidified agar (25 mL in standard Petri plates) and filled with 80 µL of test sample. Plates were incubated aerobically at 37°C for 18 h, and the diameter of the zone of inhibition was measured.

4.4.6. Metagenomic analysis

4.4.6.1. 16S rRNA sequencing

The diversity of the porcine colonic microbiome was assessed by sequencing the bacterial 16S rRNA gene in the V3-V4 region using the amplification primers 341F (5'-CCTACGGGNGGCWGCAG-3') and 805R (5'-GACTACHVGGGTATCTAATCC-3') adapted to incorporate the transposon-based Illumina Nextera adapters (Illumina, USA) and a sample barcode sequence allowing multiplexed sequencing. High-throughput sequencing was performed at the Institute for Integrative Systems Biology at Université Laval, Québec, Canada) on a MiSeq platform using 2 x 300 bp paired-end sequencing (Illumina, USA).

4.4.6.2. Sequence analysis

Demultiplexed raw data were processed using Mothur software (v1.35.1) for profiling of the 16S rRNA gene as described by Schloss *et al.* (Schloss et al., 2009). Analyzed sequences were aligned using the bacterial reference database SILVA with the align.seqs command. Chimeric sequences were identified and removed using the UCHIME algorithm (Edgar et al., 2011). Resulting sequences were clustered into operational taxonomic units (OTUs) using the OPTI parameter. Representative OTU sequences were assigned taxonomy based on the Greengenes reference database (DeSantis et al., 2006).

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4.4.7. Metabolomic analysis

4.4.7.1. Sample preparation for extracellular metabolomic analysis

Fermentation broth samples were centrifuged at 6,000 xg for 10 min at 4°C. Supernatants were micro-filtered (0.22 µm) and kept frozen at -80°C until analysis. Defrosted supernatants were extracted using Oasis® HLB SPE cartridges (30 mg, 1 cc) following the protocol described previously by Travers *et al.* (Travers et al., 2016). The cartridge was conditioned with 3 mL of methanol, 3 mL of acetonitrile and 3 mL of 0.1 % formic acid solution (in Milli-Q water), loaded with 500 µL of supernatant, washed with 3 mL of Milli-Q containing 0.1 % formic acid and then eluted with 1 ml 0.1% formic acid solution/acetonitrile 20:80 (v/v). The eluted fractions were vacuum-dried, re-suspended in 0.1% formic acid solution/acetonitrile 90:10 (v/v) and centrifuged at 13,000 rpm for 10 min at room temperature. A 50 µL aliquot was placed in a glass LC/MS analysis vial.

4.4.7.2. Sample preparation for intracellular metabolomic analysis

Samples were prepared using the quenching method described by Wellerdiek *et al.* (Wellerdiek et al., 2009). Briefly, 1 mL was drawn into a syringe containing 4 mL of aqueous methanol (60%, v/v) at -40°C and then centrifuged at 4,000 x g for 5 min at 4°C. The supernatant was discarded and the cell pellet was kept in a tube on ice and then re-suspended in methanol, chloroform and water (1:1:0.9 v/v/v) with vortex mixing after adding each solvent and then centrifuged at 8,000 x g for 10 min at 4°C. A 70 µL aliquot of the upper water layer was collected in an Eppendorf tube and stored at -80°C until LC/MS analysis (50 µL of thawed sample in the glass vial).

4.4.7.3. LC-MS analysis

5 µl of each resuspended sample was analyzed by ultra-high-performance liquid chromatography system (Ultimate 3000 RSLC, Thermo Scientific) connected to a high-resolution electrospray ionization – quadrupole – time of flight (ESI-Q-TOF) mass spectrometer (Maxis II ETD, Bruker Daltonics). Separation was achieved on an Acclaim RSLC Polar Advantage II column (2.2 µm, 2.1 × 100 mm, Thermo Scientific) at a flow rate of 300 µL/min, using the following gradient of solvent A (ultra-pure water / 0.1% formic acid) and solvent B (HPLC-MS grade acetonitrile / 0.08% formic acid) over a total run time of 17.5 min: 5 min at 0%B followed by a linear increase from 0% B to 60% B for 12 min, linear increase to 100% B for 0.2 min, decrease to 0% B for 0.5 min. The MS spectra were acquired in positive ion mode in the mass range m/z 60 – 1300. The source parameters were as follows: nebulizer gas 35 psi, dry gas 8 L/min, capillary voltage 3500 V, end plate offset 500 V, temperature 200 °C. The ESI-Q-TOF instrument was externally calibrated before each run using a

sodium formate solution consisting of 10 mM sodium hydroxide in isopropanol / 0.2% formic acid (1:1, v/v). A quality control (QC) consisting of a mix of all samples and a blank (injection of water) were recorded every 10 samples to monitor separation quality and absence of cross-contaminations.

The high-resolution LC-MS data were converted to netCDF format and analyzed using the freely available R environment version 3.4.3 (www.r-project.org). Xcms package was used for peak detection, automatic retention time alignment and peak matching, on the 0.5 – 13 min range of the LC-MS analysis. The parameters were as follows: centwave peak detection method, maximal tolerated m/z deviation in consecutive scans 10 ppm, chromatographic peakwidth between 5 and 25 s, signal to noise ratio cutoff 6, minimum difference in m/z for peaks with overlapping retention times 0.01. The multivariate generated consisted in 198 samples × 16510 peaks for the intracellular metabolome and 198 samples × 22341 peaks for the extracellular metabolome. The generated multivariate matrices were submitted to multivariate data analysis, using mixOmics package (Lê Cao et al., 2009). Unsupervised and supervised data analysis methods were used, namely principal component analysis (PCA), partial least squares discriminant analysis (PLS-DA) and sparse partial least squares discriminant analysis (s-PLS-DA).

4.5. Results

4.5.1. Inhibition of *Salmonella* Newport under porcine colonic conditions

Figure 2 shows *Salmonella* Newport washout from the colonic fermentation model in the presence of MccJ25, reuterin or rifampicin during 24 h as quantified by PMA-qPCR. Starting at 7 $\log_{10}$ cfu/mL, the count dropped by a factor of 10 within 24 h in the absence of any antimicrobial agent, which was close to the theoretical washout curve over the same period of time. When MccJ25 was added to the medium, an additional nearly 10-fold drop was observed. Neither reuterin nor rifampicin had any such effect.

MccJ25 bioavailability in the modified Macfarlane broth was tested in terms of inhibition of *Salmonella* Newport in agar diffusion assays. As shown in Figure 3, MccJ25 produced inhibition zones varying from 1.9 to 1.2 cm in diameter and was thus shown to be stable under conditions characterizing the porcine colon. Inhibition by rifampicin was weak, with an average zone diameter of 1 cm compared to 2 cm for the positive control (T+). This activity dropped steadily during the first 12 h of fermentation

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and was zero at 24 h. Reuterin failed to produce any zone of inhibition. LC/MS analysis confirmed the stability and hence bioavailability of MccJ25 during the 24 h of culture (Figure 4).

4.5.2. Impact of MccJ25 on microbiota composition

Based on analysis by PMA-qPCR, the porcine colonic microbiota cultured continuously for 15 days in modified Macfarlane broth remained remarkably stable in overall composition for the first 12 h following introduction of *Salmonella* Newport plus antimicrobial agent and appeared practically unchanged at the end of the experiment, regardless of the presence of an antimicrobial agent (Figure 5). *Bacteroides*, *Lactobacillaceae* and *Enterobacteriaceae* counts in both test reactors measured one day after connecting the inoculum reactor did not differ from those in control samples collected from the inoculum during its stabilization period (data not shown). Total 16S rRNA gene abundance shows that none of the agents had any significant effect on the microbiota overall composition.

4.5.3. Microbial diversity based on 16S rRNA MiSeq sequencing

Evaluation of the impact of the different antimicrobial compounds on porcine intestinal microbiota was extended to the relative abundance of several families of bacteria in the three reactors. In the fecal sample, 95% of the viable bacteria were *Prevotellaceae*, whereas as the inoculum reactor community became quite diverse as it stabilized during days 6–15 (Figure. 6A), with the rise of new families such as *Lachnospiraceae*, *Ruminococcaceae* and later *Succinivibrionaceae*. *Prevotellaceae* and *Porphyromonadaceae*, both belonging to the phylum Bacteroidetes, are abundant in all samples, the former dominating at 40–70%, followed by *Lachnospiraceae* and *Ruminococcaceae*. Variations were observed in both test reactors especially after adding rifampicin, most notably the conspicuous increases in *Erysipelotrichaceae* abundance after 12 h and 24 h in test reactors 1 and 2 respectively. *Salmonella* Newport alone did this in reactor 1 as well as induce an increase in *Succinivibrionaceae* abundance in reactor 2. Other than a slight effect on this family and possibly on *Ruminococcaceae*, neither MccJ25 nor reuterin had a significant impact on relative abundances in comparison with the control (the inoculum as stabilized on day 15).

4.5.4. LC-MS data analysis and metabolomic profiling

Supernatants of collected fermentation samples were analyzed using LC-MS method in order to evaluate the impact of MccJ25, reuterin and rifampicine on the metabolome of the swine colonic microbiota. The multivariate data analysis of the effect of different treatments on extracellular and

intracellular metabolome is shown on fig 7. The results obtained by PCA analysis (Figure 7. A) clearly showed data-structuring by treatment. For the intracellular metabolome, the rifampicin treatment group (Figure 7 Aa, green plots) presented the highest variability and clearly stood out from the control group corresponding to stabilization samples (brown plots). Samples treated with MccJ25 (red plots) showed a clear clustering, less segregated from the stabilization samples. For the extracellular metabolome, the segregation between treatment was less clear (Figure 7Ab). Note that for certain treatments groups (such as reuterin and *Salmonella* – treated samples), a segregation per fermenter was observed. The PLS-DA showed similar trends as PCA analysis for the intracellular metabolome (Figure 7Ba) while segregation per groups was revealed for the extracellular metabolome (Figure 7Bb). However, a segregation per fermenter was still observed for certain treatment groups. Finally, sPLS-DA analysis performed only for the intracellular metabolome (Figure 7C) permitted to assign the most discriminant peaks. The corresponding compounds are under identification, from LC-MS/MS data and database search.

4.6. Discussion

The widespread use of antibiotics in livestock production has led to the emergence of antibiotic-multiresistant bacteria, which can infect humans via the food chain. The use of antibiotics as growth promoters has been banned outright in several industrialized countries, which has increased the urgency of the search for alternatives. Researchers began decades ago to propose bacteriocins as substitutes. However, very few studies have brought clear proof that bacteriocins inhibit bacterial pathogens under the conditions encountered in the intestines of farm animals. In this study, a metagenomic approach was used to evaluate the inhibitory activity of MccJ25 against *Salmonella* Newport in a model of the pig colon and to compare this activity to that of two other antimicrobial agents. We used this approach also to generate highly original data on the impact of such agents on the composition and stability of the porcine colonic microbiota. A state-of-the-art chromatographic method was optimised for the purpose of evaluating their impact on the microbiota metabolic activity. In the lead-up to this study, a stable bacterial population representative of the porcine colonic microbiota was reproduced in vitro using the PolyFermS model developped previously by Tanner *et al.* (Tanner et al., 2014a). This model allows reproducible testing of different treatments in parallel reactors, thus providing a built-in control inoculated with the same microbiota. It was used in the present study to mimic a *Salmonella* infection in pigs and to evaluate the previously demonstrated

inhibitory activity of MccJ25 and other antimicrobial agents against this pathogen (Tanner et al., 2014b).

Salmonella enterica is known to be the principal causative of acute enteric disease in swine, a major contributor to economic loss in the industry and above all a menace to human health (Foley et al., 2008). Controlling *Salmonella* has become essential in pork production. Numerous studies suggest that bacteriocins are effective in the treatment of infections in animal models, whether delivered as pure peptides or via bacteriocin-producing probiotic bacteria (van Winsen et al., 2001; Ojha and Kostrzynska, 2007; Riboulet-Bisson et al., 2012). In a study using a porcine model, a mixture of bacteriocin-producing probiotic strains considerably reduced infection caused by *Salmonella enterica* Serovar Typhimurium through domination in the ileum, the primary attachment site of the pathogen (Casey et al., 2007). However, the link between bacteriocin production and the anti-*Salmonella* activity has never been clearly established. It has been further demonstrated that feeding pigs with *Lactobacillus salivarius* UCC118 producing the broad-spectrum class IIb bacteriocin Abp118 affected intestinal microbiota diversity in addition to inhibiting *Salmonella*. This was attributed to competitive exclusion or immunomodulation of host defense mecanisms (Riboulet-Bisson et al., 2012). In an in vitro study, *Bifidobacterium thermophilum* RBL67 was shown to inhibit colonization by *Salmonella* Typhimurium N-15 under simulated porcine intestinal conditions through a synergistic effect with selected prebiotics (Tanner et al., 2014b). Inhibition of *Salmonella* Typhimurium in pigs by oral administration of microcin-24-producing *Escherichia coli* has also been demonstrated (Frana et al., 2004). Apart from these few studies, there has been no serious investigation of the behavior and stability of bacteriocins in the gastrointestinal tract. Furthermore, no study has clearly established a direct link between the presence of bacteriocins and the antimicrobial activities observed in the digestive tract. Such studies require the prior development of sensitive and specific methods for the detection and quantification of bacteriocins in a complex environment such as the colon. In general, the bacteriocins of Gram-negative bacteria are a much more recent research topic and their inhibition of *Salmonella* remains less studied.

MccJ25 is one of the most studied Gram-negative bacteriocins. Produced by bacteria of the family *Enterobacteriaceae*, this lasso peptide of mass 2.1 kDa (Salomon and Farías, 1992b) is reportedly bactericidal to a wide range of Gram-negative species including *Escherichia coli*, *Salmonella* and *Shigella* (Blond et al., 1999b; Rintoul et al., 2001b). Intraperitoneal injections into a murine model have

been shown to decrease *Salmonella* Newport counts significantly in the spleen and liver (Lopez et al., 2007a). However, no study of the anti-*Salmonella* activity of MccJ25 under porcine gastrointestinal conditions has been published.

Using the PolyFermS model, we have provided clear evidence of the inhibitory activity of MccJ25 against *Salmonella* Newport under conditions simulating the porcine colon. This activity remained potent during 24 h of continuous culture, as shown by PMA-qPCR and agar diffusion, and was superior to those of the broad-spectrum antibiotic rifampicin and the antimicrobial compound reuterin. The inhibitory activity was shown to be correlated with the greater stability and bioavailability of MccJ25 compared to reuterin and rifampicin. Samples taken from test reactor 1 at regular intervals over 24 h further showed significant inhibition of *Salmonella* Newport using the agar diffusion assay. The stability of MccJ25 has been demonstrated in several environments and attributed to its unique lasso structure (Rosengren et al., 2003). Also known as [1]rotaxane topology, this structure confers remarkable stability at high temperatures and extreme pHs and resistance to some proteases (Salomon and Farías, 1992b; Blond et al., 1999b; Rosengren et al., 2003). Such features allow MccJ25 to resist very stringent conditions like those encountered in the GI tract, unlike other bacteriocins such us pediocin PA-1, a class IIa bacteriocin which has been found sensitive to simulated GI conditions and to lose its antibacterial activity completely in the small intestine (Kheadr et al., 2010).

Reuterin is known as a potent antimicrobial produced naturally by *Lactobacillus reuteri* and is used widely in the food industry (Cleusix et al., 2007a). It represents an interesting model for the present study since it has been shown to exert antibacterial activity against *Salmonella spp.* (Kuleaşan and Çakmakçı, 2002). Its failure to inhibit *Salmonella* Newport in the present study is due probably to non-specific interactions with various compounds in Macfarlane medium or perhaps to instability under these conditions. Meanwhile, rifampicin was slightly inhibitory for nearly 8 h of culture, based on the agar diffusion assay but not corroborated by *Salmonella* Newport counts based on PMA-qPCR. Although tylosin and chlortetracycline are the two most commonly used antibiotics as growth promoters and for therapeutic purposes in pork production (Apley et al., 2012), rifampicin was chosen as a control for this study because of its mode of antibacterial activity, which is similar to that of MccJ25 in targeting bacterial RNA polymerase (Campbell et al., 2001; Adelman et al., 2004; Mukhopadhyay et al., 2004; Alifano et al., 2015). In this study we succeeded for the first time in

developing a very specific and sensitive method using LC-MS to detect and quantify McJ25 in fermentation medium simulating the porcine colon. This method allowed us to confirm the bioavailability of MccJ25 and therefore make a clear link with its anti- *Salmonella* Newport activity under such conditions.

Another aim of the present work was to study the impact of Mcc25, rifampicin and reuterin on the composition and diversity of the pig colonic microbiota. Enumerating specific bacterial groups by PMA-qPCR at the beginning of the study gave us a general idea of the composition of the pig gut microbial community. No major changes in *Bacteroides*, *Lactobacillaceae*, *Enterobacteriaceae* and total 16S rRNA gene contents were observed when MccJ25, reuterin and rifampicin were added separately. However, 16S rRNA gene sequencing using the illumina MiSeq approach revealed some changes in microbial diversity. In published studies on swine fecal and colonic microbiomes *Firmicutes*, *Bacteroidetes* and *Proteobacteria* have been reported as the most abundant phyla (Poroyko et al., 2010; Holman et al., 2017). These prevalences are in accordance with our findings that the most abundant families of bacteria are *Prevotellaceae* and *Porphyromonadaceae* (phylum *Bacteroidetes*), *Lachnospiraceae* and *Ruminococcaceae* (phylum *Firmicutes*) and *Succinivibrionaceae* (phylum *Proteobacteria*). The present meta-analysis of a porcine intestinal bacterial community has revealed a remarkable increase in relative abundance of some bacterial families, such as *Lachnospiraceae*, *Ruminococcaceae*, *Succinivibrionaceae* and unidentified members of the order Clostridiales in comparing the inoculum reactor effluent to the initial fecal sample. These changes in microbiota profile may due to differences between feces and submerged culture and might reflect adaptation to the new environment throughout the stabilization period. Similar results have been observed in previous studies using laboratory models of the colon based on culture in liquid media. (Le Blay et al., 2008; Van den Abbeele et al., 2010; Tanner et al., 2014a). Moreover, the metagenomic analysis showed the predominance of *Prevotellaceae* both in the fecal sample and in the bioreactor effluent. Indeed, *Prevotella* has been shown to be the most abundant bacterial group in the porcine colon (Kim et al., 2011; Looft et al., 2012). This is likely related to the typical pig diet, which is relatively rich in polysaccharides requiring fibrolytic bacteria for their breakdown in the colon to provide short-chain fatty acids (SCFAs) as an energy source for other members of the microbial community (Vital et al., 2014). Species of *Prevotella* reportedly produce acetate, which is an essential SCFA for butyrate production by some bacterial species in the swine gut ecosystem (Looft et al., 2014b). Furthermore, we observed significant variations in the relative abundance of some bacterial groups, especially

following perturbation with rifampicin, which induced a remarkable increase in the prevalence of *Erysipelotrichaceae*, variations not observed following addition of MccJ25 or rifampicin to the medium. This might be due to the broad-spectrum activity of rifampicin affecting both Gram-positive and Gram-negative bacteria, unlike the narrow spectrum of MccJ25 activity against bacteria belonging to the *Enterobacteriaceae* family only. Based on what is known about reuterin, it was expected to have had a broad spectrum of action. Something in the medium appears to have interfered with it.

The LC-MS method developed as a complement to metagenomic analysis to study the metabolic activity of the colonic microbiota has rarely been mentioned in the literature on colonic microbiota but provides important clarification of the effects of natural antimicrobials on the microbial ecosystem of the swine colon. The intracellular and extracellular metabolome profiles both showed that reuterin had no significant impact compared to the control, whereas MccJ25 and rifampicin were distinguishable from other treatments and were shown to induce the production of different metabolites by bacterial communities that remain to be identified.

In conclusion, since MccJ25 appears to be a potent inhibitor of *Salmonella* Newport in the PolyFermS continuous culture model of the porcine proximal colonic microbiome, it should be considered as a potential alternative to antibiotics in pork production. MccJ25 also does not appear to affect the composition of this bacterial community, as demonstrated by PMA-qPCR and 16S rRNA MiSeq sequencing analysis. This is important since it has been shown that changes in gut microbiota composition are associated with various metabolic disorders and likely subject the animal to stress in any event. The different multivariate analyses of the LC-MS data obtained in this study show that MccJ25 does modify the metabolomic profile, as manifested in over-representation of intracellular metabolites of the colonic microbiota. A similar effect was observed with rifampicin, which proves that MccJ25 has a real impact on the metabolic activity of the microbiota. Further study is necessary in order to exploit metabolomic data such as these and identify the intracellular and extracellular metabolites involved in the normal metabolic activity of this bacterial community as well as explain changes induced by MccJ25 and thus compare it to an antibiotic control. Future *in vivo* studies should be focused on validating the effectiveness of MccJ25 as an inhibitor of *Salmonella* in farmed pigs as well as evaluating its impact on the intestinal microbiota.

4.7. Acknowledgements

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4.9. Competing interests

None declared.

4.10. Author contributions

SN designed and performed experiments, carried out data analysis and wrote the manuscript. IF managed the overall project, participated in the design of the experiments, analysis and interpretation of data and writing of the manuscript. SR contributed to manage the overall project and helped for the interpretation of data and writing the manuscript. SZ participated for the design of the experiments, performed metabolomic analysis and helped for analysis and interpretation of data and the writing of the manuscript. MBT participated for the preparation of the Macfarlane nutritive medium during the fermentation period. BF contributed to production and purification of reuterin and provided the swine fecal sample. MBT, JT and BF participated in the immobilization part of the fermentation procedure and helped for the operation of the PolyFermS system.

4.11. Tables & figures

4.11.1. Tables

Table, 1. Primers used for enumerations of swine colonic bacteria and *Salmonella* Newport by the PMA-qPCR method

Target	Bacterial strain	Primer	Sequence 5'-3'	Reference
Total 16S rRNA genes	*E. coli* ATCC25922	Eub338F	ACT CCT ACG GGA GGC AGC AG	Guo *el al.* 2008 (Guo et al., 2008)
		Eub518R	ATT ACC GCG GCT GCT GG	
Bacteroides	*B. thetaiotaomicron* ATCC29741	Bac303F	GAA GGT CCC CCA CAT TG	Ramirez-Farias *et al.* 2008 (Ramirez-Farias et al., 2008)
		Bfr-Fmrev	CGC KAC TTG GCT GGT TCA G	
Lactobacillaceae	*L. acidophilus* ATCC 4356	F_Lactos 05	AGC AGT AGG GAA TCT TCC A	Furet et al. 2009 (Furet et al., 2009)
		R_Lacto 04	CGC CAC TGG TGT TCY TCC ATA TA	
Enterobacteriaceae	*E. coli* ATCC25922	Eco1457F	CAT TGA CGT TAC CCG CAG AAG AAG C	Bartosch et al. 2004 (Bartosch et al., 2004)
		Eco1652R	CTC TAC GAG ACT CAA GCT TGC	
Salmonella	*Salmonella* Newport ATCC 6962	invAF	CGTTTCCTGCGGTACTGTTAATT	Li & Chen. 2013 (Li and Chen, 2013)
		invAR	TCGCCAATAACGAATTGCCCGAAC	

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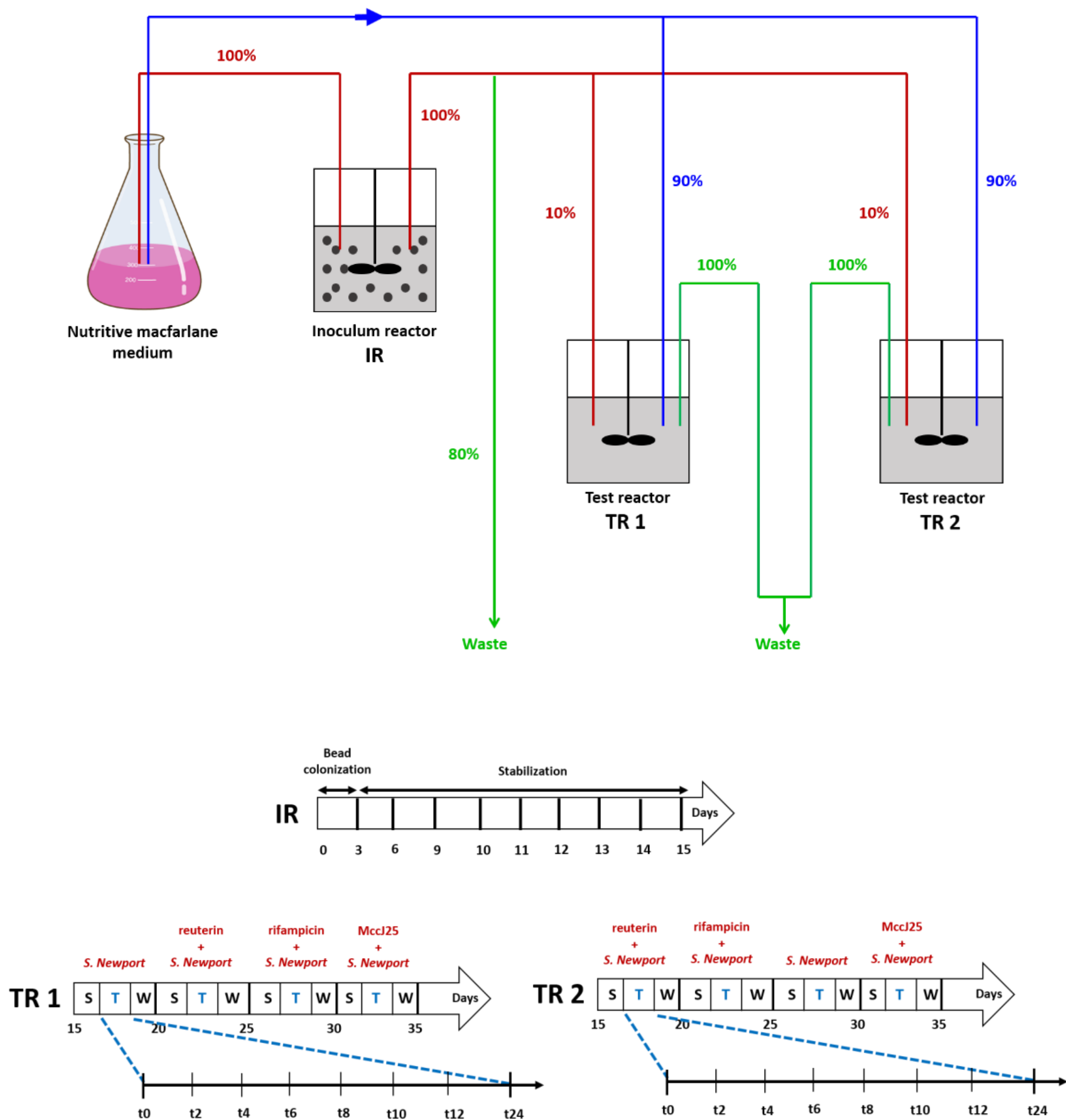
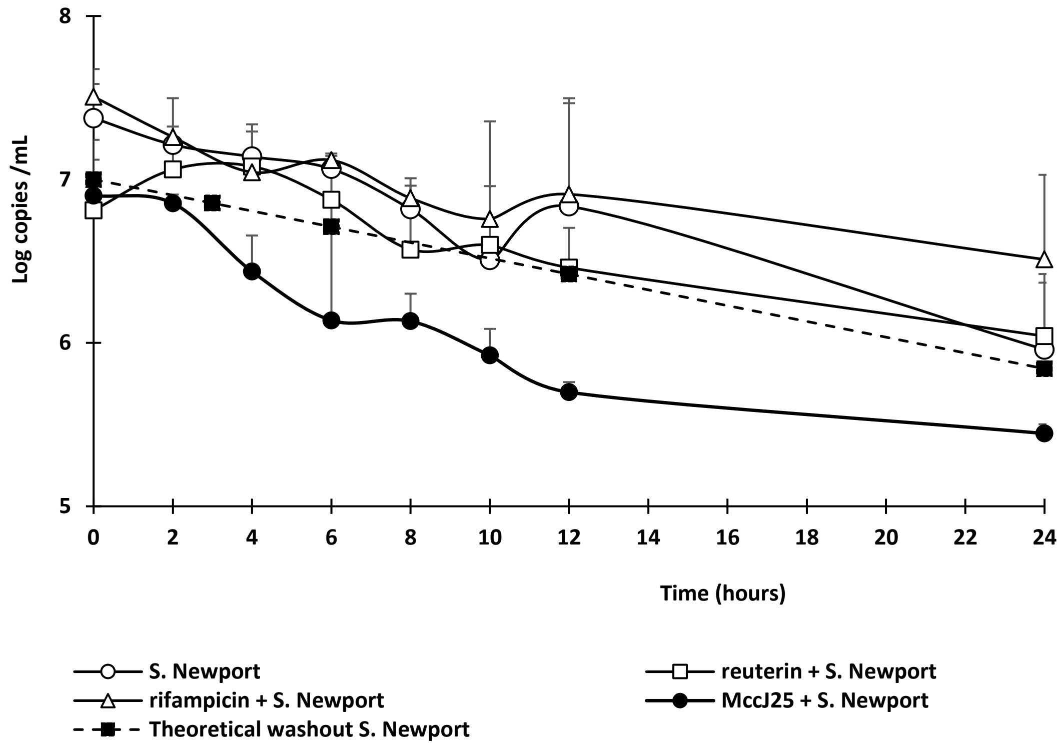
4.11.2. Figures



Figure, 1. PolyFermS model set-up and experimental treatment schedule. Relative flow rates are indicated in %. IR: inoculum reactor containing pig fecal bacteria immobilized in gel beads (30% v/v); TR1, TR2: test bioreactors; S: stabilization period; T: treatment period; W: wash period.



Figure, 2. Washout of *Salmonella enterica* subsp. *enterica* serovar Newport ATCC 6962 from continuous culture of porcine intestinal microbiota in modified Macfarlane broth in the PolyFermS model starting from 10^7 cfu/mL. The dashed line represents the theoretical washout of inert particles starting at 10^7 per mL. Error bars indicate standard deviation based on two independent repetitions in test reactors 1 and 2. Plotted values are based on PMA-qPCR analysis.

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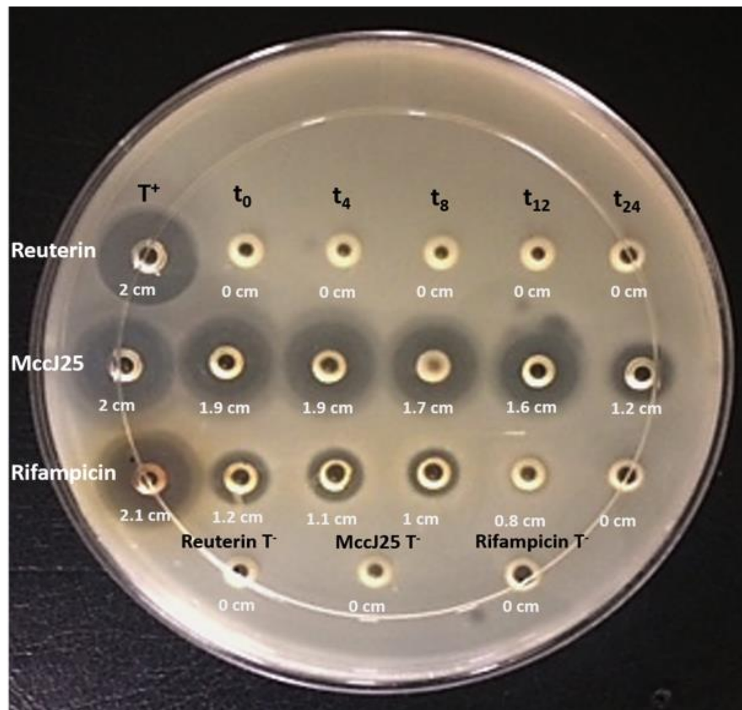
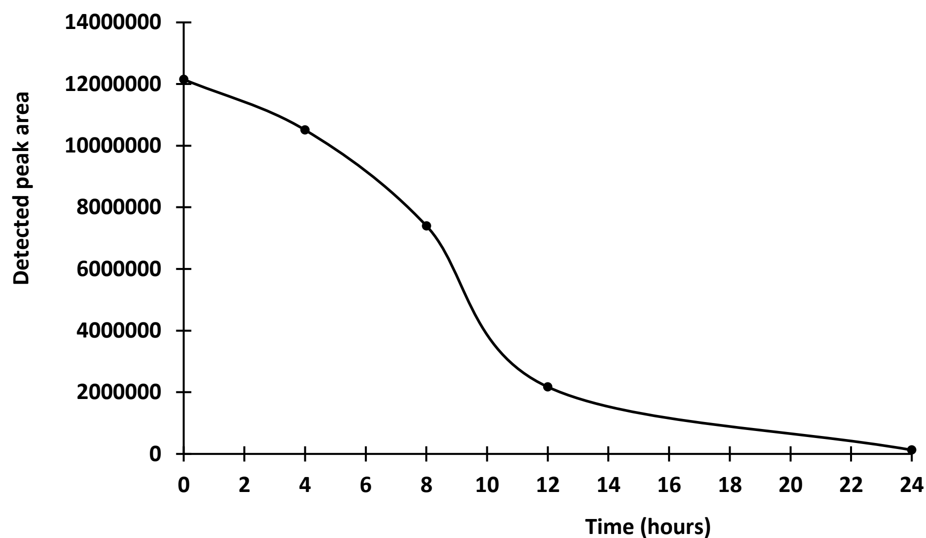
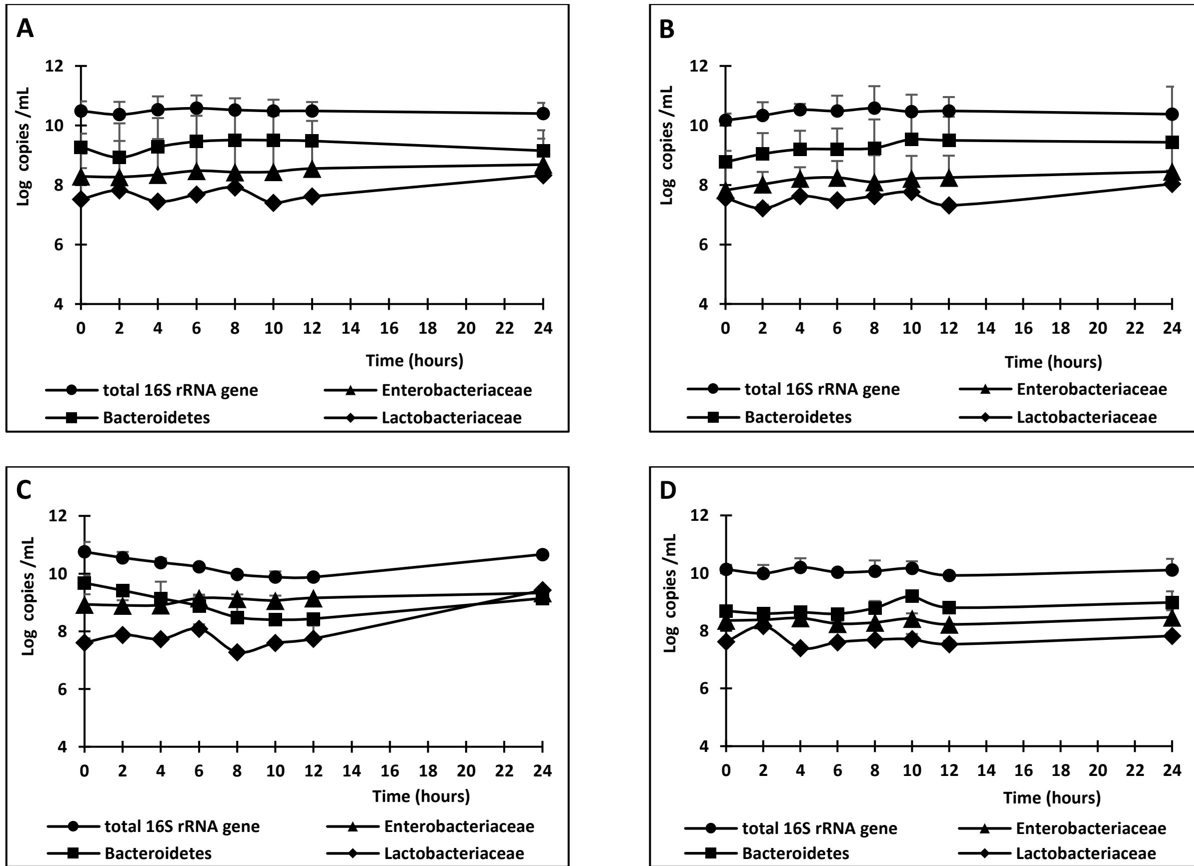


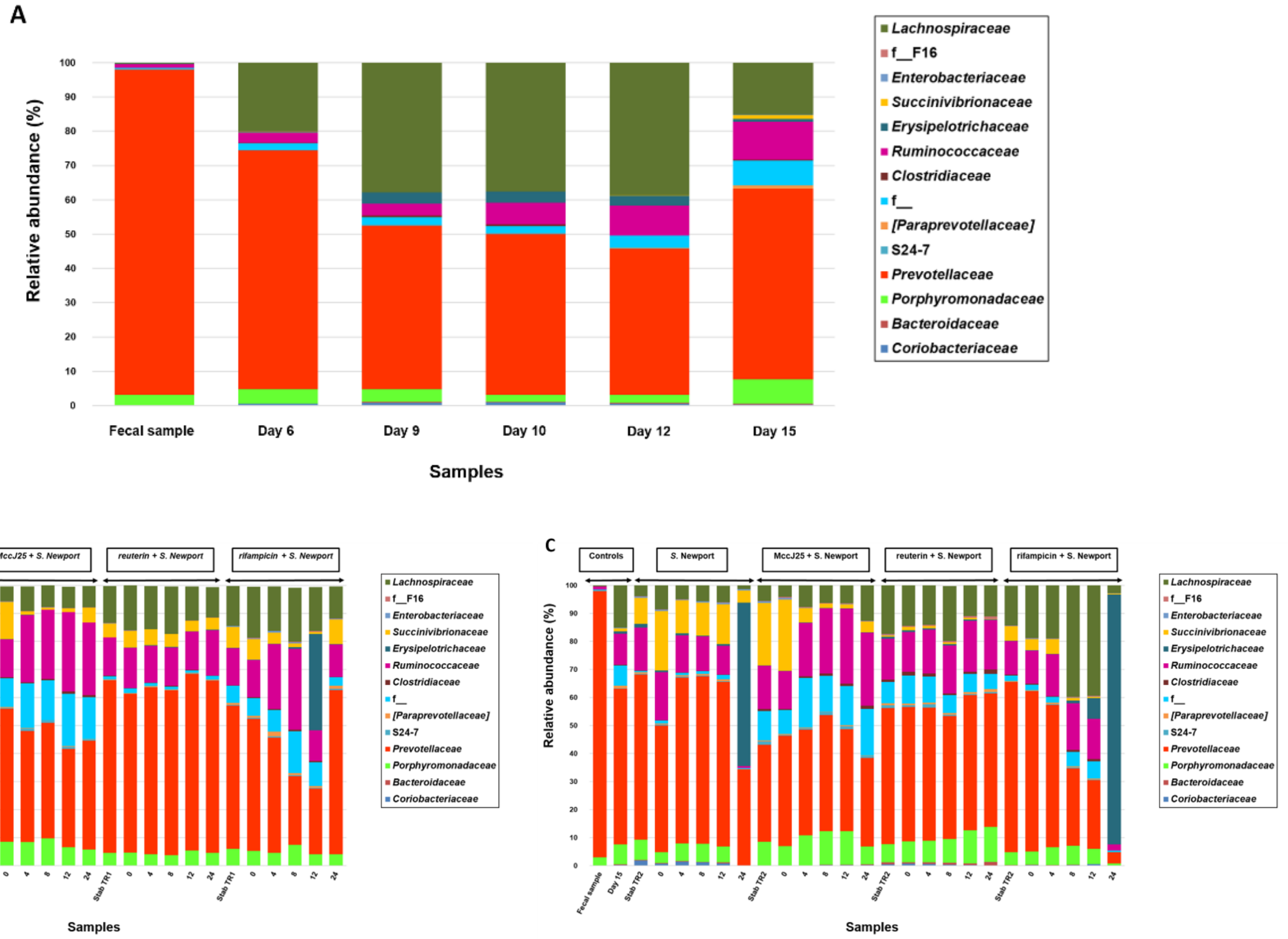
Figure, 3. Agar diffusion assay showing the inhibitory activity of MccJ25, reuterin and rifampicin against *Salmonella enterica* subsp. *enterica* serovar Newport ATCC 6962 during 24 h of culture in modified Macfarlane broth under porcine colonic conditions in test reactor 1 of the PolyFermS model.



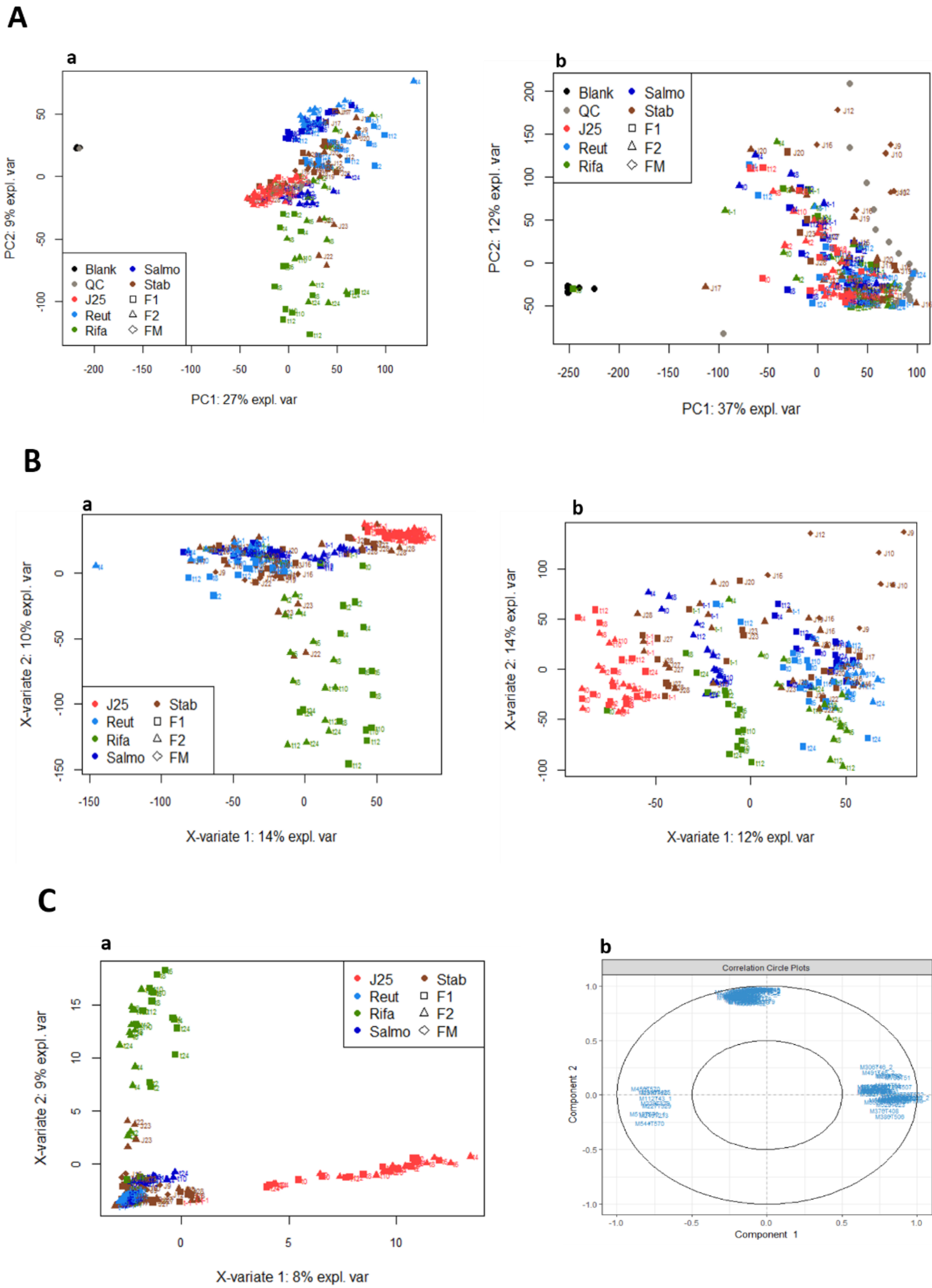
Figure, 4. Persistence of MccJ25 in continuous culture of porcine colon microbiota in modified Macfarlane broth (test reactor 1 of the PolyFermS model), based on LC/MS peak area.



Figure, 5. Mean concentration ($\log_{10}$ copy number per mL of effluent) of specific bacterial groups measured by PMA-qPCR in PolyFermS model test reactors TR1 and TR2 for 24 h after adding (A) *Salmonella* Newport at an initial concentration of 10^7 cfu/mL, (B) reuterin + *Salmonella* Newport, (C) rifampicin + *Salmonella* Newport, (D) MccJ25 + *Salmonella* Newport. Bars indicate standard deviation resulting from means of two independent repetitions in TR1 and TR2.



Figure, 6. Composition of the pig colonic microbiota as determined by sequencing of 16S rRNA extracted from fecal bacteria and effluent from (A) the inoculum reactor IR, (B) test reactor TR1 and (C) test reactor TR2 of the PolyFermS model. Bars represent the abundance of each bacterial family relative to the control samples (fecal sample, inoculum culture on day 15, TR1 or TR2 after stabilization) and after treatment with *Salmonella* Newport, MccJ25 + *Salmonella* Newport, reuterin + *Salmonella* Newport or rifampicin + *Salmonella* Newport at 0, 4, 8, 12 and 24 hours.



Figure, 7. Representation of metabolic activity of swine colonic microbiota in the PolyFermS model by analyzing all the effluent samples collected from the inoculum reactor IR and test reactors TR1 and

TR2. (A) Principal component analysis (PCA) based on (a) intracellular and (b) extracellular metabolomics data. (B) Partial least squares discriminant analysis (PLS-DA) based on (a) intracellular and (b) extracellular metabolomics data. (C) Sparse partial least squares discriminant analysis (s-PLS-DA) based on intracellular metabolomics data. (a) visualization of 50 selected variables corresponding to each presented component. (b) Correlation circle plots for the s-PLS-DA analysis.

Conclusion générale et perspectives

Depuis leur découverte dans les années 50, les antibiotiques ont été utilisés à grande échelle dans plusieurs secteurs, notamment en élevage porcin, afin de traiter et/ou prévenir les infections causées par certaines bactéries pathogènes. Ils ont également été très utilisés dès le jeune âge et à des doses subthérapeutiques pour promouvoir la croissance des animaux. Cependant, l'usage abusif de ces antibiotiques dont plusieurs possèdent un large spectre a engendré une recrudescence inquiétante du nombre de bactéries pathogènes multirésistantes qui peuvent se transmettre à l'humain et entrainer des problèmes graves de santé publique. Face à cette problématique, la recherche ne cesse de s'activer pour trouver des alternatives pouvant être utilisées en production animale, en remplacement ou en association avec les antibiotiques. Parmi les alternatives proposées, les bactériocines suscitent de plus en plus d'intérêt comme une des alternatives les plus prometteuses.

Les bactériocines sont des peptides antimicrobiens produits par plusieurs espèces bactériennes et ayant un spectre d'action assez restreint inhibant ainsi à des concentrations nanomolaires des bactéries qui sont plus généralement proches phylogénétiquement de la souche productrice (Nes, 2011). De nombreuses études ont démontré le fort potentiel des bactériocines à inhiber les bactéries pathogènes surtout dans les matrices alimentaires tel que les produits laitiers, carnés et végétaux (Hammami et al., 2013a). Par contre, très peu d'études ont porté sur leur potentiel comme alternatives aux antibiotiques chez l'humain ou chez l'animal. Leur potentiel d'utilisation comme chez les animaux d'élevage comme le porc n'a été que rarement investiguée. De plus, les quelques travaux rapportés dans la littérature se sont plus concentrés sur les bactériocines de bactéries à Gram positif (van Winsen et al., 2001; LeBel et al., 2013; Tanner et al., 2014b). Quelques travaux ont également porté sur les colicines, des bactériocines de bactéries à Gram négatif ayant des effets inhibiteurs contre des souches pathogènes d'*Escherichia coli* chez le porc (Stahl et al., 2004b; Cutler et al., 2007b).

Dans le cadre de cette thèse, nous nous sommes intéressés à la microcine J25 (MccJ25), la plus étudiée des bactériocines de bactéries à Gram négatif, et à son potentiel comme alternative aux antibiotiques en élevage porcin. Des approches originales de métagénomic et métabolomique de même que des modèles dynamiques in vitro du tube digestif porcin ont été adoptées pour évaluer la stabilité gastro-intestinale, l'activité anti-*Salmonella*, ainsi que l'impact de la MccJ25 sur le microbiote colique porcin.

Dans le premier article de cette thèse, nous avons étudié la stabilité et l'activité inhibitrice de la MccJ25 durant son transit dans le tractus gastro-intestinal en utilisant le simulateur dynamique *in vitro* du tube digestif (TIM1). La MccJ25 a été tout d'abord produite et purifiée en suivant un protocole établi dans des études précédentes (Blond et al., 1999b; Rebuffat et al., 2004) . Ensuite, la MccJ25 purifiée a été soumise à une digestion GI à l'aide du model TIM-1 puis analysée et quantifiée par HPLC et son activité contre une souche de *Salmonella enterica* a été également évaluée par les tests qualitatifs et quantitatifs de l'activité antibactérienne. Les résultats obtenus ont démontré que la MccJ25 est plus stable au niveau de l'estomac, tandis qu'une dégradation partielle a été observée lors de son entrée dans le duodénum. Ces résultats sont étonnamment en désaccord avec ceux rapportés par des études précédentes dans lesquelles la MccJ25 s'est montrée remarquablement rigide et stable sous des conditions extrêmement dénaturantes et très résistante à de nombreuses protéases (Salomon and Farías, 1992a; Blond et al., 1999b; Blond et al., 2001a; Rosengren et al., 2004). Cette remarquable stabilité de la MccJ25 est due à sa structure particulière et unique appelée structure en lasso. Malgré cette dégradation partielle démontrée dans le duodénum, la MccJ25 demeure plus résistante aux conditions gastriques comparativement à d'autres bactériocines notamment la pédiocine PA-1, qui s'est montrée beaucoup plus sensible aux conditions gastro-intestinales (GI) (Kheadr et al., 2010). De plus, nous avons pu identifier dans cette étude les formes de dégradation de la MccJ25 dans les conditions duodénales par des analyses par LC-MS/MS et aussi par l'approche des réseaux moléculaires utilisant les outils de la plateforme GNPS (Global Natural Product Social Molecular Networking) (Yang et al., 2013; Wang et al., 2016). Les principales formes de dégradations ont été également identifiées par les mêmes méthodes d'analyse en présence d'enzymes protéolytiques qui sont présentes au niveau du duodénum. Grâce à ces analyses, on a pu déterminer les principales enzymes ayant contribué à la dégradation de la MccJ25 au niveau du duodénum. Il s'agit de deux enzymes pancréatiques, l'élastase et l'α-chymotrypsine. À travers ces résultats, on peut conclure que la MccJ25 pourrait être considérée comme un bon candidat pour une éventuelle utilisation comme une alternative aux antibiotiques puisqu'elle est capable de résister en bonne partie au transit GI et donc atteindre son site d'action au niveau iliaque ou colique. L'encapsulation de la MccJ25 pourrait aussi être envisagée pour améliorer d'avantage la biodisponibilité de cette bactériocine au niveau GI.

Le deuxième article de cette thèse avait pour objectif d'évaluer l'activité inhibitrice de la MccJ25 contre une souche de *Salmonella* Newport dans différents milieux de culture dont le milieu Mcfarlane qui

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simule les conditions coliques chez le porc. Cette activité inhibitrice a été comparée à celle de deux autres composés antimicrobiens, la réutérine qui est un aldéhyde produit par une souche de *Lactobacillus reuteri* et la rifampicine, un antibiotique possédant un large spectre d'action. L'évaluation de l'activité anti-*Salmonella* des trois antimicrobiens testés a permis de déterminer leurs concentrations minimales inhibitrices (CMI) et bactéricides (CMB). Les résultats obtenus avec le milieu LB ont démontré que les valeurs de CMI et de CMB de la MccJ25 respectivement égales à 0,03 μM et 3,7 μM sont inférieures à celles de la réutérine et la rifampicine. Une valeur de CMI similaire et égale à 0,05 μM a été déterminée dans une étude précédente évaluant l'activité inhibitrice de la MccJ25 contre un isolat clinique de *Salmonella* Newport dans les mêmes conditions (Rintoul et al., 2001a). Nous avons également démontré que l'activité inhibitrice de la MccJ25 semble être influencée par le volume réactionnel final. En effet, dans un essai utilisant une microplaque de 96 puits avec un volume final égal à 125 µL, les trois antimicrobiens testés ont démontré une inhibition de la croissance de *Salmonella* Newport pendant toute la période d'incubation. Cependant, lorsque nous avons effectué la même expérience en utilisant de plus grands volumes dans une microplaque de 24 puits où le volume final est égal à 2,1 mL, seules la réutérine et la rifampicine exerçaient un effet bactéricide alors que la MccJ25 a entrainé une croissance modérée de *Salmonella* Newport après seulement 8 h d'incubation, même à la plus forte concentration testée qui est égale à 64 fois la CMB. Cette perte de l'activité inhibitrice après 8 h pourrait être due à la dégradation du peptide ou bien à l'apparition de souches mutantes résistantes à la MccJ25. Pour expliquer ce phénomène, la biodisponibilité de la MccJ25 a été évaluée à l'aide du test de diffusion sur gélose et son activité résiduelle contre *Salmonella* Newport a été étudiée dans les échantillons prélevés à partir des essais effectués dans les microplaques de 24 puits aux concentrations testées égales à 8 fois et 64 fois la CMB. Les résultats obtenus ont démontré que la MccJ25 demeure biodisponible et stable ce qui explique son activité inhibitrice contre *Salmonella* Newport même après 24h d'incubation. Pour vérifier la deuxième hypothèse, on a récupéré les cellules de *Salmonella* Newport à partir des mêmes essais en microplaque afin d'évaluer leurs sensibilités à la MccJ25 par le test de diffusion sur gélose et en les comparant aux souches sauvages n'ayant pas été en contact avec la MccJ25. Les résultats obtenus ont clairement démontré le développement de résistance à la MccJ25 chez *Salmonella* Newport pendant la période d'incubation dans le milieu LB. Bien que l'apparition des cellules bactériennes résistantes suite à leur exposition prolongée aux bactériocines n'a pas été clairement définie dans la littérature, ce phénomène a été déjà rapporté avec les antibiotiques (Egan et al., 2017). Toutefois, le

phénomène de résistance de manière générale a été déjà observé chez certaines bactériocines produites par des bactéries à Gram positif, notamment la classe des lantibiotiques (Draper et al., 2015) et chez certaines souches d'*Escherichia coli* résistantes à la MccJ25 qui serait produite à cause des mutations dans les gènes codant pour le récepteur transmembranaire FhuA et pour la sous-unité de l'ARN polymérase, qui sont tous les deux impliqués dans le mécanisme d'action de la MccJ25 (Salomón and Farías, 1993; Yuzenkova et al., 2002).

Nous avons également évalué l'activité inhibitrice de la MccJ25 dans le milieu Macfarlane qui simule les conditions physiologiques rencontrées dans le côlon porcin. Les résultats ont démontré que dans le milieu Macfarlane non fermenté, la MccJ25 inhibe la croissance de *Salmonella* Newport pendant les 4 premières heures d'incubation à une concentration correspondant à 128 fois la CMB. Cependant, lorsque le test est effectué dans le milieu Macfarlane fermenté avec la microflore fécale de porc, une inhibition totale de *Salmonella* Newport a été observée pendant les 14 h d'incubation à des concentrations de MccJ25 correspondant à 128 fois et 64 fois supérieures à la CMB. Ces résultats montrent que l'inhibition de *Salmonella* Newport dans le milieu Macfarlane nécessite des concentrations plus élevées de MccJ25 comparativement au milieu LB. Ces résultats montrent également que la faible activité inhibitrice de la MccJ25 observée dans le milieu Macfarlane non fermenté est probablement due à l'interaction non spécifique de la MccJ25 avec différents composés rencontrés dans le milieu qui affecteraient sa biodisponibilité et donc son activité. Enfin, l'inhibition totale de la croissance de *Salmonella* Newport observée dans le milieu Macfarlane fermenté est probablement due au fait que *Salmonella* Newport est capable de survivre mais pas de croitre dans le milieu fermenté et que son compte bactérien n'excède jamais 10^6 ufc/mL, contrairement à la concentration de 1,23 x 10^9 ufc/m qui est atteinte dans le milieu Macfarlane non fermenté et 6,5 x 10^8 ufc/mL dans le milieu LB.

À l'issue de ce deuxième article, nous avons clairement démontré l'activité inhibitrice de la MccJ25 contre *Salmonella* Newport dans un milieu simulant l'environnement du côlon porcin. Nous avons également démontré que des concentrations plus élevées sont nécessaire pour atteindre des activités inhibitrices significatives dans ces milieux complexes. Ces résultats suggèrent une fois de plus le grand potentiel d'utilisation de la MccJ25 comme une nouvelle alternative aux antibiotiques chez le porc.

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Dans le cadre du troisième article, l'activité de la MccJ25 contre *Salmonella* Newport et son impact sur la composition et l'activité métabolique du microbiote colique du porc ont été évalués en utilisant le modèle *in vitro* de fermentation en continu (PolyFermS) qui simule les conditions du côlon proximal porcin. Ce modèle *in vitro*, développé précédemment par Tanner *et al.* permet de reproduire fidèlement les conditions physiologiques du côlon proximal du porc et offre la possibilité de réaliser et comparer différents traitements avec le même microbiote. (Tanner et al., 2014a). Dans cette étude, nous avons dans un premier temps évalué l'activité inhibitrice de la MccJ25 contre *Salmonella* Newport au cours de la fermentation colique et de comparer cette activité à celle de la réutérine et la rifampicine. Les résultats obtenus par la méthode de PMA-qPCR et par le test de diffusion sur gélose ont démontré une remarquable activité inhibitrice de la MccJ25 contre *Salmonella* Newport pendant les 24 h de fermentation colique. Par contre, cette activité inhibitrice n'a pas été observée avec la réutérine et la rifampicine. L'activité de la MccJ25 a été corrélée avec sa stabilité et sa biodisponibilité au cours de la fermentation comparativement à la réutérine et à la rifampicine. Cette importante activité de la MccJ25 a été par la suite confirmée par une méthode très spécifique et sensible qu'on a réussi à développer pour la première fois et qui consiste à analyser les échantillons prélevés du bioréacteur par LC-MS afin de détecter et de quantifier la MccJ25 tout au long de la fermentation colique.

L'autre objectif dans cette étude était d'étudier l'impact de la Mcc25, la réutérine et la rifampicine sur la composition et la diversité bactérienne du microbiote colique porcin. La quantification des principaux groupes bactériens par la méthode de PMA-qPCR nous a permis au départ d'avoir une idée globale sur le profil général de la communauté microbienne qui compose le côlon porcin. D'après les résultats obtenus, aucun changement majeur n'a été observé chez le groupe des *Bacteroidetes*, des *Lactobacillaceae*, des *Enterobacteriaceae* lorsque la MccJ25, la réutérine et la rifampicine ont été ajoutées séparément. Cependant, on a pu détecter certaines modifications dans la composition bactérienne du microbiote colique suite à l'ajout des antimicrobiens testés par la méthode de séquençage du gène de l'ARNr 16S en utilisant l'approche illumina MiSeq. En effet, ces résultats de séquençage ont démontré que les *Firmicutes*, *Bacteroidetes* et *Proteobacteria* représentent les phyla les plus abondants au niveau du microbiote colique du porc ce qui concorde avec des études antérieures rapportées par la littérature (Poroyko et al., 2010; Holman et al., 2017). De plus, ces résultats ont révélé une prévalence plus élevée des *Prevotellaceae* appartenant au phylum des *Bacteroidetes* dans tous les échantillons de la fermentation. La prédominance de *Prevotella spp*. au

niveau du côlon porcin a été rapportée dans des études précédentes qui l'ont associées au régime alimentaire typique du porc, caractérisé par une teneur relativement élevée en hydrates de carbone (Kim et al., 2011; Looft et al., 2012). Ceci nécessite donc la présence de bactéries fibrolytiques dans l'écosystème intestinal, comme le cas des *Prevotella spp*., permettant de dégrader les polysaccharides et fournir des acides gras à chaîne courte (AGCC) tel que l'acétate, considérés comme une source d'énergie pour les autres membres de la communauté microbienne (Vital et al., 2014). Cette analyse métagénomique nous a également permis de mettre en évidence l'augmentation de l'abondance relative de certaines familles bactériennes dans les échantillons prélevés à partir du fermenteur mère, notamment les *Lachnospiraceae*, les *Ruminococcaceae*, les *Succinivibrionaceae* et une classe de Clostridies non identifiée, contrairement aux abondances relatives retrouvées dans l'échantillon fécal de départ. Ces changements dans le profil métagénomique ont été observés dans des études antérieures utilisant un modèle *in vitro* de fermentation colique et qui ont été attribués à l'adaptation des communautés bactériennes au nouvel environnement pendant toute la période de stabilisation du microbiote colique suite au transfert du *in vivo* (échantillon fécal) à l'*in vitro* (microbiote colique porcin simulé) (Le Blay et al., 2008; Van den Abbeele et al., 2010; Tanner et al., 2014a). Enfin, nous avons également observé des variations des abondances relatives de certains groupes bactériens, en particulier après l'ajout de la rifampicine qui a induit une augmentation remarquable de la prévalence des *Erysipelotrichaceae*. Cependant, ces variations n'ont pas été observées après l'ajout de la MccJ25 et de la réutérine. Cela peut s'expliquer par le fait que la rifampicine est un antibiotique à large spectre pouvant inhiber divers genres bactériens contrairement à la MccJ25 connue pour son spectre d'activité restreint. La réutérine n'a induit aucune variation dans la composition du microbiote malgré son large spectre d'action. Ceci pourrait être dû à son instabilité dans les conditions coliques qui a été démontrée précédemment lorsqu'on a évalué son activité inhibitrice contre *Salmonella* Newport au cours de la fermentation.

Parallèlement à l'analyse métagénomique, nous avons développé une méthode LC-MS permettant d'effectuer une analyse approfondie de l'activité métabolique du microbiote colique porcin. Cette approche a été rarement utilisée dans la littérature pour l'analyse du microbiote colique et nous a permis de générer des résultats uniques sur l'effet des antimicrobiens sur l'activité de l'écosystème microbien du côlon porcin. Les résultats obtenus par les différentes analyses des données multivariées effectuées à partir des données LC-MS, ont démontré que la MccJ25 et la rifampicine entrainent la variabilité la plus élevée au niveau des profils métabolomiques intracellulaires et

extracellulaires et que les échantillons traités par ces deux antimicrobiens se sont clairement démarqués des autres groupes contrôles. Cependant, aucune variation significative n'a été observée au niveau du profil métabolomique lorsque la réutérine est ajoutée. Des analyses complémetaires plus approfondies sont actuellement en cours afin d'identifier et caractériser, à partir des données LC-MS/MS et de plusieurs bases de données, les différents métabolites obtenus suite à l'ajout de la MccJ25 et la rifampicine. Ces données métabolomiques combinées aux données métagénomiques sont essentielles et permettront de mieux comprendre les effets induits par les antimicrobiens testés sur le microbiote colique du porc.

En conclusion à cette étude, et à la lumière des résultats obtenus, la MccJ25 s'est montrée remarquablement stable dans les premières étapes de digestion ainsi que dans les conditions coliques. Nous avons également démontré que la MccJ25 présente une puissante activité inhibitrice contre *Salmonella* même dans des conditions extrêmes telles que les conditions coliques. La MccJ25 ne semble pas non plus affecter la composition du microbiote colique porcin. De telles caractéristiques suggèrent un fort potentiel de la MccJ25 pour son utilisation comme alternative aux antibiotiques en production animale. Ce travail va servir de tremplin à d'autres études ayant pour objectif de confirmer et de valider ces résultats *in vivo* chez des animaux d'élevage et d'examiner les possibilités d'inclure l'utilisation de ce peptide antimicrobien dans les pratiques d'élevage, notamment dans l'élevage porcin.

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