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In Vitro Model of Colonization Resistance by the Enteric Microbiota: Effects of Antimicrobial Agents Used in Food-Producing Animals[▽]

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A bioassay was developed to measure the minimum concentration of an antimicrobial drug that disrupts the colonization resistance mediated by model human intestinal microbiota against *Salmonella* invasion of Caco-2 intestinal cells. The bioassay was used to measure the minimum disruptive concentrations (MDCs) of drugs used in animal agriculture. The MDCs varied from 0.125 μg/ml for some broad-spectrum antimicrobial drugs (e.g., streptomycin) to 16 μg/ml for drugs with limited spectra of antimicrobial activity (e.g., lincomycin). The acceptable daily intake (ADI) residue concentration calculated on the basis of the MDCs were higher for erythromycin, lincomycin, and tylosin than the ADI residue concentrations calculated on the basis of the MICs. The MDC-based ADI values for apramycin, bacitracin, neomycin, novobiocin, penicillin G, streptomycin, tetracycline, and vancomycin were lower than the reported MIC-based ADI values. The effects of antimicrobial drugs at their MDCs on the bacterial composition of the microbiota were observed by denaturing gradient gel electrophoresis of 16S rRNA sequences amplified by PCR. Changes in the population composition of the model colonization resistance microbiota occurred simultaneously with reduced colonization resistance. The results of this study suggest that direct assessment of the effects of antimicrobial drugs on colonization resistance in an in vitro model can be useful in determining ADI values.

Antimicrobial drugs are given to animals as growth promoters and therapeutic agents. If residual amounts of these drugs remain in tissues and animal products, low concentrations of the drugs may be ingested by human consumers (17). In recent years, there have been questions concerning the effects of the consumption of subtherapeutic levels of antimicrobial drugs in animal-derived foods on the human intestinal microbiota (8, 9). The presence in the gastrointestinal tract of some antimicrobial drugs at low concentrations may be a selective pressure for the growth of antimicrobial drug-resistant strains of bacteria (36).

Since antimicrobial drugs in food animals may have undesirable consequences, it has been prudent to reduce their use through the use of alternatives like competitive exclusion (21) and to limit residue concentrations so that they are as low as reasonably attainable (8). National regulatory agencies and international public health organizations assess the safety of ingested antimicrobial drug residues for the consumer by evaluating the chemical, pharmacological, toxicological, and antimicrobial properties of veterinary drugs (8). International committees determine safety limits for antimicrobial drug residues in food using in vivo and in vitro models and other relevant data (21). Those committees use a decision tree approach, starting with evaluation of the evidence that the drug is bioavailable to the microbiota and then move on to evaluation of the evidence that the drug can affect the composition of the microbiota. The next step is to determine what in vitro and in vivo test methods should be used if the effect of the drug on the microbiota perturbs the symbiotic effect of the microbiota, such as colonization resistance against enteric pathogens. For example, mice in a mouse model of human microbiota (27, 28) and rats in a rat model of human microbiota (29) were administered ciprofloxacin and then challenged with *Salmonella*. The antimicrobial drug was associated with increased *Salmonella* infections in both models. The in vivo models were useful for the determination of acceptable daily intake (ADI) limits, but such studies are resource intensive and limited in the number of gnotobiotic animals that can be included. They also do not specifically measure the effects of drugs on microbiota resistance to *Salmonella* invasion of intestinal tissues. The strengths of in vivo models are that they include host factors, such as adherence of bacteria and drugs to fecal matter and mucus, that is not accounted for in in vitro models.

In vitro model systems are less expensive and can yield more data. Using in vitro chemostat models of human intestinal microbiota, researchers reported that 4.3 μg/ml ciprofloxacin caused reductions in the numbers of *Bacteroides fragilis* group bacteria and selected for resistant variants of these species (5). Other intestinal microbiota functions, such as short-chain fatty acid concentration profiles and microbial enzyme activities, were also measured in chemostat models to assess the effects of various tetracycline, neomycin, and erythromycin concentrations on the microbial population balance (6). The in vitro chemostat models were able to detect changes in the microbiota population balance, but this is not the same as detection of the effects of antimicrobial drugs on colonization resistance to pathogens by the microbiota. Other in vitro models that do address the issue of direct drug effects on colonization resistance by the microbiota have been explored, such as the Caco-2 intestinal cell protection model (10, 20). It seems prudent to combine the strengths of in vivo and in vitro models for determination of the ADIs of antimicrobial drugs. In the present study, we addressed the issue of colonization resistance in an in

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TABLE 1. Compositions of model human microbiota mixtures[a]

Mixture 1	Mixture 2	Mixture 3
Escherichia coli ATCC 25922	*Kluyvera ascorbata* ATCC 14236	*Eubacterium biforme* ATCC 27806
Enterococcus durans ATCC 6056	*Propionibacterium granulosum* ATCC 11829	*Fusobacterium nucleatum* ATCC 25586
Enterococcus faecalis ATCC 27274	*Propionibacterium granulosum* ATCC 25564	*Anaerococcus hydrogenalis* ATCC 49630
Bacteroides fragilis ATCC 25285	*Lactobacillus ruminus* ATCC 25644	*Ruminococcus bromii* ATCC 27255
Bacteroides thetaiotaomicron ATCC 29148	*Lactobacillus salivarius* ATCC 11741	*Eubacterium hadrum* ATCC 29173
Enterococcus faecium ATCC 6569	*Lactobacillus johnsonii* ATCC 33200	*Ruminococcus productus* ATCC 27340
Lactococcus lactis ATCC 11454	*Lactobacillus reuteri* ATCC 23272	*Ruminococcus obeum* ATCC 29174
Bacteroides vulgatus ATCC 8482	*Bifidobacterium longum* ATCC 15707	*Clostridium leptum* ATCC 29065
Bacteroides distasonis ATCC 8503	*Bifidobacterium adolescentis* ATCC 15703	*Collinsella aerofaciens* ATCC 25986
Pediococcus acidilactici ATCC 8042	*Bifidobacterium bifidum* ATCC 29521	
Streptococcus infantarius ATCC BAA-103	*Morganella morganii* ATCC 25830	
	Providencia alcalifaciens ATCC 9886	
	Enterobacter aerogenes ATCC 29940	

[a] The bacteria were obtained from ATCC. Each mixture was combined to make the model human microbiota.

vitro assay by adapting the Caco-2 cell protection model of intestinal barrier function to measure the concentrations of antimicrobial drugs that disrupted colonization resistance.

MATERIALS AND METHODS

Model human intestinal microbiota. The model human microbiota was a mixture of 33 strains of facultative and obligately anaerobic bacteria (Table 1) that resembles the composition of the major bacterial species identified to be present in the human ileum and colon (2, 4, 7, 13, 22, 30, 31, 33). Since some bacteria in the model human microbiota are obligate anaerobes, their short-term aerotolerance was tested by overnight anaerobic culture of each kind of bacterium after 1 h of aerobic incubation at 37°C in a humidified atmosphere of 95% air and 5% CO_2. The bacterial species listed in Table 1 were cultured in their preferred medium, as recommended by the American Type Culture Collection (ATCC; Manassas, VA), under anaerobic conditions. Each culture was centrifuged at 10,000 × *g* for 10 min, and then the supernatants were decanted and the bacterial pellets were resuspended in sterile salmonella-shigella (SS) stabilizer (8% nonfat dry skim milk, 5% sucrose, 0.5% thiourea). Samples of each bacterial culture were serially diluted, and the bacteria were enumerated by agar plate dilution analyses on brucella blood agar supplemented with hemin and vitamin K (Remel, Lenexa, KS) and incubated at 37°C in a hydrogen-free anaerobic atmosphere with ascorbic acid sachets (Remel). The bacteria were mixed without further culture to maintain the relative abundance of each isolate in the model human microbiota. Three separate mixtures were made, and each mixture consisted of progressively more obligate anaerobes. The three mixtures were used in experiments to assess the effects of various antimicrobial drug concentrations on groups of anaerobic bacteria, as described below. The complete model microbiota was constructed by mixing equal volumes of the three mixtures. The volume of the model human microbiota was then adjusted so that the total bacterial count was 10^{11} CFU/ml. Aliquots of the model microbiota were frozen at −80°C and thawed for subsequent dilution and use for each experiment.

Culture of challenge bacteria. The challenge organism used in this study, *Salmonella enterica* serovar Cubana, was obtained from the culture collection maintained at the Microbiology Division at the National Center for Toxicological Research. The isolate was serotyped by the Arkansas Regional Laboratory of the Food and Drug Administration (FDA), Jefferson, AR, and was found to be serotype G2 (serovar Cubana). A single-colony culture was grown in brain heart infusion broth (Remel) and suspended in sterile SS stabilizer (8% powdered skim milk, 5% sucrose, 0.5% thiourea in water) at a concentration of 10^{11} CFU/ml.

Culture of Caco-2 cells in vitro. Caco-2 cells are a human colon carcinoma cell line that approximates the functions of enterocytes when the cells are grown in confluent monolayers (38). A model of colonization resistance efficacy developed in this laboratory with Caco-2 cell monolayers to assess the capacity of bacteria to competitively exclude *Salmonella* was modified for the present experiments (37). Caco-2 cells obtained from ATCC were grown to confluence in Falcon 24-well culture plates (BD Biosciences, San Jose, CA), which contained Dulbecco modified Eagle's minimal essential medium (DMEM) with 25 mM glucose, 10% heat-inactivated fetal calf serum, and an additional 1% nonessential amino acids (the medium components were from Invitrogen Corporation, Carlsbad, CA) at 37°C in an atmosphere of 10% CO_2 and 90% air. The Caco-2 cells (60 passages from the original clone) were seeded in 1 ml of medium at a concentration of 1.4×10^4 cells/cm^2. The cell monolayers were protected from microbial contaminants during growth to confluence by the addition of 2.5 μg/ml amphotericin B, 100 U/ml penicillin, and 100 U/ml streptomycin to the growth medium.

Antimicrobial drugs. The antimicrobial drugs that were tested in the present study have been used in animal agriculture (1, 11, 15, 16, 32, 34–36, 40). The following antimicrobial drugs were obtained from Sigma Chemical Company (St. Louis, MO): apramycin, bacitracin, carbadox, cephalothin, clindamycin, dihydrostreptomycin, erythromycin, lincomycin, nalidixic acid, neomycin, novobiocin, penicillin G, streptomycin, sulfamethoxazole, tetracycline, tylosin, vancomycin, and virginiamycin. Carbadox, erythromycin, tylosin, and virginiamycin were dissolved in minimal amounts of 95% ethyl alcohol and were subsequently diluted with 0.1 M sodium phosphate buffer, pH 6.0 (14, 39). Nalidixic acid and sulfamethoxazole were dissolved in water with the dropwise addition of 1 M sodium hydroxide to dissolve the drugs (14). They were subsequently diluted in 0.1 M sodium phosphate buffer. The other antimicrobial drug compounds were dissolved in water and diluted with 0.1 M sodium phosphate buffer (14, 39).

Verification of colonization resistance against *Salmonella* by the model colonization resistance microbiota. The capacity of the model microbiota and the individual bacterial mixtures with increasing anaerobicities were tested by an in vitro assay for their efficacies at promoting the colonization resistance against *S. enterica*, as described previously (37). The reductions in the numbers of *S. enterica* cells invading Caco-2 enterocyte culture cells caused by the bacterial mixtures were compared to those for a medium control and also to those for a competitive exclusion culture that has proven efficacy against *S. enterica* colonization of poultry in vivo (25).

Assay for minimal concentrations of antimicrobial drugs that disturb colonization resistance. The in vitro model used in the assay for the minimal concentrations of antimicrobial drugs that disturb colonization resistance is a modification of one involving *Salmonella* invasion of human enterocyte-like Caco-2 cells (3, 20). Confluent monolayers of Caco-2 cells were rinsed twice with sterile phosphate-buffered saline (PBS) and 1 ml DMEM. The model microbiota was diluted to a working concentration of 10^9 CFU/ml and treated with concentrations of the antimicrobial drugs ranging from 0 to 512 μg/ml for 1 h at 37°C. The treated model microbiota was subsequently centrifuged at 10,000 × *g* for 1 min and rinsed with a total of five PBS washes to remove the drugs. One milliliter of antimicrobial drug-treated model microbiota was then added to each well of a 24-well tissue culture plate containing the confluent Caco-2 cell monolayers and incubated at 37°C in a humidified 5% CO_2 atmosphere for 1 h. A challenge dose of 10^8 *Salmonella* cells was added to each test well, and the cultures were incubated again for 1 h at 37°C in a humidified 5% CO_2 atmosphere. Multiples of four test wells per treatment were used for the statistical averaging of the results. After the incubation period, the cell monolayers were rinsed twice with PBS and 1 ml of DMEM, with 50 μg/ml gentamicin added to the cell monolayers, which were incubated for 30 min at 37°C in a humidified 5% CO_2 atmosphere, to eliminate the extracellular *Salmonella* cells. The monolayers were rinsed twice with PBS, and 1 ml sterile water was added. The cell monolayers were homogenized with a Tissue Tearor for 15 s on setting no. 2 (BioSpec Products, Bartlesville, OK), and serial dilutions were spread on SS agar (Remel) plates for the enumeration of the cell-invasive *Salmonella* cells. Control wells consisted of Caco-2 cell monolayers not treated with the model microbiota and monolayers

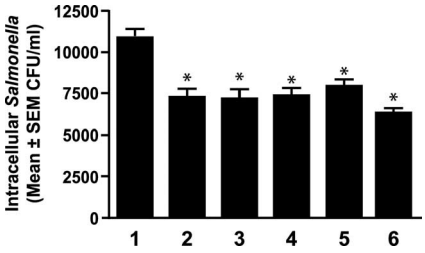


FIG. 1. In vitro analysis of colonization resistance against *S. enterica* by the model human microbiota. An in vitro assay was used to compare the numbers of *S. enterica* cells that invaded Caco-2 cells in 1 h during incubation with medium control (bar 1), a commercial competitive exclusion product (bar 2), colonization succession mixture 1 (bar 3), colonization succession bacterial mixture 2 (bar 4), colonization succession mixture 3 (bar 5), or the combined colonization succession mixtures (bar 6). The results are the mean CFU/ml intracellular *S. enterica* cells ± standard errors of the mean counted from two repetitions of three wells per treatment. *, $P < 0.05$.

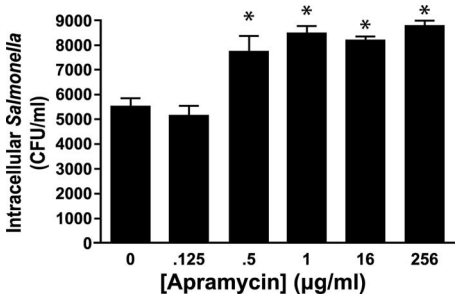


FIG. 2. Statistical determination of the MDC of apramycin. The MDC was the antimicrobial drug concentration that allowed a statistically significant increase in the number of *Salmonella* cells invading the Caco-2 cells. The result of an experiment with apramycin is shown as representative of the data acquired for each antimicrobial drug tested in this study. Probabilities of statistical significance were determined by analysis of variance. *, $P < 0.05$.

treated with model microbiota but no test antimicrobial drug. The minimal disruptive concentration (MDC) was the first dilution of antimicrobial drug that gave a statistically significant increase in intracellular *Salmonella* cell numbers compared to those for the untreated controls.

PCR-DGGE. The PCR-denaturing gradient gel electrophoresis (PCR-DGGE) method of Muyzer et al. (24) was used in this study to observe changes in the model human microbiota population resulting from exposure to antimicrobial drugs. The effects of representative drugs from each group with similar MDCs were assayed. Briefly, 1-ml aliquots of the model colonization resistance microbiota working dilution were incubated at 37°C for 1 h with 1× MDC of antimicrobial drug. The bacteria were rinsed with PBS three times, and total DNA was extracted from the final bacterial pellets by using PrepMan DNA isolation reagent (Applied Biosystems, Foster City, CA). Subsequently, 125 ng of total DNA was added to a PCR master mixture containing 2.5% acetamide, 25 pmol of forward and reverse primers (24), 0.2 mM deoxynucleotide triphosphates, 5 U *Taq* polymerase, and the PCR buffer supplied by the manufacturer (Invitrogen). PCR was conducted for 1 cycle of 94°C for 5 min, 64°C for 1 min, and 72°C for 3 min and then for 19 cycles under the same conditions, except that the annealing temperature was diminished by 0.5°C each cycle. An additional nine cycles were run with an extension temperature of 55°C for 3 min; and then one cycle was run at 94°C for 1 min, 55°C for 1 min, and 72°C for 10 min. One-quarter of the PCR product was separated on an 8% polyacrylamide 30% to 60% denaturing gradient gel at 60°C with a separation voltage of 130 V. The band patterns in the gels were photographed after incubation in 0.5 mg/liter ethidium bromide for 15 min, followed by a 10-min water rinse. The excitation wavelength for the ethidium bromide was 350 nm, and the fluorescence was electronically imaged through a 590-nm band-pass filter by using a 440CF image station (Eastman Kodak, Rochester, NY). Each lane on the gel was evaluated for the absence of bands, indicative of the bacterial groups eliminated from the colonization resistance microbiota by the antimicrobial drug at 1× MDC. Cluster analysis of pairwise similarities between band patterns was performed by using the Dice coefficient for band-based similarities, which were then displayed in a dendrogram by using Ward's algorithm in the Bionumerics software package (Applied Maths, Inc., Austin, TX).

Statistical analysis. Four wells of Caco-2 cells were used per treatment in an experiment, and the results of three separate experiments were combined for statistical robustness. Evaluation of statistically significant differences between results from the treatment groups and the control groups were determined by repeated-measures one-way analysis of variance and Tukey's posttests by using Prism (version 4.0) software (GraphPad Software, San Diego, CA). Numerical count data were $\log_{10}$ transformed prior to statistical analysis to make the data better fit a normal distribution. Statistical significance was defined by a P value of <0.05.

RESULTS

Verification of colonization resistance by the model microbiota. The model microbiota was developed from three mixtures of bacteria with successively decreasing aerotolerances for use in the present study and also for use in colonization succession of gnotobiotic mice for an in vivo study. Thus, the relative capacity of facultative and obligate anaerobes to resist colonization by *Salmonella* could be assessed. Invasion of Caco-2 intestinal cells by *Salmonella* was significantly reduced by each of the succession colonization mixtures, the combined model microbiota, and a commercial competitive exclusion product, which was used as a positive control (Fig. 1). There were significant differences in the abilities of any of the mixtures of bacteria to competitively exclude *Salmonella* from invasion of Caco-2 cells. Although the bacteria in the model microbiota were tested to be aerotolerant over the incubation times used, we tested the method in an anaerobic chamber in case the bacteria could be kept anaerobic. The Caco-2 cell monolayers became nonadherent to the culture well surfaces within 10 min of initiation of anoxic conditions and lost 70% viability, as determined by trypan blue staining. The procedure was not applicable under anaerobic conditions.

MDCs of antimicrobial drugs. The MDC results were derived by statistical analysis from the intracellular *Salmonella* counts displayed graphically in Fig. 2 and Fig. 3. The MDC data in Fig. 2 show how the apramycin concentration at 0.125 μg/ml significantly disrupted the protective effect of the model microbiota on *Salmonella* invasion of Caco-2 cells. The MDCs of each drug tested are compared in Fig. 3. The drugs tested in the present study represent many of the major drug groups used in animal agriculture. The range of MDCs of the drugs that were tested was between 0.125 and 16 μg/ml. Antimicrobial drugs are often classified by their bacterial spectrum of activity (Table 2), which correlates with their molecular classes, such as macrolides, glycopeptides, and aminoglycosides. In this study, there did not appear to be a tendency for the drugs of a given molecular class to have the same MDC. For example, the three aminoglycosides tested, dihydrostreptomycin, neomycin, and streptomycin, had MDC breakpoints of 0.5, 16, and 0.125 μg/ml, respectively (Fig. 3). A closely related drug, apramycin, had a breakpoint of 0.5 μg/ml. The drugs to which the model microbiota were the most sensitive were carbadox, novobiocin, and streptomycin (Fig. 3). The model microbiota was less susceptible to a group of drugs with an MDC of 0.5 μg/ml and consisted of apramycin, bacitracin, dihydrostreptomycin, eryth-

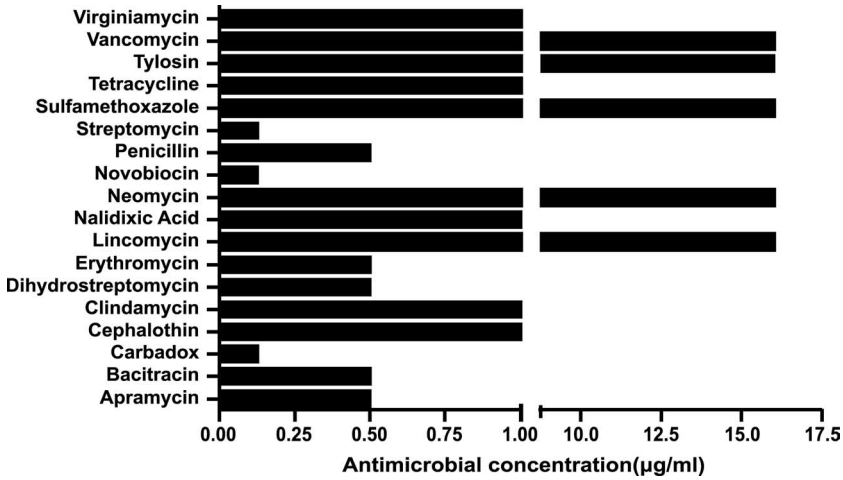


FIG. 3. MDCs of antimicrobial drugs. The MDC was the antimicrobial drug concentration that allowed a statistically significant increase in the number of *Salmonella* cells invading the Caco-2 cells.

romycin, and penicillin (Fig. 3). Another group of antimicrobial drugs that had an MDC of 1 μg/ml consisted of cephalothin, clindamycin, nalidixic acid, tetracycline, and virginiamycin (Fig. 3). The model microbiota was the least susceptible to a group of drugs with an MDC of 16 μg/ml; this group of drugs consisted of lincomycin, neomycin, sulfamethoxazole, tylosin, and vancomycin (Fig. 3).

The MICs of antimicrobial drugs are used in conjunction with other data by several national and international agencies and organizations as part of the criteria for determining their ADIs in the form of food residues. Replacement of the published MICs (12) with MDC values in ADI calculations revealed some interesting differences (Table 3). The comparison shows that colonization resistance is more susceptible to some drugs than the MIC-derived ADI values would indicate. These include apramycin, bacitracin, dihydrostreptomycin, neomycin, novobiocin, penicillin, streptomycin, and tetracycline. Colonization resistance appears to be less susceptible to erythromycin, lincomycin, and tylosin than would be assumed from MIC-based ADI values.

Microbial ecological changes observed in the model colonization resistance microbiota after antimicrobial drug treatments. The effects of select antimicrobial drugs on the microbial population balance in the model human microbiota were evaluated by PCR-DGGE analysis of 16S rRNA (Fig. 4). The first three lanes on the gel in Fig. 4 are 16S rRNA gene fragments amplified from three mixtures of bacteria that were combined to produce the complete model human microbiota applied to lane 4. The disappearance of any of the 12 apparent

TABLE 2. MDCs of antimicrobial drugs that disrupt colonization resistance in vitro compared with activity spectrum and molecular class of drugs

Drug	Spectrum of activity[a]	Drug class	MDC (μg/ml)[b]
Apramycin	G−	Aminocyclitol	0.5
Bacitracin	G+, *Clostridium difficile*	Decapeptide	0.5
Carbadox	G−	Quinoxalines	0.125
Cephalothin	G−, some G+, some Obl	Cephalosporin	1.0
Clindamycin	G+, many Obl	Lincosamide	1.0
Dihydrostreptomycin	G−, Fac	Aminoglycoside	0.5
Erythromycin	G+, some G−, some Obl	Macrolide	0.5
Lincomycin	G+, G−, some Obl	Lincosamide	2–16
Nalidixic acid	G−, Fac	Quinolone	1.0
Neomycin	G−, Fac	Aminoglycoside	2–16
Novobiocin	G+, some Obl	Novobiocin	0.125
Penicillin G	G+, some Obl	Penicillin	0.5
Streptomycin	G−, Fac	Aminoglycoside	0.125
Sulfamethoxazole	G−, some G+, Fac	Sulfonamide	2–16
Tetracycline	G+, G−, Fac	Tetracycline	1.0
Tylosin	G+	Macrolide	2–16
Vancomycin	G+, Fac, *C. difficile*	Glycopeptide	2–16
Virginiamycin	G+, some Obl	Streptogramin	1.0

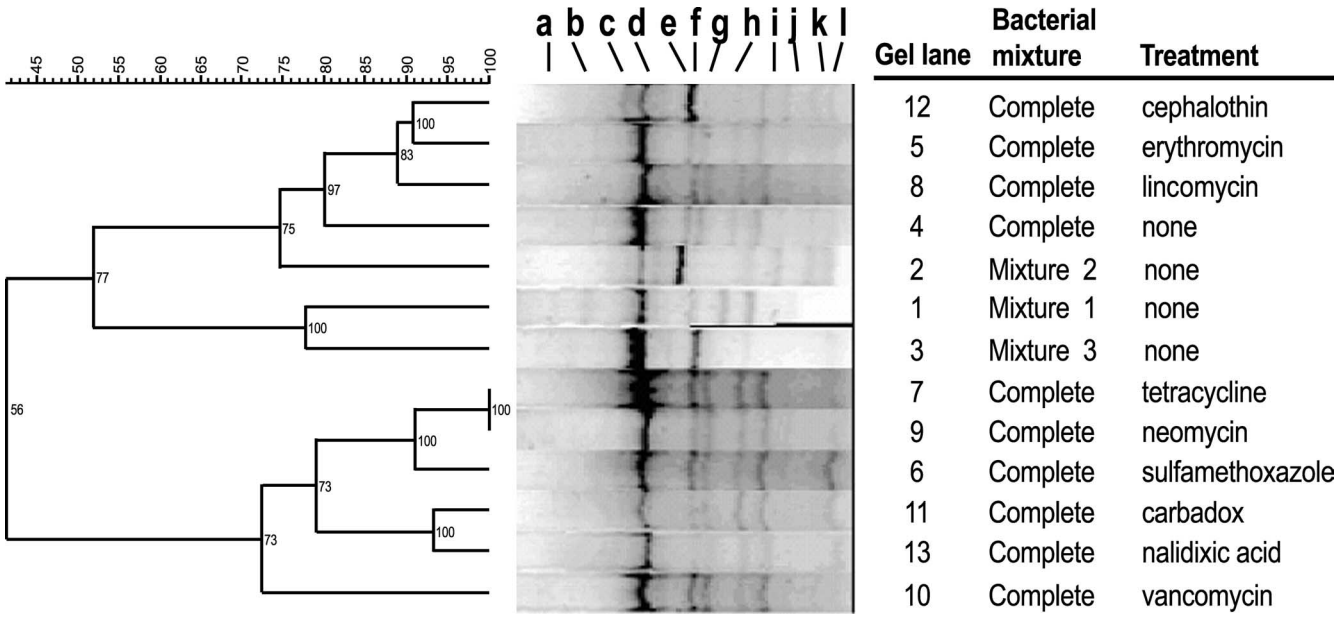
[a] Antibiotic drug class and spectrum of activity data are from references 23 and 41. Abbreviations: G+, gram-positive organism; G−, gram-negative organism; Fac, facultative anaerobe; Obl, obligate anaerobe.

[b] The MDC is the drug concentration that significantly reduces the colonization resistance of Caco-2 cell invasion from *Salmonella* by the model intestinal flora.

TABLE 3. Comparison of MDC-based ADI values calculated in this study with published MIC-based ADI values

Antimicrobial drug	ADI (μg/kg) determined on basis of:	
	MIC[a]	MDC
Apramycin	40	2.5
Bacitracin	3.9	0.34
Dihydrostreptomycin	80	1.25
Erythromycin	5	25
Lincomycin	10	800
Neomycin	160	40
Novobiocin	1.25	0.62
Penicillin	30	0.04
Streptomycin	80	0.31
Tetracycline	3	0.33
Tylosin	6.06	384

[a] ADI values were published by the EMEA-CVMP (12) at www.emea.eu.int/index/indexv1.htm.



Gel lane	Bacterial mixture	Treatment
12	Complete	cephalothin
5	Complete	erythromycin
8	Complete	lincomycin
4	Complete	none
2	Mixture 2	none
1	Mixture 1	none
3	Mixture 3	none
7	Complete	tetracycline
9	Complete	neomycin
6	Complete	sulfamethoxazole
11	Complete	carbadox
13	Complete	nalidixic acid
10	Complete	vancomycin

FIG. 4. Changes in microbial ecology of the model colonization resistance microbiota caused by antimicrobial drugs. The 16S rRNA gene amplification products from microbiota treated with antimicrobial drugs were separated by DGGE and stained with ethidium bromide. The lanes of the gel are as follows: 1, untreated mixture 1; 2, untreated mixture 2; 3, untreated mixture 3; lanes 4 to 13, the complete model human microbiota with no treatment (lane 4), erythromycin (lane 5), sulfamethoxazole (lane 6), tetracycline (lane 7), lincomycin (lane 8), neomycin (lane 9), vancomycin (lane 10), carbadox (lane 11), cephalothin (lane 12), and nalidixic acid (lane 13). The lowercase letters (a to l) marking the 16S rRNA gene PCR products in the complete model human microbiota are shown in the left column. The numbers on the dendrogram (left panel) indicate the cophenetic correlation, an expression of the consistence of each cluster.

bands in lane 4 observed in any subsequent lane indicated an inhibitory effect of the antimicrobial compound on model human microbiota component bacteria containing the rRNA sequences present in that lane. Erythromycin (lane 5) treatment caused a loss of band c, corresponding to the bacteria present in mixture 3 (refer to Table 1 for the isolates in each mixture). Mixture 3 consisted of the least aerotolerant obligate anaerobic bacteria in the mixture. Sulfamethoxazole (lane 6) treatment eliminated bands c and e, which came from mixtures 2 and 3, which represent the aerotolerant and obligate anaerobic groups of bacteria, respectively, in the colonization resistance flora. Tetracycline (lane 7) treatment appeared to eliminate bands a, b, and k from isolates in all three mixtures. Mixture 1 represents bacteria present in the most aerotolerant group in the model human microbiota. Lincomycin (lane 8), neomycin (lane 9), vancomycin (lane 10), carbadox (lane 11), and nalidixic acid (lane 13) all eliminated bands from all three mixtures; but each perturbed a different part of the model human microbiota, as seen by the different combinations of eliminated bands. The perturbed bands appeared to be bands b, c, and e in lane 8; bands a, c, e, and k in lane 9; bands a, b, c, and e in lane 10; bands b, c, e, and k in lane 12; and bands a, c, and e in lane 13. The cephalothin (lane 12) treatment appeared to eliminate bands a and b from mixtures 1 and 3.

In addition to visual inspection of the band patterns, the gel was analyzed with Bionumerics software (Applied Maths, Inc.) for similarities between the DGGE band patterns from each experiment (Fig. 4). There was a 75 to 77% cophenetic correlation between the band patterns of the complete mixture and each submixture that was used to define groups of bacteria on the basis of their anaerobicities and population succession. Complete microbiota treated with cephalothin, erythromycin, or lincomycin had a 75 to 97% correlation with the complete mixture. Another cluster that had only a 56% correlation to the complete mixture included microbiota treated with tetracycline, neomycin, sulfamethoxazole, carbadox, nalidixic acid, and vancomycin.

DISCUSSION

The International Committee on Harmonization of Technical Requirements for Registration of Veterinary Products recognizes the importance of considering the effects of antimicrobial drugs in food on colonization resistance. It wrote, "The colonization barrier is a function of the normal intestinal flora that limits colonization of the colon by exogenous microorganisms, as well as overgrowth of indigenous, potentially pathogenic microorganisms. The capacity of some antimicrobial drugs to disrupt this barrier is well established and known to have human health consequences" (12). In the same document, the authors point out that ADI calculations based on MIC data require the use of uncertainty factors that make the results overly conservative. The limitations of the current in vitro testing methods are an incentive to evaluate the use of other techniques to measure the effects of drugs on colonization resistance that might reduce the uncertainty of ADI calculations.

Although MIC determinations for drugs are very useful in calculations of the ADI levels for antimicrobial drugs, we wanted to use a drug concentration measurement that was more relevant to the colonization resistance function of the complex intestinal microbiota. The MIC technique was de-

signed and optimized for the evaluation of pathogenic clinical isolates and not indigenous intestinal bacteria (7). The MIC determination is valid only for pure bacterial cultures and does not take into account the interactions of bacteria in a climax population, such as the intestinal microbiota. The effect of an antimicrobial drug on the elimination or suppression of a single species of bacteria may not disturb the colonization resistance function of the complex bacterial population. The MICs in ADI calculations are primarily from the most susceptible groups of the predominant anaerobic bacteria known to be present in the microbiota, as recommended in the EMEA guidelines (12), which does not directly address the disruption of the barrier effect (colonization resistance) imparted by commensal bacteria. The MDC values acquired in the present study could be used in ADI calculations to correct for the overly conservative ADI values imposed on some drugs, such as lincomycin and tylosin, as shown in the present report. The ADI values calculated by the replacement of MIC with MDC numbers for most of the drugs tested in the present study were more conservative than the MIC-based values. Some antimicrobial drugs may have a more significant effect on colonization resistance than the MIC-based estimates reveal.

The model human microbiota developed in the present study consisted of 33 strains of the most abundant cultivable species of bacteria in the human intestinal tract (8). Strain selection took into account the association of the bacteria with the mucosal epithelium (13, 29, 30), the relative abundance in the population (8), and the production of essential nutrients for other members of the microbial population (18, 19, 22). The strains of bacteria were selected from human intestinal isolates of a range of genera that represent the predominant (by mass) bacteria found in the human intestinal microbiota. The in vitro model measured the sensitivity of colonization resistance to a range of concentrations of most of the antimicrobial drugs used in animal agriculture. The combination of defined bacteria used for the model microbiota in the present study is based on current knowledge of the bacterial populations in the human intestinal tract; however, future studies, like the Human Microbiome Project (nihroadmap.nih.gov/hmp/index.asp), could shed new information that could be used to improve the model. A model microbiota does not contain unculturable bacteria and many of the host factors present in an in vivo model, but it does allow simplification and a focus on a specific function of a very complex biological system, namely, colonization resistance against a specific pathogen. The advantages and disadvantages of other in vitro models and in vivo models are clearly tabulated in the review by Cerniglia and Kotarski (8). That review also explains how the decision tree methodology works to utilize all the available data from both in vivo and in vitro evaluations to arrive at the best possible ADI concentration limits. The in vitro model described in the present study was intended to provide additional information on cell invasion that is not as clearly measured with other models but that could be influential in the final ADI determination.

Although the MDCs of the drugs tested in the present study ranged from 0.125 to 16 μg/ml, antimicrobial drug concentrations up to 512 μg/ml were tested. None of the drugs tested had MDCs greater than 16 μg/ml, suggesting that most antimicrobial drugs have similar concentration ranges where a general no-effect limit on colonization resistance is reached. This limit was 16 μg/ml of drug per 8.30 $\log_{10}$ CFU of model microbiota in our experiments.

A further refinement of this assessment of drug residue effects on colonization resistance can be made by looking at the sensitivities of certain groups of bacteria from the model human microbiota to inhibition by individual antimicrobial drugs. Erythromycin and cephalothin treatment caused a loss of 16S rRNA PCR bands from the least aerotolerant obligate anaerobic bacteria in the model human microbiota. Erythromycin is more effective against gram-positive bacteria and cephalothin is more effective against gram-negative bacteria, but both are effective against obligate anaerobic species (Table 2). Since both drugs had similar MDCs of 1 and 0.5, respectively, it may be concluded that both gram-positive and gram-negative bacteria from the obligate anaerobe group are important for colonization resistance.

Sulfamethoxazole treatment eliminated the bands from the aerotolerant and obligate anaerobic groups of bacteria in the model human microbiota. It has a broad spectrum of activity against mostly facultative anaerobic gram-positive and -negative bacteria (Table 2), which agrees with the broad elimination of DGGE bands observed in these experiments. The relatively high 16-μg/ml MDC breakpoint for this drug suggests that certain species of bacteria critical for colonization resistance were less susceptible to sulfamethoxazole. Since sulfonamides are not very effective against obligate anaerobes, this result suggests that the important colonization resistance species may be one of the obligate anaerobes.

Tetracycline treatment eliminated evidence of some isolates from all three bacterial groups, including the facultative anaerobic species. This result is consistent with the broad spectrum of antimicrobial activity against facultative anaerobes that is attributed to tetracycline (Table 2).

Lincomycin, neomycin, vancomycin, carbadox, and nalidixic acid all eliminated some 16S rRNA bands from the three mixtures; but each drug perturbed a different part of the model human microbiota, as seen by different the combinations of bands eliminated on the DGGE gels. One group of antimicrobials containing cephalothin, erythromycin, and lincomycin clustered together in the similarity analysis (cophenetic correlation) along with the untreated control groups. Another group consisting of neomycin, vancomycin, carbadox, and nalidixic acid clustered together along with the other antimicrobials. Each cluster consists of drugs with narrow and broad spectra of activity and a range of MDCs from 0.5 to 16 μg/ml, which suggests that spectrum of activity may not be a good predictor of the degree of perturbation that an antimicrobial drug will have on the colonization resistance capacity of an intestinal microbiota. We can get a clearer perspective of these results by comparing them to those of an in vivo experiment. The microbiota of chickens that were fed antimicrobial drugs to enhance weight gain were evaluated for microbial population changes using DGGE (26). In the latter study, enramycin-treated birds gained more weight than avilamycin- or bacitracin methylene disalicylate-treated birds. The antimicrobial drugs induced changes in the DGGE band patterns that were clustered in groups associated with the weight gain effects. Environmental factors appeared to cause more distinct band pattern changes than the antimicrobial drugs. The experience in that study of

gram-positive bacterium-inhibiting antibiotics having little effect on the chicken microbiota is similar to the lower degree of perturbation that we observed with the cluster containing cephalothin, erythromycin, and lincomycin. This is in contrast to the greater degree of perturbation of the band patterns that we observed for drugs like sulfamethoxazole that inhibit the growth of a broader spectrum of bacteria. It may be concluded from these results that the spectrum of activity of an antimicrobial drug has little significance for determining how sensitive the model human microbiota will be to residue concentrations.

In the present study, we evaluated an in vitro model system for evaluating the susceptibility of colonization resistance by a model human intestinal microbiota to antimicrobial drugs that might be found as residues in food. This model system could be used in conjunction with in vivo animal models and bioreactor fecal culture models for the calculation of ADIs for antimicrobial drug residues. If the model system is used for this purpose, the concentration intervals would need to be smaller than those used in the present study. While in vivo models better account for host factors, the in vitro model described in this study allows a focus on resistance against tissue invasion by *Salmonella*. Further studies like the present one can be used to evaluate the degree to which specific strains of intestinal tract bacteria contribute to colonization resistance.

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ERRATUM

In Vitro Model of Colonization Resistance by the Enteric Microbiota: Effects of Antimicrobial Agents Used in Food-Producing Animals

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Volume 52, no. 4, p. 1230–1237, 2008. Page 1233, Table 3: Reference 12 is not a source of the MIC-based data. Therefore, the footnote should read "ADI values were published by the EMEA-CVMP (www.emea.europa.eu/htms/vet/mrls/a.htm)."

Page 1233, column 2, line 4: "(12)" should read "(www.emea.europa.eu/htms/vet/mrls/a.htm)."

Page 1235, column 1, line 12: "(12)" should read "(www.emea.europa.eu/htms/vet/mrls/a.htm)."