

Antibiotics in the human food chain: Establishing no effect levels of tetracycline, neomycin, and erythromycin using a chemostat model of the human colonic microflora

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Abstract

A chemostat model of the healthy human large bowel ecosystem was used to establish no effect levels for tetracycline, neomycin, and erythromycin. For each compound, the equivalent to four oral doses (0, 1.5, 15, and 150 mg/60 kg person/d) was studied. Concentrations of the test compounds in the chemostat medium were intended to simulate fecal levels that might be expected following consumption of food containing antibiotic residue and were based on published oral doses and fecal levels. We monitored the following parameters: short chain fatty acids, bile acids, sulfate reduction, azoreductase and nitroreductase activities, β -glucosidase and β -glucuronidase activities, a range of bacterial counts and, lastly, the susceptibility among sentinel bacteria to each test compound. Neomycin and erythromycin reduced bile acid metabolism. Neomycin elevated propionate levels and caused a marginal diminution in azoreductase activity. Based on our results, the no observed effect level (NOEL) of both tetracycline and erythromycin was 15 mg/60 kg person/d. The NOEL for neomycin was 1.5 mg/60 kg person/d.

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1. Introduction

The colonic ecosystem of humans is a self-righting “organ” without which we cannot survive. Our gut floras

contain about 10^{13} bacteria comprised of 400–500 different species (Carman et al., 1993). Because the bulk of the digests in our colons is bacteria, antibiotics may affect colonic ecology. Antibiotic residues can appear in the human food supply as a result of treatment or husbandry of livestock and poultry. The effect—if any—of even very low levels of antimicrobial agents found as residues in edible tissues of these animals may be a public health issue. Thus, in addition to other toxicological effects, the US Food and Drug Administration Center for Veterinary Medicine (FDA-CVM) considers effects on human intestinal flora when it evaluates the human food safety of antimicrobial drug residues. There are broadly speaking three possible unintended

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consequences to the gut flora of consuming antimicrobial. These are: (1) shifts in bacterial counts and biochemistry, (2) changes in the incidence of antibiotic resistant bacteria, and (3) changes in the ability of the resident flora to prevent colonization by potential enteropathogens.

Previously (Carman et al., 2004; Carman and Woodburn, 2001) as part of the studies to develop the chemostat system as a model for determining NOELs for antibiotics in human gut flora, we studied the effects of low levels of ciprofloxacin on large bowel flora using a chemostat model of the human colon. Employing the same chemostat model, we have now addressed the question: “What are the no observed effect levels (NOELs) of tetracycline, neomycin and erythromycin on the human colonic flora?” Tetracycline has a codified acceptable daily intake (ADI); it is 1.5 mg/person/d (21CFR 556.720). The ADI of neomycin is 0.36 mg/person/d (21CFR 556.430). An ADI for erythromycin has not been set.

2. Materials and methods

2.1. Fecal inoculum

Eight adult volunteers (6 males, 2 females; 21–52 years old) donated their first fecal motion of the day. None had received antibiotics for at least 12 weeks. None reported any signs of diarrhea or other intestinal tract problems during the same time. All considered themselves to be in good health and to be consumers of non-vegetarian diets typical of North Americans. We did not screen donors for inherent resistance to any of the test compounds. In general, resistance to drugs that have been in use for some time is typically more common than for drugs of more recent provenance. Thus, a new drug has more “room” to produce signs of increased resistance, whereas resistance for older drugs is often already so widespread, that there is not much opportunity for its increase.

The collection of feces, their pooling and dispensing into homogeneous 10 g aliquots, freezing under anaerobic gasses, storage, and the subsequent preparation of 10% wt/vol suspensions for use as inocula has been fully described before (Carman and Woodburn, 2001). Che-

mostats were inoculated with 50 mL suspensions, each containing 10 g feces, on days 1, 3, and 5 of the study by injections through a septum in the vessel lid.

2.2. Chemostat

Our one-chambered chemostat model of the human large bowel ecosystem has been described before (Carman and Woodburn, 2001). Briefly, the 500-mL culture was maintained under nitrogen, at 37°C and between pH 6.4 and pH 6.6. Medium was pumped (35 mL/h, equivalent to a dilution rate of 0.07/h) into the vessel and the culture itself was magnetically stirred.

2.3. Medium

The medium contained (g/L deionized water): starch (5.0), bovine milk casein (3.0), peptone (3.0), pectin (0.6), xylan (0.6), arabinogalactan (0.6), amylopectin (0.6), L-cysteine (0.4), hemin (0.01), cholesterol (0.25), chenodeoxycholic acid (0.25), cholic acid (0.25), KH_2PO_4 (2.0), NaH_2CO_3 (10.0), NaCl (4.5), $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (0.5), and $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ (0.45). The medium was agitated constantly to keep the particulates evenly suspended.

2.4. Antibiotics

Test compounds were added to the medium reservoirs as concentrated solutions prepared gravimetrically to achieve the intended levels. The levels of recoverable activity in medium, collected via three way valves placed between each reservoir and chemostat, were measured by bioassay. The added and recovered levels are shown in Table 1. The test compounds were tetracycline (0, 0.15, 1.5, and 15 $\mu\text{g}/\text{mL}$), neomycin (0, 1.78, 17.8, and 178 $\mu\text{g}/\text{mL}$), and erythromycin (0, 1.5, 15, and 150 $\mu\text{g}/\text{mL}$). Four different chemostats were maintained for each drug, one at each concentration. For reasons presented below, the drug concentrations tested corresponded to fecal levels reached by ingesting 0, 1.5, 15, and 150 mg/60 kg person/d.

2.5. Justification of dose levels

Our goal was to assay the effects of each test compound at concentrations in the chemostat medium approximating fecal levels that might reasonably be

Table 1
Nominal levels of test compound added to medium and measured by bioassay (mean level, $n = 3$)

Equivalent daily dose (mg/60 kg person/d)	Tetracycline ($\mu\text{g}/\text{mL}$)		Neomycin ($\mu\text{g}/\text{mL}$)		Erythromycin ($\mu\text{g}/\text{mL}$)	
	Added	Recovered	Added	Recovered	Added	Recovered
0	None	Not detected	None	Not detected	None	Not detected
1.5	0.15	Not detected	1.78	Not detected	1.5	1.0
15	1.5	1.5	17.8	3.3	15	15
150	15	8.8	178	7.4	150	149

expected by consuming 0, 1.5, 15, and 150 mg/60 kg person/d. The lowest concentration of tetracycline tested corresponded to its ADI (21CFR 556.720). The same low concentrations of neomycin and erythromycin were used because at the time that these studies were performed, the FDA had considered that less than 1.5 mg/person/day of any antibiotic would probably not cause adverse effects on the human intestinal flora. In fixing the dose levels, we assumed that the relationship between oral dose and fecal concentration for each of the test compounds was linear even at the 1.5 mg/person/d level. We have since learned that Perrin-Guyomard et al. (2001) did not see linearity with tetracycline in their mouse study. There are no equivalent data for neomycin and erythromycin. We reviewed the literature on oral doses and associated fecal concentrations and pooled individual results to derive their relationships. When a range of fecal concentrations was given, we used the upper limit. Values given as greater or less than a nominal value were used as if they were that nominal value (i.e., >0.3 g/d was 0.3 g/d and so on). We assumed 1 g of feces to be the equivalent to 1 mL of chemostat fluid.

For each of the three test compounds, the low and medium dose levels reproduced residue levels that might be reached in feces following the consumption of food containing antibiotic residues. The high doses should not be regarded as reproducing levels reached during therapy. The latter are generally higher than our high dose level.

2.6. Tetracycline levels

We considered all tetracyclines to behave similarly. Though this appears acceptable for tetracycline, chlortetracycline and oxytetracycline, data for pyrrolidinoethyltetracycline suggests that it may not always be so (van Marwyck, 1958). van Marwyck (1958) gave 10 volunteers 1 g tetracycline/d and the levels in the subjects' feces ranged from 10 to 97 µg/g feces. Since ingested tetracycline remains active (Kelly and Buyske, 1960), the higher value, rounded up to 100 µg/g, suggests that 1 g/d would lead to a fecal level of 0.1 mg/g. Thus 1.5 mg/d would produce a fecal level of 0.15 µg/g and accordingly, to achieve levels equivalent to doses of 0, 1.5, 15, and 150 mg/60 kg person/d, we tested 0, 0.15, 1.5, and 15 µg/mL chemostat medium.

2.7. Neomycin levels

Almost all of any orally ingested neomycin is excreted in feces virtually untouched and unabsorbed by host and bacterial metabolism (van Koten-Vermeulen and van Leeuwen, 1995). As no directly measured oral dose-to-fecal level ratios were available to us, we assumed that the equivalent to the daily intake of neomycin (1.5 mg/person/d) must all be in the daily flow through in

the chemostat. Thus over 24 h, a flow rate of 35 mL/h will deliver 840 mL containing 1.5 mg neomycin. This corresponds to a concentration of 1.5 mg/840 mL or 1.78 µg/mL chemostat medium. We tested neomycin at 0, 1.78, 17.8, and 178 µg/mL, corresponding respectively to daily intakes of 0, 1.5, 15, and 150 mg/60 kg person/d.

2.8. Erythromycin levels

We relied on published (Andremont et al., 1983; Hartley et al., 1978; Heimdahl and Nord, 1982; Nichols et al., 1977; Steinbakk et al., 1992) relationships of oral doses, ranging from 1 to 3 g/d, and the resulting fecal levels. Plotting daily intake versus fecal level (not shown) suggested that 1 g erythromycin by mouth each day produced a level of approximately 1 mg/g feces. A daily intake of erythromycin of 1.5 mg/person/d would therefore be equivalent to 1.5 µg/mL in the chemostat. We tested erythromycin at 0, 1.5, 15, and 150 µg/mL, corresponding respectively to daily intakes of 0, 1.5, 15, and 150 mg/60 kg person/d.

2.9. Bioassays of test antibiotic in chemostat medium

Bioassays (Carman and Woodburn, 2001) were run to measure tetracycline, neomycin or erythromycin in the medium using *Bacillus cereus* ATCC 11778, *Bacillus stearothermophilus* ATCC 10149, and *Micrococcus luteus* ATCC 9341, respectively. Standardized lawns of the appropriate indicator were overlaid with discs containing diluted medium. The *B. cereus* and *M. luteus* plates were incubated overnight at 37 °C. The *B. stearothermophilus* plates, for the assay of neomycin, were incubated overnight, at 57 °C. The diameters of any zone of inhibition were compared to standard curves to determine the amounts of drug present. The assays were run in triplicate and the mean test concentrations were reported (Table 1).

2.10. Timetable

Each chemostat was run for 16 days to reach steady state. From day 17 to 24 inclusive, the medium supplied contained no test compound. From Noon on day 25 to Noon on day 35, test compound was present in the medium at the concentrations shown in Table 1.

2.11. Sampling

In our extensive use of this chemostat system, we have repeatedly seen a steady state develop about 7 days after the final inoculation with feces. Consequently, we refrained from sampling until day 17. Thereafter, six samples (10 mL each) were aspirated daily from each chemostat at Noon of days 17 to 32. All but one sample were collected into 1 mL of sterile glycerol that was

added as a cryoprotectant (Guerin-Danan et al., 1999); the volume of added glycerol was considered in all subsequent calculations. The sample collected without added glycerol was used for the extraction of bile acids. The tubes—all glass—were flushed with anaerobic gases (90% nitrogen and 10% carbon dioxide) for 5 min before sealing and the glycerol dispersed using a vortex mixer before freezing at -70°C or lower. Samples were thawed inside the glove box at room temperature and for about 60 min prior to analysis.

Although we sampled daily we did not test every day's samples. In particular, counts and resistance levels were not measured every day simply because to do so was prohibitively expensive.

2.12. Short chain fatty acid (SCFA)

The gas chromatographic method was fully described by Carman and Woodburn (2001).

2.13. Bile acids

Bile acids from the samples not containing glycerol were assayed by gas chromatography (Scheinbach et al., 1994). Residual primary bile acids (cholic and chenodeoxycholic acids) and their major secondary metabolites (deoxycholic, and lithocholic acids respectively) were found in every sample. One or more minor secondary compounds (ursodeoxycholic, hyocholic, and ketocholanic acids) were also detected on occasions. We have reported the percentage of the total moles of bile acids recovered that were secondary metabolites.

2.14. Sulfate reduction

Sulfide, a product of dissimilatory sulfate reduction, was measured inside an anaerobic glove box with a silver/sulfide electrode according to the manufacturer's instructions (PHH253-kit: OMEGA Engineering, Stamford, CT). Sulfide levels will rise when sulfate reduction is increased and vice versa.

2.15. Azoreductase and nitroreductase

Bench top, anaerobic assays (Carman and Woodburn, 1999) were used to measure both activities.

2.16. β -Glucosidase

Chemostat sample (1.5 mL) was added to *p*-nitrophenyl- β -D-glucopyranoside (2 mL, 1 mM). Incubation (37°C) was stopped at intervals (0, 10, 20, 30, 50, and 70 min) by adding reaction mix (0.5 mL) to NaOH (2.5 mL, 0.01 M). The stopped samples were centrifuged (3 min, 15,000g) and supernatant absorbencies were read at 402 nm. The concentrations of *p*-nitrophenol

produced were determined by comparison to a standard curve. Activity was expressed as $\mu\text{mol/h/mg}$ protein.

Preliminary results showed no differences between the assay run aerobically and the identical assay run anaerobically and so, for ease, the assays were run aerobically.

2.17. β -Glucuronidase

Chemostat sample (1.5 mL) was added to *p*-nitrophenyl- β -D-glucuronide (2 mL, 1 mM). Incubation (37°C and aerobic) was stopped at intervals (0, 10, 20, 30, 50, and 70 min) by adding reaction mix (0.5 mL) to glycine buffer (2.5 mL, 0.2 M) containing NaCl (0.2 M) at pH 10.4. The stopped samples were centrifuged (3 min, 15,000g) and supernatant absorbencies read at 402 nm. The concentrations of *p*-nitrophenol produced were determined by comparison to a standard curve. Activity was expressed as $\mu\text{mol/h/mg}$ protein.

2.18. Protein assay

Total protein in chemostat samples was determined using Coomassie brilliant blue G-250 dye according to the manufacturer's instructions (Bio-Rad, Hercules, CA). Bovine serum albumin was used as a calibration standard.

2.19. Bacterial counts

In an anaerobic glove box, chemostat samples were serially diluted (1/10-fold increments) in pre-reduced and anaerobically sterilized (PRAS) dilution blanks (Holdeman et al., 1977; Randolph Biomedical, West Warwick, RI). Aliquots (0.1 mL) of each dilution were plated on three commercially prepared PRAS agars, namely bacteroides bile esculin agar, bifidobacterium selective agar and fusobacterium selective agar (Anaerobe Systems, San Jose, CA). We also used the following PRAS agars that we made ourselves using published formulations (Mitsuoka, 1980; Summanen et al., 1993): lactobacillus selective agar, eubacterium selective agar, peptococcimegasphaera selective agar, and peptostreptococci agar and veillonella-neomycin agar. Total anaerobic counts were made on a PRAS habitat stimulating medium made with Fastidious Anaerobe Agar (LabM, Bury, England) supplemented with pooled human feces (10% by weight) and neomycin (100 $\mu\text{g/mL}$) added before autoclaving. Plates were incubated anaerobically at 37°C . Lactobacillus selective agar plates were incubated for 7 days. Other plates were incubated for 72 h. To enumerate clostridial spores, 1 mL of each dilution was added to 1 mL of absolute ethanol, thoroughly mixed, incubated at room temperature for 30 min after which 200 μL was plated on egg yolk agar plates (Anaerobe Systems). Inoculated plates were incubated anaerobically for 3–5 days at 37°C .

Following anaerobic plating, the dilutions were plated (0.1 mL), aerobically on MacConkey (Carr Scarborough, Decatur, GA) and bile esculin azide agar (Carr Scarborough). These plates were incubated aerobically at 37°C for 24 h. Plates with 30–300 colonies were counted. Counts were adjusted to give count/mL chemostat fluid.

2.20. Susceptibility among sentinel bacteria to tetracycline, neomycin or erythromycin

We nominated three groups of sentinels relevant to this issue: *Bacteroides fragilis* group (BfG), *Escherichia coli*, and enterococci. They were chosen because each is common in the feces of healthy humans. BfG, the most numerous bacteria in the human colon are 20–25% of the total viable flora (Holdeman et al., 1976; Moore and Holdeman, 1974). *E. coli* and enterococci are the two most common facultative anaerobes in feces. Also, as each is an opportunistic pathogen, selective and differential media have been developed for them. Lastly, their susceptibility to antibiotics is an important clinical issue.

Our methods (Carman et al., 2001; Carman and Woodburn, 2001) were based on those developed and validated by Osterblad and her colleagues (1995), who concluded that replica plating on tetracycline-supplemented selective agar gives as accurate an indication of resistance as measuring with for example, NCCLS methods (NCCLS, 2003). Consequently we use “resistant” to mean able to grow on an agar plate containing a specified level of a test compound. In these assays, the level of antibiotic was fixed gravimetrically and was not related in any way to levels in the chemostats.

Counts of *Bacteroides fragilis* group (BfG), *E. coli*, and enterococci were made on bacteroides bile esculin agar, MacConkey agar and bile esculin azide agar, respectively. Plates with 30–300 colonies were replicated onto the homologous media with and without added tetracycline or neomycin or erythromycin using sterile velvet pads and a replicating block and collar (Replicattech, Princeton, NJ). The levels of test compound used were 8 µg tetracycline/mL (for *E. coli*, enterococci, and BfG), 8 µg neomycin/mL (for *E. coli* and enterococci), 200 µg neomycin/mL (for BfG), and 8 µg erythromycin/mL (for *E. coli*, enterococci, and BfG). The percentage of presumptive sentinel colonies growing on the no drug control plates and that were absent after 24–48 h incubation from plates supplemented with test compound was noted. We assayed the susceptibility to each compound of the sentinel bacteria in the fecal pool from which the inoculum was prepared (Table 2).

2.21. Statistical analysis

Our analytical methods were described in Carman and Woodburn (2001). Four chemostats were run for each drug and data from each pooled to create a mean pretreatment level for each parameter at steady state. The mean pretreatment levels were extrapolated into the exposure period, bordered by 95% prediction intervals (PI₉₅) that provided boundaries for assessing future individual observations. It was concluded that a test compound had no statistical impact on the parameter being followed if data for the exposure period fell within the PI₉₅ about the mean pretreatment level. It was concluded that a test compound had a statistically significant as well as a biologically significant impact on the steady-state level of that feature only when the observed value consistently fell outside the PI₉₅. When considering our results, we have discounted, but not completely ignored, the significance of single, isolated points lying outside the PI₉₅ on the grounds that a within dose trend is more likely to be of consequences than a single outlying datum point. Lastly, we gave consideration to dose-dependent trends. Also, it should be remembered that we compared our post-treatment findings with pretreatment levels that were the means of four results. On occasions the spread of the four sets of pre-treatment values about their respective single mean was large (e.g., Figs. 4 and 5) resulting in wider prediction intervals and thus making unequivocal interpretation harder than in instances in which pretreatment data clustered more tightly about the mean (e.g., Fig. 2).

3. Results

We have expressed the results either as the amount (µg/mL) of test compound added to the chemostat medium or as the intended oral doses (0, 1.5, 15, and 150 mg/60 kg person/d; this is how the figure legends present the four doses) and not on the levels determined by bioassay (Table 1). Also, it should be remembered that we compared our post-treatment findings with pretreatment levels that were the means of four results.

3.1. Tetracycline

There was a suggestion of a transitory, dose-dependent increase in the mean percentage of *E. coli* isolates able to grow on plates with 8 µg/mL tetracycline (Fig. 1). However, as resistance levels were already high in the

Table 2
Resistance to tetracycline, neomycin, and erythromycin in the fecal pool—expressed as a percentage of the total count/g

Sentinel group	Tetracycline (8 µg/mL)	Neomycin (8 µg/mL)	Neomycin (200 mg/mL)	Erythromycin (8 µg/mL)
Enterococci	99	100	Not done	72
<i>E. coli</i>	100	0	Not done	100
<i>B. fragilis</i> Group(BfG)	37	Not done	100	100

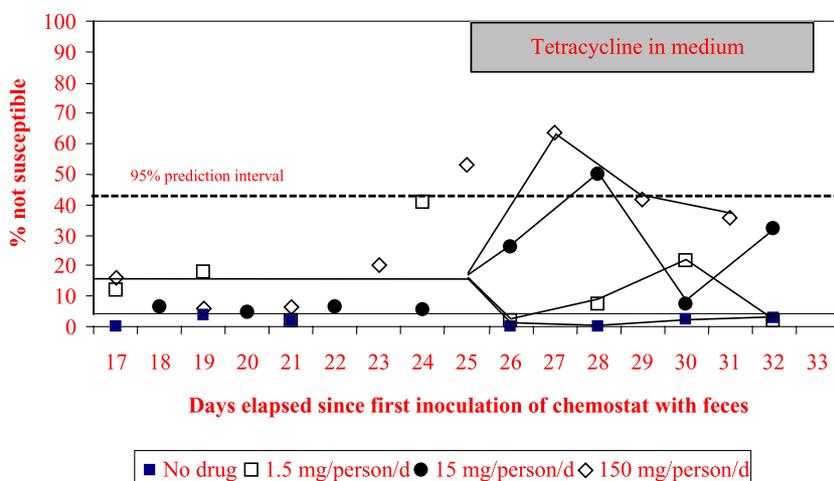


Fig. 1. Effect of tetracycline on human fecal flora growing in a chemostat: susceptibility of *E. coli* isolates to 8 µg/mL tetracycline.

150 mg/person/d chemostat before tetracycline was added to the medium, the trend should be interpreted with caution. The time for the changes to be apparent and the extent of changes were proportional to the dose delivered. The effect was significant at 15 and 1.5 µg/mL and, although apparent, it was not significant at 0.15 µg/mL. Within 48 h, the level rose from 12% to greater than 50% in the 15 µg/mL chemostat but had begun to fall 2 days thereafter. The levels of resistant bacteria in the 1.5 µg/mL chemostat rose significantly to slightly less than 50% four days after the start of the exposure period. There was a small, delayed increase in the 0.15 µg/mL chemostat. Similar responses among the enterococci and *B. fragilis* Group were not seen, possibly because even before the drug was introduced to the chemostat, levels of resistance in these two groups were already close to >90% (Table 2), eliminating room for any increase.

None of the following parameters showed a response to tetracycline: SCFA and bile acid metabolism, bacterial enzyme levels, sulfate reduction and bacterial counts (Table 3).

3.2. Neomycin

Although total SCFA production was unchanged (data not shown), the relative amounts of propionate rose while butyrate fell when expressed as a percent of total SCFA. From a pretreatment level of 25%, propionate rose

significantly at 150 mg/person/d to >30% (Fig. 2). The increase was sustained throughout the exposure period and was mirrored by a reduction in butyrate down from 8% of the total SCFA levels to <3% (data not shown). There was a similar reduction in the relative abundance of the minor SCFA (data not shown). Propionate levels at 15 mg/person/d were similarly affected in a dose-dependent manner (Fig. 2), though the corresponding reductions in butyrate and minor SCFA levels were not significant (data not shown). Acetate levels were not affected by the presence of neomycin.

Bile acid metabolism was affected by neomycin (Fig. 3). The effects, which were transitory, took at least 24 h to appear, and were eventually significant at all three levels of neomycin tested. The extent of the effects, its duration and the time it took to become apparent was dose-dependent. At 150 mg/person/d secondary bile acids fell from a pretreatment level of 80% of the total to less than 20%. The effect lasted 5 days. At 15 mg/person/d the level dropped to slightly less than 50% lasting 4 or 5 days only. At 1.5 mg/person/d the effect was insignificant until the fourth day after the addition of neomycin, at which time the level had fallen to 55%, a change that was only just significant, and lasted only 48 h. By the end of the treatment periods, secondary bile acid production had returned to their pretreatment levels despite continuous exposure to neomycin.

Azoreductase activity, though highly variable during the pretreatment period, fell from 0.4 µmol/h/mg protein

Table 3

Summary of all parameters assayed that showed no changes following exposure to antibiotics

Test compound	Parameters remaining unchanged
Tetracycline	Short chain fatty acids, bile acid metabolism, reductases, glycosidases, sulfate reduction, bacterial counts
Neomycin	Sulfate reduction, nitroreductase, glycosidases, bacterial counts and resistance levels among BfG and <i>E. coli</i>
Erythromycin	Short chain fatty acids, reductases, glycosidases, sulfate reduction, bacterial counts and resistance levels

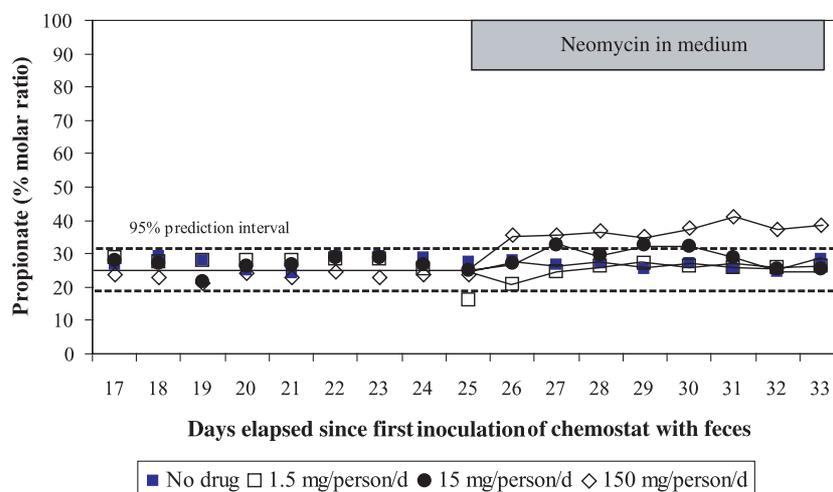


Fig. 2. Effect of neomycin on human fecal flora growing in a chemostat: propionate levels.

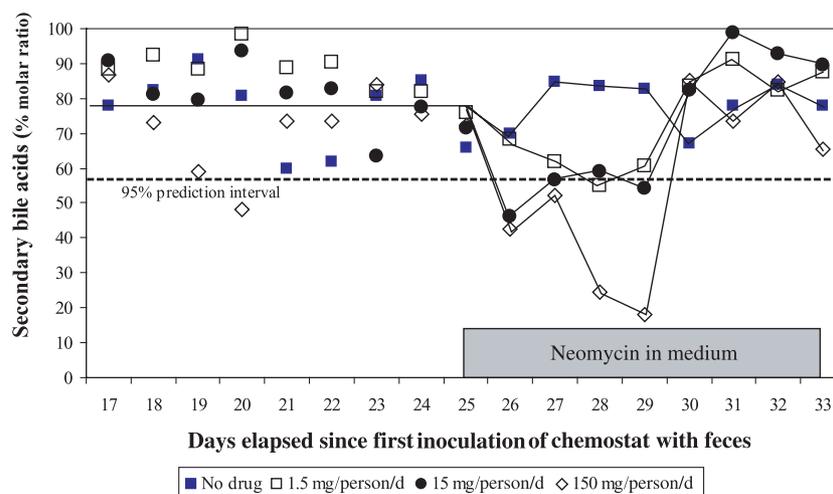


Fig. 3. Effect of neomycin on human fecal flora growing in a chemostat: secondary bile acids as a percentage of total bile acids molarity.

to below $0.1 \mu\text{mol/h/mg}$ in the presence of 15 and 150 mg/person/d. At 150 mg/person/d, this was a significant reduction; at 15 mg/person/d the reduction was marginally significant. There was no effect at 1.5 mg/person/d (Fig. 4).

Resistance levels among the enterococci were less than 10% before exposure to neomycin (Fig. 5). Levels rose significantly with 150 and 15 mg/person/d to 80% or more. The 1.5 mg/person/d dose gave rise to less consistent elevations, though these were still on occasion significant. Resistance levels in the BfG and *E. coli* were not affected.

Neomycin had no effect on sulfate reduction, on three of four bacterial enzymes (azoreductase was the exception), and on bacterial counts (Table 3).

3.3. Erythromycin

Erythromycin had a transitory, dose-dependent effect on bile acid metabolism similar to that of neomycin. Secondary compounds formed almost 90% of the pretreatment total. This fell within 48 h in the 150 and 15

mg/60 kg person/d chemostats to 40 and 55%, respectively; both were significant changes. Levels returned to their pretreatment value 4 or 5 days after the onset of erythromycin exposure (Fig. 6).

None of the following showed a response to erythromycin: SCFA, bacterial enzyme levels, sulfate reduction, bacterial counts, and resistance levels (Table 3).

4. Discussion

4.1. General

The best and most direct way to assess the effects of low levels of an antibiotic on intestinal flora would be simply to feed humans the test compound and see what happens. Unfortunately, very few such studies have been done; none is likely. Feeding trial data that have been published are usually limited by the use of therapeutic doses of older test compounds resulting in no observed

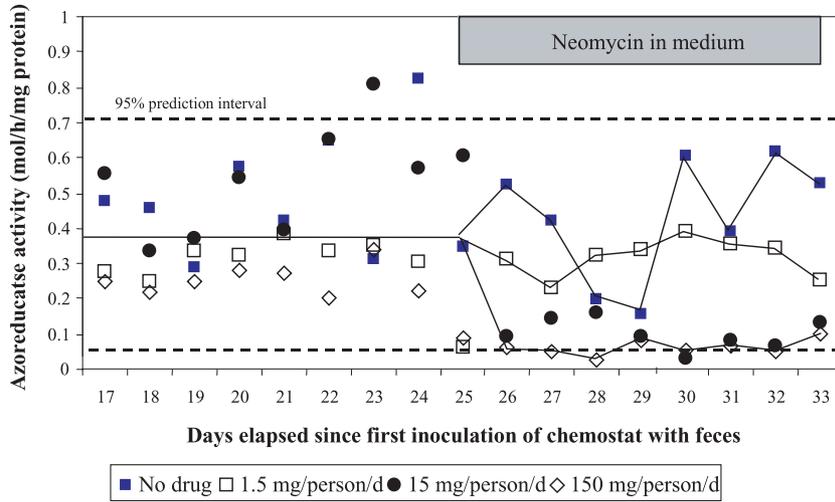


Fig. 4. Effect of neomycin on human fecal flora growing in a chemostat: azoreductase activity.

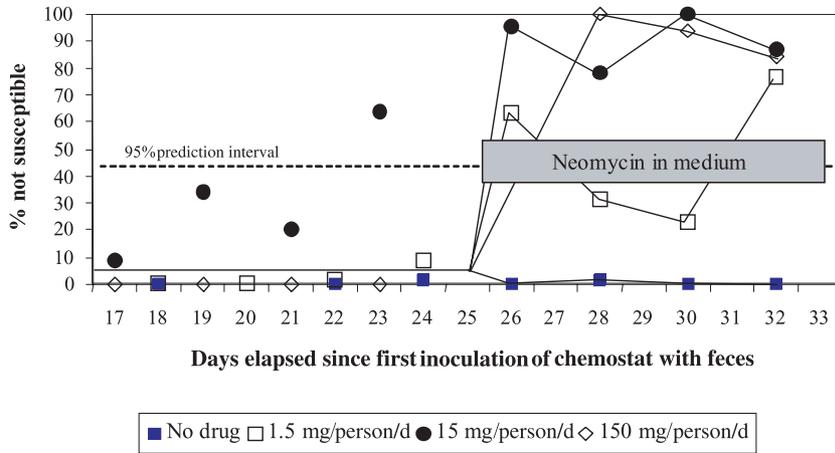


Fig. 5. Effect of neomycin on human fecal flora growing in a chemostat: susceptibility of enterococci isolates to 8 mg/mL neomycin.

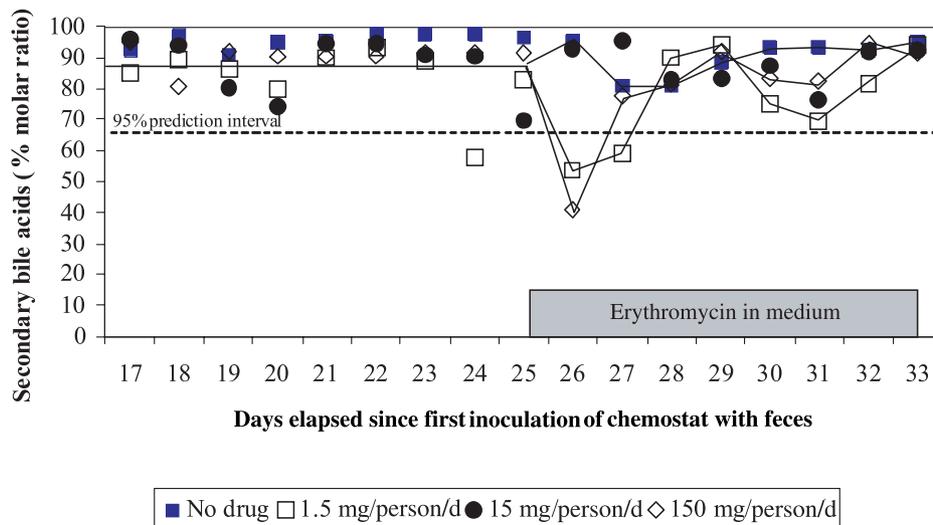


Fig. 6. Effect of erythromycin on human fecal flora growing in a chemostat: secondary bile acids as a percentage of total bile acid molarity.

effect levels (NOELs) that are high and frequently imprecise (e.g., >1 g/person/d). This makes their comparison with our work problematic. Because feeding trials with antimicrobial compounds might result in the emergence of novel or spread of already existing resistance to clinically useful compounds, it is also important at this point to draw attention to one facet of this study that may not be readily apparent. In fact, whenever most of the sentinel bacteria in the colon are already resistant, there will likely be little measurable change in this most sensitive of parameters. To illustrate this potential issue, consider that before the 1970s, about 30% of clinical or environmental bacteroides isolated in North America were resistant to tetracycline. By 2000 over 80% were resistant (Shoemaker et al., 2001). We found about 40% of the isolated BfG from the feces used as inoculum to be tetracycline-resistant (Table 1). Likewise, resistance to erythromycin among the bacteroides has also increased over the last 3 decades, up from <10% to about 30% (Shoemaker et al., 2001), though we found a higher incidence of erythromycin resistance (close to 100%) in the BfG in the inoculum than the 30% or so we might have expected from Shoemaker's work. As bacteroides, comprising 20% of the cultivable flora of feces (Holdeman et al., 1976; Moore and Holdeman, 1974), are the most abundant single group of fecal bacteria, there is presumably less and less opportunity for increased resistance to older compounds to develop among the bulk of the bacteria in the human colon. Newer compounds may not yet have elicited widespread resistance and thus may have a greater potential impact on the model than would older compounds. This was true for ciprofloxacin (Carman and Woodburn, 2001; Carman et al., 2004).

4.2. Oral doses and fecal levels

Some authors found much higher fecal concentrations over a range of therapeutic doses of tetracycline than our approach would suggest. Most notable among these is McVay (1952) whose data suggest that even for such low oral doses as we have modeled, there will still be adequate colonic tetracycline to be a problem for the resident flora. However, McVay's report must be regarded with caution. He gave 9 healthy subjects 2 g tetracycline/d for 2 days (i.e., a total of 4 g/person). On the fourth day after treatment began he found from 6400 to 102,400 µg tetracycline/g feces. (Though he actually reported data for the 1st to the 5th day after treatment, it is the fourth day's data that is most often cited by others.) So, if as McVay states, 1 g of feces from one of the donors contains 102,400 µg and if the mean daily fecal mass for an adult is 150 g (Cerniglia and Kotarski, 1999), then on day 4 alone that patient would excrete $(150 \times 102,400/1,000,000)$ g or 15.36 g while having ingested only 4 g total. McVay's acetone extraction method may have contributed to the inflated activity of

recovered tetracycline. But whatever the cause of this result, McVay's data should be received with caution.

4.3. Tetracycline

The reported in vivo effects of tetracycline at therapeutic levels are varied and sometimes contradictory. According to Tannock (1995), tetracycline is "moderate" in its effect on the fecal flora; according to Hovestad (1989) it has a small, or no effect. Finegold et al. (1983) summarized the effects of tetracyclines as ranging from an overgrowth of *E. coli*, through no effect, to the elimination of *E. coli*. Resistance levels among coliforms were either unchanged or rose. The carriage rates of enterococci and fecal streptococci either rose, remained unchanged, or fell, sometimes to complete elimination. Similarly, lactobacilli levels fell or rose and on one occasion became more resistant to chlortetracycline. Tetracyclines reduced carriage rates of *Bacteroides* spp., often to elimination. The same contradictory responses were seen among the anaerobic cocci where resistance became more common. Clostridia were adversely affected by tetracyclines.

Finegold was reviewing data collected from studies in which the subjects received from 1 to 4.5 g/d for 5 or more days, i.e., therapeutic doses only. Hovestad (1989) and Tannock (1995) do not give details but presumably they also considered data from studies using therapeutic dose levels, more than likely the same studies. In comparison, our highest test level for each of the compounds was intended to model an intake of only 150 mg/person/d. Our results indicated that counts did not change in response to tetracycline at this relatively low concentration. Not surprisingly, lower levels also had no effect. In fact, the sole response we saw to tetracycline was increased resistance in *E. coli*. It is noteworthy that despite all the *E. coli* isolates in the inoculum being resistant to 8 µg/mL, on average only 15% of the isolates from the chemostats before the introduction of tetracycline were resistant (Fig. 1). The initial low level of resistance in the chemostat suggests that sensitive strains may have thrived better in the chemostat than did their resistant counterparts, possibly because maintaining resistance genes is costly in the absence of any selective pressure. On the other hand, why this should be true only for the tetracycline resistant *E. coli* is not clear.

The in vivo selection of resistance by tetracycline and related compounds is already documented (Ahart et al., 1978; DePaola et al., 1995). Valtonen et al. (1976) gave humans with acne 100 mg/d, the equivalent of a chemostat dose of 10 µg/mL compared to the 0.15, 1.5, and 15 µg/mL we tested. Before treatment, only 27% of Valtonen's patients excreted resistant coliforms; after treatment the frequency rose to 80%. In contrast, in a chemostat study (Lebek and Eggert, 1983) involving mixed cultures of *E. coli*, 0.25 µg/mL was adequate to

select for the growth of resistant strains over their susceptible counterparts. This corresponds well with our finding of an insignificant increase in resistance at 0.15 µg/mL and a significant increase at 1.5 µg/mL (Fig. 1).

Our data suggest a NOEL of 15 mg/person/d for tetracyclines. How well does this compare with NOELs from human feeding trials? Tancrede and Barakat (1987) fed volunteers oxytetracycline (2000, 20, and 2 mg/person/d for 7 days) and monitored bacterial counts before, during, and after exposure. They saw sporadic and uncontrollable colonization among members of the 2 mg/d group by resistant strains of enterobacteria (the near equivalent of the sentinel *E. coli* in our study) that left them unable to measure resistance changes. Instead, they studied about 1500 isolates of the most dominant anaerobes isolated before, during, and after exposure. This would probably have included a high percentage of bacteroides (most similar to the sentinel *B. fragilis* Group or BfG in our study). No changes were seen in resistance levels among these anaerobes. On the other hand, in subjects fed 20 mg/d ($n=6$) or 2 g/d ($n=2$), effects were seen in resistance levels among the enterobacteria. With 20 mg/d the effect was confined to individuals rather than every member of the group. The extent of the change was reduced but not completely so by the end of the “after” period. Tancrede and Barakat (1987) assert a NOEL for tetracycline of approximately 2 mg/person/d.

Goldberg et al. (1961) reported the emergence of resistance after 10 mg/d for 26 weeks. Though because they challenged at only this one level they were not able to span any putative NOEL. In addition, the increase in the number of coliforms and yeasts observed was transitory and resistant coliforms were already present prior to the initiation of treatment.

Tetracycline (100 mg/d) given over a long period as a treatment for acne vulgaris was linked to an increased percentage of people carrying transferable *R* factor in their resident fecal bacteria and the number of multiresistant strains altered the resistance patterns of the microflora (Valtonen et al., 1976). However other workers have shown that in volunteers given 0, 50, and 1000 mg/d tetracycline for 4 days the shedding of *E. coli* from the intestine increased at the 1000 mg/d dose only, indicating that 50 mg/d the resident *E. coli* flora were retained and, by implication, colonization with foreign strains would not occur (Hirsch et al., 1973).

Though the doses given were therapeutic, there are some useful data on the effects of tetracyclines on microbial metabolism in the gut. Korpela et al. (1984) showed that administration of oxytetracycline 1 g/d for 5 days caused a reduction in the biotransformation of cholesterol and a reduction of the amount of esterified neutral sterols in the feces but did not affect the levels of serum cholesterol or triglycerides. Korpela et al. (1986) also

reported a consistent decrease in concentrations of secondary bile acids in response to oxytetracycline (1 g/d, the equivalent of a fecal level of 10.05 µg/mL). The same group of workers also saw a significant increase in fecal conjugated estrogens, possibly due to the antimicrobial activity on the responsible deconjugating bacteria (Hammilainen et al., 1987). Ingestion of tetracycline (1 g/d for 9 days) has been shown to increase not only the number of individuals excreting tetracycline resistant strains of *E. coli* but also the proportion of *E. coli* resistant isolates (Hirsch et al., 1973). Further studies in humans have shown that therapeutic doses of tetracycline caused an emergence of resistant *E. coli* strains but the level of resistant bacteria decreased with time after the last treatment (Bartlett et al., 1975; Hirsch et al., 1973).

NOELs have been generated using mice exposed to tetracycline. Perrin-Guyomard et al. (2001) seeded germ free mice with human feces and challenged them with tetracycline (0, 1, 10, and 100 µg/mL) in the drinking water. Though they counted bacteria, measured analytes and monitored the barrier effect, resistance was the most sensitive indicator of residual antibiotic in the gut. They established a NOEL of 1 µg/mL drinking water. Above the NOEL, resistance levels rose among the Gram-positive anaerobes, *B. fragilis*, enterobacteria, and enterococci. Colonization resistance was impaired at their highest dose. Perrin-Guyomard et al. (2001) estimated 1 µg/mL to be the equivalent of a NOEL of 0.125 mg/kg body weight/d based on a 40 g-mouse water intake of 5 ml/d. In a similar study (Corpet et al., 1989), in which germ free mice were associated with only two isogenic strains of *E. coli* (one of which carried an *R* plasmid), the minimum selecting concentration of tetracycline was 6.5 µg/ml (administered in drinking water over 10–15 days). This resulted in a fecal concentration of 0.5 µg/g tetracycline that is equivalent to 50% of the MIC for the plasmid free strain. This concentration did not eliminate the susceptible strains from the gut but their model lacked any of the dominant, anaerobic flora.

4.4. Neomycin

As an aminoglycoside, neomycin has an oxygen-dependent mechanism of action making it unlikely to have a major impact in an anaerobic environment such as the large bowel. This may partly explain the lack of published reports of its effects on bowel flora. Most of the literature on the effects of neomycin on the gut flora is limited to bacterial counts. The effects of therapeutic levels of neomycin on the gut flora are moderate (Tannock, 1995) and characterized primarily by slight decreases in coliforms and obligate anaerobes accompanied by slight increases in enterococci.

In their review, Finegold et al. (1983) discussed the effects of neomycin on human fecal flora. Doses between

2 and 6 g/d for 1 to 5 days caused the partial to complete elimination of coliforms, a major shift. New strains were able to colonize—a phenomenon we did not investigate. The effect on enterococci ranged from none to their elimination; again there was colonization by new strains. Lactobacilli were reduced up to 1000-fold, a minor change. Bacteroides and fusobacteria, the Gram-negative obligately anaerobic rods that dominate the human colonic flora, were either unchanged or eliminated. Usually, there was no effect on the clostridia.

The equivalent data in our study are not easy to interpret because of the variation in the counts. Even so, at 178 $\mu\text{g/mL}$ (150 mg/person/d) there was no apparent impact on *E. coli* or enterococci levels, a response that does not concur with Finegold et al. (1983). Bacterial resistance in the inocula may have obscured trends we might have seen twenty or more years ago when resistance was not common. Lactobacilli were unaffected. The Gram-negative anaerobes, the most prolific of the gut's anaerobes, showed no response to neomycin. In fact, none of the groups we monitored were demonstrably affected by the addition of test compound at any level to the medium.

In this study, neomycin had a greater impact than did either tetracycline or erythromycin. Though in common with these two other drugs, the effects we saw were not on the rates of isolation of particular bacteria. Instead, the effects of neomycin were confined to individual SCFA, bile acid metabolism, azoreductase activity, and levels of resistance among enterococci. Of these four, the first three might be thought surprising since they are likely markers of the predominantly anaerobic bacteria in the chemostat and large bowel. However in our preliminary study (Woodburn et al., Abstract N-186. Effects of antibiotics on a chemostat model of the human colon. Abstracts of the 97th Annual Meeting of the American Society for Microbiology, Miami Beach, FA. 1997; p. 411), neomycin at 178 $\mu\text{g/mL}$ led to a 5-fold reduction in total SCFA production in the chemostat and acetate became more than 80% of this total. Secondary bile acid production by bacteria suffered a 4-fold decrease. At sufficiently high enough levels, it may be that neomycin can exert an effect on even the most obligate of anaerobes. Further confirmation comes from Rubulis et al. (1970) who gave two obese patients an oral dose of neomycin that in our model would be predicted to raise the fecal level to over 5340 $\mu\text{g/mL}$. Secondary bile acid as a percentage of total bile acids fell from 74 and 100% to 17 and 51%, respectively. This supports the effect of neomycin at high doses that we saw. Admittedly though, in a cirrhotic patient the percentage remained close to 90% regardless of neomycin (Rubulis et al., 1970). Hirano et al. (1981) showed essentially the same effect of neomycin on the in vitro metabolism of primary bile acids.

Resistance to neomycin among fecal anaerobes is rarely investigated presumably because of the low prob-

ability of its occurrence. We saw none in our experiments. However, we did see a dose dependent rise in the frequency of isolation of neomycin resistant enterococci, a facultative anaerobe (Fig. 5). However, this finding is somewhat equivocal since there is a high level of variance around the pretreatment mean.

4.5. Erythromycin

Coliforms appear to be the critical indicators of erythromycin activity on gut microflora. Andremont et al. (1983) gave a single human 2 mg erythromycin daily for 35 days and the fecal level ranged from 2 to 4.5 mg/g. At the same time, human flora associated mice were treated with erythromycin. Both the human and the mice had no changes in total bacterial counts or in the levels of enterococci. On the other hand, the levels of coliforms dropped significantly from $>10^6/\text{g}$ to $\leq 10^2/\text{g}$ in both. Coliform counts were markedly reduced (10^2 - to 10^8 -fold) in humans given 100 mg erythromycin/d for a week (Heimdahl and Nord, 1982), a level that produced a mean fecal concentration of 350 $\mu\text{g/g}$ at its peak on day 5. This diminution in the *E. coli* count was a response that we did not see in the chemostats. However, our highest dose (15 $\mu\text{g/mL}$) was 20-fold or so lower than that achieved by Heimdahl and Nord (1982), and 100 times lower than by Andremont et al. (1983). In addition to a reduction in coliform counts, Heimdahl and Nord (1982) saw reductions in levels of enterococci, the *B. fragilis* group, fusobacteria and veillonella, though not in every subject.

We, however, saw only one effect of erythromycin on the chemostat ecology. This was a dose dependent and transitory reduction in the extent of metabolism of primary to secondary bile acids (Fig. 6). Koopman et al. (1987) using pooled intestinal material from erythromycin treated mice, had conversion levels fall from about 70% without treatment to <50%, a fall commensurate with our results.

Early research on resistance and erythromycin (Andremont et al., 1983; Heimdahl and Nord, 1982) indicated that a rise in resistance might be expected following exposure to erythromycin. However, comparing early results with more recent data on resistance is not always fruitful. Since erythromycin (and for that matter tetracycline and neomycin) first appeared resistance levels among hitherto susceptible bacteria have increased. In fact, 75% or more of the members of all three groups of sentinel microbes within the inoculum were resistant to erythromycin (Table 2). This left little room for the pressure exerted by exposure to select for increased frequency of resistance. This will presumably be true of many compounds with long histories of use. Conversely, it is reasonable to expect newer compounds to still have adequate potency against the bulk of the normal gut flora and to produce increased levels of resistance.

Our results showing that erythromycin had little impact on the flora are largely in agreement with other reports. Hoverstad et al. (1986) saw only insignificant effects on total and individual SCFA production. On the other hand, Hentges et al. (1989) using human-flora associated germ free mice did see a significant decrease in propionate, a response we did not observe. The same researchers (Marsh et al., 1990) saw no effect of erythromycin on SCFA in children from Texas, whereas in children from Costa Rica SCFA levels fell by half. It is not clear why their results were contradictory. Others (Clausen et al., 1991; Steinbakk et al., 1992) have reported significant drops in total SCFA production.

5. Conclusions

In conclusion, the single chambered chemostat reproduced the biology of the human bowel adequately. It reproduced many of the same changes due to antibiotics seen by other researchers. It was useful for titrating the effects of antibiotics on human fecal and colonic bacteria, particularly at lower than therapeutic doses. At such levels, bacterial counts remained unchanged regardless of the test compound used. Such changes as we did see were limited to metabolic activities, in particular bile acid metabolism and to resistance levels. Changes were generally dose-dependent and though most pronounced at the high dose level, they were often apparent and significant in the intermediate dose level chemostats. At the low dose level, though sometimes present, changes were not significant.

Based on our results, an acceptable daily intake for tetracycline and erythromycin would be 15 mg/person/d and for neomycin 1.5 mg/person/d. Other traditional toxicological studies may generate different values.

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