Competitive Exclusion of a Glycopeptide-Resistant *Enterococcus faecium* in the Presence of Vancomycin But Not Equivalent Concentrations of Tylosin or Gentamicin

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ABSTRACT The effect of subtherapeutic concentrations of antibiotics (10.0 and 40.0 μg/mL of vancomycin, gentamicin, and tylosin) on the efficacy of a mixed anaerobe culture of chicken microflora (CCF) was studied in a continuous-flow fermentation system. Efficacy of CCF posttreatment was assessed by challenge with glycopeptide-resistant *Enterococcus faecium* (GRE) at 6.0 $\log_{10}$ cfu/mL. Bacterial enumeration of endogenous CCF isolates, volatile fatty acid (VFA) analysis, and challenge with GRE indicated that CCF efficacy was affected by all antibiotic treatments. Although CCF treated with 10.0 μg/mL of vancomycin eliminated GRE13 at a rate of 0.61 $\log_{10}$ cfu/mL per day, it was unable to eliminate *E. coli*, a gram-negative challenge organism. All other antibiotic treatments allowed GRE persistence at approximately 2.0 to 6.5 $\log_{10}$ cfu/mL. All antibiotic-treated cultures had decreased concentrations of acetic and propionic acids. Our data suggest that low concentrations of antimicrobials may adversely affect the microbial ecology of gut microflora with respect to its ability to exclude exogenous bacteria. Moreover, gentamicin had an adverse effect on the inhibitory stringency of CCF even though it showed little anti-anaerobic activity against CCF strict anaerobes in pure culture. Verification of the results in live animals will be necessary to determine if antimicrobial treatment could compromise the effectiveness of normal microflora to serve as a natural host defense against infection.

(*Key words*: antibiotic resistance, competitive exclusion, glycopeptide, *Enterococcus macrolide*)

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INTRODUCTION

The microflora that inhabits the gastrointestinal tract of domestic animals consists of a balanced composition of facultative and obligate anaerobic bacteria. The profile of mature microflora varies considerably along the length of the gastrointestinal tract and may be specific to species or individuals (Havenaar and Huis in't Veld, 1992). During the life of an individual, the microflora competitively occupy the individual niches throughout the gastrointestinal tract, adapting to each other and the host. Such niche adaptation results in an immunity profile that distinguishes host from nonhost microorganisms (Klaenhammer, 2001); microorganisms not adapted to the local environment may be eliminated (Brook, 1999). Several factors may contribute to elimination of exogenous bacteria, including competition for nutrients, competition for attachment sites in the gut, production of antibacterial substances (e.g., fatty acids, bacteriocins, or hydrogen peroxide), and the host's immune response (Havenaar and Huis in't Veld, 1992; Brook, 1999; Mead, 2000).

It has been generally accepted that a naturally acquired diverse mixture of gut microflora, devoid of pathogens, contributes to animal health and well-being. Many factors influence the establishment of healthy microflora in poultry. Poultry production today uses an 'all in, all out' process in which chicks are reared in a nonnatural environment separated from their mothers. This eliminates exposure to normal flora from a maternal source, and may predispose chicks to colonization by enteropathogens (Nurmi and Rantala, 1973). Nurmi and Rantala (1973) devised a strategy to provide day-old chicks with pathogen-free commensal bacteria from adult chickens. This procedure successfully protected chicks against colonization by enteric pathogens and was termed competitive exclusion (CE). Nisbet et al. (1993) improved the stability and delivery of CE by applying continuous-flow technology to the preparation of CE cultures. Continuous-flow technology provides the ability to reproduce identical subcultures for in vitro experimentation, and allows

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Abbreviation Key: CCF = chicken continuous-flow microflora; CE = competitive exclusion; GRE = glycopeptide-resistant Enterococcus; VFA = volatile fatty acid; VL broth = viande Lévure broth.

an alternative method to study the complex ecology of mixed anaerobic bacterial populations (Poole et al., 2001a,b; Poole et al., 2003).

During experiments to study vancomycin resistance gene transfer among enterococci, we discovered that chicken continuous-flow microflora (CCF), which contains 4 endogenous species of enterococci, was quite proficient at eliminating exogenous *Enterococcus faecium*. In vivo studies in chickens, using CCF and glycopeptide-resistant *E. faecium* (GRE), produced similar results (Poole et al., 2001a). In the same study, the endogenous *Enterococcus faecalis* I.2 was found to produce a bacteriocin-like substance that inhibited all *E. faecium* isolates tested. It was believed that endogenous *E. faecium* I.3 may have co-adapted with *E. faecalis* I.2 in the chicken gastrointestinal tract, allowing it to persist at low levels in the CCF culture.

Donskey et al. (1999, 2000) have shown that third-generation cephalosporins and antibiotics with potent anti-anaerobic activity promote persistent high-density GRE colonization of humans, whereas antibiotics with little anaerobic activity do not. Gentamicin has little anti-anaerobic activity and is administered to chickens in ovo, on day of hatch, or though maternal routes, to reduce bacterial contamination of eggs. Antibiotic residue deposited in the gut of chicks affects the efficacy of CE cultures. However, adverse effects from gentamicin were shown to be less severe than from ceftiofur (McReynolds et al., 2000); these findings were consistent with observations in mice and humans (Donskey et al., 1999, 2000).

Endogenous CCF enterococci and lactobacilli have previously been determined to be sensitive to gentamicin based on the NCCLS (National Committee for Clinical Laboratory Standards, 2001) guidelines, whereas only 1 of the 15 strict CCF anaerobes was susceptible using the same criteria (Poole et al., 2001a). Tylosin is a macrolide antibiotic used widely in food animal production. Its spectrum of activity includes facultative and obligate anaerobic gram-positive and gram-negative bacteria. Subtherapeutic levels of tylosin may exhibit positive effects on the host that include inhibition of bacterial virulence and stimulation of the immune system (Shryock et al., 1998). Glycopeptide antibiotics (e.g., vancomycin, avoparcin, and teichoplanin) were never approved for nutritional use in food animal production in the US. However, vancomycin was chosen for use in this study because its spectrum of activity is limited to gram-positive microorganisms. Most antimicrobials used in poultry feed target gram-positive microorganisms.

The ability of CCF to eliminate GRE has been used as a tool to determine the efficacy of derivative cultures (Poole et al., 2001a, 2003). The CCF culture was used in this study to evaluate the effect of antibiotics with different inhibitory activities on its efficacy against GRE without the influence of the host immune response.

MATERIALS AND METHODS

Bacterial Strains and Culture Conditions

Glycopeptide-resistant *E. faecium* GRE13, *E. faecium* GRE47, and *E. faecalis* GRE14 were graciously provided by Neil Woodford.[2] *Enterococcus faecium* I.3, *E. faecalis* I.2, *Enterococcus gallinarum* I.5, *Enterococcus avium* I.6, *Lactobacillus salivarius* II.1, *Veillonella* sp. VL1, *Veillonella* sp. VL2, *Bacteroides fragilis* IV.12, and *Bacteroides ovatus* IV.5 were endogenous to CCF and have been previously described (Corrier et al., 1995). *E. faecium* (ATCC 700221), *E. faecalis* (ATCC 29212), and *Escherichia coli* (ATCC 25922) strains were obtained from ATCC.[3] All samples for bacterial enumeration from pure and mixed cultures were serially diluted in 10-fold increments in PBS (pH 7.0), and plated on selective agar medium. M-enterococcus medium[4] was used without antibiotics or with 20 μg/mL of tylosin or vancomycin to select for Enterococci. Vancomycin was also used to select for GRE in gentamicin experiments. Differentiation of enterococcal species has been described previously (Poole et al., 2001a). Briefly, morphological differences between *E. faecalis* and other enterococci can be observed on M-enterococcus medium when incubated aerobically; therefore, enumeration of enterococci was done aerobically. *L. salivarius* was selected aerobically on Rogosa agar[4] with and without 20 μg/mL of tylosin. MacConkey agar[4] was used to select *E. coli*. Veillonella agar[4] and BBE agar[5] were used to select for *Veillonella* spp., and *Bacteroides* spp., respectively. Obligate anaerobic bacteria were grown in an anaerobic chamber[6] under an atmosphere of 90% N_2, 5% CO_2, and 5% H_2. When applicable, API bacterial identification strips[7] were used for routine microbiological identification.

Experimental Design for Continuous-Flow Studies

An anaerobic continuous-flow culture of chicken intestinal microflora (CCF) was established from a commercial preparation of PREEMPT,[8] as previously described (Poole et al., 2001a). The CCF parent culture was never treated with antimicrobials or challenged with exogenous bacteria. All experimental cultures were derived from the parent culture. To initiate an experimental culture, a 100-mL aliquot of the CCF parent culture was inoculated into a fermentation apparatus containing Viande Lévure (VL) broth (10.0 g of tryptose, 5.0 g of yeast extract, 5.0 g of NaCl, 2.5 g of dextrose, 2.4 g of beef extract, and 0.6 g of L-cysteine per liter, pH 6.0), as previously described (Poole et al., 2001a). One week postinoculation, experimental CCF cultures were treated with 10.0 or 40.0 μg of a single antibiotic (vancomycin, tylosin, or gentamicin)[9] and the treatment was continued for 21 d. Twenty-four

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[3]ATCC, American Type Culture Collection, Manassas,VA.
[4]Difco Laboratories, Detroit, MI.
[5]Anaerobe Systems, Morgan Hill, CA.
[6]Bactron IV, Sheldon Manufacturing Inc., Cornelius, OR.
[7]BioMérieux Inc., Hazelwood, MO.
[8]MS Bioscience, Dundee, IL.
[9]Sigma Chemical Co., St. Louis, MO.

TABLE 1. Description of bacterial isolates used in the study

Isolate name	Species	MIC[1] (μg/mL) VM[2]	GM[3]	TY[4]	Origin
I.2	*Enterococcus faecalis*	1.0	4.0	256	Chicken ceca
I.3	*Enterococcus faecium*	0.5	0.5	2.0	Chicken ceca
I.5	*Enterococcus gallinarum*	4.0	2.0	0.2	Chicken ceca
I.6	*Enterococcus avium*	1.0	2.0	0.2	Chicken ceca
29212	*E. faecalis*	2.0	4.0	256	Human urine
700221	*E. faecium*	256	256	2.0	Human feces
13	*E. faecium*	256	8.0	256	Chicken
14	*E. faecalis*	256	8.0	256	Chicken
47	*E. faecium*	256	8.0	0.125	Chicken
II.2	Lactobacillus salivarius	NA[5]	4.0	128	Chicken ceca
IV.5	Bacteroides ovatus	NA	32	1.0	Chicken ceca
IV.12	*Bacteroides fragilis*	NA	32	0.125	Chicken ceca
VL1	*Veillonella* sp.	NA	4.0	2.0	Chicken ceca
VL2	*Veillonella* sp.	NA	16	256	Chicken ceca
25922	Escherichia coli	NA			Clinical

[1]MIC = minimal inhibitory concentration.
[2]VM = vancomycin.
[3]GM = gentamicin.
[4]TY = tylosin.
[5]NA = not applicable.

hours posttreatment, cultures were challenged with an organism resistant to the treatment antibiotic (Table 1) to a final concentration of 10^6 cfu/mL. The GRE, total enterococci, lactobacillus, veillonella, and bacteroides populations were enumerated daily. Cultures that cleared GRE were treated on d 22 with 100 μg/mL of the appropriate antibiotic to determine if GRE could be recovered.

Antimicrobial Susceptibility and Antibiotic Stability

The minimal inhibitory concentrations for tylosin, vancomycin, and gentamicin were determined using the microbroth dilution method, according to the method of the National Committee for Clinical Laboratory Standards (NCCLS, 2001). Minimal inhibitory concentrations on strict anaerobic bacteria and facultative bacteria were determined using standard VL broth medium under anaerobic conditions, or aerobic conditions, respectively.

Viande Lévure broth carboys containing individual test antibiotics (vancomycin, tylosin, or gentamicin) or untreated controls were maintained at room temperature for 11 d during continuous-flow experiments. To determine antibiotic stability throughout the experiments, medium from carboys containing 10.0 and 40.0 μg/mL of the individual antibiotics was collected after 11 d. Five milliliters of each sample was inoculated with one colony of *E. faecium* I.3 and incubated overnight at 37°C. No growth was detected in any of the treated VL samples; however, medium without antibiotics did support growth.

Volatile Fatty Acid Analysis

Samples were collected daily from the parent CCF and all experimental CCF cultures in triplicate. A sample volume of 0.2 mL was mixed with 1.8 mL of sterile water and frozen until analysis. The samples were analyzed for acetic, propionic, isobutyric, butyric, valeric, and isovaleric acids using a gas chromatograph as previously described (Nisbet et al., 1993). Total volatile fatty acid values equaled the sum of the individual VFA listed.

RESULTS

Effect of Antibiotics on CCF Microflora

Microbiological enumeration on selective medium was done daily for the following CCF microflora: total enterococci, *L. salivarius*, *Bacteroides* spp., and *Veillonella* spp. The mean population values for the final 7 d of each experiment are shown in Table 2. *Bacteroides* spp. and *Veillonella* spp. decreased by approximately 100- and 10-fold, respectively, in CCF cultures treated with 10 μg/mL of vancomycin even though both genera are intrinsically resistant to vancomycin. *L. salivarius*, also intrinsically resistant to vancomycin, increased by approximately 100-fold compared with untreated controls. Vancomycin treatment at 10.0 μg/mL was not sufficient to eliminate the susceptible CCF enterococci. *E. faecalis* I.2 and *E. avium* I.6 were identified on M-enterococcus medium without vancomycin, but not on M-enterococcus medium with 20.0 μg/mL of vancomycin. In CCF cultures treated with 40.0 μg/mL of vancomycin, *Veillonella* spp. and *L. salivarius* increased approximately 10- and 100-fold, respectively, from pretreatment levels. By d 10, the bacteroides population was 100-fold higher than pretreatment levels. Six days posttreatment, the endogenous CCF enterococci could not be cultured.

In CCF cultures treated with 10.0 or 40.0 μg/mL of tylosin, *Bacteroides* spp. could not be cultured after 6 and 3 d, respectively. There was little change in *L. salivarius* concentrations in any of the tylosin-treated cultures. By

TABLE 2. Chicken continuous-flow (CCF) bacterial populations[1] (log_{10} cfu/mL) in untreated and treated[2] CCF cultures for d 15 to 21

	No challenge isolate	Isolate GRE13[3]					Isolate GRE 700221[4]	
	Untreated	Untreated	VM10	VM40	TY10	TY40	GM10	GM40
Enterococci	6.52 ± 0.08	6.48 ± 0.02	5.69 ± 0.07	3.86 ± 0.60	6.44 ± 0.27	6.64 ± 0.02	6.29 ± 0.15	6.35 ± 0.47
Lactobacilli	4.42 ± 0.08	4.43 ± 0.12	6.40 ± 0.43	6.82 ± 0.10	3.97 ± 0.19	4.99 ± 0.26	4.07 ± 0.67	2.72 ± 1.67
Bacteroides	6.47 ± 0.26	6.64 ± 0.01	4.5 ± 0.81	6.98 ± 0.18	0.0	0.0	6.30 ± 0.32	6.58 ± 0.30
Veillonella	6.70 ± 0.05	6.40 ± 0.23	5.53 ± 0.18	7.0 ± 0.27	4.85 ± 0.18	6.50 ± 0.07	6.39 ± 0.41	6.88 ± 0.28

[1]Values are means ± SD, 3 pooled replicates, n = 21.

[2]VM10 = 10 μg/mL of vancomycin; VM40 = 40 μg/mL of vancomycin; TY10 = 10 μg/mL of tylosin; TY40 = 40 μg/mL of tylosin; GM10 = 10 μg/mL of gentamicin; GM40 = 40 μg/mL of gentamicin.

[3]GRE13 = glycopeptide-resistant *Enterococcus faecium* GRE13.

[4]GRE 700221 = glycopeptide-resistant *Enterococcus faecium* (ATCC 700221).

5 d posttreatment (10.0 μg/mL of tylosin), *Veillonella* spp. decreased approximately 100-fold; however, in the CCF treated with 40.0 μg/mL of tylosin, *Veillonella* spp. fluctuated but stabilized near pretreatment levels. By 6 d posttreatment, macrolide-resistant *E. faecalis* I.2 and GRE13 were the only enterococci present.

In CCF cultures treated with 10.0 or 40.0 μg/mL of gentamicin, bacteroides and veillonella populations were unchanged over the duration of the experiment. The *L. salivarius* population remained unchanged in the cultures treated with 10.0 μg/mL of gentamicin, but decreased at least 10-fold in cultures treated with 40.0 μg/mL of gentamicin, compared with untreated controls. Gentamicin treatment was not sufficient to eliminate the susceptible CCF enterococci. On M-enterococcus medium without antibiotic, *E. faecalis* I.2 and *E. avium* I.6 were identified by API bacterial identification strips. Although *E. faecium* I.3 and *E. gallinarum* were not selected for API identification, it does not rule out their presence in very low numbers.

Effect of Antibiotics on VFA Production

Administration of antibiotics resulted in a reduction of total VFA in all treated cultures. The mean VFA concentrations for the final 7 d of each experiment are shown in Table 3. Acetic, propionic, and valeric acids were reduced in all treated cultures. The most significant decreases were observed in tylosin-treated cultures. In the second repetition of the 40.0 μg/mL gentamicin-treated CCF culture, where the *L. salivarius* population decreased to nearly undetectable levels, the butyric acid concentration dropped to 13.56 (1.71, n = 7). In repetitions 1 and 3 (40.0 μg/mL of gentamicin), the *L. salivarius* population only exhibited a 10-fold decrease compared with untreated controls, and the butyric acid concentrations were 33.42 (1.61, n = 7) and 27.92 (2.2, n = 7), respectively.

Effect of Antibiotics on Competitive Exclusion Efficacy

Untreated CCF cultures were individually challenged with 3 GRE isolates of chicken origin and 1 isolate (700221) from ATCC. The chicken isolates GRE13, GRE14, and GRE47 were eliminated from each culture at rates of –1.22, –1.25, and –1.12 log_{10} cfu/d, respectively. Isolate 700221 was eliminated from untreated CCF at a rate of –1.36 log_{10} cfu/d. By d 6, GRE could not be cultured from any of the untreated replicate CCF cultures. Treatment of CCF with 10.0 μg/mL of vancomycin doubled the time required to reduce GRE13 to undetectable levels; GRE13 was eliminated from this culture at a mean rate of –0.61 log_{10} cfu/day. At 21 d postchallenge, GRE could not be recovered with 100.0 μg/mL of vancomycin from the untreated or 10.0 μg/mL vancomycin-treated CCF cultures. Treatment of CCF cultures with gentamicin, tylosin, or 40.0 μg/mL of vancomycin, allowed exogenous GRE to persist in the cultures over a 21-d period (Figure 1A

TABLE 3. Volatile fatty acid analysis[1] (VFA) in untreated and antibiotic-treated[2] chicken continuous-flow (CCF) cultures for d 15 to 21

	No challenge isolate	Challenge Isolate GRE13[3]					Challenge Isolate GRE 700221[4]	
	Untreated	Untreated	VM10	VM40	TY10	TY40	GM10	GM40
Acetic acid	47.54 ± 0.58	46.97 ± 5.33	19.67 ± 2.3	19.7 ± 6.66	21.52 ± 1.1	21.59 ± 1.86	25.23 ± 2.39	29.84 ± 1.83
Propionic acid	26.87 ± 2.37	27.47 ± 2.1	17.70 ± 2.07	19.01 ± 2.27	19.08 ± 1.0	19.31 ± 1.16	17.93 ± 2.35	20.23 ± 2.06
Isobutyric acid	8.21 ± 0.5	8.08 ± 0.45	5.51 ± 0.46	6.46 ± 0.48	5.12 ± 0.14	6.30 ± 0.32	6.4 ± 0.39	6.51 ± 0.49
Butyric acid	31.19 ± 2.9	31.32 ± 3.3	17.90 ± 0.83	15.17 ± 1.94	34.79 ± 2.13	30.12 ± 2.0	25.90 ± 2.7	33.42 ± 1.61
Isovaleric acid	8.66 ± 0.61	8.58 ± 0.44	5.73 ± 0.43	7.51 ± 0.71	6.75 ± 0.23	5.67 ± 0.23	7.09 ± 0.55	7.44 ± 0.47
Valeric acid	7.06 ± 0.44	7.09 ± 0.33	2.52 ± 0.35	2.99 ± 0.29	3.21 ± 0.06	2.63 ± 0.14	3.31 ± 0.27	2.54 ± 0.17
Total VFA	130.7 ± 6.47	132.35 ± 6.4	68.30 ± 4.57	70.12 ± 7.26	90.47 ± 2.5	85.64 ± 4.24	85.94 ± 8.19	99.98 ± 5.56

[1]Values are means (SD, 3 pooled replicates, n = 2 (μmol/mL).

[2]VM10 = 10 μg/mL of vancomycin; VM40 = 40 μg/mL of vancomycin; TY10 = 10 μg/mL of tylosin; TY40 = 40 μg/mL of tylosin; GM10 = 10 μg/mL of gentamicin; GM40 = 40 μg/mL of gentamicin.

[3]GRE13 = glycopeptide-resistant *Enterococcus faecium* GRE13.

[4]GRE 700221 = glycopeptide-resistant *Enterococcus faecium* (ATCC 700221).

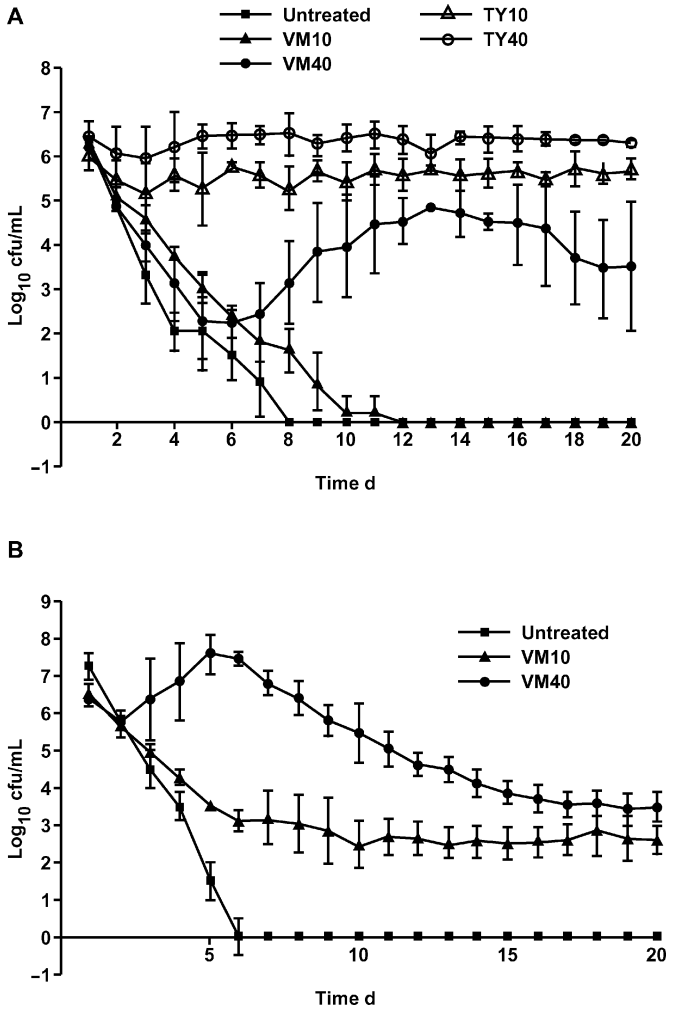

FIGURE 1. Efficacy of competitive exclusion culture (CCF) against challenge with glycopeptide-resistant *Enterococcus* (GRE) after antimicrobial treatment. Panel A: Survival of GRE13 in CCF in the presence and absence of subtherapeutic concentrations of tylosin or vancomycin. CCF cultures were untreated (×), or treated with 10 (▲), or 40 (•) μg/mL of vancomycin or treated with 10 (△), or 40 (○) μg/mL of tylosin (n = 3). Panel B: Survival of GRE 700221 in CCF in the presence and absence of subtherapeutic concentrations of gentamicin. CCF cultures were untreated (×), or treated with 10 (▲), or 40 (•) μg/mL of gentamicin (n = 3).

and 1B.) In CCF cultures treated with 40.0 μg/mL of tylosin, GRE13 remained at the challenge dose. In cultures treated with 10.0 μg/mL of tylosin, GRE 13 was present at a concentration slightly below the challenge dose. In vancomycin-treated cultures (40.0 μg/mL), GRE13 decreased 10,000-fold in 4 d, at a rate similar to untreated CCF, then increased approximately 100-fold (Figure 1A). In CCF cultures treated with 10.0 μg/mL of gentamicin, GRE 700221 was reduced 10,000-fold in approximately 4 to 5 d; then remained at 10^3 cfu/mL for the duration of the experiment (Figure 1B). In cultures treated with 40.0 μg/mL of gentamicin, GRE 700221 increased approximately 10-fold with a concurrent reduction in *L. salivarius*. The GRE 700221 population peaked at 4 d postchallenge and gradually decreased to 10^4 cfu/mL by d 21.

Due to unexpected results from the 10.0 μg/mL vancomycin-treated culture, an additional experiment was done using *E. coli* as the challenge organism. When CCF was treated with 10.0 μg/mL of vancomycin and challenged with *E. coli*, the culture allowed persistence of *E. coli* at the challenge dose (10^6 cfu/mL), in contrast to untreated control cultures, which eliminated *E. coli* in 7 d.

DISCUSSION

In addition to issues of resistance, antimicrobial use may negate many beneficial properties conferred by gastrointestinal commensal bacteria (Humbert et al., 1991; Bolder and Palmu, 1995; Takesue et al., 2002). Many authors believe that complex anaerobic microflora, including facultative anaerobes, may be required for effective CE cultures (Corrier et al., 1995; Mead, 2000). The results of this study demonstrated persistence of higher GRE populations with antibiotics that inhibited strict anaerobes and greatly decreased CCF species diversity.

Tylosin reduced the inhibitory stringency of CCF, and allowed GRE to persist in CCF at populations near the challenge dose. The 40.0 μg/mL tylosin treatment suffered the greatest loss of microbial diversity of any treatment group, with only 3 endogenous species surviving. In addition to the loss of microbial diversity, rapid decreases in acetic acid and propionic acid concentrations were also observed. Nisbet et al. (1996) have found a significant correlation between increased concentrations of propionic acid in chicken ceca with decreased *Salmonella* populations. In tylosin-treated cultures, all VFA with the exception of butyric acid decreased within 24 h of treatment. In contrast, the effect of low-dose gentamicin treatment on the inhibitory stringency of CCF was not evident until d 6, and then the GRE 700221 population was held at a reduced level. In these cultures, VFA decreased gradually over the first 7 d and then remained constant. Treatment with high-dose gentamicin had an immediate effect on the VFA levels and the inhibitory stringency of CCF; however there may have been a slight rebound of inhibitory capacity. Interestingly, endogenous CCF *L. salivarius* II.2 and enterococci were present throughout the gentamicin experiments, even though they remained susceptible to gentamicin in microbroth dilution assays. Similar observations with *L. salivarius* II.2 in tylosin-treated cultures challenged with *E. coli* O157:H7 had been observed and investigated previously (Poole et al., 2003). Tolerance of these isolates cannot be attributed to resistance or CO_2 inactivation of the antibiotics. However, it does not rule out the production of an exocellular protein by a CCF bacterium that might cause tylosin inactivation.

The data presented here elucidate the complex ecology of gastrointestinal bacteria in the absence of the host immune response or competition for attachment to the lumen wall. In this environment, survival is likely to be based on an ability to compete for nutrients, growth rate, resistance to VFA and bacteriocins produced in the niche, and tolerance of low pH.

The reduction of VFA concentrations in CCF cultures treated with 10.0 μg/mL of vancomycin was similar to

that of the 40.0 μg/mL treatment with the same drug. Using VFA concentrations as a measure of efficacy, it was thought that the 10.0 μg/mL vancomycin-treated CCF would allow persistence of GRE, but this was not the case. However, there was a difference in species diversity between the high and low dosages of vancomycin-treated cultures. A notable difference detected using our selection methods was the presence of endogenous CCF enterococci in the 10.0 μg/mL-treated culture, but not the 40.0 μg/mL-treated culture. To determine if the general stringency of CCF was reduced, an additional experiment was done using *E. coli* as the challenge organism. The CCF culture treated with 10.0 μg/mL of vancomycin was unable to inhibit or decrease the *E. coli* population below the challenge dose. The VFA concentration in the *E. coli*-challenged culture (10.0 μg/mL of vancomycin) was consistent with the same treatment challenged with GRE (data not shown). The ability of an impaired CCF culture to inhibit *E. faecium* but not *E. coli* suggests the presence of a factor that may be specific to *E. faecium*. We have previously observed a bacteriocin-like factor produced by CCF *E. faecalis* I.2. In agar overlay assays, the substance produced by *E. faecalis* I.2 only inhibited *E. faecium* and not other bacterial species or other enterococcal species (Poole et al., 2001a).

The data presented in this report may support the need for complex anaerobic microflora for effective CE cultures. It has been apparent from our studies that *E. faecium* I.3 has adapted to the hostile environment that exogenous *E. faecium* isolates have encountered in CCF. Even so, *E. faecium* I.3 could not maintain a high level in CCF (T. Poole, unpublished data). We speculate that *E. faecium* I.3 co-adapted to inhibitory factors in the chicken ceca during the initial microbial colonization or niche adaptation process. It may be more difficult for an exogenous species to adapt to the cocktail of inhibitors present in a mature ecological niche. This is analogous to the multiple attack approach used in the treatment of human immunodeficiency virus infection (Kaufmann and Cooper, 2000). Therefore, cycling of antibiotics may allow bacteria to consecutively adapt leading to multidrug resistance more rapidly than if they are treated with a multidrug cocktail.

Niche selection pressure may explain why most probiotic preparations fed to animals are not maintained after oral administration is discontinued (Netherwood et al., 1999). Many groups select probiotic microorganisms based on various inhibitory assays and short-term mixed batch cultures (Wagner et al., 2002; Bielke et al., 2003); however, these methods provide no data on the survivability of these isolates in long-term culture. Our experience suggests that mixtures of nonadapted strains would be reduced to 2 or 3 isolates in 1 wk in a continuous-flow culture. Although selected probiotic cultures may be strongly inhibitory to enteropathogens in vitro, they are of little value if they cannot become stable inhabitants of the gut. Therefore, health benefits of probiotic cultures may be greatly increased if cultures are derived from niche-adapted mixtures of bacteria.

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