Received: 4 April 2018 | Revised: 8 August 2018 | Accepted: 13 August 2018

DOI: 10.1111/jvp.12710

ORIGINAL ARTICLE

WILEY JOURNAL OF Veterinary Pharmacology and Therapeutics

Evaluation of the ability of colistin, amoxicillin (components of Potencil®), and fluoroquinolones to attenuate bacterial endotoxin- and Shiga exotoxin-mediated cytotoxicity—In vitro studies

Agnieszka Szuster-Ciesielska[1] | Renata Urban-Chmiel[2] | Andrzej Wernicki[2] | Laurent Mascaron[3] | Marek Wasak[4] | Eric Bousquet[5]

[1]Department of Virology and Immunology, Maria Curie-Skłodowska University, Lublin, Poland

[2]Department of Veterinary Prevention and Avian Diseases, Institute of Biological Bases of Animal Diseases, University of Life Sciences, Lublin, Poland

[3]Virbac Consultant, Puteaux, France

[4]Virbac, Warszawa, Poland

[5]Virbac, Carros, France

Correspondence
Agnieszka Szuster-Ciesielska, Department of Virology and Immunology, Maria Curie-Skłodowska University, Lublin, Poland.
Email: aszusterciesielska@gmail.com or szustera@hektor.umcs.lublin.pl

Funding information
Virbac France, Grant/Award Number: Project No 1/2015

Abstract

Escherichia coli is one of the major pathogens in humans and animals causing localized and systemic infections, which often lead to acute inflammation, watery diarrhea, and hemorrhagic colitis. Bacterial lipopolysaccharide (LPS) and Shiga exotoxins (Stx) are mostly responsible for such clinical signs. Therefore, highly effective treatment of *E. coli* infections should include both eradication of bacteria and neutralization of their toxins. Here, for the first time, we compared the in vitro ability of common antibiotics to decrease LPS- and Stx-mediated cytotoxicity: colistin, amoxicillin (used separately or combined), enrofloxacin, and its metabolite ciprofloxacin. Three experimental scenarios were realized as follows: (a) the direct effect of antibiotics on endotoxin, (b) the effect of antibiotic treatment on LPS-mediated cytotoxicity in an experiment mimicking "natural infection," (c) the effect of antibiotics to decrease Stx2e-mediated cytotoxicity. Two cell lines, A549 and Vero cells, were used to perform cytotoxic assays with the methyl tetrazolium (MTT) and lactate dehydrogenase leakage (LDH) methods, respectively. Colistin and amoxicillin, especially used in combination, were able to attenuate LPS toxic effect, which was reflected by increase in A549 cell viability. In comparison with other antibiotics, the combination of colistin and amoxicillin exhibited the highest boster or additive effect in protecting cells against LPS- and Stx2e-induced toxicity. In summary, in comparison with fluoroquinolones, the combination of colistin and amoxicillin at concentrations similar to those achieved in plasma of treated animals exhibited the highest ability to attenuate LPS- and Stx2e-mediated cytotoxicity.

KEYWORDS

amoxicillin, colistin, fluoroquinolones, lipopolysaccharide, Shiga exotoxins

1 | INTRODUCTION

Most *Escherichia coli* strains that colonize animal and human intestines are harmless and part of the physiological microflora. However, some pathogenic bacteria and their toxins may lead to the development of localized or systemic infections and inflammation, watery and bloody diarrhea, hemorrhagic colitis, and hemolytic-uremic syndrome (HUS). Among them, endotoxins (LPS) and Shiga exotoxins

(Stx) type 1 and 2 produced by *E. coli* O157:H7 (STEC) are highly responsible for clinical signs, including septic shock, often leading to fatal consequences (Angus & van der Poll, 2013; Burvenich, Van Merris, Mehrzad, Diez-Fraile, & Duchateau, 2003; Kolenda, Burdukiewicz, & Schierack, 2015; Nguyen & Sperandio, 2012).

Although the main options to avoid neonatal calf diarrhea are management measures like hygiene and feeding with colostrum after calving, antibiotic treatment is necessary in case of infection clinical signs appearing. Neonatal calf diarrhea caused by *E. coli* infections remains worldwide one of the most common reason of morbidity and mortality in unweaned dairy calves leading to significant economic and productivity losses in the cattle industry (Meganck, Hoflack, Piepers, & Opsomer, 2015; Picco et al., 2015; Uetake, 2013). According to the National Animal Health Monitoring System (NAHMS), 57% of unweaned calf mortality due to diarrhea in most cases occurred in calves less than 1 month old (Cho & Yoon, 2014; El-Seedy, Abed, Yanni, & Abd El-Rahman, 2016). Thus, because of diarrhea and septicemia which affect dairy and beef production, *E. coli* infections remain a substantial problem even in calves with adequate passive immunity (Gerros, Semrad, & Proctor, 1995; Kolenda et al., 2015). Moreover, as the main natural reservoir of Shiga exotoxin-producing *E. coli* (STEC), cattle and other ruminants are sources of zoonotic infections in humans—approx. 75% of human disease outbreaks were linked to bovine-derived products or cattle directly (Nguyen & Sperandio, 2012). Although virulent strains of *E. coli* O157:H7 are rarely harbored by pigs (Ferens & Hovde, 2011), studies demonstrate that the Stx2e variant represents the major virulence factor responsible for the pig edema disease, which is characterized by hemorrhagic lesions, neurological disorders, and often fatal outcomes especially for weaned piglets (Baranzoni et al., 2016; Moxley, 2000; Muthing et al., 2012; Oanh, Nguyen, De Greve, & Goddeeris, 2012).

Antibiotic treatments of *E. coli* infections are sometimes controversial as they are believed to induce bacterial cell lysis and release of stored toxins. Additionally, some antimicrobials have also been reported to enhance toxin synthesis and production (Rahal, Fadlallah, Nassar, Kazzi, & Matar, 2015). Therefore, an optimal approach for the treatment of *E. coli* infection should include not only eradication of bacteria but also a significant decrease in the Stx and LPS concentrations and/or neutralization of their toxic effects in blood and intestine of affected animals.

However, in contrast to clinical studies assessing the efficacy of commercially available antibiotics and to in vitro studies evaluating the susceptibility of bacteria to antibiotics, the direct effect on *E. coli* toxins is very occasionally documented and compared between antibiotics. The most complete recently published work about the effects of colistin on both Stx and endotoxin does not include comparison to other antibiotics (Percivalle, Monzillo, Pauletto, Marone, & Imberti, 2016). Other authors compared the effect of several antimicrobials on the production and release of Stx by enterotoxemic *E. coli* isolates from pigs; however, they did not examine endotoxin (Uemura, Sueyoshi, Taura, & Nagatomo, 2004). Due to the consequences of *E. coli* infections in both animals and humans manifesting with strong clinical signs such as blood diarrhea, high fever, apart of direct bacteria effect inhibiting of activity of endotoxin and Shiga exotoxin seems to be antibiotic treatment choice.

Therefore, the aim of this study was to compare the in vitro activity of common bactericidal antibiotics to inhibition of LPS- and Stx-mediated cytotoxicity: colistin (CST), amoxicillin (AMX) (separately used or in combination), enrofloxacin (ENR), and ciprofloxacin (CIP). These antibiotics (except ciprofloxacin, which is an active metabolite of enrofloxacin) are used as commercially available preparations licensed for the treatment of the alimentary tract and/or septicemia caused by susceptible strains of *E. coli* in young livestock. Therefore, the treatment effects of infections with a potential risk of shigatoxemia or *E. coli* septicemia and developing septic shock in the case of massive release of LPS are worth to be evaluated. To realize the study goals, we determined (a) the direct effect of antibiotics on endotoxin, (b) the effect of antibiotic treatments on LPS-mediated cytotoxicity in an experiment mimicking "natural infection," (c) the inhibiting effect of antibiotics on Stx2e-mediated cytotoxicity.

2 | MATERIALS AND METHODS

2.1 | Antibiotics

As recommended by the manufacturers, the stock solutions of antibiotics were prepared in deionized water (CST, AMX, and CIP) or in DMSO (ENR) and then filtrated through 0.22-μm pore-size membrane filters (Millex GV filter, Millipore). All antibiotics were used individually, and in some experiments, AMX and CST were combined to investigate their synergistic effects on bacterial toxins. The expected maximum serum concentrations (C_{max}) of amoxicillin and colistin in calves were estimated to range from 650 to 1,600 ng/ml for CST and from 1,900 to 6,200 ng/ml for AMX (according to an internal pharmacokinetic study of an injectable suspension combining both antibiotics at the dose regimen of 10 mg amoxicillin/kg/day and 25,000 IU colistin/kg/day once per day for 3 days via the intramuscular route (Potencil®; Virbac, authorized for sale and treatment in EU countries.). As reported in the scientific literature (McKellar, Gibson, Monteiro, & Bregante, 1999), pharmacokinetics of enrofloxacin and its metabolite ciprofloxacin after subcutaneous administration of ENR in cattle depends on antibiotic dosage. After treatment with injectable solution of enrofloxacin, the plasma C_{max} in respect to ENR dose (2.5–12.5 mg/kg) can be estimated as 0.2 ± 0.084 to 0.7 ±0.23 μg/ml for ENR and 0.13 ±0.029 to 0.45 ±0.07 μg/ml for CIP. Therefore, three concentrations of each antibiotic were extrapolated (the lowest, medium, and the highest) and tested in the in vitro assays: 1,900, 4,050, and 6,200 ng/ml for AMX, 650, 1,125, and 1,600 ng/ml for CST, 0.2, 0.45, and 0.7 μg/ml for ENR, and 0.13, 0.29, and 0.45 μg/ml for CIP. When the AMX/CST association was used, the highest, medium, and lowest concentrations of each antibiotic were combined as follows: 6,200 ng/ml AMX plus 1,600 ng/ml

CST (AMX/CST1), 4,050 ng/ml AMX plus 1,125 ng/ml CST (AMX/CST2), and 1,900 mg/ml AMX plus 650 ng/ml CST (AMX/CST3). All chemicals needed were purchased from Sigma-Aldrich (Steinheim, Germany).

2.2 | Cell cultures

The A549 cell line (human epithelial lung carcinoma, ATCC® CCL-185) sensitive to enteropathogenic *E. coli* strains (EPEC) and responding to EPEC LPS toxicity (Nishio et al., 2013) was used. Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) containing 10% heat-inactivated fetal bovine serum (FBS), 100 U/ml of penicillin, and 100 μg/ml of streptomycin in humidified atmosphere with 5% of CO_2 at 37°C (Heracell CO_2 Incubator).

The Vero cell line (*Cercopithecus aethiops* normal kidney cells, ATCC® CRL-1586) with cells bearing Gb3 (glycolipid globotriaosyl ceramide) and Gb4 (globotetraosyl ceramide) receptors is susceptible to all Stx variants (Mainil, 1999). Thus, this cell line was used to determine the ability of antibiotics to neutralize Stx2e-mediated cytotoxicity. Cells were cultured in Eagle MEM containing 10% heat-inactivated FBS, 100 U/ml of penicillin, and 100 μg/ml of streptomycin in humidified atmosphere with 5% of CO_2 at 37°C (Heracell CO_2 Incubator). Both cell lines were purchased directly from the American Type Culture Collection (Rockville, MD, USA). Culture media, FBS, and antibiotics were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Cells at a density of 3×10^4 cells/ml (A549 cell line) or 2×10^5 cells/ml (Vero cell line) were seeded into 96-well plates (100 μl/well) (Nunc, Roskilde, Denmark) and cultured in standard medium with addition of 10% FBS for 24 hr. Next, the medium was replaced with a fresh one with 2% FBS and different concentrations of the tested antibiotics and/or toxin depending on the study design. The treated cells were then maintained in a humidified CO_2-incubator at 37°C for 24–96 hr until viability determination. Depending on the experimental designs, controls included nontreated cells in standard medium or cells cultured in medium with addition of 1% DMSO or 1% Triton X-100.

2.3 | Bacterial endotoxin

Commercially available *E. coli* LPS was used (*E. coli* 0111:B4; Sigma-Aldrich). Considering relatively low sensitivity of A549 cells to LPS, and following scientific papers (Liberati, Trammell, Randle, Barrett, & Toth, 2013; Nishio et al., 2013), we used high (nonpharmacological) concentrations of LPS to detect its cytotoxic effect; to elicit the proper toxin concentration to be applied in this study, A549 cells were treated with various concentrations of LPS (0.1–750 μg/ml) for 24–72 hr. Based on MTT and lactate dehydrogenase (LDH) assays results (Supporting information Table S1), LPS at a concentration of 500 μg/ml (high toxicity) and 250 μg/ml (close to 50% cytotoxic dose values, CD_{50}) was chosen for further determination of the potential antitoxic effect of the antibiotics.

2.4 | Bacterial strain and process for Stx2e production

According to recent epidemiological data, swine may carry Stx2e-producing *E. coli* with virulence gene profiles associated with human infections (Baranzoni et al., 2016; Muthing et al., 2012). Moreover both colistin and amoxicillin were used before against *E. coli* -produced Stx2e (Konstantinova et al., 2008). Therefore, in this part of the study, the O139 ETEEC reference strain (*E. coli* ATCC 23546), which releases Stx2e, was used. Harvesting of supernatant Stx2e-rich toxin was carried out according to Uemura et al. (2004). The lyophilisate bacterial strain *E. coli* ATCC 23546 was suspended in brain heart infusion broth (BHI, Oxoid, UK) and incubated at 37°C overnight on a shaker 120 rpm. Next, the bacteria were screened on Columbia agar plates. The agar was supplemented with 5% of sheep blood to exclude potential contamination. The bacteria were then incubated in a thermostat overnight at 37°C (Memmert CO_2 incubator). Single colonies were picked from the plate, suspended in BHI broth (Oxoid), and incubated overnight at 37°C on a shaker 120 rpm. The resulting bacterial culture was brought to a density of 10^8 colony-forming units (CFUs) (OD600, SmartSpec™PLU; BioRad) and as suspension in BHI broth was cultured for 4 hr with three final concentrations for each tested antibiotic alone or the AMX/CST mixture as described above. Bacteria cultured without antibiotics served as a control (time point at 0 and 4 hr). After centrifugation (1,835 *g*/10 min Sigma, PL), the pellet was resuspended in BHI broth at an approximated concentration of 10^4 CFU and incubated at 37°C at log growth phase (about 3.5 hr). After centrifugation (1,835 *g*/15 min), cells were sonicated in cold water with ice (4 cycles/30 s, Ultrasonic disintegrator UD-20, Techpan, PL), filtrated through 0.22-μm pore-size membrane filters (Millex GV filter; Millipore), and designed to evaluation of the antibiotic effect on bacterial Stx2e with using Vero cell line. Described experiment was repeated three times, and each sonicated and filtrated bacteria culture was further used to cytotoxic assay in three independent experiments.

To assess Stx2e-rich supernatant, we exactly followed Uemura procedure (Uemura et al., 2004), however, we realized that this supernatant likely contains also endotoxin. Therefore, the content of collected Stx2e-rich supernatant reflects the presence of toxins during the infection process as was also carried out in many other studies with different bacterial exotoxin production (Aulik, Hellenbrand, Klos, & Czuprynski, 2010).

2.5 | Determination of cell viability

The cell viability was determined based on the mitochondrial activity and integrity of cell membranes using the yellow tetrazolium salt 3-(4,5 dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay and the LDH release assay, respectively.

The MTT colorimetric assay (Mosmann, 1983) was conducted as described elsewhere (Pachuta-Stec & Szuster-Ciesielska, 2015). Briefly, an MTT solution (25 μl of 5 mg/ml in PBS; Sigma-Aldrich) was added to each well containing cultured cells. The cells were

incubated at 37 °C for 3 hr, and 100 μl of 10% SDS in 0.01 M HCl was added per well to dissolve formazan crystals during overnight incubation. The spectrophotometric absorbance was measured at a 570-nm wavelength using a VICTOR X4 Multilabel Plate Reader (Perkin Elmer, Waltham, MA, USA). Data were presented as % of the viability of the control (nontreated) cells (mean ± *SD* of three independent experiments, each with eight replicates) calculated according to the following formula:

$$\text{cell viability}(\%) = \frac{\text{A570 of treated cells}}{\text{A570 of control cells}} \times 100$$

The LDH assay is based on the release of intracellular LDH through an injured cytoplasmic membrane. Therefore, detection of LDH in culture supernatants reflects the degree of cell membrane disruption (Nishio et al., 2013). The LDH assay was performed according to the protocol outlined by the manufacturer of the Cytotoxic Detection Kit (Sigma-Aldrich) using 50 μl of each culture supernatant. Maximum LDH release was determined by the incubation of cells in standard medium containing 1% of Triton X-100 (positive control). The spectrophotometric absorbance was measured at 570-nm wavelength using a VICTOR X4 Multilabel Plate Reader (Perkin Elmer). Data were presented as % of the maximum LDH release after subtraction of the background determined from the medium alone (mean ± *SD* of three independent experiments, each with four replicates) calculated according to the following formula:

$$\text{cell viability}\,(\%) = 100 - \left(\frac{\text{A570 of treated cells}}{\text{A570 of Triton X- 100 control cells}}\right) \times 100$$

2.6 | Experimental design

This experiment consisted of three parts; the first two parts referred to *E. coli* LPS, and the third one to *E. coli* Stx2e toxin. Experiments were preceded by preliminary studies to confirm the absence of a cytotoxic effect of the elicited concentrations of antibiotics in A549 cell cultures. In all designed experiments, we used culture media with no standard antibiotics addition (penicillin and streptomycin). Preliminary studies as well as experiments were independently performed for three times.

2.7 | Preliminary studies design

A549 cells were cultured for 24, 48, and 72 hr with the following final concentrations of antibiotics: amoxicillin (AMX)—1,900, 4,050, and 6,200 ng/ml, colistin (CST)—650, 1,125, and 1,600 ng/ml, enrofloxacin (ENR)—0.2, 0.45, and 0.7 μg/ml, and ciprofloxacin (CIP)—0.13, 0.29, and 0.45 μg/ml. Additionally, the combination of amoxicillin and colistin (AMX/CST) was used at the following concentrations: 6,200 ng/ml AMX plus 1,600 ng/ml CST (AMX/CST1), 4,050 ng/ml AMX plus 1,125 ng/ml COL (AMX/CST2), and 1,900 mg/ml AMX plus 650 ng/ml CST (AMX/CST3). Then, after the desired incubation time, the MTT and LDH tests were performed. Appropriate control cells were included as follows: nontreated cells and those cultured in medium with 1% DMSO or with 1% Triton X-100.

2.8 | Evaluation of the direct effect of antibiotics on LPS (before contact with cells)

To check any potential interaction between the antibiotics and LPS, each tested antibiotic, separately or in combination of AMX/CST in 2% DMEM (at final concentrations indicated in preliminary studies), was first incubated with 250 or 500 μg/ml LPS at 37°C for 1 hr. Then, the appropriate mixtures were added to A549 cell cultures for 24, 48, and 72 hr. The potential ability of the antibiotics to inhibition of activity of LPS reflected by increasing cell viability in comparison with cells treated only with LPS was evaluated using both MTT and LDH assays.

2.9 | Evaluation of the antibiotic effect after preincubation of A549 cells with LPS—"infection and treatment" mimicking studies

To mimic the real conditions of bacterial infection and treatment, the cell cultures were pretreated with 250 or 500 μg/ml LPS for 6, 12, or 24 hr (possible duration of disease development followed by diagnosis and treatment of *E. coli* infected animals). After the appropriate incubation time, the tested antibiotics at final concentrations indicated before were added during three consecutive days. Twenty-four hours after the last antibiotic treatment, the MTT and LDH tests were performed to assess the antibiotic ability to decrease LPS toxic effect and improve cell viability (Supporting information Figure S1).

2.10 | Evaluation of the antibiotic effect on bacterial Stx2e

The aim of this part of study was to determine the ability of the tested antibiotics (in concentrations indicated before) to decrease Stx2e-mediated cytotoxicity. Following 4 hr of incubation with antibiotics, bacteria cultures (*E. coli* ATCC 23546) were sonicated, filtrated, then twofold (1:2–1:2,048) diluted in 2% MEM and incubated with Vero cells for 72 hr. Next, the MTT and LDH tests were performed. We calculated CD_{50} (*cytotoxic dose)* value that reflected the toxin concentration giving 50% of its maximal cytotoxic effect. A higher value of CD_{50} means increased toxic potential as higher dilution should be performed to achieve a 50% effect. By analyzing the differences between cell viability when treated with bacterial supernatants taken at the start point (control 0) and after 4 hr, we concluded that a 4-hr duration of incubation was sufficient to produce a high level of Stx2e toxin (see Results section).

2.11 | Statistical analysis

Normally distributed continuous variables were expressed as mean ± *SD* of at least four independent experiments, each with

TABLE 1 Viability of A549 cells after incubation with the mixture of LPS and antibiotics for 24–72 hr (LPS and each antibiotic were incubated together for 1 hr in 37°C before contact with A549 cells) (results are presented as % of viable cells according to MTT and LDH assays)

		MTT assay						LDH assay					
		LPS 500 μg/ml			LPS 250 μg/ml			LPS 500 μg/ml			LPS 250 μg/ml		
Antibiotic	Concentration	24 hr	48 hr	72 hr	24 hr	48 hr	72 hr	24 hr	48 hr	72 hr	24 hr	48 hr	72 hr
AMX	6,200 ng/ml	32.6 ± 1.8*	34.4 ± 1.2*	11.6 ± 0.6	91.3 ± 2.4*	85.3 ± 2.8*	84.0 ± 2.7*	33.0 ± 5.0*	13.6 ± 3.1	4.9 ± 1.3	78.8 ± 2.7	82.0 ± 1.8*	73.9 ± 2.4*
	4,050 ng/ml	30.0 ± 3.0*	33.8 ± 2.0*	9.9 ± 2.8	90.8 ± 3.0*	86.5 ± 3.0*	82.5 ± 3.2*	28.0 ± 3.0*	16.0 ± 2.1	3.7 ± 0.8	76.4 ± 2.7	79.0 ± 3.0*	74.0 ± 2.6*
	1,900 ng/ml	25.2 ± 1.4	24.2 ± 2.9	11.4 ± 2.9	95.4 ± 1.5*	93.4 ± 2.0*	85.3 ± 2.2*	19.9 ± 1.8	8.5 ± 1.4	4.6 ± 0.7	73.5 ± 3.0	69.8 ± 2.2*	58.1 ± 2.4*
CST	1,600 ng/ml	53.0 ± 2.2*	46.0 ± 2.0*	14.4 ± 1.0	90.8 ± 2.0*	81.6 ± 1.6*	82.2 ± 3.5*	44.0 ± 3.0*	14.5 ± 1.5	6.3 ± 2.6	85.0 ± 1.6	84.3 ± 6.0*	80.6 ± 1.8*
	1,125 ng/ml	52.1 ± 1.2*	39.2 ± 1.9*	17.3 ± 1.9	95.9 ± 1.7*	82.8 ± 3.6*	82.7 ± 2.3*	46.3 ± 2.6*	15.2 ± 1.4	6.4 ± 0.5	84.5 ± 1.7	86.5 ± 2.5*	77.8 ± 1.5*
	650 ng/ml	43.2 ± 0.7*	30.5 ± 2.5*	16.4 ± 1.7	96.7 ± 1.3*	88.1 ± 1.3*	89.1 ± 2.5*	30.9 ± 3.6*	11.9 ± 2.1	5.8 ± 0.8	86.2 ± 1.9	85.2 ± 1.3*	75.0 ± 3.0*
ENR	0.7 μg/ml	12.9 ± 2.0	14.2 ± 1.6	0.1 ± 0.001	85.7 ± 3.2	83.0 ± 3.0*	85.1 ± 1.6*	9.8 ± 0.7	5.7 ± 0.6	5.0 ± 0.7	81.0 ± 3.2	75.0 ± 2.1*	83.2 ± 4.9*
	0.45 μg/ml	11.9 ± 1.0	10.8 ± 1.1	7.5 ± 0.4	80.6 ± 3.3	78.4 ± 2.1	72.3 ± 1.7*	10.5 ± 0.5	4.9 ± 0.1	5.9 ± 0.9	74.4 ± 3.1	61.1 ± 1.3*	54.6 ± 3.6*
	0.2 μg/ml	10.1 ± 1.3	15.3 ± 3.6	6.4 ± 0.8	71.3 ± 2.7	68.4 ± 2.6	61.0 ± 2.5*	11.3 ± 2.3	8.3 ± 0.7	4.4 ± 0.8	68.5 ± 3.5	50.4 ± 1.5	44.9 ± 1.8*
CIP	0.45 μg/ml	25.1 ± 1.7	17.2 ± 0.8	2.3 ± 0.7	84.2 ± 2.3	84.1 ± 2.0*	83.7 ± 2.0*	20.8 ± 3.1	13.5 ± 2.7	7.3 ± 0.6	83.7 ± 2.7	80.3 ± 1.7*	81.3 ± 2.7*
	0.29 μg/ml	21.2 ± 1.1	13.7 ± 1.7	1.8 ± 0.7	86.9 ± 3.1	86.0 ± 2.4*	71.4 ± 2.9*	16.7 ± 2.5	14.5 ± 0.6	5.8 ± 0.8	79.8 ± 2.9	80.6 ± 3.7*	83.0 ± 2.0*
	0.13 μg/ml	17.4 ± 0.9	14.2 ± 0.7	0.1 ± 0.001	88.3 ± 2.1	77.9 ± 4.0	69.6 ± 3.0*	14.9 ± 1.8	8.2 ± 0.7	5.5 ± 0.5	73.4 ± 2.7	70.0 ± 1.8*	76.6 ± 2.2*
LPS control		11.2 ± 1.3	5.3 ± 0.8	0	70.1 ± 3.4	55.5 ± 4.6	29.2 ± 2.7	12.0 ± 0.7	1.6 ± 0.4	0	74.0 ± 3.6	31.2 ± 5.5	15.1 ± 2.7

Notes. *Statistically significant in comparison with the LPS control, $p \leq 0.05$ (one-way ANOVA).

TABLE 2 Viability of A549 cells after incubation with LPS, amoxicillin, and colistin at different concentrations for 24–72 hr(LPS and the mixture of antibiotics were incubated together for 1 hr in 37°C before contact with A549 cells) (results are presented as % of viable cells according to MTT and LDH assays)

Antibiotic mixture	MTT assay						LDH assay					
	LPS 500 µg/ml			LPS 250 µg/ml			LPS 500 µg/ml			LPS 250 µg/ml		
	24 hr	48 hr	72 hr	24 hr	48 hr	72 hr	24 hr	48 hr	72 hr	24 hr	48 hr	72 hr
AMX/CST1	72.6 ± 1.2*[a,c]	64.3 ± 1.0*[a,b,c]	15.0 ± 3.0	98.2 ± 2.2*	97.1 ± 2.6*	90.3 ± 5.5*	66.2 ± 2.9*[a,c]	64.4 ± 3.4*[a,c]	10.3 ± 3.2	86.2 ± 3.2	85.0 ± 2.9*	78.5 ± 4.0*
AMX/CST2	57.2 ± 2.7*[c]	37.0 ± 3.0*[c]	14.2 ± 1.8	97.9 ± 1.1*	97.3 ± 0.8*	80.3 ± 4.0*	53.7 ± 3.4*[,c]	47.2 ± 2.7*[a,c]	11.3 ± 3.4	87.6 ± 1.8	88.4 ± 2.4*	77.4 ± 5.0*
AMX/CST3	40.9 ± 6.4*[,c]	30.5 ± 3.4*[,c]	12.5 ± 1.4	86.7 ± 1.5	83.2 ± 4.3*	75.6 ± 3.5*	33.7 ± 3.3*[,c]	15.6 ± 2.4	8.9 ± 1.7	83.6 ± 3.5	84.7 ± 3.6*	65.1 ± 4.6*
LPS control	12.3 ± 2.3	2.7 ± 0.9	0	70.8 ± 3.6	55.6 ± 4.6	32.5 ± 4.0	13.4 ± 1.8	1.9 ± 0.6	0	73.2 ± 2.7	31.9 ± 3.7	11.0 ± 2.0

Notes. $p \leq 0.05$ (one-way ANOVA).
AMX/CST1—6,200 ng/ml AMX plus 1,600 ng/ml CST.
AMX/CST2—4,050 ng/ml AMX plus 1,125 ng/ml CST.
AMX/CST3—1,900 ng/ml AMX plus 650 ng/ml CST.
*Statistically significant in comparison with the LPS control.
[a]Statistically significant in comparison with AMX/CST3 (during the same time of incubation).
[b]Statistically significant in comparison with AMX/CST2 (during the same time of incubation).
[c]Statistically significant in comparison with the longest time of incubation (72 hr) with the same AMX/CST mixture.

four or eight replicates. The statistical significance of differences between determinations was calculated with the Student *t* test or one-way Anova with Tukey post hoc test. Data were analyzed using STATISTICA software version 12 (StatSoft. Inc., Tulsa, OK, USA), and *p* value ≤0.05 was considered statistically significant. The CD_{50} value was calculated using GraphPad Prism software version 6 (GraphPad Software Inc., La Jolla, USA).

3 | RESULTS

3.1 | Preliminary studies—cytotoxicity of antibiotics in cell culture

The results of the MTT tests confirmed the lack of the toxicity of chosen concentrations of antibiotics used separately or in combination (AMX/CST) even during long-term contact with A549 cells—cell viability was generally over 90% (Supporting information Table S2A,B). These observations were supported by the results of the LDH test (Supporting information Table S3A,B).

3.2 | Evaluation of the direct effect of antibiotics on LPS (before contact with cells)

Even if none of the tested antibiotics completely neutralized the highest LPS concentration, colistin was found to be the most effective. Except for the longest time of incubation, the viability of A549 cells significantly increased by 25%–40% (depending on the CST concentration) in comparison with LPS treatment only. Amoxicillin was also able to significantly inhibit the activity of high concentrations of LPS (500 µg/ml), however, with a slightly lower efficacy than colistin (Table 1). When 250 µg/ml of LPS was used, both CST and AMX, regardless of their concentrations, almost completely neutralized the endotoxin. These differences, compared to the LPS control, were statistically significant. After 24 hr of incubation, cell viability was then comparable to that of the control cells and reached over 90%. Incubation prolonged to 72 hr only slightly decreased cell viability to over 80%. Enrofloxacin and ciprofloxacin were also able to neutralize LPS partially; however, this beneficial effect was significant only when the lower LPS concentration was used and only after 48 or 72 hr incubation (Table 1).

In comparison with CST and AMX used separately, incubation of LPS with the combination of these antibiotics caused more effective endotoxin neutralization, which was reflected by an additional increase in A549 cell viability. However, this effect strongly depended on the combined antibiotic concentration as well as on incubation time of such mixtures with the cells and was more visible when 500 µg/ml of LPS was used (Table 2). Based on the best results achieved with 500 µg/ml LPS, below we compared the neutralizing effect of this high endotoxin concentration only.

Generally, the % of viable cells (reflecting the level of endotoxin neutralization) depended mainly on the antibiotics concentration and then on the duration of treatment of the cells with the antibiotic mixtures (24–72 hr). The combination of amoxicillin and colistin

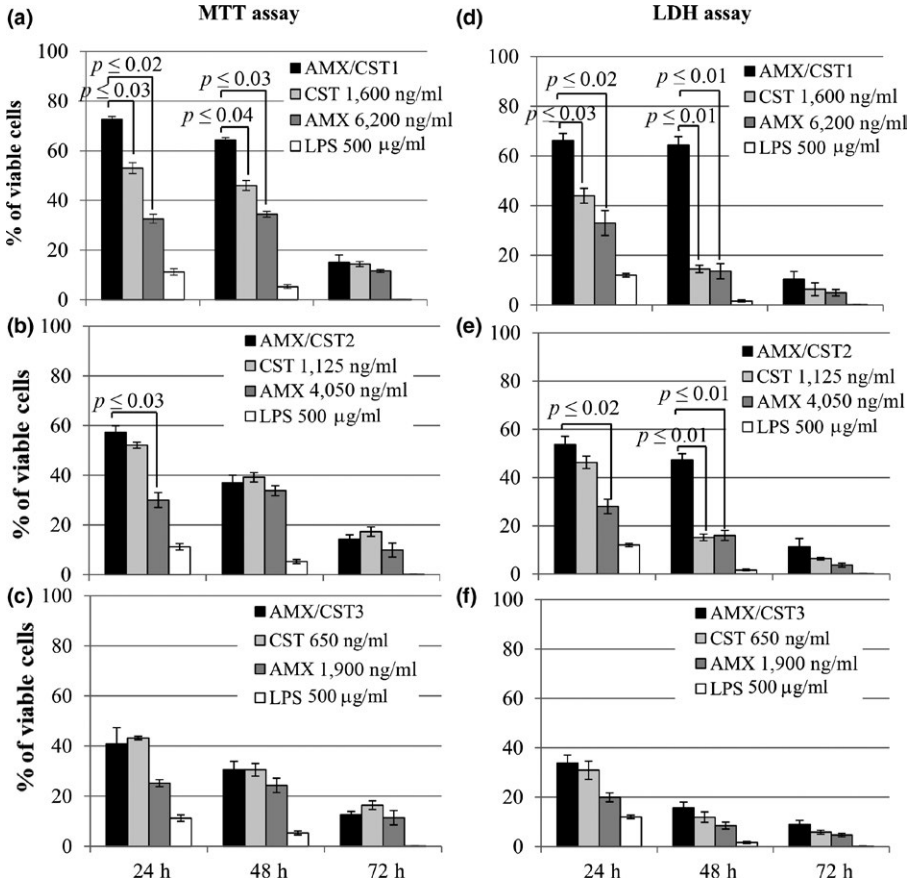

FIGURE 1 Comparison of LPS (500 µg/ml) neutralization between the amoxicillin and colistin combination (AMX/CST) and these antibiotics used separately. LPS and antibiotics were incubated together for 1 hr in 37°C before contact with A549 cells. Cytotoxicity was measured after 24, 48, or 72 hr incubation time. Results are presented as % of viable cells according to the MTT (a-c) and LDH (d-f) assays. Data are given as means ± *SD* with statistical significance (one-way ANOVA)
AMX/CST1—6,200 ng/ml AMX plus 1,600 ng/ml CST
AMX/CST2—4,050 ng/ml AMX plus 1,125 ng/ml CST
AMX/CST3—1,900 mg/ml AMX plus 650 ng/ml CST

exhibited the best ability to additively neutralization of LPS exclusively at their highest concentration—this effect was visible even after 48 hr as well. At the lowest AMX/CST concentrations, the LPS neutralization effect was comparable to the activity of separate components (Figure 1). Considering the results achieved with enrofloxacin and ciprofloxacin, it was not surprising then that the AMX/CST combination exhibited significantly higher capability of endotoxin neutralization (Figure 2).

3.3 | Evaluation of the antibiotic effect after preincubation of A549 cells with LPS—"infection and treatment" mimicking studies

In this part of the study, we considered different durations of cell exposition to LPS (6–24 hr) before the antibiotic treatment, as well as various LPS and antibiotic concentrations. The threefold administration of enrofloxacin resulted in the accumulation of the cytotoxic effect due to DMSO presence (used as an ENR solvent). Therefore, we omitted here the results concerning ENR.

At the high LPS concentration (500 µg/ml), the most critical factor for cell viability was the time of exposure. After the shortest time of preincubation (6 hr) with LPS, the tested antibiotics showed a comparable, almost complete ability to decrease the toxic endotoxin effect, which was demonstrated by high cell viability. However, when the earlier contact of cells with LPS was extended to 12 hr, CIP lost this ability. The beneficial activity of AMX and CST depended on their concentration; the best protective effect was observed when the highest concentrations of these antibiotics were used. All the aforementioned results of the beneficial activity of the drugs were statistically significant (Table 3). After 24 hr of preincubation of cells with the high LPS concentration, only CST, among the tested antibiotics, partly protected cells against endotoxin toxicity. According to the MTT method, the viability of treated cells was still 20%, while cells incubated only with LPS lost their viability, and this difference was statistically significant (Table 3). In conclusion, the protection effect strongly depended on the delay of cell exposure to LPS (6–24 hr) before the antibiotic treatment.

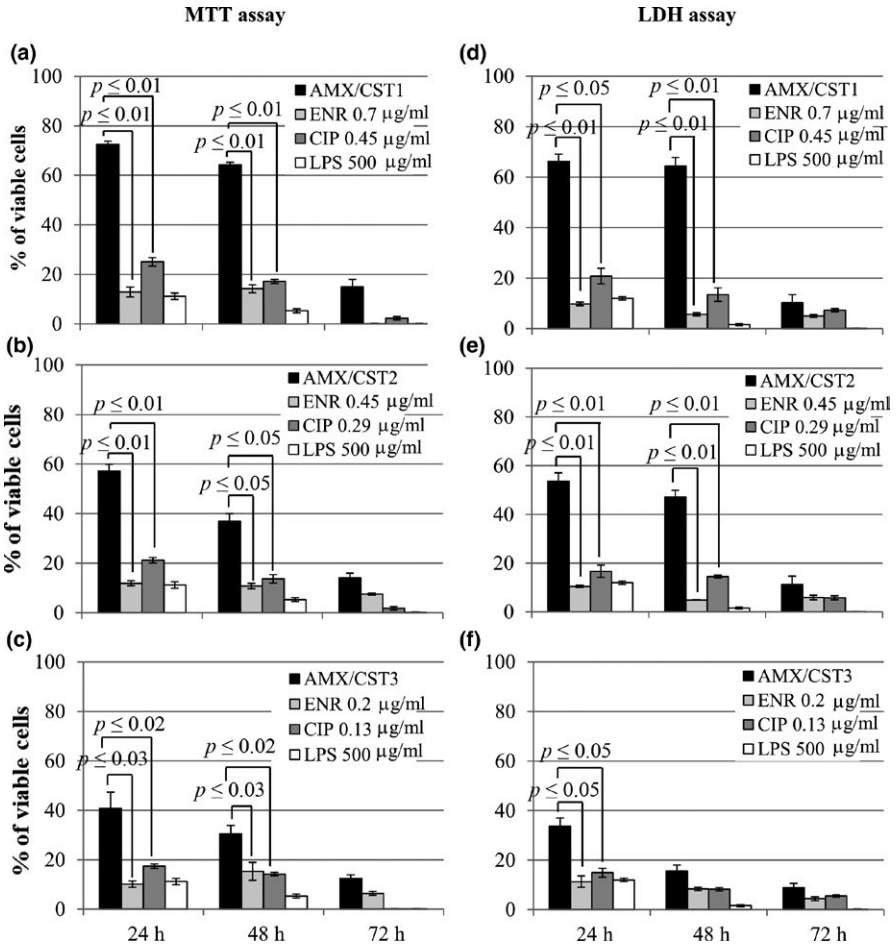

FIGURE 2 Comparison of LPS (500 μg/ml) neutralization between the amoxicillin and colistin combination (AMX/CST) and these antibiotics used separately. LPS and antibiotics were incubated together for 1 hr in 37°C before contact with A549 cells. Cytotoxicity was measured after 24, 48, or 72 hr incubation time. Results are presented as % of viable cells according to the MTT (a-c) and LDH (d-f) assays. Data are given as means ± *SD* with statistical significance (one-way ANOVA)
AMX/CST1—6,200 ng/ml AMX plus 1,600 ng/ml CST
AMX/CST2—4,050 ng/ml AMX plus 1,125 ng/ml CST
AMX/CST3—1,900 mg/ml AMX plus 650 ng/ml CST

According to the MTT method, both AMX and CST completely inhibited the cytotoxicity of lower LPS concentration (250 μg/ml), regardless of preincubation time (6–24 hr). A difference between these antibiotics appeared after LDH measurement; colistin was characterized by an activity around 20% higher than that of amoxicillin with a statistical significance in the case of the 6–12 hr cell preincubation with LPS. For CIP, also the longest cell preincubation period with LPS (250 μg/ml) was critical; its middle and lowest concentrations were revealed as significantly less beneficial for cell protection than in the case of AMX and CST (Table 3). It should be underlined that the 3-day exposure of A549 cells to all the antibiotics applied individually, regardless of their concentration, did not change the high cell viability.

The AMX/CST combination exhibited better protecting potential than its components used separately, and this effect was visible especially after the longest preincubation period of LPS (500 μg/ml) with A549 cells. Depending on the type of assay (MTT or LDH), cell viability increased additionally by 10%–20%, and these differences were statistically significant (Table 4, Figure 3). In the case of the lower LPS concentration, the efficacy of the AMX/CST combination did not differ from the activity of its individual components (Figure 4). Ciprofloxacin protected A549 cells comparably to the AMX/CST combination only after the shortest (6 hr) preincubation of cells with the higher concentration of LPS (Figure 3).

3.4 | Evaluation of the antibiotic effect on bacterial Stx2e

The results of the MTT assay demonstrated that, under the lowest dilutions of the bacterial supernatant (1:2 and 1:4 where the concentration of Stx2e was high), the cell viability was unexpectedly higher than that of cells incubated with further dilutions of the bacterial supernatant. The likely cause of this phenomenon is a fact that, in the 1:2 and 1:4 dilutions of bacteria supernatant, the concentration of antibiotics was also high and protected the Vero cells against Stx2e

TABLE 3 Viability of A549 cells after 6, 12, or 24 hr preincubation with LPS (500 μg/ml or 250 μg/ml) and antibiotic exposure during three consecutive days (results are presented as % of viable cells according to MTT and LDH assays). Data are presented as means ± *SD* with statistical significance (one-way ANOVA)

		6 hr			12 hr			24 hr		
Antibiotic	Concentration	LPS 500	LPS 250	Antibiotic control	LPS 500	LPS 250	Antibioticcontrol	LPS 500	LPS 250	Antibiotic control
MTT assay										
AMX	6,200 ng/ml	93.3 ± 3.5*,**	91.1 ± 1.2*	92.0 ± 2.0	68.7 ± 4.0*,a,**	93.0 ± 2.6*	92.6 ± 2.5	12.3 ± 2.5	94.0 ± 1.0*	92.3 ± 3.2
	4,050 ng/ml	92.0 ± 2.6*,**	92.6 ± 1.5*	90.6 ± 2.1	65.3 ± 4.5*,b,**	91.3 ± 3.2*	95.0 ± 2.0	10.6 ± 1.5	94.0 ± 2.6*,b	95.0 ± 2.6
	1,900 ng/ml	93.4 ± 3.0*,**	92.0 ± 2.0*	96.0 ± 2.5	59.8 ± 2.7*,c,**	93.3 ± 3.5*	93.6 ± 2.6	8.3 ± 1.6	95.1 ± 2.6*,c	93.8 ± 2.8
CST	1,600 ng/ml	91.7 ± 3.0*,**	94.0 ± 3.6*	95.2 ± 2.3	71.3 ± 4.1*,a,**	90.5 ± 2.0*	92.3 ± 3.5	21.3 ± 2.5*,a	92.3 ± 2.0*	94.6 ± 4.5
	1,125 ng/ml	90.7 ± 2.5*,**	91.6 ± 1.5*	92.3 ± 3.0	64.0 ± 4.0*,b,**	94.0 ± 1.0*	95.5 ± 1.9	23.0 ± 2.6*,b	94.5 ± 1.7*,b	94.0 ± 0.9
	650 ng/ml	91.6 ± 4.2*,**	93.6 ± 3.5*	95.2 ± 4.0	59.7 ± 5.5*,c,**	92.3 ± 2.4*	94.2 ± 1.3	20.0 ± 3.6*,c	94.0 ± 3.6*,c	92.3 ± 2.0
CIP	0.45 μg/ml	92.7 ± 1.5*,**	92.6 ± 2.5*	91.2 ± 1.6	0	96.0 ± 2.0*	95.6 ± 2.2	0	85.9 ± 2.7*	92.2 ± 2.6
	0.29 μg/ml	91.6 ± 3.8*	92.7 ± 2.4*	91.6 ± 3.3	0	94.6 ± 1.2*,**	93.5 ± 2.5	0	66.7 ± 3.5*	91.6 ± 5.0
	0.13 μg/ml	88.7 ± 2.5*,**	90.3 ± 3.2*	93.0 ± 2.1	0	95.6 ± 2.2*,**	95.3 ± 2.2	0	60.5 ± 2.0*	91.9 ± 4.4
LPS control		13.1 ± 1.9	49.6 ± 1.3	–	4.8 ± 1.0	42.6 ± 2.1	–	0	33.0,±4.7	–
LDH assay										
AMX	6,200 ng/ml	72.4 ± 2.4*	81.9 ± 1.7*	84.4 ± 0.7	58.0 ± 3.6*,a,**	66.9 ± 1.2*	82.0 ± 1.0	9.2 ± 0.7	69.0 ± 3.6*	82.3 ± 1.9
	4,050 ng/ml	68.7 ± 1.1*	82.6 ± 1.1*,**	84.8 ± 1.8	54.0 ± 1.1*,b,**	63.6 ± 1.2*	81.1 ± 3.5	7.2 ± 0.7	65.9 ± 1.8*	83.0 ± 3.3
	1,900 ng/ml	58.4 ± 2.3*	81.9 ± 1.9*,**	84.2 ± 1.9	52.6 ± 2.1*,c,**	60.4 ± 1.5*	82.7 ± 2.3	5.5 ± 0.4	65.2 ± 1.6*	82.1 ± 3.2
CST	1,600 ng/ml	79.3 ± 2.1*,**	80.3 ± 1.5*	80.5 ± 0.9	60.6 ± 2.6*,a,**	85.5 ± 2.8*	84.9 ± 2.4	5.9 ± 1.0	82.6 ± 1.5*	82.2 ± 1.4
	1,125 ng/ml	74.3 ± 1.6*,**	82.6 ± 2.5*	83.0 ± 2.1	51.0 ± 2.0*,b,**	84.6 ± 1.2*,d	85.0 ± 1.2	5.4 ± 0.6	84.6 ± 2.1*,d	82.6 ± 2.5
	650 ng/ml	70.0 ± 1.0*,**	83.1 ± 2.6*	81.9 ± 1.2	41.9 ± 1.9*,c,**	84.8 ± 2.3*,e	84.2 ± 2.3	5.6 ± 0.9	82.3 ± 2.5*,c, e	81.9 ± 1.4
CIP	0.45 μg/ml	81.0 ± 1.2*	80.7 ± 1.1*	84.2 ± 3.8	0	80.7 ± 1.7*	83.3 ± 2.7	0	70.8 ± 3.1*	80.0 ± 4.2
	0.29 μg/ml	70.4 ± 1.8*	80.4 ± 4.7*	84.3 ± 2.3	0	75.3 ± 3.1*	82.3 ± 2.4	0	67.5 ± 2.5*	82.3 ± 3.0
	0.13 μg/ml	60.3 ± 2.6*	69.9 ± 1.2*	83.8 ± 3.5	0	73.4 ± 3.2*	81.9 ± 1.8	0	61.7 ± 2.7*	80.6 ± 1.2
LPS control		17.9 ± 1.5	41.3 ± 2.8	–	9.1 ± 1.7	33.3 ± 2.5	–	0	24.7 ± 2.1	–

Notes. *Statistically significant in comparison with the LPS control, $p \leq 0.05$. **Statistically significant in comparison with the longer time of preincubation with LPS, $p \leq 0.01$.
[a,b,c]Statistically significant in comparison with ciprofloxacin at the highest (a),—middle (b), and the lowest concentration (c), $p \leq 0.05$. [d,e]Statistically significant in comparison with amoxicillin at the middle (d), and lowest concentration (e), $p \leq 0.05$.

TABLE 4 Viability of A549 cells after 6, 12, or 24 hr preincubation with LPS (500 or 250 μg/ml) and then exposure to the amoxicillin and colistin combination during three consecutive days (results are presented as % of viable cells according to MTT and LDH assays). Data are presented as means ± *SD* with statistical significance (one-way ANOVA)

	6 hr			12 hr			24 hr		
Antibiotic combination	**LPS 500**	**LPS 250**	**Antibiotic control**	**LPS 500**	**LPS 250**	**Antibiotic control**	**LPS 500**	**LPS 250**	**Antibioticcontrol**
MTT assay									
AMX/CST1	95.6 ± 2.8*	92.1 ± 2.6*	92.8 ± 1.7	80.1 ± 3.9*,***	93.8 ± 3.0*	99.3 ± 5.5	41.7 ± 4.5*,**	91.8 ± 3.0*	90.6 ± 3.5
AMX/CST2	93.7 ± 3.5*,***	93.9 ± 3.5*	92.7 ± 2.8	72.9 ± 1.8*,**,***	92.5 ± 4.1*	96.7 ± 4.9	42.6 ± 3.6*,**	92.3 ± 2.6*	93.7 ± 3.9
AMX/CST3	94.9 ± 5.0*,***	95.1 ± 4.0*	94.5 ± 3.2	63.4 ± 4.5*,**,***	90.6 ± 4.4*	93.6 ± 2.5	38.2 ± 3.1*,**	91.6 ± 4.0*	92.0 ± 4.0
LPS control	13.1 ± 1.9	49.6 ± 1.3	–	4.8 ± 1.0	42.6 ± 2.1	–	0	33.0 ± 4.7	–
LDH assay									
AMX/CST1	78.6 ± 3.5*	82.4 ± 3.6*	81.8 ± 3.7	66.9 ± 2.9*,***	80.3 ± 4.3*	83.2 ± 3.5	25.3 ± 4.3*,**	80.1 ± 3.3*	80.8 ± 3.8
AMX/CST2	74.2 ± 2.5*	83.1 ± 2.5*	83.3 ± 2.6	60.3 ± 3.8*,**,***	80.0 ± 3.1*	82.8 ± 3.9	24.8 ± 3.2*,**	81.3 ± 2.7*	81.6 ± 2.6
AMX/CST3	72.8 ± 4.1*,***	81.9 ± 3.7*	82.6 ± 3.9	54.7 ± 3.6*,**,***	80.8 ± 4.0*	82.6 ± 2.9	22.2 ± 4.1*,**	80.9 ± 4.5*	81.9 ± 4.3
LPS control	17.9 ± 1.5	41.3 ± 2.8	–	9.1 ± 1.7	33.3 ± 2.5	–	0	24.7 ± 2.1	–

Notes. AMX/CST1—6,200 ng/ml AMX plus 1,600 ng/ml CST.
AMX/CST2—4,050 ng/ml AMX plus 1,125 ng/ml CST.
AMX/CST3—1,900 ng/ml AMX plus 650 ng/ml CST.
*Statistically significant in comparison with the LPS control, $p \leq 0.05$.
**Statistically significant in comparison with the antibiotic control, $p \leq 0.05$.
***Statistically significant in comparison with the longer time of preincubation with LPS, $p \leq 0.01$.

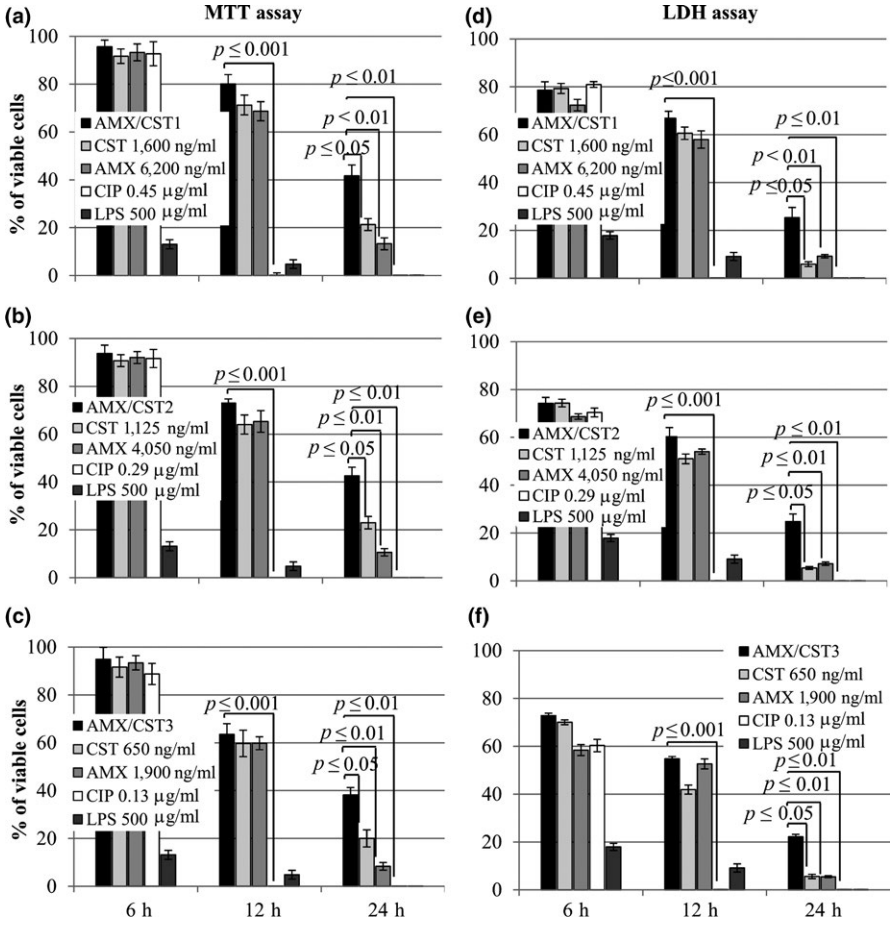

FIGURE 3 Comparison of LPS (500 µg/ml) toxicity neutralization between the amoxicillin and colistin combination (AMX/CST), its components used separately (AMX and CST), and ciprofloxacin (CIP). A549 cells were preincubated with LPS (for 6, 12, or 24 hr) before the antibiotic treatment (during three consecutive days). Results are presented as % of viable cells according to the MTT (a-c) and LDH (d-f) assays. Data are given as means ± *SD* with statistical significance (one-way ANOVA)
AMX/CST1—6,200 ng/ml AMX plus 1,600 ng/ml CST
AMX/CST2—4,050 ng/ml AMX plus 1,125 ng/ml CST
AMX/CST3—1,900 mg/ml AMX plus 650 ng/ml CST

toxicity (data not shown). Therefore, these dilutions of the bacterial supernatants were excluded from the presentation of the results.

All studied antibiotics were able to minimize Stx2e toxic effect. However, their efficacy varied depending on drug concentrations (Tables 5 and 6). Considering the CD_{50} values, the most effective antibiotics used individually were amoxicillin and ciprofloxacin (at their highest concentrations), which led to a decrease in the CD_{50} value from over 1,400 (calculated for the bacterial control) to about 500 (according to both MTT and LDH assays) (Tables 5 and 6). In comparison with AMX and CIP, the AMX/CST mixture appeared more effective—the highest concentration of its components inhibited Stx2e-mediated cytotoxicity, decreasing the CD_{50} value to about 365 (average of MTT and LDH results) (Tables 5 and 6). Additionally, this combination was the only antibiotics that statistically improved the Vero cell viability (reflecting a decrease in the Stx2e level) when the 1:256 dilution of the bacterial supernatant was used (Tables 5 and 6). Then, we performed statistical comparison between AMX/CST effectiveness in attenuation of Stx2e toxic effect with other antibiotics used individually. The first significant difference appeared when the 1:512 dilution of the bacterial supernatant was analyzed. Therefore, we presented below the graphical results of this experiment with the statistical analyses only for the 1:512 and 1:1,024 dilutions of the bacterial supernatant. In comparison with amoxicillin and colistin used separately, the AMX/CST combination exhibited significantly higher ability to inhibiting Stx2e-mediated toxicity, which was detected in the case of the 1:512 dilution of the bacterial supernatants (Figure 5a,b,d). When the 1:1,024 dilutions were analyzed, the activity of the AMX/CST combination was still higher in comparison with COL, but comparable to AMX (Figure 5a,b,d,e). Additionally, the statistical analyses strongly confirmed that the AMX/CST combination more effectively decreased Stx2e-mediated cytotoxicity than fluoroquinolones (ENR and CIP) (Figure 6a,b,d,e). The combination of AMX and CST appeared to have the best efficiency when the highest and middle concentrations of particular antibiotics were applied.

4 | DISCUSSION

It has been established that the course of *E. coli* infection and induced clinical signs depends on the presence and activity of bacterial toxins such as LPS and Stx2e (Cornick, Matise, Samuel, Bosworth, & Moon, 2000; Dean-Nystrom, Bosworth, & Moon, 1997; Kolenda et al., 2015; Nguyen & Sperandio, 2012). The clinical diarrhea treatment with colistin or amoxicillin in young calves caused by *E. coli* pathogenic strains has been often reported (Catry et al., 2016; Constable, 2004). During antibacterial therapy, the concentration of these toxins may increase and lead to fatal complications due to bacterial membrane disruption and/or bactericidal activity (Hurley, 1995). The critical consequence of the activity of these toxins, particularly

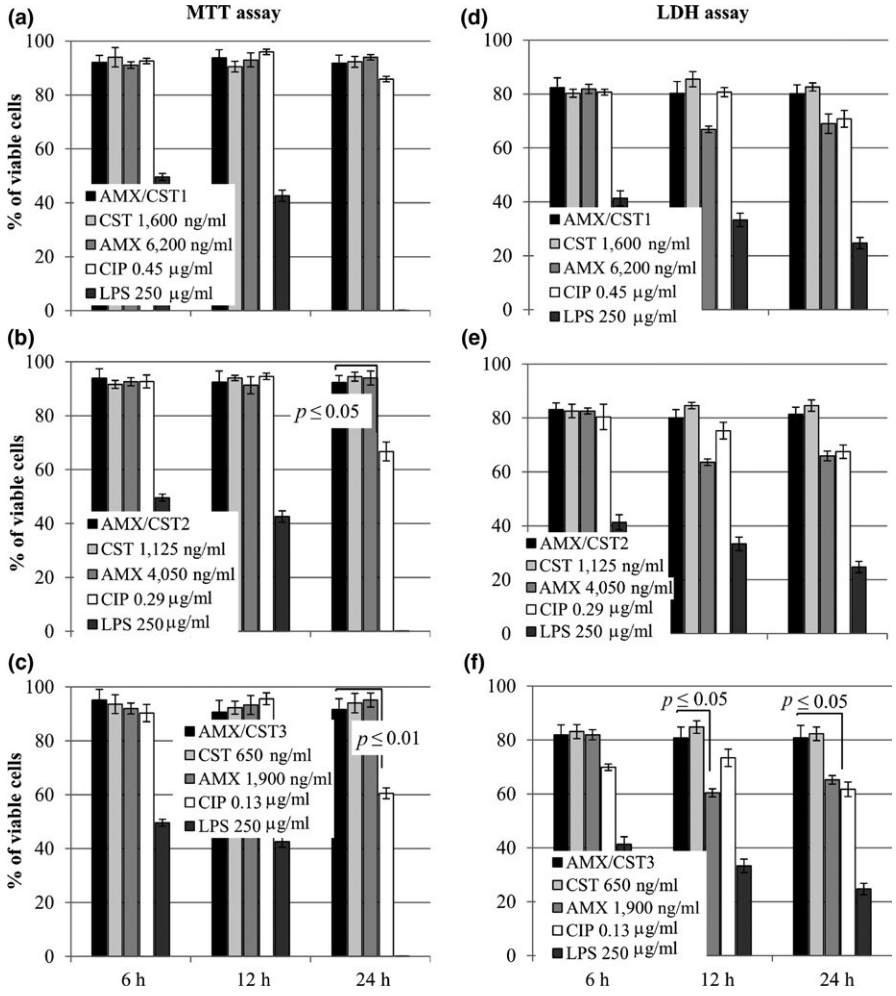

FIGURE 4 Comparison of LPS (250 µg/ml) toxicity neutralization between the amoxicillin and colistin combination (AMX/CST), its components used separately (AMX and CST), and ciprofloxacin (CIP). A549 cells were preincubated with LPS (for 6, 12, or 24 hr) before the antibiotic treatment (during three consecutive days). Results are presented as % of viable cells according to the MTT (a-c) and LDH (d-f) assays. Data are given as means ± *SD* with statistical significance (one-way ANOVA)
AMX/CST1—6,200 ng/ml AMX plus 1,600 ng/ml CST
AMX/CST2—4,050 ng/ml AMX plus 1,125 ng/ml CST
AMX/CST3—1,900 mg/ml AMX plus 650 ng/ml CST

LPS, is endotoxic shock that causes sudden death during the few first hours from the beginning of the antibiotic therapy (Hardie & Kruse-Elliott, 1990; Holzheimer, 2001). Therefore, antibiotic treatment during the strong bacterial infection caused intensive clinical signs followed with toxemia in animals should be targeted at not only eradication of the bacterial infection, but also a decrease in Stx and LPS concentrations or neutralization of their effects in blood and intestine. Hence, for the first time, we evaluated and compared the cell protecting potential of three commonly used bactericidal antibiotics (and one active metabolite—CIP) at concentrations close to those achieved in the blood of treated animals against bacterial endo- and exotoxin. Considering different study designs, the main conclusion from this study is that the combination of both amoxicillin and colistin (used in concentrations achieved in calve plasma after Potencil® administration) exhibited the best cell protecting potential against both LPS and Stx2e toxins. Although Stx2e is swine associated, the mechanism of its toxic activity is very similar to other toxins in the Stx toxin family. Therefore, we decided to use more safety for human toxin as the basis in our experiment.

The assessment of the antibiotic efficacy in in vitro studies was based on the significant increase in cell viability following their contact with bacterial LPS or Stx2e during different experimental scenarios. To determine cell viability, at least two methods should be chosen (Bopp & Lettieri, 2008). The MTT and LDH assays are commonly used to assess cytotoxicity (Fotakis & Timbrell, 2006) and have already been described to be appropriate to evaluate the viability of A549 cells (Uboldi et al., 2009). Generally, the results of both the MTT and LDH tests were comparable. Some differences resulted from the sensitivity of the LDH and MTT methods, as LDH can be released outside cells upon damage of the plasma membrane earlier than mitochondrial disruption (Weyermann, Lochmann, & Zimmer, 2005). Additionally, some papers reported that nonspecific intracellular reduction of tetrazolium can appear, which may lead to underestimated results of cytotoxicity by the MTT method (Jo et al., 2015).

In the presented experimental conditions reflecting high endotoxemia (500 µg/ml of LPS) and in comparison with individual used antibiotics, the AMX/CST mixture had the highest additive efficacy in inhibition of LPS cytotoxic effect on cell cultures viability by direct contact outside the cells. However, in case of moderate endotoxemia (250 µg/ml of LPS), both AMX and AMX/CST mixture presented similar effect in neutralizing this lower LPS concentration. Additionally, in laboratory conditions mimicking natural infection and in vivo treatment, CST and AMX as well as their combination exhibited the highest cell protecting potential against LPS toxicity, especially after a 24-hr preincubation of the cells with endotoxin.

TABLE 5 Viability of Vero cells after 72 hr incubation with Stx2etoxin-rich bacterial supernatants collected after treatment of bacteria with antibiotics for 4 hr (results are presented as % of viable cells according to MTT assay)

		Dilution factor									
Antibiotic	Concentration	8	16	32	64	128	256	512	1,024	2,048	CD_{50}
AMX	6,200 ng/ml	11.5 ± 1.7	15.3 ± 0.8	14.1 ± 2.0	18.3 ± 1.0	19.4 ± 1.3	19.0 ± 2.1	56.5 ± 1.4***	94.8 ± 1.1***	95.8 ± 1.4	492.8
	4,050 ng/ml	14.0 ± 0.2	11.1 ± 2.0	11.2 ± 2.4	11.6 ± 1.5	12.5 ± 1.8	14.8 ± 1.4	34.9 ± 1.0*	86.5 ± 1.9***	95.7 ± 1.3	694
	1,900 ng/ml	12.0 ± 1.9	10.9 ± 1.8	10.6 ± 1.3	11.9 ± 1.1	11.7 ± 1.2	13.0 ± 1.4	17.4 ± 1.8	72.8 ± 3.4***	91.9 ± 4.0	827
CST	1,600 ng/ml	11.6 ± 1.8	10.5 ± 1.1	10.1 ± 0.8	19.7 ± 1.7	18.6 ± 0.4	19.5 ± 2.4	38.7 ± 2.8**	73.4 ± 5.2***	95.3 ± 2.9	718.6
	1,125 ng/ml	12.6 ± 1.8	11.1 ± 1.6	11.6 ± 2.5	12.5 ± 3.7	17.7 ± 2.0	17.7 ± 1.6	20.8 ± 1.7	61.1 ± 2.0**	87.4 ± 3.2	873
	650 ng/ml	13.6 ± 0.5	12.9 ± 1.2	13.0 ± 1.3	14.4 ± 1.9	15.7 ± 1.5	14.2 ± 0.9	20.5 ± 2.2	58.6 ± 1.9**	74.9 ± 2.6	827
ENR	0.7 μg/ml	12.4 ± 2.3	11.3 ± 1.6	11.3 ± 1.4	14.6 ± 0.8	16.2 ± 1.0	15.6 ± 1.2	22.6 ± 1.3	73.2 ± 3.9***	90.2 ± 2.9	795
	0.45 μg/ml	14.4 ± 2.8	11.0 ± 0.8	11.7 ± 1.6	12.6 ± 1.2	17.2 ± 1.4	17.0 ± 0.8	19.5 ± 1.5	70.1 ± 3.3***	89.8 ± 4.0	826
	0.2 μg/ml	13.3 ± 0.5	13.3 ± 0.7	13.8 ± 1.6	16.0 ± 0.2	18.7 ± 1.2	18.2 ± 0.8	19.1 ± 1.5	63.6 ± 3.5**	81.3 ± 4.6	827
CIP	0.45 μg/ml	14.0 ± 1.6	12.8 ± 0.6	12.1 ± 1.5	20.5 ± 1.6	20.3 ± 0.9	22.0 ± 3.0	53.4 ± 2.6**	76.3 ± 2.5***	91.5 ± 2.8	495
	0.29 μg/ml	12.0 ± 0.9	12.0 ± 1.3	12.8 ± 1.5	18.2 ± 1.8	19.4 ± 1.5	19.8 ± 1.2	24.2 ± 2.0	69.5 ± 3.6***	83.1 ± 2.0	776
	0.13 μg/ml	13.9 ± 0.8	13.0 ± 0.7	13.2 ± 1.2	17.9 ± 1.6	19.6 ± 1.1	19.6 ± 1.8	26.4 ± 1.3	50.9 ± 2.6*	73.8 ± 1.7	868
AMX/CST1		13.6 ± 0.9	12.7 ± 1.3	13.2 ± 0.9	16.7 ± 0.6	25.6 ± 0.7	35.9 ± 1.1*	72.4 ± 2.2***	91.9 ± 1.4***	94.5 ± 1.4	387.6
AMX/CST2		13.4 ± 1.2	12.4 ± 0.8	12.6 ± 1.5	16.5 ± 2.0	19.3 ± 1.6	18.3 ± 1.5	42.3 ± 2.4*	85.3 ± 2.8***	92.4 ± 1.7	630
AMX/CST3		13.0 ± 0.8	12.8 ± 0.9	12.0 ± 0.4	13.2 ± 1.1	18.0 ± 1.0	18.3 ± 1.2	20.5 ± 1.9	84.4 ± 1.4***	91.1 ± 1.7	756
Control 0		13.7 ± 1.8	12.9 ± 0.4	13.7 ± 2.2	13.1 ± 0.9	13.1 ± 1.3	28.2 ± 0.8	60.4 ± 2.8	81.0 ± 2.9	88.7 ± 2.0	432
Control 4 hr		13.6 ± 2.1	12.7 ± 0.7	12.1 ± 0.5	12.1 ± 0.5	13.8 ± 1.4	14.0 ± 1.0	15.7 ± 0.6	25.0 ± 4.1	82.5 ± 3.2	1,420

Notes. Data are given as means ± *SD*.
AMX/CST1—6,200 ng/ml AMX plus 1,600 ng/ml CST.
AMX/CST2—4,050 ng/ml AMX plus 1,125 ng/ml CST.
AMX/CST3—1,900 ng/ml AMX plus 650 ng/ml CST.
Statistically significant in comparison with the bacterial control (4 hr), *$p \leq 0.01$, **$p \leq 0.001$, ***$p \leq 0.0001$ (Student *t* test). Statistical differences among the antibiotics are depicted in Figure 5.

TABLE 6 Viability of Vero cells after 72 hr incubation with Stx2etoxin-rich bacterial supernatants collected after treatment of bacteria with antibiotics for 4 hr (results are presented as % of viable cells according to LDH assay)

		Dilution factor									
Antibiotic	Concentration	8	16	32	64	128	256	512	1,024	2,048	CD_{50}
AMX	6,200 ng/ml	14.4 ± 1.5	11.6 ± 2.1	14.7 ± 2.8	15.2 ± 0.4	17.0 ± 0.1	19.9 ± 1.3	45.2 ± 1.6*	65.6 ± 1.5***	79.1 ± 2.0	511
	4,050 ng/ml	11.9 ± 0.9	14.5 ± 0.7	12.1 ± 1.8	12.7 ± 0.3	13.6 ± 0.3	15.2 ± 0.4	22.3 ± 1.6	65.2 ± 0.8***	72.9 ± 3.1	741
	1,900 ng/ml	11.8 ± 1.2	11.2 ± 1.4	10.4 ± 1.0	13.8 ± 1.1	15.3 ± 1.5	17.3 ± 0.5	19.6 ± 1.2	59.1 ± 2.1***	71.7 ± 2.2	787
CST	1,600 ng/ml	14.3 ± 0.9	14.8 ± 2.7	11.4 ± 1.8	12.9 ± 0.1	12.9 ± 0.5	14.7 ± 0.3	30.4 ± 1.0*	54.6 ± 2.9***	78.4 ± 1.0	814
	1,125 ng/ml	11.7 ± 1.5	12.6 ± 1.0	13.0 ± 3.0	14.4 ± 2.1	14.2 ± 1.0	14.5 ± 0.7	24.4 ± 0.7	50.5 ± 2.1***	61.3 ± 3.8	742
	650 ng/ml	15.0 ± 1.1	12.5 ± 1.6	15.6 ± 0.7	16.3 ± 1.5	17.1 ± 0.5	14.0 ± 0.3	20.0 ± 1.1	42.3 ± 2.1**	59.7 ± 2.7	868
ENR	0.7 μg/ml	9.4 ± 1.2	10.5 ± 0.8	11.6 ± 0.6	10.9 ± 1.1	12.9 ± 2.3	21.3 ± 1.5	22.3 ± 0.4	63.3 ± 2.6***	72.3 ± 2.0	743
	0.45 μg/ml	11.3 ± 1.5	12.6 ± 1.1	11.6 ± 1.2	11.6 ± 1.7	15.4 ± 1.5	20.6 ± 0.7	19.7 ± 1.2	61.5 ± 3.7***	74.6 ± 1.2	762
	0.2 μg/ml	7.6 ± 0.7	10.4 ± 1.0	11.2 ± 0.8	11.5 ± 1.4	13.5 ± 1.7	15.7 ± 0.5	19.3 ± 1.1	53.5 ± 2.5**	68.2 ± 2.7	788
CIP	0.45 μg/ml	10.5 ± 0.9	11.0 ± 1.2	10.9 ± 1.8	14.2 ± 0.9	18.4 ± 1.0	20.5 ± 2.0	45.1 ± 1.7**	58.7 ± 2.1***	73.0 ± 2.5	475
	0.29 μg/ml	11.4 ± 0.9	12.6 ± 1.1	11.8 ± 1.0	11.2 ± 1.6	11.7 ± 1.2	17.8 ± 0.9	25.5 ± 3.3	55.5 ± 3.3***	73.0 ± 4.0	785
	0.13 μg/ml	11.9 ± 1.3	11.0 ± 1.3	11.2 ± 2.8	12.4 ± 0.5	12.9 ± 0.7	12.6 ± 1.4	24.3 ± 2.6	54.0 ± 0.6***	69.3 ± 1.9	782
AMX/CST1		10.6 ± 1.3	12.5 ± 0.6	13.5 ± 0.6	14.4 ± 1.4	17.1 ± 0.8	30.4 ± 1.7*	63.2 ± 3.0***	67.0 ± 2.0***	73.3 ± 2.8	343
AMX/CST2		12.5 ± 0.9	13.4 ± 0.6	15.6 ± 0.9	15.6 ± 2.1	17.6 ± 1.2	17.7 ± 1.3	34.6 ± 3.4*	63.6 ± 1.7***	70.5 ± 1.2	632
AMX/CST3		9.8 ± 0.8	11.4 ± 1.1	11.1 ± 0.6	12.9 ± 0.8	14.7 ± 0.9	16.7 ± 1.3	18.1 ± 0.3	50.4 ± 1.8**	68.3 ± 2.5	848
Control 0		10.8 ± 0.7	11.7 ± 0.5	12.4 ± 1.4	14.1 ± 0.4	16.4 ± 1.1	17.4 ± 1.3	51.3 ± 2.0	64.4 ± 1.1	71.8 ± 1.6	436.7
Control 4 hr		11.6 ± 1.5	11.9 ± 1.1	13.0 ± 1.7	13.2 ± 1.4	12.0 ± 1.2	10.9 ± 1.2	17.9 ± 0.8	20.2 ± 1.2	66.5 ± 1.5	1,437

Notes. Data are given as means ± *SD*.
AMX/CST1—6,200 ng/ml AMX plus 1,600 ng/ml CST.
AMX/CST2—4,050 ng/ml AMX plus 1,125 ng/ml CST.
AMX/CST3—1,900 ng/ml AMX plus 650 ng/ml CST.
Statistically significant in comparison with the bacterial control (4 hr), $^{*}p \leq 0.01$, $^{**}p \leq 0.001$, $^{***}p \leq 0.0001$ (Student *t* test). Statistical differences among the antibiotics are depicted in Figure 6.

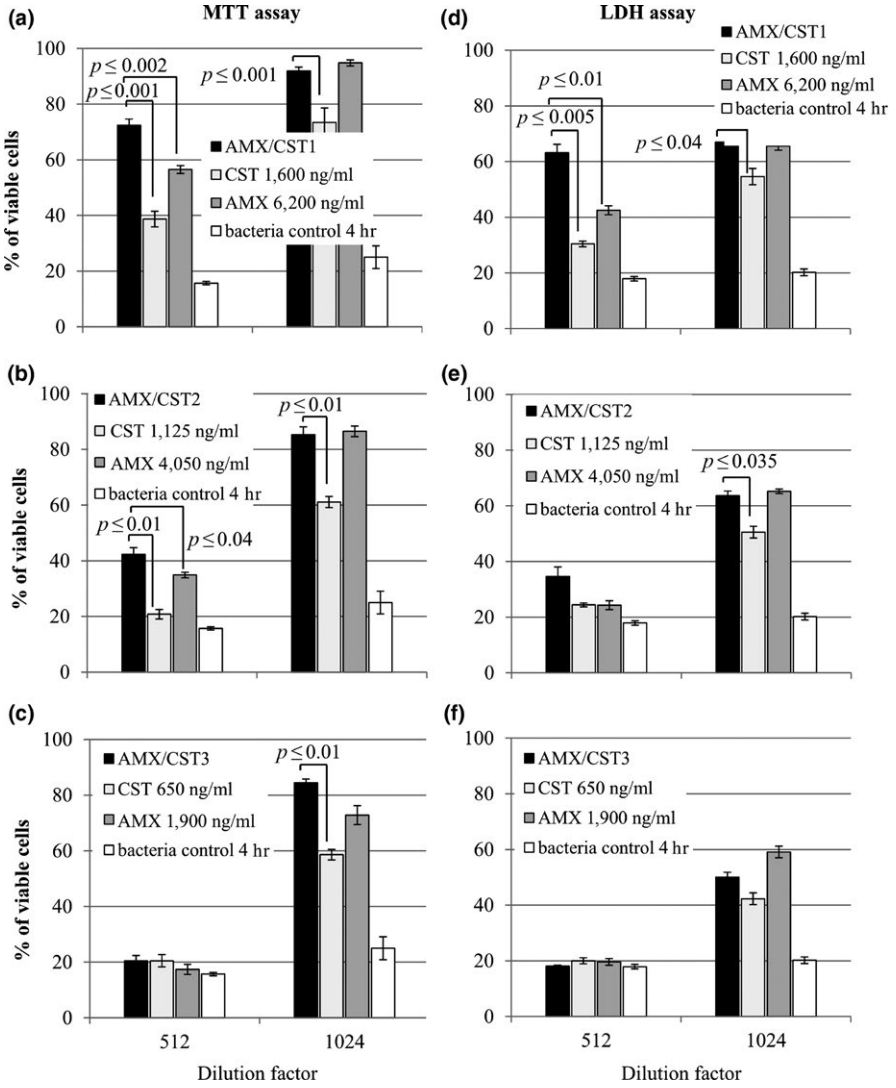

FIGURE 5 Statistical comparison of Stx 2e neutralization (1:512 and 1:1,024 dilutions of bacterial supernatants are presented) between the amoxicillin and colistin combination (AMX/CST) and its components used separately (AMX and CST). Results are presented as % of viable Vero cells according to the MTT (a-c) and LDH (d-f) assays. Data are given as means ± *SD* with statistical significance (one-way ANOVA)
AMX/CST1—6,200 ng/ml AMX plus 1,600 ng/ml CST
AMX/CST2—4,050 ng/ml AMX plus 1,125 ng/ml CST
AMX/CST3—1,900 mg/ml AMX plus 650 ng/ml CST

Under field conditions, animal treatment is usually delayed by at least 6–24 hr considering the onset of clinical signs detected by farmers—the incubation time of any bacterial infection or toxemia lasting for a few hours or days. Therefore, the antibiotic treatment following earlier contact of LPS with cells in the presented experimental conditions (6, 12 or 24 hr) reflected an optimal in vivo antimicrobial therapy timeline. It should be underlined that all antibiotics, regadless of their concentration, did not changed high viability of A549 cells even after 3 days of treatment—this fact confirms the safety of used antibiotics.

Binding of cationic polypeptide CST to negatively charged lipid A (the toxic component of LPS) has been well documented before in in vitro studies (Morrison & Jacobs, 1976; Rhouma, Beaudry, Theriault, & Letellier, 2016; Warren, Kania, & Siber, 1985), with following inhibition of inflammatory cytokine release, especially TNF-α. Moreover, the endotoxin-neutralizing activity of CST has been confirmed by in vivo challenge trials (Dosogne et al., 2002; Giacometti et al., 2003) and in veterinary clinical studies concerning cases with naturally occurring septic shock (Senturk, 2005). Those experiments also demonstrated the ability of CST to alleviate the clinical implications of endotoxemia and even to protect against lethality in a rat model (Giacometti et al., 2003; Senturk, 2005). Contrarily to colistin, there have been no reports of binding of amoxicillin to LPS. Moreover, amoxicillin used in a rat model and simulating the treatment of otitis media in infected dogs resulted in an increase in the proinflammatory cytokine level, probably due to the bactericidal effect of amoxicillin and endotoxin release. This could participate in the development of septic shock. Evidently, in

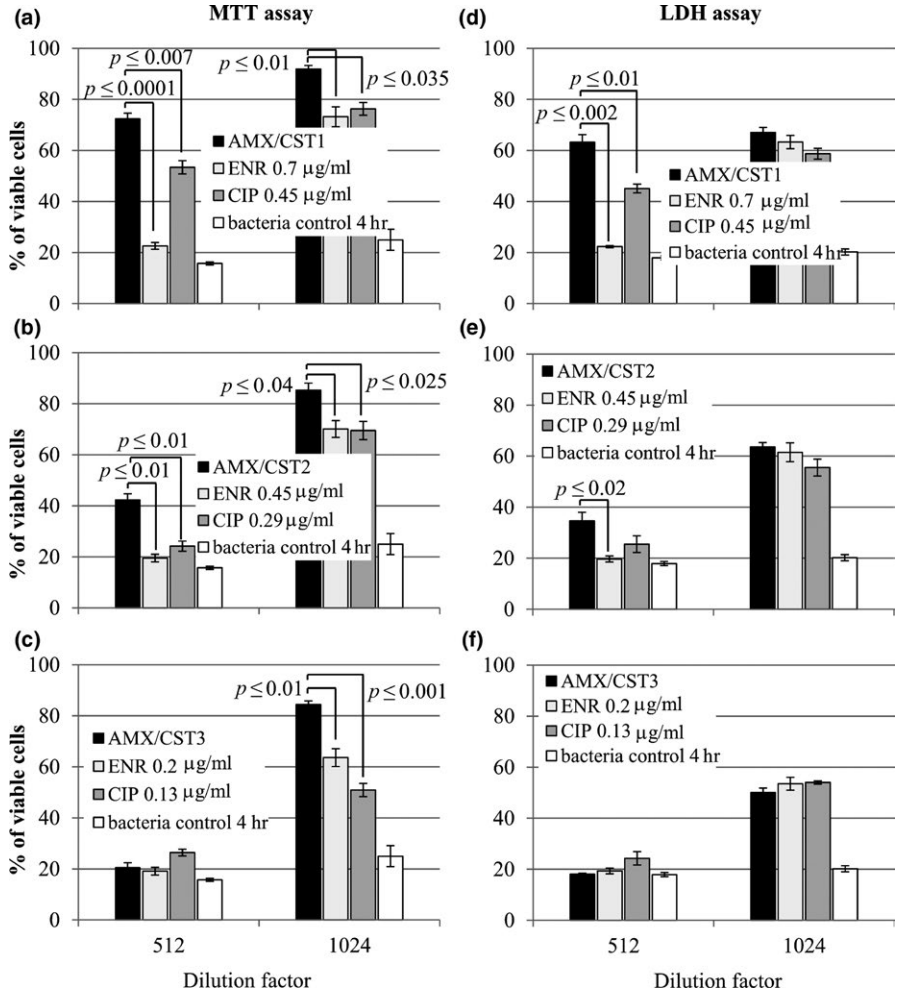

FIGURE 6 Statistical comparison of Stx 2e neutralization (1:512 and 1:1,024 dilutions of bacterial supernatants are presented) between the amoxicillin and colistin combination (AMX/CST), enrofloxacin (ENR), and ciprofloxacin (CIP). Results are presented as % of viable Vero cells to the according MTT (a-c) and LDH (d-f) assays. Data are given as means ± *SD* with statistical significance (one-way ANOVA)
AMX/CST1—6,200 ng/ml AMX plus 1,600 ng/ml CST
AMX/CST2—4,050 ng/ml AMX plus 1,125 ng/ml CST
AMX/CST3—1,900 mg/ml AMX plus 650 ng/ml CST

that study, the endotoxin was not neutralized (Melhus, 2001). On the other hand, other β-lactams, cefaclor, and cefpodoxime decreased synthesis of IL-6 and TNF-α in human granulocytes phagocytizing *E. coli* (Scheffer & Konig, 1993).

Thus, even inferior to colistin, the endotoxin-neutralizing activity of amoxicillin demonstrated in the present study is very original, not only as individually used antibiotic but especially in combination with colistin, where an additive beneficial effect was observed. Similar additive effect was observed by Ghiselli et al. (2002) who indicated that the combination of amoxicillin-clavulanate in experimental rats model influenced on LPS activity. However, further investigations are needed to explain the nature of such interactions.

A challenge study with *E. coli* mastitis in cattle revealed that enrofloxacin treatment significantly limited bacteria in milk but did not reduce LPS concentration in animal plasma (Dosogne et al., 2002). This fact could suggest that enrofloxacin is unable to neutralize endotoxin. Indeed, further research clearly indicated a lack of physicochemical interactions between fluoroquinolones and LPS (Lindner, Wiese, Brandenburg, Seydel, & Dalhoff, 2002).

In our experiments, we decided also to include ciprofloxacin as model substance, because it is a major metabolite of enrofloxacin. In fact, in treated cattle, about 35% of enrofloxacin is metabolized to ciprofloxacin. Although ciprofloxacin is dedicated for humans, in EU countries, it is registered also for treatment of calves of less than 1 year of age (The European Union summary report on antimicrobial resistance in zoonotic and indicator bacteria from humans, animals and food in 2015, https://www.efsa.europa.eu).

Ciprofloxacin is probably able to at least partly neutralize of LPS as THP-1 mononuclear cells released less TNF after exposure to filtrates of *E. coli* cultures treated with this antibiotic, however, the nature of such interaction was not described (Simon, Koenig, & Trenholme, 1991). Additionally, in in vivo experiment ciprofloxacin (250 mg/kg) protected 25% of mice challenged with a lethal dose of *E. coli* LPS against death (Khan, Slifer, Araujo, Suzuki, & Remington, 2000).

In the present study mimicking natural infection and treatment, when the low concentration of LPS was used, ciprofloxacin protected cells against LPS-mediated cytotoxicity comparably to amoxicillin and colistin. The differences among these antibiotics appeared in the case of the high LPS concentration—in contrast to AMX and CST, ciprofloxacin was efficient exclusively after the shortest contact of the cells with endotoxin. Such an effect of CIP was probably caused by limiting proinflammatory cytokine production as other in vitro and in vivo experiments supported that ciprofloxacin at a pharmacological concentration above 25 pg/ml significantly inhibited production of IL-1α, IL-1β, IL-6, and TNF-α in human monocytes after stimulation with LPS (Bailly, Fay, Ferrua, & Gougerot-Pocidalo, 1991). Additionally, recent studies described the immunomodulatory activity of another fluoroquinolone used in humans—moxifloxacin – as a beneficial regulator of the expression of TLRs and proinflammatory cytokines in response to LPS (Bode et al., 2014). Weiss et al. reported that those anti-inflammatory properties of moxifloxacin, including the reduction of the proinflammatory cytokines IL-1β and IL-6, could be explained through its inhibitory effect on the TLR4/LPS-signaling pathway (Weiss et al., 2004). However, in vivo effects of antibiotics on LPS-induced cytokine production could be more complex as in some aspects, it differed from in vitro reported results (Bailly et al., 1991).

Escherichia coli O157:H7 releasing Shiga toxins may cause mild to severe intestinal pathology in cattle (Gyles, 2007; Lowe et al., 2009). Additionally, the possible synergistic effect of Stx and LPS observed in vivo (Proulx, Seidman, & Karpman, 2001) could be responsible for enhancing clinical signs. Therefore, the therapeutic interest is limiting not only the bacterial growth but also the released endotoxin toxicity. In the presented in vitro studies, the *E. coli* O139 ETEEC reference strain releasing Stx2e was used instead of the highly pathogenic *E. coli* O157:H7 strain. The design of this experiment allowed quantification of the Stx2e neutralization by the tested antibiotics using a susceptible Vero cell line. When higher bacteria supernatant dilutions (1,024–2,048) were incubated with Vero cells, their viability was comparable regardless of individual AMX or AMX/CST combination treatment. However, when lower bacteria supernatant dilutions were used (256–512), treatment with AMX/CST combination resulted in the higher cells viability in comparison with individual used antibiotics with the first significant value in case of AMX/CST1. Concluding, the AMX/CST combination exhibited the better ability to inhibiting Stx2e cytotoxic effect in Vero cells than individual antibiotics, especially in case of high Stx2e concentration. This fact probably results from the additive effect of AMX and CST. In this experiment, also ciprofloxacin appeared as an effective antibiotic. Colistin and enrofloxacin showed a similar activity, however, lower than that of amoxicillin and ciprofloxacin.

Interaction of colistin and Stx toxin was already documented last year by Percivalle et al. (2016), who described the ability of this drug to inhibit *E. coli* 0157:H7 Shiga-like toxin release, as well as binding thereof, thereby protecting Vero cells. Similarly to LPS, there is no information in scientific papers about the Stx-neutralizing activity of amoxicillin or other penicillins. Although the ability of enrofloxacin to reduce production and release of Stx by enterotoxaemic ETEC is well known (Uemura et al., 2004), its neutralizing potential on Shiga toxins has not been described. However, another fluoroquinolone, ciprofloxacin, exhibited an ability to either increase or decrease Stx production, depending on its concentration (McGannon, Fuller, & Weiss, 2010). Subinhibitory concentrations of the antibiotic increased Stx expression, while no Stx was detected at a concentration at or above MIC (*minimal inhibitory concentration*). In the present experiment, ciprofloxacin was used at concentrations above MIC, which is determined for *E. coli* as 0.12 μg/ml (Baudry-Simner, Singh, Karlowsky, Hoban, & Zhanel, 2012).

To sum up, the main observation of this study is the significant beneficial in vitro effects demonstrated by colistin and amoxicillin (especially used in combination of concentrations found during Potencil® therapy of bacterial diarrhea in young calves and piglets) to attenuate LPS- and Stx-mediated cytotoxicity (in concentrations reflecting high endotoxemia), in contrast to the fluoroquinolones, enrofloxacin, and ciprofloxacin. However, in model of mild endotoxemia, effectiveness of all studied antibiotics was comparable indicating that concentration of antibiotics was enough to completely neutralization of low concentration of LPS or its toxic effect in the cells culture.

It should also be emphasized that this effect was exhibited by antibiotics used at concentrations within the range of achievable maximum serum concentrations following their administration to animals. However, the complexity of the cytokine effects as well as the complexity of the interaction between the host immune system and antibiotics would require further studies to determine possible beneficial effects on the course of the infectious and inflammatory process in affected animals. To confirm these in vitro results, an in vivo comparative treatment study would be necessary in controlled young livestock challenged with endo- or shigatoxemia.

ACKNOWLEDGMENTS

This study was partly supported by Virbac France (https://corporate.virbac.com)—project No 1/2015 to RUC and ASC. The authors would like to thank Audrey Brunet and Denis Jarrin from Virbac, for their insight and expertise that greatly assisted this research.

CONFLICT OF INTEREST

The authors declare no conflict of interests.

AUTHOR CONTRIBUTION

ASC: Conceived and designed the experiments; Performed in vitro experiments (LPS and partly Stx 2e); Performed statistical analysis, interpretation, and graphical presentation of results; Wrote the manuscript. RUC: The main scientific coordinator of the whole project. Conceived and designed the experiments; Performed in vitro experiments (partly Stx 2e); Participation in interpretation of the

collected results and writing of the manuscript. AW: Conceived and designed the experiments. LM and MW: One of the scientific coordinators—conceived and monitored the research project for Virbac. EB: Reviewed the manuscript; Participation in the editorial part of the manuscript.

ORCID

Agnieszka Szuster-Ciesielska http://orcid.org/0000-0003-4046-1920

REFERENCES

Angus, D. C., & van der Poll, T. (2013). Severe sepsis and septic shock. *New England Journal of Medicine*, *369*(9), 840–851. https://doi.org/10.1056/NEJMra1208623

Aulik, N. A., Hellenbrand, K. M., Klos, H., & Czuprynski, C. J. (2010). Mannheimia haemolytica and its leukotoxin cause neutrophil extracellular trap formation by bovine neutrophils. *Infection and Immunity*, *78*(11), 4454–4466. https://doi.org/10.1128/IAI.00840-10

Bailly, S., Fay, M., Ferrua, B., & Gougerot-Pocidalo, M. A. (1991). Ciprofloxacin treatment in vivo increases the ex vivo capacity of lipopolysaccharide-stimulated human monocytes to produce IL-1, IL-6 and tumour necrosis factor-alpha. *Clinical and Experimental Immunology*, *85*(2), 331–334.

Baranzoni, G. M., Fratamico, P. M., Gangiredla, J., Patel, I., Bagi, L. K., Delannoy, S., … Pepe, T. (2016). Characterization of Shiga Toxin Subtypes and Virulence Genes in Porcine Shiga Toxin-Producing *Escherichia coli*. *Frontiers in Microbiology*, *7*, 574.

Baudry-Simner, P. J., Singh, A., Karlowsky, J. A., Hoban, D. J., Zhanel, G. G. & Canadian Antimicrobial Resistance, A. (2012) Mechanisms of reduced susceptibility to ciprofloxacin in *Escherichia coli* isolates from Canadian hospitals. *Canadian Journal of Infectious Diseases and Medical Microbiology*, *23*(3), e60–e64.

Bode, C., Diedrich, B., Muenster, S., Hentschel, V., Weisheit, C., Rommelsheim, K., … Baumgarten, G. (2014). Antibiotics regulate the immune response in both presence and absence of lipopolysaccharide through modulation of Toll-like receptors, cytokine production and phagocytosis in vitro. *International Immunopharmacology*, *18*(1), 27–34. https://doi.org/10.1016/j.intimp.2013.10.025

Bopp, S. K., & Lettieri, T. (2008). Comparison of four different colorimetric and fluorometric cytotoxicity assays in a zebrafish liver cell line. *BMC Pharmacology*, *8*, 8. https://doi.org/10.1186/1471-2210-8-8

Burvenich, C., Van Merris, V., Mehrzad, J., Diez-Fraile, A., & Duchateau, L. (2003). Severity of *E. coli* mastitis is mainly determined by cow factors. *Veterinary Research*, *34*(5), 521–564. https://doi.org/10.1051/vetres:2003023

Catry, B., Dewulf, J., Maes, D., Pardon, B., Callens, B., Vanrobaeys, M., … Haesebrouck, F. (2016). Effect of Antimicrobial Consumption and Production Type on Antibacterial Resistance in the Bovine Respiratory and Digestive Tract. *PLoS ONE*, *11*(1), e0146488. https://doi.org/10.1371/journal.pone.0146488

Cho, Y. I., & Yoon, K. J. (2014). An overview of calf diarrhea - infectious etiology, diagnosis, and intervention. *Journal of Veterinary Science*, *15*(1), 1–17. https://doi.org/10.4142/jvs.2014.15.1.1

Constable, P. D. (2004). Antimicrobial use in the treatment of calf diarrhea. *Journal of Veterinary Internal Medicine*, *18*(1), 8–17. https://doi.org/10.1111/j.1939-1676.2004.tb00129.x

Cornick, N. A., Matise, I., Samuel, J. E., Bosworth, B. T., & Moon, H. W. (2000). Shiga toxin-producing *Escherichia coli* infection: Temporal and quantitative relationships among colonization, toxin production, and systemic disease. *Journal of Infectious Diseases*, *181*(1), 242–251. https://doi.org/10.1086/315172

Dean-Nystrom, E. A., Bosworth, B. T., & Moon, H. W. (1997). Pathogenesis of O157:H7 *Escherichia coli* infection in neonatal calves. *Advances in Experimental Medicine and Biology*, *412*, 47–51. https://doi.org/10.1007/978-1-4899-1828-4

Dosogne, H., Meyer, E., Sturk, A., van Loon, J., Massart-Leen, A. M., & Burvenich, C. (2002). Effect of enrofloxacin treatment on plasma endotoxin during bovine *Escherichia coli* mastitis. *Inflammation Research*, *51*(4), 201–205. https://doi.org/10.1007/PL00000293

El-Seedy, F. R., Abed, A. H., Yanni, H. A., & Abd El-Rahman, S. A. A. (2016). Prevalence of Salmonelle and E.coli in neonatal diarrheic calves. *Beni-Suef University Journal of Basic and Applied Sciences*, *5*(1), 45–51.

Ferens, W. A., & Hovde, C. J. (2011). *Escherichia coli* O157:H7: Animal reservoir and sources of human infection. *Foodborne Pathogens and Disease*, *8*(4), 465–487. https://doi.org/10.1089/fpd.2010.0673

Fotakis, G., & Timbrell, J. A. (2006). In vitro cytotoxicity assays: Comparison of LDH, neutral red, MTT and protein assay in hepatoma cell lines following exposure to cadmium chloride. *Toxicology Letters*, *160*(2), 171–177. https://doi.org/10.1016/j.toxlet.2005.07.001

Gerros, T. C., Semrad, S. D., & Proctor, R. A. (1995). Alterations in clinical, hematological and metabolic variables in bovine neonatal endotoxemia. *Canadian Journal of Veterinary Research*, *59*(1), 34–39.

Ghiselli, R., Giacometti, A., Cirioni, O., Mocchegiani, F., Viticchi, C., Scalise, G., & Saba, V. (2002). Cationic peptides combined with betalactams reduce mortality from peritonitis in experimental rat model. *Journal of Surgical Research*, *108*(1), 107–111. https://doi.org/10.1006/jsre.2002.6518

Giacometti, A., Cirioni, O., Ghiselli, R., Orlando, F., Mocchegiani, F., D'Amato, G., … Scalise, G. (2003). Antiendotoxin activity of antimicrobial peptides and glycopeptides. *Journal of Chemotherapy*, *15*(2), 129–133. https://doi.org/10.1179/joc.2003.15.2.129

Gyles, C. L. (2007). Shiga toxin-producing *Escherichia coli*: An overview. *Journal of Animal Science*, *85*(13 Suppl), E45–E62. https://doi.org/10.2527/jas.2006-508

Hardie, E. M., & Kruse-Elliott, K. (1990). Endotoxic shock. Part II: A review of treatment. *Journal of Veterinary Internal Medicine*, *4*(6), 306–314. https://doi.org/10.1111/j.1939-1676.1990.tb03128.x

Holzheimer, R. G. (2001). Antibiotic induced endotoxin release and clinical sepsis: A review. *Journal of Chemotherapy*, *13*(1), 159–172. https://doi.org/10.1179/joc.2001.13.Supplement-2.159

Hurley, J. C. (1995). Antibiotic-induced release of endotoxin. A therapeutic paradox. *Drug Safety*, *12*(3), 183–195.

Jo, H. Y., Kim, Y., Park, H. W., Moon, H. E., Bae, S., Kim, J., … Paek, S. H. (2015). The unreliability of MTT assay in the cytotoxic test of primary cultured glioblastoma cells. *Experimental Neurobiology*, *24*(3), 235–245. https://doi.org/10.5607/en.2015.24.3.235

Khan, A. A., Slifer, T. R., Araujo, F. G., Suzuki, Y., & Remington, J. S. (2000). Protection against lipopolysaccharide-induced death by fluoroquinolones. *Antimicrobial Agents and Chemotherapy*, *44*(11), 3169–3173. https://doi.org/10.1128/AAC.44.11.3169-3173.2000

Kolenda, R., Burdukiewicz, M., & Schierack, P.. (2015). A systematic review and meta-analysis of the epidemiology of pathogenic *Escherichia coli* of calves and the role of calves as reservoirs for human pathogenic *E. coli*. *Frontiers in Cellular and Infection Microbiology*, *5*, 23.

Konstantinova, L., Hamrik, J., Kulich, P., Kummer, V., Maskova, J., & Alexa, P. (2008). The effect of intramuscular administration of colistin on the development and course of experimentally induced oedema disease in weaned piglets. *Veterinary Microbiology*, *128*(1–2), 160–166. https://doi.org/10.1016/j.vetmic.2007.09.021

Liberati, T. A., Trammell, R. A., Randle, M., Barrett, S., & Toth, L. A. (2013). Cytokine and chemokine responses of lung exposed to surrogate viral and bacterial infections. *Comparative Medicine*, *63*(2), 114–126.

Lindner, B., Wiese, A., Brandenburg, K., Seydel, U., & Dalhoff, A. (2002). Lack of interaction of fluoroquinolones with lipopolysaccharides.

Antimicrobial Agents and Chemotherapy, *46*(5), 1568–1570. https://doi.org/10.1128/AAC.46.5.1568-1570.2002

Lowe, R. M., Baines, D., Selinger, L. B., Thomas, J. E., McAllister, T. A., & Sharma, R. (2009). *Escherichia coli* O157:H7 strain origin, lineage, and Shiga toxin 2 expression affect colonization of cattle. *Applied and Environment Microbiology*, *75*(15), 5074–5081. https://doi.org/10.1128/AEM.00391-09

Mainil, J. (1999). Shiga/verocytotoxins and Shiga/verotoxigenic *Escherichia coli* in animals. *Veterinary Research*, *30*(2–3), 235–257.

McGannon, C. M., Fuller, C. A., & Weiss, A. A. (2010). Different classes of antibiotics differentially influence shiga toxin production. *Antimicrobial Agents and Chemotherapy*, *54*(9), 3790–3798. https://doi.org/10.1128/AAC.01783-09

McKellar, Q., Gibson, I., Monteiro, A., & Bregante, M. (1999). Pharmacokinetics of enrofloxacin and danofloxacin in plasma, inflammatory exudate, and bronchial secretions of calves following subcutaneous administration. *Antimicrobial Agents and Chemotherapy*, *43*(8), 1988–1992.

Meganck, V., Hoflack, G., Piepers, S., & Opsomer, G. (2015). Evaluation of a protocol to reduce the incidence of neonatal calf diarrhoea on dairy herds. *Preventive Veterinary Medicine*, *118*(1), 64–70. https://doi.org/10.1016/j.prevetmed.2014.11.007

Melhus, A. (2001). Effects of amoxicillin on the expression of cytokines during experimental acute otitis media caused by non-typeable Haemophilus influenzae. *Journal of Antimicrobial Chemotherapy*, *48*(3), 397–402. https://doi.org/10.1093/jac/48.3.397

Morrison, D. C., & Jacobs, D. M. (1976). Binding of polymyxin B to the lipid A portion of bacterial lipopolysaccharides. *Immunochemistry*, *13*(10), 813–818. https://doi.org/10.1016/0019-2791(76)90181-6

Mosmann, T. (1983). Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. *Journal of Immunological Methods*, *65*(1–2), 55–63. https://doi.org/10.1016/0022-1759(83)90303-4

Moxley, R. A. (2000). Edema disease. *Veterinary Clinics of North America: Food Animal Practice*, *16*(1), 175–185. https://doi.org/10.1016/S0749-0720(15)30142-0

Muthing, J., Meisen, I., Zhang, W., Bielaszewska, M., Mormann, M., Bauerfeind, R., … Karch, H. (2012). Promiscuous Shiga toxin 2e and its intimate relationship to Forssman. *Glycobiology*, *22*(6), 849–862. https://doi.org/10.1093/glycob/cws009

Nguyen, Y., & Sperandio, V.. (2012). Enterohemorrhagic *E. coli* (EHEC) pathogenesis. *Frontiers in Cellular and Infection Microbiology*, *2*, 90.

Nishio, K., Horie, M., Akazawa, Y., Shichiri, M., Iwahashi, H., Hagihara, Y., … Niki, E. (2013). Attenuation of lipopolysaccharide (LPS)-induced cytotoxicity by tocopherols and tocotrienols. *Redox Biology*, *1*, 97–103. https://doi.org/10.1016/j.redox.2012.10.002

Oanh, T. K., Nguyen, V. K., De Greve, H., & Goddeeris, B. M. (2012). Protection of piglets against Edema disease by maternal immunization with Stx2e toxoid. *Infection and Immunity*, *80*(1), 469–473. https://doi.org/10.1128/IAI.05539-11

Pachuta-Stec, A., & Szuster-Ciesielska, A. (2015). New norcantharidin analogs: Synthesis and anticancer activity. *Archiv der Pharmazie*, *348*(12), 897–907. https://doi.org/10.1002/ardp.201500255

Percivalle, E., Monzillo, V., Pauletto, A., Marone, P., & Imberti, R. (2016). Colistin inhibits *E. coli* O157:H7 Shiga-like toxin release, binds endotoxins and protects Vero cells. *New Microbiologica*, *39*(2), 119–123.

Picco, N. Y., Alustiza, F. E., Bellingeri, R. V., Grosso, M. C., Motta, C. E., Larriestra, A. J., … Vivas, A. B. (2015). Molecular screening of pathogenic *Escherichia coli* strains isolated from dairy neonatal calves in Cordoba province, Argentina. *Revista Argentina de Microbiología*, *47*(2), 95–102. https://doi.org/10.1016/j.ram.2015.01.006

Proulx, F., Seidman, E. G., & Karpman, D. (2001). Pathogenesis of Shiga toxin-associated hemolytic uremic syndrome. *Pediatric Research*, *50*(2), 163–171. https://doi.org/10.1203/00006450-200108000-00002

Rahal, E. A., Fadlallah, S. M., Nassar, F. J., Kazzi, N., & Matar, G. M. (2015). Approaches to treatment of emerging Shiga toxin-producing *Escherichia coli* infections highlighting the O104:H4 serotype. *Frontiers in Cellular and Infection Microbiology*, *5*, 24.

Rhouma, M., Beaudry, F., Theriault, W., & Letellier, A. (2016). Colistin in Pig Production: Chemistry, Mechanism of Antibacterial Action, Microbial Resistance Emergence, and One Health Perspectives. *Frontiers in Microbiology*, *7*, 1789.

Scheffer, J., & Konig, W. (1993). Cephalosporins and inflammatory host reactions. *Respiration*, *60*(Suppl 1), 25–31. https://doi.org/10.1159/000196246

Senturk, S. (2005). Evaluation of the anti-endotoxic effects of polymyxin-E (colistin) in dogs with naturally occurred endotoxic shock. *Journal of Veterinary Pharmacology and Therapeutics*, *28*(1), 57–63. https://doi.org/10.1111/j.1365-2885.2004.00634.x

Simon, D. M., Koenig, G., & Trenholme, G. M. (1991). Differences in release of tumor necrosis factor from THP-1 cells stimulated by filtrates of antibiotic-killed *Escherichia coli*. *Journal of Infectious Diseases*, *164*(4), 800–802. https://doi.org/10.1093/infdis/164.4.800

Uboldi, C., Bonacchi, D., Lorenzi, G., Hermanns, M. I., Pohl, C., Baldi, G., … Kirkpatrick, C. J. (2009). Gold nanoparticles induce cytotoxicity in the alveolar type-II cell lines A549 and NCIH441. *Particle and Fibre Toxicology*, *6*, 18. https://doi.org/10.1186/1743-8977-6-18

Uemura, R., Sueyoshi, M., Taura, Y., & Nagatomo, H. (2004). Effect of antimicrobial agents on the production and release of shiga toxin by enterotoxaemic *Escherichia coli* isolates from pigs. *Journal of Veterinary Medical Science*, *66*(8), 899–903. https://doi.org/10.1292/jvms.66.899

Uetake, K. (2013). Newborn calf welfare: A review focusing on mortality rates. *Animal Science Journal*, *84*(2), 101–105. https://doi.org/10.1111/asj.12019

Warren, H. S., Kania, S. A., & Siber, G. R. (1985). Binding and neutralization of bacterial lipopolysaccharide by colistin nonapeptide. *Antimicrobial Agents and Chemotherapy*, *28*(1), 107–112. https://doi.org/10.1128/AAC.28.1.107

Weiss, T., Shalit, I., Blau, H., Werber, S., Halperin, D., Levitov, A., & Fabian, I. (2004). Anti-inflammatory effects of moxifloxacin on activated human monocytic cells: Inhibition of NF-kappaB and mitogen-activated protein kinase activation and of synthesis of proinflammatory cytokines. *Antimicrobial Agents and Chemotherapy*, *48*(6), 1974–1982. https://doi.org/10.1128/AAC.48.6.1974-1982.2004

Weyermann, J., Lochmann, D., & Zimmer, A. (2005). A practical note on the use of cytotoxicity assays. *International Journal of Pharmaceutics*, *288*(2), 369–376. https://doi.org/10.1016/j.ijpharm.2004.09.018

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

How to cite this article: Szuster-Ciesielska A, Urban-Chmiel R, Wernicki A, Mascaron L, Wasak M, Bousquet E. Evaluation of the ability of colistin, amoxicillin (components of Potencil®), and fluoroquinolones to attenuate bacterial endotoxin- and Shiga exotoxin-mediated cytotoxicity—In vitro studies. *J vet Pharmacol Therap.* 2018;00:1–19. https://doi.org/10.1111/jvp.12710