Minimizing human infection from *Escherichia coli* O157:H7 using GUMBOS

Marsha R. Cole1, Min Li1, Ravirajsingh Jadeja2, Bilal El-Zahab1, Daniel Hayes3, Jeffery A. Hobden4, Marlene E. Janes2 and Isiah M. Warner1*

1Department of Chemistry, Louisiana State University, Baton Rouge, LA 70803, USA; 2Department of Food Science, Louisiana State University Agricultural Center, Baton Rouge, LA 70803, USA; 3Department of Agricultural and Biological Engineering, Louisiana State University, Baton Rouge, LA 70803, USA; 4Department of Microbiology, Immunology, and Parasitology, Louisiana State University Health Sciences Center, New Orleans, LA 70112, USA

*Corresponding author. Tel: +1-225-578-2829; Fax: +1-225-578-3971; E-mail: iwarner@lsu.edu

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**Objectives:** Reduction in faecal shedding of Shiga toxin-producing enterohaemorrhagic *Escherichia coli* (EHEC) in food-producing animals is a viable strategy to minimize human disease initiated by exposure to these microorganisms. To this end, an intervention strategy involving the electrostatic hybridization of two commonly used anti-infective agents for veterinary practice (i.e. chlorhexidine and ampicillin) was evaluated to curtail EHEC-transmitted disease from ruminant sources. Chlorhexidine di-ampicillin is a novel group of uniform material based on organic salts (GUMBOS) with inherent in vitro antibacterial activity that comes from its parent antimicrobial ions, chlorhexidine and ampicillin.

**Methods:** Antibacterial activities for chlorhexidine diacetate, sodium ampicillin, chlorhexidine di-ampicillin and stoichiometrically equivalent 1:2 chlorhexidine diacetate:sodium ampicillin were assessed using the serial 2-fold dilution method and time–kill studies against seven isolates of *E. coli* O157:H7 and one non-pathogenic *E. coli* 25922. Further studies to investigate synergistic interactions of reacted and stoichiometrically equivalent unreacted antimicrobial agents at MICs and possible mechanisms were also investigated.

**Results:** Synergism and in vitro antibacterial activities against EHEC were observed in this study, which suggests chlorhexidine di-ampicillin could be a useful reagent in reducing EHEC transmission and minimizing EHEC-associated infections. Likewise, chlorhexidine di-ampicillin reduced HeLa cell toxicity as compared with chlorhexidine diacetate or the stoichiometric combination of antimicrobial agents. Further results suggest that the mechanisms of action of chlorhexidine di-ampicillin and chlorhexidine diacetate against *E. coli* O157:H7 are similar.

**Conclusions:** Reacting antimicrobial GUMBOS as indicated in this study may enhance the approach to current combination drug therapeutic strategies for EHEC disease control and prevention.

**Keywords:** antibacterial activity, combination drug therapy, chlorhexidine, ampicillin

**Introduction**

Infections caused by Shiga toxin-producing enterohaemorrhagic *Escherichia coli* (EHEC) are associated with bloody diarrhoea, thrombocytic thrombocytopenic purpura, haemorrhagic colitis and haemolytic uraemic syndrome.1,2 Although strains of EHEC are represented by several serotypes, the vast majority of severe infections are produced by serotype O157.1,3 Cattle and other ruminants are known to be reservoirs of EHEC and are therefore known sources for faecal contamination in food and beverages. As a result, faecal waste from ruminants is associated with large outbreaks of disease caused by EHEC.4,5 Thus, aside from enforcing good hygiene practices for all aspects of food handling, i.e. from harvest to preparation, EHEC transmission may be better controlled by reducing faecal shedding from food-producing animals.2 Several methods have been tested in attempts to control the colonization of pathogenic microbes in food-producing animals, including regulation of animal diet and vaccination. To date, studies of such control measures to reduce or eliminate faecal shedding of EHEC in cattle have been inconclusive.4,6–8

The current approach to minimizing EHEC faecal shedding in a food-producing animal involves the use of antiseptics. In this
regard, various compounds have been used, some of which have demonstrated efficacy in the reduction of EHEC in faeces of food-producing animals. However, many of these compounds are inherently toxic and are not approved for such use in animals.9 One such compound is the dicationic biguanide, chlorhexidine. Most approved uses of chlorhexidine for cattle include extralabel topical applications, such as intramammary or terminal rectal de-colonization therapeutic agents. Under the provisions of the US Animal Medicinal Drug Use Clarification Act of 1996, various pharmaceutical agents can be freely used in food-producing animals beyond the approved labelling at the discretion of the treating veterinarian, if residues transferred to edible tissues are below levels of toxicological concern.9 Although subsequent leaching into adjacent tissues was not part of the study conducted by Naylor et al.,7 chlorhexidine enemas were shown to eliminate high-level faecal shedding and reduce low-level shedding of E. coli O157:H7 at the terminal rectum. Unfortunately, it is rather challenging to balance effective concentrations approved for use in food-producing animals without concern about possible human exposure to either EHEC infection or toxic reagents.10–13 Therefore, it is important to implement a therapeutic method that minimizes chlorhexidine toxicity and maintains its powerful antibacterial activity.

Many antibiotics, such as ampicillin, are also commonly used in animal feedlots to selectively reduce faecal shedding of EHEC in ruminants.5 However, the rapid rise of antimicrobial resistance severely curtails the widespread use of antibiotics in the control of microbe colonization in food-producing animals.14 Therefore, approaches that can reduce the concentration of antibiotics used in animal feedlots would be ideal to minimize the current contributions they have in the development of antibiotic resistance. Other methods of reducing EHEC faecal shedding have been explored using FDA-approved ionophores as feed additives. However, ionophores, such as monensin, lasalocid, and laimomycin propionate and bambermycin, are known to be ineffective in reducing EHEC shedding in faecal samples, particularly those isolated from sheep.15,16

With the goal of developing a safe and effective compound that could be administered to food-producing ruminants, such as cattle, goats and sheep, to reduce faecal shedding of EHEC, chlorhexidine and ampicillin were combined to form the salt, chlorhexidine di-ampicillin. These two compounds were chosen primarily for their history of use in veterinary practice. Integrating pharmaceutically active ions into entities with other ions that can modify the properties of pharmaceutically active agents has been shown to be a realistic approach to resolving many issues associated with antibiotics, anaegiesics and anti-inflammatory drug therapy.17–21

The implementation of designer salts known as a ‘group of uniform material based on organic salts’ (GUMBOS) is a new strategy outlined in this article for minimizing the effects of EHEC. GUMBOS are organic salts composed of organic and/or inorganic counterions with melting points between 25 and 250°C. These compounds have unique architectural platforms, in that multimodal properties innate to the desired application can be incorporated into the salt via judicious selection of the ions.22 In this article, we apply this approach to remedy the toxicity of chlorhexidine and loss of efficacy present in ampicillin in order to reduce EHEC faecal shedding in food-producing animals.23 A chlorhexidine di-ampicillin GUMBOS composed of the antiseptic chlorhexidine and an antibiotic, ampicillin, was stoichiometrically synthesized and evaluated for antimicrobial activity and acute cytotoxicity in comparison with the parent compounds and the mixture of parent compounds in stoichiometric equivalents to the GUMBOS.

Since chlorhexidine di-ampicillin GUMBOS is composed of two separate and distinct antimicrobial agents, it is important to understand the rationale of how the use of GUMBOS differs from the mixture of the two parent antimicrobials, such as in conventional combination drug therapy. Essentially, the two parent antimicrobial ions (e.g. sodium ampicillin and chlorhexidine diacetate) are strong electrolytes that dissociate completely in aqueous and some organic solvents. However, the differing dielectric constants of the solvents used to react the parent antimicrobial ions govern the probability of ion association or GUMBOS formation. For example, higher solvent dielectric constants cause more solvent–ion interactions, whereas solvents with lower dielectric constants cause more ion–ion associations.24,25 Due to the large dielectric constant of water, aqueous media create large solvation spheres around antimicrobial ions, which can limit the interaction and subsequent reaction of the parent ions.24,25 By pre-reacting drug mixtures in solvents with dielectric constants lower than water, the formation of new compounds (i.e. GUMBOS or other salts with a low reactivity) can be facilitated if the parent ions are coadministered using the combination drug therapy approach. In this regard, the observed antimicrobial synergy against EHEC and improvements in in vitro chlorhexidine mammalian cytotoxicity observed with the chlorhexidine di-ampicillin GUMBOS support why conventional combination drug therapy may not achieve similar results.

Materials and methods

Synthesis of chlorhexidine di-ampicillin GUMBOS

A methanolic solution containing stoichiometric amounts of chlorhexidine diacetate and sodium ampicillin, with the latter in slight excess, was stirred for 2 days at room temperature to ensure the complete formation of chlorhexidine di-ampicillin. After removing methanol using rotary evaporation, the unreacted starting materials and by-products were removed from the product by washing several times with cold deionopisere water. The white product was dried under high vacuum overnight. The identity and purity of chlorhexidine di-ampicillin were confirmed by use of various analytical techniques. The structures of chlorhexidine and ampicillin are shown in Figure 1. See the Supplementary data at JAC Online for the structural characterization acquired by 1H-NMR (Figure S1), 13C-NMR (Figure S2), mass spectroscopy (Figure S3 and Table S1), elemental analysis, circular dichroism (Figure S4) and absorbance spectroscopy (Figure S5).

Antimicrobial activity

Seven strains of E. coli O157:H7 were used in this study (Table S2, available as Supplementary data at JAC Online). Each isolate was grown individually on MacConkey agar with sorbitol for 24 h at 37°C. E. coli ATCC 25922 was used as a non-pathogenic strain. All E. coli O157:H7 isolates were obtained from a collection maintained in the Food Safety/Food Microbiology Laboratory of Louisiana State University.

Each MIC was determined in triplicate by use of microbroth dilution essentially as described by Motyl et al.26 Test inocula were prepared with colonies suspended in saline (0.85% NaCl) and matched to a 0.5 McFarland standard. Cation-adjusted Mueller–Hinton broth (Difco,
Figure 1. Molecular structures of chlorhexidine diacetate (left) and sodium ampicillin (right). Chlorhexidine di-ampicillin consists of two ampicillin molecules electrostatically tethered to one chlorhexidine molecule. Molecular numbering follows IUPAC nomenclature for each ion, with the exception of chlorhexidine, where only half is labelled for simplification.

Results

Antimicrobial activity

The antibacterial activities of the reacted chlorhexidine di-ampicillin GUMBOS were compared with those of chlorhexidine diacetate, sodium ampicillin and the stoichiometric combination of the parent salts against EHEC (Table 1). Chlorhexidine di-ampicillin inhibited the growth of E. coli O157:H7 at concentrations ranging between 0.06 and 0.12 mg/L, with an average 15 min prior to fluorescence detection. Time–kill behaviour was performed in triplicate and statistically analysed (P<0.05) using SAS 9.2 (SAS Institute Inc.).

Cytotoxicity assay

To determine cell viability, the colorimetric MTS dye assay (CellTiter 96® AQueous One Solution Cell Proliferation Assay, Promega, Madison, WI, USA) was used as an indicator of cell viability. HeLa cells (ATCC CCL-2) grown in Dulbecco’s modified Eagle’s medium-reduced serum supplemented with 3% fetal bovine serum were plated at a density of 1×10⁴ cells/well into 96-well culture plates (Falcon, Franklin Lakes, NJ, USA). Doubling dilutions ranging from 3 to 350 µM chlorhexidine di-ampicillin, chlorhexidine diacetate and sodium ampicillin were used to treat cells for 24 h at 37°C. Cells treated with only medium served as a negative control. After 24 h of incubation, 40 µL of MTS solution was added to each well and incubated for an additional 1 h. The absorbance intensity was measured using a Perkin Elmer Wallac Victor2 V Fluorescence/Luminescence Plate Reader (Boston, MA, USA) at 490 nm. All experiments were performed in quadruplicate and the relative cell viability (%) was expressed as a percentage relative to the untreated control cells after converting doubly diluted molar concentrations into mg/L. The cytotoxicity was statistically analysed (P<0.05) using SAS 9.2 (SAS Institute Inc.).
Our study shows that chlorhexidine di-ampicillin successfully inhibited E. coli O157:H7 with greater antibacterial activity than chlorhexidine diacetate, sodium ampicillin and a stoichiometric mixture of the two precursor ions when challenged with several EHEC isolates. When the MIC of chlorhexidine di-ampicillin was compared with that of the stoichiometric mixture of commercial chlorhexidine diacetate and sodium ampicillin, a 2.25-fold improvement in MIC was observed.

**Discussion**

Cytotoxicity towards HeLa cells is shown in Table 3. The acute toxicity (LD₅₀) of chlorhexidine di-ampicillin was determined to be 142 mg/L. The toxicity of chlorhexidine diacetate, sodium ampicillin and the stoichiometric chlorhexidine diacetate and sodium ampicillin mixture was 26, >200 and 98 mg/L, respectively. Thus, exchanging diacetate with di-ampicillin, as evident in chlorhexidine di-ampicillin, was able to attenuate the associated cytotoxicity of chlorhexidine salts nearly 3–5× when used alone or when stoichiometrically combined with sodium ampicillin. The effective bactericidal and bacteriostatic concentrations of chlorhexidine di-ampicillin are, respectively, 16× and 1775× less than its LD₅₀.

**Table 1.** MICs (mg/L) of antibacterial agents

<table>
<thead>
<tr>
<th>Bacterial strain</th>
<th>Chlorhexidine diacetate</th>
<th>Sodium ampicillin</th>
<th>Combination</th>
<th>GUMBOS</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. coli non-pathogenic strain</td>
<td>0.19±0.06</td>
<td>2.6±0.37</td>
<td>1.1±0.14</td>
<td>0.36±0.12</td>
</tr>
<tr>
<td>E. coli O157:H7 strains</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>chicken 301C</td>
<td>0.38±0.03</td>
<td>1.5±0.19</td>
<td>2.7±0.41</td>
<td>0.07±0.02</td>
</tr>
<tr>
<td>pork 204P</td>
<td>0.06±0.01</td>
<td>1.9±0.04</td>
<td>0.55±0.14</td>
<td>0.06±0.01</td>
</tr>
<tr>
<td>beef 933</td>
<td>0.25±0.04</td>
<td>2.6±0.04</td>
<td>1.4±0.41</td>
<td>0.09±0.05</td>
</tr>
<tr>
<td>apple cider C7929</td>
<td>0.19±0.04</td>
<td>3.7±0.11</td>
<td>9.6±1.4</td>
<td>0.12±0.05</td>
</tr>
<tr>
<td>hamburger 43895</td>
<td>0.13±0.02</td>
<td>0.7±0.07</td>
<td>0.96±0.14</td>
<td>0.10±0.02</td>
</tr>
<tr>
<td>human 43889</td>
<td>0.19±0.03</td>
<td>3.7±0.04</td>
<td>2.7±0.04</td>
<td>0.07±0.02</td>
</tr>
<tr>
<td>human 43890</td>
<td>0.13±0.02</td>
<td>2.6±0.11</td>
<td>2.7±0.08</td>
<td>0.08±0.02</td>
</tr>
</tbody>
</table>

Standard deviations are from three measurements and statistically analysed at 95% confidence. Combination results refer to the stoichiometric mixture (i.e. 1 mol chlorhexidine diacetate:2 mol sodium ampicillin) containing fractional inhibitory concentrations equivalent to chlorhexidine di-ampicillin (GUMBOS) stoichiometry used to compare conventional combination drug therapy approaches with the novel GUMBOS approach. GUMBOS refers to the reacted chlorhexidine diacetate and sodium ampicillin (i.e. chlorhexidine di-ampicillin) free of sodium acetate by-products.
the GUMBOS approach. GUMBOS refers to the reacted chlorhexidine diacetate and sodium ampicillin (i.e. chlorhexidine di-ampicillin) stoichiometry used to compare conventional combination drug therapy approaches with the novel GUMBOS di-ampicillin (GUMBOS) stoichiometry used to compare conventional fractional inhibitory concentrations equivalent to chlorhexidine 1 mol chlorhexidine diacetate:2 mol sodium ampicillin) containing acetate by-products.

Table 2. Calculated interaction indices for chlorhexidine di-ampicillin GUMBOS and the stoichiometric mixture of combined parent salts

<table>
<thead>
<tr>
<th>Combination</th>
<th>GUMBOS</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. coli non-pathogenic strain</td>
<td></td>
</tr>
<tr>
<td>E. coli 25922</td>
<td>2.8 (N) 0.9 (N)</td>
</tr>
<tr>
<td>E. coli O157:H7 strains</td>
<td></td>
</tr>
<tr>
<td>chicken 301C</td>
<td>1.4 (N) 0.1 (S)</td>
</tr>
<tr>
<td>pork 204P</td>
<td>1.9 (N) 0.5 (S)</td>
</tr>
<tr>
<td>beef 933</td>
<td>1.2 (N) 0.1 (S)</td>
</tr>
<tr>
<td>apple cider C7929</td>
<td>10.1 (A) 0.5 (S)</td>
</tr>
<tr>
<td>hamburger 43895</td>
<td>1.5 (N) 0.3 (S)</td>
</tr>
<tr>
<td>human 43889</td>
<td>2.0 (N) 0.2 (S)</td>
</tr>
<tr>
<td>human 43890</td>
<td>5.1 (A) 0.3 (S)</td>
</tr>
</tbody>
</table>

Standard deviations are from three measurements and statistically analysed at 95% confidence. Classification denoted in parentheses, where A = antagonism, N = neutral and S = synergy, according to universal limits established in Odds FC. Synergy, antagonism, and what the chequerboard puts between them. J Antimicrob Chemother 2003; 52: 1. Combination results refer to the stoichiometric mixture (i.e. 1 mol chlorhexidine diacetate:2 mol sodium ampicillin) containing fractional inhibitory concentrations equivalent to chlorhexidine di-ampicillin (GUMBOS) stoichiometry used to compare conventional combination drug therapy approaches with the novel GUMBOS approach. GUMBOS refers to the reacted chlorhexidine diacetate and sodium ampicillin (i.e. chlorhexidine di-ampicillin) free of sodium acetate by-products.

<table>
<thead>
<tr>
<th>Antimicrobial agent</th>
<th>LD₅₀ (mg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chlorhexidine diacetate</td>
<td>26 ± 2</td>
</tr>
<tr>
<td>GUMBOS</td>
<td>142 ± 3</td>
</tr>
<tr>
<td>Combination</td>
<td>98 ± 3</td>
</tr>
<tr>
<td>Sodium ampicillin</td>
<td>&gt;200</td>
</tr>
</tbody>
</table>

Standard deviations are from four measurements and statistically analysed at 95% confidence.

ampicillin, a maximum 80-fold improvement in antibacterial activity was observed. This observation advocates that chlorhexidine di-ampicillin GUMBOS would be a more effective antibacterial agent than simply using the two parent agents in combination or separately for the application of reducing the transmission of EHEC from the terminal recta of infected ruminants. Likewise, examination of the interaction indices allows for classification of chlorhexidine di-ampicillin as a synergistic ionic pair in comparison with the additive and antagonistic action of the parent salts in mixture. As such, our findings indicate that the coadministration of sodium ampicillin and chlorhexidine diacetate will not achieve similar synergy as observed with the stoichiometrically reacted chlorhexidine di-ampicillin GUMBOS. The MBCs of chlorhexidine di-ampicillin and chlorhexidine diacetate were 8.7 and 4.6 mg/L, respectively, which are concentrations comparable to the effective concentration used to eradicate EHEC from cattle in a study by Naylor et al.⁺ Time–kill profiles at the chlorhexidine di-ampicillin MBC (8.7 mg/L, 108× MIC) suggested an initial rapid interaction of the biocide with cells, resulting in 40% viability in 30 min for both GUMBOS and chlorhexidine diacetate. Such a quick reduction in cell number has been previously reported for chlorhexidine salts,³⁰,³¹ albeit with greater toxicity than our GUMBOS. Examination of the literature supports the rapid yet potent activity of chlorhexidine salts at inhibitory and bactericidal concentrations. It is suggested that chlorhexidine salts have a broad concentration range of antibacterial activity in which increasing concentrations cause greater disruption of the bacterium’s osmotic equilibrium and subsequent intracellular leakage.³²

Research from previous antibacterial studies has shown that the presence of other anionic salts and detergents antagonizes the antibacterial activity of chlorhexidine salts.³³ This was also found in our antibacterial results consisting of the stoichiometric mixtures of chlorhexidine diacetate and sodium ampicillin. This implies that anionic ampicillin can also interfere with chlorhexidine activity when used as an unreacted mixture.³¹,³⁴ However, this is not the case with reacted chlorhexidine di-ampicillin, since synergistic and increased antimicrobial activity over the mixture was observed. The MICs obtained in this study show that ampicillin does not interfere with chlorhexidine when reacted and suggest that the two ions must be functional without inhibiting the activity of the other, i.e. synergistic.

We also evaluated the effects of divalent cations on the susceptibility of EHEC to chlorhexidine di-ampicillin. It was found that EHEC susceptibility to chlorhexidine di-ampicillin was attenuated by Ca²⁺ and Mg²⁺. These findings suggest that the antibacterial mechanism of action for chlorhexidine di-ampicillin may involve the displacement of divalent cations, such as Mg²⁺ and Ca²⁺, as similarly observed for chlorhexidine salts.³⁰ Moreover, extraneous divalent cations have been described to inhibit the outer membrane activity of polycationic salts such as chlorhexidine. Therefore, we hypothesize that the presence of excess divalent cations may also repel GUMBOS by inhibiting its interaction with the lipopolysaccharide (LPS) membrane layer, disallowing effective cation displacement. Since the MICs of chlorhexidine diacetate and chlorhexidine di-ampicillin were comparable for some isolates, we hypothesize that their activities resemble the already established mechanism of action for chlorhexidine salts containing a bio-inactive counterion (i.e. dihydrochloride, diacetate or digluconate).³⁰,³¹,³³–³⁹ Studies are ongoing to identify differences in the mechanisms of action between chlorhexidine di-ampicillin GUMBOS and chlorhexidine diacetate and their relationship to the resulting antibacterial activities obtained in this study.

Acute toxicities of chlorhexidine diacetate, sodium ampicillin and chlorhexidine di-ampicillin were determined in vitro using HeLa cells. In this study, the LD₅₀ value against HeLa cells was 26 mg/L for chlorhexidine diacetate, which agrees with previously published values. Such concentrations of chlorhexidine have caused mild to severe inflammatory responses as well as induced apoptosis, necrosis and overexpression of cellular stress indicators when used systemically.⁴⁶–⁶³ Reports indicate that cytotoxicity results in mild to severe discomfort when increasing quantities of chlorhexidine are ingested, although it is poorly absorbed. Additionally, its use intravenously has caused hypotonic-induced haemolysis.⁵⁰ Since examination of the literature suggests that a 2% aqueous concentration of chlorhexidine...
may detrimentally affect host tissues, reducing the apparent toxicity associated with chlorhexidine in the chlorhexidine di-ampicillin structure is of high priority.

At the LD$_{50}$ for chlorhexidine diacetate, HeLa cells treated with chlorhexidine di-ampicillin resulted in 93% viability. More specifically, the acute toxicity of chlorhexidine di-ampicillin (i.e. 142 mg/L) towards HeLa cells was 5× less toxic than chlorhexidine diacetate. Treating HeLa cells with a stoichiometric equivalent mixture of chlorhexidine diacetate and sodium ampicillin resulted in a worse LD$_{50}$ (i.e. 98 mg/L), as compared with the reacted chlorhexidine di-ampicillin GUMBOS. Thus, our results suggest that HeLa cells were more sensitive to the toxic effects of chlorhexidine diacetate when used alone and when used with sodium ampicillin in the stoichiometric combination. Interestingly, the addition of two ampicillin molecules to the chlorhexidine structure as a mixture or GUMBOS was still able to reduce the cytotoxic effects of chlorhexidine towards HeLa cells. This approach demonstrates that the reacted salt, chlorhexidine di-ampicillin, has the potential to extend the antibacterial efficacy of sodium ampicillin while reducing toxicities associated with chlorhexidine diacetate.

In conclusion, examination of the results of this study suggests that chlorhexidine di-ampicillin may be a viable alternative to antisepsics and antibiotics in the prevention of E. coli O157:H7 colonization from ruminant reservoirs of infection. More importantly, the levels of potency, reduced toxicity and improved synergy observed in chlorhexidine di-ampicillin GUMBOS were not exceeded by the equivalent combination of chlorhexidine diacetate and sodium ampicillin. Our data also indicate that chlorhexidine di-ampicillin has bactericidal activity against EHEC by directly disrupting the outer membrane. This activity is premised on the displacement of divalent cations from their binding sites on LPS in the outer membrane. The direct action of GUMBOS may contribute to its bactericidal activity against Gram-negative bacteria. Other applications beyond reducing faecal shedding of EHEC in cattle might be found wherever chlorhexidine is used, e.g. in the prevention of meningitis in neonates by the eradication of group B streptococci in the vaginas of pregnant women or in the reduction of resistant infections associated with catheter-induced bacteraemia. Ultimately, the GUMBOS approach represents an alternative strategy to traditional pharmaceutical drug design and conventional combination drug therapy.

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Transparency declarations

None to declare.

Supplementary data

Supplementary information, Tables S1 and S2, and Figures S1 to S5 are available as Supplementary data at JAC Online (http://jac.oxfordjournals.org/).

References