Gastric stability and oral bioavailability of colistin sulfate in pigs challenged or not with *Escherichia coli* O149: F4 (K88)

Mohamed Rhouma a,b,c, Francis Beaudry d, William Thériault a,b,c, Nadia Bergeron a,b,c, Sylvette Laurent-Lewandowski a,b,c, John Morris Fairbrother b,e, Ann Letellier a,b,c,d,e⁎

a Chaire de recherche en salubrité des viandes (CRSV), Canada
b Groupe de recherche et d'enseignement en salubrité alimentaire (GRESA), Canada
c Centre de recherche en infectiologie porcine et avicole (CRCPA), Canada
d Groupe de recherche en pharmacologie animale du Québec, Canada
e OIE Reference Laboratory for *Escherichia coli* (ECL), Faculté de médecine vétérinaire — Université de Montréal, 3200 rue Sicotte, Saint-Hyacinthe, QC J2S 7C6, Canada

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ABSTRACT

The aim of the present study was to investigate the *in vitro* gastric stability of colistin sulfate (CS) and its antimicrobial activity against *Escherichia coli* and to study the impact of ETEC O149: 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 O149: 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 (P WD).

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1. 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. 1). The amino groups mediate both the bactericidal effect and toxicity to human cells (Claussel et al., 2007; 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 (Claussel et al., 2007). 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 ≤2 mg/L as the susceptibility breakpoint and >2 mg/L as the resistance breakpoint, whereas the British Society for Antimicrobial Chemotherapy selected ≤4 mg/L and ≥8 mg/L as the susceptibility and resistance breakpoints, 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* (Bello et al., 2008; Callens et al., 2012; Casal et al., 2007). 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 mediates bacterial adherence to the intestinal mucosa and mediates 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 diarrheaa (Bellloc 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 (Committee for Medicinal Products for Veterinary Use (CVMP), 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 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 O149: F4 (K88). The second objective was to study the impact of experimental infection of piglets with ETEC O149: 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 O149: F4 (K88) and the total E. coli population were determined.

2. Material and methods

2.1. 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 IU 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 min, 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 min) in duplicate was 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 5.105 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 the inhibition of bacterial growth. Additionally, antimicrobial activity of acetonitrile and SGF without CS was tested to ascertain whether these two compounds interfere with CS antimicrobial activity.

2.2. 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 biosafety 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 based on the guidance of the Canadian Council on Animal Care (CCAC).

2.3. Jugular catheterization of pigs, blood sampling, and analytic methods

After 2 days of acclimatization (23 days 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 days old) until euthanasia (32 days 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 days), 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 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 tylosin 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 12,000g 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 (3 μm) 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.

2.4. 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) groups. The challenge strain for experimental infection of pigs was a nalidixic acid-resistant (Nal') variant of ETEC O149: F4 strain ECL8559 (O149: LT: STa: STb: East1: paa: hem:). F4 was and hemolytic when grown on blood agar, as previously described (Daudelin et al., 2011). The ETEC O149: 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 10⁵ CFU of strain ECL8559 following the administration of 10 mL CaCO₃. Both administrations were performed using a syringe attached to a polyethylene tube. CaCO₃ 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 Jamaludeen et al. (2009), at 24, 48, 72, 96, and 120 h post-challenge by a person with no prior knowledge of the treatment assignment.

2.5. Microbiological analysis of fecal samples and ileal mucosa

Fecal samples from the two challenged groups were collected before challenge and 24, 48, 72, 96, 108, and 120 h post-challenge. These samples were used to examine the presence of the challenge strain ETEC O149: 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 hemolytic ETEC O149: 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 O149: 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 1 min. 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.

2.6. Statistical analysis

Bacterial counts were log₁₀ transformed prior to data analysis to normalize distributions. Colonization of the ileal mucosa by ETEC O149: F4...
in the two groups was compared with the equal variance t-test. Total *E. coli* counts as well as ETEC O149: 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 diarrheal 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. Results

3.1. 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 the addition of pepsin) and reached the maximum at around 15 min after pepsin addition with 50% CS degradation (Fig. 2). At least four other peaks other than the CS peak were detected on the HPLC-MS/MS mass chromatogram, probably corresponding to CS degradation products (M1, M2, M3, and M4) (Fig. 3). These 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 was 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. 4). Thus, gastric degradation of CS was not accompanied by a loss but rather slight 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. 4). 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.

3.2. CS plasma quantification and pharmacokinetic analysis

CS levels in plasma and in SGF were quantified by HPLC–MS/MS. The lower limit of quantitation (LOLO) 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 (20 ng/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 LOD after 30 min of CS administration (Fig. 5). However, in the challenged treated group, the concentration of CS was less than the LOD at all sampling times (Fig. 6). 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.

3.3. Analysis of bacterial shedding and health status of challenged pigs

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 O149: 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. 7. After challenge, there was a rapid initial increase in ETEC O149: F4 shedding in the feces of all challenged pigs that persisted for the 5 days post-challenge (Fig. 7). The administration of a single oral dose of CS showed a tendency to reduce fecal ETEC O149: 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 O149: F4 excretion load increased to regain baseline values. However, at 48 h post-treatment, ETEC O149: F4 counts were not different between the two challenged groups (*P* = 0.052) (Fig. 7). In both challenged groups, total *E. coli* counts demonstrated the same trend of decline as observed for ETEC O149: 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 group as compared with those of the non-challenged group. Treatment with CS did not result in a reduction in the attachment of ETEC O149: 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 was not affected by CS administration, with no statistical difference compared with challenged untreated pigs (Fig. 8). The severity of diarrhea in challenged pigs was not affected by CS administration, with no statistical difference compared with challenged untreated pigs (Fig. 8). The severity of diarrhea in challenged pigs was not affected by CS administration, with no statistical difference compared with challenged untreated pigs (Fig. 8).
Fig. 3. 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).

Fig. 4. 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).
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. 8). 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 feces after challenge, with maximum softening being observed at 72 h post-challenge and a mean diarrhea score of 2 (Fig. 8). 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. 8). In the current study, a reduction in fecal shedding of ETEC O149: F4 was correlated with a reduction of diarrhea score after 24 h of CS treatment.

4. 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 O149: 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, the 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 O149: 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 \( (\lambda) \), 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 (Committee for Medicinal Products for Veterinary Use (CVMP), 2010).

In our study, healthy pigs showed a trace plasmatic concentration of CS at 30 min 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 systemic concentrations are usually undetectable (Guyonnet et al., 2010). Moreover, in challenged treated groups, CS systemic concentrations were not detected in any of the

![Fig. 5. 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 quantification (20 ng/mL). Colistin sulfate was not detected at other time points.](image1)

![Fig. 6. 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.](image2)
samples analyzed. Thus, the different withdrawal periods in various countries are not related to the presence of CS systemic residues but are 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$_2$O$_2$), 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 O149: 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. (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 O149: 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 O149: F4 (Jensen et al., 2006, 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 O149: 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 O149: F4 and total *E. coli*, ETEC O149: 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., 2007; Guyonnet et al., 2010).

5. 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 O149: 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 O149: 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 O149: 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.

Conflict of interest statement

None of the authors of this paper has a financial or personal relationship with other people or organizations that could inappropriately influence or bias the content of the paper.

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