Université de Montréal

Évaluation *in vivo* de l'efficacité thérapeutique, de la résistance et la pharmacocinétique de la colistine sulfate lors du traitement de la diarrhée colibacillaire post sevrage chez le porc

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Résumé

La diarrhée colibacillaire post-sevrage (DCPS) est une infection intestinale endémique dans les fermes porcines à l'échelle mondiale. Cette maladie est causée principalement par la présence et la multiplication au niveau de l'intestin des porcelets d'un pathotype d'*Escherchia coli*, nommé *E. coli* entérotoxinogène (ETEC) et en particulier celui qui exprime l'adhésine F4 (K88) (ETEC: F4). Le sérogroupe ETEC: O149 a été le plus isolé à partir des cas de DCPS à travers le monde. Plusieurs études ont rapporté un taux de résistance important des souches O149: F4 contre les antibiotiques qui sont classiquement utilisés pour traiter cette infection et en particulier les aminoglycosides. Ainsi, pour remédier aux échecs thérapeutiques observés dans les fermes porcines au Canada, les vétérinaires ont commencé à utiliser, sous leurs responsabilités, un antibiotique, la colistine sulfate (CS), qui n'est pas homologué en production animale au Canada.

Cette étude avait pour buts d'étudier la pharmacocinétique de la CS *in vitro* et *in vivo*, de développer une technique sensible pour une quantification plasmatique de la CS, de déterminer son efficacité thérapeutique *in vivo* dans un modèle d'infection expérimentale de DCPS et de caractériser la résistance d'*E. coli* consécutive à l'utilisation thérapeutique de la CS chez le porc.

Une simulation du liquide gastrique (SLG) a été préparée, et après l'ajout de la CS et de la pepsine à cette solution, les concentrations de la CS ont été mesurées par chromatographie liquide à haute performance couplée à la spectrométrie de masse en tandem (HPLC-MS/MS). Une dégradation rapide de CS a été constatée dans la SLG et a été accompagnée par la formation de produits de dégradation qui ont démontré une activité microbienne plus importante par comparaison avec la molécule mère (CS). Dans un volet *in vivo*, l'infection expérimentale des

porcelets sevrés par une souche ETEC: F4 n'a pas augmenté l'absorption digestive de la CS dans un modèle subclinique de DCPS chez le porc.

L'administration orale de la CS à la dose thérapeutique de 50,000 UI/kg à raison de 2 fois par jour pendant 5 jours pour traiter la DCPS dans des conditions expérimentales a entraîné une réduction significative de l'excrétion fécale de la souche infectieuse (ETEC : F4), de la population totale d'*E. coli* et des scores de diarrhée, uniquement pendant la période du traitement. Cependant, ces résultats ont été accompagnés par une légère augmentation dans l'excrétion fécale des *E. coli* résistants à la colistine, et le traitement n'a pas empêché la perte de poids des porcs infectés. En revanche, l'infection expérimentale des porcelets par ETEC: F4 a augmenté l'absorption digestive de la CS dans un modèle clinique de diarrhée colibacillaire chez le porc.

Cette étude a permis de générer pour la première fois des données scientifiques concernant l'efficacité thérapeutique, la pharmacocinétique et la résistance à la colistine dans un modèle de DCPS chez le porc. Elle a également remis en doute la pertinence économique d'augmenter la dose de CS pour accélérer le rétablissement clinique des porcs. Finalement, elle a indiqué que des conditions d'élevage optimales, sans autres facteurs prédisposants, étaient aussi efficaces que la CS dans l'amélioration des symptômes cliniques de la DCPS.

Mots-clés: Colistine sulfate; *E. coli;* diarrhée post-sevrage; porc; pharmacocinétique ; résistance.

Abstract

Post-weaning diarrhea (PWD) caused by *Escherichia coli* is an endemic intestinal infection in pig farms worldwide. This disease is mostly the consequence of the presence and the multiplication in piglet's gut of an *Escherchia* pathotype, named enterotoxigenic *E. coli* (ETEC) and in particular those that express the F4 (K88) fimbrial adhesin (ETEC: F4). The predominant serogroup of *E. coli* isolated from piglets with PWD worldwide is O149. Several studies have reported a significant resistance rate of O149 ETEC strains against commonly used antibiotics for the treatment of PWD, particularly, aminoglycosides. Thereby, to address therapeutic failures observed in pig farms during PWD treatment, veterinarians in Canada started using, under their responsibilities, the colistin sulfate (CS), an antibiotic not approved for farm animals in Canada.

The objectives of this thesis were: to study the pharmacokinetics of CS *in vitro* and *in vivo*, to develop a sensitive method for the quantification of CS plasma concentrations in pigs, to determine the therapeutic efficacy of CS in an experimental model of PWD, and to characterize the resistance of *E. coli* to colistin consecutive to its therapeutic use in pigs.

Simulated gastric fluid (SGF) was prepared, and after the addition of CS and pepsin to this solution, the concentrations of CS were followed by liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS). A rapid degradation of CS in the SGF was observed, and the degradation products showed a greater antimicrobial activity compared to the native CS. On the other hand, the experimental challenge of piglets with an ETEC: F4 strain has not increased the CS intestinal absorption in a subclinical model of PWD in pigs.

The oral administration of a therapeutic dose of CS at 50,000 IU/kg twice a day for 5 successive days to treat an experimental PWD in pigs, resulted in a significant reduction of fecal ETEC: F4 and total *E. coli* shedding, and in diarrhea scores but only during the treatment period. However, CS treatment resulted in a slight increase in fecal shedding of CS resistant *E. coli* and

did not prevent weight loss in challenged pigs. In addition, challenge with ETEC: F4 resulted in an increase of CS intestinal absorption in a clinical model of PWD.

This study has generated, for the first time, scientific data regarding CS therapeutic efficacy, its pharmacokinetic and the selection of *E. coli* colistin resistant in an experimental model of PWD in pigs. It also challenged the economic relevance of increasing CS oral doses to accelerate the clinical recovery of pigs. Finally, it indicated that optimal housing conditions were without other predisposing factors, effective as CS in improving clinical symptoms of experimental PWD in pigs.

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Liste des sigles et des abréviations

ADG: *Average Daily Gain* / gain moyen quotidien

AIDA-1: *Adhesin-involved-in-diffuse-adherence* / adhésine impliquée dans l'adhérence diffuse

AUC: *Area under curve* / *aire sous la courbe*

BPW: Buffered peptone water

Cmax: *Maximum concentration* / concentration maximale

CMI: *Minimum inhibitory concentration* / concentration minimale inhibitrice

CMS: *Colistin methanesulphonate sodium* / colistine méthanesulfonate sodique

CS: *Colistin sulfate* / colistine sulfate

DCPS: Diarrhée colibacillaire post-sevrage

E. coli: Escherichia coli

EAST1: *Enteroaggregative E. coli heat-stable enterotoxin 1* / entérotoxine résistante à la chaleur 1 *E. coli* entéro-agrégatifs)

EMA: European Medicines Agency / Agence européenne des médicaments

EPEC*: Enteropathogenic E. coli / E. coli* entéropathogène

ESBL*: Extended-spectrum beta lactamases / bêta-lactamase à spectre étendu*

ETEC*: Enterotoxinogenic E. coli* / *Escherichia coli* entérotoxinogène

EUCAST: *European Committee on Antimicrobial Susceptibility Testing*

F4 (K88): Fimbriae F4

GMQ : Gain Moyenne Quotidien

HPLC: *High performance liquid chromatography* / chromatographie en phase liquide à haute performance

IM: Intramuscular

L-Ara4N: 4-amino-4-deoxy-L-arabinose

LB: Luria-Bertani

LLOQ: *Lower limit of quantitation* / limite inférieure de quantification

LOD : *Limit of detection* / limite de détection

LPS: *Lipopolysaccharide*

LT: *Heat-labile enterotoxin* / entérotoxine labile à la chaleur

MDR-GNB: Multidrug-resistant Gram-negative bacteria

mg: Milligramme

MH: Mueller-Hinton

min: Minute

mL: Millilitre

MRGN: Multi-resistant Gram negative

MRLs: Maximum Residue Limits

MS/MS: *Tandem mass spectrometry /* spectrométrie de masse en tandem

ng: Nanogramme

nmol: Nanomole

PBS : *Phosphate Buffered Saline* / tampon phosphate salin

PCR: *Polymerase chain reaction* / réaction en chaîne par polymerase

PD: Pharmacodynamics

PEtN: *Phosphoethanolamine* / phosphoéthanolamine

PK: Pharmacokinetics

PK/PD: *Pharmacokinetic-Pharmacodynamics* / pharmacocinétique-pharmacodynamie

PWD: Post-weaning diarrhea

SDD: *Selective Decontamination of the Digestive tract* / décontamination digestive sélective

SEM: *Standard error of the mean* / erreur standard à la moyenne

SGF: *Simulated gastric fluid* / simulation du liquide gastrique

STa: *Heat-stable enterotoxin a* / entérotoxine résistante à la chaleur a

STb: *Heat-stable enterotoxin b* / entérotoxine résistante à la chaleur b

T1/2: *Time half-life* / temps de demi vie

TNF-α: *Tumor necrosis factor alpha* / facteur de nécrose tumorale alpha

UFC/g: Unité formatrice de colonie / gramme

UI: *International unit* / Unité international

WHO: *World Health Organization* / organisation mondiale de la santé

x g: Force g

μg: Microgramme

μL; Microlitre

%: Pourcentage

°C: Degré Celsius

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Introduction

La diarrhée colibacillaire post-sevrage (DCPS) est une maladie qui cause des pertes économiques considérables dans les fermes porcines à l'échelle mondiale (Amezcua et al., 2002a; Fairbrother et al., 2005). La colonisation par *Escherichia coli* entérotoxinogène (ETEC) de la muqueuse intestinale des porcelets sevrés représente le facteur le plus déterminant pour l'apparition des signes cliniques de la DCPS (Fairbrother and Gyles, 2012). Plusieurs études ont rapporté que ETEC F4-positif (ETEC: F4) représente la principale cause des DCPS chez les porcs à l'échelle mondiale (Gyles and Fairbrother, 2010). Le sérogroupe prédominant de ETEC associée à la DCPS chez le porc au Canada et dans le monde est O149, et les virotypes les plus impliqués dans cette maladie sont O149: LT: STb: F4 ou O149: LT: STa: STb: F4 (Fairbrother et al., 2005). Au Canada, les souches ETEC isolées à partir des porcelets diarrhéiques ont montré un taux de résistance très élevé contre plusieurs antibiotiques et particulièrement ceux qui sont couramment utilisés pour le traitement de la DCPS tel que les aminoglycosides (Amezcua et al., 2002b; Maynard et al., 2003). Cette résistance est à l'origine de plusieurs échecs thérapeutiques qui ont été constatés sur le terrain. Ainsi pour remédier à cette situation, des vétérinaires ont opté pour l'utilisation, sous leur responsabilité, d'un antibiotique, la colistine sulfate (CS) qui n'est pas homologué en médecine vétérinaire au Canada.

La colistine (polymyxine E) est un antibiotique polypeptidique de la famille des polymyxines (Yu et al., 2015). Cet antibiotique est largement utilisé en production porcine dans plusieurs pays pour le traitement des infections intestinales à *E. coli* (Kempf et al., 2013).

Plusieurs études récentes qui ont été menées en production porcine ont rapporté que des *E. coli* isolés à partir des porcs présentant des infections intestinales, avait un taux de résistance élevé à la CS (Harada et al., 2005; Morales et al., 2012).

En médecine porcine, la CS est principalement administrée par la voie orale, à la dose de 50,000 UI/kg à raison de 2 fois par jour pendant une période de 3 à 5 jours consécutifs, pour le traitement des infections intestinales causées par des entérobactéries tel qu'*E. coli* ou *Salmonella* (Guyonnet et al., 2010). Cependant, plusieurs études ont rapporté que la posologie de CS (dose, durée de traitement) utilisée dans les fermes porcines a été souvent différente des posologies recommandées par les monographies (Chauvin et al., 2002). En revanche, aucune étude n'a évalué l'efficacité du régime thérapeutique classique à base de colistine (50,000 UI/kg) dans le traitement de la DCPS. En plus, il n'y a pas de données disponibles dans la littérature sur le rôle de ce schéma thérapeutique dans l'évolution de la résistance d'*E. coli* à la colistine chez le porc.

L'utilisation des techniques classiques de quantification systémique de la colistine chez le porc tel que la chromatographie en phase liquide à haute performance (CLHP), avec une limite de quantification de 250 ng/mL, a pu confirmer que cet antibiotique est faiblement absorbé au niveau digestif après son administration orale chez le porc (Guyonnet et al., 2010). Cependant, aucune étude n'a utilisé une technique très sensible pour la quantification systémique de la colistine chez le porc, afin de confirmer cette faible biodisponibilité orale. En plus, l'effet d'une infection intestinale à ETEC sur la modification de l'absorption digestive de la CS n'a pas été investigué.

Le délai d'attente appliqué à la CS en production porcine suite à son utilisation thérapeutique dépend des pays où elle est utilisée, de 1 à 7 jours (Official Journal of the European Union, 2010). Une augmentation de l'absorption intestinale de la CS suite à une infection digestive bactérienne pourrait avoir un impact sur le temps d'attente après l'administration orale de cet antibiotique.

Tous ces facteurs démontrent bien l'importance de la recherche en production porcine pour générer des données spécifiques à la CS dans cette production.

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Cette thèse de doctorat s'inscrit dans cette perspective. Elle a comme hypothèse que la CS subit une dégradation digestive dans le tractus gastro-intestinal du porc et l'utilisation orale de cet antibiotique pour le traitement clinique de la DCPS pourrait améliorer les symptômes cliniques de la maladie, une réduction de l'excrétion fécale d'*E. coli* et des gènes de virulence de ETEC : F4 (STa, STb, LT et F4), améliorer la croissance des animaux et exacerber la résistance d'*E. coli* à la CS. En plus, l'infection expérimentale à ETEC : F4 pourrait augmenter l'absorption intestinale de la CS chez des porcelets sevrés.

Les objectifs spécifiques de l'étude étaient de déterminer la stabilité de la CS dans une simulation du liquide gastrique chez le porc et d'évaluer l'activité antibactérienne *in vitro* des produits de dégradation de la colistine, de mesurer l'efficacité thérapeutique de deux doses de CS dans le traitement oral de la DCPS induite expérimentalement, de suivre l'évolution de la résistance d'*E. coli* à la colistine consécutive à son utilisation thérapeutique pour le traitement de la DCPS, d'évaluer l'effet d'une infection à ETEC :F4 dans un modèle d'infection expérimentale de DCPS, sur la modification de l'absorption intestinale de la CS chez le porc et de générer des données pharmacocinétiques relatives à la CS, suite à son administration orale chez des porcelets sains comparativement à des porcelets infectés par ETEC : F4.

Cette thèse est rédigée sous forme d'articles et inclut des articles de revue qui ont été publiés ou qui ont été soumis à des journaux scientifiques et qui font l'objet de la revue de littérature dans ce travail.

Recensement de la littérature

Colistin in pig production: Chemistry, Applications, Mechanism of antibacterial action, Microbial resistance emergence, and One Health Perspectives

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Contribution du candidat:

J'ai participé à l'élaboration du plan de cette revue en proportion équivalente de celle des autres coauteurs. J'ai effectué les collectes de toutes les données bibliographiques. J'ai analysé les résultats de la littérature pour proposer des perspectives à l'utilisation de la colistine en médecine porcine. J'ai mis au point un concept One Health pour la gestion de la résistance à la colistine dans l'interface Homme-porc-environnement. J'ai rédigé la revue conformément aux exigences du journal, et j'ai intégré les commentaires faits par les coauteurs ainsi que ceux formulés par les réviseurs de l'article.

1. Colistin in pig production: Chemistry, Mechanism of antibacterial action, Microbial resistance emergence, and One Health Perspectives

1.1.1 Abstract

Colistin (Polymyxin E) is one of the few cationic antimicrobial peptides commercialized in both human and veterinary medicine. For several years now, colistin has been considered the last line of defense against infections caused by multidrug-resistant (MDR) Gram-negative such as *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Klebsiella pneumoniae*. Colistin has been extensively used orally since the 1960s in food animals and particularly in swine for the control of *Enterobacteriaceae* infections. However, with the recent discovery of plasmid-mediated colistin resistance encoded by the *mcr-1* gene and the higher prevalence of samples harboring this gene in animal isolates compared to other origins, livestock has been singled out as the principal reservoir for colistin resistance amplification and spread. Co-localization of the *mcr-1* gene and Extended-Spectrum-β-Lactamase (ESBL) genes on a unique plasmid has been also identified in many isolates from animal origin. The use of colistin in pigs as a growth promoter and for prophylaxis purposes should be banned, and the implantation of sustainable measures in pig farms for microbial infection prevention should be actively encouraged and financed. The scientific research should be encouraged in swine medicine to generate data helping to reduce the exacerbation of colistin resistance in pigs and in manure. The establishment of guidelines ensuring a judicious therapeutic use of colistin in pigs, in countries where this drug is approved, is of crucial importance. The implementation of a microbiological withdrawal period that could reduce the potential contamination of consumers with colistin resistant bacteria of porcine origin should be encouraged. Moreover, the management of colistin resistance at the human-pig-environment interface requires the urgent use of the One Health approach for effective control

and prevention. This approach needs the collaborative effort of multiple disciplines and close cooperation between physicians, veterinarians, and other scientific health and environmental professionals. This review is an update on the chemistry of colistin, its applications and antibacterial mechanism of action, and on *Enterobacteriaceae* resistance to colistin in pigs. We also detail and discuss the One Health approach and propose guidelines for colistin resistance management.

1.1.2 Introduction

Antibiotics in the polymyxin family include 5 different chemical compounds (polymyxin A, B, C, D, and E) (Falagas et al., 2005; Gallardo-Godoy et al., 2016), of which polymyxin B and colistin (also called polymyxin E) are the only two polymyxins used clinically (Cassir et al., 2013; Landman et al., 2008). For humans, two forms of colistin are commercially available: colistin methanesulfonate sodium (CMS) for parenteral use and aerosol therapy; and colistin sulfate (CS) for oral and topical use (Brink et al., 2014; Li et al., 2006). Colistin is used in human medicine for the treatment of infections due to multidrug-resistant (MDR) Gram-negative bacteria (GNB) such as *Pseudomonas aeruginosa*, *Acinetobacter baumannii*, *Klebsiella pneumoniae*, and carbapenemase-producing *Enterobacteriaceae* (Azzopardi et al., 2013; Velkov et al., 2009) and is used as a last-resort treatment option against these infections (Biswas et al., 2012; Falagas and Rafailidis, 2008). Recently, the World Health Organization (WHO) and several government agencies such as Health Canada have reclassified colistin in the category of very high importance in human medicine (Government of Canada, 2014; WHO, 2011). Colistin's mechanism of action is mainly related to its attachment to the lipopolysaccharides (LPS) of GNB, leading to membrane-permeability disturbance and cell death (Biswas et al., 2012; Falagas and Rafailidis, 2008).

Colistin sulfate is the only form of colistin approved in pig production in some countries for the control of *Enterobacteriaceae* infections, particularly for those caused by *E. coli* (Guyonnet et al., 2010; Rhouma et al., 2016a). Since its introduction on the market in the 60s, colistin was used in pig production in several countries with different purposes; therapeutically, prophylactically, and even for growth promotion (Katsunuma et al., 2007; Rhouma et al., 2016a). Interestingly, in the late 2000s and after decades of colistin use in swine, several studies began reporting a significant resistance rate of *Enterobacteriaceae* to colistin *in* pigs (Enne et al., 2008; Harada et al., 2005; Lu et al., 2010; Rhouma et al., 2016a). The most common mechanism of colistin resistance in *E. coli* and *Salmonella* involves a modification of the lipid A portion of LPS through the addition of phosphoethanolamine (PEtN) and/or a 4-amino-4-deoxy-L-arabinose (L-Ara4N), which reduces its binding to colistin and leads to bacterial resistance (Bergen et al., 2012; Olaitan et al., 2014). This chromosomal mechanism of colistin resistance is the result of the activation of the two-component systems PhoP/PhoQ and PmrA/PmrB by specific mutations or environmental stimuli leading to an overexpression of LPS-modifying genes (Olaitan et al., 2014). However, several studies have reported the isolation of colistin resistant *E. coli* strains in the absence of chromosomally encoded mechanisms (Olaitan et al., 2015b; Quesada et al., 2015). At the end of 2015, researchers identified a stable plasmid mediated *mcr-1* gene encoded for phosphoethanolamine transferase conferring resistance to colistin in some GNB isolated from food animals, raw meat, and humans in several countries (Liu et al., 2016; Rhouma et al., 2016a). The discovery of this horizontal mechanism of colistin resistance raised alarm bells about the impact of colistin use on colistin resistance spread in animal production, especially in swine. In fact, the link between pigs and humans in terms of colistin resistant *E. coli* strain transfer following direct contact has recently been confirmed (Olaitan et al., 2015b). These findings have led to a serious fear about the possible loss of colistin effectiveness in the treatment of MDR-

GNB in humans. Hence, it is urgent to establish close cooperation between physicians, veterinarians, and countries to ensure judicious use of colistin in both veterinary and human medicine. The application of the One Health concept could be a solution for the management of colistin resistance in the human-pig-environment interface.

This review is an update on colistin chemistry, its applications and antibacterial mechanism of action, and on *Enterobacteriaceae* resistance in pigs. We also detail and examine the One Health concept to arrive at proposed guidelines for rational use of colistin in swine and humans and to find ways to prevent bacterial resistance spread in the human-pig-environment interface.

Please refer to our recent review for rates of colistin resistance in pigs, the possible link between colistin pharmacokinetic/pharmacodynamic (PK/PD), and colistin use and *Enterobacteriaceae* resistance emergence in swine (Rhouma et al., 2016a).

1.1.3 Chemical structure of colistin and its antibacterial mechanism of action

1.1.3.1 Colistin chemical structure

Colistin (polymyxin E) is a polymyxin antibiotic produced by *Paenibacillus polymyxa* var *colistinus* (Tambadou et al., 2015) consisting of a cyclic heptapeptide ring with three positively charged amine groups, a tail tripeptide moiety with two positively charged amine groups, and a hydrophobic acyl chain tail (Figure 1) (Azzopardi et al., 2013; Bergen et al., 2012; Dijkmans et al., 2015; Li et al., 2006; Rhouma et al., 2015). Colistin is an amphipathic molecule, with hydrophobicity mainly attributable to the fatty acyl moiety and hydrophilicity due to the five L-diaminobutyric acid (L-Dab) amino groups (Li et al., 2006). The L-Dab molecules are positively charged in positions 1, 3, 5, 8, and 9 (Figure 1). These amino groups are responsible for the electrostatically interaction between colistin and the lipid A portion of LPS molecules of GNB and play a central role in the bactericidal activity of colistin (Azzopardi et al., 2013). The

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polymyxins family includes 5 chemically distinct compounds (polymyxin A-E) and only colistin (polymyxin E) and polymyxin B have been used in clinical practice (Dijkmans et al., 2015). Polymyxin B and colistin share a similar primary sequence with the only difference being one amino acid in position 6 in which D- phenylalanine in polymyxin B is replaced by D- leucine in colistin (Figure 1) (Biswas et al., 2012; Gallardo-Godoy et al., 2016; Li et al., 2006; Velkov et al., 2009; Yoshino et al., 2013).

Two different forms of colistin are available commercially: CS, which is administered either orally for bowel decontamination or topically as a powder for skin infections, and CMS, which is commonly administered intravenously and used exclusively in human medicine (Bergen et al., 2012; Michalopoulos et al., 2011). Both can be delivered by inhalation (Li et al., 2006). CS is the only active ingredient of the polymyxin family and is approved in some countries for the control of *Enterobacteriaceae* infections in pigs (Official Journal of the European Union, 2010; Rhouma et al., 2016a; Wan et al., 2016) and is used mostly in monotherapy or sometimes in combination with other substances. Researchers found at least thirty different components in commercially available colistin, 13 of which were separated using the isocratic liquid chromatography (LC) method (Orwa et al., 2000). The two major components of colistin are colistin A (polymyxin E1) and colistin B (polymyxin E2), which differ only in the fatty acid side chain (Orwa et al., 2000). In fact, colistin A and colistin B are acylated by (S)-6-methyloctanoic acid and (S)-6-methylheptanoic acid, respectively (Li et al., 2006). The proportion of these 2 major components in commercial products differs between the different pharmaceutical preparations of colistin available on the market (Bergen et al., 2012; Brink et al., 2014). This could be due to the fact that colistin is a natural product produced by fermentation, so its composition can vary considerably between manufacturers (Brink et al., 2014). In fact, no pure colistin A and B reference standards are commercially available (Dotsikas et al., 2011) and no certificates of analysis that include

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chemical characterization are available in veterinary medicine to adequately establish the purity of the marketed CS formulations (Rhouma et al., 2016a). CS is a polypeptide antibiotic with a chemical structure characterized by the presence of multiple peptide bonds documented to predispose CS to chemical and enzymatic degradation (Chihara et al., 1973; Rhouma et al., 2015). In fact, in pig simulated gastric fluid (SGF), CS led to the formation of degradation products that have a significant antimicrobial activity compared to non-degraded CS (Rhouma et al., 2015).

1.1.3.2 Colistin antibacterial mechanism of action on *Enterobacteriaceae* in pigs

Colistin has a narrow antibacterial spectrum with an effect limited to GNB; Gram-positive bacteria do not contain LPS in their cell wall and, as a consequence, are excluded from the spectrum of activity of polymyxins (Dijkmans et al., 2015).

The initial target of colistin is the LPS component of the outer membrane (OM) of GNB. The most documented steps of colistin antibacterial activity are described below ((Biswas et al., 2012; Deris et al., 2014b; Hancock, 1997; Martis et al., 2014; Nation et al., 2014; Powers and Hancock, 2003; Velkov et al., 2009; Yu et al., 2015)).

1- Colistin initially binds to LPS and specifically to lipid A, a key component of the LPS, through electrostatic interaction between positively charged Dab residues of colistin and the negatively charged phosphate groups of lipid A. Lipid A plays a crucial role in the control of bacterial permeability (Velkov et al., 2009).

2- Colistin competitively displaces divalent cations calcium (Ca^{2+}) and magnesium (Mg^{2+}) that normally stabilize the LPS and as a consequence the 3-dimensional structure of the LPS is altered. In fact, colistin has affinities for LPS that are at least three times higher than those for divalent cations (Hancock, 1997).

3- Colistin causes an expansion of the OM monolayer by the insertion of its hydrophobic terminal fatty acyl chain or the D-Leu[6] -L-Leu[7] segment into the OM.

4- Colistin leads to a permeabilization of the OM by the formation of destabilized areas through which colistin will transit the OM via a self-promoted uptake mechanism (Hancock and Scott, 2000; Straus and Hancock, 2006). This mechanism explains how colistin acts in synergy with conventional antibiotics (Hancock, 1997). In fact, hydrophilic antibiotics such as rifampicin, vancomycin, meropenem, β-lactam, tigecycline, and gentamicin can work synergistically due to this disruption of membrane integrity by colistin (Bolla et al., 2011).

5- Colistin destroys the physical integrity of the phospholipid bilayer of the inner membrane (IM) through membrane thinning by straddling the interface of hydrophilic head groups and fatty acyl chains (Velkov et al., 2009).

6- This leads to inner membrane lysis, leakage of intracellular contents and cell death.

Colistin also exerts an anti-endotoxin activity because it binds to the lipid A component of LPS (Falagas et al., 2005; Şentürk, 2005). In this way, colistin prevents endotoxin's ability to induce shock through the release of cytokines such as tumor necrosis factor-alpha (TNF-α) and IL-8 (Baeuerlein et al., 2009; Şentürk, 2005).

It should be stressed here again that colistin's antibacterial mechanism of action based on membrane lysis death was the most documented explanation for the effectiveness of this antibiotic in the treatment of GNB infections. However, its ultimate mechanism of action is still unknown (Biswas et al., 2012; Nation et al., 2014). Other mechanisms of polymyxin bactericidal activity have been proposed such as a vesicle-vesicle contact pathway (Cajal et al., 1996; Clausell et al., 2007a) and a hydroxyl radical death pathway (Sampson et al., 2012; Yu et al., 2015). The vesicle-vesicle contact antimicrobial mechanism described involves the polymyxin B molecule

with a hydrophobic acyl tail that can enter into and cross the OM and induce a lipid exchange between leaflets of the IM and OM; this leads to membrane osmotic instability due to the change in the phospholipid composition, thereby inducing cell lysis (Clausell et al., 2007a). However, this mechanism of action has not been studied with colistin. It has been shown that polymyxin B and colistin exert a rapid antimicrobial activity against the sensitive and multidrug-resistant isolates of *A. baumannii* and *E. coli* through hydroxyl radical production by the Fenton reaction (Sampson et al., 2012), leading to the formation of hydroxyl radicals through the reduction of hydrogen peroxide by ferrous iron (Fe^{2+}). The production of this reactive oxygen species (ROS) might lead to oxidative damage in the bacterial DNA, proteins, and lipids and cause cell death (Sharma et al., 2016). However, this feature of colistin has not yet been evaluated in clinical practice. Most recently, it was shown that colistin was able to inhibit the vital respiratory enzyme NADH-quinone oxidoreductase in the bacterial inner membrane of GNB (Deris et al., 2014a). This mechanism was regarded as a secondary mode of action of polymyxins.

1.1.4 Pharmacokinetics and pharmacodynamics (PK and PD) of colistin in pigs

1.1.4.1 Clinical PK and PD studies of colistin in pigs

Unlike for human medicine, only a few studies have been conducted in pigs to evaluate the PK of colistin following oral (Guyonnet et al., 2010; Rhouma et al., 2016b; Rhouma et al., 2015) or intramuscular (IM) administration (He et al., 2011; Lin et al., 2005; Tang et al., 2009a) (Table I). These studies were performed using CS, since this is the only form of colistin approved in swine medicine, and were conducted in healthy pigs. It is reasonable to think that the PK can be different in sick animals. The oral CS PK data in pigs were obtained using either a high-pressure liquid chromatography (HPLC) assay (Guyonnet et al., 2010) or a liquid chromatography coupled with the tandem mass spectrometry (HPLC–MS/MS) method (Rhouma et al., 2016b; Rhouma et

al., 2015). CS PK data in pigs after parenteral administration were obtained using mostly microbiological assays (Lin et al., 2005; Tang et al., 2009a) (Table I); these data should be viewed with caution because of the limited sensitivity of this method and the descriptions of the experiment conditions.

After oral CS administration in pigs and despite the use of a very sensitive analytical method, CS plasma concentrations were very difficult to quantify in healthy pigs (Guyonnet et al., 2010; Rhouma et al., 2015). A concurrent oral challenge of pigs with an ETEC: F4 strain did not increase CS intestinal absorption in a subclinical induction model of *post-weaning diarrhea (*PWD) (Rhouma et al., 2015). However, in pigs with clinical PWD following an experimental oral challenge with the ETEC: F4 strain, CS plasma concentrations were higher in the challenged groups compared to the unchallenged one (Rhouma et al., 2016b). These studies confirm that colistin is poorly absorbed through pig's gastrointestinal tract even in infected animals and corroborates the involvement of oral CS administration in exacerbating colistin resistance by exerting selection pressure on pig's intestinal flora (Rhouma et al., 2016a).

Parenteral CS PK studies in pigs were mainly conducted to study the safety of IM CS administration. The CS intestinal concentrations through the biliary system elimination were not determined following IM administration to assess whether or not colistin exerts a selective pressure on pig's intestinal microflora after its parenteral administration. There is no available data in the literature concerning the possible renal tubular reabsorption of CS in pigs as previously demonstrated in rats through a carrier-mediated process (Ma et al., 2009); if this is the case, it would justify an extension of the colistin withdrawal period in pigs.

Even though some studies have been able to quantify colistin in the pig's systemic circulation following its oral administration using very sensitive methods (Rhouma et al., 2016b), these concentrations were very low compared to the Maximum Residue Limits (MRLs) for this

molecule in pigs, which supports the short withdrawal period of one to seven days for oral CS in pigs (Official Journal of the European Union, 2010). In fact, the EMEA Committee for Medicinal Products for Veterinary Use (CVMP) has established the MRLs for colistin in swine: 150, 150, 150, and 200 μg/kg in muscle, liver, fat, and kidney, respectively (Tang et al., 2009a). However, no study has been performed in pigs to assess CS degradation product toxicity, and no screening tests are available in the market to detect these products in pig meat (Rhouma et al., 2015). It was shown that *E. coli* experimental infection in pigs increased CS intestinal absorption (Rhouma et al., 2016b), and authors have claimed that this information should be taken into consideration when determining the CS withdrawal period in pigs. Even with intestinal infection, CS systemic concentrations in pigs remain below MRLs, thus adjusting the withdrawal period after *E. coli* infection in pigs should be considered for antibiotics that are characterized by high oral bioavailability.

The potential for the emergence of *E. coli* resistance in pigs during therapy with CS has been shown following its use at the recommended regimen (100,000 IU/kg/day), as demonstrated previously (Rhouma et al., 2016b). In this study, despite a rapid initial reduction in *E. coli* fecal excretion following CS oral treatment, the emergence of CS resistance among commensal *E. coli* was observed starting from the third day of treatment. CS selection pressure resistance disappeared after 5 days of CS treatment and CS resistant *E. coli* strains were isolated 6 days after the last treatment (Rhouma et al., 2016b). This is of significant importance in food safety and public health perspective because this means pigs that are treated with CS and given a one day withdrawal period as recommended (Official Journal of the European Union, 2010) are shipped to slaughter with potential colistin resistant *E. coli* in their gut. Therefore, applying a microbiological withdrawal time for CS resistant bacteria in addition to the chemical one is of crucial importance to reduce the risk of passage of these bacteria in pig slaughterhouses to

humans through the handling of raw meat or the consumption of undercooked meat.

In order to monitor *E. coli* colistin resistance in pigs subsequent to the therapeutic use of this antibiotic in the treatment of PWD, our team used MacConkey agar medium supplemented with CS at 2 µg/mL, which represents the breakpoint value (Rhouma et al., 2016b). We confirmed that this medium overestimated the number of CS resistant *E. coli* and that the isolation of putative resistant bacteria on this medium requires confirmation by MIC determination using a Mueller–Hinton broth media. To overcome this problem related to the absence of a selective medium for the screening of colistin resistant bacteria, Nordmann and collaborators developed a screening medium that is able to detect intrinsic and acquired polymyxin-resistant bacteria without the need to confirm resistance isolates by MIC determination (Nordmann et al., 2016). The implementation of this medium will facilitate the monitoring of colistin *Enterobacteriaceae* resistance in food-producing animals.

1.1.4.2 Perspectives for colistin (PK/PD) studies in pigs

While great advances in colistin research have occurred in the last decade in both human and veterinary medicine (Rhouma et al., 2016a), colistin PK/PD data are very limited in pigs. To successfully combat the development and dissemination of bacterial resistance against this antibiotic in swine, we believe that specific CS clinical PK/PD data are of crucial importance (Table II).

Furthermore, the recent discovery of a plasmid mediated *mcr-1* gene encoding for *Enterobacteriaceae* colistin resistance in farm animals and in humans (Liu et al., 2016) has prompted several regulatory agencies such as the European Medicines Agency (EMA) to re-evaluate colistin in farm animals (European Medicines Agency, 2016a). More data on colistin PK/PD will be essential to ensuring judicious use of colistin in pigs (Table II).

It should be stressed here again that the CS commercially available is obtained by a bacterial fermentation process (Brink et al., 2014; Tambadou et al., 2015). Consequently, its composition may vary between commercially available CMS products (He et al., 2013), although no study in veterinary medicine has verified this variability. In addition, the unit of CS dosing in pig production is not standardized; some practitioners use international units whereas others use milligrams per kg of body weight (Guyonnet et al., 2010; Rhouma et al., 2016a; Trauffler et al., 2014; Ungemach et al., 2006). We believe that the standardization of CS composition and dosage in pigs worldwide is critical to ensuring judicious use of this antibiotic, and it would allow comparison between studies in terms of therapeutic efficacy and resistance rate.

Only one study has determined the CS concentrations in clinical healthy intestinal tracts of pigs after a single oral administration of this molecule (Guyonnet et al., 2010). In this study, colistin concentrations were not detectable in fecal samples, from the duodenum to ileum, after 4 h of its oral administration regardless of doses used (25,000, 50,000, or 100,000 IU/kg) (Guyonnet et al., 2010). However, CS is usually administrated in swine medicine to treat sick animals at a dose of 50,000 IU/kg body weight every 12 h for three to five days (Official Journal of the European Union, 2010), and the intestinal C_{max} concentrations of colistin were not determined after a repetitive CS oral treatment to justify the efficacy of this therapeutic regimen in the treatment of pig's diseases associated with *Enterobacteriaceae*. The duration of CS oral treatment in pig farms is far longer than three to five days as recommended on product monographs (Chauvin et al., 2002; Van Rennings et al., 2015). Nevertheless, no study in field conditions has evaluated the impact of CS treatment duration on its clinical efficacy in pigs and on bacterial resistance emergence. Our team showed in experimental conditions that 3 days of CS oral treatment of pigs challenged with an ETEC: F4 strain was enough to treat clinical symptoms of PWD in pigs (Rhouma et al., 2016b), and a positive correlation was observed between CS treatment duration

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and CS selection pressure on commensal *E. coli.*

It has previously been demonstrated that antimicrobial activity is related to inoculum size and stage of infection. Specifically, researchers found that antimicrobial activity may be higher for a lower bacterial inoculum, and treating experimental animals at an early stage of infection reduced both the required dose of antimicrobials and the amplification risk of bacterial resistance in the intestine (Ferran et al., 2011; Vasseur et al., 2014). The impact of the inoculum on the bactericidal activity of colistin has been investigated *in vitro* for some strains of *P. aeruginosa* of human origin (Bulitta et al., 2010). In this study, killing of the susceptible population was 23-fold slower for the 10^9 CFU and 6-fold slower for the 10^8 CFU than for the 10^6 CFU. These findings require further investigation in pigs to study the efficiency of an early use of CS in the treatment of infections associated with *Enterobacteriaceae* in swine and to examine the impact of such practice on the resistance amplification risk among pig's intestinal bacteria and on colistin amounts used at the farm level.

Numerous *in vitro* and *in vivo* studies using colistin and various other antibiotics have provided evidence for increased bacterial killing and decreased emergence of resistance with the use of certain colistin combinations against MDR Gram-negative bacteria (Bergen et al., 2012; Li et al., 2006). Using colistin with other antimicrobial agents (aztreonam, piperacillin, ceftazidime, imipenem, ampicillin-sulbactam, ciprofloxacin, carbapenems, and rifampicin) is the most used combination treatment in human medicine (Li et al., 2006; Martis et al., 2014). Nevertheless, optimal combinations are not defined, and the relative value of a combination may vary between bacterial strains (Clancy et al., 2013).

In swine, and despite the use of some combinations of colistin with other antimicrobial agents (Table III), no study has demonstrated the effectiveness of such association and its role in colistin *Enterobacteriaceae* resistance occurrence.

Several susceptibility testing methods are used in pigs to determine colistin MIC against bacterial strains of porcine origin (Rhouma et al., 2016a), without specific clinical breakpoints for colistin against *Enterobacteriaceae* after its oral use in swine medicine (Boyen et al., 2010; Richez and Burch, 2016). Such information is of crucial importance for identifying the colistin PD index that is predictive of microbiological efficacy and outcome and to establish the quantitative relationship between PK and PD parameters (Papich, 2014).

Recently, the plasmid-mediated colistin resistance gene *mcr-1* was detected in some Extended - Spectrum- β-Lactamase (ESBL, *bla* $_{CTX-M}$) producing *E. coli* isolates from pigs in Germany and in Vietnam (Falgenhauer et al., 2016; Malhotra-Kumar et al., 2016b). These findings highlight the importance of the active surveillance of colistin resistance in pigs. The suggested strategies to reduce colistin use in pigs should never be associated with an increase in the use of third and fourth generation fluoroquinolones or cephalosporins or the overall use of antimicrobials on farms as claimed in the last report of the EMA (European Medicines Agency, 2016b).

Recommended points of investigation to generate essential PK/PD data for judicious use of colistin in pig production are summarized in Table II.

1.1.5 Clinical use and indications of colistin in pig production

1.1.5.1 Indications and use of colistin in pigs

The main indication of colistin in pigs is the treatment of digestive infections caused by *Enterobacteriaceae*, especially for those caused by *E. coli* (Guyonnet et al., 2010). Colistin is widely used for the control of PWD in piglets in Europe (Callens et al., 2012b; Kempf et al., 2013). Some epidemiological surveys have been reported that colistin is sometimes used off-label in pig farms to treat infections other than intestinal diseases such as respiratory disease (Catry et al., 2015; Chauvin et al., 2002; Van Rennings et al., 2015). Approximately 99% of colistin use in

pig production is carried out orally for mass treatment in intensive husbandry systems (European Medicines Agency, 2016b).

Colistin sulfate is used therapeutically, prophylactically, and even as a growth promoter in swine in some countries (Rhouma et al., 2016a). The CS is not approved in pig production in Canada and in the USA, and this antibiotic is not used as a feed additive for growth promotion in Europe for at least two decades, (Kempf et al., 2013). However, CS is used in Canada, in some cases under veterinarian responsibility, as a last resort option for the treatment of PWD in farms with high rates of resistance to aminoglycosides (Rhouma et al., 2016b).

However, the most common use of colistin in pig production worldwide is oral, metaphylactic use (Casal et al., 2007b; Trauffler et al., 2014). This practice involves treating all animals belonging to the same pen – animals with clinical symptoms as well as clinically healthy ones (Ferran et al., 2011). In its last report, the EMA recommended using colistin only for therapy or metaphylaxis purposes in food-producing animals. All indications for prophylactic use of this molecule should be prohibited and indications of colistin should be restricted only for the treatment of enteric infections caused by susceptible non-invasive *E. coli* (European Medicines Agency, 2016b).

Colistin is used in pigs at the dose of 100,000 IU per kg of body weight for three to five consecutive days and divided into two administrations per day (European Medicines Agency, 2016b). This therapeutic regimen is recommended for colistin veterinary formulations administered in drinking water. However no recommendation has been made for CS products administered in feed or by an injectable route in pigs. It is important to stress the lack of standardization of therapeutic regimen and its impact on the judicious use of colistin in swine (Catry et al., 2015).

It is difficult to determine the real quantities of colistin used in pig production worldwide because

these data vary considerably from one country to another, and sometimes colistin amounts used in pigs in some countries are very high relative to the size of swine herds (European Medicines Agency, 2016b; Mayor, 2016). Even within the same country, quantities of colistin in pigs vary from one survey to another due to the absence of standardized methods for data collection (Casal et al., 2007b; Moreno, 2014).

1.1.5.2   Combination therapy

In vitro and clinical investigations examining synergism of colistin combined with other antimicrobials in human medicine has been investigated recently and reviewed (Bergen et al., 2015a; Bergen et al., 2015b). The ultimate objective of this combination is to overcome the suboptimal exposure and the resistance emergence associated with the use of colistin in monotherapy. Indeed, the combination of colistin with other antibiotics is intended to extend the CS spectrum of activity to cover Gram-positive bacteria and to prevent the emergence of antibiotic resistance (Zhanel et al., 2006). However, a considerable controversy regarding the effectiveness of these combinations to counter the spread of MDR bacteria has been discussed in human medicine (Tamma et al., 2012). Most recently, Lagerbäck and collaborators showed that colistin and rifampicin combinations were active *in vitro* against all NDM-1-producing *K. pneumoniae* strains used in their study. However they claimed that such effectiveness should be further explored *in vivo* to be considered for clinical use (Lagerbäck et al., 2016). Parchem and collaborators confirmed that colistin combination therapy should be considered in critically ill patients with MDR Gram-negative pneumonia (Parchem et al., 2016).

In swine, CS is typically used in monotherapy for the oral treatment of infections associated with *Enterobacteriaceae* (Rhouma et al., 2016a). However, there are some commercial formulations where CS is associated with others antimicrobial agents, mostly with β-lactam antibiotics (He et

al., 2011) such as ampicillin or amoxicillin (Table III). In fact, it has been shown that the combination of amoxicillin with colistin has a synergy and additive affect *in vitro* against pathogenic *E. coli* of avian origin, without antagonism between the two antibiotics (Hamouda et al., 2011). Colistin combinations were used exclusively for the curative therapy of pig bacterial infections (Table III). Moreover, it has been reported that in the weaning period, colistin was frequently applied in combination therapy with amoxicillin against symptoms of arthritis and/or meningitis and PWD in pigs (Timmerman et al., 2006). Combinations of colistin and amoxicillin plus zinc oxide (ZnO) in the pre-weaning and growing stages in feed were also reported in pigs (Moreno, 2014).

Given the lack of appropriately conducted randomized controlled clinical trials, reliable data on the efficiency of colistin combination use for the treatment of *E. coli* in pigs and its impact on bacterial resistance evolution are very limited or non-existent. In a recent study, Li and collaborators showed that a combination of CS with bacitracin zinc and chlortetracycline suppressed the increase of *tet* genes in fecal samples of weaned pigs (Li et al., 2016b). In this study, the relative fecal abundances of four *tet* genes (*tet*X, *tet*C, *tet*L, and *tet*W) were reduced in pigs treated with a combination of chlortetracycline, bacitracin zinc, and CS compared with the group treated only with chlortetracycline (Li et al., 2016b). However, in this study no information was reported regarding the evolution of resistance to colistin following the combination use of these antibiotics.

With the lack of solid microbiological evidence on the effectiveness and the impact on bacterial resistance evolution of colistin combination therapy in pigs, the CVMP recommended the withdrawal of marketing authorizations for all veterinary formulations containing colistin in combination with other antimicrobial substances (European Medicines Agency, 2016b).

Heavy metals such as zinc are widely used in pigs, especially for the control of PWD in

combination with colistin, and is incorporated into swine feed at levels of 125 to 3000 mg/kg of feed (Holman and Chénier, 2015). Zinc oxide fed at pharmacological levels reduces diarrhea and mortality and improves growth in pigs (Fairbrother et al., 2005). However, there are two major concerns regarding the use of ZnO in swine. On the one hand, there is environmental pollution because of the high levels of supplementation, and on the other there is co-selection and co-resistance where antibiotic resistance genes are located on the same mobile genetic element as ZnO resistance genes (Holman and Chénier, 2015). To the best of our knowledge, no study has investigated whether or not resistance genes associated with colistin and heavy metals could be carried on the same mobile genetic element. Such information is crucial since ZnO is among the proposed strategies to reduce colistin quantities used for the control of PWD in pig production (European Medicines Agency, 2016b).

In addition to colistin combination therapy used in field conditions (Table III), there are other combinations with this antibiotic that have been used in several scientific studies to evaluate the efficacy of some colistin alternative substances (Table IV).

These studies (Table IV) that evaluated the therapeutic efficacy of colistin combination therapy in pigs were carried out in China and focused primarily on clinical effectiveness not the emergence of antimicrobial resistance. Therefore, no information was available concerning the evolution of colistin bacterial resistance subsequent to the use of these combination therapies in swine, and we do not know whether these combinations are used in practice on pig farms in China.

1.1.6   Mechanisms of *Enterobacteriaceae* resistance to colistin

Owing to an excessive use of colistin in pig production for many decades, several studies conducted with swine reported the isolation of *E. coli* and *salmonella* strains with high percentages of resistance to colistin (Rhouma et al., 2016b). In the present review we will detail

the mechanisms of resistance to colistin for *Salmonella* and *E. coli*, due to the importance of these two bacteria in both swine and human health.

1.1.6.1 Chromosomal resistance

An initial and essential step in colistin action on GNB is the electrostatic interaction between the positively charged peptide of this antibiotic and the negatively charged lipid A of LPS (Deris et al., 2014b). Chromosomal resistance to colistin in *Salmonella* and *E. coli* is most often mediated by modifications of LPS, which result in alterations in the target and reduced binding of the antimicrobial (Biswas et al., 2012). Changes in LPS consist in a modification of lipid A with the addition of a 4-amino-4-deoxy-L-arabinose (L-Ara4N) and/or phosphoethanolamine (PEtn). These molecules reduce the net negative charge of LPS and as a consequence increase the resistance to colistin (Needham and Trent, 2013). In *Salmonella* and *E. coli*, the biosynthesis of L-Ara4N and/or PEtn is mediated by PmrA/PmrB and PhoP/PhoQ two-component response regulators and sensor kinase systems (Falagas et al., 2010). In fact, the PhoPQ and PmrAB two-component systems (TCS) in *Salmonella* and *E. coli* have been reviewed extensively elsewhere (Needham and Trent, 2013; Olaitan et al., 2014). A brief overview is provided here with a focus on the more recent discoveries.

PmrB and PhoQ are sensor cytoplasmic membranes activated respectively by high concentrations of Fe^{3+} and low pH and by low concentrations of Mg^{2+} and Ca^{2+} or certain antimicrobial peptides (McPhee et al., 2003; Rubin et al., 2015). In colistin resistant *Salmonella*, apart from an environmental stimuli such as low Mg^{2+} concentration, a mutation in the PmrA/PmrB and/or PhoP/PhoQ TCS is the major mechanism involved in LPS modification (Olaitan et al., 2014). In *Salmonella*, PhoPQ further influences lipid A modification by activating the PmrAB system through the activation of PmrD (Kato et al., 2012). However, it was proposed in *E. coli* that the

two systems are not coupled because PmrD does not activate the PmrA/PmrB system (Winfield and Groisman, 2004). This hypothesis was initially justified by a high divergence between the *Salmonella* and *E. coli* PmrD proteins (Winfield and Groisman, 2004). However, it was later found that *E. coli* PmrB possesses higher phosphatase activity that exceeds the same activity of the *Salmonella* homolog, and the replacement of the *E. coli pmrB* gene with the *Salmonella* homolog was able to render *E. coli* resistant to polymyxin under PmrD-inducing conditions with low concentrations of Mg^{2+} (Chen et al., 2011). Moreover, it has been demonstrated that the sRNA MgrR of *E. coli* was also involved in the regulation of lipid A modification (Moon and Gottesman, 2009). Most recently, Rubin and collaborators have shown that in *E. coli*, another unknown bacterial system activates PmrD under low $Mg2^{+}$ conditions to promote lipid A modification, even in the absence of PhoPQ (Rubin et al., 2015).

Mutations in TCS corresponding to *E. coli* and *Salmonella* can cause their constitutive over expression, leading to permanent modification of lipid A by L-Ara4N and PEtN (Olaitan et al., 2014). Recently, various mutations have been identified in both *pmrA* and *pmrB* genes of colistin-resistant *E. coli* isolated from healthy pigs and pigs with intestinal disease (Table V). Mutations in the PmrAB TCS are mostly involved in the development of resistance to colistin in *E. coli* (Quesada et al., 2015).

For PmrA, mutations mostly occurred in the phosphate accepter domain, while for PmrB, mutations most commonly occurred in the kinase domain (Quesada et al., 2015).

Of note, regardless of the mutation location in PmrA or PmrB genes, there was no association with a difference in MIC of these colistin resistant *E. coli* strains (Table V).

Despite the fact that polymorphism in the PmrAB system has been reported *in vitro* in *Salmonella* (Sun et al., 2009), Quesada and collaborators did not detect any of the protein polymorphisms of PmrA and PmrB sequences in colistin resistant *Salmonella* isolates from swine lymph nodes

(Quesada et al., 2015). However, the polymorphism of genes encoding the PhoPQ system in colistin-resistant *Salmonella* has not been investigated in this study. Recently, an in-depth investigation of these *Salmonella* isolates showed that 100% of them harboured the plasmid carrying the *mcr-1* gene (Quesada et al., 2016).

Furthermore, the effects of colistin resistance on virulence and on *in vitro* and *in vivo* fitness costs have been extensively studied in other GNB such as *A. baumannii* and *K. pneumoniae* (Beceiro et al., 2014; Choi and Ko, 2015). A study of the fitness costs of colistin resistant *Salmonella* pmrAB mutants *in vitro* and in a mouse model showed low fitness costs for these strains (Sun et al., 2009). Nevertheless, to the best of our knowledge no study has followed the fitness costs of colistin resistant *E. coli* mutants. Many studies have discussed the factors affecting the fitness cost of colistin resistance, including growth retardation, impaired virulence, increased susceptibility to other antibiotics, and substantially reduced clinical invasiveness (López-Rojas et al., 2011; Pournaras et al., 2014). In swine, it has been reported that oral colistin treatment is accompanied by a selection pressure on the colistin resistant *E. coli* commensal population (Rhouma et al., 2016b). Further investigations are required to study the fitness costs of colistin resistant *E. coli* and *Salmonella* of porcine origin.

1.1.6.2   Plasmid-encoded colistin resistance

Before November 2015, several studies in human and in swine medicine confirmed the isolation of *E. coli* isolates confirmed resistant to colistin without having a mutation in *pmrA* and/or *pmrB* genes (Olaitan et al., 2015b; Quesada et al., 2015). The discovery for the first time in early November 2015 in China of a plasmid mediated colistin resistance-1 (MCR-1) protein in *Enterobacteriaceae* (Liu et al., 2016) has provided explanation for the other potential colistin resistance mechanisms in GNB. Initially, this plasmid was considered to be a phenomenon

relegated to China (Paterson and Harris, 2016), however the *mcr-1* gene was soon after isolated in several countries on 4 continents: Asia, Africa, Europe, and the Americas (Rhouma et al., 2016a; Schwarz and Johnson, 2016; Skov and Monnet, 2016).

Very recently, in June 2016, a novel plasmid-mediated colistin resistance gene, *mcr-2*, was identified in colistin resistance *E. coli* isolates from porcine and bovine origin in Belgium (Xavier et al., 2016b). The *mcr-2* gene was detected with higher prevalence than of *mcr-1* gene among colistin-resistant *E. coli* of porcine origin.

MCR-1 and MCR-2 proteins showed 80.65% of identity and are members of the phosphoethanolamine transferase enzyme family that promotes the addition of a phosphoethanolamine group to lipid A, leading to a decreased affinity of colistin for the LPS (Liu et al., 2016; Xavier et al., 2016b). In Liu and collaborator's study, the *mcr-1* associated plasmid, designated pHNSHP45, is approximately 64 Kb in length and is an IncI2-like plasmid that harbors a predicted 83 open reading frames (ORFs) with a G+C content of 42.7% (Liu et al., 2016). The plasmid pHNSHP45 carrying *mcr-1* gene was initially isolated in July 2013 from an *E. coli* strain recovered from a pig farm (Shanghai, China) and showed resistance to most antibiotic families except the carbapenems (Liu et al., 2016). Subsequently, *mcr-1* has been reported in different plasmid incompatibility groups from different animal species, including IncHI2 (200-290 Kb), pVT553 (62 Kb), IncX4 (30 Kb), and IncP (79 Kb) plasmids in *E. coli* from broilers poultry, bovine, and swine origin (Anjum et al., 2016; Falgenhauer et al., 2016; Malhotra-Kumar et al., 2016a; Perreten et al., 2016; Veldman et al., 2016) and IncX4 (30 Kb) plasmids in *Salmonella* from chicken and turkey meat (Veldman et al., 2016; Webb et al., 2016). Xavier and collaborators isolated the *mcr-1* gene in pKP81-BE plasmid (91 Kb) from colistin resistant *E. coli* of porcine origin (Xavier et al., 2016a). The pKP81-BE plasmid showed a G+C content of 44.9% and belonged to IncFII incompatibility type with 4% similarity compared to

pHNSHP45. These findings showed that *mcr-1* has horizontally transferred to other plasmid types, leading to an increase in its target bacterial range (Li et al., 2016a; Tse and Yuen, 2016).

The *mcr-2* associated plasmid, designated pKP37-BE, is approximately 35 Kb in length and is an IncX4 incompatibility type, with a G+C content of 41.3%, and did not carry any other resistance genes (Xavier et al., 2016b).

The *mcr-1* gene has been identified in *Enterobacteriaceae* derived from humans, food, farm animals (Liu et al., 2016), vegetables (Zurfuh et al., 2016), the environment including water (Petrillo et al., 2016), and even wild migratory bird (Ruzauskas and Vaskeviciute, 2016). The *mcr-1* gene has also been identified in several multidrug resistant bacteria such as ESBL producing and carbapenemase-producing *E. coli* of chicken and swine origin (Falgenhauer et al., 2016; Yao et al., 2016). In colistin resistant *E. coli*, a co-localization of *mcr-1* and bla_{CTX-M} genes on a unique IncHI2-type plasmid was also reported in chickens (Grami et al., 2016; Sun et al., 2016) and in calves (Haenni et al., 2016). The co-localization of *mcr-1* with an ESBL gene on a conjugative plasmid increases the possibility of bacterial resistance to colistin and of broad-spectrum cephalosporins being maintained, even without the use of theses antibiotics in food animals. This finding poses significant challenges for successful clinical treatment of GNB and for resistance control strategies in both veterinary and human medicine. Veldman and collaborators reported for the first time a chromosomally located *mcr-1* gene in two colistin resistant *E. coli* isolated from veal calves (Veldman et al., 2016). In this study, the *mcr-1* gene was associated with the insertion sequence (IS) IS*Apl1-mcr-1* (or an *mcr-1*-containing mobile element) located immediately upstream of *mcr-1*, as also reported in plasmid pHNSHP45 (Liu et al., 2016). IS*Apl1* is a member of the IS*30* family, which was initially identified in *Actinobacillus pleuropneumoniae* (Tegetmeyer et al., 2008). The presence of this IS in association with the *mcr-1* gene strongly suggests that this gene is able to translocate to the chromosome and to different

plasmid backbones – as well as between bacterial strains. Furthermore, the *mcr-2* gene was associated with an IS of the IS_{1595} superfamily (Xavier et al., 2016b).

In swine, to the best of our knowledge, the plasmid-borne *mcr-1* gene has been observed in at least 2 enterobacterial species, *E. coli* and *Salmonella*, in ~12 countries on four different continents (Rhouma et al., 2016a; Schwarz and Johnson, 2016). Pig-to-human transmission of MCR-1 colistin resistance has already been reported (Olaitan et al., 2016a; Olaitan et al., 2015b), raising serious concerns about the consequences of the use of this antibiotic in pig productions on human healthcare.

In pigs, the *mcr-1* gene was isolated mainly from colistin resistant *E. coli* strains with variable prevalence between countries; China (20.6%), Vietnam (22%), Belgium (13.2%), Brazil (2%) Spain (0.68%), Germany (0.51%), and France (0.50%) (Falgenhauer et al., 2016; Fernandes et al., 2016; Liu et al., 2016; Malhotra-Kumar et al., 2016a; Nguyen et al., 2016; Perrin-Guyomard et al., 2016; Quesada et al., 2016). Most recently, in the USA pig production, the *mcr-1* gene was identified for the first time in a colistin resistant *E. coli* strain isolated from a pig from South Carolina (Meinersmann et al., 2016). In these studies, despite using the same technique (PCR) for *mcr-1* gene screening, it is difficult to compare these results between countries because of the lack of data on previous antibiotic treatments in sampled pigs, on the quantities of colistin used at the farm level, on the potential combination of antibiotics with colistin, and on the health status of the pigs. Moreover, there are no published longitudinal studies on pigs that quantify the link between colistin quantities used on farms and the evolution of bacterial resistance against this antibiotic.

Almost all studies conducted on pigs worldwide to screen *mcr-1* gene presence in enterobacterial species reported that colistin resistant strains harboring this gene also showed resistance to one or several classes of antibiotics conventionally used in swine such as: Aminoglycoside,

Sulphonamide, Trimethoprim, Tetracycline, Quinolone, Lincosamide, β-lactam, and third generation cephalosporin (Anjum et al., 2016; Falgenhauer et al., 2016; Malhotra-Kumar et al., 2016b; Nguyen et al., 2016). This multi-resistance of *mcr-1* positive *E. coli* strains in pigs was associated with the presence of a *sul3*-containing class 1 integron, In*640*, in the plasmid's mediated *mcr-1* gene. This integron showed the presence of genes encoding resistance to trimethoprim (*dfrA12*), aminoglycosides (*aadA1a* and *aadA2*), sulphonamides (*sul3*), and phenicols (*cmlA1*) (Xavier et al., 2016a). Furthermore, IncX4 plasmids have been shown to harbour *mcr-1 and mcr-2* genes a swell as ESBL genes (Xavier et al., 2016b).

In the study of Quesada and collaborators, the *mcr-1* gene was screened and detected in three colistin resistant *Salmonella* strains isolated from 122 lymph nodes and in two colistin resistant *E. coli strains* isolated from 439 swine fecal samples (Quesada et al., 2016). This study was the first in swine to demonstrate the existence of a plasmid carrying *mcr-1* gene, in addition to a mutation in PmrAB TCS, in two colistin resistant *E. coli* strains. The coexistence of these two colistin resistance mechanisms in *E. coli* was not associated with a difference in the MIC of these strains compared to resistant *Salmonella* strains that expressed only the plasmid carrying *mcr-1* gene (Quesada et al., 2016). It should be stressed here that the *mcr-1* gene found in colistin resistant enterobacterial strains of porcine origin was often associated with low levels of resistance; the MICs of 4 or 8 mg/L observed for most isolates are only 2 to 4 times higher than the EUCAST clinical breakpoint (2 mg/L) (Anjum et al., 2016; Liu et al., 2016; Quesada et al., 2016). Fernandes and collaborators reported the isolation of a colistin-susceptible *E. coli* strain carrying the *mcr-1* gene from the fecal sample of a healthy pig (Fernandes et al., 2016). This finding, *suggests* that *mcr-1*-positive isolates may be difficult to detect if only the *mcr-1* gene is screened in colistin resistant isolates. Further studies are needed to examine the expression of *mcr-1* gene in *E. coli* and to determinate the promoter and the operon responsible for this expression.

1.1.7　One Health Perspectives

1.1.7.1　Importance of the one health concept in colistin resistance management

Currently, colistin is an antibiotic widely used in veterinary medicine, particularly in pigs, for the oral treatment of intestinal infections caused by *Enterobacteriaceae* (Rhouma et al., 2016a). In humans, colistin is *used for the treatment* of infections caused by *MDR-GNB and is considered to be a last-resort antibiotic* treatment *option* for carbapenemase-producing *Enterobacteriaceae* infections (Gurjar, 2015). During the last decade, research on colistin experienced a significant increase, especially regarding the mechanism of resistance of colistin and the optimization of its therapeutic regimen using the PK/PD relationship (Michalopoulos and Falagas, 2011; Olaitan et al., 2014).

Recently, the *mcr-1* gene was isolated from colistin resistant *E. coli* strains from several farm animals: pigs (Rhouma et al., 2016a), piglets (Malhotra-Kumar et al., 2016a), chickens (Shen et al., 2016), cattle (Suzuki et al., 2016), and veal calves (Haenni et al., 2016). A strong similarity was found between the different classes of plasmid carried *mcr-1* genes in these animal productions, and the successful gene-plasmid combination was mainly attributed to the presence of IS*Apl1* upstream in the *mcr-1* gene (Falgenhauer et al., 2016). These findings are in favor of a possible movement of this mobile genetic element between the various animal productions (Falgenhauer et al., 2016) (Figure 2). In addition to their use in pigs, polymyxins and especially polymyxin B are used in some countries for the treatment of coliform and *Pseudomonas* mastitis in cows (Du Preez, 2000), and this antibiotic is sometime used for this purpose as an extra-label drugs in cattle such as in Canada and in the United States (Smith et al., 2005). Intramamary infusions of 1 to 2 million units of polymyxin B/quarter gave an efficiency for the treatment of cows with severe cases of coliform mastitis (Smith et al., 2005). Although some studies have

reported the isolation of colistin resistant *E. coli* strains harbouring the *mcr-1* gene from cow with mastitis (Suzuki et al., 2016), the role of polymyxin B, used for the treatment of mastitis, in colistin resistance still unknown. Furthermore, colistin is used outside of North America orally in calves and lambs at a dose of 100.000 IU/kg b.w day divided in two identical doses for 3 consecutive days for the treatment of gastrointestinal diseases caused by GNB (Official Journal of the European Union, 2010). This use could explain the isolation of bacteria resistant to colistin in calves, even despite the lack of data on an preliminary treatment of these animals with colistin (Haenni et al., 2016).

On the other hand, colistin is used in some countries such as China for the control of intestinal infection caused by GNB in chicken, turkeys, rabbits and ducks (Dowling, 2013). Colistin was incorporated into the feed of these animals at the dose of 3.33 mg/kg b.w for turkeys, 3.8 mg/kg b.w for rabbits and chickens, and 20 mg/kg for ducks (Zeng et al., 2010). Colistin was also used in the drinking water in laying hens at the dose of 3.8 mg/ kg b.w (Goetting et al., 2011). Furthermore, colistin is widely used in Europe for the oral treatment of *E. coli* infections in chicken and laying hens at the dose of 75.000 IU/kg b.w day for 3 to 5 consecutive days in the drinking water (Le Devendec et al., 2015; Official Journal of the European Union, 2010). Although several studies have confirmed the isolation of bacteria resistant to colistin harbouring *mcr-1* gene from avian origin, however, to the best of our knowledge no scientific study has investigated the resistance of GNB to colistin in turkeys, rabbits and ducks.

In addition, the *mcr-1* gene was also isolated from wild migratory birds such as the European herring gull (*Larus argentatus*) in Lithuania (Ruzauskas and Vaskeviciute, 2016) and the kelp gulls (*Larus dominicanus*) in Argentina (Liakopoulos et al., 2016). The role of these migratory birds in the spread of the *mcr-1* gene between continents should not be underestimated.

The *mcr-1 gene* was identified in resistant *E. coli* strains isolated from environmental samples

such as river water (Zurfuh et al., 2016), chicken feed in trough (Yu et al., 2016), and ready-to-eat vegetables (Zurfuh et al., 2016). Therefore, the role of animal manure used in the fertilization of agricultural lands in the environmental dissemination of the *mcr-1* gene needs to be verified. Several studies have reported the isolation of colistin resistant bacteria from pig manure (Hölzel et al., 2010).

In addition, the *mcr-1* gene was identified in resistant *E. coli* strains isolated from food samples such as chicken and pork meat (Liu et al., 2016), ground beef (Mulvey et al., 2016), and retail meats (chicken, pork and beef) (Kuo et al., 2016). These foods of animal origin represent a major route of contamination with the *mcr-1* gene for slaughterhouse workers and consumers (Figure 2).

The gene encoding plasmid-mediated colistin resistance, was also identified in resistant *E. coli* strains isolated from humans with gastroenteritis or wound infections (Doumith et al., 2016; Falgenhauer et al., 2016) and from asymptomatic people (Olaitan et al., 2016a). The *mcr-1* gene was isolated from humans from 4 continents, showing that plasmid-mediated colistin resistance has already spread worldwide.

It was reported that food animals are the main source of human contamination by the MCR-1 and MCR-2 (Nordmann and Poirel, 2016; Rhouma et al., 2016a; Xavier et al., 2016b). However Ruppé and collaborators isolated the *mcr-1* gene in colistin resistant *E. coli* from five children with ages ranging between 2 months and 27 months who did not have pets or a history of animal contact (Ruppé et al., 2016). Moreover, despite the fact that colistin is not approved in animal production in the USA, McGann and collaborators reported for the first time in the USA, the identification of *mcr-1* gene in a colistin resistant *E. coli* strain cultured from a woman with a urinary tract infection (UTI). However this strain remained susceptible to several other antimicrobial agents (McGann et al., 2016). These findings suggest that *mcr-1* is already

widespread in the environment and transmissible via various routes to humans. Thereby, there is also a potential risk of the transfer of *mcr-1* gene from human to animal. However such transfer should be investigated in future studies.

Most recently, *mcr-1*-harboring *E. coli was* isolated from healthy dogs and cats in a pet shop in Guangzhou, China (Zhang, 2016). An interesting finding in this study was that the *mcr-1* gene in colistin resistant *E. coli* was isolated from a worker at this pet shop – and it was the same *E. coli* strain clonally related to those originating from dogs. This finding is in favor of a possible transmission of *mcr-1*–harboring *E. coli* between dogs and humans.

Polymyxins are used in dogs and cats mostly for topical indications (De Briyne et al., 2014; Mateus et al., 2011). In fact, polymyxin B is used in the treatment of canine otitis externa, and it showed synergy with miconazole against *E. coli* and *P. aeruginosa* (Pietschmann et al., 2013). Polymyxin B used also in ophthalmic suspension for the treatment of keratitis in dogs (Beckwith-Cohen et al., 2015). For the treatment of this ophthalmic disease, polymyxin B is commonly associated with other drugs such as neomycin, and dexamethasone (Beckwith-Cohen et al., 2015), or chloramphenicol (Hindley et al., 2016). Furthermore, it was shown that colistin used at the dose of 12. 500 IU/kg IM for 5 days in combination with ampicillin had demonstrated an anti-endotoxic effects in dogs with naturally occurring endotoxic shock (Şentürk, 2005). Despite the isolation of *E. coli* resistant to polymyxins harbouring the *mcr-1* gene from dogs and cats, it is difficult to determine the role of polymyxin B administered topically in the exacerbation of colistin resistance in dog's or cat's intestine.

Neither the role of waste and contaminants from the pharmaceutical industry nor the role of fish farms has been documented as a source of colistin resistance amplification in the environment. In fact, it has been reported that administration of colistin sulfate with other antibiotics in the diets of fish significantly improved feed conversion and promoted their growth rate (Hao et al., 2014).

To the best of our knowledge, no study has documented the isolation of colistin resistant *E. coli* strains or *mcr-1* gene from fish.

Transmission of *mcr-1* gene resistance from animals to humans can take place through a variety of routes (Figure 2). Therefore, the management of colistin resistance requires global and coordinated action between the different actors in order to intercept this resistance spread and preserve the efficacy of colistin for the treatment of MDR-GNB in human medicine.

We believe that the One Health concept is more important than ever to better manage the impact of colistin resistance in human and veterinary medicine. Such a concept needs a global strategy to develop collaborations and interdisciplinary communication between concerned specialists (Figure 3).

1.1.7.2   Action in swine medicine

The use of colistin in swine has contributed to the intensification of modern pig productions by assuring successful weaning, higher animal densities, and most likely helped to reduce economic losses caused by *E. coli* infections such as PWD and *edema disease (Rhouma et al., 2016a)*. Economic *gains have come* at a considerable *cost,* which is being borne, *in particular, by public health and other* stakeholders such as the environment and the animals themselves. *In fact,* the recent discovery of a plasmid-mediated *mcr-1* gene encoding for colistin resistance in *Enterobacteriaceae* has aroused great concern about the possible loss of colistin effectiveness for the treatment of MDR- GNB in humans. Because of the high rate of isolates carrying the *mcr-1* gene isolated from animals compared to humans, livestock production has been pinpointed as a reservoir of the *mcr-1* determinant (Nordmann and Poirel, 2016), hence the need for rapid action in food animals to prevent the spread of colistin resistance (Figure 3). This section will focus on interventions in swine medicine but is applicable to all animal production where colistin is used.

The use of colistin as a growth promoter: This practice should be banned internationally. In addition to the fact that antimicrobials for growth promotion can generally be purchased without veterinary involvement, low subinhibitory concentrations of antibiotics used to improve animal growth has been shown to promote antibiotic resistance emergence (Aminov and Mackie, 2007; Andersson and Hughes, 2010; Nosanchuk et al., 2014). No recent studies have been able to clearly establish a link between the use of antibiotics as growth promoters and the improvement of animal performance in modern farming conditions with a high level of sanitation (Diarra and Malouin, 2014).

The use of colistin for prophylaxis and metaphylactic purposes: This usage is involved in the increase of colistin quantities used in pigs and increases its prevalence as waste in the environment (Rhouma et al., 2016a). Such usage of an antibiotic of very high importance in human medicine should be strictly avoided in swine. Intestinal disease prevention in pigs should be based mainly on livestock preventive management measures (optimal temperature, vaccination, sanitation, housing conditions, applying biosecurity rules, etc.) (Aarestrup et al., 2008a; Fairbrother et al., 2005).

The use of colistin for therapeutic purposes: Nevertheless that colistin is a cheap therapeutic strategy with certain efficacy against Enetrobacteria associated disease in swine, it has been shown that the oral use of colistin for the treatment of pigs in an experimental PWD model was associated with a pressure selection on *E. coli* populations (Rhouma et al., 2016b). Therefore, the use of colistin as the first therapeutic choice to treat intestinal infections in pigs should be avoided. The therapeutic alternative to colistin should not be an antibiotic belonging to β-lactam family because of the co-localization of *mcr-1* and ESBL genes in the same mobile genetic element. In addition, in its very recent advice, the EMA required that the reduction of colistin use in farm animals should not be associated with an increase in the consumption of

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fluoroquinolones, 3rd- and 4th-generation cephalosporins, or the overall use of antimicrobials (European Medicines Agency, 2016b).

Requirements for colistin therapeutic use: Clinical diagnoses of the disease by veterinarians and the isolation of pathogen agent linked with the antibiogram tests to determinate bacterial susceptibility to colistin are essential to justifying its therapeutic use. Isolation from the animal husbandry of bacteria harboring the *mcr-1* gene should be considered a strong reason not to use colistin on that farm.

Moreover, the veterinarian should ensure that colistin prescribed is used in farms only for the treatment of sick pigs as recommended; compliance with label instructions (no underdosing or prolongation of dosing interval, withdrawal period) is of paramount importance. Any deviations from the guideline recommendations must be justified and recorded. In this context, extra-label use of colistin in some countries where this antibiotic is not approved in swine such as in Canada, must take place within a valid veterinarian-client-patient relationship. An analysis of the specific situation at farms and a determination that there are no alternatives to this antibiotic for the treatment of this case is required. Research is very important in order to establish a microbiological withdrawal period that could reduce the risk that pigs sent to slaughter contain colistin resistant bacteria or *mcr* genes in their gut.

Surveillance and monitoring of colistin use on farms: Veterinarians should ensure that colistin use targets clinical disease, should consider reduction of its use whenever practical, and should direct management and husbandry issues at the same time. Veterinarians should also consider laboratory examination as a routine practice to evaluate the effectiveness of colistin treatment and to monitor the sensitivity of infectious strains on the farm. Educational and awareness campaigns for employers and pig farmers are essential to generate an understanding that can support the veterinarian to withhold colistin. The professional organization of each country should develop

clinical-practice guidelines on the judicious use of colistin. Data on colistin usage in food animals are critically important because they provide a basis for the development of national policies and they guide the risk of colistin resistance management and assess the effect of possible interventions (Aarestrup et al., 2008b). At a minimum, these data should include national use of colistin in kilograms of active ingredient on an annual basis and data should be stratified by animal species (Merle et al., 2012). The OIE and WHO recommend collecting the amount of antibiotics in food animals (WHO, 2004). Finally, the standardization of a data collection method regarding the use of colistin in farms between countries is very important to evaluate the effectiveness of such interventions to manage colistin resistance spread.

Monitoring of colistin resistance: There are many national antimicrobial resistance monitoring and surveillance programs that already exist and are well established in many countries (Gelbrand et al., 2015) (Table VI). Among the principles of the One Health approach is the improved use of existing natural resources and implementation, which includes the monitoring of colistin resistance spread in both human and veterinary medicine. However, regulations and practices vary widely between these surveillance programs and are influenced by the economic and social context of each country (Laxminarayan et al., 2013).

Coordination between the various stakeholders is paramount for effective surveillance systems at the country level. In Canada, a new initiative to better manage the dissemination of antimicrobial resistance at the human-animal interface was established by the Public Health Agency of Canada in 2015. The aim of this program, called the Canadian Antimicrobial Resistance Surveillance System (CARSS), is to strengthen the coordination and integration of antimicrobial resistance and antimicrobial use activities and information in Canada and to consolidate surveillance from seven existing systems (Gelbrand et al., 2015).

Practical conditions for the reduction of colistin use on farms: Governments should fund

research that enhances our understanding of environmental and genetics factors that facilitate the development of infectious disease in food animals, and that examines alternative strategies for the use of antibiotics on farms. Financial assistance for farmers in the implementation of sustainable practices and interventions to prevent infections, such as sanitation, housing, improvement of nutritional programs, and immunization, is very important for the reduction of the use of colistin or other antibiotics on farms. In addition, the preparation of guides and educational material for veterinarians and farmers on appropriate disease management and treatment based on the recent results of research is crucial for the responsible use of antimicrobials in farms. Efforts to improve microbiological laboratories are vital to help veterinarians undertake rapid therapeutic action with the most appropriate antibiotic and at an early stage of the disease (Årdal et al., 2016). Finally, the competent authorities should clearly define guidelines for colistin marketing, sales, and use on farms.

1.1.7.3 Action in the environment

In addition to the isolation of colistin resistant bacteria from manure, water, migratory birds, and vegetables (Hölzel et al., 2010; Schwarz and Johnson, 2016), the toxicity impact of colistin on the environment is a topic of concern (Bressan et al., 2013; Guo et al., 2014). Indeed, it has been shown that the presence of colistin at therapeutic concentrations in swine farm wastewater was associated with a toxicity against ammonia-oxidizing bacteria (AOB) (Bressan et al., 2013). These AOB are involved in the biodegradation of xenobiotic compounds and in the conversion of ammonia to nitrites in wastewater treatment plants (Bressan et al., 2013). The ecotoxicity effect of colistin was demonstrated in the earthworm *Eisenia fetida*; colistin caused significant damage to its intestinal epithelium and caused the induction of stress-related gene expressions (Guo et al., 2014).

In addition, it has been reported that colistin-resistant *E. coli* were isolated from wild rabbits (*Oryctolagus cuniculus*) and wild hares (*Lepus europaeus europaeus*) that have not been previously treated with colistin (Dotto et al., 2014). Consequently, wildlife may represent another potential reservoir of colistin resistance bacteria in the environment that could contaminate humans through contaminated food and water or by direct human and animal contact (Gelbrand et al., 2015).

This section will be devoted to the possible interventions to limit the spread of colistin resistant bacteria and genes in the environment via pig manure (Figure 3).

*R*educing the use of antibiotics on farms: It has been estimated that about 75% of the administered antibiotics is not absorbed by animals but is excreted via the feces or urine (Chee-Sanford et al., 2009). This finding is even more pronounced with colistin, which is very poorly absorbed in animal's gastrointestinal tract (Rhouma et al., 2016a). It has also been reported that the frequency of bacteria carrying antimicrobial resistance genes is high in pig manure compared to other farm animals (Heuer et al., 2011), and a high frequency and concentration of antibiotic resistance genes (ARG) was detected around swine farms (Chen et al., 2010). Therefore, the role of pig manure is not to be underestimated in the dissemination of colistin resistance in the environment. It is crucial to consider reducing the use of antibiotics on farms, especially critically important antimicrobials, in favor of other measures such as the improvement of nutritional programs, housing, and animal immunization (Pruden et al., 2013).

Biological management of manure: Some studies have reported that composting eliminates on average 50–70% of some antimicrobials such as chlortetracycline, monensin, and tylosin (Pruden et al., 2013) and reduces the relative quantities of the bla_{TEM}, *sul3*, and *erm*(B) genes in manure (Le Devendec et al., 2015). However, to the best of our knowledge, no study has shown the efficacy of this technique in reducing amounts of colistin or *mcr* genes in pig manure.

The effectiveness of reducing antibiotic resistance genes in pig manure depends mostly on the method manure is handled; aerobic biofiltration of manure has been reported to reduce *erm*(X) more effectively than other ARG such as *erm*(F), *erm*(B), and *tet*(G), while mesophilic anaerobic digestion and lagoon storage reduced none of these AR genes (Chen et al., 2010). There has been much controversy concerning the efficiency of these biological manure treatments, such as lagoons and composting, in ARG reduction (Pruden et al., 2013), which is why more research is needed into assessing the effectiveness of swine waste treatment processes in the destruction of resistant bacteria and ARG in pig manure. With the lack of regulation worldwide or international guidelines to control the release of pig manure containing antibiotics (Wei et al., 2011), it is difficult to reduce the spread of colistin resistance into the environment by manure land applications.

1.1.7.4 Action in human medicine

Colistin is currently considered to be one of the last-resort antibiotics used for the treatment of infections caused by MDR-GNB in humans (Bergen et al., 2015a). Maintaining the effectiveness of this antibiotic is a challenge for both scientists and physicians. Nevertheless, there are several possible proposals to optimize the use of colistin in human medicine (Figure 3).

Screen for colistin resistance in patients: This step is crucial before undertaking a therapeutic intervention using colistin, and screening should be done in both patients with and without prior history of colistin usage (Olaitan et al., 2016b). Hospitals should know whether or not their laboratories have the ability and the necessary equipment to perform colistin resistance testing and *mcr-1* screening tests among admitted patients who needed colistin as a treatment.

Prevention of contamination by colistin resistant bacteria in hospital: Hand hygiene plays a crucial role in achieving this goal (Mathur, 2011). Interactive educational programs are important

to explain the steps of hand hygiene technique as well as its rationale. Given the coproduction of *mcr-1* genes and NDM enzymes by the same colistin resistant isolates, as reported by (Du et al., 2016), we believe that the guide for the control of healthcare-associated infections due to carbapenem-resistant *Enterobacteriaceae*, published in 2012 by the U.S. Centers for Disease Control and Prevention (CDC) and updated in 2015 (available from: http://www.cdc.gov/hai/organisms/cre/cre-toolkit/index.html), would be a very good tool to prevent contamination by colistin resistant strains in hospitals. In addition, the identification of a patient carrying isolates that produce *mcr-1* gene in association with carbapenemases should be strictly considered a reason for patient isolation (Nordmann and Poirel, 2016).

Prevention of contamination of humans following direct contact with animals or meat: Epidemiological studies have described a possible horizontal transmission of a colistin resistant *E. coli* strain from pigs (Olaitan et al., 2015b) or from companion animals (Zhang, 2016) to humans following close contact. It has been shown that colistin-resistant E. coli was isolated from healthy individuals without prior colistin usage (Olaitan et al., 2016b). Better hygiene, particularly hand washing with soap or using alcohol disinfectant after handling animals at a farm, pet shop, or slaughterhouse is obligatory. Also, using gloves during pig or manure handling and taking a shower at the exit of a piggery are mandatory practices that should be enforced. As well, employees must be particularly familiar with hand hygiene techniques and their purpose. Considering that a high percentage of colistin resistant *E. coli* is isolated from retail meat (Liu et al., 2016), consumers should avoid any type of cross contamination between meat and salad or other raw foods.

Re-evaluation of colistin use for selective digestive decontamination: In the intensive care unit, colistin is sometimes used orally for selective decontamination of the digestive tract (SDD), mainly to target resistant gram-negative aerobic bacteria, along with a short course of a parenteral

broad-spectrum antimicrobial such as cefotaxime (a third generation cephalosporin) (Silvestri et al., 2007). This practice has been shown through meta-analysis of randomized control trials to reduce the occurrence of respiratory tract infections, mortality, and overall bloodstream infections in critically ill patients (de Jonge et al., 2003; Silvestri et al., 2007). However, it has been demonstrated that prolonged use of colistin as part of SDD is associated with the emergence of colistin resistance among ESBL producing *K. pneumoniae* isolates (Halaby et al., 2013). The long-term effects of colistin use in SDD was singled out as a possible source of colistin resistance amplification, therefore the re-evaluation of this practice is a topic of concern for intensive care units (Rawson et al., 2016).

Evaluation and optimization of colistin combination therapy: Several *in vitro* and in mouse model studies have shown that combination of colistin with other antimicrobials such as rifampicin and imipenem may be more effective than colistin monotherapy in the treatment of MDR-GNB (Aoki et al., 2009; Lagerbäck et al., 2016). A review of 15 studies involving 55 unique patient cases found that clinical success was lower for colistin monotherapy compared with colistin combination therapy for treatment of infections caused by *K. pneumoniae* carbapenemases (KPCs) producers (Hirsch and Tam, 2010). However, another review reported considerable controversy regarding the clinical efficacy of colistin combination therapy during the treatment of MDR-GNB (Tamma et al., 2012). This interesting therapeutic approach needs to be clinically studied in depth to assess its effectiveness and its impact in MDR-GNB resistance occurrence.

1.1.8 Conclusion

Colistin is an antibiotic widely used in pigs for the oral control of bacterial infections caused by *E. coli* and *Salmonella*. The recent discovery of a plasmid-mediated *mcr-1* gene encoding for

colistin resistance in *Enterobacteriaceae* has generated great concern about the possible loss of effectiveness of colistin for the treatment of MDR-GNB in humans. Because of the large amounts of colistin used in food animals and particularly in pigs, pig production has been pointed to as the greatest cause of colistin resistance amplification and spread. Consequently, experts, scientists, and government agencies have called for a reduction of colistin use in pigs and stressed that this antibiotic should be used only for the treatment of diseased animals as a last-resort treatment under strict circumstances. The *mcr-1* gene has been isolated on 4 continents from sources other than food animals, such as the environment and human origins, and some *E. coli* isolates carrying a plasmid-encoded *mcr-1* gene were associated with ESBL or carbapenemases enzymes. This highlights the need for an overarching approach on the judicious use of all antibiotics, especially those of critical importance for human health. The One Health concept is more important than ever to better manage colistin resistance at the human- animal-environment interface through the use of adequate science-based risk management policies that respect interdisciplinary regulations. Finally, we should start thinking beyond colistin therapy in swine and begin evaluating the effectiveness of other alternative strategies against infections caused by *Enterobacteriaceae*.

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 potential conflicts of interest.

1.1.9 Tables

Table I: Colistin sulfate PK data in pigs following its oral or intramuscular administration

Colistin sulfate route of administration/pigs health status	Dose used (mg/Kg)	Quantification method/LLOQ	Plasma C_{max} (ng/mL) Intestine C_{max} (mg/Kg)	T_{max} (h)	References
Oral/Clinical healthy	1.2	HPLC 250 ng/mL 0.5 µg/g	Plasma: NA* Intestine: 26.97	Plasma: NA Intestine: 2	(Guyonnet et al., 2010)
	2.4	HPLC 250 ng/mL 0.5 µg/g	Plasma: NA* Intestine: 43.57	Plasma: NA Intestine: 1	
	4.8	HPLC 250 ng/mL 0.5 µg/g	Plasma: NA* Intestine: 91.75	Plasma: NA Intestine: 1	
Oral/Clinical healthy	2.4	LC–MS/MS 20 ng/mL	Plasma: NA* Intestine: NA	Plasma: NA Intestine: NA	(Rhouma et al., 2015)
Oral/Clinical healthy	2.4	LC–MS/MS 1 ng/mL	Plasma: 10.3 Intestine: NA	Plasma: 0.5 Intestine: NA	(Rhouma et al., 2016b)
Oral/Experimental PWD	2.4	LC–MS/MS 1 ng/mL	Plasma: 122.3 Intestine: NA	Plasma: 0.5 Intestine: NA	
Oral/Clinical healthy	4.8	LC–MS/MS 1 ng/mL	Plasma: 32.2 Intestine: NA	Plasma: 0.5 Intestine: NA	
Oral/Experimental PWD	4.8	LC–MS/MS 1 ng/mL	Plasma: 338.3 Intestine: NA	Plasma: 0.5 Intestine: NA	

IM/Clinical healthy	2.4	HPLC 150 ng/mL	Plasma: 2780 Intestine: NA	Plasma: 0.5 Intestine: NA	(He et al., 2011)
IM/Clinical healthy	2.5	Microbiological assay	Plasma: NA Intestine: NA	Plasma: NA Intestine: NA	(Tang et al., 2009a)
IM/Clinical healthy	2.5	Microbiological assay	Plasma: 3730 Intestine: NA	Plasma: 0.5 Intestine: NA	(Lin et al., 2005)
IM/Clinical healthy	5	Microbiological assay	Plasma: 6400 Intestine: NA	Plasma: 0.5 Intestine: NA	(Lin et al., 2005)

PWD, post-weaning diarrhea. **LLOQ**, Lower limit of quantitation. **C_{max}**, maximum plasma or intestinal colistin concentration. **T_{max}**, time at which the C_{max} is observed. **NA**, information not available. ***** Concentrations of CS were less than the LLOQ of the method.

Table II: Topics that should be investigated to ensure judicious use of colistin in pigs

- Uniform composition and dosing of commercial CS formulations

- Studies to establish specific clinical breakpoints of oral colistin against *Enterobacteriaceae*

- Clinical trials in field conditions to define the optimum dosing strategies, including total daily dose and treatment duration

- Generate more data regarding the PK/PD of colistin in animals with intestinal diseases

- Clinical trials to evaluate the effectiveness of CS treatment at an early stage of disease to reduce colistin quantities used on farms

- Studies to evaluate the effectiveness of CS parenteral formulations and their potential risks on resistance occurrence within intestinal microflora

- Clinical controlled trials to evaluate the potential risks and benefits of combining colistin with other antimicrobial agents

•	Studies to elucidate mechanisms of the development of co-resistance to colistin on farms
•	Studies to evaluate the efficacy and toxicity of colistin degradation products
•	Studies to determine a microbiological withdrawal period for colistin resistant bacteria in addition to the chemical withdrawal period
•	Studies toevaluatetheexpressionof *mcr* genes on *Enterobacteriaceae* in pigs

Table III: Colistin sulfate combination with other antimicrobial agents used in pig production in France (ANSES, 2016)

Combination*	Route of administration	Indications	Withdrawal Time (days)
Colistin- Ampicillin	IM	Septicemia, gastrointestinal, respiratory and genitourinary infections	21
Colistin- Amoxicillin	IM	Septicemia, gastrointestinal, respiratory infections	10
Colistin-Erythromycin	Oral	Intestinal infections	21
Colistin- Neomycin	Oral	Intestinal infections	14
Colistin- Oxytetracycline	Oral	Intestinal infections	7
Colistin- Spiramycin	Oral	Intestinal infections	10
Colistin- Trimethoprim	Oral	Intestinal infections	7
Colistin- Ampicillin- Dexamethasone	IM	Septicemia, gastrointestinal, respiratory infections	21

* Colistin is always used as colistin sulphate. **IM**: intramuscular

Table IV: Colistin combination with other antimicrobial agents in scientific studies conducted in pigs

Combination	Doses in feed (mg/kg)	Treatment duration (days)	*E. coli* (log10 CFU/g of caecal digesta)	Weight gain (g/d)	References
Kitasamycin – Colistin sulfate- Olaquindox	50- 100- 60	14	N/A	307[b]	(Li et al., 2008)
Kitasamycin - Colistin sulfate- Chlortetracycline	50- 80- 150	35	4.69[a]	505[a]	(Li et al., 2012)
Kitasamycin - Colistin sulfate	100- 800	19	3.09[a]	367[a]	(Wu et al., 2012)
Kitasamycin- Colistin sulfate	100- 40	28	N/A	528[a]	(Huang et al., 2015)
Enramycin- Colistin sulfate- Zinc oxide	200- 200- 2000	28	N/A	787[b]	(Kuang et al., 2015)

N/A: not available. **a:** Statistically significant compared to the control group. **b:** Not statistically significant compared to the control group.

Table V: Mutations in two-component systems conferring resistance to colistin in *E. coli* of pig origin

Bacteria	Health status /Samples	Gene	Mutation in aa	MIC (mg/L)	References
E. coli	Clinical healthy	*pmrA*	S39I R81S	4	(Quesada et al., 2015)

	/Feces				
E. coli	Clinical healthy /Feces	*pmrB*	V161G	4	(Quesada et al., 2015)
E. coli	Experimental PWD/Feces	*pmrA*	G53R	8	(Thériault, 2015)
E. coli	Experimental PWD/Feces	*pmrB*	T156M	8	(Thériault, 2015)

aa: Amino acid. **PWD**: Post weaning diarrhea. **MIC**: Minimum inhibitory concentration.

Table VI: Examples of antimicrobial resistance monitoring and surveillance programs in some countries

Countries	Name of surveillance program	Directed by	Target
European Union	The European Antimicrobial Resistance Surveillance System (EARSS)	European Centre for Disease Prevention and Control (ECDC)	Humans
Denmark	The Danish Antimicrobial Resistance Monitoring and Research Program (DANMAP)	Danish Ministry of Food, Agriculture and Fisheries and the Danish Ministry of Health	Humans, animals, and food
Canada	The Canadian Integrated Program for Antimicrobial Resistance Surveillance (CIPARS)	Health Canada	Humans, animals, and meat
United States	National Antimicrobial Resistance	Food and Drug	Humans,

	Monitoring System (NARMS)†	Administration Center for Veterinary Medicine (FDACVM)	animals, and meat
Norway	The Norwegian AMR surveillance program (NORM)	The Norwegian Ministry of Health and Social Affairs	Humans, animals,
Japan	The Japanese Veterinary Antimicrobial Resistance Monitoring Program (JVARM)	Ministry of Agriculture, Forestry and Fisheries	Animals

† In collaboration with the United States Department of Agriculture (USDA) and the Center for Disease Control and Prevention (CDC).

1.1.10 Figures
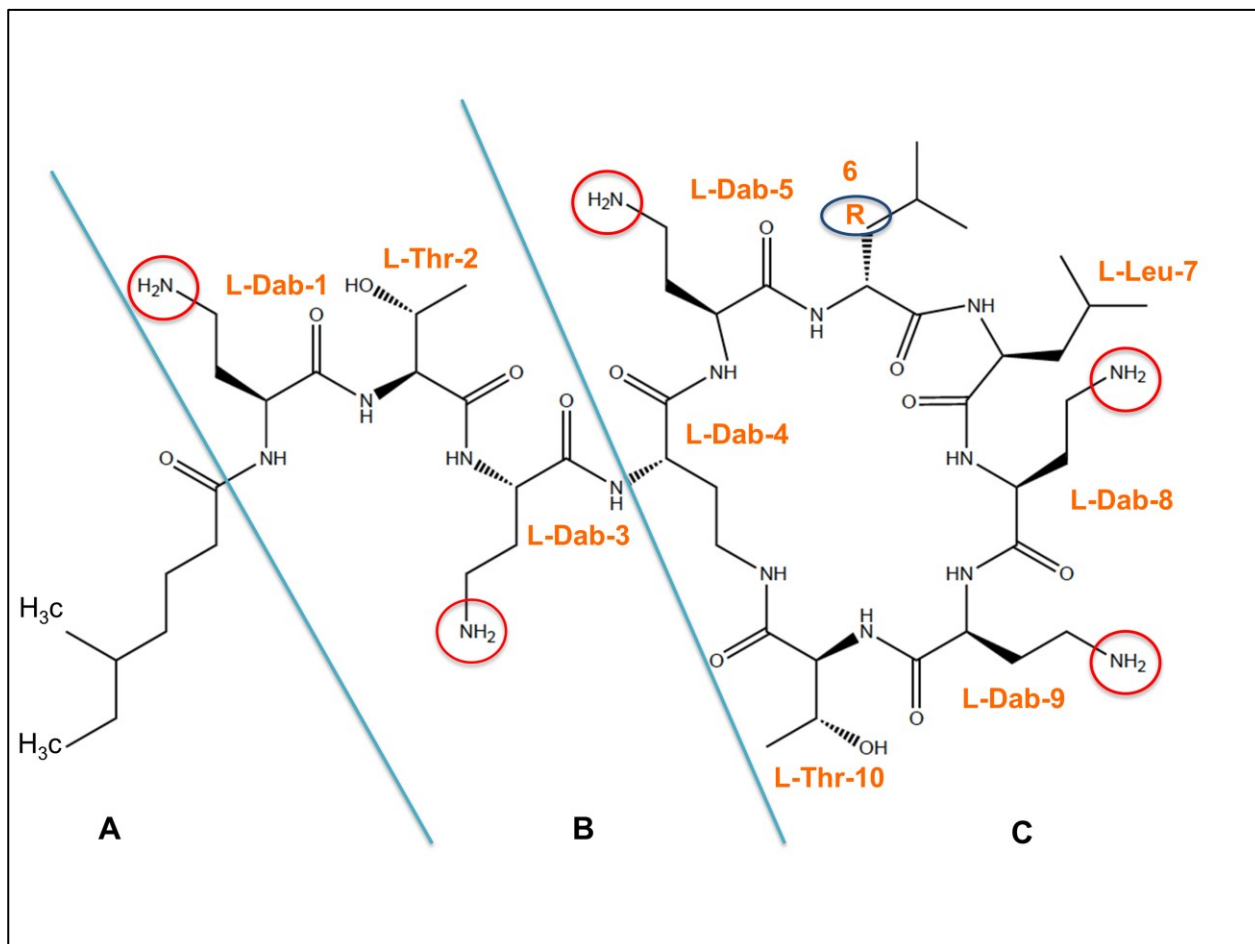
Figure 1: Chemical structure of colistin is composed of three parts: (A): hydrophobic acyl tail, (B): linear tripeptide segment (C): hydrophilic, heptapeptide ring.

Arabic numeral indicates the position of amino acids on the structure and the reactive amino groups are encircled. R6: D- phenylalanine in polymyxin B or D- leucine in polymyxin E (colistin). L-Dab: L-diaminobutyric acid.
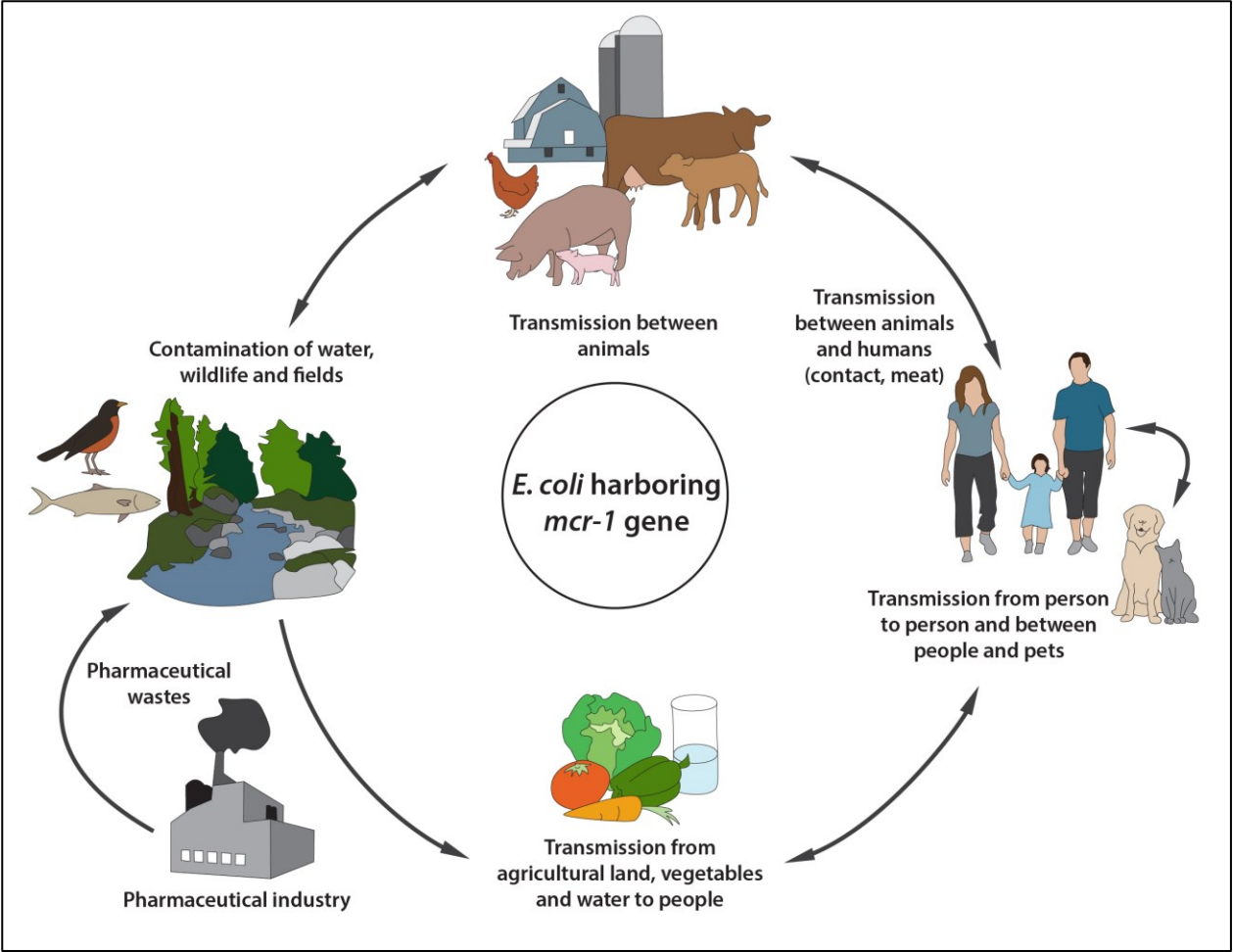
Figure 2: Circulation of colistin resistant *E. coli* harboring *mcr-1* gene between animals-environment-food and humans.
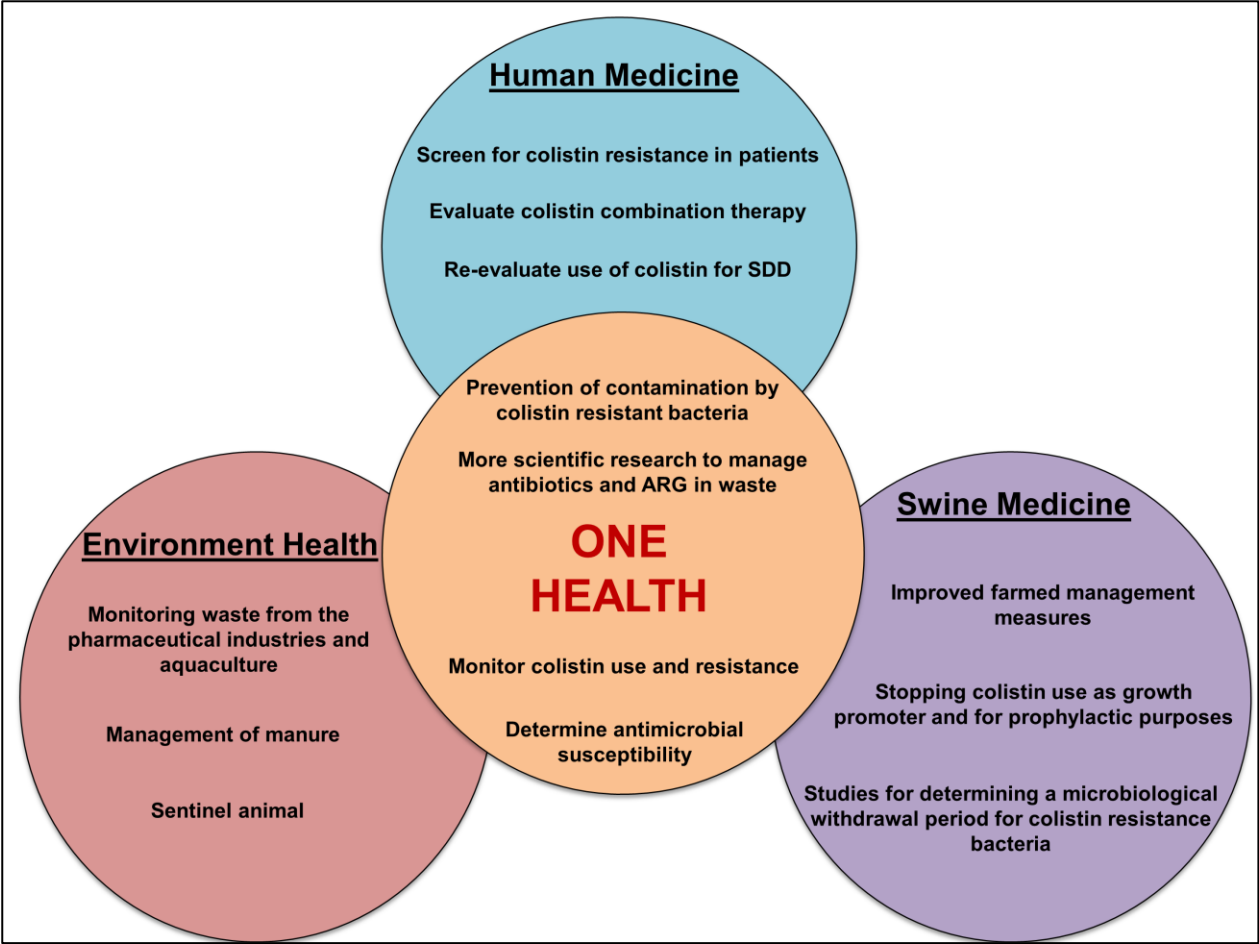
Figure 3: Schematic representation of various actions to be undertaken to ensure reliable management of colistin resistance in a One Health perspective.

SDD: Selective decontamination of the digestive tract. ARG: antibiotic resistance genes.

Resistance to colistin: What is the fate for this antibiotic in pig production?

Cet article de revue a été publié dans le journal; *International Journal of Antimicrobial Agents*.

mai 2016, volume 48, pages 119-126.

Contribution du candidat:

J'ai participé à l'élaboration du plan de cette revue en proportion équivalente de celle des autres coauteurs. J'ai effectué les collectes des toutes les données bibliographiques. J'ai analysé les résultats de la littérature pour établir un lien entre la pharmacocinétique et la modalité d'administration de la colistine chez le porc et la dissémination de la résistance à la colistine chez *E. coli*. J'ai rédigé la revue conformément aux exigences du journal, et j'ai intégré les commentaires faits par les coauteurs ainsi que ceux formulés par les réviseurs de l'article.

1.2   Resistance to colistin: What is the fate for this antibiotic in pig production?

1.2.1   Abstract

Colistin, a cationic polypeptide antibiotic, has reappeared in human medicine as a last-line treatment option for multidrug-resistant Gram-negative bacteria (GNB). Colistin is widely used in veterinary medicine for the treatment of gastrointestinal infections caused by *Enterobacteriaceae*. Colistin-resistant GNB due to chromosomal mutations have already been reported in both human and veterinary medicine, however several recent studies have just identified a plasmid mediated *mcr-1* gene encoding for *E. coli* colistin resistance. The discovery of a non-chromosomal mechanism of colistin resistance in *E. coli* has led to strong reactions in the scientific community and concerns among physicians and veterinarians. Colistin use in food animals and particularly in pig production has been singled out as responsible for colistin resistance emergence. The present review will focus mainly on the possible link between colistin use in pigs and the spread of colistin resistance in *Enterobacteriaceae*.

First, we demonstrate a possible link between *Enterobacteriaceae* resistance emergence and oral colistin pharmacokinetic/pharmacodynamic (PK/PD) and its administration modalities in pigs. We then discuss the potential impact of colistin use in pigs on public health with respect to resistance.

We believe that colistin use in pig production should be re-evaluated and its dosing and usage optimized. Moreover, the search for competitive alternatives to using colistin in swine is of paramount importance to preserve the effectiveness of this antibiotic for the treatment of multidrug-resistant GNB infections in human medicine.

Keywords

Colistin; pigs; *Escherichia coli*; resistance; pharmacokinetic; human

1.2.2 Introduction

Colistin is an antibiotic from the polymyxins family – a group of cationic polypeptide antibiotics consisting of 5 chemically different compounds (Polymyxins A-E). Only polymyxin E (colistin) and polymyxin B are currently available in the market (Bergen et al., 2006). Two forms of colistin (polymyxin E) are used for the treatment of infection caused by Gram-negative bacteria (GNB) in humans: colistin sulfate (CS) for oral and topical use; and colistin methanesulfonate sodium (CMS) for parenteral use (Gurjar, 2015). Among the two forms commercially available, CS is the only approved product in pig production in some countries to control pig intestinal infections caused by *Enterobacteriaceae* (Official Journal of the European Union, 2010; Tang et al., 2013; Wu et al., 2012).

The colistin mechanism of antibacterial action is based essentially on the electrostatic interaction between positively charged amino groups of colistin and the negatively charged phosphate groups of lipid A subunits present on the structure of lipopolysaccharides (LPS) (Biswas et al., 2012; Gallardo-Godoy et al., 2016; Gurjar, 2015). Colistin alters the structure of LPS and leads to the increased permeability of the cell membrane, which results in leakage of the cell contents and bacterial death (Hancock, 1997; Martis et al., 2014).

The lack of new antibacterial chemical entities commercialized over the last several years, and the rapid development of resistance in GNB to current antibiotics, has led to an overuse of colistin in both human and veterinary medicine (Kempf et al., 2013; McClure and Day, 2014). During the last decade, research on colistin experienced a very significant increase (Fig. 4). Despite its high toxicity, colistin has replaced aminoglycosides in humans for the treatment of multidrug-resistant GNB and it is considered a last-line treatment option for carbapenemase-producing *Enterobacteriaceae* (Gurjar, 2015). Given the importance this antibiotic has taken on since 2012, the World Health Organization (WHO) has reclassified colistin as critically important

for human medicine (WHO, 2011).

Concurrent with the excessive use of colistin over the last few years in both human and veterinary medicine worldwide is a reported increase in resistance to colistin of bacteria that were normally susceptible to this antibiotic (Kempf et al., 2013; Olaitan et al., 2014). The most documented mechanism of colistin resistance in *Salmonella* and *E. coli* involves a mutation in the two-component systems PhoP/PhoQ and/or PmrA/PmrB that results in structural modifications of the lipid A subunit, which affects the LPS negative charge and leads to less electrostatic interaction with positive charges of colistin (Needham and Trent, 2013; Olaitan et al., 2014). However, the mechanisms of colistin resistance are multifaceted and do not involve just one molecular origin (Olaitan et al., 2014); despite the fact that this mutation is an important cause of colistin resistance in *E. coli*, it appears that it is not the exclusive resistance mechanism (Liu et al., 2016; Olaitan et al., 2016a; Olaitan et al., 2015b).

The study of Liu and collaborators in *The Lancet Infectious Diseases* was the first to show the involvement of a stable plasmid mediated *mcr-1* gene encoded for phosphoethanolamine transferase conferring resistance to colistin in *E. coli* (Liu et al., 2016). This study contributed to our understanding of other potential *E. coli* resistance mechanisms to colistin and described for the first time a *mcr-1* gene on a mobile genetic element involved in colistin resistance dissemination between animals and humans. Additionally, because of the high rate of colistin resistant *E. coli* carrying the *mcr-1* gene isolated from food animals compared to humans (Liu et al., 2016), livestock production was pinpointed as the greatest cause of colistin resistance amplification and spread. It has been reported in several studies that the *E. coli* colistin resistance rate was higher in swine compared with other animal productions (De Jong et al., 2012; Enne et al., 2008; Harada et al., 2005; Lu et al., 2010; Malhotra-Kumar et al., 2016a). We conducted the following literature review to determine a possible link between colistin use in pig production

and colistin *Enterobacteriaceae* resistance emergence and to discuss its potential impact on public health with respect to resistance.

1.2.3 Colistin sulfate use in pig production and *Enterobacteriaceae* resistance rate

Among the two forms of colistin commercially available, the only approved product in pig production is CS, which is used for the control of pig's intestinal infections caused by *E. coli* and *Salmonella* (Callens et al., 2012b; Guyonnet et al., 2010; Official Journal of the European Union, 2010). Indeed, CS is used therapeutically, prophylactically, and even as a growth promoter in pig industries in some countries (Casal et al., 2007b; Catry et al., 2015; Katsunuma et al., 2007; Kim et al., 2013; Trauffler et al., 2014).

In human medicine, the use of colistin was abandoned in the 1970s mainly because of its ability to cause human nephrotoxicity (Falagas et al., 2005; Li et al., 2006; Spapen et al., 2011). However, in the late 90s, GNB resistance development against aminoglycosides led to the resurgence of the clinical use of colistin (Biswas et al., 2012; Falagas et al., 2005). Nowhere in the literature is there an indication that colistin usage was also interrupted in pig production when it was withdrawn in human medicine between 1970 and 2000. Furthermore, colistin is used orally in pigs and it is characterized by a low oral bioavailability (Guyonnet et al., 2010; Rhouma et al., 2015), therefore the risk of side effects associated with CS systemic exposure in pigs is negligible.

Colistin is used in massive quantities in pig production worldwide (Catry et al., 2015; Kim et al., 2013; Liu et al., 2016). In France, 90% of the farms in the pig industry reported using colistin during the post-weaning period, 48% used it to treat sows during gestation and lactation, and 19% used it at the finishing level (Kempf et al., 2013). In Belgium, more than 30% of prophylactic and metaphylactic oral treatment in 50 randomly chosen fattening pig farms was based on colistin use

(Callens et al., 2012b). In Spain, Casal and collaborators reported that colistin was the most frequently used antibiotic for metaphylactic intestinal disease control in 107 pigs farms and that there was a high rate of prophylactic use of this antibiotic without defined diagnosis (Casal et al., 2007b). In Austria, 49 pig farrow-to-finish farms chosen for antibiotic monitoring showed that 34.4% of farms used colistin for metaphylactic/prophylactic purposes (Trauffler et al., 2014). In 60 Swedish farrow-to-finish pig herds, Sjolund and collaborators reported that the use of colistin accounted for 18% of all antibiotic treatments in weaned piglets (Sjolund et al., 2015). In Germany, Van Rennings and collaborators reported that in 495 pig farms, colistin was among the most used antibiotics in piglets (Van Rennings et al., 2015) and of the 20,373.6 kg of antimicrobial agents used on these farms in 2011, polypeptides (colistin) represented 4.2% of all antibiotics used (Van Rennings et al., 2015). In the Red River Delta region of Vietnam, Kim and collaborators reported that 210 pig husbandry entities representing 3 different systems (farm household, semi-industrial and industrial) have been using colistin for several purposes (Kim et al., 2013). Indeed, in 78 entities colistin was used for growth promotion, in 12 for disease prevention, and in 56 for therapy (Kim et al., 2013). In the Netherlands, Bos and collaborators reported that colistin has been little used in piglets and sows compared to its use in starter calves (Bos et al., 2013). In an Australian national survey of antimicrobial use in the pig industry conducted in 2006, Jordan and collaborators did not report any colistin use in pig production during that period (Jordan et al., 2009).

China remains the largest user worldwide of colistin in agriculture with 11 942 tonnes per year by the end of 2015. Given the expansion and intensification of animal husbandry and a 4.75% average annual increase of colistin use in this country, the annual quantity used will be 16 500 tonnes by 2021 (Liu et al., 2016). Furthermore, CS in pig production in some countries outside North America and the European Union was used as a feed additive for growth promotion

(Katsunuma et al., 2007; Kim et al., 2013; Makita et al., 2016). In 2015, the European Union and North America imported 480 tonnes and 700 tonnes of colistin from China respectively (Liu et al., 2016). However, CS is an unapproved antibiotic in veterinary medicine in some countries including Canada, and it is used under the veterinarian liability (dose, withdrawal period) for the treatment of *Enterobacteriaceae* infections in pigs (Rhouma et al., 2015). It should be stressed here again that it is often difficult to determine the exact amount of colistin used in pig production in the world, and it is even sometimes difficult to compare the results of two studies conducted in the same country in terms of CS quantities used in pigs (Casal et al., 2007b; Moreno, 2014).

Additionally, several studies have reported isolation of *Enterobacteriaceae* resistance to colistin from pigs with different rates across countries (Table VII). Augmenting this complexity of comparing data between countries are the variances between studies such as number of samples, methods used, animal health status, and usage or not of antibiotics at the farm level. The existence has been demonstrated of a relationship between the extent of CS resistance among *Enterobacteriaceae* in pigs and the CS amount used in pig production in some countries (Catry et al., 2015; Mateu and Martin, 2000). In their 2011 study based in Croatia, Habrun and collaborators reported a low rate of colistin resistance *E. coli* in weaned pigs and they linked this finding with the recent use of colistin in pig production in this country (Habrun et al., 2011). One study has reported that some *E. coli* isolates of porcine origin were confirmed resistant to CS without having a mutation in *pmrA* and/or *pmrB* genes (Olaitan et al., 2015b). Recently, an in-depth investigation of these isolates showed that 90% of them were harbouring the *mcr-1* gene (Olaitan et al., 2016a). Research into this antibiotic has increased significantly in swine medicine in response to the increased use of colistin in pig production (Fig. 5).

1.2.4 Potential link between oral colistin sulfate pharmacokinetic/pharmacodynamic (PK/PD) and *Enterobacteriaceae* resistance emergence in pigs

Unlike for human medicine, CS is the only approved form of colistin in pig production (Guyonnet et al., 2010; Official Journal of the European Union, 2010). Colistin sulfate is mostly used in monotherapy by oral administration for the treatment of *Enterobacteriaceae* infections in pigs (Guyonnet et al., 2010; Official Journal of the European Union, 2010; Rhouma et al., 2015). Very few PK studies have been done after an oral CS administration in pigs (Guyonnet et al., 2010; Rhouma et al., 2015). In one study, despite the use of a sensitive analytical method, CS systemic concentrations were below the lower limit of quantitation (LLOQ) (250 ng/mL) of a high pressure liquid chromatography assay (HPLC-UV) (Guyonnet et al., 2010). By using this analytical method, the observed maximum gastrointestinal tract concentration (C_{max}) of oral CS in pigs were 43.57 mg/kg and 91.75 mg/kg after CS oral administration of 50,000 and 100,000 IU/kg respectively (Guyonnet et al., 2010). These C_{max} concentrations were obtained after only 1 h (T_{max}) of CS oral administration regardless of the dose used (Guyonnet et al., 2010). Additional work was performed using a significantly more sensitive high-performance liquid chromatography coupled with tandem mass spectrometry (HPLC-MS/MS), which achieved an LLOQ of 20 ng/mL; the peaks observed were still below the limit of quantification (Rhouma et al., 2015). A concurrent oral challenge of pigs with an ETEC O149: F4 strain did not increase CS intestinal absorption in a subclinical induction model of post-weaning diarrhea (PWD) (Rhouma et al., 2015). Nevertheless, it should be stressed here again that PK studies in pigs after oral CS administration (Guyonnet et al., 2010; Rhouma et al., 2015) were carried out under controlled breeding conditions using a limited number of animals and CS was administered by oral gavage; the outcome of oral CS PK can differ depending on farm conditions such as antibiotic

administration modality (i.e. water, food), interaction with feed, and livestock management as described for other antibiotics in pigs (Bibbal et al., 2007; Soraci et al., 2014).

Considering that CS is not absorbed in the digestive tract, feces are the main route of CS excretion and the pig's digestive microflora is therefore exposed to large concentrations of CS following an oral administration (Guyonnet et al., 2010; Rhouma et al., 2015). In this way, pig's intestinal microbiota could be associated with amplification and persistence of CS resistance genes (*mcr-1*) and bacteria. Indeed, Makita and collaborators reported a link between pigs' exposure to colistin within the previous 6 months of sampling and the increase of *E. coli* resistance to this antibiotic (Makita et al., 2016). However, to the best of our knowledge, no study has followed the evolution of colistin *Enterobacteriaceae* resistance during an oral CS treatment in pigs. Thus, the role of CS to exert a selection pressure in pig's gut microflora should be confirmed in future studies. Additionally, it has been reported that CS undergoes digestive degradation in pigs, leading to the formation of CS metabolites with significant *in vitro* antimicrobial activity (Rhouma et al., 2015). However, no information is available in the literature about the effect of these degradation products on *E. coli* resistance emergence.

Moreover, to the best of our knowledge, no study in swine medicine has investigated the possible variability in CS intestinal bioavailability between commercially available oral formulations. In fact, the PK of 4 commercial formulations of CMS were investigated after intravenous administration in rats and results showed inconsistent colistin bioavailability *in vivo* between these formulations (He et al., 2013). In addition, Li and collaborators reported a varying and confusing product content labelling of CMS used in many countries (Li et al., 2006). This variability between formulations can involve the composition of colistin products. Indeed, colistin is composed of at least 30 components, with the main components being colistin A

(polymyxin E1) and colistin B (polymyxin E2), which differ only in the fatty acid side chain (Dotsikas et al., 2011).

No formal certificates of analysis that include molecular characterisation are available in veterinary medicine to adequately establish the purity of CS commercial formulations. In addition, as no pure colistin A and B reference standards are available, it is difficult to assess the purity of CS commercial formulations used in swine medicine (Zhao et al., 2014).

Very little work has been conducted on oral CS PD in pigs, and only one study has investigated this topic (Guyonnet et al., 2010). It was shown in this study that CS acts on pigs' *E. coli* strains by a concentration-dependent mechanism; this suggests a correlation between bactericidal activity and CS intestinal exposure (area under the curve (AUC)) (Guyonnet et al., 2010). Lin and collaborators reported that CS bioavailability after an IM administration in pigs was inversely proportional with the administered CS dose, with a systemic bioavailability of 95.94% and 88.45% for 2.5 mg/kg and 5 mg/kg body weight respectively (Lin et al., 2005). These findings contradict the reported concentration-dependent mechanism of CS. Further research is essential to better illustrate the link between oral CS PK and PD in order to optimize effectiveness of this antibiotic against susceptible pathogens and to minimise the emergence of *Enterobacteriaceae* resistance in pigs.

In another study, it was reported that the AUC/MIC is the PK/PD index that best predicts colistin antibacterial activity against *E. coli* isolates in pigs (Guyonnet et al., 2010). However, the *E. coli* strains used in this study had a colistin MIC value of 0.5 μg/mL (three strains) and 1 μg/mL (one strain), which indicates that these strains were sensitive to colistin (MIC ≤ 2 μg/mL) (Bergen et al., 2012; Li et al., 2005). It would be difficult to use these results to make predictions of *in vivo* oral CS efficacy in clinical cases and difficult to avoid the risk of CS resistance emerging in pigs, given that some *E. coli* isolates from sick pigs showed a colistin MIC value of 32 μg/mL (Boyen

et al., 2010; Morales et al., 2012). Further, with a feed concentration of 66 mg/kg of colistin, this antibiotic will reach porcine jejunum with CS concentrations that allow eradication of *E. coli* strains with a MIC of 8 μg/mL, but not strains with an MIC of 16 μg/mL or 32 μg/mL (Burch, 2007). Otherwise, even though CS degradation products have a high antimicrobial activity *in vitro* against some *E. coli* strains compared to pure colistin (Rhouma et al., 2015), these products have not been characterized and identified, and no study has been conducted to assess their effect on CS resistant *E. coli* in pigs.

Antimicrobial susceptibility testing for colistin in pigs was performed using disc diffusion, an E-test, agar dilution, and broth dilution (Table VII), although the accuracy of the disc diffusion method compared to other methods has been questioned (Boyen et al., 2010). Boyen and collaborators described that colistin clinical breakpoint values used to predict clinical efficiency of this antibiotic in oral pig formulations were determined based on human CLSI breakpoints from colistin parenteral formulations (Boyen et al., 2010). And despite the massive use of colistin in veterinary medicine (Catry et al., 2015), no specific clinical breakpoints for this antibiotic are available for pigs or for other farm animals (Boyen et al., 2010). It must be remembered here that with the actual level of available data on clinical CS PK/PD in pigs, and because of the absence of specific CS clinical breakpoints, it would be difficult to optimize colistin dosing and counter *Enterobacteriaceae* colistin resistance spread in pigs worldwide. Furthermore, the therapeutic regimen of CS in pig production should be re-evaluated to preserve the effectiveness of this antibiotic for the treatment of multidrug-resistant GNB infections both in human and veterinary medicine.

1.2.5 Involvement of colistin sulfate administration modality in pigs in colistin *Enterobacteriaceae* resistance emergence

In the pig industry, CS is administered most often collectively in water or feed, and this method of administration usually leads to a variable amount of colistin in pigs' intestines as a consequence of the hierarchical behaviour of farm animals (Soraci et al., 2014). Even if many countries are claiming to not use colistin as a growth promoter in pigs, it is very difficult to ensure that CS doses that reach animals' intestines are bactericidal and not subtherapeutic.

Practitioners in swine medicine use very different dosage regimens for CS treatments (Guyonnet et al., 2010). Some practitioners use international units whereas others use milligrams per kg of body weight to select CS doses in pigs (Guyonnet et al., 2010; Trauffler et al., 2014; Ungemach et al., 2006). Colistin sulfate is widely used by the oral route in pigs because of the practicality of this pathway for mass antimicrobial administration and its low toxicity compared with the intramuscular (IM) route (Lin et al., 2005). Indeed, the injection of 10 mg/kg/day of CS solution for 5 days by IM route in piglets was associated with local irritation at the injection site and a granular degeneration in hepatocytes and renal tubular epithelial (Lin et al., 2005).

The actual oral dose of CS used in drinking water for the treatment of swine intestinal diseases is 50.000 IU/kg body weight every 12 h for 3 or 5 days, though recommended doses found in monographs differ (Catry et al., 2015; Chauvin et al., 2002; Postma et al., 2015a). In fact, several monitoring studies of CS in pig farms showed that the CS classic regimen is sometimes overdosed (Chauvin et al., 2002) or underdosed (Callens et al., 2012b; Timmerman et al., 2006; Trauffler et al., 2014), and often the duration of CS treatment is far longer than the 3 or 5 days recommended by monographs (Chauvin et al., 2002; Van Rennings et al., 2015). In some countries where CS is not approved for pig production such as Canada, CS therapeutic regimen is defined by transposition of application data (dosage, withdrawal time) from countries where CS

is approved (Rhouma et al., 2015).

CS doses incorporated into pig feed for the treatment of *Enterobacteriaceae* infections were variable between studies, with a range of 66 to 800 per kg of feed (Burch, 2007; Torrallardona et al., 2003; Wu et al., 2012). In other studies where colistin was used to increase pig feed efficiency, CS doses incorporated in the diet varied from 20 to 60 mg per kg of feed (Wan et al., 2016; Wang et al., 2016b).

It must be remembered here that in the absence of a standardised colistin regimen in pigs, it is difficult to ensure a judicious use of this antibiotic for pig farms and to counter CS resistance emergence.

Furthermore, pig farmers and employees are largely unaware of their involvement in the problem of antibiotic resistance and this could contribute to colistin resistance dissemination (Visschers et al., 2015). In fact, some studies conducted on pigs showed that colistin has been used for indications other than those for which it is authorised, e.g., respiratory disease (Callens et al., 2012b; Catry et al., 2015; Van Rennings et al., 2015). In addition, deviations from leaflet dosage recommendations for CS were frequently encountered in pig farms in many countries (Callens et al., 2012b; Chauvin et al., 2002; Timmerman et al., 2006; Trauffler et al., 2014). It was also reported that selection for bacterial resistance would be greatly advantaged if adequate applications of antibiotics were not respected (Ungemach et al., 2006).

In addition, the traffic and sale of antibiotics at markets and pharmacies is largely unregulated in several countries, thus farmers can obtain their antibiotics without prescription and even without involvement of a person with pharmaceutical training (Kim et al., 2013; Laxminarayan et al., 2013; Maron et al., 2013). Moreover, in countries where the use of antibiotics in animal production requires a prescription, veterinarians are influenced by their peers, pharmaceutical promotion, and perceived demands of farmers. Therefore, it is often difficult to comply with the

antibiotic's treatment guidelines (Radyowijati and Haak, 2003). In some cases, CS was used for treating intestinal disease in pig farms without a defined diagnosis of the involved pathogens (Casal et al., 2007b). Additionally, CS is often used in pig farms for metaphylactic/prophylactic purposes (Trauffler et al., 2014). However, the 2013 guidelines of the European Medicines Agency (EMA) recommended removing all indications for preventive or prophylactic use of colistin and using this antibiotic only for the treatment of infected animals and those in contact with them (European Medicines Agency, 2016a).

We consider that the major use of colistin in pig production worldwide is metaphylactically through the oral route (Casal et al., 2007b; Trauffler et al., 2014; Van Rennings et al., 2015), which involves treatment of clinically healthy animals belonging to the same pen as animals with clinical symptoms (Aarestrup, 2005; Ferran et al., 2011). It has been demonstrated that treatment of an early infection with low pathogenic inoculum in an animal model (metaphylactically) with certain antibiotics (marbofloxacin, cefquinome) was able to fully cure an infection without any measurable amplification of intestinal *Enterobacteriaceae* resistance (Ferran et al., 2009; Ferran et al., 2011; Vasseur et al., 2014). However, no study has shown this effect for CS that could justify the efficiency of CS metaphylactic use in pig production. Therefore we consider that this practice in swine could contribute to a waste of colistin, to environmental contamination, and to the emergence of CS bacterial resistance. It was shown in human medicine that the use of oral colistin for selective decontamination of the digestive tract (SDD) to eradicate GNB was associated with an increase in colistin-resistant GNB strains (Halaby et al., 2013). In the same study, an association between prolonged use of colistin as part of SDD and the emergence of colistin resistance among ESBL producing *K. pneumoniae* isolates was found (Halaby et al., 2013). It should be stressed here again that colistin is used most often by oral route in pigs, with a treatment period that can reach 15 days (Chauvin et al., 2002). Furthermore, it was demonstrated

that the risk of antimicrobial resistance in *E. coli* was increased in swine with the oral administration of antimicrobial agents (Burow et al., 2014) and, by extension, the risk of transfer of this resistance to humans (Barton, 2014).

Several studies in pigs have reported a fecal presence of colistin *Enterobacteriaceae* resistant (Table VII). However, we don't know whether or not sampled animals in these studies were treated with colistin. A significant increase of colistin *E. coli* resistance rate was reported in the only study to note that a previous treatment with colistin had been performed in pigs, with 13.1% and 66.7% in untreated and treated pigs respectively (Makita et al., 2016).

Despite its crucial importance in human medicine worldwide, colistin is used in some countries as a growth promoter in pigs (Gavioli et al., 2013; Katsunuma et al., 2007; Kim et al., 2013; Makita et al., 2016; Yen et al., 2015). Indeed, antibiotics used for growth promotion can generally be purchased without veterinary involvement (Laxminarayan et al., 2013; Maron et al., 2013). The effect of low doses of antimicrobials for growth promotion on antimicrobial resistance apparition has been documented for several antimicrobials (Andersson and Hughes, 2014). The economic benefits of antimicrobial growth promotion in modern farms has been questioned (Graham et al., 2007), and the ban on antimicrobial growth promoters in the pig industries of some countries has not been associated with a decrease in the expansion and development of pig productions (Aarestrup et al., 2010) or with an increase in antimicrobial consumption per kilogram of pig produced (Aarestrup et al., 2010; Arnold et al., 2004).

1.2.6 The use of colistin in pigs and potential impact on public health

As mentioned in the previous sections, oral colistin in pigs is characterized by low gastrointestinal absorption and bioavailability. Consequently, resistant CS bacteria and genes along with CS and its degradation products could be found in manure from treated pigs, which

can be spread in the environment. Moreover, the low efficiency of composting to reduce concentrations of antibiotics and of genes coding for resistance in the manure (Le Devendec et al., 2015; Wei et al., 2011), and the lack of regulation worldwide to control the release of livestock wastewater containing antibiotics, make it difficult to reduce the spread of colistin resistance to humans via the environment (Van den Meersche et al., 2016; Wei et al., 2011). On the other hand, in a recent study, Van den Meersche and collaborators found a positive correlation between colistin amounts used in pig farms and the quantity of this antibiotic found in manure derived from treated pigs (Van den Meersche et al., 2016). Hölzel and collaborators reported that 1.6% of *E. coli* isolated from liquid pig manure were resistant to colistin (Hölzel et al., 2010) and Costa and collaborators found that 10% of *E. coli* isolated from the environment of pig farms (feed, facility waste) were resistant to colistin (Costa et al., 2010). Therefore the risk of distributing colistin resistant bacteria to agricultural land by fertilization with pig manure seems to be significant. However, to the best of our knowledge, no study has investigated if the colistin resistant *E. coli* strains isolated from pigs' environments carried the *mcr-1* gene or not.

The plasmid mediated *mcr-1* gene was discovered for the first time in China (Liu et al., 2016). This gene was found in colistin resistant commensal *E. coli* strains from food animals during a surveillance project on antimicrobial resistance between April 2011 and November 2014. In this survey, 14.9% of *E. coli* isolates from raw meat, 20.6% of *E. coli* isolated from pigs, and 1.4% of *E. coli* isolated from in-patients carried the *mcr-1* gene (Liu et al., 2016). With 56.7 million tonnes of pork meat produced in 2014 (Liu et al., 2016), China is the world's largest pig producer. This also means that they have to contend with an estimated 618 billion kilograms of manure each year (Larson, 2015) and 29.000 to 87.000 tons of antibiotic residues annually in livestock waste (Hao et al., 2015). Thus, the role of manure is not to be underestimated in the dissemination of colistin resistance. Several publications have reported the isolation of colistin-

resistant *E. coli* from healthy individuals without prior colistin usage (Olaitan et al., 2016b).
Another scientific study described colistin resistance in *E. coli* from a pig and a boy in Laos, for
which no known chromosomally encoded colistin resistance mechanisms were identified (Olaitan
et al., 2015b). In fact, the pig belonged to the boy's family and the boy (with no history of
antibiotic therapy) was responsible for feeding the pig (Olaitan et al., 2015b). This observation
indicates for the first time a possible horizontal transmission of colistin resistance from pig to
human.

Ghimire and collaborators reported a very high rate of colistin resistance in *Campylobacter* spp,
isolated from dressed porcine carcasses in Nepal (Ghimire et al., 2014). In Portugal, Figueiredo
and collaborators reported that 7.2% of *Salmonella* spp. isolated from swine processed food were
resistant to colistin (Figueiredo et al., 2015). It should be stressed here that *mcr-1* gene was found
in *E. coli* isolated from raw pig meat and a high rate of colistin resistance among the most
important foodborne pathogens of porcine origin (*Salmonella* and *Campylobacter*) was reported.
These findings indicate a major public health issue with respect to resistance. Additionally,
Olaitan and collaborators reported for the first time the isolation of colistin-resistant *Salmonella*
Newport strains in humans, and they did not exclude the possible transmission of these strains to
humans from farm animals (Olaitan et al., 2015a).

In animal production, the *mcr-1* gene has already been detected in colistin resistant *E. coli* and
Salmonella isolates from several countries (Table VIII). A recent survey in China carried out in
chicken colistin resistant *E. coli* strains isolated between 1970 and 2014 showed that *mcr-1* gene
was detected for the first time in three *E. coli* strains isolated in the 1980s (Shen et al., 2016). The
mcr-1 gene was also present in colistin resistant *E. coli* strains isolated from swine in Belgium
during 2011-2012 (Malhotra-Kumar et al., 2016a), in colistin resistant *E. coli* strains isolated
from swine in Germany in 2009 (Falgenhauer et al., 2016), and in French swine farms in 2011

(Perrin-Guyomard et al., 2016). These studies confirmed that the *mcr-1* gene was already present in gut flora of food animals before its discovery in 2015.

With the detection of the *mcr-1* gene in several countries in the world, we can confirm that plasmid-mediated colistin resistance is not solely a Chinese phenomenon, and with air travel and trade exchanges between countries, we believe that no country is immune from the spread of colistin resistance. As a consequence of this discovery in food animals in several countries and the risk of widespread dissemination of the *mcr-1* gene as reported previously for other antibiotic resistance genes such as bla_{KPC} and bla_{NDM-1} (Rolain and Olaitan, 2016), the EMA has been asked by the European Commission to update its advice on the use of colistin in animals (European Medicines Agency, 2016a). Furthermore, the Responsible Use of Medicines in Agriculture Alliance (RUMA) of the British livestock industry announced that its members had agreed to voluntarily restrict the use of colistin in food animals pending the outcome of a European Commission risk assessment (VMD, 2016).

1.2.7 Conclusion

The rapid loss of antibiotic effectiveness as a consequence of bacterial resistance is a challenge for both public and animal health and requires a concerted global action. The recent discovery and dissemination of the *mcr-1 gene* is a serious threat to colistin as the last resort antibiotic treatment option for multidrug-resistant GNB in human medicine. The lack of colistin specific clinical PK/PD data in swine makes it difficult to ensure judicious use of this antibiotic on pig farms. Hence, it is of paramount importance to conduct a reassessment of colistin use in pig productions and to establish an international monitoring system of its bacterial resistance. The search for alternative competitive therapies to colistin that act on the digestive flora of pigs is a necessity in order to preserve the effectiveness of this crucial antibiotic in human medicine.

Funding

1.2.8 Tables

Table VII: Percentage of colistin resistance in *Enterobacteriaceae* isolated from pigs according to their health status.

Countries	Bacterial species	Health status of pigs	Method of analysis	Percentage of resistance	References
Belgium	*E. coli*	Symptoms of *E. coli* infection	Agar dilution	9.6%	(Boyen et al., 2010)
Belgium	*E. coli*	Diarrhea	Broth dilution	13.2%	(Malhotra-Kumar et al., 2016a)
Brazil	*E. coli*	Postweaning diarrhea or oedema disease	Agar dilution	6.3%	(Morales et al., 2012)
Brazil	*Salmonella enterica*	Enterocolitis	Agar dilution	21%	(Morales et al., 2012)
China	*E. coli*	NA	Broth microdilution	33.3%	(Lu et al., 2010)
Croatia	*E. coli*	Clinical signs of diarrhea	E-test	3%	(Habrun et al., 2011)
Europe (DK, Fr, GE, NL, ES)	*E. coli*	Clinical healthy (slaughterhouse)	Agar dilution	0.2%	(De Jong et al., 2012)
Europe (Fr, NL, ES)	*Salmonella spp.*	Clinical healthy (slaughterhouse)	Agar dilution	6.3%	(De Jong et al., 2012)
France	*E. coli*	Clinical healthy (Farm)	Disc diffusion	0.5%	(Belloc et al., 2008)
France	*E. coli*	Clinical healthy (slaughterhouse)	Broth microdilution	1%	(Perrin-Guyomard et al., 2016)
Greece	*Salmonella enterica*	Clinical healthy (slaughterhouse)	Disc diffusion	21.4%	(Evangelopoulou et al., 2014)
Italy	*Salmonella Typhimurium*	Clinical healthy (slaughterhouse)	Disc diffusion	8%	(Lomonaco et al., 2009)
Japan	*E. coli*	Symptoms of *E. coli* infection	Agar dilution	35.6%	(Harada et al., 2005)
Japan	*E. coli*	Clinical healthy (Farm)	Agar dilution	0.8%	(Kijima-Tanaka et al., 2003)
Lithuania	*E. coli*	Clinical symptoms	Disk diffusion	2%	(Ruzauskas et al., 2006)
Lithuania	*Salmonella choleraesuis*	Clinical symptoms	Disk diffusion	17%	(Ruzauskas et al., 2006)
Portugal	*Salmonella spp.*	NA	Broth microdilution	6.9%	(Figueiredo et al., 2015)
Spain	*E. coli*	Clinical healthy (slaughterhouse)	Broth microdilution	0.4%	(Quesada et al., 2015)
Spain	*Salmonella enterica*	Clinical healthy (slaughterhouse)	Broth microdilution	1.5%	(Quesada et al., 2015)

Spain	*E. coli*	Diarrhea, oedema disease	Disc diffusion	26.7%	(Mateu and Martin, 2000)
UK	*E. coli*	Clinical healthy (slaughterhouse)	Disc diffusion E-test	34.1%	(Enne et al., 2008)

NA, information not available; DK, Denmark; Fr, France; GE, Germany; NL, The Netherlands; ES, Spain.

Table VIII: Countries where the *mcr-1* gene was isolated from *Enterobacteriaceae* in pigs and other farm animals (February 2016).

Countries	Animal production	Bacterial species	References
China	Pigs, Chicken	*E. coli*	**(Liu et al., 2016; Shen et al., 2016)**
Laos	Pigs	*E. coli*	**(Olaitan et al., 2015b)**
Algeria	Chicken	*E. coli*	**(Olaitan et al., 2016a)**
Vietnam	Pigs	*E. coli*	**(Malhotra-Kumar et al., 2016b)**
Denmark	Chicken	*E. coli*	**(Hasman et al., 2015)**
France	Veal calves	*E. coli*	**(Haenni et al., 2016)**
Germany	Pigs	*E. coli*	**(Falgenhauer et al., 2016)**
Malaysia	Pigs	*E. coli*	**(Petrillo et al., 2016)**
Japan	Cattle, Pigs	*E. coli*, *Salmonella*	**(Suzuki et al., 2016)**
United Kingdom	Pigs	*E. coli*	**(VMD, 2016)**
Belgium	Pigs, Calves	*E. coli*	**(Malhotra-Kumar et al., 2016a)**
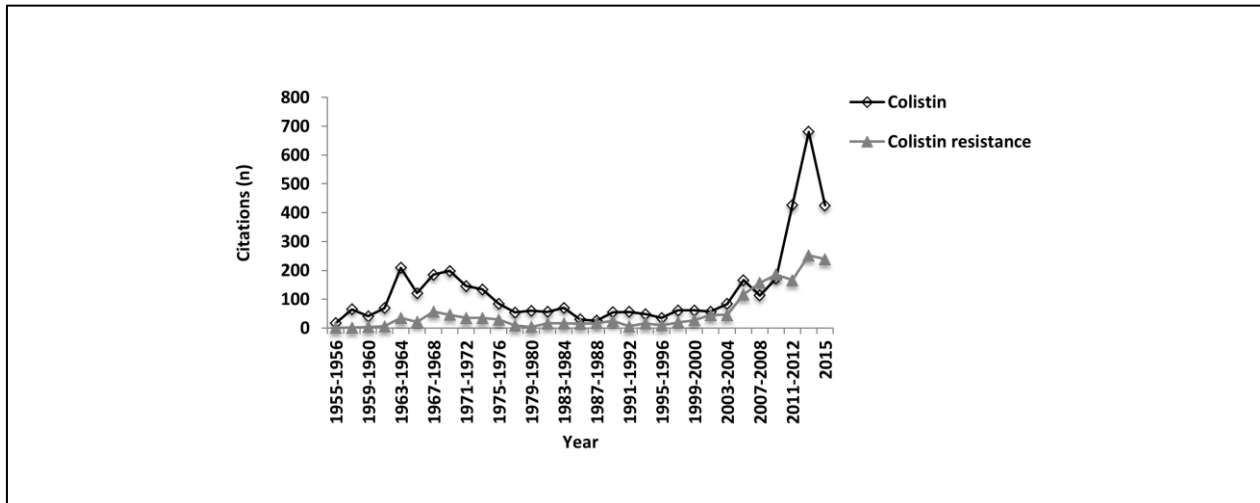
Figure 4: The number of citations found in the PubMed database from 1955 to 2015 using either the search phrase 'colistin' or 'colistin resistance.'
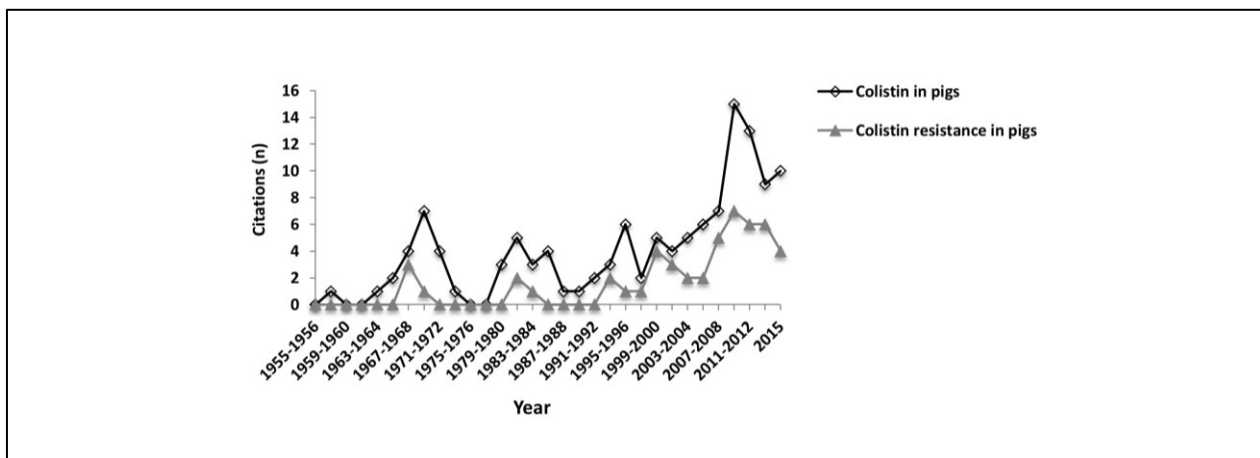
Figure 5: The number of citations found in the PubMed database from 1955 to 2015 using either the search phrase 'colistin in pigs' or 'colistin resistance in pigs.'

Factors of post weaning diarrhea and alternative strategies to colistin for the control of this disease in pigs

Cet article de revue a été soumis au journal; *Acta Veterinaria Scandinavica*.

Mohamed Rhouma, John Morris Fairbrother, Francis Beaudry, Ann Letellier

Contribution du candidat:

J'ai participé à l'élaboration du plan de cette revue en proportion équivalente de celle des autres coauteurs. J'ai effectué les collectes des toutes les données bibliographiques. J'ai analysé les résultats de la littérature pour mettre en place une approche pratique pour le contrôle de la diarrhée colibacillaire post sevrage chez le porcelet. J'ai rédigé la revue conformément aux exigences du journal, et j'ai intégré les commentaires faits par les coauteurs de l'article.

1.3 Factors of post weaning diarrhea and alternative strategies to colistin for the control of this disease in pigs

1.3.1 Abstract

Post-weaning diarrhea (PWD) is one of the most serious threats for the swine industry worldwide. It is commonly associated with the proliferation of enterotoxigenic *Escherichia coli* (ETEC) in the pig intestine. Colistin, a cationic antibiotic, is widely used in swine for the oral treatment of intestinal infections caused by *E. coli*, and particularly of PWD. However, despite the effectiveness of this antibiotic in the treatment of this disease, several studies have reported high rates of colistin resistant *E. coli* in swine. Furthermore, this antibiotic is considered of very high importance in humans, being used for the treatment of infections due to multidrug-resistant (MDR) Gram-negative bacteria (GNB). Moreover, the recent discovery of the *mcr-1* gene encoding for colistin resistance in *Enterobacteriaceae* on a conjugative stable plasmid has raised great concern about the possible loss of colistin effectiveness for the treatment of MDR-GNB in humans. Consequently, it has been proposed that the use of colistin in animal production should be considered as a last resort treatment only. Thus, to overcome the economic losses, which would result from the restriction of use of colistin, especially for prophylactic purposes in PWD control, we believe that an understanding of the factors contributing to the development of this disease and the putting in place of practical alternative strategies for the control of PWD in swine is crucial. Such alternatives should improve animal gut health and reduce economic losses in pigs without promoting bacterial resistance. The present review begins with an overview of risks factors of PWD and an update of colistin use in PWD control worldwide in terms of quantities and microbiological outcomes. Subsequently, alternative strategies to the use of colistin for the

control of this disease were described and discussed. Finally, a practical approach for the control of PWD in its various phases was proposed.

Keywords: Post-weaning diarrhea, pigs, *E. coli*, colistin, resistance, alternatives.

1.3.2   Introduction

Post-weaning diarrhea (PWD) due to *Escherichia coli* is an economically important disease in pig production worldwide, affecting pigs during the first two weeks after weaning and characterized by sudden death or diarrhea, dehydration, and growth retardation in surviving piglets (Amezcua et al., 2002b; Fairbrother et al., 2005). Furthermore, many stress factors associated with the weaning period such, as removal from the sow, dietary changes, adapting to a new environment, mixing of pigs from different farms and histological changes in the small intestine, may negatively affect the response of immune system and lead to an intestinal gut dysfunction in pigs (Lallès et al., 2007; Lallès et al., 2004; McCracken et al., 1999). Post-weaning diarrhea is usually associated with proliferation of enterotoxigenic *E. coli* (ETEC) (Fairbrother et al., 2005; Luppi et al., 2016). This pathotype is characterized by the production of enterotoxins and adhesins, both essential for disease development (Nagy and Fekete, 2005), the predominant adhesins in PWD being F4 and F18 (Delisle et al., 2012; Luppi et al., 2016). Small intestinal adhesion and subsequent colonization by ETEC in pigs is mediated by F4 or F18 specific receptors, the existence and function of these receptors being crucial to determine the susceptibility of pigs to ETEC infections (Nagy and Fekete, 2005). The predominant serogroup of ETEC associated with PWD in pig worldwide is O149, commonly in the combination O149: LT: STa: STb: EAST1: F4ac (Fairbrother et al., 2005). Colistin, a polymyxin antibiotic produced by *Paenibacillus polymyxa* var *colistinus* (Tambadou et al., 2015)*,* is widely used for the control of PWD in pigs (Kempf et al., 2013). However, this antibiotic is now considered as the last therapeutic option for

the treatment of infections caused by multidrug-resistant Gram-negative bacteria (MDR-GNB) such as *Pseudomonas aeruginosa*, *Acinetobacter baumannii*, *Klebsiella pneumoniae* and *Enterobacter* species in humans (Michalopoulos et al., 2011; Walkty et al., 2009).

On the other hand, in the last several years, studies have reported the isolation of colistin-resistant *E. coli* from pigs (Boyen et al., 2010; Morales et al., 2012), the proportion reaching 35% in some countries (Harada et al., 2005). Until recently, resistance to colistin had only been associated with chromosomally mediated mutations. However, in 2015, a stable plasmid-mediated gene, *mcr-1,* encoding a phosphoethanolamine transferase conferring resistance to colistin was identified in certain GNB isolated from various origins including farm animals, raw meat and humans, in several countries (Liu et al., 2016; Rhouma et al., 2016a; Schwarz and Johnson, 2016). The discovery of a mechanism for horizontal transfer of colistin resistance, and hence the potential for interspecies transfer, gave rise to a strong reaction in the scientific community regarding the potential reduction of colistin effectiveness in human medicine (Nordmann and Poirel, 2016). Food producing animals, and in particular pigs, have been singled out as the most potential reservoirs for spread and amplification of colistin resistance (Nordmann and Poirel, 2016). Thus, scientists and regulatory agencies such as the European Medicine Agency (EMA) have recommended to reduce the use of colistin in animal production and to restrict its use to the treatment of sick animals as a last resort option (European Medicines Agency, 2016b). In addition, several studies have reported coexistence of *mcr-1* gene with genes encoding the production of Extended-Spectrum β-Lactamase (ESBL) and carbapenemase enzymes (Du et al., 2016; Haenni et al., 2016). This constitutes an additional degree of concern about the risk of spread of resistance against antimicrobials of very high importance in human medicine. Furthermore, a high prevalence of ESBL-positive *E. coli* isolated from PWD piglets has been reported (Xu et al., 2015). Taken together, these findings underline the need to better understand

PWD risk factors and to find alternatives to antimicrobials and particularly to colistin in pigs for the control of PWD in order to manage antimicrobial resistance and maintain at the same time livestock productivity. Hence, the aim of the present review was to provide an overview of risk factors of PWD as well as an update of information on the extent of colistin use in PWD control worldwide in terms of quantities and microbiological outcomes. In addition, alternative strategies to the use of colistin for the control of this disease were described and discussed. Finally, a practical approach was proposed for the control of the PWD in its various phases.

For more information on the prevalence of colistin resistance in pigs and the possible link between colistin pharmacokinetic/pharmacodynamic (PK/PD) and emergence of resistance in *Enterobacteriaceae* in swine, please refer to our recent review (Rhouma et al., 2016a).

1.3.3 Risk factors for post-weaning diarrhea in pigs

Post-weaning diarrhea is an economically important **enteric disease in pigs** due to financial losses (Amezcua et al., 2002b). This disease occurs most frequently within the two weeks **after weaning** and is characterized by a profuse diarrhea, dehydration, significant mortality and loss of body weight of surviving pigs (Fairbrother et al., 2005). Mortality associated with this disease may reach 20% -30% over a 1- to 2-month time span among infected weaned pigs during acute outbreaks of PWD (Amezcua et al., 2002b).

PWD is a multifactorial disease where the exact cause has not yet been identified (Jensen et al., 2006) (Figure 6). The occurrence of PWD in pigs involves interactions between the sow, piglet, environment, ETEC bacteria and livestock management (Hong et al., 2006).

1.3.3.1 Predisposing factors

Post-weaning diarrhea is usually associated with proliferation of one or more strains of β-hemolytic ETEC in the small intestine of pigs, in particular those that express fimbrial adhesins

F4 (K88) or F18 (Fairbrother et al., 2005). Thus, small intestinal epithelial cell adhesion and subsequent colonization by ETEC is mediated by F4- or F18-specific receptors (F4R or F18R), the existence and function of which are crucial in determining the susceptibility of pigs to ETEC infection (Fairbrother et al., 2005; Nagy and Fekete, 2005). The genetic predisposition of the pig is primordial for the development of PWD (Rhouma et al., 2016b).

In addition, conditions related to pregnancy and parturition of the sow such as litter size, parity, and postpartum dysgalactia syndrome are significant in the predisposition of piglets to microbial infection (Hong et al., 2006; Muns et al., 2016). The sow placenta is not permeable to maternal immunoglobulin transport and therefore newborn piglets acquire maternal immunoglobulin from colostrum during the first 24 h to 48 h of life (Lallès et al., 2007). It was reported that weaning age and preweaning health play a key role in the onset of PWD (Madec et al., 1998). Moreover, the post-weaning period is a critical phase in the pig's life when the intestinal immune system is immature, and the removal of IgA and other bioactive compounds derived from sow milk contributes to susceptibility of pigs to microbial infections (Heo et al., 2013). Studies investigating the profitability of weaning pigs at an early age, below 21 days, have further encouraged moves away from this practice to weaning pigs no earlier than 26 days of age to reduce the occurrence of PWD (Madec et al., 1998; Main et al., 2004). In the European Union (EU), welfare legislation encourages weaning no earlier than 28 days of age in the absence of cleaned housing sections to ensure that healthy pigs are transferred into nursery accommodation (Baxter et al., 2013). Moreover, studies suggest that increasing weaning age reduces stress associated with this period and allows pigs to have a more mature gastrointestinal tract and become increasingly familiar with solid feed during lactation with an improvement in growth performance and in immune response (Baxter et al., 2013; McLamb et al., 2013).

Feed intake is usually reduced initially after weaning and the pig may develop anorexia of

variable duration and extent between farms, depending on livestock management and the nature

of the feed (Le Dividich and Seve, 2000). Madec and collaborators reported that the feed intake

over the first week after weaning is strongly correlated to the risk of disease occurrence over the

post-weaning period (Madec et al., 1998). Underfeeding during weaning reduces growth

performance of pigs, and contributes to intestinal inflammation and adversely affects villous

height and crypt depth (McCracken et al., 1999). This morphological disruption of the intestinal

mucosa promotes the creation of an ideal environment for the multiplication of bacteria such as

E. coli and allows toxins and bacteria to cross the epithelium as a result of this inflammation

(Campbell et al., 2013) (Figure 7).

1.3.3.2 Contributing factors

Housing factors, population density, parity segregated production and the feeding regimen after

weaningplay a role in the development of PWD (Laine et al., 2008).

It is beyond the scope of this review to discuss in detail all the ideal conditions for pig housing

during the post-weaning period, but to highlight the most important. As reviewed by Le Dividich

and Herpin (Le Dividich and Herpin, 1994), it is essential to provide the correct environmental

temperature, 26–28°C, to maintain pigs in their thermo-neutral zone. Chilling reduces intestinal

peristaltic activity and consequently increases bacterial colonization, and low temperatures in

weaner facilities appears to be responsible for a more severe course of PWD (Fairbrother and

Gyles, 2012). Also, it has been shown that automatic temperature control in the weaners housing

reduces considerably the prevalence of PWD (Laine et al., 2008). Wathes and Whittemore

reviewed several recommendations to prevent pig diseases by appropriate housing and

environment management (Wathes and Whittemore, 2007). These approaches involve avoiding

drafts while removing moisture and gases by the use of adequate ventilation. Moreover, the

removal of manure and soiled bedding on a regular basis is also important to reduce the microbial load on farms.

An increase in herd size was associated with a higher prevalence of PWD (Laine et al., 2008). However, it was reported elsewhere that PWD occurred on a variety of farm types and regardless of the herd size (Amezcua et al., 2002a). Mixing piglets from different farms is a common practice in pig husbandry, particularly at weaning. This mixing can result in fighting as the pigs strive to establish dominance relationships, with most aggressive interactions being typically shown during the first few hours after grouping (Coutellier et al., 2007). It has been reported that the hierarchical behaviour among pigs leads to very significant differences in food and water consumption on farms (Soraci et al., 2014). Production based on segregated sow parities was proposed as a solution to reduce the impact of the social hierarchy. This system of grouping according to the sow's farrowing rank reduces disease challenge by reducing variation in the immune status of the piglets (Boyd et al., 2002).

It was shown that the prevalence of PWD was higher on farms that fed weaned piglets only twice a day with a restricted amount of feed than on farms that provided more than two meals per day with or without feed restriction (Laine et al., 2008). In addition, Amezuca and collaborators reported that the occurrence of PWD was greater with pelleted feed and inadequate feeder space per piglet in the pen (Amezcua et al., 2002a).

A previously mentioned, PWD is a complex disease that may result from interaction between several infectious agents. However, most epidemiological studies have focussed on monitoring the effect of only one pathogen in the occurrence of this disease, and there is inadequate information concerning other relevant enteric pathogens such as viruses and parasites. Some investigations of mixed infections in PWD showed that rotavirus was considered to be an important enteric pathogen in weaned piglets with a prevalence of 77.5%, followed by *E. coli*,

Coccidia, Sapovirus and *Cryptosporidium parvum* with prevalence of 55%, 10%, 2.5% and 2.5% respectively (Katsuda et al., 2006). In addition, infection by the Porcine Reproductive and Respiratory Syndrome virus (PRRSv) results in an impairment of the immune response of piglets, permitting ETEC to cause a septicemia leading to death (Nakamine et al., 1998b). However, these data were reported more than 10 years ago and are unlikely to reflect the current epidemiologic situation.

1.3.3.3 Determining factors

ETEC is the most common cause of PWD in pigs. This pathotype is, characterized by the production of enterotoxins and adhesins, both essential for disease development. Enterotoxins produced by ETEC may be heat stable (STa, STb, or enteroaggregative *E. coli* heat stable enterotoxin 1 [EAST1]) or heat labile (LT) (Fairbrother et al., 2005). Enterotoxins are plasmid-mediated secreted proteins or peptides of ETEC bacteria acting on the intestinal epithelium of pigs (Nagy and Fekete, 2005).

In pigs, the most frequently found fimbrial adhesins of ETEC are K88 (F4), K99 (F5), 987P (F6), F41, and F18 (Fairbrother and Gyles, 2012). F4-positive and F18 ETEC (ETEC: F4 and ETEC: F18) strains represent the major cause of PWD in pigs. F4 are flexible fimbriae that occur as the F4ab, F4ac, or F4ad variant, the F4ac variant being by far the most important type encountered in PWD (Schroyen et al., 2012). The F4 fimbriae mediate bacterial attachment to F4 receptors (F4R), present on the small intestinal brush borders of villous enterocytes allowing ETEC to survive and persist in the intestine and cause diarrhea (Xia et al., 2015). Thus, attachment of ETEC to the pig intestinal mucosa is a crucial step in the pathogenesis and the initiation of PWD. Two antigenic variants of F18 fimbriae exist: F18ab (F107) and F18ac (2134P and 8813). F18ac is commonly associated with ETEC causing PWD, whereas F18ab is often involved in oedema

disease (Byun et al., 2013). No cross protection between F18ab and F18ac was observed on vaccination against F18 variants (Bertschinger et al., 2000). A non-fimbrial adhesin identified as AIDA (adhesin involved in diffuse adherence) has been observed to be associated with ETEC strains recovered from pigs with PWD (Moredo et al., 2015). In this study, 50.0% of isolates were ETEC-$aidA^+$, moreover it has been demonstrated that the expression of AIDA by a diarrheagenic *E. coli* strain (AIDA-I$^+$, STb$^+$) was essential for pig's intestinal colonization and for *in vitro* bacterial autoaggregation and biofilm formation (Ravi et al., 2007).

Porcine pathogenic *E. coli* involved in PWD typically belong to serogroups O8, O138, O139, O141, O147, O157 and O149, the latter being the predominant serogroup in most countries (Gyles and Fairbrother, 2010; Noamani et al., 2003). The most implicated virotype in PWD is ETEC: LT: STb: F4 (Luppi et al., 2016). However, O serogroup and virulence gene patterns vary from region to region and over time (Fairbrother et al., 2005).

Pathogenesis of porcine ETEC has been reviewed extensively elsewhere (Fairbrother and Gyles, 2012; Gyles and Fairbrother, 2010). Piglets ingest ETEC found in their environment, especially derived from mammary glands of their mother and from the farrowing room or from the pen environment on arrival in the nursery (Figure 7). These ETEC originate from the gut of piglets with ETEC diarrhea, or asymptomatic carrier animals at the farm (Gyles and Fairbrother, 2010). ETEC bacteria adhere to the small intestinal epithelium without inducing significant morphological changes, and the secretion of water and electrolytes into the intestinal lumen generated by the release of enterotoxins, alter the functions of enterocytes by increasing secretion and reducing absorption (Nagy and Fekete, 2005). Excessive secretion of electrolytes and water leads to dehydration, metabolic acidosis, and possible death (Gyles and Fairbrother, 2010) (Figure 7). Moreover, intestinal ETEC infections may also result in secondary septicemia and manifestations of icterus, petechial hemorrhages in the mucosal membranes, a syndrome referred

to as enteric colibacillosis complicated by shock (Nagy and Fekete, 2005). ETEC isolates from pig farms with PWD may show a high frequency of resistance to multiple antimicrobials (Amezcua et al., 2002b; Maynard et al., 2003). Nevertheless, there is no indication that drug resistance enhances the virulence of ETEC, although virulence genes are sometimes associated with drug resistance genes (Noamani et al., 2003).

Porcine attaching and effacing *E. coli* (AEEC) induce intestinal lesions similar to those produced by enteropathogenic *E. coli* (EPEC) in humans, and this pathotype is found in pigs with PWD (Fairbrother and Gyles, 2012). These *E. coli* carry the *eae* gene encoding a 94 kDa outer membrane protein (intimin) which is responsible for intimate attachment to epithelial cells. However, the pathogenic significance of porcine EAE positive isolates in weaned pigs is still unknown (Fairbrother et al., 2005). Furthermore, identification of porcine EPEC is difficult and many veterinary diagnostic laboratories do not routinely screen for this pathotype of *E. coli*, isolates of which do not usually possess any of virulence factors of classic PWD or oedema strains (Fairbrother and Gyles, 2012).

1.3.4 Extent of colistin use in weaned pigs worldwide

The global demand for colistin in agriculture is expected to reach 16 500 tonnes by the year 2021, this being one of the least expensive classes of antimicrobials available in veterinary medicine in some countries (Liu et al., 2016). Thus, the pricing structure makes colistin particularly attractive for use in pig production. Since the inception of its clinical use in 1960, colistin has been used in pig production in many countries for the treatment and prevention of digestive disorders caused by *Enterobacteriaceae*, and even sometimes for growth promotion over long periods, to improve growth rate and feed conversion efficiency in pigs (Catry et al., 2015; Katsunuma et al., 2007; Rhouma et al., 2016a). In certain countries such as Canada, where colistin has not been approved

for use in pigs, a rapid increase in resistance of ETEC to a wide range of antimicrobials has prompted the use of colistin in weaned pigs under the veterinarian's responsibility (Rhouma et al., 2015). However, current data on the total quantities of colistin used in pigs worldwide have been difficult to acquire (Catry et al., 2015). Some data, for example in Denmark, indicate that the use of colistin for the treatment of sows increased between 2002 and 2008 (Jensen et al., 2012). Of the two forms of colistin commercially available, colistin sulfate (CS) and colistin methanesulphonate sodium (CMS), the only approved product in pig production is CS, usually administrated orally in the drinking water at the dose of 50 000 IU/kg body weight every 12 h for 3 or 5 days (European Medicines Agency, 2010). Colistin is mostly used in monotherapy in pigs, although it may be combined with other antimicrobials, such as amoxicillin, for the treatment of PWD (Timmerman et al., 2006).

1.3.4.1   Colistin use in post weaning diarrhea on farms

Due to its activity directed against GNB, colistin is widely used for the control of PWD in pigs (Callens et al., 2012b; Kempf et al., 2013). Two surveys conducted in pig farms in Belgium, in 2006 (Timmerman et al., 2006), and 2012 (Callens et al., 2012b) confirmed that colistin was the most frequently used antimicrobial for the control of PWD, being mostly used prophylactically. However, colistin was underdosed in 90% and 53% of the cases, in the first and the second survey respectively. In Germany, it was reported that intestinal diseases in weaners were commonly treated with colistin, pigs being treated 9.7 days (median) per 100 days with this antibiotic, although tetracycline and tylosin were also used in approximately equal amounts (Van Rennings et al., 2015). In a study in France, it was reported that 90% of pig farms used colistin during the post-weaning period (Kempf et al., 2013). In Vietnam, a survey conducted on pig farms representing three different animal production systems (farm household, semi-industrial

and industrial) showed that colistin was the most commonly used antimicrobial for prevention and therapy of gastrointestinal disorders in pigs (Kim et al., 2013).

It has been reported that China is worldwide the country with the greatest use of Colistin in pigs worldwide (Liu et al., 2016), although we did not find any reports in the literature on surveys of colistin use in this country in the post-weaning period. Overall, colistin is widely used in the management of the PWD, with a lot of difference between countries in terms of quantities used and modality of administration (Rhouma et al., 2016a).

1.3.4.2 Microbiological and clinical outcomes of colistin use in controlled conditions

Most of the recent studies conducted in pigs have used CS in experimental conditions for the control of diarrhea in the post-weaning period (Table IX). Several of these studies were performed to examine the effectiveness of alternative substances to colistin in the treatment of PWD (Tang et al., 2013; Torrallardona et al., 2007).

It is often difficult to compare results between studies, because of the variability in the dose of CS used, treatment duration, and the experimental design of the study. In Table IX, we have summarized the main results reported in the literature concerning fecal *E. coli* shedding and pig performance following oral CS treatment. Several studies have also followed histological (intestinal mucosa morphology) and biochemical (d-lactate, nitric oxide, xylose, etc) parameters subsequent to CS use in the post- weaning period in pigs (Tang et al., 2013; Wan et al., 2016). In order to evaluate the effect of colistin on fecal *E. coli* shedding, bacterial quantification was performed in most studies using culture methods (Rhouma et al., 2016b; Torrallardona et al., 2007), whereas other studies used real-time PCR (Fleury et al., 2016; Wan et al., 2016). Furthermore, the oral use of a high dose of colistin in healthy piglets was not associated with a significant perturbation in the pig gut microbiota as demonstrated by a high-throughput

sequencing method (Fleury et al., 2016).

Although colistin has been used in some studies to promote animal growth, data were not conclusive to support the effectiveness of this practice (Yen et al., 2015). In this study, no difference was observed between the CS treated and the control group in terms of ADG/d. Also, the economic benefits of antimicrobial growth promotion in modern farms has been questioned (Graham et al., 2007), he benefit of this use being associated with poor hygiene on farms.

1.3.5 Alternative strategies to colistin for post-weaning diarrhea control

Reduced colistin usage in livestock and particularly in swine is highly promoted worldwide and is required in Europe as a public health measure to reduce colistin resistance spread, and to prevent the loss of colistin effectiveness in human medicine (Rhouma et al., 2016c). Furthermore, concurrent treatment with colistin in piglets was associated with the isolation of resistant bacteria from the earliest days of treatment (Rhouma et al., 2016b). Almost all studies conducted on isolates from pigs worldwide to screen *mcr-1* gene presence in enterobacterial species reported that colistin resistant isolates harboring this gene also showed resistance to one or several classes of antimicrobials conventionally used in swine such as: Aminoglycoside, Sulphonamide, Trimethoprim, Tetracycline, Quinolone, Lincosamide, β-lactam, and third generation cephalosporin (Anjum et al., 2016; Falgenhauer et al., 2016; Malhotra-Kumar et al., 2016b; Nguyen et al., 2016).

However, to ensure swine welfare, productivity and reduced mortality associated with PWD, alternatives to colistin and other antimicrobials, especially those of critical importance for human health, are essential in pigs. There is major debate over the terminology 'alternative to antibiotics' because we do not propose substances with antibacterial activity but rather substances that act on bacteria indirectly, either by stimulating the host immune system, by the release of

substances that have anti-bacterial activity or by improving the host gut health and consequently growth performance (Cheng et al., 2014). Thus, we will use the terminology «strategies» or «measures» to describe alternatives to antimicrobials. Due to the multifactorial etiology of PWD, finding case-specific preventive measures against this disease is a challenge for both researchers and veterinarians. Here we give an overview of these preventive strategies, focusing on the most practical and promising ones for the control of PWD in pigs.

1.3.5.1 Preventive measures

In the literature, many alternatives to antimicrobial usage in food producing animals have been reported and discussed (Allen et al., 2014; Caly et al., 2015; Cheng et al., 2014; Wang et al., 2016a). The most promising way to mitigate the development of colistin resistance is to reduce the use of antimicrobials at the farm level (Table X). There are documented relationships between housing conditions and incidence of PWD in pig herds; Madec and collaborators claimed that prevention of PWD disorders could be based solely on the control of zootechnical conditions (Madec et al., 1998). We have demonstrated in controlled experimental conditions with a high level of biosecurity, that pigs challenged with ETEC: F4 and not treated demonstrated a reduction in the signs of PWD within the same interval as the colistin treated group (Rhouma et al., 2016b). The management strategies around weaning should focus on measures that avoid any kind of stress for pigs. These measures include preventing the spread of infection, providing the pigs with good thermal comfort, giving them adapted feed and allowing access to this feed for all pigs. Considerable research has been performed into developing diets for weaners and there is now a range of high quality diets that are readily digested by the early-weaned pig (Heo et al., 2013). The main purposes of these diets are to achieve high post-weaning feed intakes and minimize duration of post-weaning anorexia and consequently growth retardation. It has been reported that

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the presence of some ingredients in the feed for weaners, such as soybeans, seems to favor the occurrence of PWD (Dreau et al., 1994). This could be due to the presence of trypsin inhibitors or antigens inducing a localized immune response (Fairbrother et al., 2005). Furthermore, it was shown that soya bean meal (SBM) reduced duodenal specific activities of most intestinal enzymes and increased crypt depth in pigs (Salgado et al., 2002). Thus, such ingredients should be avoided in feed of early-weaned pigs and could be replaced by pea and faba bean seeds. In addition, feeds with decreased protein content and the addition of organic acid to reduce gastric pH were found to decrease *E. coli* colonization and to minimize PWD prevalence (Heo et al., 2013).

The scientific community increasingly recognizes the importance of communication and awareness among farmers in relation to antimicrobial resistance, as reflected by the growing number of publications in this area in recent years (Rhouma et al., 2016a; Visschers et al., 2015). This suggests that farmers' perceptions, and the factors affecting their behaviour, need to be better understood if effective measures associated responsible and prudent use of antimicrobials are to be implemented successfully.

Moreover, effective diagnostic tools are essential for veterinarians to confirm the bacterial etiology of PWD and to determine the antimicrobial susceptibility of the identified bacterial strain. The laboratory diagnosis is particularly important in PWD to avoid the inappropriate use of antimicrobials. DNA-based molecular detection methods such multiplex PCR based on the detection of ETEC virulence genes are rapidly becoming part of the routine laboratory diagnosis of PWD, and these genes are used as a biomarkers of ETEC strains (Nagy and Fekete, 2005).

In several countries, implementation of financial penalties for high antimicrobial users is proposed as a method to reduce antimicrobial usage and pig farmers would receive a financial bonus when they use alternative methods or when they greatly reduce antimicrobial use on their

farms (Visschers et al., 2015). Vaccination seems to be an effective approach to reduce the occurrence of PWD and to reduce infection pressure and increase immunity in the pig population (Fairbrother et al., 2005). Several studies conducted in pigs confirm a reduction of antimicrobial usage after vaccination (Postma et al., 2015b). In fact, vaccination against the porcine proliferative enteropathy caused by *Lawsonia intracellularis* reduced the need for therapeutic oxytetracycline administration in Danish pigs (Bak and Rathkjen, 2009). Live attenuated and wild type avirulent *E. coli* vaccines appear to be promising for the control of ETEC infections and live vaccine against ETEC: F4, is now available in Canada and Europe (Melkebeek et al., 2013). This vaccine is added to the drinking water and recommended for the vaccination of healthy weaned pigs of 17 days or more. Clinical studies confirmed that administration of this vaccine significantly reduced intestinal colonization by virulent ETEC: F4 and the accumulation of fluid in the intestines after an experimental challenge (Nadeau and Fairbrother, 2011). The immunity in piglets begins 7 days after oral vaccination, however, since PWD caused by ETEC: F4 occurs shortly, in the first week, after weaning, an immune trough may exist in the first days after weaning during which the pigs are not protected (Melkebeek et al., 2013). Thus, the time of the administration of this vaccine should be adjusted. In addition, clinical trials of vaccination against ETEC: F18 have been carried out in pigs. Genetically susceptible pigs were vaccinated orally on three consecutive days, beginning 10 days before weaning with a live F18ac-positive *E. coli* vaccine (Bertschinger et al., 2000). In this study, a significant rise in F18ac-specific serum IgA and a 3 Log CFU decrease in fecal shedding of the F18ac-positive challenge strain was observed compared to the unvaccinated group. However, this vaccine did not induce protective immunity against ETEC: F18. On the other hand, it was shown that a minor subunit of F18 (FedF) alone or genetically fused to F4 FaeG subunit or conjugated to F4 fimbriae induced protective anti-F18 antibodies in pigs (Tiels et al., 2008). In general, the success of a vaccine

against PWD depends largely on the identification of the most prevalent ETEC pathotype present in the farm, resulting in matching of the appropriate protective antigens with the adhesin produced by the ETEC present on the farm, and administering it at the optimal time (Nagy and Fekete, 2005). For vaccines consisting of live F4 or F18ac-positive *E. coli,* it is often recommended to vaccinate suckling pigs to obtain a strong mucosal immunity production, IgA, before weaning. However, our knowledge is very limited about the effect of maternal antibodies on the survival of these vaccine strains in the intestine of pigs of this age. Also, there is no cross protection against ETEC strains expressing a different fimbria or toxin. Recently, plant-based vaccines for protection of pigs against ETEC were investigated. A rice-based cholera vaccine expressing the choleratoxin (CT) subunit B (CTB) (MucoRice-CTB) was tested in pigs for protection against LT-ETEC infection (Takeyama et al., 2015). CTB-based vaccines can target not only F4-type but also F18-type ETECs, and this vaccine also induced maternal CTB-specific IgG and IgA in the colostrum and milk of sows after farrowing. CTB-specific antibodies were also secreted into the gut lumen of weaned pigs and reduced intestinal loop fluid accumulation upon ETEC challenge, indicating a protective effect of this vaccine against ETEC diarrhea (Takeyama et al., 2015). However, the cost of these vaccines is very high and, unlike open-air farming, the production of transgenic plants for biotherapeutic use is very demanding, and the procedures for manufacturing and processing of plant-based pharmaceuticals are not well defined. Thus, a large-scale production of these vaccines not envisaged, at least in the near future. Current progress in the development of subunit vaccines against ETEC associated with diarrhea in humans and animals has been reviewed extensively elsewhere (Melkebeek et al., 2013; Zhang and Sack, 2015). However, none of these subunit vaccines has been marketed in swine.

The selection of animals genetically resistant to ETEC F4 and/or F18 is considered as a radical solution to eliminate the PWD in a swine herd. However, progress in this area is very limited or

even non-existent. Pigs that are resistant to ETEC: F4 and/or F18 do not express intestinal receptors for these fimbrial types (Fairbrother et al., 2005). The expression of these receptors is genetically determined and inherited in a dominant way and the loci controlling F4R and F18R expression are located on separate chromosomes. The gene underlying resistance to F4ab/ac ETEC has been assigned to porcine chromosome 13, whereas the F4ad ETEC receptor is localized on another chromosome that was not identified (Rasschaert et al., 2007). A PCR-RFLP test has been developed to allow genotyping for F4ab/ac ETEC resistance/susceptibility (Daudelin et al., 2011). Three different genotypes were observed and were identified as resistant (RR), susceptible heterozygote (SR) and susceptible homozygote (SS). However, it cannot be predicted if additional types of adhesive fimbriae or new variants of known types will emerge which could bind to yet unidentified receptors and could cause outbreaks of diarrhea and mortality in the nursery (Fairbrother et al., 2005). It is difficult to understand the reasons behind the non-exploration of the genetic breeding for ETEC resistant pigs to reduce economic loss associated with PWD and to reduce the use of antimicrobials on farms. It was shown in an early study that F4 susceptible piglets tend to have better growth performance then F4 resistant ones (Edfors-Lilja et al., 1986). Also, heterozygous F4R$^-$ piglets are not passively protected from infection by ETEC: F4 strains (Zhou et al., 2015).

1.3.5.2 Feed additives

In pigs, PWD can be controlled by the use of various preventive strategies without using antimicrobials (Table XI). Feed supplements such as pre-probiotics, synbiotics, organic acids, antimicrobial peptides, dehydrated porcine plasma, specific egg yolk, bacteriophages and zinc oxide (Heo et al., 2013; Kim et al., 2016; Pérez-Bosque et al., 2016; Suiryanrayna and Ramana, 2015; Thacker, 2013; Wang et al., 2016a; Wittish et al., 2014) have been used in weanling pigs to

enhance growth, feed efficiency and to reduce PWD. Here, we give an overview of these feed strategies, focusing on the most used practices showing clinical effectiveness in reducing symptoms of PWD and ETEC attachment to enterocytes.

Prebiotics are selectively fermented components of feed, indigestible by the host animal, that modulate the gut microbiota to benefit host health. Resulting effects include the stimulation of short-chain fatty acid (SCFA) production and the proliferation of bifidobacteria and lactic acid bacteria such as *Lactobacillus* spp. and *Bifidobacterium* spp (Allen et al., 2013; Callaway et al., 2008). Common prebiotics include inulin and oligosaccharides such as galactooligosaccharides (GOS) and fructooligosaccharides (FOS) (Slavin, 2013). Pigs fed with chito-oligosaccharides (COS) showed better overall intestinal health (based on villi height), improved performance (measured by body weight gain and feed conversion ratio) and higher *Lactobacillus* counts than those found in control pigs or pigs receiving diets supplemented with chlortetracycline (Liu et al., 2008). Also, fermented ingredients, such as non-starch polysaccharide hydrolysis products of soybean meal (SBM) in weaned pig feed, were found to interfere with attachment of ETEC to enterocytes and were beneficial in maintaining fluid balance during ETEC infection (Kiarie et al., 2008). It was shown that the prebiotic β-galactomannan (βGM) inhibited the *in vitro* adhesion of ETEC on the cell surface of porcine intestinal IPI-2I cells, and decreased the mRNA ETEC-induced gene expression of pro-inflammatory cytokines such as TNF-α, IL-6, GM-CSF and chemokines on intestinal IPI-2I cells (Badia et al., 2012).

Probiotics such as *Lactobacillus*, *Streptococcus*, *Bifidobacterium*, *Bacillus*, and yeasts are live microbial feed supplements (Allen et al., 2013). Probiotic bacteria have also been shown to produce antimicrobial molecules, such as bacteriocins, and to inhibit the production of bacterialtoxins or the adhesion of pathogens to the intestinal mucosa (Callaway et al., 2008). Several studies demonstrated that pretreatment with certain probiotics, such as *Lactobacillus*

rhamnosus, was effective in reducing diarrhea in experimental ETEC: F4 PWD in pigs, possibly via modulation of the intestinal microbiota, enhancement of intestinal antibody defence, and regulation of production of systemic inflammatory cytokine (Zhang et al., 2010). A *Bacillus licheniformis* and *Bacillus subtilis* spore mixture (BLS-mix) was effective in preventing loss of intestinal epithelial barrier integrity after challenge with ETEC: F4 in experimental PWD (Yang et al., 2016). In addition, it was shown that the feeding of pigs with live yeast *Saccharomyces cerevisiae* enhanced their growth and reduced the duration and the severity of PWD caused by ETEC (Trckova et al., 2014). It has been demonstrated that the administration of a mixture of two probiotics, *Pediococcus acidilactici* and *Saccharomyces cerevisiae boulardii*, in the feed of challenged weaned pigs reduced ETEC: F4 attachment to the ileal mucosa in comparison with the group treated with chlortetracycline and tiamulin (Daudelin et al., 2011).

Synbiotics refers to a combination of probiotic and prebiotic approaches; it is possible that a prebiotic that confers gastrointestinal health benefits could selectively increase the population and/or activity of probiotics in the gut (Vondruskova et al., 2010). Synbiotics can be either complementary or synergistic. Complementary synbiotics consist of a probiotic and a prebiotic selected independently to confer benefits to the host. On the other hand, synergistic synbiotics are comprised of a prebiotic chosen specifically for the selected probiotic to potentiate its effect in the gut (Krumbeck et al., 2015). It was shown that the combination of raw potato starch and a probiotic had a beneficial effect on pig growth performance and resulted in a reduction of diarrhea and increased microbial diversity in the gut of weaned pigs challenged with an ETEC: F4 strain (Krause et al., 2010). Also, Guerra-Ordaz and collaborators showed that following a challenge of pigs with pathogenic *E. coli* (O149:K91:H10), administration of lactulose in the feed resulted in improved weight gain, increased lactobacilli and the proportion of butyric acid in the colon, and less inflammation due to a reduction of the pig major acute-phase

protein (Pig-MAP) in serum. Administration of *Lactobacillus plantarum* in the feed promoted lactobacilli growth, modulated fermentative activity, reduced inflammation, and improved intestinal mucosa function and showed a tendency to reduce diarrhea. The application of a synbiotic diet resulted in the benefits of both diet regimes, thus being an example of a complementary synbiotic (Guerra-Ordaz et al., 2014).

Organic acids such as citric, fumaric, lactic, propionic, benzoic and formic acids showed beneficial effects in the pig gastrointestinal tract. Their most important mechanism of action is to inhibit microorganisms through a decrease of pH in the intestine but they also stabilize the nutritional quality of the feed (Suiryanrayna and Ramana, 2015). Addition of organic acids to weaned pig diets improved growth performance and health (Heo et al., 2013). It was reported that regardless of the organic acids used in the feed, these compounds reduced the incidence and severity of diarrhea in pigs, and improved the performance of the treated group compared to that of the negative control group (Tsiloyiannis et al., 2001).

Antimicrobial peptides (AMPs) are small molecules constituting an important part of the innate immune system. They may present antibacterial, antifungal, antiparasitic, and antiviral activities, and are increasingly of interest as alternatives to classic antibiotics (Allen et al., 2014). AMPs such as lactoferrin, cecropin, defensin, plectasin and bacteriocin showed beneficial effects on growth performance, nutrient digestibility, small intestinal morphology and gut microbiota in pigs (Wang et al., 2016a). Available data on the effect of AMPs on swine health and especially in the control of PWD have been reviewed extensively elsewhere (Wang et al., 2016a; Xiao et al., 2015). Antimicrobial lactoferrin peptides are one of the most commonly used AMPs in pig feeds. More recently, it was shown in a murine model of intestinal inflammation that treatment with porcine lactoferrin-derived peptide LFP-20 was effective in the prevention of histological damage, the inflammatory response and the disruption of tight junction structure induced by LPS

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in the intestine (Zong et al., 2016). Colicins, a class of bacteriocins produced by *E. coli* and closely related species, have been shown to inhibit the activities of ETEC: F4 and F18 strains *in vitro* and *in vivo*, and improve the growth performance, reduce the incidence of PWD and the expression of the interleukin 1β and tumor necrosis factor beta genes in ileal tissues of pigs (Cutler et al., 2007). On the other hand, resistance to AMPs has been observed *in vitro* in GNB such as *E. coli* (Guilhelmelli et al., 2013). Thus, the use of AMPs in pig farms needs careful and controlled implementation to limit possible resistance development and cocktails of AMPs might be useful to mitigate selection for resistance (Allen et al., 2014).

Spray dried plasma (SDP) is a protein rich product obtained from the industrial fractionation of blood from healthy animals (Pérez-Bosque et al., 2016). It was shown that addition of SDP to the feed improved growth performance, and protects pigs against ETEC: F4 infection by reducing the intestinal expression of inflammatory cytokines such as TNF-α and interleukin-8 and maintaining mucosal integrity, and enhancing specific antibody defense (Adewole et al., 2016). Spray dried plasma (SDPP) of porcine origin has been pinpointed as a potential source for the coronavirus in a recent epidemic of Porcine Epidemic Diarrhea (PED) (Lee, 2015). Thus, spray-dried chicken plasma (SDCP) has been evaluated as a replacement for SDPP in weaned pigs. Indeed, the effect of SDCP on serum biochemistry, intestinal barrier function, immune parameters, and the expression of intestinal development–related genes in piglets was similar to SDPP (Zhang et al., 2016). Nevertheless, a study has provided evidence that PED virus is inactivated during the SDPP production process (Gerber et al., 2014).

Specific egg yolk antibodies: The chicken egg yolk is a source of large quantities of relatively inexpensive IgY antibodies (Fairbrother et al., 2005). Several studies reported that specific chicken antibodies provide protection against ETEC infections in pigs (Adewole et al., 2016). Despite the effectiveness of this practice, we have not found in the recent literature (last 5 years)

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any studies evaluating the use of specific egg yolk antibodies in PWD control. This is probably the consequence of the non- profitability in pig production of this practice, or the lack of protection against ETEC challenge or PWD occurrence, possibly because the antibodies contained in the eggs are not specific against the infected ETEC strains present on the farm (Chernysheva et al., 2003).

Bacteriophages are highly species-specific viruses that can infect and kill bacteria. They have been widely evaluated in clinical trials to treat bacterial infections in pigs as an alternative to antibiotics use (Zhang et al., 2015). Recently, it was reported that dietary supplementation with bacteriophages for the treatment of PWD caused by an ETEC: F4 strain in an experimental model, was effective in reducing rectal temperature, faecal consistency score, *E. coli* adhesion score in the ileum and caecum, and villous height: crypt depth (VH: CD) ratio in the duodenum and jejunum (Lee et al., 2016). However there are several disadvantages associated with the use of phage therapy in swine. Phages have a narrow spectrum of activity directed against a limited number of bacteria and the possible development of bacterial resistance against phages has to be considered (Zhang et al., 2015). To overcome the narrow spectrum of activity, some recent studies have reported beneficial effects of a bacteriophage cocktail used in the feed for weanling pigs. This combination resulted in enhanced growth performance and gut health of pigs, although the combination of phages with probiotics did not show any additional effect (Kim et al., 2016). Some authors have considered that the development of phage-resistant bacteria could be positive for the host (Levin and Bull, 2004). In fact, resistance to phages can reduce the fitness of the bacteria and could thereby impair their competitive capacity and consequently their ability to colonize the intestinal mucosa of the host (Levin and Bull, 2004).

Zinc oxide: it has been shown that the addition of zinc (Zn) as zinc oxide (ZnO) at the levels of 2400 to 3000 ppm in pig feed was effective in the reducing of PWD and mortality and in

improving growth performance in weaned pigs (Adewole et al., 2016; Zhu et al., 2016). However, Amezcua and collaborators reported an important proportion of farms with PWD occurrence using high levels of ZnO (Amezcua et al., 2002b). Also, several studies reported an increased proportion of *E. coli* isolates resistant to tetracycline and sulfonamides in pigs fed with high zinc doses (Bednorz et al., 2013; Vahjen et al., 2015). This may explain why antimicrobial resistance persists even in the absence of antimicrobial exposure (Holman and Chénier, 2015). Moreover, the use of high zinc levels in pig feeds has led to heavy metal contamination in the soil, raising environmental concerns (Holman and Chénier, 2015).

Others: Several studies have documented a significant improvement of weight gain, and feed conversion, as well as the reduction of the incidence, severity and duration of diarrhea in weaned pigs fed diets supplemented with substances such as: exogenous enzymes (Tactacan et al., 2016), milk products (De Greeff et al., 2016), clay minerals (Subramaniam and Kim, 2015), and medicinal plants (Ayrle et al., 2016). Although a large number of peer-reviewed studies about these substances are available in the scientific literature, most of the clinical studies were performed in experimental conditions. More research is needed to evaluate the potential effectiveness of these substances under field conditions for the control of PWD in pigs.

1.3.6 Results of comparative studies

Several studies have been carried out in experimental conditions to assess the effectiveness of alternatives to colistin for the control of PWD in pigs (Table XII). Herein, we give an overview of studies published in 2015 or 2016.

A number of recent experimental studies have now shown that some alternatives (Table XII) resulted in similar or superior clinical outcomes compared to colistin for improving growth performance and intestinal integrity and in reducing of incidence of diarrhea in weaned pigs.

However, these studies were conducted in experimental conditions and in most cases in healthy weaned pigs. Thus, further research is needed to demonstrate the stability and the efficacy of such alternatives (probiotics, AMPs, medicinal plants) in field conditions. Also, work is needed to optimize the doses of these substances to incorporate in the feed to ensure their effectiveness in PWD control. The financial cost and the ease of administration of such alternatives are the other important criteria that should be taken into consideration in pig production.

1.3.7 Limits and perspectives

A long and growing list of compounds have been tested for their ability to replace colistin or other antibiotics for the control of PWD in pigs. However, it is difficult to identify a single "ideal" solution for PWD management. Also, as was discussed above, PWD is a multifactorial disease and the exact overall etiology has not yet been fully elucidated, making it difficult to choose suitable alternatives. Moreover, the most of these alternatives produce inconsistent results regarding their effectiveness in field conditions (Thacker, 2013). Oral administration of specific-antibody-containing egg yolk, or SDP to weaned piglets showed in some cases no protection against ETEC strains or PWD outcomes, likely because the contained antibodies were not specific against the infecting ETEC strains present on the farm (Fairbrother et al., 2005). The composition of plant extracts, organic acids and probiotics is complex and knowledge regarding their mechanisms of action is poor, resulting in variable results and safety risks (Cheng et al., 2014). Synergy mechanisms of probiotics and prebiotics are not very well known nor well studied (Krumbeck et al., 2015). Although AMPs and bacteriophages helped in the treatment of PWD, the bacterial resistance risk, the high cost and the narrow antibacterial spectrum of these alternatives reduce their practical use on farms (Allen et al., 2014). Vaccination is one of the most promising strategies for the control of PWD in pigs both in terms of preventive ability and cost-

effectiveness (Melkebeek et al., 2013). The control of production parameters (temperature, ventilation, density, sanitation, biosafety, improvement of feed quality) is crucial factors for the control of PWD and the reducing of the use of antimicrobials during the post-weaning period (Rhouma et al., 2016b). However, the improvement of farm conditions and management requires investment and awareness of pig farmers. Furthermore, the use of regular diagnostic testing is crucial to ensure an appropriate choice of the antimicrobial and to monitor its effectiveness on farms. Thus, efforts to improve microbiological laboratory detection methods are of paramount importance to help the veterinarian to act rapidly at an early stage of the disease (Årdal et al., 2016).

For the management of PWD in different stages of its evolution, we propose a comprehensive approach that involves producers, the nutrition industry, veterinarians, the diagnostic laboratory, and researchers (Figure 8). The absence of a well-identified etiology of PWD and of an effective alternative to antimicrobials, requires a close collaboration between the different stakeholders to reduce antibiotic resistance and economic losses caused by this disease in swine.

1.3.8   Conclusion

Despite the progress that has been observed in modern pig farms during the last decade to prevent infectious diseases and improve global animal health, PWD remains a problem that causes significant economic losses and represents a barrier for the development of intensive and large-scale pig industry. Antibiotics have contributed significantly to mitigate the economic losses caused by infectious diseases and particularly PWD in swine. However, increasing bacterial resistance leading to therapeutic failures on farms as well as the greater vigilance of consumers regarding antimicrobial residues, have resulted in more intensive research and several clinical trials for the development of alternatives to antimicrobials. Thus, several alternatives have been

developed, some of which have been commercialized for the management of PWD in pigs. However, the effectiveness of these compounds has been variable from one farm to another due to the management of livestock and farm conditions. Although some alternatives have showed comparable efficacy to antimicrobials or colistin in the control of PWD, there is still a considerable gap between these alternatives and antibiotics concerning their effectiveness in PWD control. Control of housing conditions and vaccination are the most promising strategies for the prevention of PWD in pigs and for reducing of the overall use of antimicrobials on farm. However, the establishment and the effectiveness of these strategies depend on the involvement of all stakeholders in pig farming. Judicious use of antimicrobials in pigs and continued development of alternatives to antimicrobials and colistin remains a priority to ensure a long-term sustainable development in pigs.

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 potential conflicts of interest.

1.3.9 Tables

Table IX: Microbiological and clinical outcomes of monotherapy with colistin in pigs

Bacterial agents/condition	Dose per day	Duration (days)	Sample type	Reduction in *E. coli* (log cfu/g)*	Performance (ADG, g/d)	Reference
E. coli K99 /Experimental PWD	300 mg/kg of diet	7	-Ileum -Cecum	6.55 6.63	122[a]	(Torrallardon a et al., 2003)
E. coli K99 /Experimental PWD	300 mg/kg of diet	10	-Ileum -Cecum	2.3 3.2	128[b]	(Torrallardon a et al., 2007)
Weaned pigs (clinically healthy)	200 mg/kg of diet	7	N/A	N/A	229[b]	(Yin et al., 2009)
ETEC mixture /Experimental PWD	200 mg/kg of diet	21	-Ileum -Cecum -Colon	1.54 1.65 0.65	292[a]	(Tang et al., 2009b)
ETEC mixture /Experimental PWD	2.5 mg/ animal (Oral- Water)	21	Fecal samples	3	283[a]	(Tang et al., 2013)
Weaned pigs (clinically healthy)	40 mg/kg of diet	14	-Ileum -Cecum -Colon	N/A	142.2[b]	(Yen et al., 2015)
E. coli K88 /Experimental PWD	4,8 mg/kg (Oral- Water)	5	Fecal samples	4	214[b]	(Rhouma et al., 2016b)
E. coli K88 /Experimental PWD	9,6 mg/Kg (Oral- Water)	5	Fecal samples	4	N/A	(Rhouma et al., 2016b)
Weaned pigs (clinically	2,4 mg/kg (Oral-	5	Fecal samples	2††	N/A	(Fleury et al., 2016)

healthy)	Water)				
Weaned pigs (clinically healthy)	172,8 mg/Kg of diet	14	Fecal samples	4.5††	N/A	(Fleury et al., 2016)

PWD: Post-Weaning Diarrhea. **ADG**: Average Daily Weight Gain. **N/A**: not available. *****Reduction compared to the control group. **ETEC**: Enterotoxigenic *Escherichia coli*. **a**: Statistically significant compared to the control group. **b**: Not statistically significant compared to the control group.†: log (copies/g). ††: log cfu of *Enterobacteriaceae* /g.

Table X: Preventive strategies to reduce the use of antimicrobials during the post-weaning period

Strategies	Benefits	Limitations	References
Control of housing environment and improved biosecurity	-Very effective approach -Significantly reduces PWD occurrence - Reduces the use of antimicrobials in farm - Sustainable approach	-Significant cost -Extreme weather conditions in some countries -Acceptability of farmers to change some management techniques -Financial support is required	(Madec et al., 1998; Rhouma et al., 2016b)
Diet management (reducing the amount of soybean)	-Reduces the severity and frequency of PWD and oedema disease -Reduction of histological changes in intestinal crypt and villi	-Growth retardation - Increase production - Considerable controversy between studies	(Heo et al., 2013)
Communicative advisory tools for pig farmers	-Improving breeding management -Farmers feel concerned by the problem of antibiotic resistance -Raised awareness and responsibility	-Requires a lot of field work - Farmers worried mostly about infectious diseases and financial issues -Financial bonus is required	(Visschers et al., 2015)
Laboratory diagnosis to confirm etiology of PWD	-Avoid the use of antimicrobials to treat viral diarrhea - Allows an appropriate choice for antibiotics	- Significant cost - Lack of rapid diagnostic techniques	(Postma et al., 2015b)
Policy measures	-Reduce the sale and the use of	-Requires penalties	(Visschers

	antimicrobials on farm -Reduce self-medication	-Financial bonus is required	et al., 2015)
Immunoprophylaxis: Live attenuated and live wild type avirulent *E. coli*	-Specific protection against ETEC: F4 or F18 -Easy to administer on farms (drinking water) -Reduces antimicrobial use in the PW period -ᴍarketed in swine	-Interference with the lactogenic immunity of piglets -Absence of cross-protection between F18ab strains - Limited availability in some countries	(Melkebeek et al., 2013)
Immunoprophylaxis: Subunit vaccines (Purified F4 fimbriae)	- A powerful oral immunogen - Leads to a specific mucosal immune response -Leads to a significant reduction in fecal excretion of ETEC: F4	-The proposed immunization procedure required large quantities of F4 - Antigen degraded by the pH of the stomach and by digestive enzymes -Usually required mucosal adjuvant such as Cholera toxin	(Delisle et al., 2012)
Breeding of resistant pigs	-Very effective approach -Greatly reduces the total amount of antimicrobials use on farms -Reduces the selection pressure	-Expensive process -Lack of techniques for a large-scale selection	(Fairbrother et al., 2005)

PWD: Post weaning diarrhea

Table XI: Benefits and limitations of the major alternative feed strategies for the control of post weaning diarrhea in pigs

Strategies	Benefits	**Limitations**	**References**
Prebiotics, Probiotics and Synbiotics	-Improved intestinal health -Improved growth performance - Reduced ETEC: F4 attachment to the ileal mucosa -Reduced diarrhea	- Sometimes contradictory studies on their effectiveness -Lack of information on the potential synergism between pre- and probiotics	(Badia et al., 2012; Yang et al., 2016)
Organic acids	-Decreased pH in the intestine -Improved growth performance	- Exact modes of action still unknown -Anti microbial activities is different	(Suiryanrayna and Ramana, 2015)

	- Reduced PWD	between acids	
Antimicrobial peptides (AMPs)	-Improved growth performance -Decreased diarrhea -Reduced the markers of intestinal inflammation -Enhance immune function	-The pharmacokinetics *in vivo* is unknown -Bacterial resistance -Cocktails of AMPs might be used to mitigate selection for resistance	(Wang et al., 2016a; Xiao et al., 2015)
Spray dried plasma (SDP)	-Improved growth performance -Reduced incidence and severity of diarrhea - Reduced the markers of intestinal inflammation -Maintained mucosal integrity	- High cost - Required rigorous control during the preparation process - Potential source of pathogens?	(Adewole et al., 2016)
Specific egg yolk antibodies	-Improved growth performance -Decreased diarrhea -Maintained intestinal mucosal integrity	-High cost -Antibodies are sometime not specific against the infecting ETEC strains on farms	(Adewole et al., 2016)
Bacteriophages	-Reduced *E. coli* mucosal adhesion -Maintained intestinal mucosal integrity -Decreased diarrhea	-Narrow spectrum of activity -Development of bacterial resistance -A combination of phages is needed	(Zhang et al., 2015)
Zinc oxide	-Inhibition of bacterial adhesion to the intestinal mucosa -Stimulated growth rate - Maintained intestinal mucosal integrity -Modulated immune functions	-High levels increased PWD - Soil heavy metal contamination -Bacterial resistance -Co-resistance	(Holman and Chénier, 2015; Zhu et al., 2016)

Table XII: Effects of colistin compared to alternatives measures on growth performance, on intestinal morphology, on *E. coli* shedding and diarrhea of weaned pigs.

Trials	ADG (g/day)	Ileum Villus height (μm)	Ileum Crypt depth (μm)	*E. coli* (log 10 CFU/g)	Diarrhea	References
Study 1: HP	**d0–35**	**d35**	**d35**		**d0-21***	
Hop β-acids†(360 mg/kg**)**	441	337	214	NA	1.51	(Sbardella et al., 2016)
Colistin sulfate (40 mg/kg**)**	425	366	230	NA	1.51	
Control	387[a]	349	219	NA	1.72	
Study 2: HP	**d21**	**d21**	**d21**	**d21**		
Two *Macrocephala* Flavored Powder (3000 mg/kg**)**	NA	121	66.30	7.93[a]	NA	(Ye et al., 2015)
Colistin sulfate (300 mg/kg**)**	NA	107	57.63	6.48[a]	NA	
Control	NA	120.49	64.75	6.63	NA	
Study 3: HP	**d1-21**	**d21**	**d21**	**Ileum d21††**	**d1-7***	
Recombinant plectasin (Ple) (60 mg/kg**)**	311.43	227.69	95.53	6.61	10.48	(Wan et al., 2016)
Colistin sulfate (60 mg/kg**)**	333.57	195.57	88.48	5.86	8.57	
Control	193.10[a]	160.45	105.82	6.29	36.19	
Study 4: HP	**d0-14**				**d0-14**	
Medium-chain triglyceride (MCT) (3000 mg/kg**)**	141.2	NA	NA	NA	0.91	(Yen et al., 2015)
Colistin sulfate (40 mg/kg**)**	142.2	NA	NA	NA	0.91	
Control	130.7	NA	NA	NA	1.01	

Study 5: HP	d28-56	d42	d42		d28-56**	
Freshwater microalgae *Chlorella vulgaris* (1000 mg/kg)	395	435	278	NA	24[b]	(Furbeyre et al., 2016)
Colistin sulfate (20 mg/kg)	400	440	283	NA	34[a]	
Control	393	415	299	NA	36[a]	
Study 6: CP		**† d1 post challenge**	**† d1 post challenge**			
Live yeast (5×10^{10} CFU/kg)	NA	322	246	NA	NA	(Trevisi et al., 2016)
Colistin sulfate (1000 mg/kg)	NA	334	236	NA	NA	
Control	NA	294	199	NA	NA	

HP: Healthy pigs. **CP**: Challenged pigs. † Jejunum. **NA**: not available.* Diarrhea occurrence was calculated as the proportion of days in which pigs showed clinical signs of diarrhea.** Number of pig days with diarrhoea score ≥2. **a.b** : values within a row with different superscripts differ significantly at $P < 0.05$. ††: log (copies/g).

1.3.10 Figures
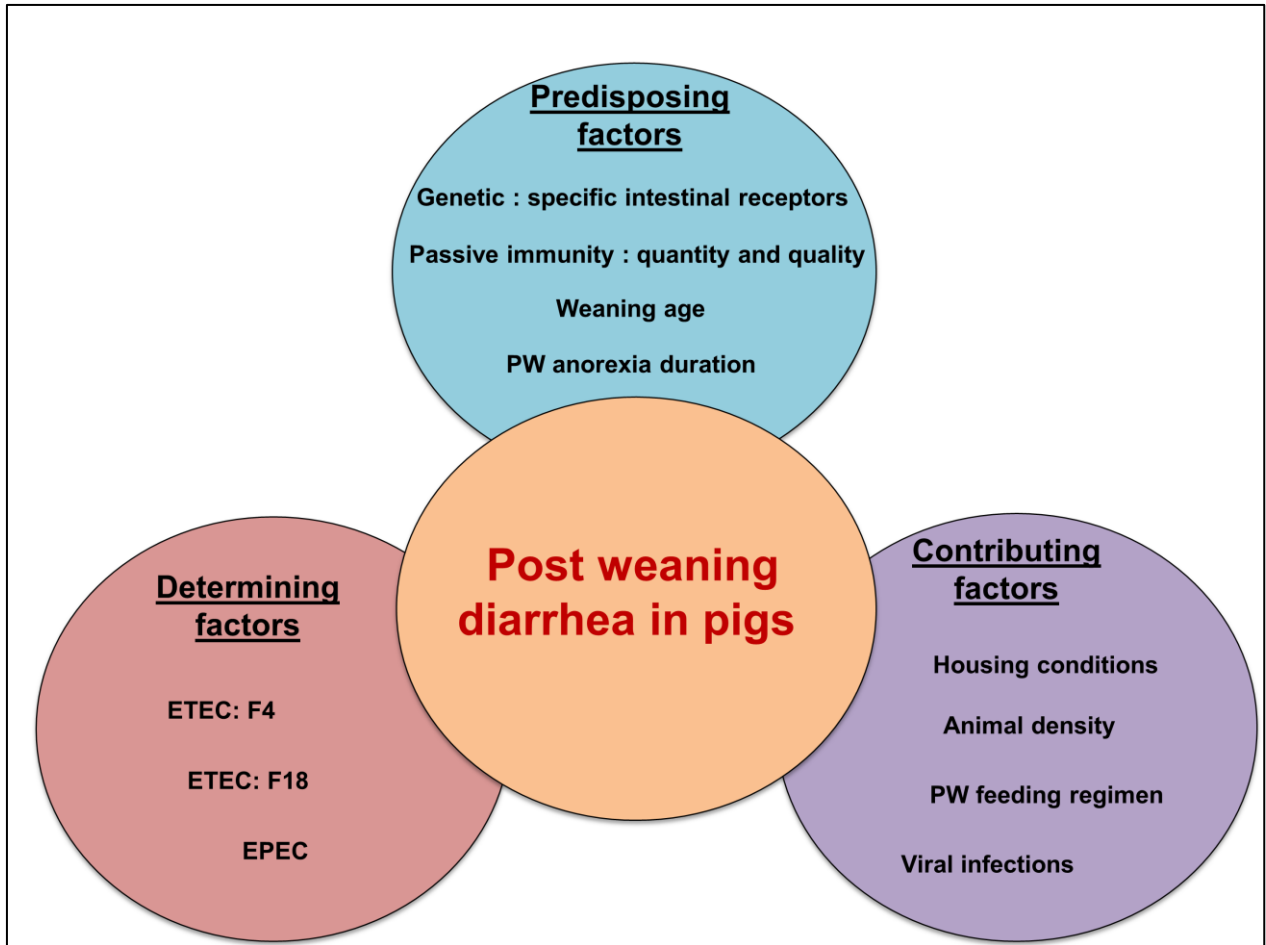
Figure 6: The multifactorial genesis of post weaning diarrhea (PWD) in pigs involves interaction between predisposing, contributing and determining factors.

PW: Post weaning.
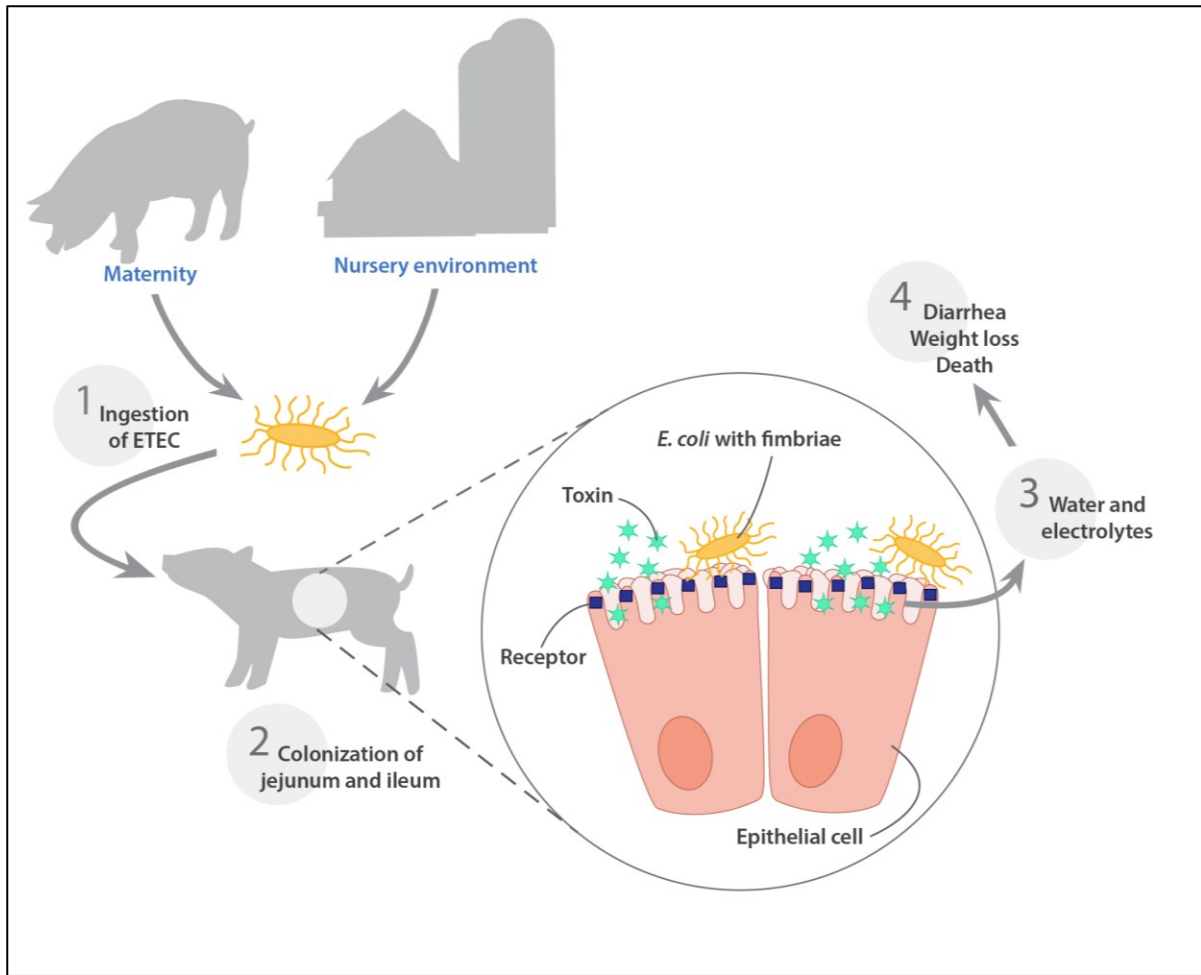
Figure 7: Schematic representation of the steps involved in the pathogenesis of post weaning diarrhea in pigs
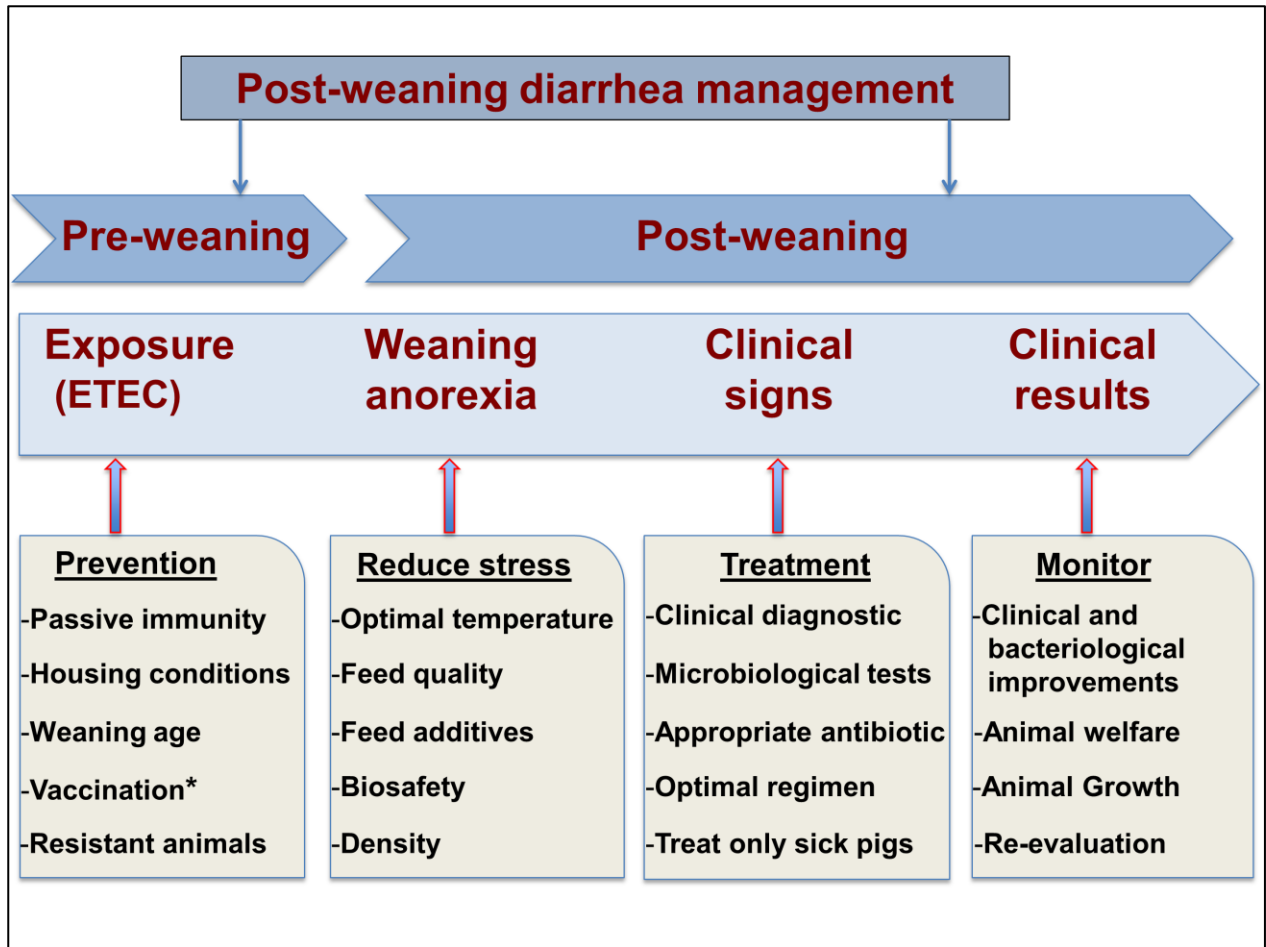
Figure 8: Illustrative interventions for the management of post-weaning diarrhea in pig farms. Inspired from (Kirby, 2011).

*Vaccination just prior to or at weaning

2. Problématiques, hypothèses et objectifs

Les problématiques de ce projet de recherche sont les suivantes : 1) les doses thérapeutiques de la CS utilisées dans les fermes porcines sont très différentes des doses recommandées par les monographies, 2) il n'a y pas de données microbiologiques et pharmacocinétiques dans la littérature scientifique pour l'utilisation de la CS lors du traitement de la DCPS chez le porc, 3) il n'y a pas de description de l'évolution de la résistance d'*E. coli* à la CS consécutive à son utilisation thérapeutique chez le porc dans la littérature scientifique.

Diverses hypothèses ont été élaborées en lien avec les problématiques du projet, 1) la CS subit une dégradation digestive dans le tractus gastro-intestinal du porc, 2) l'utilisation orale de la CS pour le traitement clinique de la DCPS améliore les symptômes cliniques de la maladie, réduit l'excrétion fécale d'*E. coli* et des gènes de virulence de ETEC : F4 (STa, STb, LT et F4), et améliore la croissance des animaux traités, 3) l'utilisation thérapeutique de la CS augmente l'excrétion fécale des isolats d'*E. coli* résistants à cet antibiotique, 4) l'infection expérimentale à ETEC : F4 augmente l'absorption intestinale de la CS chez des porcelets sevrés.

Les objectifs spécifiques de l'étude étaient : 1) de déterminer la stabilité de la CS dans une simulation du liquide gastrique chez le porc et d'évaluer l'activité antibactérienne *in vitro* des produits de dégradation de la CS, 2) de mesurer l'efficacité thérapeutique de deux doses de CS dans le traitement oral de la DCPS induite expérimentalement, 3) de suivre l'évolution de la résistance d'*E. coli* à la CS consécutive à son utilisation thérapeutique pour le traitement de la DCPS, 4) d'évaluer l'effet d'une infection à ETEC : F4 dans un modèle d'infection expérimentale de DCPS sur la modification de l'absorption intestinale de la CS chez le porc, 5) de générer des données pharmacocinétiques relatives à la CS, suite à son administration orale chez des porcelets sevrés sains comparativement à des porcelets infectés par ETEC : F4.

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3. Gastric stability and oral bioavailability of colistin sulfate in pigs challenged or not with *Escherichia coli* F4 (K88)

Contribution du candidat:

J'ai participé à l'élaboration du protocole de recherche en proportion équivalente de celle des autres coauteurs. J'ai effectué les collectes des échantillons (sang et matières fécales) et j'ai effectué toutes les analyses de laboratoire, à l'exception des analyses par HPLC-MS/MS qui ont été effectuées par Dr Francis Beaudry. J'ai analysé les résultats. J'ai rédigé l'article conformément aux exigences de la revue et j'ai intégré les commentaires faits par les coauteurs ainsi que ceux formulés par les réviseurs de l'article.

3.1   Abstract

The aim of the present study was to investigate the *in vitro* gastric stability of colistin sulfate (CS) and its antimicrobial activity against *E. coli* and to study the impact of ETEC: F4 (K88) infection in pigs on CS intestinal absorption. The stability profile of CS was evaluated in a simulated gastric fluid (SGF). Antimicrobial activity of CS and its degradation products were examined in a 96-well polystyrene microplate model. The effect of experimental infection with ETEC: F4 on CS intestinal absorption was determined by quantification of CS systemic concentration using a validated LC–MS/MS method. A rapid degradation of CS accompanied by an increase in CS antimicrobial activity by comparison with non-degraded CS ($p < 0.0001$) was observed in SGF. Additionally, CS levels were not quantifiable in systemic circulation using a highly sensitive method and concurrent oral challenge did not affect CS absorption in an induction model of subclinical post-weaning diarrhea (PWD).

Keywords: Colistin sulfate, pigs, *E.coli*, gastric stability, antimicrobial activity, intestinal absorption.

3.2   Introduction

Colistin, also known as polymyxin E, is a polypeptide antibiotic with significant *in vitro* activity against several multi-resistant Gram-negative (MRGN) pathogens, in particular *Pseudomonas aeruginosa* (Tunyapanit et al., 2013; Walkty et al., 2009), *Acinetobacter baumannii* (Liu et al., 2014) and *Klebsiella pneumoniae* (Ku et al., 2013). For these bacterial species, polymyxins are sometimes the only available active antibiotics in human medicine (Bergen et al., 2012). Given the importance of colistin for treatment of serious bacterial infections in humans and the limited availability of alternative antimicrobials for effective treatment of MRGN pathogens, Health Canada has classified this antibiotic in the category of very high

importance in human medicine (Category I) (Government of Canada, 2014).

The chemical structure of colistin consists of a hydrophilic cycloheptapeptide ring with three positively charged amine groups, a tail tripeptide moiety with two positively charged amine groups, and a hydrophobic acyl chain tail (Azzopardi et al., 2013; Biswas et al., 2012) (Fig. 9). The amino groups mediate both the bactericidal effect and toxicity to human cells (Clausell et al., 2007b; Mares et al., 2009). The target of antimicrobial activity of colistin is the bacterial cell membrane. This antibiotic has a strong positive charge and a hydrophobic acyl chain allowing a high binding affinity for lipopolysaccharide (LPS) molecules (Azzopardi et al., 2013). Colistin interacts electrostatically with LPS and competitively displaces divalent cations, causing disruption of the outer cell membrane that results in an increase in the permeability of the cell envelope, leakage of intracellular contents and, subsequently, bacterial death (Clausell et al., 2007b). Antimicrobial susceptibility testing for colistin can be performed using disc diffusion, E-test, agar dilution, and broth dilution (Balaji et al., 2011). Different susceptibility breakpoints for colistin have been used by different organizations (Bergen et al., 2012). The *Société Française de Microbiologie* has selected $\leq$ 2 mg/L as the susceptibility breakpoint and >2 mg/L as the resistance breakpoint, whereas the British Society for Antimicrobial Chemotherapy selected $\leq$ 4 mg/L and $\geq$ 8 mg/L as the susceptibility and resistance breakpoint, respectively (Li et al., 2005).

Colistin sulfate (CS) has been used in the livestock industry in many countries and is the recommended treatment in swine medicine for gastrointestinal tract infections, particularly for those caused by *Escherichia coli* (Belloc et al., 2008; Callens et al., 2012a; Casal et al., 2007a). Post-weaning diarrhea (PWD) is an economically important disease in pigs due to financial losses as a result of mortalities, morbidity, reduced growth performance of surviving pigs, and cost of medication (Fairbrother et al., 2005). The predominant cause of PWD in pigs worldwide and in Canada is Enterotoxigenic *E. coli* (ETEC) of O group 149 (Fairbrother et al., 2005; Jamalludeen

et al., 2007). ETEC O149 is characterized by the production of fimbriae F4 (K88) that mediate bacterial adherence to the intestinal mucosa and mediate heat stable and heat labile enterotoxins. Both families of enterotoxins enhance the secretion of sodium, chloride, and water into the intestinal lumen causing secretory diarrhea (Fairbrother and Gyles, 2012; Fairbrother et al., 2005). In pigs, CS is mainly used *per os* at a dosage of 50,000 IU/kg every 12 h for a period of 5 consecutive days for the treatment of intestinal infections caused by *E. coli*. This drug regimen has shown significant efficacy in the treatment of *E. coli* diarrhea (Belloc et al., 2008; Guyonnet et al., 2010). Colistin sulfate is used "off-label" in Canada for the treatment of PWD by transposition of data (dose, route of administration, dosing frequency) from countries where CS is approved.

In healthy pigs receiving therapeutic doses *per os*, it has been shown that CS is poorly absorbed. CS concentrations in the plasma were below the lower limit of quantitation (0.250 µg/mL) as determined by HPLC-UV (Guyonnet et al., 2010). Thus, the pig's intestinal microflora is exposed to the full dose of CS administered orally. On the other hand, there is little published data on the effect of bacterial gut infection in pigs on CS intestinal absorption. Such infections may affect bioavailability of oral antibiotics as a result of changes in intestinal hyperemia, tissue permeability, or intestinal peristalsis. Furthermore, there is no available information in the literature concerning the possible degradation of CS throughout a pig's digestive tract. This degradation may partly explain the low levels of CS systemically. In addition, there are differences in the withdrawal time between countries where this drug is approved for the treatment of colibacillosis in pigs (Official Journal of the European Union, 2010) due to the lack of data on CS intestinal absorption in pigs. Thus, understanding the stability of CS in the pig gastrointestinal tract is very important for interpreting results from pharmacokinetic and pharmacodynamic studies.

The first objective of this study was to investigate the *in vitro* gastric stability of CS and its antimicrobial activity with respect to two *E. coli* strains: the non-virulent strain ATCC 25922 and the virulent strain ETEC: F4 (K88). The second objective was to study the impact of experimental infection of piglets with ETEC: F4 (K88) on CS intestinal absorption levels using a highly sensitive analytical method (HPLC-MS/MS). Finally, the effect of a single oral dose of colistin (50,000 IU/kg) on the level of fecal shedding of ETEC: F4 (K88) and the total *E. coli* population were determined.

3.3 Material and methods

3.3.1 Stability of CS in simulated gastric fluid and antimicrobial activity of degradation products

The stability and degradation profiles of CS in simulated gastric fluid (SGF), prepared according to the United States Pharmacopoeia (United States Pharmacopeial Convention, 2009), were evaluated. Briefly, SGF was composed of 3.2 g/L pepsin and 2 g/L NaCl at a pH of 1.2. A quantity of 50,000 UI of CS (Daniel Bond & Frédéric Beaulac Inc., QC, Canada) was added to 500 ml of SGF when this solution reached 37°C. At each time point of 0 (before adding pepsin), 5, 10, 15, 30, 45, and 60 minutes, three samples were taken out. Each sample was composed of 333 µl of sample solution and 666 µl of acetonitrile. Samples were centrifuged at 12.000 g for 5 min. The supernatant was transferred into an injection vial. Colistin sulfate concentrations were determined at each time point using an HPLC-MS/MS method. Comparatively, a concentration of 32 µg of CS was used as a stock solution to evaluate antimicrobial activity of CS after acetonitrile neutralization by evaporation. Antimicrobial assays were conducted in a sterile 96-well polystyrene microplate and 100 µL of fresh Mueller Hinton broth was added to each well. Then, 100 µL of each time point sample (0, 5, 10, 15, 30, 45, and 60 minutes) *in duplicate* were

removed from the first well and double diluted from 8 µg/ml to 15 ng/ml. Two rows without CS in each plate were used as controls. One row was used as a positive control and contained *E. coli* ATCC 25922 or ECL8559 and the other row, without bacterial inoculum but containing 200 µL of Mueller Hinton broth, was the negative control. Finally, 100 µl of a bacterial count of 5.10^5 CFU/ml of *E. coli* ATCC 25922 or ECL8559 suspensions was inoculated in each well. Bacterial inocula were prepared from overnight cultures of *E. coli* ATCC 25922 and ECL8559 and were diluted in sterile saline solution (0.9%) standardized to a 0.5 McFarland standard. The bacterial cultures were then diluted one hundred-fold in Mueller Hinton broth and 100 µL of the final solution was added to each well of the 96-well plate within 10 min of inoculum preparation. In order to demonstrate the reproducibility of results, three digests (SGF) were used in this experiment and for each digest, two microplates were prepared for each bacterial strain. The microplates were incubated at 37°C for 24 h. The minimum inhibitory concentration (MIC) was then determined as the lowest concentration that resulted in inhibition of bacterial growth. Additionally, antimicrobial activity of acetonitrile and SGF without CS were tested to ascertain whether these two compounds interfere with CS antimicrobial activity.

3.3.2 Animals

Twenty-one healthy piglets 21 days of age at the beginning of experimentation were used in this study. Piglets were selected for the presence of the F4 receptor gene by PCR-RFLP as previously described (Daudelin et al., 2011). Each pig was individually housed in a pen, fed a standard non-medicated ration for post-weaning pigs and received water *ad libitum*. The room temperature was kept at 24-26°C. This experimental study was conducted in the biosecurity level 2 agro-environment platform for farm animals at the Faculté de Médecine Vétérinaire (FMV, Saint-Hyacinthe, QC, Canada) of the Université de Montréal. All procedures were approved by

the ethics committee of the FMV base on the guidance of the *Canadian Council on Animal Care* (*CCAC*).

3.3.3   Jugular catheterization of pigs, blood sampling, and analytic methods

After 2 days of acclimatization (23 d old), animals were restrained on a V-shaped table and were non-surgically cannulated as previously described (Matte, 1999). Each cannula was fitted with a flexible catheter that allowed the pig to freely move within the pen and permitted blood collection without handling the jugular vein. Blood samples (3 mL) from the catheter were collected in potassium EDTA tubes from one day after catheter placement (24 d old) until euthanasia (32 d old). These samples were used to assess the dehydration level following challenge and CS treatment, as determined by measuring changes in blood packed-cell volume (PCV) and plasma total protein (TP) as described elsewhere (Santiago-Mateo et al., 2012). Briefly, blood samples were placed in 75-mm capillary tubes and centrifuged for TP and PCV analysis. PCV was determined with a standard hematocrit total percentage chart. Plasma TP content was determined with a standard medical refractometer. An increase in PCV and plasma TP from pre-inoculation sample collection to post-inoculation sample collection served as an indication of dehydration.

After CS oral administration (30 d), blood samples (3 mL) were collected from the cannula at 30 min and 1, 2, 4, 6, 8, 12, 24, 36, and 48 h in potassium EDTA tubes. Plasma was separated by centrifugation at 2000 g for 10 min and stored at -20°C prior to analysis. Colistin sulfate plasma concentrations were determined by a liquid chromatography coupled with tandem mass spectrometry (HPLC-MS/MS). The HPLC system was a series 200 liquid chromatography apparatus (Perkin–Elmer, Boston, MA, USA) and the spectrometry system used was an API 2000 QTRAP AB-SCIEX (Concord, ON, Canada). Colistin sulfate was extracted from the pig plasma

using a protein precipitation method; 200 μL of plasma was mixed with 200 μL of internal standard solution (500 ng/mL tylosine in acetonitrile) in a 1.5 mL centrifuge tube. Samples were vortexed and allowed to rest 10 min at room temperature prior to centrifugation. Samples were centrifuged at approximately $12,000 \times g$ for 5 min and 200 μL of supernatant was transferred into an injection vial.

Chromatographic separation was performed using an isocratic mobile phase with a Thermo Aquasil C18 100×2.1 mm (3μm) column (Thermo Scientific, Waltham, MA, USA). The mass spectrometer was operated in the positive ion mode and the analysis was performed by multiple-reaction monitoring (MRM), as previously described (Ma et al., 2008). Data were acquired and analyzed with Xcalibur 1.4 (San Jose, CA, USA) and regression analyses were performed with PRISM (version 5.0d) GraphPad software (La Jolla, CA, USA) using a nonlinear curve-fitting module with an estimation of the goodness of fit. Calibration curves were calculated by using the equation $y = ax + b$, as determined by weighted $(1/x)$ linear regression of the calibration line constructed from the peak-area ratios of the drug and the internal standard.

3.3.4   Experimental challenge, antibiotic administration, and health status

Animals were divided into three groups of 7 pigs each: challenged treated (CT), challenged untreated (CNT), and non-challenged treated (NCT) group. The challenge strain for experimental infection of pigs was a nalidixic acid-resistant (Nal[r]) variant of ETEC: F4 strain ECL8559 (0149:LT: STa: STb: East1: paa: hemβ: F4) and was hemolytic when grown on blood agar, as previously described (Daudelin et al., 2011). The ETEC: F4 strain was kindly provided by the *OIE Reference Laboratory for Escherichia coli* at the Faculté de médecine vétérinaire (Saint-Hyacinthe, QC, Canada) of the Université de Montréal.

At 27 days of age, 14 pigs were orally challenged with 5 mL of trypticase soy broth

(Difco Laboratories, Inc., Detroit, MI, USA) containing 10^9 CFU of strain ECL8559 following the administration of 10 mL $CaCO_3$. Both administrations were performed using a syringe attached to a polyethylene tube. $CaCO_3$ was used in order to increase bacterial survival in the stomach and to aid safe transfer of the inoculum into the small intestine. At 30 days of age, pigs in the 2 treated groups received a single oral dose of CS at 50,000 IU/kg by oral gavage using a syringe attached to a polyethylene tube. Pigs in the untreated group received the same quantity of water. The single dose of CS was used to determine the area under the curve (AUC) of CS systemic concentration and to permit subsequent extrapolation of the AUC value to characterize the terminal phase following repeated CS administration. Fecal consistency, rectal body temperature measured using a digital thermometer, anorexia, and lethargy were monitored daily. Severity of diarrhea was quantified using a fecal consistency score (0, normal; 1, soft feces; 2, mild diarrhea; and 3, severe diarrhea), as described by (Jamalludeen et al., 2009), at 24, 48, 72, 96, and 120 h post challenge by a person with no prior knowledge of the treatment assignation.

3.3.5 Microbiological analysis of fecal samples and ileal mucosa

Faecal samples from the two challenged groups were collected before challenge and 24, 48, 72, 84, 96, 108, 120 h post challenge. These samples were used to examine the presence of the challenge strain ETEC: F4 and total *E. coli* population. A quantity of 10 g of feces was diluted 10-fold in peptone water and selected dilutions were plated on Petrifilm *E. coli*/Coliform count plates (3M, St Paul, MN, USA) and on 5% bovine blood agar plates containing 50 µg/mL of nalidixic acid (Sigma-Aldrich Canada Ltd., Oakville, ON, Canada) in order to count the total *E. coli* population and the haemolytic ETEC: F4 challenge strain, respectively. Plates were incubated aerobically for 24 h at 37°C.

Immediately after euthanasia at 5 days post-challenge, pigs of the two challenge groups

were necropsied and a 15-20 cm segment of the ileum of each pig was cleaved, placed in a sterile container with ice, and transferred to the laboratory within 30 min for evaluation of colonization of the ileal mucosa by the ETEC: F4 challenge strain, as previously described (Nyachoti et al., 2012). Briefly, ileal segments were opened longitudinally and feces removed by scraping the mucosal surface with a clamp. Ileal segments were then weighed diluted 10-fold in peptone water and mixed with a stomacher for one minute. Selected dilutions were plated on 5% bovine blood agar plates containing 50 μg/mL of nalidixic acid. The plates were incubated aerobically for 24 h at 37°C.

3.3.6   Statistical Analysis

Bacterial counts were $\log_{10}$ transformed prior to data analysis to normalize distributions. Colonization of the ileal mucosa by ETEC: F4 in the two groups was compared with the equal variance *t*-test. Total *E. coli* counts as well as ETEC: F4 counts were analyzed with a repeated-measures ANOVA with time (6 levels) as a within-subject factor and group as the between-subject factor. A priori contrasts were performed to compare group means at different time periods and to compare pre- and post-infection means in each treatment. For these multiple comparisons, the alpha level was adjusted downward using the Bonferroni sequential procedure. A similar procedure was used to analyze PCV and plasma TP with additional contrasts to investigate changes before and after challenge and before and after treatment. Ordinal diarrhea scores were analyzed with the Cochran-Mantel-Haenszel test at each time period. Quantitative MIC values were transformed into base 2 logarithms of dilution factor to reduce variability and then submitted to analysis with a linear mixed model with time as a fixed factor and trial as a random factor. Tukey's post-hoc tests were used to compare the mean value at time 0 with the mean values at each other time period. Statistical analyses were carried out with SAS v.9.3.

(Cary, N.C. USA) and the level of statistical significance was set at 0.05 throughout.

3.4 Results

3.4.1 *In vitro* CS gastric stability and its antimicrobial activity

Concentrations of CS from each time point obtained by HPLC-MS/MS showed a rapid degradation of CS in SGF. This deterioration started quickly (from the 5th minute after addition of pepsin) and reached the maximum at around 15 minutes after pepsin addition with 50% CS degradation (Fig. 10). At least four other peaks other than the CS peak were detected on HPLC-MS/MS mass chromatogram, probably corresponding to CS degradation products (M1, M2, M3, and M4) (Fig. 11). Theses metabolites were not identified and separated in this study. However, the antimicrobial activity of CS and its degradation products was tested in *vitro*.

The *in vitro* antimicrobial activity of CS and its potential degradation products were evaluated against *E. coli* ATCC 25922 and ECL8559 using a micro-broth dilution assay. Samples taken from SGF at each time point sampling showed antimicrobial activity against *E. coli* ATCC 25922 and ECL8559 that increased significantly ($P < 0.005$) over time after pepsin addition for the two *E. coli* strains compared with the non-degraded CS. These results were found for all three digest tests, conducted with the same protocol and the same experiment conditions (Fig. 12). Thus, gastric degradation of CS was not accompanied by a loss but rather an increase in antimicrobial activity. Nevertheless, the antimicrobial activity of the degraded CS did not increase over time and no statistically significant difference between MIC from each time point sampling after pepsin addition to the SGF ($P = 0.93$) was observed (Fig. 12). No statistically significant difference in the antimicrobial activity of CS and its degradation products was observed with respect to the bacterial strain of *E. coli* tested ($P = 0.99$). In addition, acetonitrile and SGF without CS did not demonstrate any antimicrobial activity.

3.4.2 CS plasma quantification and pharmacokinetic analysis

CS levels in plasma and in SGF were quantified by HPLC-MS/MS. The lower limit of quantitation (LLOQ) of the method was 20 ng/mL of plasma. The calibration curve was constructed by plotting the peak area ratio of colistin to the internal standard versus the nominal concentration (C) of the analyte. The linearity was determined by weighted (1/X) linear regression analysis. The regression equation of the calibration curve was then used to calculate the concentration of colistin in the plasma and in gastric fluid. In the 2 treated groups (challenged and non-challenged), the plasma concentration of CS was less than the lower limit of quantitation (20ng/mL) for all samples. In the non-challenged treated group, the concentration of CS was greater than the limit of detection (LOD) but less than LLOQ after 30 minutes of CS administration (Fig. 13). However, in the challenged treated group, the concentration of CS was less than the LOD at all sampling times (Fig. 14). Thus, based on the results found of this study, pharmacokinetic variables (C_{max}, AUC and $T_{1/2}$) for absorbed CS could not be determined. Bioavailability of CS could not be determined but would likely be negligible, based on these results.

3.4.3 Analysis of bacterial shedding and health status of challenged piglets

None of the animals in the challenged groups manifested severe clinical diarrhea or other clinical signs and no difference in plasma TP and PCV values between the two challenged groups following challenge or treatments was observed. However, one animal in the challenged treated group was not infected due to poor feed intake and was consequently removed from the experiment. The excretion of ETEC: F4 recovered from the feces throughout the experimental period for the experimentally challenged treated group compared with the challenged untreated group was expressed in log $_{10}$ CFU/g and shown in Fig. 15. After challenge, there was a rapid

initial increase in ETEC: F4 shedding in the feces of all challenged pigs that persisted for the 5 days post challenge (Fig. 15). The administration of a single oral dose of CS showed a tendency to reduce fecal ETEC: F4 counts during the next day following treatment, with a maximum effect at 24 h post-treatment (96 h post challenge). This reduction was not significantly lower than those of baseline values (before CS administration) ($P= 0.20$) and compared with the challenged untreated group ($P= 0.66$). After 24 h post treatment, the fecal ETEC: F4 excretion load increased to regain baseline values. However, at 48 h post treatment, ETEC: F4 counts were not different between the two challenged groups ($P= 0.052$) (Fig. 15). In both challenged groups, total *E. coli* counts demonstrated the same trend of decline as observed for ETEC: F4 (i.e. maximum effect was observed at 24 h post-treatment, 96 h post challenge) (data not shown). This reduction in the treated group was not significantly lower than those of baseline values (72 h post challenge) ($P= 0.20$) and compared with the challenged untreated group ($P= 0.06$). A similar trend was observed at 48 h post treatment, with no difference in total *E. coli* counts between the two challenged groups ($P= 0.13$).

At necropsy, no weight difference was observed between challenged and non-challenged pigs. Examination of the large intestine did not reveal watery or softened contents and macroscopic lesions in the intestinal mucosa were not observed in any of the pigs. Mesenteric lymph nodes did not show a difference in size in pigs of the challenged groups as compared with those of the non-challenged group. Treatment with CS did not result in a reduction in the attachment of ETEC: F4 to the ileal mucosa compared with the challenged untreated group ($P= 0.72$). None of the pigs developed diarrhea before being challenged and neither the challenge strain nor any other ETEC was detected by multiplex PCR in pigs prior to the challenge nor in any of the non-challenged pigs post challenge. The severity of diarrhea in challenged pigs assigned to the treated and untreated groups, based on their diarrhea scores at 24, 48 and 72 h

after challenge but before treatment, was not significantly different between the two challenged groups (Fig. 16). After challenge, only one pig in the challenged treated group had severe diarrhea at days 2 and 3 (score 4) after challenge but before treatment. On the other hand, challenged pigs showed a slight softening of faeces after challenge, with maximum softening being observed at 72 h post challenge and a mean diarrhea score of 2 (Fig. 16). Baseline mean values of diarrhea score at 72 h post challenge were not different between the two challenged groups (Cochran-Mantel-Haenszel; $P = 0.13$). However after CS treatment, there was a tendency of diarrhea score reduction in the treated group, especially at 120 h after challenge, compared with the untreated group (Cochran-Mantel-Haenszel; $P = 0.06$) (Fig. 16). In the current study, a reduction in fecal shedding of ETEC: F4 was correlated with a reduction of diarrhea score after 24 h of CS treatment.

3.5   Discussion

The aim of the present work was to investigate the *in vitro* gastric stability of CS to explain the low systemic concentrations obtained after oral administration of this drug in pigs and to determine the effect of CS gastric passage on its antimicrobial activity. We subsequently studied the impact of ETEC: F4 (K88) infection on the CS intestinal absorption level in pigs, using a new high sensitivity analytical method (HPLC-MS/MS). The results of the *in vitro* gastric simulation test showed that less than 50% of CS could be delivered to the intestine for potential absorption as an intact molecule. Indeed, presence of peptide bonds in CS side chains may predispose this structure to pepsin enzymatic degradation (Motyan et al., 2013). On the other hand, *in vitro* simulation of the gastric passage of CS results in the formation of a number of degradation products, depending on the number of peptide bonds cleaved in CS side chains (number of free amino groups formed following pepsin action). The retention of high

antimicrobial activity of the degradation products (M1, M2, M3, and M4) in comparison with the non-degraded CS may be explained by the loss of CS side chains, which generate several metabolites in the mixture with significantly less steric hindrance; this favors more interaction with LPS, resulting in the higher antimicrobial activity that was observed in this study. In addition, the structure of CS comprises a cyclic heptapeptide ring attached to a tripeptide which in turn is attached to a hydrophobic acyl chain, resulting in an amphiphilic structure (Govaerts et al., 2003; Orwa et al., 2002). Thus, a hydrophilic, polycationic cyclic heptapeptide with three positively charged amino groups – which remain after side chain removal – plays a central role in bactericidal activity (Azzopardi et al., 2013).

In order to determine the impact of ETEC: F4 (K88) infection on CS intestinal absorption in pigs, we used a sensitive analytical method (HPLC-MS/MS) for the quantification of CS in pig plasma to determine pharmacokinetic parameters. A single dose of CS was used to determine AUC and the elimination rate constant of (λ_z), an important parameter in order to determine CS elimination half-life ($T_{1/2}$), which is an index of drug persistence in the body (Toutain and Bousquet-Melou, 2004). These parameters will allow us to determine the most appropriate withdrawal period to protect consumers against the potential risk of the presence of CS residues in pig meat. Indeed, the recommended withdrawal period of CS in pigs after oral administration of the same molecule, dose, and route of administration differs between countries (Official Journal of the European Union, 2010).

In our study, healthy pigs showed a trace plasmatic concentration of CS at 30 minutes after a single oral administration of CS at 50,000 IU/Kg, and these trace concentrations were below the LLOQ (20 ng/mL) but above the limit of detection. Thus, the bioavailability of CS in healthy pigs following oral administration is not quantifiable despite the use of a highly sensitive analytical method, confirming previous reports demonstrating that colistin is poorly absorbed and

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systemic concentrations are usually undetectable (Guyonnet et al., 2010). Moreover, in challenged treated groups, CS systemic concentrations were not detected in any of the samples analyzed. Thus, the different withdrawal periods in various countries are not related to the presence of CS systemic residues but is rather a choice for public health consideration. Our results correlate with those of Jensen *et al.* (Jensen et al., 2004). These authors demonstrated that oral infection with *E. coli* O149:F4 was responsible for a decrease of systemic amoxicillin bioavailability compared with the non-infected group. In addition, *E. coli* may induce a mild intestinal inflammatory response in pigs during PWD (Bosi et al., 2004). This intestinal inflammation may also contribute to CS degradation. Indeed, during the inflammatory response, inflammatory cells, particularly leukocytes and macrophages, are able to produce highly reactive oxygen species (ROS) such as superoxide (O_2-), hydrogen peroxide (H_2O_2), and nitric oxide (NO) (Labow et al., 2002). The oxidative effect of these species may cause CS chain side breakdown, contributing to CS intestinal degradation, and may explain in part the absence of CS systemic concentrations in challenged treated piglets. The role of intestinal inflammation and the effect of ROS in the alteration of CS oral bioavailability were not investigated in this study but remain hypotheses to be studied in future studies.

Enterotoxins produced by the ETEC: F4 cause secretion of water and electrolytes leading to diarrhea with few microscopic lesions (Fairbrother and Gyles, 2012; Neog et al., 2011). Also, after weaning in pigs, villous height is generally reduced and crypt depth increased, which may be associated with increased occurrence of diarrhea and decrease of intestinal absorption (Vente-Spreeuwenberg et al., 2004). Thus, the presence of diarrhea, the effect of enterotoxins, and intestinal mucosal changes may partially explain the non-detection of CS systemic concentrations in challenged pigs. Our results were coherent with those of Nabuurs *et al*. (Nabuurs et al., 1994), who found that weaning and *E. coli* infection decreased absorption of fluid, potassium, or

chloride (Nabuurs et al., 1994). In addition, hepatic first pass metabolism effect may alter CS oral bioavailability, although little information about this subject is available in the literature.

In intensive livestock production such as in pig herds, metaphylactic antimicrobials are often used by oral route (Phillips et al., 2004). Our study demonstrates that in the case of metaphylactic administration of CS, this antibiotic was not quantifiable using a sensitive analytical method (HPLC-MS/MS), although it was difficult to conclude that CS degradation products were absent in systemic circulation. Further studies are needed to characterize CS degradation products in plasma and meat after CS oral treatment of bacterial intestinal infections in pigs.

In the current study, maximum ETEC: F4 shedding and diarrhea score were observed 72 h post challenge. This result is inconsistent with other experimental studies in which higher frequency of watery diarrhea was observed after the first day of the oral challenge with ETEC: F4 (Jensen et al., 2006; Jensen et al., 2004; Wellock et al., 2008). In addition, PWD is a multifactorial disease, where the combination of factors necessary to induce diarrhea has not yet been fully elucidated (Jensen et al., 2006). The oral challenge of pigs with pathogenic *E. coli* has been used widely as a model of PWD (Bhandari et al., 2008; Jensen et al., 2006). Similarly, our challenge experiments using a clinical strain of ETEC: F4 revealed various degrees of pig scouring depending on the response of each animal following challenge. Finally, the lack of difference between the CS treated and untreated challenge groups with respect to fecal shedding of ETEC: F4 and total *E. coli*, ETEC: F4 colonization of the ileal mucosa, and diarrhea score may be explained by the use of a single oral dose of CS at a concentration of 50,000 IU/kg. Indeed, CS is used clinically in pigs for the treatment of colibacillosis at a dosage of 50,000 IU/kg every 12 h for 5 days (Casal et al., 2007a; Guyonnet et al., 2010).

3.6   Conclusion

To our knowledge, this is the first study of the *in vitro* gastric stability of CS showing that this antibiotic was highly degraded in SGF, which led to the formation of degradation products that have a significant antimicrobial activity compared with non-degraded CS. The oral bioavailability of CS in pigs was monitored by a new highly sensitive method. However, the results indicate that CS levels were not quantifiable in the systemic circulation following oral administration in pigs and that concurrent oral challenge with an ETEC: F4 strain did not increase CS absorption in a subclinical induction model of PWD. In addition, a single oral dose of CS resulted in slightly reduced bacterial counts of ETEC: F4 and total *E. coli* populations in the feces. Knowing that CS is very poorly absorbed by pigs, further studies are needed to evaluate the effect of oral CS on ETEC: F4 and total *E. coli* populations in a complete treatment model and to characterize the impact of CS treatment on antimicrobial resistance of pathogenic and commensal *E. coli* in pigs.

3.7 References

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3.8 Figures

Chemical Formula: $C_{52}H_{98}N_{16}O_{13}$
Molecular Weight: 1155,43
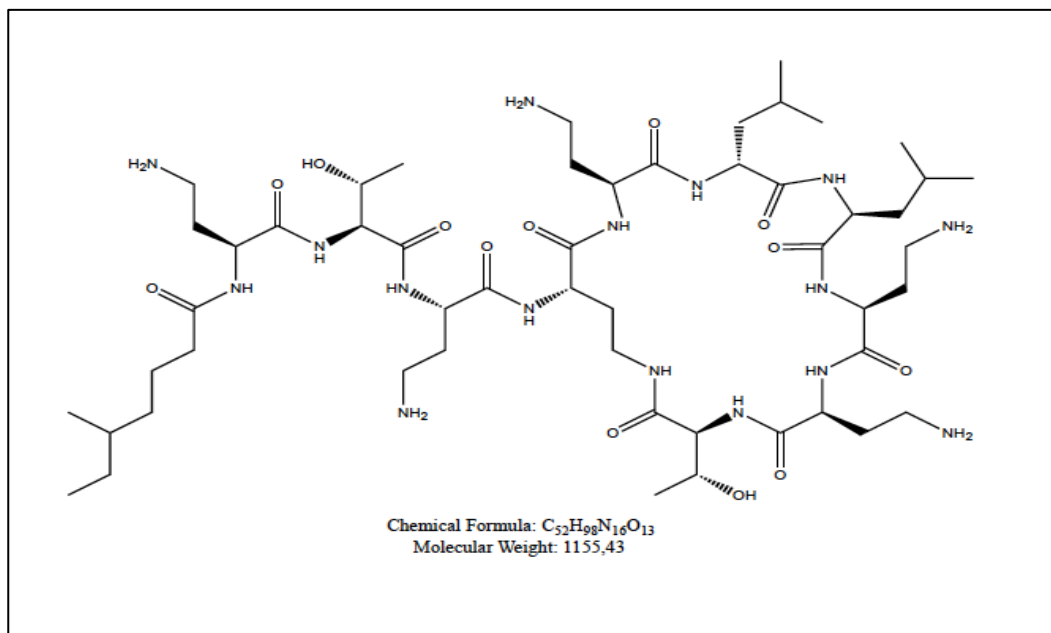
Figure 9: The colistin structure is composed of a hydrophilic cycloheptapeptide ring with three positively charged amine groups, a tail tripeptide moiety with two positively charged amine groups, and a hydrophobic acyl chain tail.
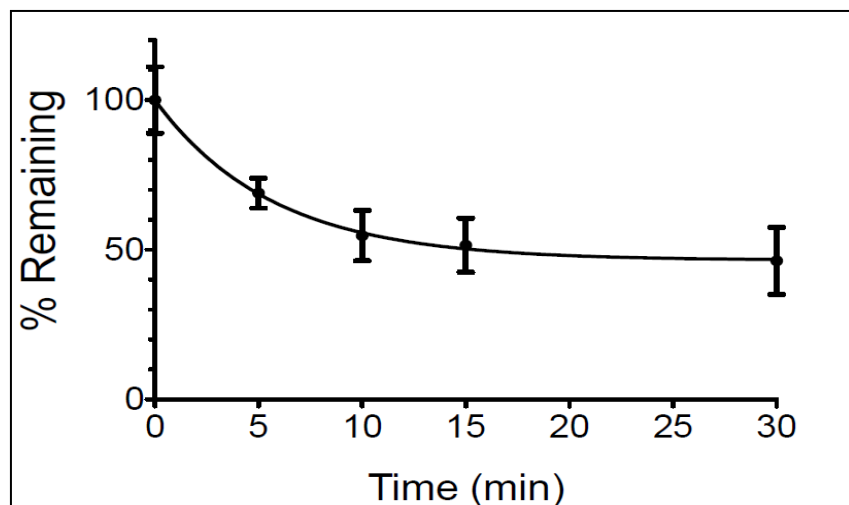
Figure 10: Degradation profile of colistin sulfate (CS): Evolution of CS concentrations over time in a simulated gastric fluid (SGF) as obtained by HPLC-MS/MS.

Degradation of CS started rapidly at 5 minutes after pepsin addition and reached the maximum around 15 minutes with 50% CS degradation.
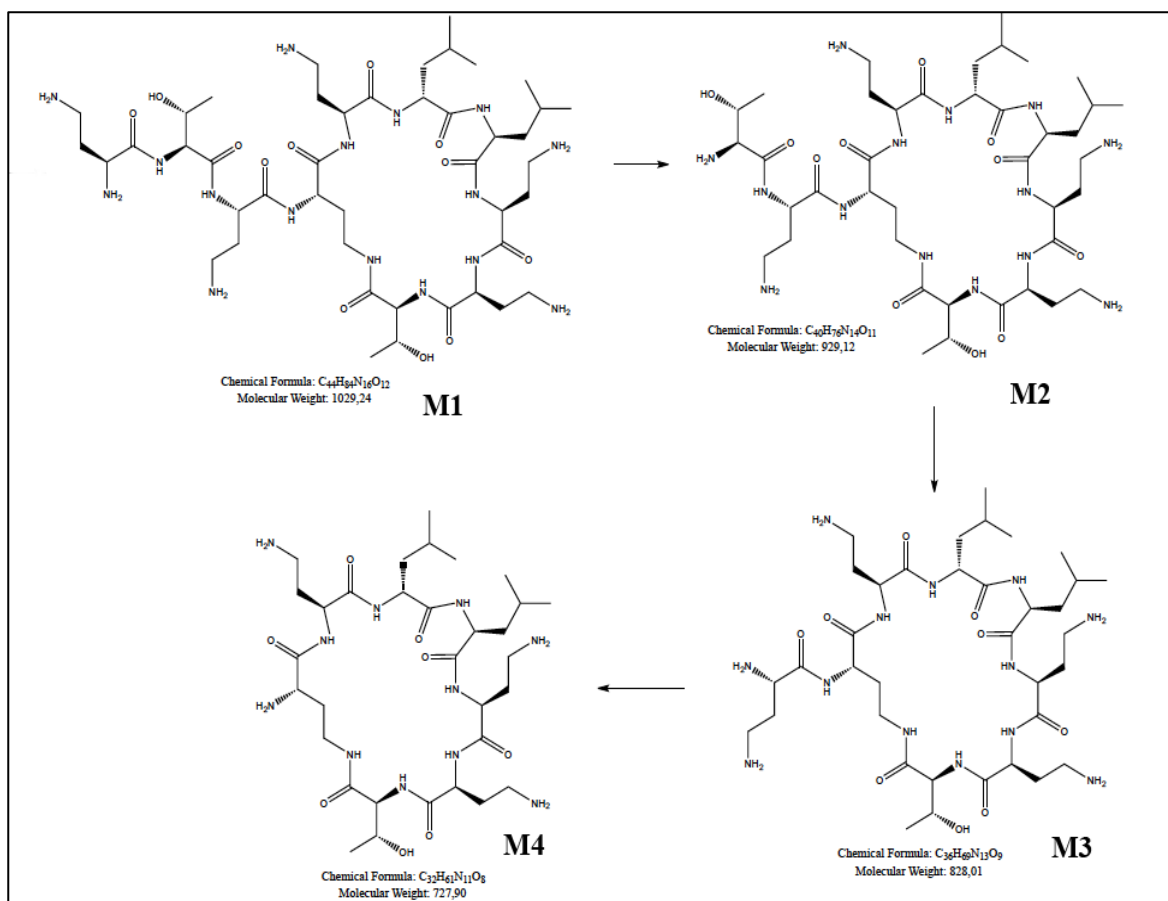
Figure 11: Degradation products (M1, M2, M3, and M4) of colistin sulfate (CS) formed by the enzymatic action of pepsin on peptide bonds in the CS side chain.

The number of degradation products formed is a function of the number of peptide bonds cleaved in the CS side chain (number of free amino groups formed following pepsin action). Adapted from (Biswas et al., 2012).
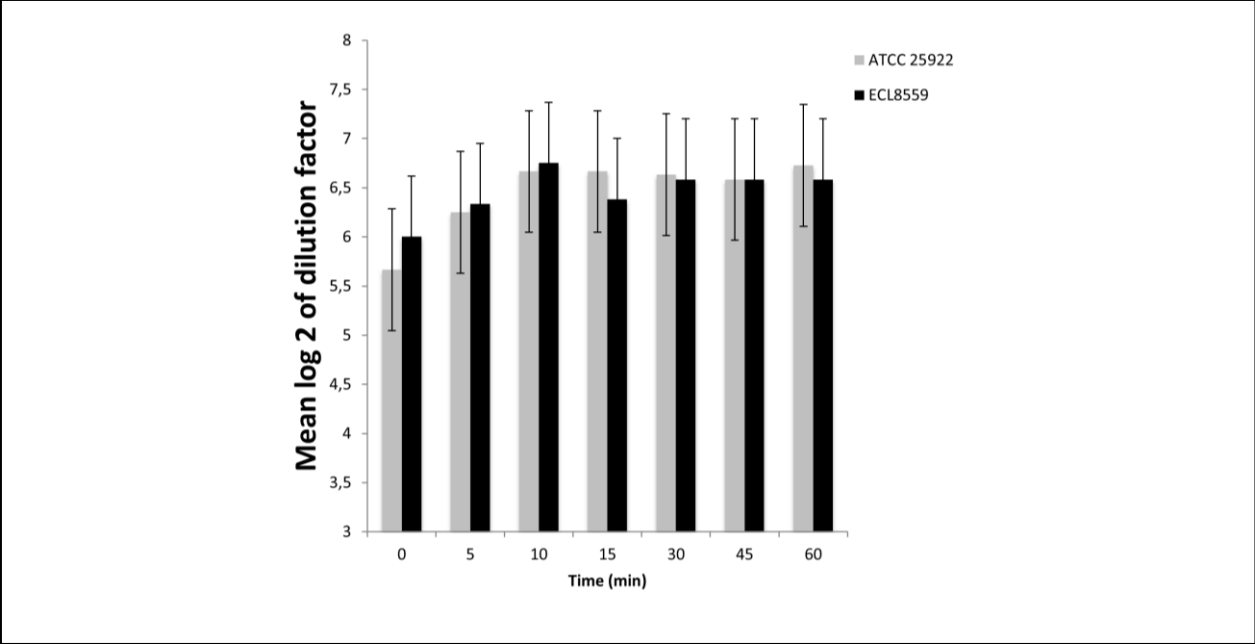
Figure 12: Mean log 2 of dilution factor ± standard deviation (SD) of minimum inhibitory concentration (MIC) value distributions of non-degraded (t=0) and degraded colistin sulfate (CS) against *E. coli* ATCC 25922 or ECL8559 over time.

Mean log 2 of dilution factor values increased significantly ($P < 0.005$) over time for the two *E. coli* strains tested by comparison with the non-degraded CS (t=0).

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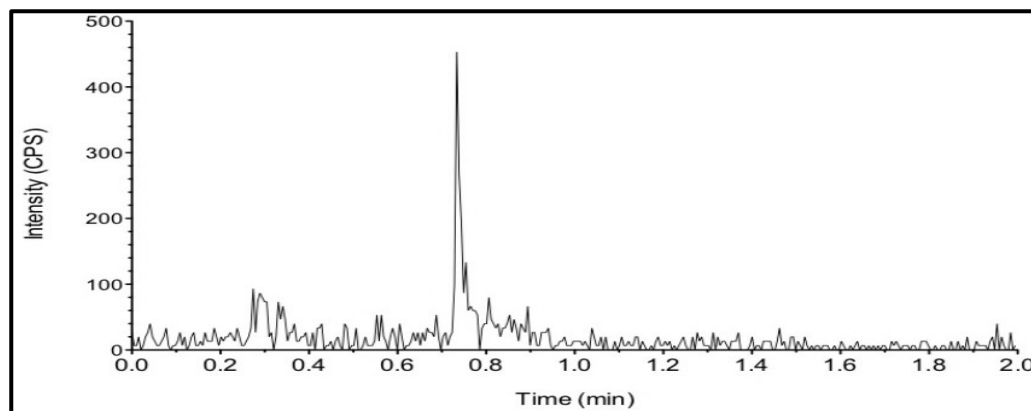
Figure 13: HPLC-MS/MS mass chromatogram of a typical sample from the non-challenged group at 30 min following oral colistin sulfate (CS) administration.

Plasmatic concentrations of CS were above the limit of detection but significantly less than the limit of quantitation (20 ng/mL). Colistin sulfate was not detected at other time points.
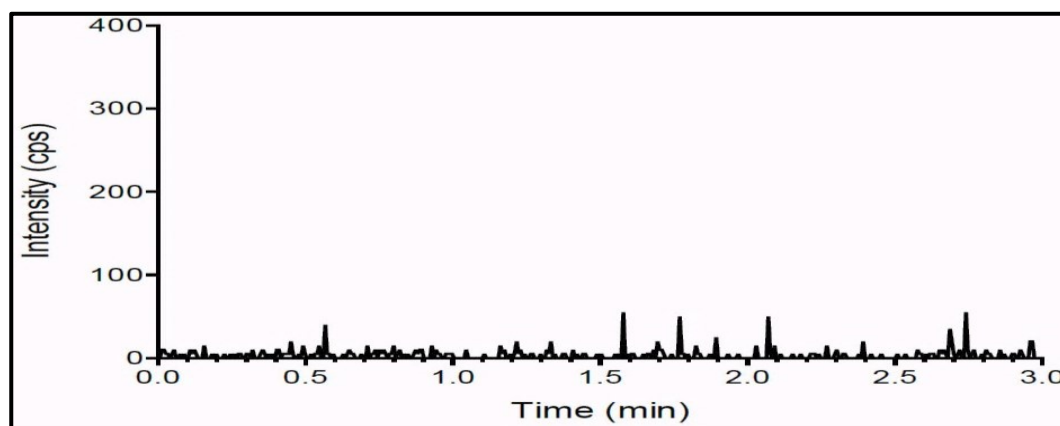
Figure 14: HPLC-MS/MS mass chromatogram of a typical sample from the challenged group at 30 min following oral colistin sulfate (CS) administration.

Plasmatic concentrations of CS were less than the limit of detection. CS was not detected at other time points.
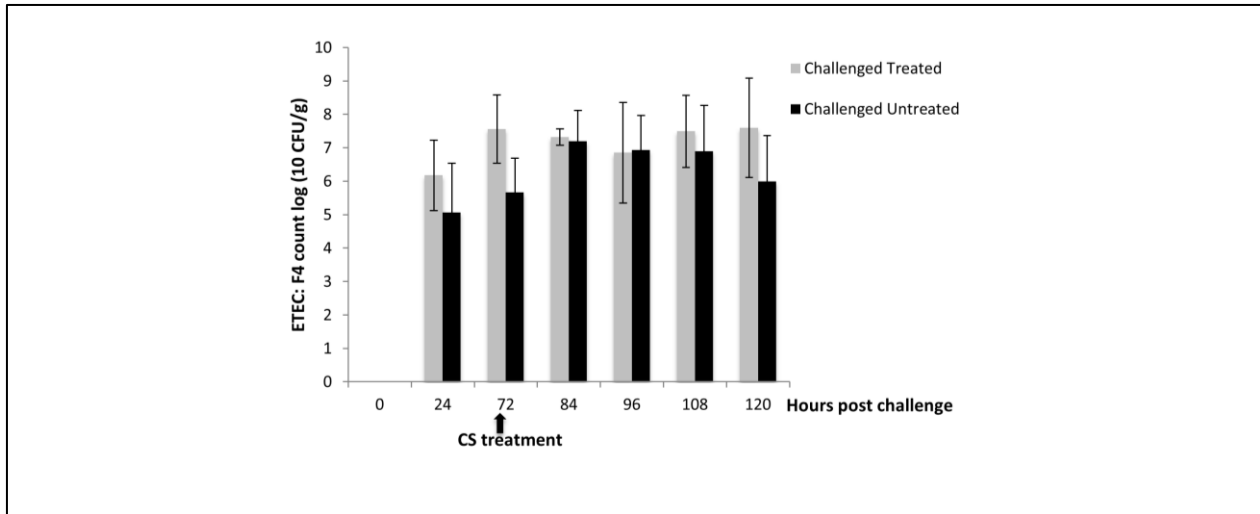
Figure 15: Evolution of fecal ETEC: F4 counts (means ± standard deviation [SD]). Challenge was performed at 0 hour and treatment with colistin sulfate (CS) was carried out at 72 hours post challenge.

Maximum effect of CS was observed at 24 h post-treatment (96 h post challenge).
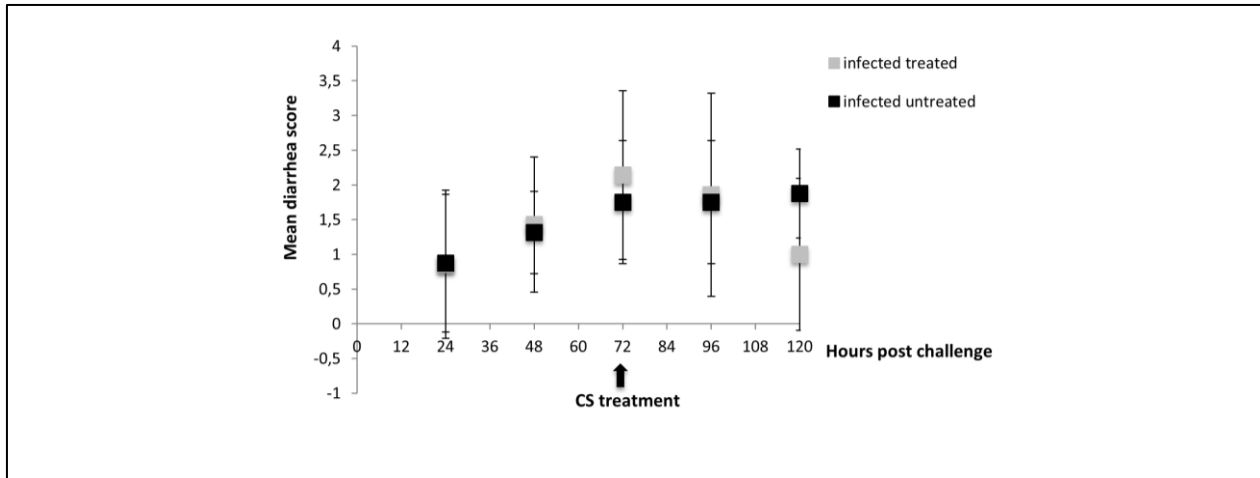
Figure 16: Effect of colistin sulfate (CS) treatment (72 h) on mean diarrhea score (± standard deviation [SD]) of weaned pigs challenged with ETEC: F4 (0 h).

A trend towards a reduction of diarrhea score was observed after CS treatment (120 h) compared with the untreated group. Mean diarrhea score = sum of daily diarrhea score/number of animals.

4. ***In vivo*** **therapeutic efficacy and pharmacokinetics of colistin sulfate in an experimental model of enterotoxigenic *Escherichia coli* infection in weaned pigs**

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Contribution du candidat:

J'ai participé à l'élaboration du protocole de recherche en proportion équivalente de celle des autres coauteurs. J'ai effectué les collectes des échantillons (sang et matières fécales) et j'ai effectué toutes les analyses de laboratoire, à l'exception des analyses par HPLC-MS/MS qui ont été effectuées par Dr Francis Beaudry. J'ai analysé les résultats. J'ai rédigé l'article conformément aux exigences de la revue et intégré les commentaires faits par les coauteurs ainsi que ceux formulés par les réviseurs de l'article.

4.1   Abstract

Enterotoxigenic *Escherichia coli* (ETEC: F4) associated with post-weaning diarrhea (PWD) in pigs has developed resistance against several antimicrobial families, leading to increased use of colistin sulfate (CS) for the treatment of this disease. The objective of this study was to determine the efficacy of oral CS treatment in experimental PWD due to ETEC: F4 challenge and determine the effect of this challenge on CS intestinal absorption. In this study, 96 pigs were divided into two trials based on CS dose (100,000 IU/kg or 50,000 IU/kg). Fecal shedding of ETEC: F4, total *E. coli*, and CS-resistant *E. coli*, diarrhea scores, and weight changes were evaluated. Colistin sulfate plasma concentrations were determined by HPLC-MS/MS.

Regardless of the dose, CS treatment resulted in a reduction of fecal ETEC: F4 and total *E. coli* shedding, and in diarrhea scores but only during the treatment period. However, CS treatment resulted in a slight increase in fecal shedding of CS resistant *E. coli* and did not prevent weight loss in challenged pigs. In addition, challenge with ETEC: F4 resulted in an increase of CS intestinal absorption.

Our study is among the first to demonstrate that under controlled conditions, CS was effective in reducing fecal shedding of ETEC: F4 and total *E. coli* in experimental PWD. However, CS treatment was associated with a slight selection pressure on *E. coli* and did not prevent pig weight loss. Further studies are needed in field conditions, to better characterize CS therapeutic regimen efficacy and bacterial resistance dissemination.

Keywords: Colistin sulfate, post-weaning diarrhea, pigs, *E. coli*, resistance, intestinal absorption, HPLC-MS/MS.

4.2   Introduction

Escherichia coli post-weaning diarrhea (PWD) is an economically important disease in pig production worldwide (Amezcua et al., 2002b; Fairbrother et al., 2005; Frydendahl et al., 2003). This disease affects pigs mostly during the two weeks after weaning and is characterized by a reduction in feed intake, poor growth rate, diarrhea and mortality (Fairbrother et al., 2005). These disturbances are most commonly associated with the proliferation of enterotoxigenic F4-positive *E. coli* (ETEC: F4) (Fairbrother et al., 2005), the most predominant sero-virotypes being O149: LT: STb: F4 and O149: LT: STa: STb: F4 [3, 5]. Small intestine epithelial cell adhesion and subsequent colonization by ETEC: F4 is mediated by the F4 fimbriae via specific receptors (F4R), crucial in determining the susceptibility of pigs to ETEC infection (Fairbrother et al., 2005; Nagy and Fekete, 2005). Because ETEC: F4 isolates from PWD have shown a high frequency of resistance to multiple antimicrobials (Amezcua et al., 2002b; Maynard et al., 2003), therapeutic failure is common and alternative molecules need to be found. Colistin sulfate (CS), a cationic antimicrobial peptide, is one possible candidate for the treatment of PWD, which is approved for use in pigs in several countries (Catry et al., 2015; Official Journal of the European Union, 2010). However, CS is not yet approved for use in pigs in other countries such as Canada and is used under veterinarian responsibility for the treatment of PWD (Rhouma et al., 2015).

The bactericidal effect of CS is the result of an electrostatic interaction between the cationic elements of CS and anionic lipopolysaccharide (LPS) molecules in the membrane of Gram-negative bacteria, leading to the displacing of magnesium (Mg^{2+}) and calcium (Ca^{2+}) – stabilizers of LPS molecules – from the LPS (Yu et al., 2015). This process results in an increase in the permeability of the cell envelope, leakage of cell contents, and subsequent cell death (Azzopardi et al., 2013; Theuretzbacher et al., 2015).

Several studies from different countries have reported isolation from pigs of *E. coli* resistant to

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colistin (Boyen et al., 2010; Costa et al., 2010; Harada et al., 2005; Kempf et al., 2013; Mateu and Martin, 2000; Morales et al., 2012). The most common mechanisms of resistance to CS in *E. coli* are modifications of the LPS with the addition of positively charged groups, such as L-4-aminoarabinose (L-Ara4N) and/or phosphoethanolamine (pEtN) (Breazeale et al., 2005; Needham and Trent, 2013; Olaitan et al., 2014). More recently, Liu and collaborators have demonstrated the presence of a stable plasmid mediated *mcr-1* gene that encodes for *E. coli* colistin resistance (Liu et al., 2016).

In pigs, CS is mainly administered *per os*, at the recommended dose of 50,000 IU/kg b.w. every 12 h for a period of 3 to 5 consecutive days for the treatment of intestinal infections caused by *Enterobacteriaceae* (Guyonnet et al., 2010; Official Journal of the European Union, 2010). However, this dose regimen is often not respected on farms (Official Journal of the European Union, 2010). Several reports have shown that the recommended dose (Casal et al., 2007b; Chauvin et al., 2002; Van Rennings et al., 2015) or duration (Chauvin et al., 2002; Van Rennings et al., 2015) of CS treatment is often surpassed.

In addition, the efficacy of CS at the dose of 50,000 IU/kg for the clinical treatment of PWD has not been investigated and no data are available in the literature on the role of this therapeutic regimen in exacerbating of *E. coli* resistance in pigs. Several studies have confirmed that CS is poorly absorbed in pigs after oral administration (Guyonnet et al., 2010; Rhouma et al., 2015). However, little is known of the effect of ETEC: F4 infection with clinical PWD on CS intestinal absorption, following the use of CS in a conventional therapeutic regimen. An increase of CS intestinal absorption could have an impact on the withdrawal time following oral administration of this antibiotic. Moreover, in countries where CS is approved in pig, this varies from 1 to 7 days (Official Journal of the European Union, 2010).

Hence, the main objective of the present study was to evaluate the effect of CS treatment in an

experimental PWD model on fecal ETEC: F4 and total *E. coli*, on *E. coli* resistance to CS, on fecal consistency, growth rates, and rectal body temperature of weaned pigs. In addition, the effect of ETEC: F4 infection on CS intestinal absorption levels was determined using a high-performance liquid chromatography coupled with tandem mass spectrometry (HPLC-MS/MS).

4.3 Material and methods

The experimental protocol (14-Rech-1729), was reviewed and approved by the Ethics Committee on Animal Use of the Faculty of Veterinary Medicine (FVM) of the University of Montreal, and it was performed in accordance with the guidelines of the Canadian Council on Animal Care (CCAC).

4.3.1 Animals, experimental design and housing

A total of 96 Duroc-Yorkshire-Landrace pigs were used to carry out the experiment, animals were housed at a biosecurity level 2 agro-environmental platform for farm animals of the FVM. Pigs were selected based on the presence of the F4 receptor gene by PCR-RFLP as previously described (Daudelin et al., 2011) at 4 days of age. Two trials of 48 pigs were conducted using different doses of CS (100,000 IU/kg (trial 1) or 50,000 IU/kg (trial 2)). In each trial, four groups of 12 pigs were constituted: challenged treated, challenged untreated, unchallenged treated, and unchallenged untreated.

After weaning (21 d old), pigs were fed a standard non-medicated ration for post-weaning pigs and had unlimited access to feed and water throughout the seven weeks of the study. The temperature of the room was kept at 24–26°C. In both trials, challenged groups were placed in the same room, although each group (n=12) was housed in a separate pen. The two unchallenged groups were placed in two different rooms. Each pen had a stainless-steel feeder and a low-pressure nipple drinker. In order to avoid contamination of control groups, biosecurity measures

were applied, including use and changing of boots, coveralls and gloves before entering each room.

4.3.2 ETEC: F4 Oral challenge and antimicrobial administration

For experimental infection of pigs, a nalidixic acid-resistant (Nal^r) variant of ETEC: F4 strain ECL8559 (O149: LT: STa: STb: East1: paa: hemβ: F4), kindly provided by the *E. coli* Laboratory as described previously (Rhouma et al., 2015), was used. The strain was passaged in a weaned pig to enhance its pathogenicity. A hemolytic, Nal^r colony isolated from the feces of this pig was confirmed to be positive for O149 and the virulence genes F4, STa, STb, LT by multiplex PCR as previously described (Longpré et al., 2016). This strain, designated ECL8559A, was used in the experimental challenge in this study. After 1-wk of acclimatization, 28-day-old pigs in the challenge groups were orally gavaged with 10^9 CFU of the ETEC: F4 strain in 5 mL of trypticase soy broth (Difco Laboratories, Inc., Detroit, MI, USA) following the administration of 10 mL of $CaCO_3$ to neutralize gastric acid.

Colistin sulfate (Bond & Beaulac Inc., QC, Canada) was administered by oral gavage in 5 mL of water using a polyethylene tube attached to a syringe, at a dose of 100,000 IU/kg or 50,000 IU/kg in trials 1 and 2 respectively. CS administration was started when at least 2 pigs from the challenged groups showed PWD symptoms (i.e.: score 2 of diarrhea, lethargy and anorexia), and continued twice a day for 5 successive days.

4.3.3 Fecal sampling and microbiological analysis

Fresh fecal samples were obtained from pigs using pre-weighed sterile rectal swabs (Puritan Medical Products, Guilford, Maine, USA). Bacteriological examination of fecal samples was performed one day before and 1, 2, 3, 5, 6, 7, 8, 10, 13, 20, 27, 36 days after oral challenge to evaluate fecal excretion of the challenge ETEC: F4 strain and total *E. coli* count. One mL of

buffered peptone water solution (BPW) was added to each swab and selected dilutions were plated on MacConkey agar and 5% bovine blood agar plates containing nalidixic acid at 50 μg/mL (Sigma-Aldrich Canada Ltd., Oakville, ON, Canada) to count the total *E. coli* population and the hemolytic challenge ETEC: F4 strain respectively, as previously described (Daudelin et al., 2011; Rhouma et al., 2015). In parallel, 5% bovine blood agar plates containing nalidixic acid at 50 μg/mL and CS at 2 μg/mL and MacConkey agar plates containing CS at 2 μg/mL were used to enumerate the CS resistant hemolytic challenge ETEC: F4 and total *E. coli* population respectively. The plates were incubated aerobically for 24 h at 37°C. Isolates recovered from media containing 2 μg/mL of colistin were considered to be putative CS-resistant, as previously described (Boyen et al., 2010). All samples were processed on the day of collection. Rectal swabs were weighed before and after sampling of pigs for individual fecal material quantification.

The isolates on MacConkey agar were confirmed as *E. coli* by colony morphology and biochemical analysis (Farmer et al., 1985). Hemolytic colonies on blood agar were confirmed as ETEC: F4 by multiplex PCR using published primers (Furrer et al., 1990; Ngeleka et al., 2003; Ojeniyi et al., 1994). The minimum inhibitory concentration (MIC) was determined as the lowest CS concentration that resulted in the inhibition of bacterial growth. The MIC was determined for the challenge strain before animal inoculation, and for confirmed *E. coli* isolates recovered from agar plates containing CS at 2 μg/mL after challenge. The MIC was carried out by microdilution method using a sterile 96-well polystyrene microplate, as previously described (Rhouma et al., 2015). The MIC was only evaluated on isolates from trial 2 (50,000 IU/kg), representing the most common dosage used in PWD treatment worldwide.

At 36 days post-challenge, pigs were euthanized and necropsies were performed.

4.3.4 Health status assessment

After the oral challenge, pigs were observed daily for signs of anorexia, lethargy and diarrhea. The severity of diarrhea was assessed visually by using a fecal consistency scoring (0, normal; 1, soft feces; 2, mild diarrhea; 3, semi liquid diarrhea and 4, liquid diarrhea) as described by Jamalludeen et *al*., (Jamalludeen et al., 2009). The rectal body temperature was monitored daily using a digital thermometer.

Pigs were weighed individually using an electric scale prior to inoculation and at 6, 19, and 35 days after beginning CS treatment.

4.3.5 Blood sampling and pharmacokinetic analysis

Blood samples (3 mL) were collected using potassium EDTA tubes, from the jugular vein of 8 pigs in each treated group, challenged or not of the two trials, at 0.5, 12, 24, and 48 hours after the last CS oral administration on day 5.

Plasma was separated by centrifugation at 3000 g for 10 min and stored at -20°C prior to analysis. These samples were used to determine CS plasma concentrations by high performance HPLC-MS/MS, in order to determine the slope of the terminal phase (λ_z). The λ_z was calculated as the negative of the slope of the log-linear regression of the natural logarithm concentration-time curve during the terminal phase. The λ_z is an important parameter used to determine CS elimination half-life ($T_{1/2}$), which is an index of drug persistence in the body (Toutain and Bousquet-Melou, 2004). Bioanalyses and pharmacokinetic analyses were performed as previously described (Rhouma et al., 2015). The quantification of CS was based on the peak area ratio of the analyte with the internal standard. A calibration curve was used for determining the concentration of CS in all unknown samples by comparing the peak area ratio of the unknown samples to a set of standard samples of known concentration. It is important to note that a linear

regression (weighted 1/concentration) produced the best fit for the concentration–detector relationship and consequently, the change of CS ionization states had a minimal effect within the analytical range used. The method precision and accuracy was well within acceptable figure of merits (CDER and CVM., 2001).

4.3.6 Statistical Analysis

Bacterial counts and CS plasma concentrations were $\log_{10}$ transformed prior to data analysis to normalize distributions. Total *E. coli* counts, ETEC: F4 counts, rectal temperature, and body weight were analyzed with repeated-measures ANOVA, with time as a within-subject factor and group as the between-subject factor. A priori contrasts were performed to compare group means at different time periods and to compare pre- and post-infection means in each treatment. For these multiple comparisons, the alpha level was adjusted downward using the Benjamini-Hochberg sequential procedure. A similar procedure was used to analyze CS plasma concentration to determine effect of ETEC: F4 oral challenge on CS intestinal absorption in pigs. Ordinal diarrhea scores were analyzed with the Cochran-Mantel-Haenszel test at each time period.

Statistical analyses were carried out with SAS v.9.4. (Cary, N.C.). The level of statistical significance was set at $p < 0.05$ for all analyses.

4.4 Results

During the acclimation period, none of the pigs in the two trials showed clinical signs of PWD. In trial 1, there were no deaths among pigs throughout the experiment. However, in trial 2, one pig in the challenged treated group died 2 days after the oral challenge and two pigs in the challenged untreated group died at 4 and 6 days after the challenge following presentation of a profuse diarrhea (score 4). Necropsies were not performed for dead pigs, due to the presence of advanced

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post-mortem bacterial invasion. However, no mortality occurred in the unchallenged groups of the two trials.

As the two trials were not performed at the same time for technical reasons, the two CS doses were only compared when the course of infection was similar for the challenged untreated groups (control groups) of the two trials. Thus, the effect of CS dose was compared between the two trials only for shedding of ETEC: F4 and total *E. coli*.

4.4.1 Analysis of ETEC: F4 bacterial shedding (trial 1 and trial 2)

After the challenge, there was a rapid initial increase in ETEC: F4 shedding in the feces of all challenged pigs (Figure 17). There were no significant differences between the groups in the recovery of ETEC: F4 on the first day post challenge but on the following day after CS first dose administration (d1), there was a reduction in the treated group compared to the untreated group in the trial 2 ($p < 0.0001$). In both trials, CS treatment resulted in a significant reduction in fecal ETEC: F4 shedding between d2 and d6 ($p < 0.0001$), and the levels of ETEC: F4 dropped below our detection limit for most pigs between d4 and d6. However, after d6, fecal excretion of ETEC: F4 increased in the treated groups to the same level of excretion as in the untreated groups, with a significant increase at d19 in trial 2 ($p = 0.007$). However, a significant reduction in fecal excretion of ETEC: F4 was observed in trial 2 compared to trial 1 between d1 and d3, inclusively ($p < 0.0001$) (Figure 17).

In the two trials, during the acclimation period, no *E. coli* were isolated on the blood agar plates containing nalidixic acid from any fecal samples, nor from unchallenged pigs throughout the experiment.

4.4.2 Analysis of shedding of total *E. coli* population (trial 1 and trial 2)

Mean total fecal *E. coli* counts of the challenged treated group and the challenged untreated

group were similar on d-3 (before challenge) in each trial and increased in both challenged groups of the two trials on d-1 (24 h after challenge) (Additional file 1). However, the ETEC: F4 challenge did not significantly increase the total *E. coli* population fecal shedding.

Colistin sulfate treatment at a dose of 100,000 IU/kg (trial 1) induced a significant reduction in fecal total *E. coli* shedding between d1 and d5 in the challenged treated group compared to the challenged untreated group ($p < 0.0001$) (Additional file 1). The same therapeutic regimen (100,000 IU/kg) also resulted in a significant reduction in fecal total *E. coli* shedding between d2 and d6 in the unchallenged treated groups compared to the unchallenged untreated group ($p < 0.0001$) (Additional file 2).

Colistin sulfate treatment at a dose of 50,000 IU/kg (trial 2) induced a significant reduction in fecal total *E. coli* shedding between d1 and d6 in the challenged treated group compared to the challenged untreated group ($p < 0.0007$) (Additional file 1). This therapeutic regimen also resulted in a significant reduction in fecal total *E. coli* shedding between d2 and d5 in the unchallenged treated group compared to the unchallenged untreated group ($p < 0.0001$) (Additional file 2). However, in both trials, starting from d7 (two days after CS cessation), fecal excretion of total *E. coli* increased in the treated groups to reach the same level of excretion as in the untreated groups (Additional file 1 and 2).

A significant reduction in fecal excretion of total *E. coli* was observed in trial 2 compared to trial 1 at d2 and d3 inclusively ($p = 0.003$ and $p < 0.0001$, respectively). Consequently, the highest reduction in total *E. coli* fecal shedding was observed in trial 2 (lower dose) between d2 and d3 (Additional file 1 and 2).

4.4.3   Isolation of *E. coli* resistant to colistin sulfate

In trial 2, before the challenge period and exposure to CS at a dose of 50,000 IU/kg, fecal

shedding of putative CS-resistant *E. coli* in the challenged treated group and the untreated group was very similar, as shown by the ratios of log putative CS-resistant E. coli /log total *E. coli* (Additional file 3). A low number of cultivable resident putative CS resistant *E. coli* were observed in all pigs used in this study.

Following CS administration, there was a significant decrease in the total *E. coli* population (Additional file 1). From d2 post CS treatment, the challenged treated pigs demonstrated a slight increase (15%) in the proportion of putative CS-resistant *E. coli* compared with the challenged untreated pigs. This difference was observed throughout CS administration, being significant between d3 and d5 ($p < 0.0005$) and gradually diminishing from the first day (day 6) of CS discontinuation (Additional file 3).

Among 80 putative CS resistant *E. coli* isolates on MacConkey plates, 72 were identified as *E. coli* by biochemical analyses, only one isolate being identified as ETEC: F4 by multiplex PCR. No putative CS resistant colonies were isolated on blood agar plates containing nalidixic acid.

Among 72 putative CS resistant *E. coli* isolates, 9 (8 in the challenged treated group and one in the challenged untreated group) were confirmed resistant to CS with an MIC > 2 μg/mL (Table XIII).

The CS resistant ETEC: F4 isolate, probably originating from the challenge strain as it was confirmed by multiplex PCR, demonstrated an MIC of 8 μg/mL, as compared to < 0.06 μg/mL for the challenge strain (ECL8559A). This ETEC: F4 isolate was found in the challenged untreated group 4 days after the oral challenge (Table XIII).

4.4.4 Analysis of health status and growth performance

Prior to bacterial challenge, no pig in either trial showed any indication of severe diarrhea or loose stools. None of the unchallenged pigs in the two trials showed any illness or diarrhea during

the experiment.

Following challenge, all challenged pigs in the two trials showed high diarrhea scores with no statistically significant difference between treated and untreated groups (Figure 18 and Additional file 4). After 2 days of CS administration (d2), diarrhea scores were significantly decreased in the challenged treated compared to the challenged untreated groups, and shown in Figure 18 for trial 2 ($p < 0.0001$). The decrease was also observed at d3 and d4 in the two trials (Figure 18 and Additional file 4). From d5 (6 days post challenge), diarrhea scores in the challenged untreated groups of both trials decreased and no statistically significant difference in the diarrhea scores between challenged untreated and challenged treated groups was observed in either trial.

Some challenged pigs in both trials developed hypothermia, several days post challenge, occasionally followed by death.

The body weight of the pigs in trial 2 in the pre-challenge period did not differ among the four groups ($p > 0.71$). Following oral challenge with ETEC: F4 and CS treatment discontinuation (d6), no difference was detected in the body weight of all pigs in both trials (Figures 19 and 20) with $p > 0.05$ and $p > 0.07$ for trials 1 and 2 respectively. After 2 weeks of CS treatment discontinuation (d19), a significantly higher body weight was observed in trial 1 for the unchallenged untreated (control) compared to the challenged untreated group ($p < 0.001$) (Figure 19). However, in trial 2 for the same time (d19), the unchallenged treated group presented a higher mean weight compared to the challenged untreated group ($p < 0.001$) (Figure 20). After 30 days of CS treatment discontinuation (d35) in both trials, the unchallenged treated and the control groups presented a higher mean weight compared to the challenged untreated groups (Figures 19 and 20). In addition, in trial 2 at d35, the unchallenged treated group and the control group presented a higher mean weight compared to the challenged treated group, with $p < 0.0001$.

Overall, the ETEC: F4 challenge resulted in decreased growth rate of the challenged groups in both trials and treatment with CS at the doses used in this study did not affect this decreased growth rate.

4.4.5 Quantification of plasma concentration of colistin sulfate and pharmacokinetic analysis

In order to determine whether ETEC: F4 challenge affects CS intestinal absorption, an HPLC-MS/MS was used for CS quantification in pig plasma. The lower limit of quantitation (LLOQ) of our method was 1 ng/mL of plasma. The pharmacokinetic analyses were performed using a non-compartmental model. In both trials, CS plasma concentrations were detected in all treated groups (challenged or not), although they were higher in challenged treated groups compared to the unchallenged treated groups for all sampling times (Figure 21).

In the challenged treated groups, the mean of C_{max} (± SD) (the observed maximum plasma concentration of CS) was 338.3 (± 676.37) ng/mL and 122.3 (±161.97) ng/mL at 0.5 h post CS treatment discontinuation in trials 1 and 2 respectively (Figure 21). In trial 1, at 0.5, 12 and 24 hours after CS treatment discontinuation, CS plasma concentrations were statistically higher in the challenged treated group compared to the unchallenged treated group with $p < 0.001$, $p < 0.0001$, and $p < 0.001$ respectively. The same finding was observed in trial 2, the CS plasma concentrations were higher in challenged treated compared to the unchallenged treated group at 0.5h ($p < 0.001$), and at 12 hours ($p = 0.04$). Thus, ETEC: F4 oral challenge exacerbated the intestinal absorption of CS in challenged compared to unchallenged weaned pigs. In both trials, at 48 h following the last CS administration, plasma concentrations were below the LLOQ of our method. We were not able to determine the λ_z and $T_{1/2}$ of CS following its oral administration

even in challenged treated pigs. Based on our sampling plan it was not possible to characterize the CS elimination phase and make a linear regression of the last CS plasma concentrations.

4.5 Discussion

The aim of the present study was to evaluate the impact of CS on the *E. coli* populations and pig health status in experimental *E. coli*-induced diarrhea in weaned pigs. We also studied the impact of ETEC: F4 oral challenge on CS intestinal absorption level in pigs using a highly sensitivity analytical method (HPLC-MS/MS).

The duration of the experiment was 35 days in each trial, to cover the withdrawal period of 30 days applied in Canada for CS in pig farms. Indeed, in the absence of scientific explanation for the difference in the withdrawal period for CS oral formulations in pigs between countries (Official Journal of the European Union, 2010), veterinarians use this long time period of 30 days as a safety measure for consumer protection against potential CS chemical residues in pig meat.

We used two doses of CS in our study in order to more closely reflect farm practices. In fact, the lower dose (50,000 IU/kg) is the recommended therapeutic dose in pigs, whereas the higher dose (100,000 IU/kg) was used to take into consideration a more realistic portrait of CS use on pig farms, where this antibiotic is often overdosed (Chauvin et al., 2002), and the social rank and heterogeneity observed among pigs in the same pens which may increase antimicrobial consumption for some pigs (Soraci et al., 2014).

In the current study, maximum ETEC: F4 shedding and diarrhea scores were observed one-day post challenge. This result is consistent with other experimental studies in which a higher frequency of watery diarrhea was observed after the first day of the ETEC: F4 oral challenge (Jensen et al., 2006; Wellock et al., 2008).

In our study, regardless of the dose, CS treatment led to a decrease of nearly 4 log cfu/g in fecal

shedding of ETEC: F4 and total *E. coli,* but only during the treatment period. This finding corroborates the study of Torrallardona et *al*., who showed that the use of CS at a dose of 300 mg/kg of diet in the treatment of weanling pigs challenged with *E. coli* K99 for a period of 7 or 14 days was associated with a reduction of the number of *E. coli* in both ileal and cecal digesta by 5.30 and 4.38 log cfu/g, respectively (Torrallardona et al., 2003). In our study, the effect of CS on the decrease of ETEC: F4 and total *E. coli* population was greater with the low dose of CS (50,000 IU/kg) used in trial 2. This finding is in disagreement with the known pharmacodynamics (PD) of CS as an antibiotic that exhibits its bactericidal activity in a concentration-dependent manner *in vitro* (Guyonnet et al., 2010). However, Lin et *al*., reported that CS bioavailability after an intramuscular (IM) administration in pigs, was inversely proportional with the administered CS doses, with a systemic bioavailability of 95.94% and 88.45% for 2.5 mg/kg and 5 mg/kg b.w. respectively (Lin et al., 2005).

In the current study, no difference was noted between low and high CS doses given to pigs, regarding *E. coli* recovery and on health status. Nevertheless, it would have been interesting to quantify colistin in pig gut, to link the microbiological effects determined to the real CS concentrations in intestinal segments. However, for logistic reasons associated with the design of the experiment and due to the low number of pigs in each group, it was not possible to sacrifice animals to recover the digestive contents, in this study.

In the present study, after CS treatment discontinuation in the two trials, there was no difference in fecal shedding of ETEC: F4, total *E. coli* population, and diarrhea scores between challenged treated and challenged untreated groups. However, it should be noted that our experiment was carried out in controlled conditions, and that the outcome of CS treatment may differ during natural infections in farm conditions associated with specific factors such as livestock management, presence of other infections in the farm, feed additives, vaccination or other factors.

In our study, 12.5% of *E. coli* isolates originating from growth on MacConkey agar plates with 2 μg/mL of CS were confirmed resistant to colistin, most (8/9) following the treatment with CS at 50,000 IU/kg, suggesting a CS selection pressure on *E. coli*. Our results corroborate those of Boyen et *al*., who determined that approximately 10% of the 157 investigated porcine *E. coli* isolates from sick pigs showed resistance to colistin (Boyen et al., 2010). However, it is not clear whether sampled animals were treated with colistin in this study. On the other hand, the MICs of CS *E. coli* resistant isolates determined in our study were in the same range as those of resistant *E. coli* isolated from sick pigs in farm conditions (Boyen et al., 2010; Morales et al., 2012).

In the present study, the CS resistance was observed in 3 *E. coli* isolates even 6 days after CS treatment discontinuation, and in an isolate confirmed ETEC: F4 in the challenged untreated group 4 days after the oral challenge. Further investigations are ongoing to explain if this CS resistance is associated with chromosomal mutations or a plasmid resistance gene, and to determine the origin of the higher MIC observed for the ETEC: F4 isolate compared to the challenge strain by determining of its natural mutation rate.

Although we observed a lower proportion of CS *E. coli* resistant isolates than reported by other authors (Harada et al., 2005; Lu et al., 2010), it is premature to confirm that the use of this CS regimen in pigs is associated with a low resistance among *E. coli*. It would be interesting to determine in a future study the effect of CS in a mass treatment (drinking water or in feed) on CS resistance in *E. coli* in pig farm conditions and following a repetitive CS treatment.

In our study, the MacConkey agar plates supplemented with 2 μg/mL of CS overestimated the number of resistant *E. coli* since a small percentage of the *E. coli* recovered from these MacConkey agar plates could be confirmed resistant to CS by MIC determination using Mueller Hinton broth. This is probably due to the culture media change between the two experiments as well as the difference in the matrix used: fecal material for MacConkey agar plates versus pure

culture for Mueller Hinton. In our study, the use of the MacConkey supplemented with 2 μg/mL of CS was useful for reducing the numbers of isolates potentially no resistant to CS and thus limiting the number of isolates to be tested on Mueller Hinton for CS resistance confirmation. Our study clearly shows the importance of confirming putative CS isolates on MacConkey agar when non-standardized culture media are used for assessing the resistance levels of a given bacterial population.

In the present study, a growth retardation was observed in surviving animals of the challenged groups compared with the unchallenged groups in the two trials. This finding corroborates the study of Bontempo et *al*., who showed that *E. coli* challenge significantly impairs performance, resulting in a reduction of average daily gain for pigs (Bontempo et al., 2014). Colistin sulfate treatment in the two trials did not prevent pig weight losses in challenged treated compared to challenged untreated pigs. In addition, we have not noticed a difference in pig body weight between unchallenged treated and unchallenged untreated groups in both trials. To the best of our knowledge, our study is the first to report these results following an oral CS administration at 50,000 IU/kg or 100,000 IU/kg b.w in pigs. Nevertheless, it will be interesting to investigate in a long-term field trial with more pigs and in field conditions the effect of CS therapeutic regimen on pig weight loss prevention in the post-weaning period.

In our study, ETEC: F4 oral challenge increased the passage of CS from the intestine to the blood in the challenged pigs compared to the unchallenged weaned pigs in the two trials. Several studies have shown that administration of bacterial lipopolysaccharide (LPS) results in the production and release of TNF-α and IL-1; these pro-inflammatory cytokines increased epithelial tight junction permeability *in vitro* in Caco-2 cells (Ma et al., 2005). In another study, it was demonstrated that IL-1, activated endothelial cells (EC) to induce vascular leakage via loss of vascular endothelial (VE)-cadherin (Dagvadorj et al., 2015). The role of LPS release by the

challenge ETEC: F4 strain in increasing pig intestinal tight junction permeability and pro-inflammatory cytokine production needs to be confirmed in a future study.

Our results demonstrated that *E. coli* intestinal infection in weaned pigs with clinical PWD symptoms, resulted in increased of CS intestinal absorption. This finding should be taken into consideration when determining CS withdrawal time, bearing in mind that withdrawal times are mostly determined in healthy animals (Buur et al., 2006), even though antibiotics are currently used to treat clinically sick pigs.

In conclusion, this is the first report on the use of CS for the treatment of experimental *E. coli*-induced diarrhea in weaned pigs. In our study, we determined that under controlled conditions in pigs, CS reduced ETEC: F4 and *E. coli* fecal shedding and diarrhea scores during treatment period. However, CS treatment did not prevent pig weight losses due to the diarrhea and exerted a slight selection pressure on the CS resistant *E. coli* commensal population. In addition, we demonstrated that oral challenge of pigs using an ETEC: F4 strain increased passage of CS from the intestine to the blood. This observation should be taken into consideration when determining the oral CS withdrawal time in pigs.

A longer duration field trial investigation is recommended to better understand the relationship between CS effectiveness and CS bacterial resistance following the use of oral CS in PWD control in commercial farm conditions and lead to a prudent use of antimicrobials in swine medicine.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

MR designed the study, carried out clinical and microbial analysis, analyzed the data, and wrote the manuscript. FB participated in the design of the study and was involved in the development of

HPLC-MS/MS assays for CS plasma concentrations determination. WT participated in the design of the study, in sampling of animals, and was involved in bacterial analysis. NB participated in the design of the study, in sampling of animals, and in bacterial analysis. GB performed statistical analysis. SLL participated in the design of the study. JMF participated in the design of the study and was involved in the challenge protocol with ETEC: F4. AL conceived and designed the study, coordinated and assisted in the acquisition of data and its interpretation, and wrote the manuscript. All authors have read and approved the final manuscript.

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4.7 Table

Table XIII: Distribution of minimal inhibitory concentrations of porcine CS resistant *E. coli* isolates in trial 2

			Colistin sulfate MIC values (µg/mL)									
			0.06	0.12	0.25	0.5	1	**[2]**	4	8	16	32
Isolates	**Time**	**Groups**										
M4A3	D3	CT								+		
M4B3	D3	CT								+		
M4C3	D3	CT								+		
M4D3	D3	CT								+		
M6A11	D11	CT									+	
M6C11	D11	CT								+		
M6B11	D11	CT								+		
L10A4*	D4	CU								+		
L1B1	D1	CT							+			

Definition of acronyms: CU = Challenged Untreated; CT= Challenged Treated.

D3= 3 days post CS treatment; D11= 11 days post CS treatment; D4= 4 days post challenge;

D1= 1 day post CS treatment.

* Isolate confirmed ETEC: F4 by multiplex PCR

The isolates with MIC values higher than resistance breakpoint (MIC > 2 µg/mL) as described by Li and collaborators (Li et al., 2005) were considered resistant.

MIC of ECL8559A < 0.06 µg/mL

4.8   Figures

Figure legends

Fig. 17 Evolution of fecal ETEC: F4 bacterial counts (means ± standard deviation [SD]). Challenge was performed at d-2 and treatment with colistin sulfate (CS) at a dose of 100,000 IU/kg (trial 1) or 50,000 IU/kg (trial 2) was started at d0 (36 hours post challenge) and administered twice daily for a period of 5 days. In the two trials, CS treatment resulted in a significant reduction in fecal ETEC: F4 shedding between d2 and d6 ($p < 0.0001$). A significantly lower fecal excretion of ETEC: F4 was observed in trial 2 compared to trial 1 between d1 and d3 inclusive ($p < 0.0001$).

Fig. 18 Mean diarrhea score (± standard deviation [SD]) of weaned pigs challenged with ETEC: F4. Challenge was performed at d-2 and treatment with colistin sulfate (CS) at the dose of 50,000 IU/kg (trial 2) was started at d0 (36 hours post challenge) and administered twice daily for a period of 5 days. Treatment with oral CS resulted in a statistically significant reduction in the diarrhea score of the challenged treated group compared to the challenged untreated group ($p < 0.0001$) between d2 and d4. Mean diarrhea score = sum of daily diarrhea score/number of animals. *: $p < 0.0001$

Fig. 19 Evolution of body weight in pigs receiving colistin sulfate (CS) orally at a dose of 100,000 IU/kg (means ± standard deviation [SD]). Challenge was performed at d-2 and treatment with colistin sulfate (CS) at the dose of 100,000 IU/kg. For each sampling time, means with different letters on a given day are statistically different. At d6 there was no significant difference between groups.

Fig. 20 Evolution of body weight in pigs receiving colistin sulfate (CS) orally at a dose of 50,000 IU/kg (means ± standard deviation [SD]). Challenge was performed at d-2 and treatment with colistin sulfate (CS) at the dose of 50,000 IU/kg. For each sampling time, means

with different letters on a given day are statistically different. At d-3 and d6 there was no significant difference between groups.

Fig. 21 Evolution of plasma CS concentrations over time in pigs challenged with an ETEC: F4 strain and receiving colistin sulfate (CS) orally (means ± standard deviation [SD]). Colistin sulfate concentrations were obtained by HPLC-MS/MS after 0.5, 12, 24 and 48 hours of CS treatment discontinuation at a therapy regimen of 100,000 IU/kg (trial 1) or 50,000 IU/kg (trial 2). In trial 1, at 0.5, 12 and 24 hours, CS concentrations were statistically higher in the challenged treated group compared to the unchallenged treated group with $p < 0.001$, $p < 0.0001$ and $p < 0.001$ respectively. In trial 2, at 0.5 and 12 hours, CS concentration was statistically higher in the challenged treated group compared to the unchallenged treated group with $p < 0.001$ and $p = 0.04$ respectively (n = 8 per group).

Additional file. 1 Evolution of fecal total _E. coli_ counts (means ± standard deviation [SD]) in challenged groups. Challenge was performed at d-2 and treatment with colistin sulfate (CS) at the dose of 100,000 IU/kg (trial 1) or 50,000 IU/kg (trial 2) was started at d0 (36 hours post challenge) and administered twice daily for a period of 5 days. CS treatment resulted in a significant reduction in fecal total _E. coli_ shedding between d2 and d5 in trial 1 and between d1 and d6 in trial 2 in the challenged treated group compared to the challenged untreated group ($p < 0.0001$).

Additional file. 2 Evolution of fecal total _E. coli_ counts (means ± standard deviation [SD]) in unchallenged groups. Treatment with colistin sulfate (CS) at a dose of 100,000 IU/kg (trial 1) or 50,000 IU/kg (trial 2) was started at d0 (36 hours post challenge) and administered twice daily for a period of 5 days. CS treatment resulted in a significant reduction in fecal total _E. coli_ shedding between d2 and d6 in trial 1 and between d2 and d4 in trial 2 in the unchallenged treated groups compared to the unchallenged untreated (control) groups ($p < 0.0001$).

Additional file. 3 Evolution of fecal ratio of putative CS-resistant *E. coli* /total *E. coli* counts (mean ± standard deviation [SD]). Challenge was performed at d-2 and colistin sulfate (CS) was administered at the dose of 50,000 IU/kg twice daily for 5 days, starting at d0 (36 hours post challenge). CS treatment induced a significant increase in fecal putative CS-resistant *E. coli* (selective pressure) shedding between d3 and d5 in the challenged treated group compared to the challenged untreated group *: $p < 0.0001$. **: $p < 0.001$.

Additional file. 4 Mean diarrhea score (± standard deviation [SD]) of weaned pigs challenged with ETEC: F4. Challenge was performed at d-2 and treatment with colistin sulfate (CS) at a dose of 100,000 IU/kg (trial 1) was started at d0 (36 hours post challenge) and administered twice daily for a period of 5 days. Treatment with oral CS had led to a statistically significant reduction in the diarrhea score of the challenged treated group compared to the challenged untreated group ($p < 0.0001$) on d2 and d4. Mean diarrhea score = sum of daily diarrhea score/number of animals (n = 12 per group). *: $p < 0.0001$.
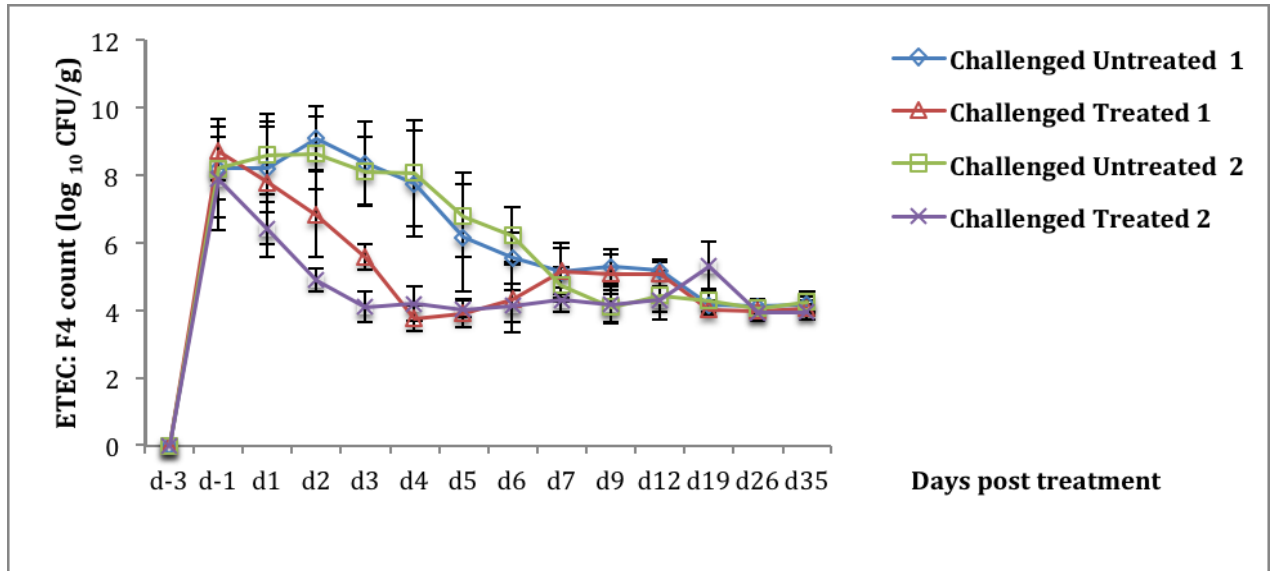
Figure 17: Evolution of fecal ETEC: F4 bacterial counts (means ± standard deviation [SD]).
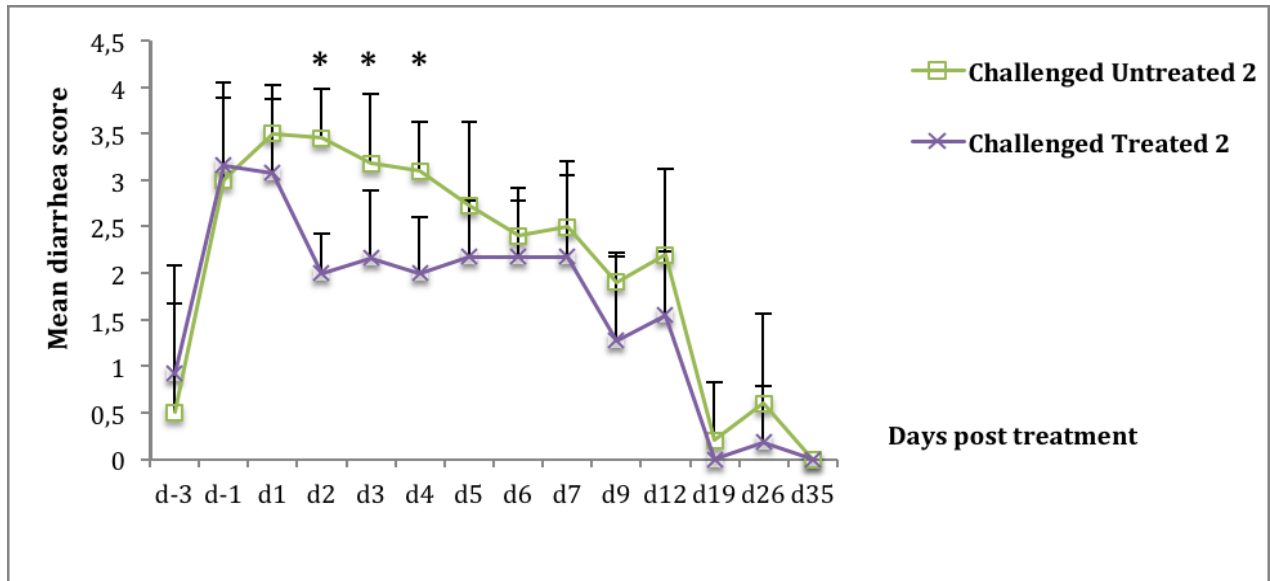
Figure 18: Mean diarrhea score (± standard deviation [SD]) of weaned pigs challenged with

ETEC: F4
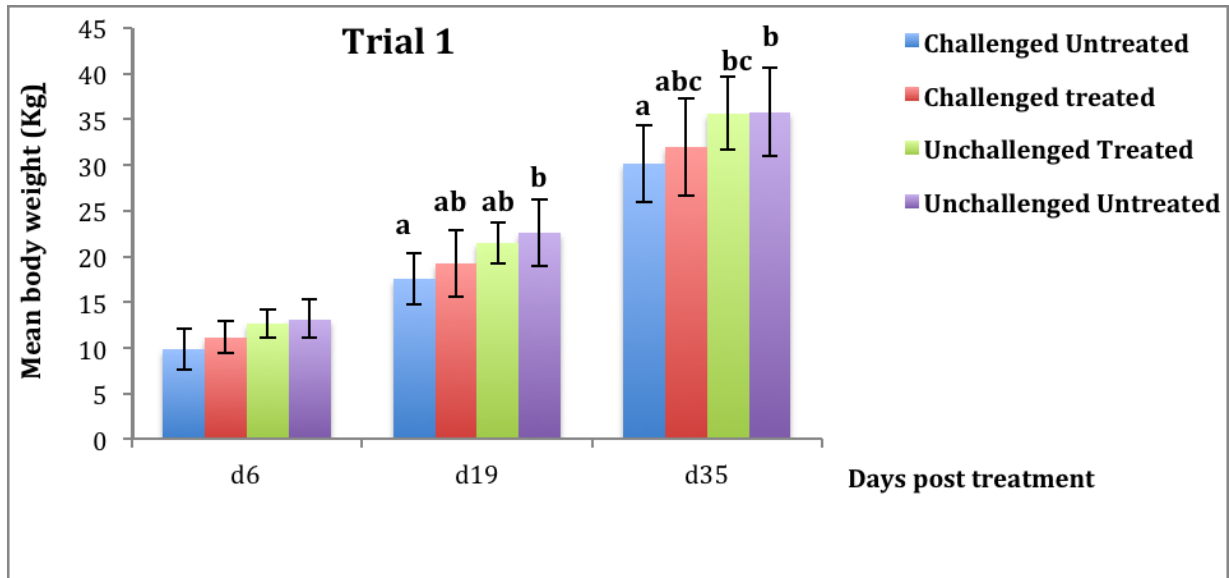
Figure 19: Evolution of body weight in pigs receiving colistin sulfate (CS) orally at a dose of 100,000 IU/kg (means ± standard deviation [SD]).
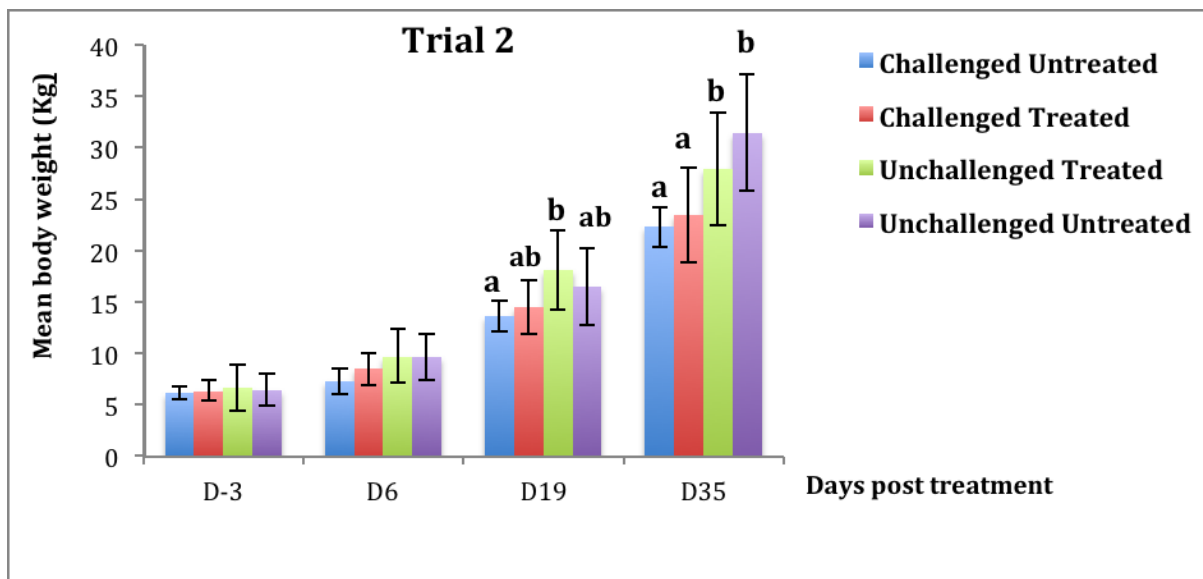
Figure 20: Evolution of body weight in pigs receiving colistin sulfate (CS) orally at a dose of 50,000 IU/kg (means ± standard deviation [SD]).
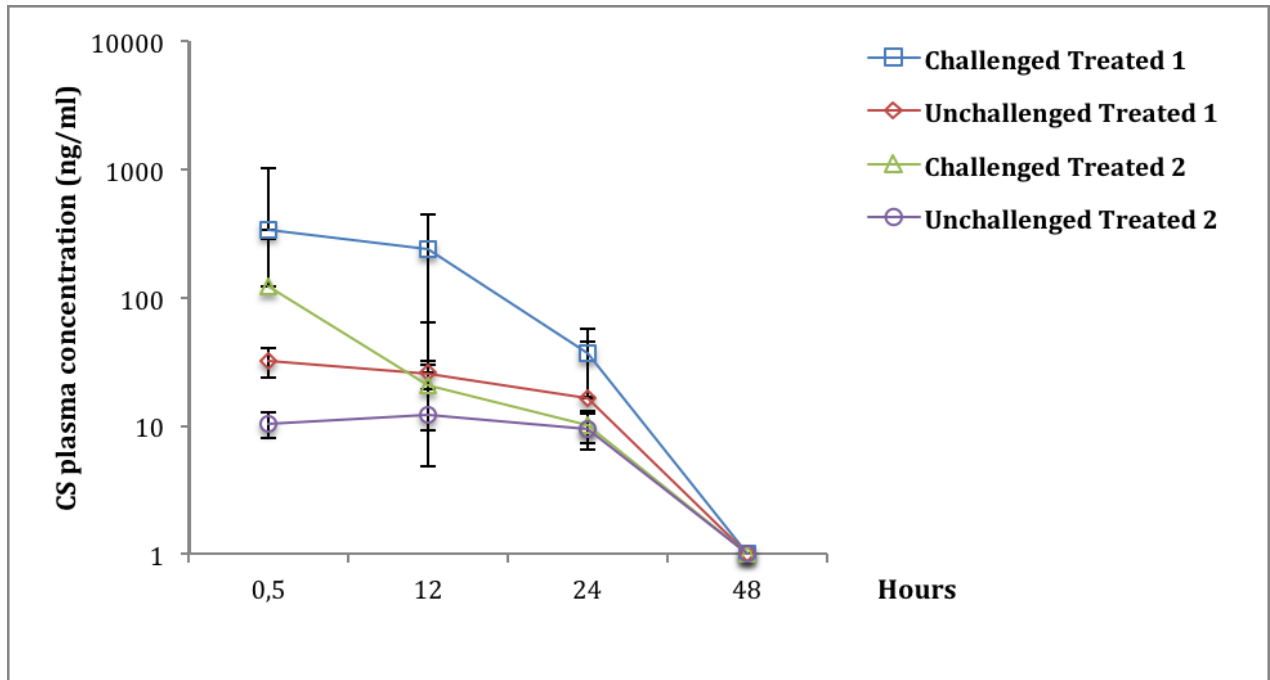
Figure 21: Evolution of plasma CS concentrations over time in pigs challenged with an ETEC: F4 strain and receiving colistin sulfate (CS) orally (means ± standard deviation [SD]).

4.9   Additional files
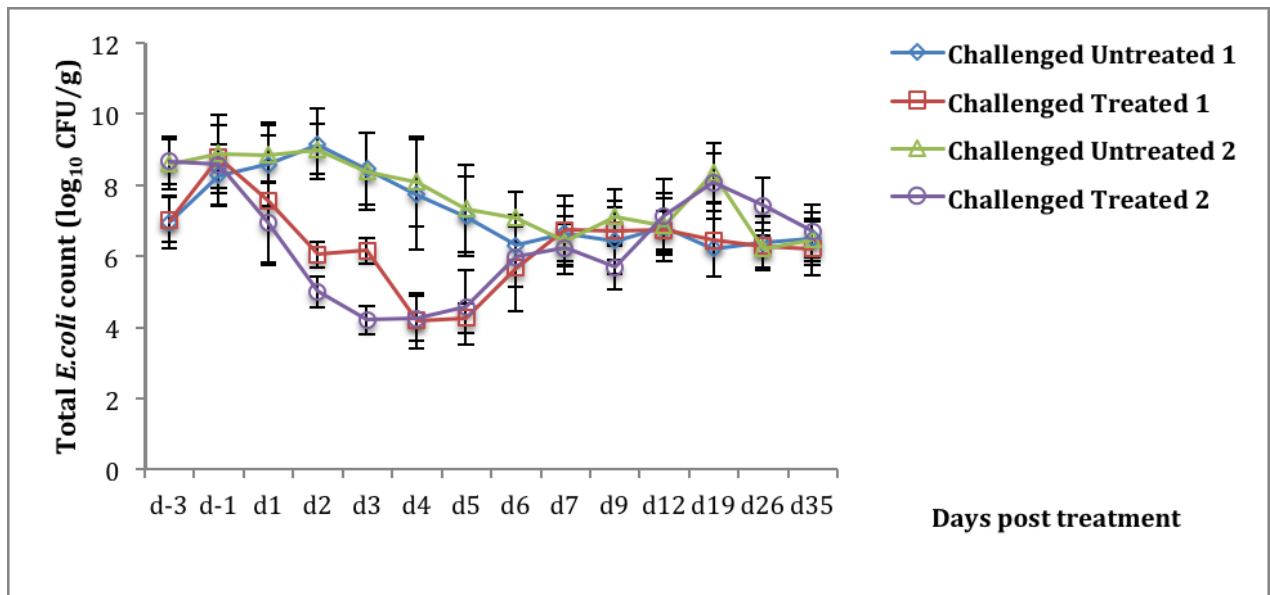
Additional file 1: Evolution of fecal total *E. coli* counts (means ± standard deviation [SD]) in challenged groups
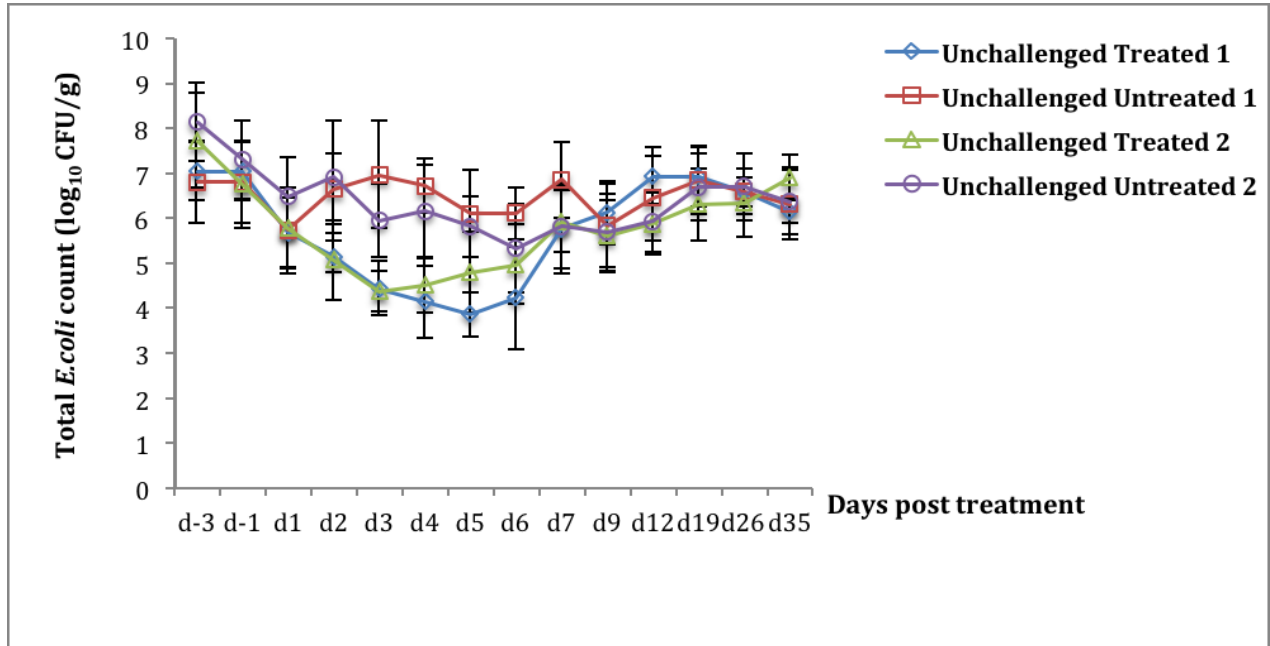
Additional file 2: Evolution of fecal total *E. coli* counts (means ± standard deviation [SD]) in unchallenged groups.
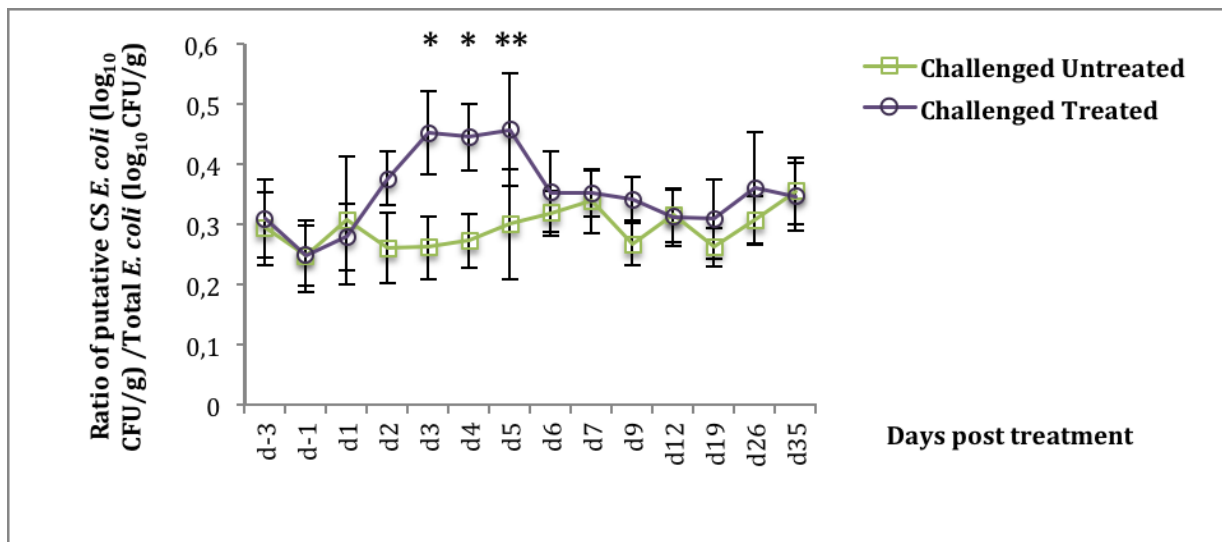
Additional file 3: Evolution of fecal ratio of putative CS-resistant *E. coli* /total *E. coli* counts (mean ± standard deviation [SD]).
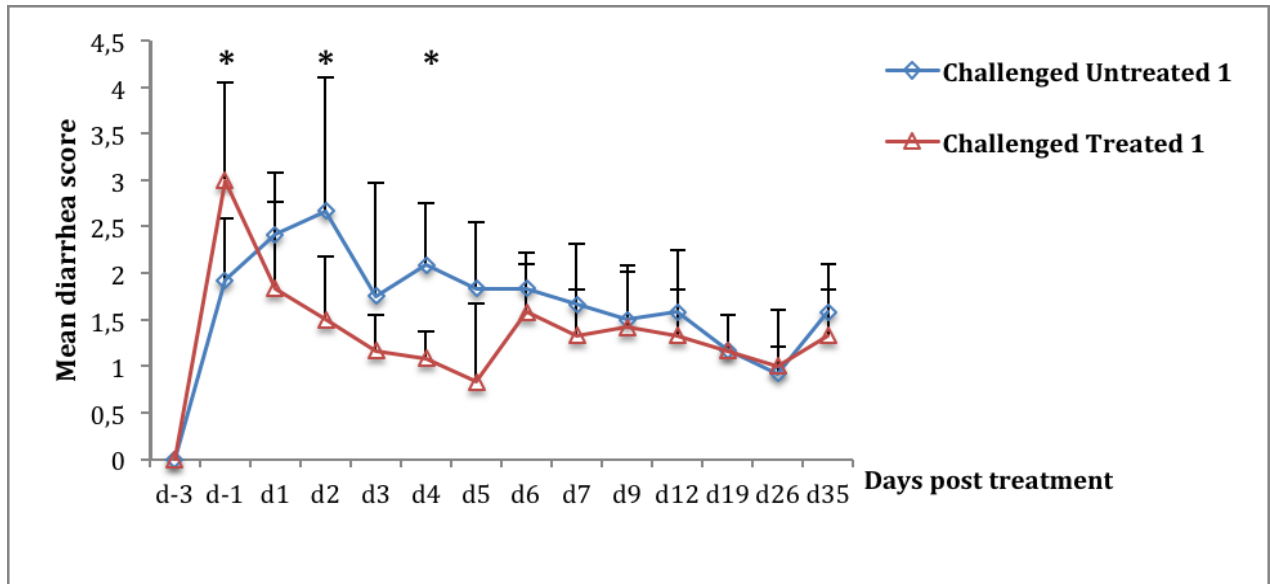
Additional file 4: Mean diarrhea score (± standard deviation [SD]) of weaned pigs challenged with ETEC: F4.

5. The fecal presence of enterotoxin and F4 genes as an indicator of efficacy of treatment with colistin sulfate in pigs

Mohamed Rhouma, John Morris Fairbrother, William Thériault, Francis Beaudry, Nadia Bergeron, Sylvette Laurent-Lewandowski, Ann Letellier

Contribution du candidat:

J'ai participé à l'élaboration du protocole de recherche en proportion équivalente de celle des autres coauteurs. J'ai effectué les collectes des échantillons et j'ai effectué toutes les analyses de laboratoire. J'ai analysé les résultats. J'ai rédigé l'article conformément aux exigences de la revue et j'ai intégré les commentaires faits par les coauteurs dans la version finale pour la soumission.

5.1   Abstract

Background: Enterotoxigenic *Escherichia coli* (ETEC) strains producing multiple enterotoxins are important causes of post-weaning diarrhea (PWD) in pigs. The aim of the present study was to investigate the fecal presence of ETEC enterotoxin and F4 genes as an indicator of colistin sulfate (CS) efficacy for treatment of PWD in pigs. Forty-eight piglets were weaned at the age of 21 d, and were divided into four groups: challenged treated, challenged untreated, unchallenged treated, and unchallenged untreated. Challenge was performed using 10^9 CFU of an ETEC: F4 strain, and treatment was conducted using oral CS at the dose of 50,000 IU/kg. The fecal presence of genes encoding for STa, STb, LT and F4 was detected using a multiplex PCR.

Results: The PCR amplification of ETEC virulence genes showed that nearly 100% of pigs excreted genes encoding for STa and STb toxins in the feces before the challenge. These genes, in the absence of the gene encoding F4, were considered as a marker for F4-negative ETEC. One day after ETEC: F4 oral challenge, pigs in the two challenged groups excreted the genes encoding LT and F4 in the feces. These genes were considered as a marker for F4-positive ETEC. After only 3 days of successive oral treatment with CS, a significant reduction in both the F4-positive and negative ETEC populations was observed in the challenged treated group compared to the challenged untreated group ($p < 0.0001$).

Conclusions: Our study is among the first to report that under controlled farming conditions, oral CS treatment had a significant effect on both fecal F4-positive and F4-negative ETEC in pigs. However, CS clinical efficiency was correlated with non-detection of F4-positive ETEC in the feces. Furthermore the fecal presence of F4-negative ETEC was not associated with clinical symptoms of post-weaning diarrhea in pigs.

Keywords: ETEC, virulence gene, fecal, colistin sulfate, diarrhea, pigs.

5.2 Background

Post-weaning diarrhea (PWD) is an economically important disease in pigs due to financial losses as a result of mortalities, morbidity, diarrhea, reduced growth performance, and medication costs (Amezcua et al., 2002b; Fairbrother et al., 2005). This disease is usually associated with proliferation of one or more strains of Enterotoxigenic *Escherichia coli* (ETEC) in the pig gastrointestinal tract (Fairbrother et al., 2005). ETEC strains are characterized by the production of enterotoxins and adhesins, both essential for disease development (Fairbrother and Gyles, 2012). Enterotoxins produced by ETEC may be heat stable (STa, STb or enteroaggregative *E. coli* heat stable enterotoxin 1 [EAST1]) or heat labile (LT). In pigs, the most frequently observed fimbrial adhesins of ETEC are K88 (F4), K99 (F5), 987P (F6), F41, and F18 (Fairbrother and Gyles, 2012). F4-positive ETEC (ETEC: F4) infections represent the major cause of PWD in pigs worldwide (Luppi et al., 2016; Nagy and Fekete, 2005). Furthermore, the most predominant serovirotypes of ETEC associated with PWD in pigs are O149: LT: STb: F4 and O149: LT: STa: STb (Gyles and Fairbrother, 2010). The diagnosis of PWD in pigs is based on clinical signs, microscopic lesions and bacteriological testing (Fairbrother and Gyles, 2012). Bacteriological tests remains the most effective method to confirm the etiology of PWD, and to assess the effectiveness of antimicrobials used in its treatment. Determination of ETEC virulence genes, is the most reliable method to identify the presence of pathogenic *E. coli* associated with PWD (Nagy and Fekete, 2005). Colistin sulfate (CS), a cationic antimicrobial peptide, is one of the most frequently used antibiotics for the treatment of PWD (Kempf et al., 2013), being mostly used *per os*, at a recommended dose of 50,000 IU/kg body weight (bw) every 12 h for a period of 3 to 5 consecutive days (Rhouma et al., 2016a). However, with the increase of the rate of CS resistance *E. coli* in pigs (Rhouma et al., 2016a), the monitoring of the therapeutic efficacy of CS appears very important. The aim of the present study was to examine the fecal presence of ETEC

enterotoxin and F4 genes in an experimental infection model as an indicator of the effectiveness of CS oral treatment to control the ETEC population in PWD in pigs.

5.3 Methods

The experimental protocol was reviewed and approved by the Ethics Committee on Animal Use of the Faculty of Veterinary Medicine (FVM) of the Université de Montréal and was performed in accordance with the guidelines of the Canadian Council on Animal Care (CCAC).

5.3.1 Animals and experimental design

The present study was conducted as part of a project designed to assess the pharmacokinetic of CS during the treatment of PWD and its effect on the exacerbation of *E. coli* resistance in pigs (Rhouma et al., 2016b). Briefly, 48 Duroc-Yorkshire-Landrace pigs were selected at 4 days of age for the presence of the F4 receptor gene by PCR-RFLP as previously described (Daudelin et al., 2011). Animals were obtained from 11 different litters.

After weaning (21 d), pigs were randomly divided into four groups of 12 pigs each: challenged treated (originated from 7 litters), challenged untreated (originated from 8 litters), unchallenged treated (originated from 5 litters), and unchallenged untreated (originated from 6 litters). Animals were fed a standard non-medicated ration for post-weaning pigs and had unlimited access to feed and water throughout the experiment.

After one week of acclimatization (28-day), pigs in the challenged groups were orally gavaged with 10^9 CFU of ETEC: F4 strain ECL8559A (O149: LT: STa: STb: F4: Nal^R) kindly provided by the Reference Laboratory for *Escherichia coli* (EcL, Faculty of Veterinary Medicine from the Université de Montréal) as described previously (Rhouma et al., 2016b). The day of the challenge corresponds to d0 in our experimentation.

Colistin sulfate (Bond & Beaulac Inc., QC, Canada) was administered by oral gavage to the challenged/treated and unchallenged/treated groups, one day after challenge, at a dose of 50,000 IU/kg bw twice a day for 5 successive days.

The rectal body temperature was monitored daily using a digital thermometer. The severity of diarrhea was assessed visually by using a fecal consistency scoring (0, normal; 1, soft feces; 2, mild diarrhea; 3, semi liquid diarrhea and 4, liquid diarrhea) as previously described (Rhouma et al., 2016b).

5.3.2 Fecal sampling and microbiological analysis

Fresh fecal samples were obtained from pigs using pre-weighed sterile rectal swabs (Puritan Medical Products, Guilford, Maine, USA). Sampling of fecal material was performed one day before (d-1) and 1, 4, 8, 13, 36 days after the oral challenge.

Fecal swabs were diluted 1:10 in buffered peptone water solution (BPW) and were incubated at 37°C overnight. A volume of 500 μl of this enrichment was placed in a 4.5 ml of Luria-Bertani (LB) broth and incubated at 37°C overnight. One ml (in duplicate) of each tube was stored at -80°C for subsequent analysis.

Rectal temperatures were taken at the same time as the fecal samples.

5.3.3 DNA extraction and multiplex PCR procedure

Fecal presence of genes encoding ETEC virulence factors STa, STb, LT and F4 was evaluated using a multiplex PCR as previously described (Longpré et al., 2016). DNA was extracted by heat lysis. Briefly, 1 ml of each sample was pelleted by centrifugation at 11,750 g for 5 min and 1 ml of Phosphate Buffered Saline (Becton Dickinson and Company, Sparks, MD, USA) was added; samples were pelleted by centrifugation at 11,750 g for 2 min and 500 μl of sterile Milli-Q water was added. Tubes were boiled for 10 min and immediately placed on ice. The boiled cell

suspensions were centrifuged at 11,750 g for 2 min, and the supernatant were used for PCR. The genes encoding STa, STb, LT and F4 were detected by multiplex PCR using published primers (Ngeleka et al., 2003; Ojeniyi et al., 1994). PCR positive and negative controls were ECL8559 (Rhouma et al., 2015), and *Listeria monocytogenes* of porcine origin respectively (Larivière-Gauthier et al., 2014).

Multiplex PCR procedures were performed according to a protocol of the EcL, available at http://www.apzec.ca/en/APZEC/Protocols/APZEC_PCR_en.aspx. The PCR reactions were performed in a 25 µl volume and comprised 2 µl of $MgSO_4$ (20 m*M*), 2.5 µl dNTP (2 m*M*), 2.5 µl of Taq buffer (10×), 1µl of STa and LT primers (5µ*M* and 10 µ*M* respectively), 1.25 µl of STb and F4 primers (10 µ*M* each), 1U Taq DNA polymerase (Bio Basic Inc., ON, Canada), and 5 µl of the DNA sample. Sterile water was used to bring the final reaction volume to 25 µl. After amplification, a 10 µl aliquot was submitted to electrophoresis in a 1.8% agarose gel stained with SYBR® Safe (Invitrogen, Burlington, ON, Canada). Amplification products were visualized and photographed under UV illumination.

5.3.4 Statistical Analysis

Percentage of pigs shedding each virulence gene (number of positive pigs/number total of pigs) for each sampling time in the 4 groups was analyzed with exact chi-square at each time period. Statistical analyses were carried out with SAS v.9.4. (Cary, N.C.). Rectal temperature was analyzed with repeated-measures ANOVA, with time as a within subject factor and group as the between-subject factor. Ordinal diarrhea scores were analyzed with the Cochran-Mantel-Haenszel test at each time period. The level of statistical significance was set at $p < 0.05$ for all analyses.

5.4 Results

Prior to the bacterial challenge (d-1), none of the pigs in any of the 4 groups showed signs of diarrhea or anorexia. The PCR amplification of ETEC virulence genes showed that nearly 100% of pigs (28 day) had a fecal presence of excreted genes encoding for STa and STb toxins in the feces before the challenge (d-1) (Fig. 22 and 23), whereas no pig had a fecal presence of excreted genes encoding LT or F4 (Fig. 24 and 25). This finding indicated that all clinically healthy pigs used in this study were infected at weaning with STa- and/or STb-positive bacteria, most likely *E. coli,* which we refer to as putative F4-negative ETEC isolates. In addition, genes encoding LT and F4 were not detected present in any fecal samples in the unchallenged groups throughout the experiment (Fig. 24 and 25).

One day after ETEC: F4 oral challenge (d1), pigs of the two challenge groups excreted the genes encoding LT and F4 in the feces (Fig. 24 and 25), with no statistically difference in prevalence between these genes and those encoding STa and STb ($p = 1$). These results indicate that the genes encoding LT and F4 were derived exclusively from the challenge and were considered as marker genes for the challenge strain (F4-positive ETEC). Thus, at d1, a significant fecal presence of putative F4-positive ETEC was observed in the challenged groups compared with the unchallenged groups ($p < 0.0001$). In addition, no fecal presence of putative F4-positive ETEC was observed in the unchallenged groups at d1 and throughout the experiment.

After three days of successive oral treatment with CS (d4), a significant reduction in the prevalence of fecal presence of putative F4-positive ETEC was observed in the challenged treated group compared to the challenged untreated group ($p < 0.0001$) (Fig. 24 and 25). Similarly, a significant reduction in the prevalence of fecal presence of putative F4-negative ETEC in fecal samples was observed in the unchallenged treated group compared with the unchallenged untreated group ($p < 0.0001$) (Fig. 22 and 23).

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From d8, corresponding to 2 days after CS oral treatment discontinuation, the genes encoding for LT and F4 were not detected in the feces of any challenged treated pigs although no difference was observed in the prevalence of fecal presence of putative F4-positive ETEC in fecal samples between the challenged treated and the challenged untreated group ($p = 0.07$) (Fig. 24 and 25). At d8 and d13, no difference was observed in the prevalence of of fecal presence of the putative F4-negative ETEC in fecal samples between the 2 unchallenged groups ($p = 0.07$) (Fig. 22 and 23). At d36, which corresponds to 30 days after CS oral treatment discontinuation, a significant reduction in the prevalence of fecal presence of putative F4-negative ETEC was observed in the challenged untreated group compared to the unchallenged untreated group as demonstrated by the presence of the gene encoding STa ($p < 0.001$) (Fig. 22). At d13 and d36, no fecal presence of putative F4-positive ETEC in fecal samples was observed in either challenged group.

Prior to the bacterial challenge (d-1), no difference was found between the 4 groups regarding diarrhea scores ($p = 0.33$) (Fig. 26). At d1, a significant increase in diarrhea score was observed in the challenged groups compared with the unchallenged groups ($p < 0.0001$). At d4, a significant reduction in diarrhea score was observed in the challenged treated group compared with the challenged untreated group ($p < 0.0001$). Moreover, this finding was associated with a significant reduction in genes encoding for LT and F4 in the fecal samples of the challenged treated group.

Starting from d8, no significant difference was established between the challenged untreated group and the challenged treated one regarding diarrhea scores (Fig. 26).

Furthermore, all challenged and unchallenged pigs, had rectal temperatures ranging mainly between 38.75°C and 39.55°C before challenge, and the oral challenge with ETEC: F4 did not cause an increase in rectal temperature of challenged piglets compared to the control groups (Additional file 1). In challenged pigs, some piglets developed hypothermia (36°C) that was

observed several days post challenge; this hypothermia was sometimes followed by death of the pig.

Mean rectal temperatures in the two challenged groups at d4 post challenge were significantly lower compared to those of the unchallenged groups ($p < 0.001$). Other than at d4, no difference was observed for other days between challenged and unchallenged groups regarding rectal temperatures ($p > 0.15$) (Additional file 5).

Mortality has been noted only in the challenged groups. In fact, one pig in the challenged treated group died 2 days after the oral challenge, after it received a single oral dose of CS, and two pigs in the challenged untreated group died at 4 and 6 days after the challenge. All pigs died after they presented acute diarrhea and anorexia.

5.5   Discussion

In this study, the fecal presence of genes encoding STa, STb, LT and F4 in pigs challenged with an ETEC: F4 strain was determined in order to follow the fecal ETEC population, as an indicator of oral CS treatment efficacy in experimental PWD. The presence of ETEC virulence genes was investigated in enriched fecal samples rather than in E. coli isolates, as in other studies (Kagambega et al., 2012). Hence, we used the terminology ''putative'' to describe the F4-positive or F4-negative ETEC populations. Nevertheless, we consider that our method is specific, as it has been reported in several studies that STa, STb, LT and F4 were found only in *E. coli* (Fairbrother et al., 2005; Ngeleka et al., 2003).

In the present study, close to 100% of pigs excreted putative F4-negative ETEC in the feces before the oral challenge. To our knowledge, our study is the first to report such a finding in clinically healthy pigs in the post-weaning period. In fact, other studies have associated the presence of isolates possessing STa and STb genes with clinical PWD in farm conditions

(Chapman et al., 2006; Kim et al., 2010; Zhang et al., 2007). Nevertheless, Casey and collaborators constructed ETEC strains expressing either STa or STb, and diarrhea was only demonstrated following the inoculation of piglets with the STa construct expressing the fimbriae F41 (Casey et al., 1998). In the current study, we have shown that in controlled conditions (optimal temperature, good sanitation, biosecurity procedures), the presence of putative F4-negative ETEC in the intestine is not always associated with clinical PWD in pigs.

In our study, three successive days of oral CS treatment, d4, was associated with a significant reduction in the fecal presence of both the putative F4-positive ETEC population and of the putative F4-negative ETEC population. At the same time, there was a significant reduction in diarrhea scores and in ETEC: F4 counts in the challenged treated pigs, as previously described (Rhouma et al., 2016b). In addition, at d4, putative F4-positive ETEC were detected in 100% of pigs belonging to the challenged untreated group, at the same time as high diarrhea scores and of the greatest fecal shedding of ETEC: F4 bacteria was observed in this group, as previously described (Rhouma et al., 2016b). These findings highlight the primary role of the F4-positive ETEC population in the occurrence of clinical PWD symptoms in our study.

In the current study, we noted that challenged groups did not develop febrile responses in the days that followed the oral challenge. On the other hand, it has been shown Yi and collaborators showed that the maximum increase in the rectal temperature of pigs challenged with an ETEC: F4 strain was observed at 6 and 12 h post-challenge (Yi et al., 2005). However, in our study, rectal temperatures were not taken during the hours that followed challenge hence we did not characterize the acute-phase response of challenged pigs.

Interestingly, after only 3 days (d4) of oral administration of CS at 50,000 IU/kg bw, a significant reduction in both the F4-positive and F4-negative ETEC populations as well as in diarrhea scores PWD symptoms was observed in the challenged treated group. Indeed, this duration of CS

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treatment is used in several countries compared to the period of 5 days (Official Journal of the European Union, 2010). We consider that our finding is important, having observed an association between CS treatment duration and CS pressure selection on the *E. coli* population during the treatment of pigs in the experimental PWD model (Rhouma et al., 2016b). Nevertheless, the effectiveness of the treatment period, 3 or 5 days, of CS oral treatment in reducing the fecal excretion of F4-positive ETEC and its role in CS resistance *E. coli* amplification, needs to be confirmed in farm conditions with more animals and in the presence of other infection pressures. Such clinical data will be very relevant in the determination of oral CS effectiveness in PWD treatment and help in the re-evaluation of colistin treatment in pigs as undertaken by some regulatory agencies such as the European Medicines Agency (EMA) (European Medicines Agency, 2016a).

Even though pigs were clinically healthy when they excreted F4-negative ETEC before the challenge, we cannot exclude the role of this population in the potentiation of F4-positive ETEC isolates in the development of PWD. In fact, a tendency in the reduction of genes encoding for STb prevalence in fecal samples was observed in association with a reduction in diarrhea scores in the challenged treated pigs. MoreoverIndeed, it is recognised that PWD is a multifactorial disease, for which the many factors necessary to induce diarrhea have not yet been fully identified (Jensen et al., 2006).

In the present study, starting from day two after termination of CS oral treatment (d8) and up to the end of the experiment, no fecal presence of F4-positive ETEC was detected in the challenged treated group. On the other hand, a significant reduction in the F4-positive ETEC population and diarrhea scores PWD symptoms was observed in the challenged untreated group, even in the absence of CS treatment. These findings could be explained by the effective immune response against ETEC: F4 in the challenged groups. Indeed, several studies have shown that oral

immunization of weaned piglets with F4 fimbriae induced a systemic F4-specific antibody response and an increase in mucosal F4-specific antibody (IgA, IgM, IgG) in intestinal tissues (Delisle et al., 2012; Luo et al., 2015; Van den Broeck et al., 2002). On the other hand, after the termination of CS oral treatment, the fecal F4-negative ETEC population reappeared in the unchallenged treated group to the same extent as observed in the unchallenged untreated group. This finding confirmed the role of the immune response following the oral challenge with ETEC: F4 in a long lasting protection of pigs against this pathogen.

In our study, 30 days after termination of CS treatment (d36), pigs in the four experimental groups showed a fecal presence of an F4-negative ETEC population, with a lower prevalence than observed at d1, but usually without clinical symptoms of PWD. Once again, these findings should be considered when determining the cause of diarrhea in pigs using PCR to monitor ETEC virulence genes. Hence, the fecal presence of F4-negative ETEC in diarrheal pigs should not confirm the ETEC etiology of the PWD. Thus, this finding contributes to avoiding the use of antimicrobials to treat viral or parasitic diarrhea in the post-weaning period.

5.6   Conclusion

The use of enriched fecal samples to investigate the fecal presence of ETEC enterotoxin and F4 genes by multiplex PCR, gave information about *E. coli* virulence profiles found in the gut of weaned pigs.

Under controlled conditions, CS oral treatment significantly reduced both the fecal F4-positive and F4-negative ETEC populations in treated groups, and this finding was associated with a significant reduction in diarrhea scores. Furthermore, the fecal presence of F4-negative ETEC was not associated with clinical PWD in pigs. A long-term field trial investigation with more animals would be helpful to confirm the effect of CS on fecal ETEC populations in farm

conditions.

Acknowledgments

Availability of data and materials

All supporting data for our findings are presented in the main paper and in the supplementary file.

5.7   References

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5.8 Figures

Fig. 22. Percentage of fecal presence of the gene encoding STa enterotoxin in weaned pigs challenged or not with ETEC: F4. Challenge was performed at d0 and treatment with colistin sulfate at the dose of 50,000 IU/kg was started at d1 (24 h post challenge) and administered twice daily for 5 days. At d4 a significant reduction in the fecal presence of the gene encoding STa was found in the unchallenged treated group compared to the challenged untreated and the unchallenged untreated groups ($p < 0.0001$). At d36, the fecal presence of the gene encoding STa was statistically lower in the challenged untreated group compared with the unchallenged untreated group ($p < 0.001$). The percentage was calculated by dividing the number of positive pigs by the total number of pigs in each group.

Fig. 23. Percentage of fecal presence of the gene encoding STb enterotoxin in weaned pigs challenged or not with ETEC: F4. Challenge was performed at d0 and treatment with colistin sulfate at the dose of 50,000 IU/kg was started at d1 (24 h post challenge) and administered twice daily for 5 days. At d4 a significant reduction in the fecal presence of the gene encoding STb was found in the unchallenged treated group compared to the unchallenged untreated group ($p < 0.001$). The percentage was calculated by dividing the number of positive pigs by the total number of pigs in each group.

Fig. 24 Percentage of fecal presence of the gene encoding LT enterotoxin in weaned pigs challenged or not with ETEC: F4. Challenge was performed at d0 and treatment with colistin sulfate at the dose of 50,000 IU/kg was started at d1 (24 h post challenge) and administered twice daily for 5 days. At d4 a significant reduction in the fecal presence of the gene encoding LT was found in the challenged treated group compared to the challenged untreated group ($p < 0.0001$). The percentage was calculated by dividing the number of positive pigs by the total number of pigs in each group.

Fig. 25 Percentage of fecal presence of the gene encoding F4 in weaned pigs challenged or not with ETEC: F4. Challenge was performed at d0 and treatment with colistin sulfate at the dose of 50,000 IU/kg was started at d1 (24 h post challenge) and administered twice daily for 5 days. The percentage was calculated by dividing the number of positive pigs by the total number of pigs in each group.

Fig. 26 Evolution of diarrhea scores (mean ± standard deviation [SD]) in pigs challenged or not with an ETEC: F4 strain. Challenge was performed at d0 and treatment with colistin sulfate at the dose of 50, 000 IU/kg was started at d1 (24 h post challenge) and administered twice daily for 5 days. For each sampling time, means with different letters on a given day are statistically different. At d-1 and d36 there was no significant difference between groups.

5.9 Additional file

Additional file 5. Rectal temperatures (mean ± standard deviation [SD]) of weaned pigs challenged or not with ETEC: F4. Challenge was performed at d0 and treatment with colistin sulfate at the dose of 50,000 IU/kg was started at d1 (24 h post challenge) and administered twice daily for 5 days.
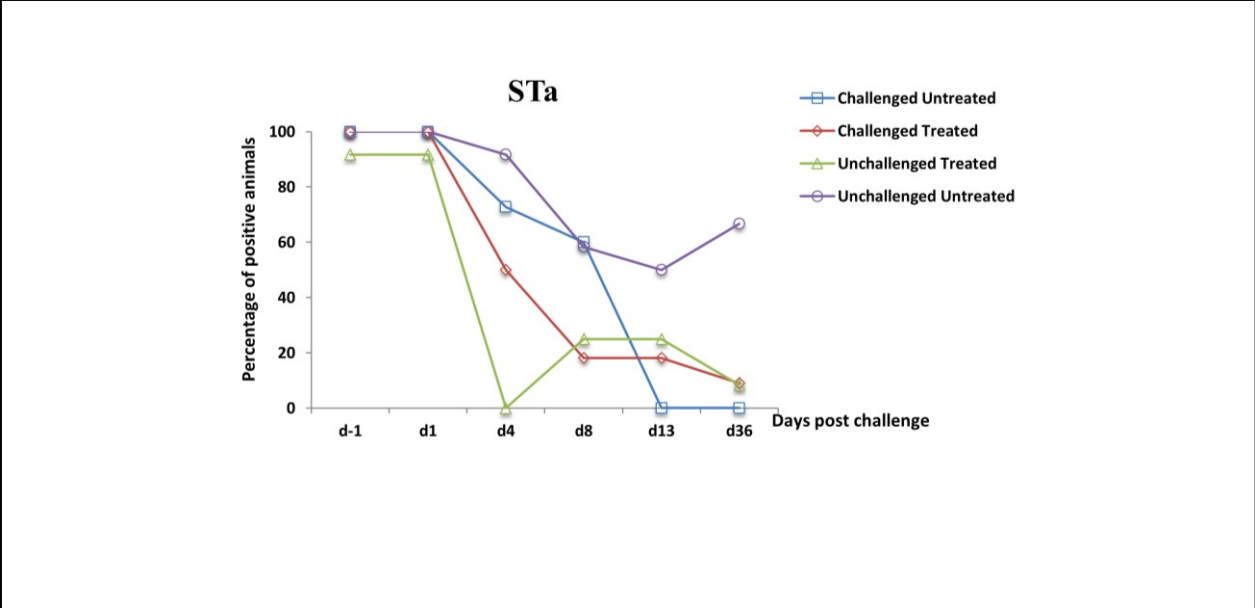
Figure 22: Percentage of fecal presence of the gene encoding STa enterotoxin in weaned pigs challenged or not with ETEC: F4.
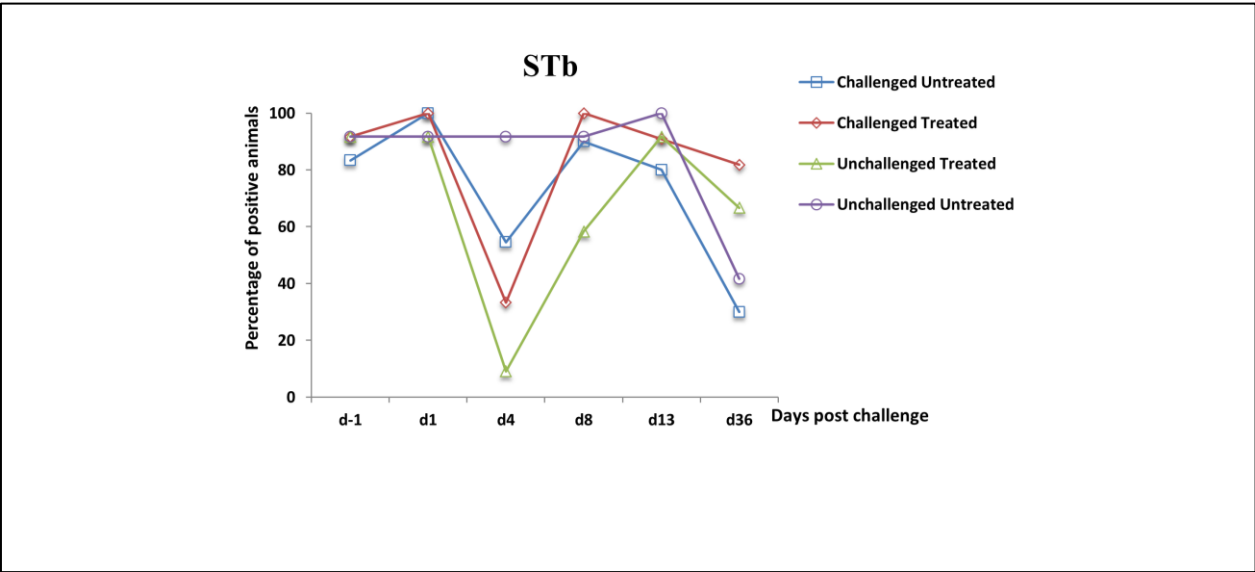
Figure 23: Percentage of fecal presence of the gene encoding STb enterotoxin in weaned pigs challenged or not with ETEC: F4
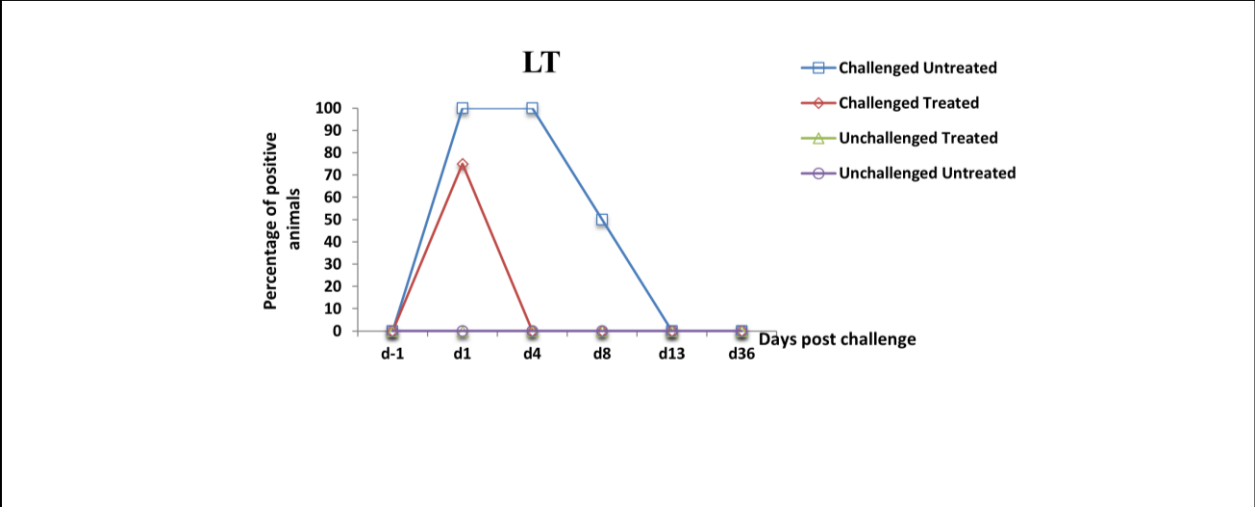
Figure 24: Percentage of fecal presence of the gene encoding LT enterotoxin in weaned pigs challenged or not with ETEC: F4.
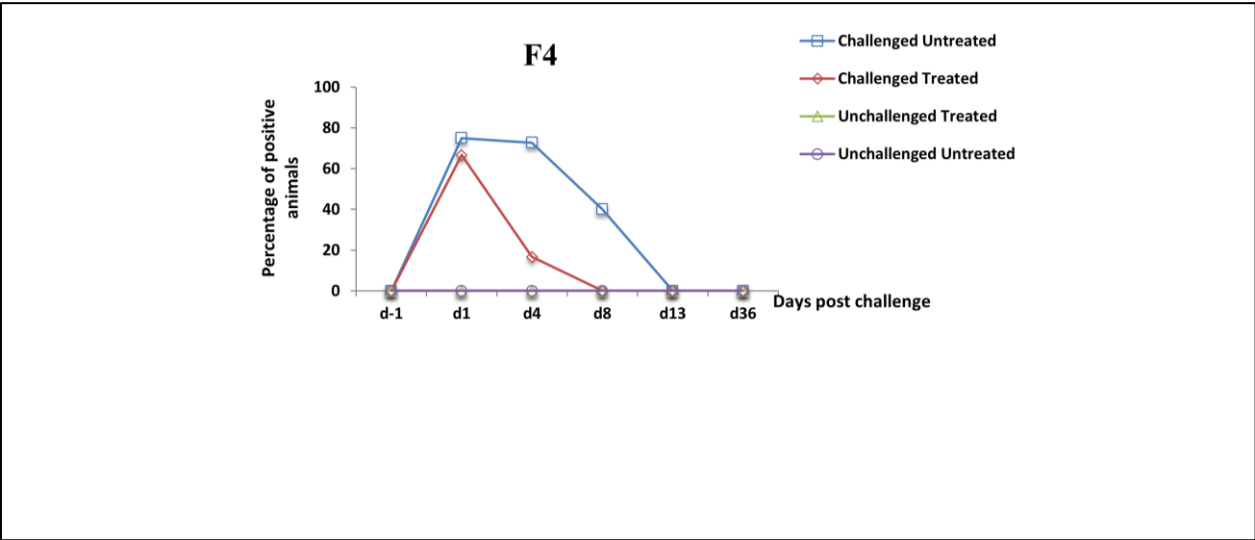
Figure 25: Percentage of fecal presence of the gene encoding F4 in weaned pigs challenged or not with ETEC: F4.
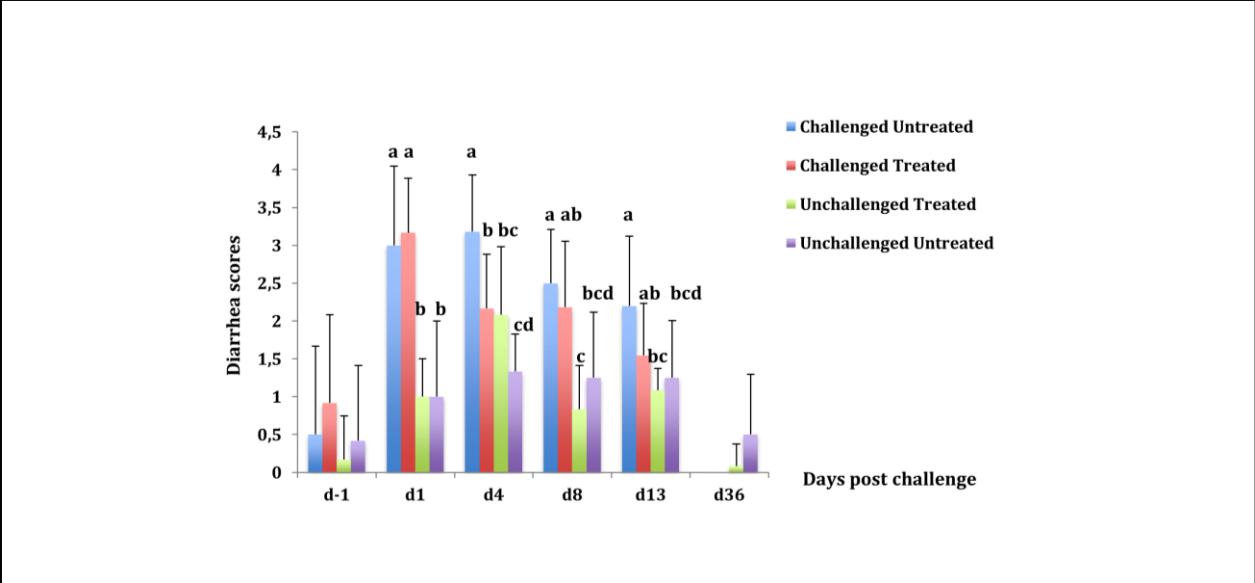
Figure 26 : **Evolution of diarrhea scores (mean ± standard deviation [SD]) in pigs challenged or not with an ETEC: F4 strain.**
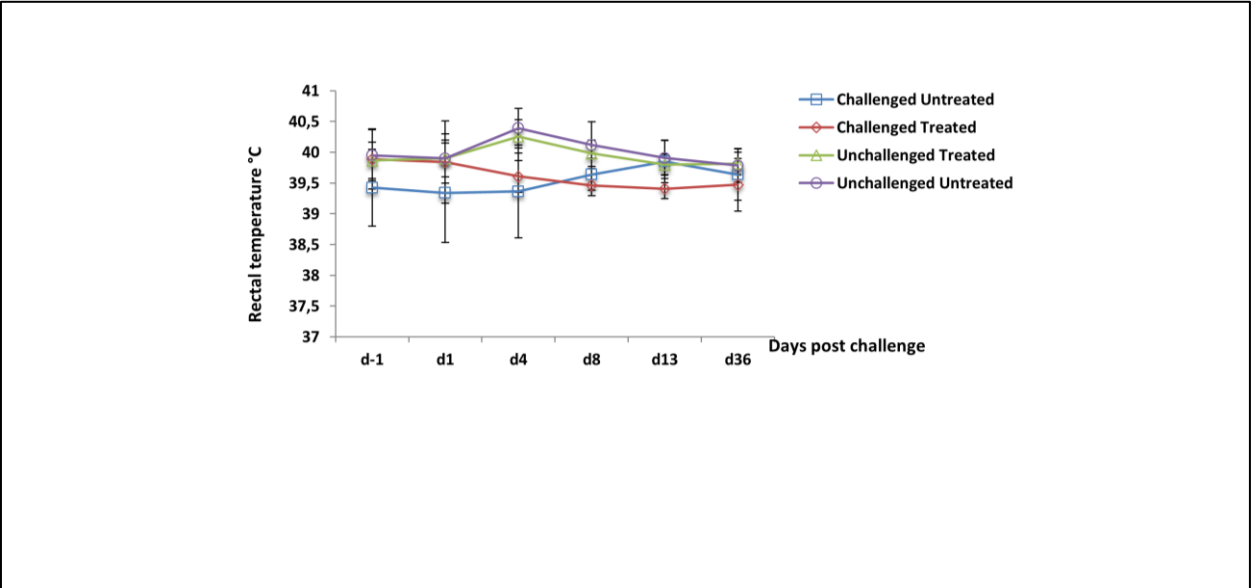
Additional file 5: Rectal temperatures (mean ± standard deviation [SD]) of weaned pigs challenged or not with ETEC: F4

6. Discussion

La diarrhée colibacillaire post-sevrage (DCPS) chez le porc est souvent associée à la présence et la multiplication au niveau intestinal de ETEC O149: F4.

6.1 Reproduction expérimentale de la diarrhée colibacillaire post-sevrage

En raison de la nature multifactorielle de la DCPS, il est souvent difficile de mettre en place un modèle expérimental fiable pour reproduire expérimentalement la DCPS chez le porc (Madec et al., 2000). Dans cet objectif, des études ont rapporté l'importance d'infecter les porcelets avec des virus, tel que le virus de la gastro-entérite transmissible ou le virus du syndrome reproducteur et respiratoire porcin avant leur inoculation avec une souche ETEC (VSRRP) (Cox et al., 1991; Nakamine et al., 1998a). Cependant, ces expériences ont abouti à de graves septicémies et un taux de mortalité très élevé. D'autres études ont recommandé l'utilisation d'un prétraitement oral avec des antibiotiques tel que le florfénicol avant l'inoculation des animaux avec ETEC, cependant malgré ce traitement l'utilisation d'un inoculum de 10^{10} CFU/animal n'a pas été associée à l'apparition des symptômes cliniques de la DCPS (Verdonck et al., 2005). Il a été démontré que l'identification des porcelets porteurs des récepteurs F4 (RF4$^+$) a mené à un taux de succès important dans la reproduction expérimentale de la DCPS par inoculation avec ETEC: F4 (Madec et al., 2000). Dans cette étude, bien que le pourcentage de porcs ayant développé une diarrhée suite à une inoculation par ETEC: F4, était plus élevé chez les animaux (RF4$^+$) par rapport à ceux qui étaient (RF4$^-$) (56% contre 34%), il est surprenant de constater que le tiers des porcs (RF4$^-$) ont développé eux aussi une diarrhée (Madec et al., 2000).

Dans notre étude, nous avons utilisé des porcelets porteurs des récepteurs RF4 pour reproduire expérimentalement la DCPS, ces animaux ont été sélectionnés à la ferme à l'âge de 3 à

4 jours en utilisant la méthode décrite par (Daudelin et al., 2011). Dans l'étude préclinique, l'inoculation des animaux à l'aide de la souche ECL8559 (O149: LT: STa: STb: East1: paa: hemβ: F4) hémolytique et résistante à l'acide nalidixique dans un modèle de DCPS n'a pas engendré de signes cliniques de la maladie, quelques porcelets ont développé une diarrhée 3 jours après l'inoculation (Rhouma et al., 2015). Une souche possédant les mêmes caractéristiques génotypiques et phénotypiques que ECL8559, a été isolée à partir d'un porcelet diarrhéique de l'étude préclinique, et identifiée ECL8559A. Cette souche a été utilisée ultérieurement dans l'étude clinique (Rhouma et al., 2016b). Contrairement à l'étude préclinique, un jour après l'inoculation orale des porcelets avec ECL8559, plus de la moitié des animaux ont développé une diarrhée associée avec une altération de l'état général. Ce résultat a été en corrélation avec d'autres études expérimentales dans lesquelles une fréquence plus élevée de diarrhée aqueuse était observée le premier jour post inoculation des porcelets avec ETEC: F4 (Jensen et al., 2006; Wellock et al., 2008). Ainsi, le passage digestif de ECL8559 dans un porcelet a permis d'augmenter sa pathogénicité. Il serait intéressant d'investiguer, dans un futur travail les éléments potentiels qui ont contribué à la hausse de pathogénicité de ECL8559A. En effet, l'étude *in vitro* de la résistance de ECL8559A par comparaison avec ECL8559 au désoxycholate de sodium et l'étude de la capacité comparative des deux souches à adhérer à des cellules épithéliales d'intestin de porc IPEC-J2, donnera une idée sur la résistance de ces deux souches aux sels biliaires et leur capacité d'adhérer à la muqueuse intestinale et ainsi indiquera leur viabilité au niveau digestif chez le porc (Almofti et al., 2011; Zhou et al., 2014).

6.2 Dégradation gastrique de la colistine sulfate

Dans notre étude, nous avons démontré pour la première fois que la CS subit une dégradation gastrique pour générer des produits de dégradation. En effet, la présence de liaisons

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peptidiques au niveau de la structure de CS la prédispose à l'action enzymatique des enzymes peptidiques telle que la pepsine, mais aussi à la dégradation chimique sous l'action du HCl gastrique. Cependant, cette dégradation a été associée avec une légère augmentation de l'activité antimicrobienne de ces produits de dégradation par comparaison avec la CS non dégradée (Rhouma et al., 2015). Ce résultat pourrait s'expliquer par la perte des chaines latérales de la CS sous l'action enzymatique et chimique, ce qui va donner naissance à plusieurs métabolites avec moins d'encombrements stériques et favorise ainsi une plus grande interaction avec les LPS des bactéries. En revanche, ces produits de dégradation nécessitent d'être identifiés pour étudier leurs activités antimicrobiennes et leur implication dans l'évolution de la résistance à la colistine chez les entérobactéries. Dans notre étude, la quantification de ces métabolites n'a pas été possible au niveau de la circulation systémique des porcelets à cause de l'absence des standards de référence. Ainsi, la caractérisation de ces produits de dégradation est indispensable pour les études toxicologiques et les études pharmacocinétiques dans un objectif de santé publique afin de déterminer l'innocuité et l'impact sur la santé du consommateur de la présence de ces produits dans la viande porcine.

Les produits de dégradation de la colistine peuvent aussi se retrouver dans le lisier du porc et continueront d'exercer une pression de sélection sur les bactéries dans l'environnement. En effet, les produits de dégradation des tétracyclines ont été retrouvés dans le lisier du porc à des quantités supérieures à celles des composés non dégradés (Solliec et al., 2016). Ainsi, il serait intéressant de suivre le processus de dégradation de la colistine dans le lisier du porc et d'évaluer les différents processus de traitements de ce dernier sur la réduction de la quantité de CS et ses métabolites.

6.3 L'utilisation de la colistine sulfate en production porcine

Dans notre étude, nous avons utilisé deux doses de CS afin de mieux refléter les conditions du terrain. En effet, la dose faible de 50,000 UI/kg était la dose thérapeutique recommandée chez les porcs, tandis que la dose élevée de 100,000 UI/kg a été utilisée pour prendre en considération le portrait réel de l'utilisation de la CS dans les élevages porcins, où cet antibiotique est souvent surdosé (Chauvin et al., 2002), considérant la hiérarchie sociale et l'hétérogénéité observée entre les animaux dans les mêmes enclos étant à l'origine d'une plus grande consommation d'antibiotiques chez certains porcs (Soraci et al., 2014).

La dose de CS utilisée en production porcine n'est pas standardisée à l'échelle mondiale. En effet, certains praticiens utilisent l'unité internationale/kg tandis que d'autres utilisent le mg/kg comme unité de mesure pour sélectionner la dose de CS à utiliser chez le porc (Guyonnet et al., 2010; Trauffler et al., 2014). Les doses de CS incorporées dans l'alimentation des porcs pour le traitement des infections à entérobactéries étaient très variables entre les études, avec des doses allant de 66 jusqu'à 800 mg/kg d'aliment (Burch, 2007; Torrallardona et al., 2003; Wu et al., 2012). En plus, lorsque la colistine est utilisée comme promoteur de croissance dans l'alimentation du porc, les doses utilisées varient entre 20 et 60 mg par kg d'aliment (Wan et al., 2016; Wang et al., 2016b).

Ainsi, en absence d'une posologie standardisée de CS chez le porc, il est difficile d'assurer une utilisation judicieuse de cet antibiotique en production porcine et de lutter efficacement contre l'émergence de la résistance à la CS.

6.4 La pharmacocinétique de la colistine sulfate chez le porc

Dans l'étude préclinique, l'utilisation d'une dose orale unique de CS avait pour objectif de déterminer l'aire sous la courbe (ASC) des concentrations plasmatiques et la constante de vitesse

d'élimination (λz) de cet antibiotique, cette dernière étant un paramètre particulièrement important afin de déterminer la demi-vie d'élimination de la CS ($T_{1/2}$), qui est un indice de persistance du médicament dans le corps. Les concentrations de CS ont été détectées chez les porcs sains avec un pic qui a été observé 30 min après l'administration orale de la CS à 50,000 UI/kg. Cependant, ces concentrations étaient inférieures à la limite de quantification de notre technique (20 ng/mL), mais au-dessus de sa limite de détection (Rhouma et al., 2015). De plus, l'inoculation des animaux avec la souche ECL8559 n'a pas augmenté l'absorption intestinale de la CS dans un modèle subclinique de DCPS. Ainsi, la biodisponibilité orale de la CS chez le porc est très faible. Notre étude confirme les résultats des études précédentes qui ont démontré que la CS est faiblement absorbée au niveau intestinal chez le porc et ces concentrations systémiques sont habituellement indétectables (Guyonnet et al., 2010).

Dans notre étude clinique, la souche ECL8559A a engendré une augmentation de l'absorption intestinale de la CS chez les porcs inoculés versus les porcs sains dans les deux essais (Rhouma et al., 2016b). En effet, plusieurs études ont démontré que l'ajout du LPS à des cellules Caco-2 induit une augmentation de la production et la libération de TNF-α et d'IL-1; ces cytokines pro-inflammatoires ont augmenté la perméabilité des jonctions serrées de ces cellules (Ma et al., 2005). En plus, il a été démontré que l'IL-1, active la perte du VE-cadhérine au niveau de l'endothélium vasculaire pour induire une fuite sanguine (Dagvadorj et al., 2015). Cependant, le rôle des LPS libérés par la souche ETEC: F4 dans l'augmentation de la perméabilité de jonctions serrées et dans la production des cytokines pro-inflammatoires chez le porc doit être confirmé dans une étude future. Ce résultat devrait être pris en considération lors de la détermination des temps d'attente des antibiotiques en production porcine, et particulièrement pour ceux qui sont caractérisés par une biodisponibilité orale importante.

Étant donné que la CS est très faiblement absorbée dans le tractus digestif du porc, les matières fécales constituent la principale voie d'élimination de cet antibiotique et la microflore est donc exposée à de fortes concentrations de CS suite à son administration orale (Rhouma et al., 2016a). Ainsi, le microbiote intestinal du porc pourrait jouer un rôle dans l'amplification et la persistance des gènes et des bactéries résistantes à la CS.

Dans une étude récente, il a été rapporté que l'administration de la CS par gavage oral à la dose de 50,000 UI/kg/jour a donné lieu à des concentrations très différentes de cet antibiotique dans les échantillons de matières fécales (MF) des porcs, et ainsi au niveau de leurs tubes digestifs (Fleury et al., 2016). En effet, les concentrations de CS variaient entre $15,11 \pm 5,42$ et $13,66 \pm 11,33$ μg de colistine/g de MF respectivement après 2 et 4 jours du traitement. Cependant, ces concentrations sont très inférieures aux concentrations bactéricides requises au niveau intestinal pour aboutir à une activité bactéricide de la CS, favorisant ainsi une exposition des bactéries à des doses subthérapeutiques de CS. Dans un modèle PK/PD, Guyonnet et collaborateurs (2010) ont déterminé la dose de 37.2 μg de colistine/g de contenu intestinal du porc comme une concentration de référence pour garantir une activité bactéricide de la CS (Guyonnet et al., 2010).

Parmi les limites de notre étude clinique, les concentrations intestinales de la CS n'ont pas été mesurées étant donné la difficulté de collecte totale des matières fécales. Une telle information aurait été intéressante pour déterminer si les concentrations de CS qui arrivent au niveau de l'intestin sont bactéricides. Cependant, cette expérience nécessitait l'euthanasie de quelques porcelets après chaque administration de CS pour récupérer la totalité du contenu intestinal, ce qui aurait réduit significativement le nombre d'animaux destinés aux autres analyses. Ainsi, une étude avec un design expérimental spécifique pour déterminer la relation PK/PD est indispensable pour optimiser la dose thérapeutique de la CS chez le porc.

Malgré les progrès qui ont été faits pour quantifier la CS chez le porc dans différentes matrices (MF, plasma), il reste encore indispensable de mettre sur le marché un standard interne qui permet la comparaison des résultats entre les études et permet d'évaluer la pureté de la CS utilisée en médecine vétérinaire (Zhao et al., 2014).

6.5 Efficacité thérapeutique de la colistine sulfate

Dans notre étude clinique, nous avons constaté que, quelle que soit la dose de CS utilisée (50,000 ou 100,000 UI/kg) dans le traitement orale de la DCPS, elle a induit une diminution significative dans l'excrétion fécale des ETEC: F4, de la population totale d'*E. coli*, et des scores de diarrhée, mais seulement pendant la période du traitement. Ces résultats corroborent l'étude de Fleury et collaborateurs (2016) réalisée chez des porcs sains traités avec une dose de CS à 50,000 UI/kg/Jour pendant 5 jours (Fleury et al., 2016). Notre étude a démontré pour la première fois que l'augmentation de la dose orale de CS n'a pas été associée avec une réduction significative de l'excrétion fécale d'*E. coli.* En effet, cette constatation est en désaccord avec la PD connue de la CS comme antibiotique « concentration-dépendant » (Nation et al., 2014). Il a été démontré *in vitro* que la vitesse de l'activité bactéricide de la CS observée sur des souches *E. coli* d'origine porcine augmente avec la concentration de colistine présente dans le milieu (Guyonnet et al., 2010). Par conséquent, pour obtenir une activité bactéricide maximale de la CS et réduire le risque potentiel de sélectionner des bactéries résistantes, le rapport: aire sous la courbe (ASC)/CMI et Cmax/CMI devrait être optimisé pour cet antibiotique (Ahmad et al., 2016; Dijkmans et al., 2015). Cependant, dans notre étude, l'utilisation de la dose élevée de CS (100.000 UI/kg) n'a pas été associée à la réduction bactérienne la plus importante, et ainsi il est impossible de confirmer l'activité bactéricide « concentration-dépendante » de cet antibiotique *in vivo* tel qu'il a été confirmé *in vitro*. Malgré le fait que le mécanisme antibactérien exact de la CS

n'est pas encore bien connu et que plusieurs études ont mentionné de nombreux mécanismes d'action antibactériens de cet antibiotique (Yu et al., 2015), nous ne pouvons pas nous baser sur une seule étude *in vivo* pour confirmer la nature de l'activité antibactérienne de la CS chez le porc. Par contre, dans une autre étude *in vivo*, il a été rapporté que la biodisponibilité de la CS après une administration intramusculaire (IM) chez le porc, était inversement proportionnelle aux doses administrées, avec une biodisponibilité systémique de 95,94 et 88,45% pour une dose de 2,5 et 5 mg /kg respectivement (Lin et al., 2005). Pour confirmer l'activité bactéricide « concentration-dépendente » de la CS chez le porc, il serait intéressant de quantifier les concentrations de cet antibiotique au niveau intestinal et de les rapprocher des résultats microbiologiques observés.

En outre, après trois jours de traitement consécutif à la CS, une réduction significative au niveau de la présence fécale des gènes qui codent pour LT et F4 a été observée. Ce résultat a été accompagné par une réduction significative des scores de diarrhée et du compte d'ETEC: F4 sur les géloses pour les animaux inoculés. Nous considérons que ce résultat devrait être pris en considération lors de la réévaluation de la CS en production porcine telle qu'entreprise par l'Agence européenne des médicaments (European Medicines Agency, 2016a). En effet, la durée de traitement des infections intestinales bactériennes par la CS chez le porc varie entre 3 et 5 jours suivant les pays de l'Union européenne (Official Journal of the European Union, 2010). De plus, une corrélation a été démontrée entre la durée du traitement oral à la CS et la pression de sélection exercée par cet antibiotique sur la population d'*E. coli* chez le porc (Rhouma et al., 2016b) et sur la population de *Klebsiella pneumoniae* lors de l'utilisation de la CS pour la décontamination digestive sélective (DDS) en médecine humaine (Halaby et al., 2013).

D'autre part, selon nos résultats, l'aspect économique de l'augmentation de la dose de CS dans le traitement de la DCPS est remis en question. En plus nous avons constaté que les groupes

infectés non traités dans les deux essais ont été capables de neutraliser l'infection à ETEC: F4 dans le même intervalle du temps que les groupes infectés traités (Rhouma et al., 2016b). Il faut prendre en considération que, notre expérimentation a été réalisée dans des conditions expérimentales contrôlées (température optimale, faible densité, une bonne hygiène), qui pourraient jouer un rôle crucial dans l'élimination de toutes sortes de stress supplémentaires pour l'animal. Nos résultats sont en corrélation avec les constatations de Madec et collaborateurs (1998) qui ont démontré que l'optimisation des conditions zootechniques, particulièrement la température, dans les fermes porcines, jouait un rôle aussi important que les antibiotiques dans le contrôle de la DCPS (Madec et al., 1998).

Dans notre étude, l'efficacité thérapeutique de la CS a été évaluée cliniquement par la mesure des scores de diarrhée, la température, le niveau d'anorexie, les paramètres de croissance des animaux et par les analyses microbiologiques (dénombrement, PCR multiplex). Il serait intéressant aussi de confirmer les résultats obtenus concernant les populations d'*E. coli* par PCR quantitative (qPCR), en plus de cibler quelques populations bactériennes indicatrices de la santé digestive chez le porcelet afin de déterminer l'évolution du rapport entre ces populations suite au traitement. En plus, le séquençage haut débit serait une technique très importante pour compléter les résultats obtenus et permettre de générer des données à propos de la composition bactérienne de l'intestin du porc suite à l'infection et au traitement.

6.6   Les gènes de virulence de ETEC

Dans la présente étude, tous les porcs utilisés ont excrété au niveau de leur MF, des gènes de virulence codant pour les deux entérotoxines STa et STb avant leur inoculation par ETEC: F4. Le même résultat a aussi été observé à la fin de notre expérimentation (30 jours après le début du traitement) où tous les animaux excrètent ces 2 gènes de virulence. Notre étude a été la première

à rapporter la présence concomitante de ces deux gènes de virulence chez des porcs cliniquement sains dans la période post-sevrage. En effet, d'autres études ont associé la présence fécale des gènes qui codent pour STa et STb avec des symptômes cliniques de DCPS dans des conditions d'élevage (Chapman et al., 2006; Kim et al., 2010). Ici nous soulignons encore une fois l'importance des conditions dans lesquelles s'est déroulée la présente expérimentation, où la présence intestinale des gènes codant pour STa et STb (marqueurs de ETEC: F4 négatif) dans l'intestin des porcelets n'a pas été associée avec une DCPS. Ses résultats devraient être pris en considération lors du diagnostic de la DCPS en utilisant la PCR multiplex. Ainsi, la présence fécale des gènes de virulence qui codent uniquement pour STa et STb chez des porcelets diarrhéiques ne confirme pas l'implication de ETEC comme étiologie de la DCPS. Cependant, un autre pathotype, *E. coli* entéropathogène (EPEC), capable de causer des lésions d'attachement et d'effacement (lésions A/E) au niveau des microvillosités intestinales du porc, a été rapporté comme étant impliqué dans 6% des cas de DCPS (Fairbrother et al., 2005). Dans notre étude, les gènes de virulence, qui codent pour EPEC, intimine (*eae*), n'ont pas été investigués dans les matières fécales des porcs. Il serait intéressant de confirmer, dans une future étude, chez des porcelets diarrhéiques le rôle de la coprésence fécale des gènes qui codent pour STa, STB et l'intimine dans l'apparition clinique de la DCPS, ou bien le rôle des infections virales concomitantes dans les fermes dans la potentialisation de l'effet intestinal de STa et STb.

Dans l'essai 2, à partir de deux jours après la fin du traitement par la CS (8 jours après l'inoculation) et jusqu'à la fin de l'expérience, aucune présence fécale de ETEC F4-positif n'a été détectée dans le groupe infecté-traité. Cependant ce même résultat a été observé pour le groupe infecté non traité à partir du 13e jour après l'inoculation. Ce résultat montre qui la CS a accéléré l'élimination fécale de ETEC F4-positif. En plus, la présence fécale des gènes qui codent pour LT et F4 (marqueurs de ETEC: F4 positif) pourrait être utilisée à la fois pour confirmer le diagnostic

clinique d'une DCPS et pour évaluer l'efficacité d'un traitement antimicrobien. Toutefois, même si les porcs étaient cliniquement sains quand ils excrètent ETEC: F4 négatif avant l'inoculation, nous ne pouvons pas exclure le rôle de cette population dans la potentialisation de ETEC: F4 positif qui sont impliqués dans le développement de la DCPS. En effet, il est reconnu que cette maladie est multifactorielle, et des nombreux facteurs sont nécessaires pour son apparition clinique (Jensen et al., 2006).

Toutefois, le groupe infecté non traité était lui aussi capable de neutraliser l'infection. Ce dernier résultat pourrait s'expliquer par la réponse immunitaire importante contre ETEC: F4 après inoculation. En effet, plusieurs études ont montré que l'immunisation par voie orale des porcelets avec l'adhésine F4 purifiée a induit une réponse immunitaire mucosale spécifique à F4 par la production des IgA, IgM, et IgG (Delisle et al., 2012; Luo et al., 2015).

6.7 Résistance bactérienne à la colistine sulfate chez le porc

Dans notre étude nous avons démontré que suite à l'administration orale de la CS à la dose de 50,000 UI/kg, il y avait une importante diminution de l'excrétion fécale de la population d'*E. coli* qui a été constatée depuis le premier jour du traitement. En parallèle, à partir du jour 2 du traitement (d2), une légère augmentation (15%) de la proportion d'*E. coli* présumée résistante à la colistine a été constatée chez le groupe infecté traité par comparaison avec le groupe infecté non traité. Cette différence entre ces deux groupes a été observée durant toute la période du traitement, et a diminué progressivement à partir de la première journée après l'arrêt du traitement (Rhouma et al., 2016b). Ces résultats sont en faveur d'une pression de sélection qui s'est exercée sur la population d'*E. coli* lors du traitement. Cependant, uniquement 12,5% des isolats d'*E. coli* provenant des géloses MacConkey supplémentées avec 2 µg/ml de CS ont été confirmés résistants à la colistine, dont la plupart (8/9) proviennent du groupe traité. Ces résultats

confirment ainsi la pression de sélection exercée par la CS sur la population d'*E. coli*. Nos résultats corroborent ceux de Boyen et collaborateurs (2010) qui ont rapporté que 10% des isolats cliniques d'*E. coli* d'origine porcine ont été confirmés résistants à la colistine, cependant aucune information sur un potentiel traitement de ces animaux avec la CS n'a été fournie dans cette étude (Boyen et al., 2010). Ainsi, notre étude était la première qui a rapporté que l'utilisation thérapeutique de la CS a été associée avec la sélection des *E. coli* résistant chez des animaux infectés avec une souche ETEC : F4 dans un modèle de DCPS. Cependant, il a été rapporté que suite à l'exposition des porcelets sains à une dose 50,000 UI/kg/j, il n'y avait pas d'isolats d'*E. coli* qui ont été confirmés résistants à la colistine dans cette étude (Fleury et al., 2016). Dans notre étude, nous avons trouvé une faible proportion d'isolats d'*E. coli* résistants à la colistine par comparaison avec d'autres études effectuées dans d'autres pays (Lu et al., 2010; Mateu and Martin, 2000). Ainsi, il serait intéressant de déterminer dans une future étude l'effet de la CS sur l'évolution de la résistance d'*E. coli* dans des conditions de fermes porcines au Canada, et suite à un traitement de masse (eau de boisson ou alimentation), en tenant compte de la hiérarchie dans le troupeau, du comportement alimentaire des porcs et de leur condition de santé.

Dans notre étude, des *E. coli* présumés résistants à la colistine ont été identifiés sur les géloses MacConkey supplémentées avec la CS avant le traitement oral à base de CS. En effet, le taux de mutation naturelle *in vitro* en absence de colistine était de 3,4 x 10^{-8} pour la souche *E. coli* ATCC 25922 et de 2,7 x 10^{-8} pour la souche ECL8559A. En présence d'une concentration sub-inhibitrice (0,01 µg/ml) de la CS le taux de mutation était de 8 x 10^{-8} pour la souche *E. coli* ATCC25922 et 1,2 x 10^{-7} pour la souche ECL8559A (Thériault, 2015). Ainsi, la présence de quelques colonies résidentes présumées résistantes à la colistine avant le traitement pourrait s'expliquer par le taux de mutation naturelle d'*E. coli*. L'exposition sub-thérapeutique à la CS augmente le taux de mutation d'*E. coli.* Il serait intéressant de tester dans une future étude, si

l'exposition des *E. coli* pathogènes à des doses thérapeutiques de CS *in vitro* augmente le taux de mutation, ce qui pourrait expliquer la pression de sélection observée *in vivo.*

En outre, notre étude a contesté la pertinence d'utiliser le milieu MacConkey supplémenté avec la CS pour isoler des bactéries résistantes à cet antibiotique. En effet, nous avons confirmé que ce milieu surestime le nombre d'*E. coli* réellement résistants à la CS, et une telle résistance nécessite d'être confirmée par la détermination de la CMI. En effet, il a été démontré que les sels biliaires présents dans le milieu MacConkey, induisaient une résistance aux polymyxine chez *E. coli* (Kus et al., 2011). Pour surmonter ce problème lié à l'absence d'un milieu sélectif pour le criblage de bactéries résistantes à la colistine, Nordmann et collaborateurs ont mis au point très récemment un milieu de dépistage qui pourrait être utilisé pour l'isolement des bactéries résistantes aux polymyxines sans la nécessité de confirmer cette résistance par détermination de la CMI (Nordmann et al., 2016). En effet, ce milieu est composé principalement, par la colistine sulfate, la daptomycine pour inhiber la croissance des bactéries à Gram positif, et l'amphotéricine B qui est un antibiotique qui possède des propriétés antifongiques. L'utilisation de ce milieu facilitera le suivi de la résistance des entérobactéries à la colistine chez les animaux de rente dans les études à venir.

Dans notre étude, nous avons confirmé que sur les 9 isolats d'*E. coli* résistants à la colistine, seulement 4 avaient une mutation dans le système à doubles composantes (SDC) PmrA/PmrB (Thériault, 2015). Notre constatation est en corrélation avec l'étude de Quesada et collaborateurs, qui a décrit pour la première fois les types de mutations dans le SDC PmrA/PmrB observées chez des isolats d'*E. coli* résistants à la colistine chez le porc (Quesada et al., 2015).

En novembre 2015, une publication scientifique a décrit pour la première fois, un nouveau gène, appelé *mcr-1*, porté par un plasmide et qui code pour la résistance à la colistine chez les entérobactéries et permet ainsi le transfert de la résistance à cet antibiotique entre les bactéries

(Liu et al., 2016). Le gène a été découvert sur un plasmide conjugatif stable et a été isolé à partir de plusieurs sources incluant les animaux de ferme et domestiques, les végétaux, les oiseaux migrateurs, l'environnement et les humains (Schwarz and Johnson, 2016). En effet, la résistance à la colistine en médecine porcine n'est pas un phénomène nouveau, plusieurs études ont rapporté l'isolement des bactéries résistantes à la colistine chez le porc depuis plusieurs années (Rhouma et al., 2016a). La découverte de ce mécanisme de résistance plasmidique à la colistine a conduit à de fortes réactions au sein de la communauté scientifique et a généré de l'inquiétude des médecins concernant le risque potentiel de la perte de l'efficacité de cet antibiotique de dernier recours en médecine humaine pour le traitement des infections bactériennes à BGN multirésistantes.

La découverte de ce plasmide contribue à la compréhension des autres mécanismes de résistance à la colistine et explique la résistance de certaines bactéries à la colistine en absence de mutation dans le SDC PmrA/PmrB. Les travaux de recherche se poursuivent pour déterminer la nature du gène (*mcr-1* ou *mcr-2*) présent dans les isolats d'*E. coli* résistants à la colistine qui ont été isolés durant la phase expérimentale dans le cadre de cette étude.

Il a été rapporté que les bactéries d'origine porcine qui ont été confirmées résistantes à la colistine et qui contiennent le plasmide qui héberge le gène *mcr-1*, ont souvent été associées à un niveau de résistance faible à la colistine. En effet, les CMI de ces isolats étaient entre 4 à 8 mg/L, ce qui correspond à une augmentation de 2 à 4 fois les valeurs du seuil cliniques (2 mg/L) définies dans les directives de l'EUCAST (*European Committee on Antimicrobial Susceptibility Testing*) (Anjum et al., 2016; Liu et al., 2016; Quesada et al., 2016). En effet, le gène *mcr-1* code pour l'ajout d'une phosphoéthanolamine (PEtN) au lipide A et conduit ainsi à une diminution d'affinité de la colistine pour les LPS (Nordmann and Poirel, 2016). Cependant, il a été démontré que l'ajout d'un groupement 4-amino-4-deoxy-L-arabinose (L-Ara4N) au lipide A des

Salmonella confère un niveau plus élevé de résistance contre les polymyxines par comparaison avec les modifications engendrées par l'ajout du PEtN (Olaitan et al., 2014). De plus, Fernandes et ses collaborateurs ont rapporté l'identification du gène *mcr-1* dans un isolat d' *E. coli* sensible à la colistine provenant d'un porc sain (Fernandes et al., 2016). Cette constatation indique la difficulté de repérer tous les isolats *mcr-1* positifs si uniquement le gène a été recherché sur des isolats confirmés résistants. En plus, cela pourrait contribuer aussi à la diffusion silencieuse du *mcr-1* entre les bactéries.

Plusieurs études ont rapporté une colocalisation plasmidique entre *mcr-1* et les β-lactamases à spectre étendu (BLSE) dans des isolats d'*E. coli* d'origine animale (Grami et al., 2016; Haenni et al., 2016). En plus, un lien historique a été établi entre *mcr-1* et BLSE (Annexe 1). Ces constatations indiquent la nécessité d'une intervention rapide chez les animaux de rente pour réduire l'utilisation non seulement de la colistine, mais aussi de tous les antimicrobiens qui ont une importance très élevée en médecine humaine.

Conclusions

Cette thèse de doctorat avait comme objectif de déterminer la pharmacocinétique de la colistine sulfate chez le porc et d'étudier l'impact de cet antibiotique sur l'excrétion fécale et la résistance d'*E. coli* dans un modèle d'infection expérimentale de DCPS. L'hypothèse principale de cette étude était que la CS subit une dégradation digestive dans le tractus gastro-intestinal du porc et l'utilisation orale de cet antibiotique pour le traitement clinique de la DCPS pourrait être associée à une amélioration des symptômes cliniques de la maladie, une réduction de l'excrétion fécale d'*E. coli* et des gènes de virulence de ETEC : F4, une amélioration de la croissance des animaux et une exacerbation de la résistance d'*E. coli* à la CS.

Tout d'abord, l'ensemble des travaux réalisés lors de cette thèse est très original puisque très peu d'études portent sur la PK, l'efficacité thérapeutique et la résistance à CS en médecine porcine.

Cette thèse a permis de décrire, pour la toute première fois, une dégradation gastrique importante de la CS, qui a été associée avec la formation des produits de dégradations qui ont démontré une activité antimicrobienne importante en comparaison avec la CS non dégradée.

De plus, dans le cadre de nos travaux, une technique HPLC-MS/MS a été mise au point et a permis pour la première fois de quantifier des concentrations systémiques de CS chez le porc. En utilisant cette technique très sensible, nous avons démontré que l'inoculation orale des animaux par une souche ETEC: F4 a augmenté l'absorption intestinale de la CS dans un modèle expérimental de DCPS.

Notre étude a permis de démontrer que la CS était efficace dans la réduction des symptômes de DCPS et l'excrétion fécale d'*E. coli* uniquement durant la période du traitement. Également pour la première fois, notre étude a démontré que l'augmentation de la dose orale de CS n'était pas associée à une réduction plus rapide des symptômes de DCPS. Et ainsi, a remis en

question le mécanisme d'action « concentration-dépendant » de la CS qui a été démontrée *in vitro*.

Dans notre étude, nous avons isolé pour la première fois des *E. coli* résistants à la colistine dans un modèle d'infection expérimentale de DCPS, suite à l'utilisation de la CS telle que recommandé par les monographies. Cette constatation devrait être considérée lors de la réévaluation de la CS en médecine porcine dans un objectif d'optimiser le dosage de cet antibiotique.

Notre étude a démontré que l'amélioration des conditions d'élevage pour des porcelets en période post-sevrage et qui ont été inoculés avec une souche ETEC: F4, a été aussi efficace que la CS dans la réduction des symptômes de la DCPS. Ainsi dans une perspective de développement durable en production porcine, pour réduire les quantités des antimicrobiens utilisées dans les fermes, l'amélioration des conditions zootechniques pour les animaux est cruciale.

Finalement, nous avons démontré pour la première fois que la présence fécale concomitante des gènes qui codent pour STa et STb (marqueurs de ETEC: F4 négatif) chez des porcelets en période post-sevrage n'a pas été associée avec des symptômes cliniques de DCPS. Cependant, la présence fécale des gènes qui codent pour LT et F4 (marqueurs de ETEC: F4 positif) pourrait être utilisée à la fois pour confirmer le diagnostic clinique d'une DCPS causée par ETEC et pour évaluer l'efficacité d'un traitement antimicrobien.

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Annexe 1: Extended-spectrum β-lactamase, carbapenemase, and the *mcr-1* gene: Is there a historical link?

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The recent discovery of a plasmid-mediated *mcr-1* gene encoding for colistin resistance in *Escherichia coli* and *Klebsiella pneumoniae* from animals, food, and humans in China [1] has initiated the global research of this plasmid in different hosts and different Gram-negative bacteria (GNB) [2].

The *mcr-1* gene has been identified in five continents from bacteria isolated from several origins, including animals, food, the environment, and humans [3, 4]. Several studies, conducted mostly in animals, have reported the identification of the *mcr-1* gene among Extended-Spectrum β-Lactamases (ESBL) producing *E. coli* [5-8]. In a retrospective study, Shen and collaborators reported the identification of the *mcr-1* gene in three *E. coli* strains from chickens in China isolated in the 1980s [9]. To the best of our knowledge, this is the oldest identification of the *mcr-1* gene reported in scientific literature.

Is it possible there is a simultaneous coexistence between ESBL, carbapenemase enzymes, and the *mcr-1* gene?

Historical events concerning the discovery and emergence of plasmid-mediated colistin-resistant bacteria as well as ESBL and carbapenemase genes are traced in Figure 27. Colistin was discovered in 1949 and became available for clinical use in the 1960s for the treatment of GNB [10]. Colistin use was very restricted between 1970 and the late 1990s in humans due to its reported nephrotoxicity and the development of less-toxic antimicrobial agents. However no restriction was reported on colistin use in veterinary medicine during this period [3].

Extended-spectrum (or third-generation) cephalosporins (e.g., cefotaxime, ceftriaxone, ceftazidime) were introduced into clinical use in the early 1980s [11]. These β-lactam antibiotics were regarded as a major advance in the treatment of infection caused by β-lactamase-producing bacteria [12]. However, the emergence of resistance against these antibiotics was observed, with the first report on plasmid-encoded β-lactamase enzymes capable of hydrolyzing the extended-

spectrum cephalosporins in *K. pneumoniae* published in 1983 [11]. This seems to correspond to the first identification of the *mcr-1* gene in *E. coli*, according to Shen and collaborators [9], which indicates a temporal concurrence between the first identification of ESBL enzymes and that of the *mcr-1* gene.

In 1985, the first carbapenems (imipenem) were marketed for the treatment of infections caused by *Enterobacteriaceae*, particularly those producing ESBLs [13, 14]. After a decade of practical use of carbapenems, a strain carrying the plasmid *K. pneumoniae* carbapenemase (KPC-1) was first observed in North Carolina in 1996 before progressively appearing worldwide [15].

The presence of ESBL and carbapenemase genes in the same bacterial strains was reported for the first time in *Klebsiella spp.* collected from October 2006 to November 2007 by the Emory University Hospital Microbiology Laboratory, Atlanta, GA, USA [16]. In this study, authors reported the presence of an ESBL in 19 of 26 (73%) of the KPC isolates [16]. Knowing the technical challenges in identifying ESBL and carbapenemase genes among resistant bacterial strains [17], it is difficult to affirm the absence of these genes before its first description.

The emergence of multidrug-resistant (MDR) GNB and the lack of new antimicrobial agents occurred concurrently with a resurgence of interest in colistin use in human medicine starting in the late 1990s [10].

The first identification of a co-localization of *mcr-1* and ESBL genes on a unique plasmid dates back to 2005 [6]. From 2006 to 2014, Haenni and collaborators reported an increase of the proportion of *mcr-1* genes among ESBL-producing *E. coli* in French calves, from 4.76% to 21.28% in 2006 and 2014 respectively [8]. In these two old bacterial collections, the *mcr-1* gene was detected in ESBL producing isolates likely because these previously identified ESBL isolates or sequences were available in the laboratories, which was not the case for non-ESBL isolates [6, 8]. This may have resulted in the preferential detection of the *mcr-1* gene in these identified

ESBL isolates; non-ESBL isolates in existence could not be tested because they were not available in laboratories [7]. The oldest collection of *E. coli* strains harboring the *mcr-1* gene was collected in China between 1970 and 2014, however we have no information if these isolates are ESBL producing bacteria or not [9].

The prevalence of the *mcr-1* gene among ESBL producing isolates from farm animals was not statistically higher than that found in ESBL-positive *E coli* isolates from humans [7, 18]. In 2009, the New Delhi metallo-beta-lactamase-1 (NDM-1) was discovered – a novel broad-spectrum carbapenemase with the ability to inactivate all β-lactams except aztreonam and with the characteristic of not being inhibited by clavulanic acid [19]. Since 2009, there have been two studies, the first carried out in China [9] and the second in Japan [20], that have both reported a significant increase in *mcr-1* gene prevalence in *E. coli* strains obtained from food animals. This finding was explained by the increased use of colistin in animal production in these two countries over the last few years. The sudden and permanent increase of the *mcr-1* gene over time presents a striking similarity to the increase in the numbers of β-lactamase enzymes identified globally, as previously presented by Davies [21]. More recently, two *E. coli* strains harboring *mcr-1* and carbapenemase genes were isolated from the urine samples of two patients in the United States. The first strain was harboring *mcr-1* and *bla*$_{\text{CTX-M}}$ genes [22] and the second strain was harboring *mcr-1* and *bla*$_{\text{NDM-5}}$ genes [23]. In China, two *E. coli* strains coproducing MCR-1 and NDM-1, were recovered from two patients with bloodstream infections [24]. MCR-1 producing *E. coli* coproducing either ESBL, AmpC (CMY-2) cephalosporinase, or NDM-9 enzymes were also isolated from chicken meat [7, 25]. However, in the absence of therapeutic historical data in these studies, it is difficult to determine whether β-lactam or colistin use had greater involvement in the exacerbation of ESBL and carbapenemase enzyme spread. Interestingly, Haenni and collaborators showed an increasing prevalence of the *mcr-1* gene in ESBL isolates from French

calves in spite of a decrease in colistin use in animal husbandry in France [8]. Likewise in Brazil, the *mcr-1* gene was identified at a prevalence of 3 % in *E. coli* strains in poultry that had not been exposed to polymyxin at any point in their lives (around 40 days) [26].

Moreover, in countries where colistin is not approved for veterinary use, such as the United States, it is difficult to accuse animal productions of being responsible for colistin resistance transfer to humans. Even in Europe, studies could not confirm a causal link between animals and humans regarding colistin resistance transfer [7].

Some studies reported that the prevalence of the *mcr-1* gene is more significant in ESBL positive isolates compared to non-ESBL ones [8]. However, given that the identification of ESBL and/or carbapenemase genes in bacteria harboring the *mcr-1* gene was not performed in over 50% of the scientific studies [27], it is difficult to establish a link between ESBL positive or negative isolates and the prevalence of the *mcr-1* gene identified worldwide. Several studies have reported that the prevalence of the *mcr-1* gene was more significant in ESBL positive isolates compared to carbapenemase positive ones [27, 28].

We believe that a historical link has existed between *mcr-1,* ESBL, and carbapenemase genes since the 1980s, however this historical evidence requires confirmation through the identification of the *mcr-1* gene present in several old collections of ESBL-positive strains to trace the kinetics over time between ESBL, carbapenemase, and *mcr-1* genes.

It is reasonable to consider that the use of broad-spectrum cephalosporins or other β-lactam antibiotics in either veterinary or human medicine may have led to colistin resistance. This fact might explain the identification of the *mcr-1* gene in patients in countries where colistin is not approved for farm animals, such as the United States. Moreover, some studies raised the possibility of acquiring ESBL, carbapenemase*,* and *mcr-1* genes following a stay in endemic

countries and a subsequent human transmission of these genes [29], which might be the case in the United States and others countries.

The re-evaluation of colistin use in livestock, as initiated by several regulatory agencies such as the European Medicines Agency (EMA), needs an overall approach that includes not only colistin use reduction but also the reduction of all antibiotic use, especially those of critical importance for human health.

Fig. 27. Schematic illustration of some historical events that combine ESBL and carbapenemase enzyme identification with colistin resistance *mcr-1* gene emergence. ESBL: Extended-Spectrum β-Lactamases. KPC-1: *Klebsiella pneumoniae* carbapenemase-1. **GNB**: Gram-negative bacteria. **NDM-1**: New Delhi metallo-beta-lactamase-1. **WHO**: World Health Organization. Dashed lines indicate a retrospective study.

Competing interests

The authors declare that they have no competing interests.

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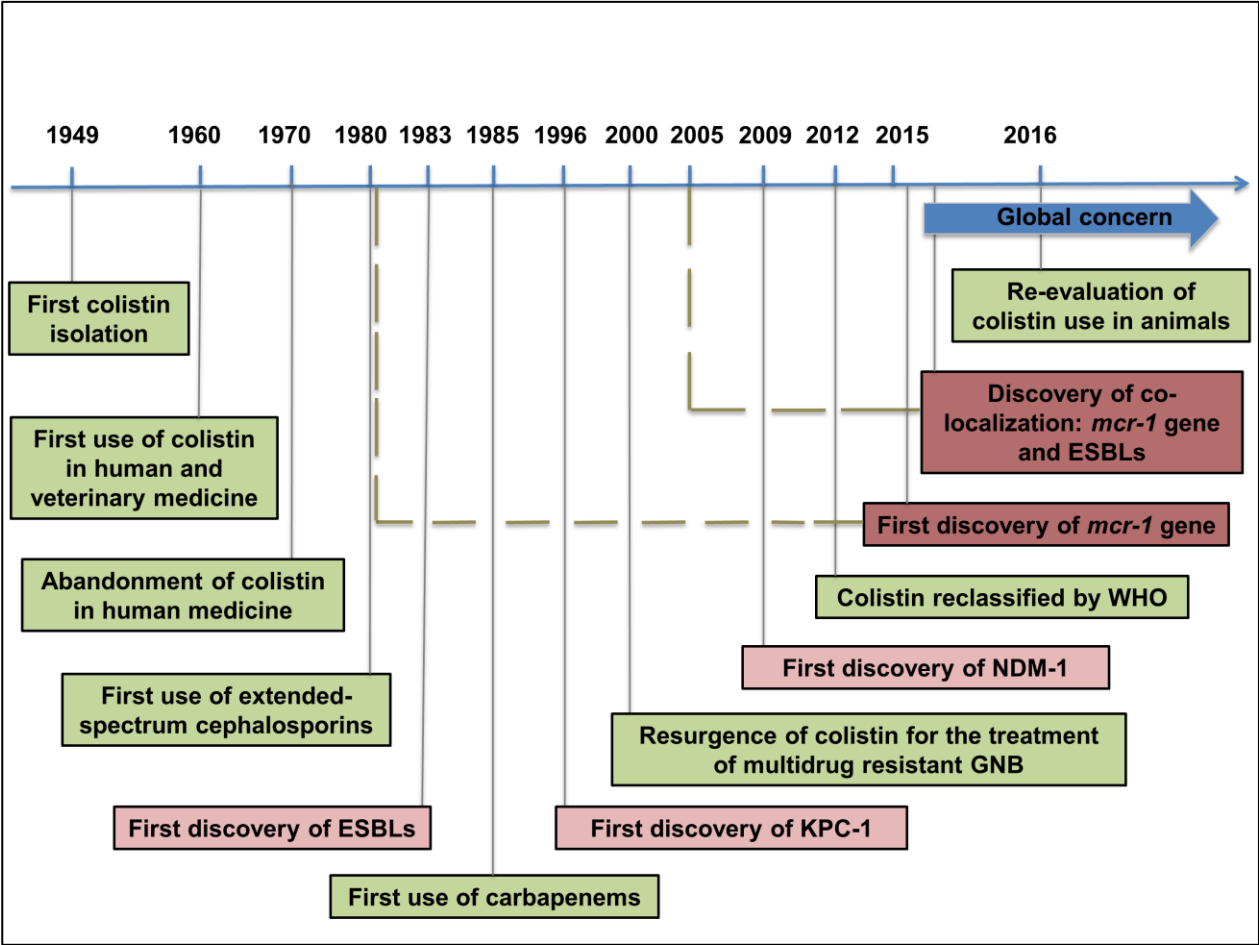
Figure 27: Schematic illustration of some historical events that combine ESBLs and carbapenemase enzyme identification with colistin resistance *mcr-1* gene emergence.

Annexe 2: Mechanisms of colistin resistance in *Escherichia coli* O149 strain *in vitro* and in an experimental model of post-weaning diarrhea in pigs

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Abstract

Several studies have reported the isolation of *Escherichia coli* colistin-resistant strains from pigs worldwide, and various mechanisms have been described to explain this resistance. Mutation in the two-component system PmrA/PmrB has been reported historically as the most resistance mechanism to colistin in *E. coli*. However, with the identification of plasmids carrying *mcr* genes encoding for *Enterobacteriaceae* colistin resistance, a new mechanism of resistance to colistin is already identified.

The main objective of this study was to investigate the genetic polymorphism in *pmrA/pmrB* and the presence of *mcr* genes in *E. coli* O149 strains acquired *in vitro* and in an experimental model of PWD in pigs. 22 mutants resistant to colistin from clinical strains were created. MIC was determined by standard double dilution method and compared to the EUCAST breakpoint. The sequencing of *pmrA* and *pmrB* of these mutants showed seven new genetic polymorphisms. Three were located in the *pmrA* gene: A80V, N128I, and S144G and four were located in the *pmrB* gene: V87E, D148Y, D148V, and T156M. The sequencing of *pmrA* and *pmrB* of *E. coli* colistin resistant strains from pigs showed two polymorphisms, G15R and T156M. However, neither *mcr-1* nor *mcr-2* gene was identified among these strains.

Our study is among the first to demonstrate the isolation of *E. coli* colistin resistant strains without having a mutation in PmrA/PmrB two-component and without harboring a *mcr* genes. This finding is in favor of the existence of other potential mechanisms of colistin resistance in *E. coli*.