MECHANISMS RESPONSIBLE FOR THE MAINTENANCE OF HIGH PREVALENCE OF ANTIMICROBIAL DRUG RESISTANT *ESCHERICHIA COLI* IN DAIRY CALVES

By

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To the Faculty of Washigton State University:

The members of the Committee appointed to examine the dissertation/thesis of ARTASHES RUBEN KHACHATRYAN find it satisfactory and recommend that it be accepted.

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Chair

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It is said that a butterfly flapping its wings in the Amazon can cause a hurricane in the Atlantic. While an oversimplified explanation of chaos theory, this can also be applied to my academic career and the impact many people had on it. I would like to take this opportunity to convey my deepest appreciation to everyone who helped shape this amazing journey in my life.

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Abstract

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The prevalence of antimicrobial drug resistant bacteria is typically highest in younger animals, and the resistance traits can theoretically be maintained in a population through (a) direct antimicrobial selective pressure, (b) secondary advantage conferred by the resistance genes in the absence of antimicrobial selective pressure, and (c) linkage to other selectively advantageous genes. We assessed the prevalence of antimicrobial resistant *Escherichia coli* at a Holstein dairy farm and confirmed a higher prevalence of tetracycline resistance in younger calves. The most common phenotype among calf isolates included resistance to streptomycin, sulfa-drugs and tetracycline (SSuT), which was most prevalent during the period when calves were on milk diet. We inoculated animals with strains of SSuT and susceptible *E. coli* and found a higher frequency of SSuT strains in neonatal calves ($P < 0.001$), but no difference in older animals ($P = 0.5$). We also fed calves a milk supplement with and without oxytetracycline and found that calves receiving the oxytetracycline had slightly lower prevalence of tetracycline resistant and SSuT *E. coli*. These data indicate that direct selection was not critical to maintain SSuT strains at the dairy.

We then inoculated newborn calves with cured-SSuT (SSuT strains cured from antimicrobial resistance genes) and progenitor SSuT strains. On average the cured-SSuT strains were as competitive as the progenitor strains indicating that any selective advantage was not a secondary by-product of the resistance genes themselves.

We observed from the above studies that there might be a relationship between the presence of SSuT *E. coli* and a milk supplement. Using a feeding trial we demonstrated that milk supplement selected for significantly more SSuT *E. coli* in calves compared with the no-supplement control group (≈ 40% vs. ≈ 20% respectively). The presence of oxytetracycline in the milk supplement did not add to this effect. *In vitro* studies demonstrated that the milk supplement supported a significantly higher cell density of SSuT strains compared with other strains. From these results we speculate that the SSuT resistance genes are genetically linked to gene(s) that confer a selective advantage in the presence of milk supplement.

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DEDICATION

To my parents Hasmik Sahakyan and Ruben Khachatryan, and my sister Rubina.

Thank you for your unconditional love and steadfast belief in me.

To my grandparents who inspired me with their impeccable ways.

In the memory of the innocent victims of the 1915 Armenian Genocide.

CHAPTER 1

Role of calf-adapted *Escherichia coli* in maintenance of antimicrobial drug resistance in dairy calves

ABSTRACT

The prevalence of antimicrobial drug resistant bacteria is typically highest in younger animals and prevalence is not necessarily related to recent use of antimicrobial drugs. In dairy cattle, we hypothesize that antimicrobial drug resistant, neonate-adapted bacteria are responsible for the observed high frequencies of resistant *E. coli* in calves. To explore this issue we examined the age-distribution of antimicrobial drug resistant *Escherichia coli* from Holstein cattle at a local dairy and conducted an experiment to determine if low doses of oxytetracycline affected the prevalence of antimicrobial drug resistant *E. coli*. Isolates resistant to 4 μg/ml tetracycline were more prevalent in < 3 month old calves (79%) compared with lactating cows (14%). In an experimental trial where calves received diets supplemented with or without oxytetracycline, the prevalence of tetracycline resistant *E. coli* was slightly higher for the latter group ($P = 0.039$) indicating that drug use was not required to maintain a high prevalence of resistant *E. coli*. The most common resistance pattern among calf *E. coli* included resistance to streptomycin (>12μg/ml), sulfadiazine (>512μg/ml), and tetracycline (>4 μg/ml) (SSuT) and this resistance pattern was most prevalent during the period when calves were on milk diets. To determine if prevalence was a function of differential fitness, we orally inoculated animals with nalidixic acid resistant strains of SSuT *E. coli* and susceptible *E. coli*. Shedding of SSuT *E. coli* was significantly greater than susceptible strains in neonatal

calves ($P < 0.001$), whereas there was no difference in older animals ($P = 0.5$). These data support the hypothesis that active selection for traits linked to the SSuT phenotype are responsible for maintaining drug resistant *E. coli* in this population of dairy calves.

INTRODUCTION

The increasing prevalence of antimicrobial drug resistant bacteria is a major concern to human and veterinary medicine (39). Resistant bacteria include both pathogens and commensal organisms, with the latter serving as a potential reservoir for mobile resistance elements (49). Bacteria become resistant through mutations of target genes or horizontal transfer of genes encoding efflux pumps, degradative enzymes, alternative housekeeping enzymes, or ribosomal protection proteins. Horizontal gene transfer is shown to occur even among different species of bacteria and within multiple environments (9, 38, 40, 42). Maintenance of antimicrobial drug resistance genes is governed by continued exposure to antimicrobial drugs (25, 54), plasmid addiction mechanisms (16), or close genetic linkage to other selectively advantageous genes (26).

In the United States, agriculture could be responsible for as much as 70% of antimicrobial drug consumption (36) and therefore agricultural animals are considered an important reservoir and arena for emerging antimicrobial drug resistance (1-3, 6, 51, 53, 55, 56). Reducing consumption of antimicrobial drugs in some cases is associated with decreasing prevalence of resistant organisms (27, 28, 46), but in other cases the resistance level remains constant or it even increases independent of antimicrobial drugs (8, 17, 45, 50). Persistence of antimicrobial resistance after removal of the selective agent has been correlated with prolonged duration and amount of antimicrobial drugs used (15).

Extended application of antimicrobial drugs selects for resistant clones, and after removal of the selective agent(s) these clones may be sufficiently adapted to the genetic load of resistance genes that they cannot be easily displaced by susceptible flora (31). We are unaware of documented cases of complete loss of resistance following withdrawal of antimicrobial drugs.

The prevalence of resistant organisms is not equally distributed across all age groups, with younger animals usually having higher levels (4, 19-22, 24, 29, 35, 44, 57). We can speculate that one reason younger animals shed more resistant organisms is that this cohort may be exposed to greater amounts of antimicrobial drugs for medication or growth promotion. In addition, the intestinal physiology of younger animals is different from older animals and thus there may be niche-specific clones that are better suited to the calf intestinal environment. If antimicrobial drug resistance in young animals results from linkage between resistance genes and genes conferring selective advantage in neonatal intestines, then removal of antimicrobial drug selection pressure is unlikely to reduce this potentially important resistance reservoir in the short-term. In this paper we examined the relationship between age and antimicrobial drug resistance at a research dairy in Washington State. Prevalence of resistant bacteria was significantly greater in younger animals compared with older animals. Experimental manipulations demonstrated that resistant *E. coli* have higher fitness in the calf enteric environment independent of exposure to antimicrobial drugs.

MATERIALS AND METHODS

Herd survey.

The study was performed at a Holstein dairy farm (WSU, Pullman, WA). This operation has been using an oxytetracycline (Terramycin, TM-50) containing milk supplement for at least 10 years. The supplement, for calves less than 6 weeks old, included 4.54% TM-50 (final concentration in the milk ≈ 26 μg/ml), 2.65% vitamin A, 1.72% vitamin D, and 91.1% dried skim milk and was added directly to bulk or waste milk at feeding time. Bulk milk conformed to FDA standards for residual antimicrobials for human consumption. Calves were fed milk twice a day, but supplement was added only once (daily). We initially examined the prevalence of antimicrobial drug resistant *Escherichia coli* at the dairy. Sixty fecal samples (5 g) were collected weekly from fresh fecal pats from pens containing calves < 3 month old (10 samples), heifers 3-6 month old (10 samples), heifers 6-14 month old (10 samples), lactating cows (20 samples) and non-lactating cows (10 samples). Samples were processed as described below.

Terramycin supplement study.

To assess the impact of TM-50 supplemented diet on the prevalence of resistant bacteria, we performed the following experiment. Newborn calves were consecutively assigned to either control or experimental group. Calves were physically separated from each other in individual pens and control and treatment group calves were held in different locations within the calf facility to avoid any chance of physical contact. Calves in the control group (n = 9) were fed bulk milk twice daily, which was supplemented once daily but contained no TM-50 (-TM-50). The diet of the experimental group (n = 9)

was identical except it included TM-50 in the milk supplement (+TM-50). The calves left the experiment at 12 weeks of age. The calf diet included, in addition to the milk, a grain concentrate free of antimicrobials. Calves were weaned at the age of 4 to 6 weeks of age. There were no documented instances of use of other antimicrobial drugs (therapeutic) during this study.

Sampling, bacterial isolation, and characterization for herd survey and supplement studies.

Fresh fecal samples (20 g) were taken 2-3 times per week from each calf. Feces were collected with sterile tongue depressors and placed into sterile bags. The collected samples were streaked for isolation on Violet Red Bile Agar with 4-Methylumbelliferyl-β-D-glucuronide (VRB-MUG) plates (VRB from Remel, USA; MUG from Biosyth Ag, Switzerland) within 4 hr after collection and were incubated overnight at 37°C. Eight presumptive *E. coli* colonies (pink coloration and luminescence under UV light) per animal per sample were used to inoculate EC (*E. coli*) medium (Remel, USA) with MUG (EC-MUG) broth (200 µl) in a 96-well plate format, leaving 8-24 non-inoculated, negative control wells. The 96-well plates were then incubated at 44.5°C overnight and the MUG reaction was reconfirmed under UV light.

Presumptive *E. coli* were tested for antimicrobial drug susceptibility using agar dilution at breakpoint concentrations. Antimicrobial drug (Sigma, USA) susceptibilities were tested using IsoSensitest agar (IA) medium (Oxoid, USA) supplemented with ampicillin (16 µg/ml), tetracycline (4 µg/ml), chloramphenicol (16 µg /ml), streptomycin (12 µg/ml), sulfadiazine (512 µg/ml). Replicated test plate series included a final plate of

antimicrobial drug free media to confirm inoculum delivery. The results of replicator assays were recorded after overnight incubation at 37°C. Results for antimicrobial drug plates were coded as a dichotomous variable: zero for no growth and one for growth. These results were used to calculate the frequencies for different resistance patterns. Accuracy of the replicator assay was validated by testing 2-4 samples from each 96 well plate by disk diffusion assay conforming to NCCLS guidelines (n = 173) (32). Quality control organisms used for the disk diffusion assay included: *E. coli* ATCC 25922; *S. aureus* ATCC 25923; *P. aueroginosa* ATCC 27853; and *E. coli* ATCC 10536.

Data was entered into Microsoft Access (Microsoft Corp, Redmond, WA) and was analyzed using Microsoft Excel and NCSS 2001 (NCSS Statistical Software, Kaysville, UT). An exact binomial test was used to test the distribution of resistance phenotypes for calves vs. cows and for treatment vs. control groups. Proportionality was adjusted for sample size differences and alpha was adjusted for multiple comparisons using a Bonferroni correction.

Molecular characterization of SSuT isolates.

Fifty SSuT (resistant to streptomycin (>12µg/ml), sulfadiazine (>512µg/ml), and tetracycline (>4 µg/ml)) isolates from calves were examined for the prevalence of specific resistance genes, shared plasmids, and clonal diversity. Antimicrobial drug resistance genes were screened using PCR (*tet*A and *tet*B (5), *str*A and *str*B (17), *sul*2 *and sul*2 (17)). Plasmids were isolated by alkaline lysis method (43) and the profiles were visualized on 1% agarose gels stained with ethidium bromide. Pulsed-field gel

electrophoresis (PFGE) was used to examine clonal diversity using the *Xba*I enzyme (12), and band profiles were analyzed using Bionumerics (Applied Maths, Austin, TX).

In vitro *and* in vivo *competition experiments.*

SSuT (resistant to streptomycin, sulfadiazine and tetracycline) *E. coli,* being the dominant resistance pattern isolated from calves, were tested *in vitro* and *in vivo* to determine their differential fitness compared to susceptible (susceptible to above tested antimicrobials) *E. coli*. For *in vivo* studies the *E. coli* strains of interest were selected for nalidixic acid resistance (Nal^r) (20 μg/ml) following the methods of Marshall et al. (33). The design for the *in vitro* competitions included mixing 10^5 CFUs (LB media) of five SSuT and five susceptible *E. coli*. All 10 (ten) *E.coli* were isolated from individual calves, on different dates and represented 3 distinct SSuT and 3 distinct susceptible fingerprints (defined by PFGE). We used mixtures of strains in the event that a particular lineage might be responsible for the pattern of high SSuT prevalence. Similar *in vitro* experiments were accomplished using Nal^r strains of SSuT and susceptible *E. coli.* The mixtures were incubated overnight at 37.0˚C, on a shaker (200 rpm). Overnight culture (10 μl) was transferred into fresh LB broth (3 ml) for eight consecutive days. On days zero, four and eight, a competition index was calculated by estimating the colony forming units (CFU)/ml for the SSuT and susceptible strains. The number of SSuT colonies was counted on fresh MacConkey agar supplemented with sulfadiazine (512 μg/ml), whereas the number of susceptible colonies was determined by subtracting the number of SSuT colonies from the same dilution grown on MacConkey agar media without antimicrobial drugs. Three replicate counts were made at each time point and averaged. The

competition index (CI) was calculated as (X-Y)/(X+Y), where X was the number of SSuT colonies and Y was the number of susceptible colonies. CI values near +1 indicated dominance by SSuT strains, whereas CI values near -1 indicated dominance by susceptible strains.

In vivo experiments involved neonatal calves (2-3 days old; n = 7) and heifers (12-14 mo; n = 12). For *in vivo* studies, five distinct SSuT Nal^r and five distinct susceptible Nal^r *E. coli* were mixed (10^8 CFUs of each for calves and 10^{10} CFUs of each for heifers) and administered *per os* (oral administration was used for neonatal calves and gastric tube for heifers). Neonatal calves were sampled every other day until shedding of inoculum bacteria was not detectable (up to 21 day PI (post inoculation)). Heifers were sampled every three days for 27 days PI. *In vivo* experiments with neonatal calves were performed in a vivarium, whereas experiments with the heifers were conducted in the farm setting. The ten strains of *E. coli* were identical for both *in vitro* and *in vivo* studies. To determine CFU/ml for SSuT and susceptible *E. coli in vivo* experiments, 1 gram of freshly collected fecal samples was serially diluted in PBS (peptone buffered saline) and ten fold dilutions were plated on specific selective media. Competition index (CI) values were calculated as described above where colony counts were averaged within test tube (*in vitro*) or within animal (*in vivo*) across time points and the null hypothesis (CI = 0) was tested using a Student's *t* test. This analysis eliminated pseudoreplication and temporal variance by averaging within animals across time points. To determine whether any particular SSuT strain dominated *in vivo* in calves, PFGEs were done on a number of random Nal^r *E. coli* (n = 29) isolated from all the calves on the final days of the

experiment. All *in vivo* inoculation studies were approved by the WSU IACUC (Institutional Animal Care and Use Committee).

RESULTS

Herd survey.

Survey results from a dairy herd at Washington State University reflected a pattern of high prevalence of antimicrobial drug resistance in younger animals for *E. coli*, which in most instances decreased with age (Table 1). The predominant pattern for neonatal calves was resistance to streptomycin, sulfadiazine and tetracycline (SSuT; Fig. 1). In contrast, most *E. coli* from cows were susceptible to all tested antimicrobial drugs.

Experimental Results.

The addition of oxytetracycline was associated with a higher prevalence of several antimicrobial resistance phenotypes relative to *E. coli* isolated from calves that received no antimicrobial supplement (Fig. 2). Nevertheless, *E. coli* collected from calves receiving oxytetracycline had a statistically larger proportion of susceptible *E. coli* and significantly smaller proportion of *E. coli* having an SSuT phenotype (Fig. 2). In addition, when data from all tetracycline resistant phenotypes was pooled (3,750 isolates), removing oxytetracycline from the diet was associated with a slightly higher than expected proportion of tetracycline resistant *E. coli* (observed = 0.55; expected = 0.533; $P = 0.039$). We interpreted this result as indicating that removing oxytetracycline did not result in a reduction in the proportion of tetracycline resistant *E. coli*. The

prevalence of the SSuT resistance pattern peaked during the period when calves were fed milk, after which the prevalence began to decline (Fig. 3).

The agar dilution antimicrobial drug susceptibility results were validated with disk diffusion method (97.1% congruence) (n = 173). Based on bacterial growth in negative control wells, we estimated that the contamination rate associated with the agar dilution assay was 4%. The approximate rate of failure to inoculate the plates with replicator was measured as growth on antimicrobial drug plate and no growth on carbohydrate plate for the same isolate and was equal to 3.15%; these samples were excluded from the analysis. Finally, our assay showed a 100% correspondence between resistance for tetracycline and oxytetracycline, at different MICs starting from 4 to 40 μg/ml (data not shown).

In vitro *competition results.*

SSuT *E. coli* consistently out-competed susceptible strains *in vitro* in the absence of antibacterial selective pressure (Student's *t* test; $P < 0.001$). There was no significant difference between competition results for Nal^r and Nal^s (Nalidixic acid sensitive) strains (Student's *t* test; $P = 0.41$). Finally, all Nal^r strains had approximately equal MICs for nalidixic acid (200 μg/ml). These latter results were consistent with similar mutations conferring the Nal^r phenotype and with negligible impact on differential fitness due to the Nal^r phenotype.

In vivo *competition results.*

SSuT *E. coli* consistently out-competed susceptible strains in neonatal calves in the absence of antibacterial selective pressure (Student's *t* test; $P < 0.001$; Fig. 4). In older cows (heifers) SSuT *E. coli* did not show any competitive advantage over susceptible *E. coli* (Student's *t* test; $P = 0.54$; Fig. 5). Throughout the experiment all heifers presented both positive and negative CI values. None of the animals in the *in vivo* studies shed detectable fecal Nal^r *E. coli* prior to inoculation (0-3 days). All strains of Nal^r *E. coli* present in the SSuT inoculum were also identified in feces as demonstrated by pulsed-field gel electrophoresis (PFGE; data not shown).

Molecular characteristics of SSuT isolates.

From 50 SSuT isolates obtained from calves, 49 harbored a large plasmid (~140 kb). All the SSuT isolates encoded *str*A, *sul*2, and *tet*B antimicrobial resistance genes. PFGE profiles revealed a very diverse population of SSuT *E. coli* in calves. Given a similarity coefficient of 90% (Dice index), there were 18 distinct band patterns for these 50 isolates (data not shown).

DISCUSSION

We studied antimicrobial drug resistance in commensal *E. coli*, which are considered a potential reservoir for resistance genes in farm animals (41). On-farm reservoirs of resistant bacteria provide a potential source for resistance gene transfer between bacteria as well as an environment for dissemination to new animals, environments, and food products. Therefore, identifying these reservoirs and mechanisms

of persistence will be a key to reducing the load of resistant bacteria in commercial facilities.

In this study, pre-weaned calves had the greatest prevalence of resistant *E. coli*, and we found an inverse relationship between prevalence of resistant *E. coli* and animal age. We also demonstrated experimentally that this high degree of resistance could be maintained in the absence of antimicrobial drug selection. In the absence of antimicrobial drugs, high prevalence of antimicrobial drug resistant *E. coli* could occur if the resistant strains had a fitness advantage in calves, but this could also occur if the animals received high doses of these strains from the environment. To test the former hypothesis we compared fitness of SSuT strains with susceptible strains *in vivo*. It was clear from these experiments that the SSuT strains had a fitness advantage in calves (Fig. 4), but not in older animals (Fig. 5). In the latter case the data were consistent with random colonization by either SSuT or susceptible strains. Given these results, environmental sources probably play a role in initial inoculation, but active competition in the calf gut leads to expansion of SSuT populations in calves. Finally, we found a very interesting correlation between highest prevalence of the SSuT resistance pattern and the period when calves received milk, suggesting that the selective advantage in calves might be related to diet. *In vitro* experiments showed that SSuT *E. coli* had higher fitness than susceptible strains under no specific selective pressure. We have no *a priori* reason to expect that similar mechanisms were responsible for *in vitro* and *in vivo* results, but they may be related.

The prevalence of resistant bacteria is usually correlated with previous use of antimicrobial drugs, but withdrawal of antimicrobial drugs does not always result in an

immediate decrease in the prevalence of resistant bacteria (8, 17, 45, 50). Our experimental results support the observation that antimicrobial drug selection is not required for short-term maintenance of resistant organisms. In the absence of antimicrobial pressure, resistance genes probably represent a metabolic burden. Nevertheless, it is possible that this burden is so low that the half-life for persistence can be measured in years or decades (e.g., 47). It is also possible that occult selection pressure exists in a direct ecological context (e.g. antibiotic or colicin producing bacteria) or via some other effective advantage deriving from resistance genes other than antimicrobial drug resistance. Finally, it is plausible that resistance genes could persist if they were closely linked to other selectively advantageous genes. For example, resistance genes might be linked to adhesin or siderophore genes. Others have shown that some adhesin genes have higher prevalence in the *E. coli* flora of younger calves (10, 23, 34) and adhesin genes have also been found on plasmids that also encode antimicrobial drug resistance genes (14, 30, 47, 48). Lactoferrin is an abundant iron chelator in milk and iron is required for bacterial growth (37, 52). Iron acquisition genes (aerobactin and enterobactin) have been associated with plasmids that also encode antimicrobial drug resistance genes (7, 11, 13, 18).

In our system, the correspondence between milk diet and highest prevalence of SSuT *E. coli* in the feces suggests either a direct benefit of the resistance genes themselves or linkage to other genes that are adaptive in this environment. A subset of SSuT isolates were characterized and we found a diverse group of clones (based on PFGE) that harbored a common set of resistance genes (*tet*B, *sul*2, and *str*A) and a common plasmid (~140 kb). It is possible that this plasmid harbors the gene(s)

responsible for selective advantage in calves. Studies are underway to identify the mechanisms responsible for the increased fitness of SSuT *E. coli* in calves and with this information we expect to devise measures to promote displacement of antimicrobial drug resistant bacteria in this age group.

REFERENCES

1. **Aarestrup, F. M.** 1999. Association between the consumption of antimicrobial agents in animal husbandry and the occurrence of resistant bacteria among food animals. Int J Antimicrob Agents **12:**279-85.
2. **Aarestrup, F. M., Y. Agerso, P. Gerner-Smidt, M. Madsen, and L. B. Jensen.** 2000. Comparison of antimicrobial resistance phenotypes and resistance genes in *Enterococcus faecalis* and *Enterococcus faecium* from humans in the community, broilers, and pigs in Denmark. Diagn Microbiol Infect Dis **37:**127-37.
3. **Aarestrup, F. M., H. Kruse, E. Tast, A. M. Hammerum, and L. B. Jensen.** 2000. Associations between the use of antimicrobial agents for growth promotion and the occurrence of resistance among *Enterococcus faecium* from broilers and pigs in Denmark, Finland, and Norway. Microb Drug Resist **6:**63-70.
4. **Brophy, P. O., P. J. Caffrey, and J. D. Collins.** 1977. Sensitivity patterns of *Escherichia coli* isolated from calves during and following prophylactic chlortetracycline therapy. Br Vet J **133:**340-5.

5. **Call, D. R., M. Bakko, M. Krug, and M. Roberts.** In Press. Identifying antimicrobial resistance genes using DNA microarrays. Antimicrob Agents Chemother.

6. **Caprioli, A., L. Busani, J. L. Martel, and R. Helmuth.** 2000. Monitoring of antibiotic resistance in bacteria of animal origin: epidemiological and microbiological methodologies. Int J Antimicrob Agents **14:**295-301.

7. **Cercenado, E., F. Baquero, A. Delgado-Iribarren, and J. L. Martinez.** 1986. Epidemiology of aerobactin production in Enterobacteriaceae. Ann Inst Pasteur Microbiol **137B:**297-303.

8. **Chaslus-Dancla, E., G. Gerbaud, M. Lagorce, J. P. Lafont, and P. Courvalin.** 1987. Persistence of an antibiotic resistance plasmid in intestinal *Escherichia coli* of chickens in the absence of selective pressure. Antimicrob Agents Chemother **31:**784-8.

9. **Chee-Sanford, J. C., R. I. Aminov, I. J. Krapac, N. Garrigues-Jeanjean, and R. Mackie.** 2001. Occurrence and diversity of tetracycline resistance in lagoons and groundwater underlying two swine production facilities. Appl Environ Microbiol **67:**1494-1502.

10. **China, B., V. Pirson, and J. Mainil.** 1998. Prevalence and molecular typing of attaching and effacing *Escherichia coli* among calf populations in Belgium. Vet Microbiol **63:**249-59.

11. **Colonna, B., L. Ranucci, P. A. Fradiani, M. Casalino, A. Calconi, and M. Nicoletti.** 1992. Organization of aerobactin, hemolysin, and antibacterial

resistance genes in lactose-negative *Escherichia coli* strains of serotype O4 isolated from children with diarrhea. Infect Immun **60:**5224-31.

12. **Davis, M. A., D. D. Hancock, T. E. Besser, and D. R. Call.** 2003. Evaluation of Pulsed-Field Gel Electrophoresis as a tool for determining the degree of genetic relatedness between strains of *Escherichia coli* O157:H7. J Clin Microbiol **41:**1843-9.

13. **Der Vartanian, M.** 1988. Differences in excretion and efficiency of the aerobactin and enterochelin siderophores in a bovine pathogenic strain of *Escherichia coli*. Infect Immun **56:**413-8.

14. **Di Martino, P., D. Sirot, B. Joly, C. Rich, and A. Darfeuille-Michaud.** 1997. Relationship between adhesion to intestinal Caco-2 cells and multidrug resistance in *Klebsiella pneumoniae* clinical isolates. J Clin Microbiol **35:**1499-503.

15. **Dunlop, R. H., S. A. McEwen, A. H. Meek, R. C. Clarke, W. D. Black, and R. M. Friendship.** 1998. Associations among antimicrobial drug treatments and antimicrobial resistance of fecal *Escherichia coli* of swine on 34 farrow-to-finish farms in Ontario, Canada. Prev Vet Med **34:**283-305.

16. **Engelberg-Kulka, H., and G. Glaser.** 1999. Addiction modules and programmed cell death and antideath in bacterial cultures. Annu Rev Microbiol **53:**43-70.

17. **Enne, V. I., D. M. Livermore, P. Stephens, and L. C. Hall.** 2001. Persistence of sulphonamide resistance in *Escherichia coli* in the UK despite national prescribing restriction. Lancet **357:**1325-1328.

18. **Harel, J., J. Fairbrother, C. Forget, C. Desautels, and J. Moore.** 1993. Virulence factors associated with F165-positive *Escherichia coli* strains isolated from piglets and calves. Vet Microbiol **38:**139-55.

19. **Hinton, M.** 1985. The sub-specific differentiation of *Escherichia coli* with particular reference to ecological studies in young animals including man. J Hyg (Lond) **95:**595-609.

20. **Hinton, M., A. J. Hedges, and A. H. Linton.** 1985. The ecology of *Escherichia coli* in market calves fed a milk-substitute diet. J Appl Bacteriol **58:**27-35.

21. **Hinton, M., A. H. Linton, and A. J. Hedges.** 1985. The ecology of *Escherichia coli* in calves reared as dairy-cow replacements. J Appl Bacteriol **58:**131-8.

22. **Hinton, M., P. D. Rixson, V. Allen, and A. H. Linton.** 1984. The persistence of drug resistant *Escherichia coli* strains in the majority faecal flora of calves. J Hyg (Lond) **93:**547-57.

23. **Holland, R. E., R. A. Wilson, M. S. Holland, V. Yuzbasiyan-Gurkan, T. P. Mullaney, and D. G. White.** 1999. Characterization of eae+ *Escherichia coli* isolated from healthy and diarrheic calves. Vet Microbiol **66:**251-63.

24. **Howe, K., and A. H. Linton.** 1976. A longitudinal study of *Escherichia coli* in cows and calves with special reference to the distribution of O-antigen types and antibiotic resistance. J Appl Bacteriol **40:**331-40.

25. **Jorgensen, S. E., and B. Halling-Sorensen.** 2000. Drugs in the environment. Chemosphere **40:**691-9.

26. **Kehrenberg, C., and S. Schwarz.** 2001. Occurrence and linkage of genes coding for resistance to sulfonamides, streptomycin and chloramphenicol in bacteria of the genera Pasteurella and Mannheimia. FEMS Microbiol Lett **205:**283-90.

27. **Klare, I., D. Badstubner, C. Konstabel, G. Bohme, H. Claus, and W. Witte.** 1999. Decreased incidence of VanA-type vancomycin-resistant enterococci isolated from poultry meat and from fecal samples of humans in the community after discontinuation of avoparcin usage in animal husbandry. Microb Drug Resist **5:**45-52.

28. **Langlois, B. E., K. A. Dawson, T. S. Stahly, and G. L. Cromwell.** 1984. Antibiotic resistance of fecal coliforms from swine fed subtherapeutic and therapeutic levels of chlortetracycline. J Anim Sci **58:**666-74.

29. **Larsen, H. E., and J. L. Larsen.** 1975. Persistance of resistant *Escherichia coli* in a calf herd. Nord Vet Med **27:**65-76.

30. **Le Bouguenec, C., and Y. Bertin.** 1999. AFA and F17 adhesins produced by pathogenic *Escherichia coli* strains in domestic animals. Vet Res **30:**317-42.

31. **Lipsitch, M., and M. H. Samore.** 2002. Antimicrobial use and antimicrobial resistance: a population perspective. Emerging Infectious Diseases (Print) **8:**347-54.

32. **Lorian, V.** 1996. Antibiotics in Laboratory Medicine, 4th ed. Williams and Wilkins.

33. **Marshall, B., D. Petrowski, and S. B. Levy.** 1990. Inter- and intraspecies spread of *Escherichia coli* in a farm environment in the absence of antibiotic usage. Proc Natl Acad Sci U S A **87:**6609-13.

34. **Martin, C., E. Rousset, and H. De Greve.** 1997. Human uropathogenic and bovine septicaemic *Escherichia coli* strains carry an identical F17-related adhesin. Res Microbiol **148:**55-64.

35. **Mathew, A. G., A. M. Saxton, W. G. Upchurch, and S. E. Chattin.** 1999. Multiple antibiotic resistance patterns of *Escherichia coli* isolates from swine farms. Appl Environ Microbiol **65:**2770-2.

36. **Mellon, M., C. Benbrook, and K. L. Benbrook.** 2001. Hogging it: estimates of antimicrobial abuse in livestock. UCS Publications., Cambridge.

37. **Mevissen-Verhage, E. A., J. H. Marcelis, W. C. Harmsen-Van Amerongen, N. M. de Vos, and J. Verhoef.** 1985. Effect of iron on neonatal gut flora during the first three months of life. Eur J Clin Microbiol **4:**273-8.

38. **Miller, R. V.** 1998. Bacterial Gene Swapping in Nature. Scientific American**:**67-71.

39. **NAS.** 1999. The use of drugs in food animals: benefits and risks. National Academy Press., Washington, DC.

40. **Nikolich, M., H. G, N. B. Shoemaker, and A. A. Salyers.** 1994. Evidence for natural horizontal transfer of tetQ between bacteria that normally colonize humans and bacteria that normally colonize livestock. Appl Environ Microbiol **60:**3255-3260.

41. **O'Brien, T. F.** 2002. Emergence, spread, and environmental effect of antimicrobial resistance: how use of an antimicrobial anywhere can increase resistance to any antimicrobial anywhere else. Clin Infect Dis **34(Suppl 3):**S78-84.

42. **Ochman, H., J. G. Lawrence, and E. A. Groisman.** 2000. Lateral gene transfer and the nature of bacterial innovation. Nature **405:**299-304.

43. **Portnoy, D. A., S. L. Moseley, and S. Falkow.** 1981. Characterization of plasmids and plasmid-associated determinants of Yersinia enterocolitica pathogenesis. Infect Immun **31:**775-82.

44. **Raška, K., H. Rašková, Z. Urbanová, D. Matejovská, V. Matejovská, V. Palounek, and L. Polák.** 1979. Resistance of gram-negative bacteria to antibiotics in large calf agglomerations. Acta Trop **36:**163-70.

45. **Rollins, L. D., S. A. Gaines, D. W. Pocurull, H. D. Mercer, and L. T. Frobish.** 1976. Persistence of transferable drug resistance in the lactose-fermenting enteric flora of swine following antimicrobial feeding. Can J Comp Med **40:**175-83.

46. **Seppala, H., T. Klaukka, J. Vuopio-Varkila, A. Muotiala, H. Helenius, K. Lager, and P. Huovinen.** 1997. The effect of changes in the consumption of macrolide antibiotics on erythromycin resistance in group A streptococci in Finland. Finnish Study Group for Antimicrobial Resistance. N Engl J Med **337:**441-6.

47. **Shimizu, M., T. Sakano, and S. Satoh.** 1988. Linkage of K99 production and STa activity in a plasmid of an *Escherichia coli* porcine isolate. Microbiol Immunol **32:**635-9.

48. **Shimizu, M., and T. Terashima.** 1982. Appearance of enterotoxigenic *Escherichia coli* in piglets with diarrhea in connection with feed changes. Microbiol Immunol **26:**467-77.

49. **Shoemaker, N. B., H. Vlamakis, K. Hayes, and A. A. Salyers.** 2001. Evidence for extensive resistance gene transfer among *Bacteroides spp*. and among *Bacteroides* and other genera in the human colon. Appl Environ Microbiol **67:**561-568.

50. **Smith, H. W.** 1975. Persistence of tetracycline resistance in pig *E. coli*. Nature **258:**628-30.

51. **van den Bogaard, A. E., and E. E. Stobberingh.** 2000. Epidemiology of resistance to antibiotics. Links between animals and humans. Int J Antimicrob Agents **14:**327-35.

52. **Wharton, B. A., S. E. Balmer, and P. H. Scott.** 1994. Faecal flora in the newborn. Effect of lactoferrin and related nutrients. Adv Exp Med Biol **357:**91-8.

53. **White, D. G., L. J. V. Piddock, J. J. Maurer, S. Zhao, V. Ricci, and S. G. Thayer.** 2000. Characterization of fluoroquinolone resistance among veterinary isolates of avian *Escherichia coli*. Antimicrob Agents Chemother **44:**2897-2899.

54. **Winckler, C., and A. Grafe.** 2001. Use of veterinary drugs in intensive animal production evidence for persistence of tetracycline in pig slurry. J of Soils and Sedim **1(2):**66-70.

55. **Witte, W.** 2000. Ecological impact of antibiotic use in animals on different complex microflora:environment. Int J Antimicrob Agents **14:**321-5.

56. **Witte, W.** 2000. Selective pressure by antibiotic use in livestock. Int J Antimicrob Agents **16:**S19-S24.

57. **Zhang, X. L., F. Wang, D. M. Zhu, S. Wu, P. C. Wu, Y. D. Chen, Y. Q. Wang, and L. Zhou.** 1998. The carriage of *Escherichia coli* resistant to antibiotics in healthy populations in Shanghai. Biomed Environ Sci **11:**314-20.

TABLES

Table 1. Percent of antimicrobial drug resistant *E. coli* from the Washington State University dairy distributed by the age of cattle, 2001.

Age	N*	Amp[a]	Tet	Strep	Sulfa	Chlor
Pre-weaned	3,221	14.9	79.2	58.0	58.1	9.8
3-6 mo Heifers	3,224	14.5	35.7	26.4	26.2	1.2
6+ mo Heifers	3,245	5.9	17	10.9	10.9	0.1
Lactating cows	6,321	4.6	14.2	6.2	10.7	0.1
Dry cows	2,877	2.4	13.3	7.3	12.3	0.5

[a] Amp = ampicillin; Tet = tetracycline; Strep = streptomycin; Sulfa = sulfamethoxazole; Chlor = chloramphenicol.

*number of isolates tested.

FIGURES

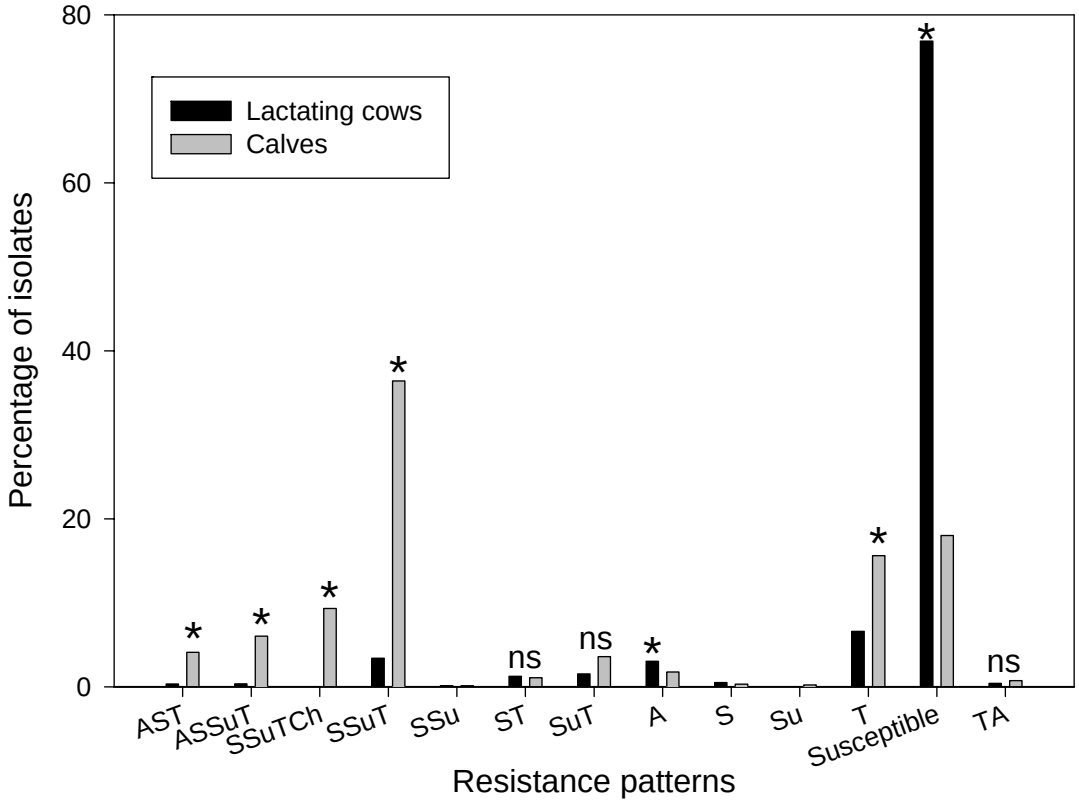


Figure 1. Frequency of resistance pattern for all fecal *E. coli* shed from pre-weaned calves (open bars; n = 3,221) or cows (closed bars; n = 6,321). Resistance patterns are denoted by letters where A = ampicillin, Ch = chloramphenicol, S = streptomycin, Su = sulfamethoxazole, and T = tetracycline. Susceptible isolates were susceptible to all antimicrobial drugs tested (see methods). * = statistically significant binomial test ($P < 0.0006$ with Bonferroni correction for 10 tests); ns = non-significant. Only phenotypes having >50 isolates were compared.

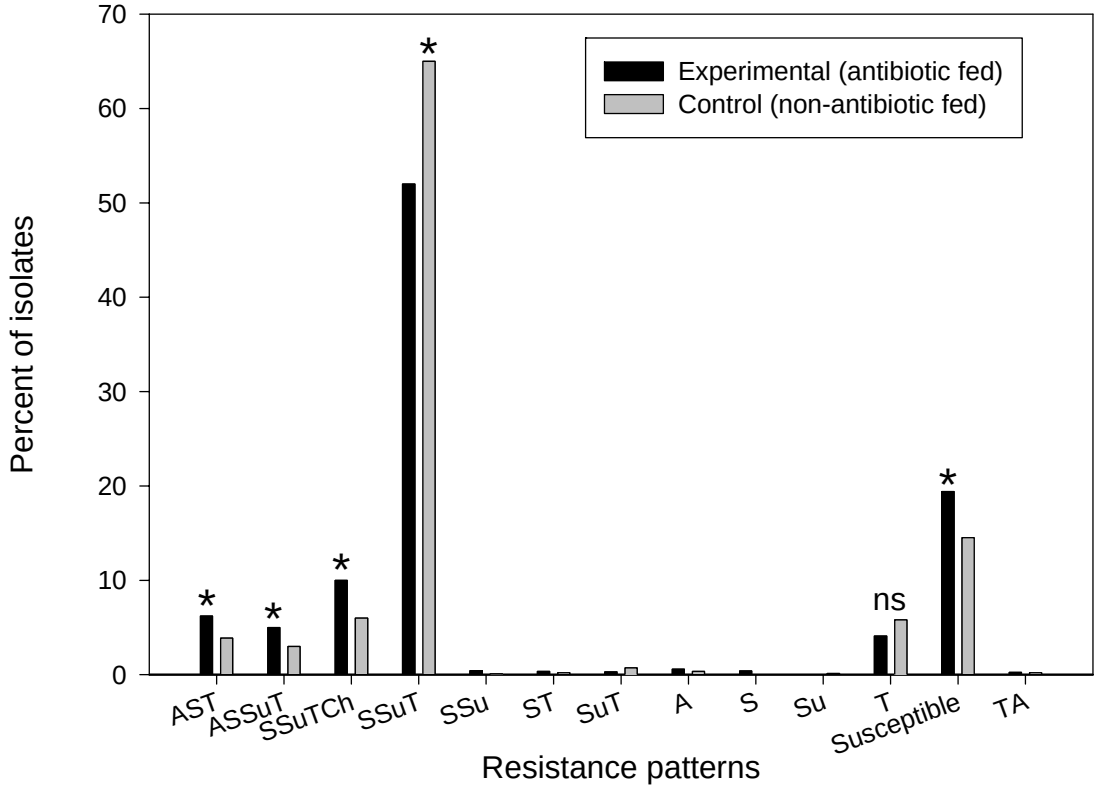


Figure 2. Frequency of resistance patterns for all fecal *E. coli* shed from calves; Experimental group (antibiotic fed; closed bars; n = 2,129) and control group (non antibiotic fed; open bars; n = 2,432). See Fig.1 for code legend. * = statistically significant binomial test ($P < 0.0017$ with Bonferroni correction for 6 tests); ns = non significant. Only phenotypes having >50 isolates were compared.

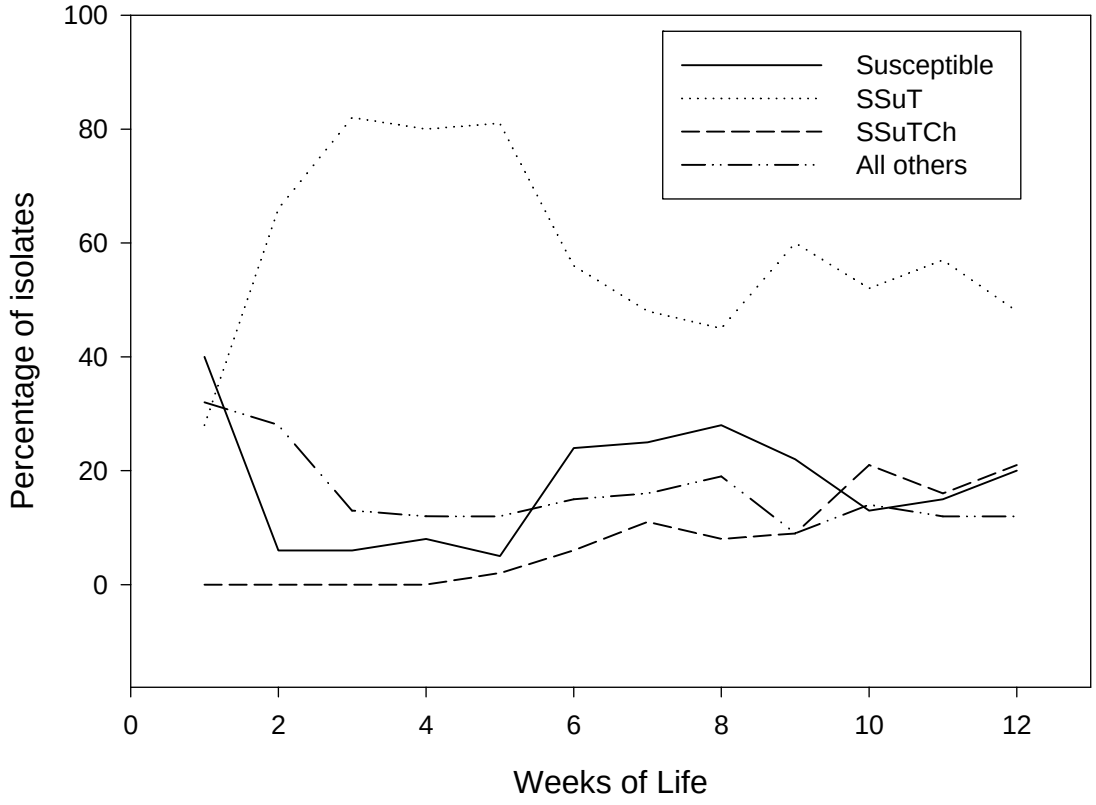


Figure 3. Resistance pattern of fecal *E. coli* over weeks of life for all calves (n = 18). See Fig. 1 for code legend.

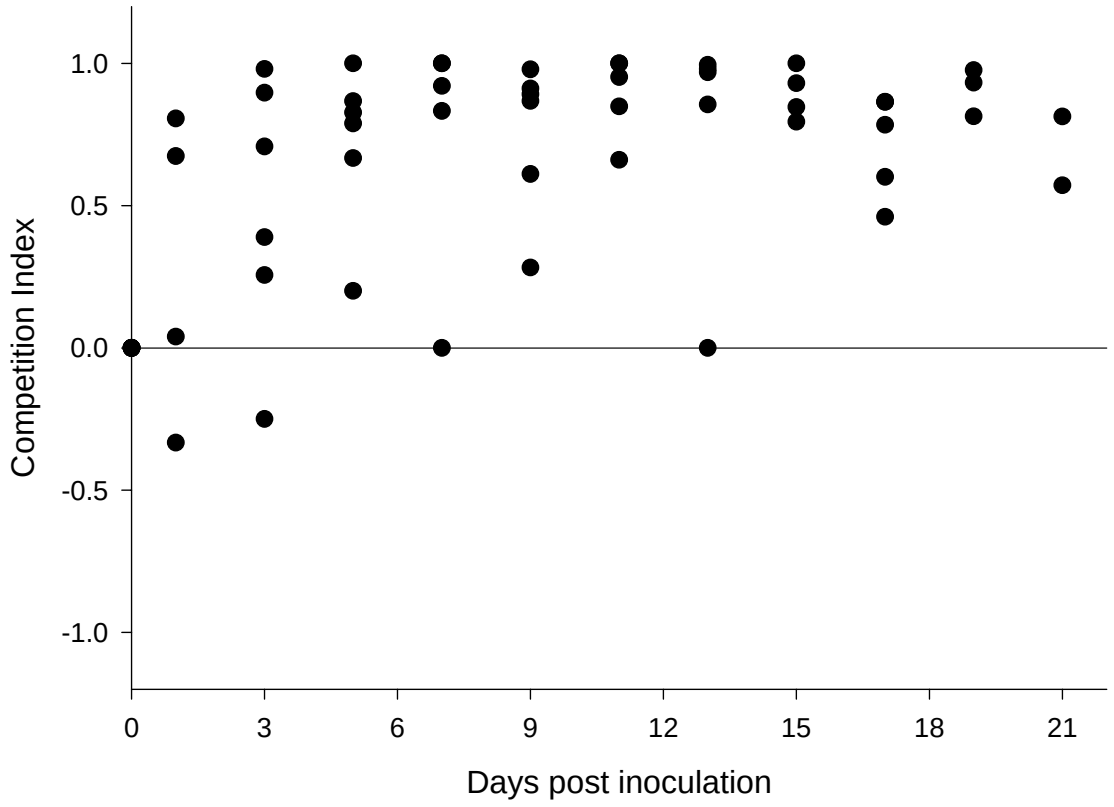


Figure 4. *In vivo* competition experiments in neonatal calves (n = 6) between Nal^r SSuT and Nal^r susceptible strains. Each circle represents a sample from a single calf. CI =(X-Y)/(X+Y) where X – number of SSuT colonies and Y- number of susceptible colonies. When CI = 0 there is an equal proportion of SSuT and Nal^r susceptible strains in the sample.

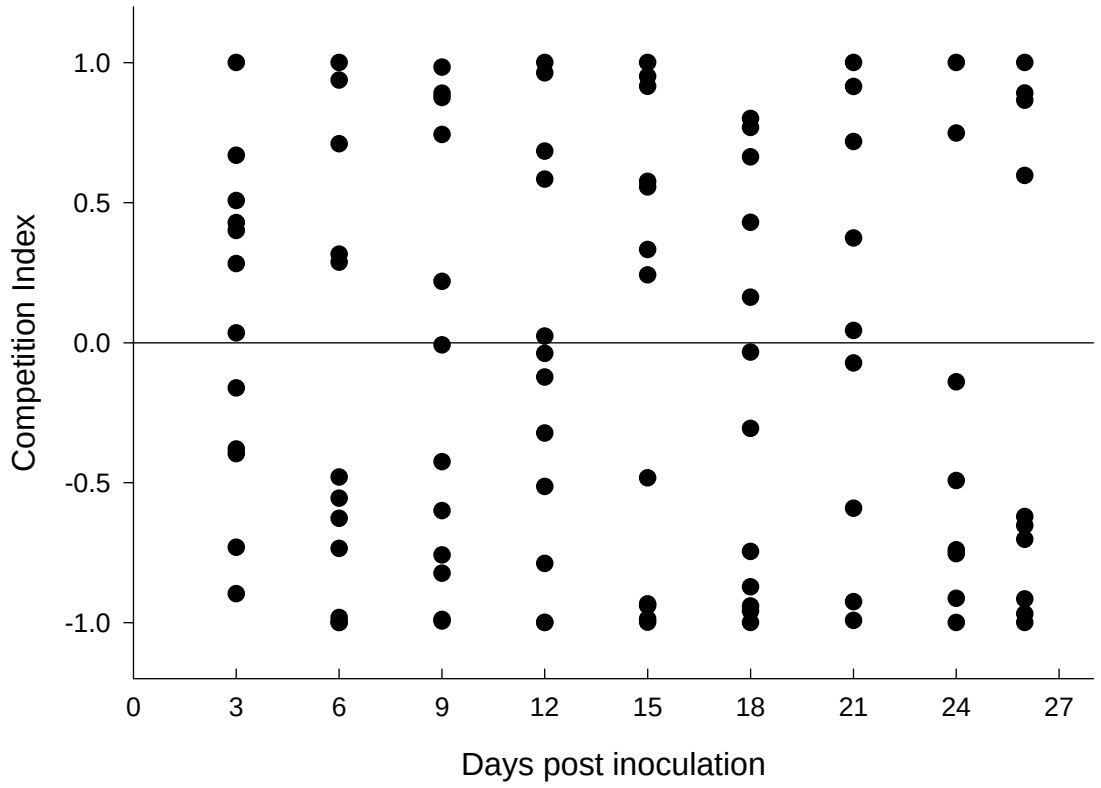


Figure 5. *In vivo* competition experiments in heifers (n = 12) between Nal^r SSuT and Nal^r susceptible strains. Each circle represents a sample from a single heifer.

CI =(X-Y)/(X+Y) where X – number of SSuT colonies and Y- number of susceptible colonies. When CI = 0 there is an equal proportion of SSuT and Nal^r susceptible strains in the sample.

CHAPTER 2

Antimicrobial Drug Resistance Genes do not Convey Secondary Fitness Advantage to Calf-Adapted *Escherichia coli*

ABSTRACT

Maintenance of the antimicrobial drug resistance in bacteria can be influenced by factors unrelated to direct selection pressure such as close linkage to other selectively advantageous genes, and secondary advantage conveyed by antimicrobial resistance genes in the absence of drug selection. Our previous trials at a dairy showed that the maintenance of the antimicrobial resistance genes is not influenced by specific antimicrobial selection, and that the most prevalent antimicrobial resistance phenotype of *E. coli* is specifically selected for in young calves. In this paper we examine the role of secondary advantages conveyed by antimicrobial resistance genes. We tested antimicrobial susceptible null mutant strains for their ability to compete with their progenitor strains *in vitro* and *in vivo*. The null mutant strains were generated by selection for spontaneous loss of the resistance genes in broth supplemented with fusaric acid or nickel chloride. On average, the null mutant strains were as competitive as the progenitor strains *in vitro* and in newborn calves (*in vivo*). Inoculation of newborn calves at the dairy with antimicrobial susceptible strains of *E. coli* did not impact the prevalence of antimicrobial resistant *E. coli*. Our results demonstrate that the antimicrobial resistance genes are not responsible for the greater fitness advantage of antimicrobial resistant *E. coli* in calves, but the farm environment and the diet clearly exert critical selective pressures responsible for the maintenance of antimicrobial resistance genes. Our current

hypothesis is that the antimicrobial resistance genes are linked to other genes responsible for differential fitness in dairy calves.

INTRODUCTION

Antimicrobial drug resistant bacteria present a significant risk to public health, and consequently there is great interest in reducing the prevalence of antimicrobial drug resistance (AR) genes in both commensal and pathogenic bacteria. One strategy to decrease the prevalence of AR bacteria is to discontinue using antimicrobial drugs for growth promotion and prophylaxis in food animals (4, 11, 22, 43, 44). Unfortunately, this strategy has produced mixed results that are dependent on the duration and amount of antimicrobial drug use (10, 16, 24, 25, 37, 38, 41). While pathogenic bacteria are the primary concern, commensal bacteria are an important reservoir for AR genes. It is hypothesized that because AR genes can easily transfer among diverse bacterial species (6, 29, 32, 35, 39, 45), then they can also move easily from commensal to pathogenic bacteria. Consequently abundance and ease of acquisition of the antimicrobial drug resistance genes ultimately complicates the orthodox treatment of diverse bacterial diseases (13, 18).

It is generally accepted that the prevalence of AR bacteria is directly correlated with antimicrobial drug use (19, 20, 40), but mechanisms unrelated to specific antimicrobial drug selection have also been implicated in wide distribution and maintenance of AR genes. Some of these mechanisms include plasmid addiction (14, 17), close linkage to other selectively advantageous genes (2, 9, 16, 21), and in some

instances secondary advantage can be conveyed by the AR genes in the absence of specific antimicrobial drug selection (15, 27, 36).

Previously we have shown that a young cohort of dairy cattle harbor a higher prevalence of antimicrobial drug resistant *E. coli* than older cattle at the Washington State University (WSU) dairy. In that study the predominant AR phenotype was SSuT (resistant to streptomycin, sulfadiazine, tetracycline and susceptible to ampicillin, chloramphenicol, and nalidixic acid) with these strains comprising ~60% of all *E. coli* isolated from calves (< 3 mo old) (23). We also demonstrated that the absence of the antimicrobial drug selection did not impact the level in antimicrobial drug resistant *E. coli*, at least short-term. Furthermore, when compared to antimicrobial susceptible strains, the SSuT *E. coli* had a significant fitness advantage in dairy calves but not in older cows. Thus younger calves represented a clear reservoir for antimicrobial drug resistance genes on the dairy farm, but the mechanism(s) responsible for maintenance of these strains was not immediately apparent.

Clearly, determining mechanisms responsible for the selection and maintenance of AR *E. coli* may allow formulation of novel strategies to decrease the prevalence of AR bacteria. Our previous study showed that antimicrobial drug selection pressure is not needed to maintain AR resistant strains of *E. coli*. In the present study we test the hypothesis that the AR genes harbored by the SSuT strains convey secondary fitness advantage(s) that permit maintenance of these strains in the dairy population. To test this hypothesis we generated null mutants and tested their ability to compete with other strains both *in vitro* and *in vivo.*

MATERIALS AND METHODS

Bacterial strains.

The strains used for the inoculation experiments were isolated from calves (1-45 days old) and cows (>1 year). The strains represented two main AR patterns: "susceptible" (susceptible to ampicillin 16 μg/ml, chloramphenicol 16 μg/ml, nalidixic acid 18 μg/ml, streptomycin 12 μg/ml, sulfadiazine 512 μg/ml and tetracycline 10 μg/ml) and SSuT (resistant to streptomycin, sulfadiazine and tetracycline in the same concentrations). Resistance phenotypes were determined as described below. In some cases strains with spontaneous mutations for nalidixic acid resistance (18 ug/ml) were used because the Nal^r phenotype is a useful marker for these experiments. Selection of Nal^r strains followed procedures described by Khachatryan et al. (23). Table 1, shows the distribution of these strains among three different experiments.

Selection for null mutants.

Because media supplemented with fusaric acid and heat inactivated chlortetracycline hydrochloride is toxic to *E. coli* when a functional tetracycline efflux pump is expressed (5, 28), we used this media to select for strains of *E. coli* that spontaneously lose tetracycline and other antimicrobial drug resistance genes. Specifically two distinct SSuT Nal^r (nalidixic acid resistant) *E. coli* strains were separately incubated in the above mentioned broth media at 37.0ºC on a shaker (200 rpm). Overnight culture (100 ul) was transferred into fresh media (5 ml) for 14 consecutive days. On days 3, 6, 9, and 14, a dilution of overnight culture from each serial passage experiment was plated on a non-selective media and grown overnight. The

following day 96 colonies from each passage experiment were picked and tested for antimicrobial drug susceptibility using agar dilution at breakpoint concentrations (23). Antimicrobial drug (Sigma, USA) susceptibilities were tested using IsoSensitest agar (IA) medium (Oxoid, USA) supplemented with tetracycline (10 μg/ml), streptomycin (12 μg/ml), sulfadiazine (512 μg/ml). Replicated test plates included a final plate of antimicrobial drug free medium to confirm inoculum delivery. Three isolates from each SSuT that became susceptible to the above mentioned antimicrobial drugs (called "cured-SSuTs"), were chosen for further analysis. The analysis included validation of antimicrobial drug susceptibility by disk diffusion assay conforming to NCCLS guidelines (26), screening for absence of AR genes by PCR (*tet*B (8), *str*A and *str*B (16), and *sul*2 (16)) and pulsed-field gel electrophoresis (PFGE) using the *Xba*I enzyme (12) to confirm similarity to the progenitor resistant strain. Quality control organisms used for the disk diffusion assay included: *E. coli* ATCC 25922; *S. aureus* ATCC 25923; *P. aeruginosa* ATCC 27853; and *E. coli* ATCC 10536.

Media supplemented with nickel chloride and heat inactivated chlortetracycline hydrochloride has also been shown to be effective for selecting tetracycline resistant null mutants (34, 42). Therefore, we also used this media to select for susceptible strains of *E. coli* that had spontaneously lost tetracycline and other antimicrobial drug resistance genes. Ten distinct SSuT *E. coli* strains were separately incubated in LB (Luria Bertani) broth supplemented with 0.6 mM nickel chloride and 50 ug/ml heat inactivated (121º C for 20 min) chlortetracycline hydrochloride at 37.0ºC on a shaker (200 rpm). Overnight culture (100 ul) was transferred into fresh media (5 ml) for 6 consecutive days. Three

antimicrobial drug susceptible isolates (cured-SSuTs) from each strain were selected and confirmed as described in the fusaric acid selection method.

In vitro competition experiments.

Each cured-SSuT isolate that was generated from SSuT strains was subjected to *in vitro* competition (23) (a) with a mixture of 5 wild-type susceptible *E. coli* isolated from calves; (b) with its progenitor resistant SSuT strain; and (c) with progenitor resistant SSuT strain that was acquired from the final passage in corresponding selective media ("media adapted progenitor"). After overnight incubation at 37.0ºC on a shaker (200 rpm), equal mixtures of cured-SSuT and competitor strains were combined and serially passaged (10 ul) into fresh LB broth (3 ml) for 8 consecutive days. On day eight, a competition index was calculated by estimating the colony forming units (CFU)/ml for the resistant and susceptible strains. The number of resistant colonies was counted on fresh LB agar supplemented with streptomycin (12 μg/ml), whereas the number of susceptible colonies was determined by subtracting the number of resistant colonies from the same dilution grown on Luria Bertani agar media without antimicrobial drugs. Three replicate counts were made and averaged. The competition index (CI) was calculated as (X-Y)/(X+Y), where X was the number of resistant colonies and Y was the number of susceptible colonies. CI values approaching +1 indicated dominance by resistant strains, whereas CI values approaching -1 indicated dominance by susceptible strains.

In vivo competition experiments between SSuT and cured-SSuT strains.

In vivo competition experiments were conducted in BL-2 isolation rooms and included six neonatal calves in the experimental group (24-48 hours old) and three neonatal calves in the control group (24-48 hours old). Neonatal calves in the experimental group were inoculated *per os* with two strains of cured-SSuT (antimicrobial drug susceptible null mutant) Nal^r and their progenitor SSuT Nal^r *E. coli* strains (10^9 CFU of each). Neonatal calves in the control group were inoculated *per os* with two wild-type antimicrobial drug susceptible (Nal^r) strains and two SSuT (Nal^r) *E. coli* strains (10^9 CFU of each). Neonatal calves were sampled every other day until shedding of inoculum bacteria was not detectable (up to 21 day post inoculation). To determine CFU/ml for SSuT and susceptible *E. coli in vivo* experiments, 1 gram of freshly collected fecal samples was serially diluted in PBS (peptone buffered saline) and ten fold dilutions were plated on specific selective media. Competition index (CI) values were calculated as described above where colony counts were averaged within animal (*in vivo*) across time points and the null hypothesis (CI = 0) was tested using a Student's *t* test. All *in vivo* inoculation studies were approved by the WSU Institutional Animal Care and Use Committee.

In vivo inoculation of antimicrobial drug susceptible strains at a dairy farm.

In vivo inoculation experiments were carried out at the Holstein dairy farm (Washington State University [WSU], Pullman, Wash.). The farm is a closed dairy herd (n = 167), where all the replacement heifers are raised on site. Calves are separated from their mothers 24-48 hours after birth and moved to a separate building where they are

housed in individual pens. Calves are weaned at 4-6 weeks of age, but stay in the calf pens for 90 day, after which they are moved to a separate heifer barn. The dairy follows accepted bio-security standards in order to prevent introduction of pathogens from outside. All the food is raised off site and purchased commercially. No antimicrobial drugs were used for chemotherapeutics throughout the duration of the experiment in calves.

The *in vivo* inoculation experiment at the farm involved 30 neonatal calves randomly assigned to one of the three groups. Group one received six cured-SSuT strains *per os* (2×10^9 CFU of each) once within first two days of life (24-36 hours) and second time seven days later. Group two received six antimicrobial drug susceptible wild-type strains in a similar way to group one. Group three received volumetric equivalent of sterile media similar to the other two groups. Each calf was sampled every five days for 3 months. Fecal samples were collected with sterile tongue depressors, and placed into sterile bags. Collected samples were streaked for isolation on 3 separate Violet Red Bile Agar with 4-Methylumbelliferyl-β-D-glucuronide (VRB-MUG) plates (VRB from Remel, USA; MUG from Biosyth Ag, Switzerland) within 4 hr after collection and were incubated overnight at 37°C. Twenty one (21) presumptive *E. coli* colonies (pink coloration and fluorescence under UV light) per animal, 7 per sample plate were used to inoculate EC (*E. coli*) medium (Remel, USA) with MUG (EC-MUG) broth (200 µl) in a 96-well plate format, leaving 8-24 non-inoculated, negative control wells. Each 96-well plate also included two positive control isolates, Q-89 and Q-90, resistant and susceptible to all tested antimicrobial drugs respectively. The 96-well plates were then incubated at 44.5°C overnight and the MUG reaction was confirmed under UV light.

Presumptive *E. coli* were tested for antimicrobial drug susceptibility using agar dilution at breakpoint concentrations (23). Antimicrobial drug (Sigma, USA) susceptibilities were tested using Mueller Hinton agar (MH) medium (Hardy Diagnostics, USA) supplemented with ampicillin (16 μg/ml), tetracycline (10 μg/ml), chloramphenicol (16 μg /ml), streptomycin (12 μg/ml), sulfadiazine (512 μg/ml), and nalidixic acid (18 ug/ml). Replicated test plate series included a final plate of antimicrobial drug free media to confirm inoculum delivery. The results of replicator assays were recorded after overnight incubation at 37°C. Results for antimicrobial drug plates were coded as a dichotomous variable: zero for no growth and one for growth. These results were used to calculate the frequencies for different resistance patterns.

Data was entered and analyzed with Microsoft Excel and NCSS 2001 (NCSS Statistical Software, Kaysville, UT). Student-Newman Keuls multiple comparison test was used to tests for statistical differences in the prevalence of resistant bacteria between groups.

RESULTS

Spontaneous loss of antimicrobial drug resistance genes in strains of E. coli.

Media supplemented with either fusaric acid or nickel chloride allowed selection for loss of tetracycline and associated streptomycin and sulfadiazine resistance genes. Ten SSuT strains were passaged and on days 6, 10, and 14, 96 isolates from each tube were assessed for antimicrobial resistance. Media supplemented with 0.6 mM nickel chloride was more efficient (90%; 6 days) in selecting detectable levels of antimicrobial drug susceptible strains (cured-SSuTs), compared to media supplemented with fusaric

acid (21%; 14 days). These results were confirmed by PCR for *tet*(B), *sul*2, *str*A and *str*B resistance genes. Media supplemented with fusaric acid also selected for isolates missing only the tetracycline resistance gene (72%; 14 days), isolates missing tetracycline and streptomycin resistance genes (2%; 14 days), and isolates missing tetracycline and sulfadiazine resistance genes only (1%: 14 days). These results were confirmed using a disk diffusion assay. The close correspondence between loss of tetracycline and other AR genes suggests close physical linkage between these genes on a genetic unit (plasmid, transposon, phage).

In vitro competition experiments with cured-SSuT strains.

In vitro competition experiments tested the cured-SSuT strains that were selected in two different selective mediums. From each SSuT strain three susceptible isolates were tested to control for spontaneous changes unique to individual bacteria. The three cured-SSuT isolates were tested in competition experiments individually and as a mixture.

Cured-SSuT isolates (n = 2) selected in media supplemented with fusaric acid (cured-SSuT (Fus)) consistently outcompeted progenitor SSuT strains, progenitor SSuT strains that retained their AR genes after 14 day passage in broth culture with fusaric acid ("media adapted progenitors"), and wild type susceptible strains (Student's one sample *t* test; $P < 0.001$) (Fig 1). The cured-SSuT isolates (n = 8) selected in media supplemented with nickel chloride (cured-SSuT (NiCl)) outcompeted wild type susceptible strains (Student's one sample *t* test; $P < 0.001$), but were equal in competitive ability with progenitor AR SSuT strains (Student's one sample *t* test; $P = 0.413$) and with media adapted progenitors (6 day passage in broth with nickel chloride) (Student's one sample *t*

test; $P = 0.021$) (Fig 1). At the individual strain level, competition results were variable (Table 2). This variation is consistent with a diverse background of *E. coli* harboring the SSuT genes.

In vivo competition between cured-SSuT and SSuT strains in isolation.

We used a calf challenge model to determine if cured-SSuT strains maintained their competitive advantage *in vivo*. Newborn calves (24-48 hours) were inoculated with Nal^r strains of cured-SSuT and Nal^r strains of SSuT (with 10^9 CFU of each) (Table 1). Consistent with previously published findings (23), SSuT Nal^r *E. coli* in the control group out-competed wild type susceptible Nal^r strains in neonatal calves (Student's *t* test; $P < 0.001$) (Fig.2). Cured-SSuT Nal^r *E. coli* were generally more competitive than parent SSuT strains in the neonatal intestinal environment (Student's *t* test; $P = 0.002$) (Fig. 2). A direct comparison of cured-SSuT and susceptible strains would only be possible if we introduced an additional antimicrobial selection marker. Nevertheless, because cured-SSuT strains were at least as competitive as progenitor strains, we inferred that the cured-SSuT strains would also out-compete susceptible strains in this calf model. Shedding of Nal^r *E. coli* from the experimental animals was not detectable 21 days after inoculation. None of the animals in the *in vivo* studies shed detectable fecal Nal^r *E. coli* prior to inoculation (0-3 days).

Inoculation of antimicrobial drug susceptible strains at a dairy farm.

Because the select cured-SSuT strains from the isolation experiment maintained competitive fitness *in vivo*, we tested the hypothesis that the cured-SSuT strains (selected

in broth supplemented with nickel chloride) could displace naturally occurring SSuT strains in a farm environment. New cured-SSuT strains were used because (a) nalidixic acid susceptible strains were to be used in the field environment and (b) since nickel chloride supplemented media selects for cured-SSuT strains more efficiently, there is less time for adaptation of the strains to the *in vitro* media environment. Cured-SSuT strains that were generally more competitive than their progenitor strains *in vitro* were used *in vivo*.

Neonatal calves were inoculated with either 6 cured-SSuT strains (Nal^S) (Table 1), or 6 wild-type susceptible strains (Nal^S) (Table 1), or "media only" within 24-36 hours of birth and 7 days later. We assessed percentage of resistant bacteria 2 times weekly for 3 months under the hypothesis that competition exclusion would lead to a significant decrease in SSuT and strains within the cured-SSuT treatment group. Among the three inoculation groups, however, there was no statistically significant difference in the level of antimicrobial drug resistance to ampicillin, chloramphenicol, streptomycin, sulfadiazine and tetracycline (Fig. 3). There was no statistically significant difference among the three inoculation groups for main resistance patterns (< 2%), except for the group inoculated with cured-SSuTs had significantly more AST (resistant to ampicillin, streptomycin and tetracycline) resistant *E. coli* (Fig. 4). Thus it appears that the cured-SSuT strains were not effective in displacing native SSuT strains at the dairy. One possible explanation for no treatment effect was a selective sweep where our cured-SSuT strains could have contaminated and dominated all treatment groups. To test this hypothesis we compared 30 susceptible isolates (acquired at 2-4 weeks post inoculation from the three groups (3 animals per group) to the inoculum ex-SSuT isolates by PFGE.

All "susceptible" isolates (n = 10) from the group inoculated with cured-SSuT *E. coli* were indistinguishable from the cured-SSuT inoculum strain SSuT-98. None of the "susceptible" isolates from the other two groups shared a distinct fingerprint with any of the cured-SSuT inoculum strains. Also there were no identical fingerprints between the groups receiving wild-type susceptible *E. coli* and "media only" (data not shown).

DISCUSSION

Antimicrobial drug resistance is a significant public health concern that calls for reducing the factors that contribute to the emergence and maintenance of antimicrobial drug resistance. Food animal production consumes large amounts of antimicrobial drugs and thus can serve as an important arena for emergence and maintenance of AR (1, 3, 46). Nevertheless, it is not clear that eliminating all non-therapeutic antimicrobial use in animal production will result in significant reduction in prevalence of AR bacteria (7, 10, 24, 25, 37, 41). This is because many factors unrelated to antimicrobial use are also implicated in the increase in prevalence of AR; including animal stress (30, 31), close linkage to other selectively advantageous genes (2, 9, 16, 21), plasmid addiction (14, 17), and secondary advantage conveyed by the AR genes (15, 27, 36).

Our previous work at the WSU dairy showed that calves shed more antimicrobial drug resistant *E. coli* compared to older cattle, making them a clear reservoir of AR genes on a farm. In that study *E. coli* with the SSuT resistance pattern were shed at much higher level compared to older cattle (~60% vs. ~5%). A short term removal of oxytetracycline from a dietary supplement did not affect the high prevalence of AR *E. coli* in calves, suggesting that other mechanisms were responsible for maintaining the SSuT strains at

the dairy. That is, it is possible that the AR genes were being selected in the presence of naturally occurring antimicrobial analogs or that these genes conferred a secondary, but unrecognized selective advantage in this environment.

Secondary advantages might include cases where the *tet*(B) proton pump interacts with substrates other than tetracycline and its analogs, or there might be a need for increased folate synthesis conveyed by the *sul*2 gene. There might also be nonspecific but important phosphotransferase activity of the *str*A/*str*B genes. These potential benefits must be balanced with potential costs. For example, pioneering experiments by Dr. Lenski's group showed a fitness reduction for *E. coli* when the *tet*(B) gene is expressed and very little cost associated with the gene when it is not expressed (33). We determined that the SSuT strains from our study do not express the *tet*(B) gene *in vitro* except in the presence of a tetracycline analog (data not shown). This does not exclude the possibility that the gene will be expressed in the farm environment. There is very little data in the literature that specifically examines the effect of *sul*2 (15) and *str*A/*str*B resistance genes on fitness of bacteria.

To test the hypothesis that resistance genes convey secondary fitness advantages, we developed null mutant strains of SSuT *E. coli* (cured-SSuT) and tested these in competition experiments (*in vitro*) and in isolation (*in vivo*). There was variation in the competition outcome for individual strains (Table 2), but on average the cured-SSuT strains were at least as competitive as parental SSuT strains for *in vitro* and *in vivo* competition experiments. This data suggested that there are no significant secondary advantages attributable to the SSuT genes in calves. The study only tested the interaction between the calf and the *E. coli* in isolation, and did not account for possible

environmental selective pressures that are probably present in a farm environment. It is also worth noting that the innoculum strains were only observed for 21 days post inoculation.

Based on the finding that the AR genes do not influence the competitive outcome *in vitro* and *in vivo*, we tested the hypothesis that inoculation of ex-SSuT and other susceptible strains would decrease the level (via competition exclusion) of AR *E. coli* shed from calves in the farm environment. Contrary to our prediction, the results of the study did not show any treatment effect. Interestingly, when compared to prevalence of AR *E. coli* in year 2001(23), there was nearly 50% decrease in number of SSuT *E. coli* in all inoculation groups for the present study and this may have significantly reduced the statistical power of our experiment. PFGE fingerprinting of susceptible isolates from all three groups (n = 30) showed that the reduced level of SSuT *E. coli* was not due to a selective sweep by any of the cured-SSuT or "susceptible" strains.

In conclusion, we have now dismissed two of the three hypotheses for the maintenance of SSuT strains at the WSU dairy. Neither direct selection from antimicrobial drugs or secondary benefits from the AR genes are responsible for maintenance of SSuT strains at our study facility. The remaining hypothesis is that the gene(s) conferring selective advantage to SSuT strains in dairy calves is located proximally to the resistance genes, probably associated with a horizontally transmissible element.

REFERENCES

1. **Aarestrup, F. M.** 1999. Association between the consumption of antimicrobial agents in animal husbandry and the occurrence of resistant bacteria among food animals. Int. J. Antimicrob. Agents **12:**279-285.
2. **Aarestrup, F. M.** 2000. Characterization of glycopeptide-resistant *Enterococcus faecium* (GRE) from broilers and pigs in Denmark: genetic evidence that persistence of GRE in pig herds is associated with coselection by resistance to macrolides. J. Clin. Microbiol. **38:**2774-2777.
3. **Aarestrup, F. M., H. Kruse, E. Tast, A. M. Hammerum, and L. B. Jensen.** 2000. Associations between the use of antimicrobial agents for growth promotion and the occurrence of resistance among *Enterococcus faecium* from broilers and pigs in Denmark, Finland, and Norway. Microb. Drug Resist. **6:**63-70.
4. **Aarestrup, F. M., A. M. Seyfarth, H. D. Emborg, K. Pedersen, R. S. Hendriksen, and F. Bager.** 2001. Effect of abolishment of the use of antimicrobial agents for growth promotion on occurrence of antimicrobial resistance in fecal enterococci from food animals in Denmark. Antimicrob. Agents Chemother. **45:**2054-2059.
5. **Bochner, B. R., H. C. Huang, G. L. Schieven, and B. N. Ames.** 1980. Positive selection for loss of tetracycline resistance. J. Bacteriol. **143:**926-933.
6. **Boyd, E. F., and D. L. Hartl.** 1997. Recent horizontal transmission of plasmids between natural populations of *Escherichia coli* and *Salmonella enterica*. J. Bacteriol. **179:**1622-1627.

7. **Bywater, R. J.** 2004. Veterinary use of antimicrobials and emergence of resistance in zoonotic and sentinel bacteria in the EU. J. Vet. Med. B. Infect. Dis. Vet. Public Health **51:**361-363.

8. **Call, D. R., M. K. Bakko, M. J. Krug, and M. C. Roberts.** 2003. Identifying antimicrobial resistance genes with DNA microarrays. Antimicrob. Agents Chemother. **47:**3290-3295.

9. **Calomiris, J. J., J. L. Armstrong, and R. J. Seidler.** 1984. Association of metal tolerance with multiple antibiotic resistance of bacteria isolated from drinking water. Appl. Environ. Microbiol. **47:**1238-1242.

10. **Chaslus-Dancla, E., G. Gerbaud, M. Lagorce, J. P. Lafont, and P. Courvalin.** 1987. Persistence of an antibiotic resistance plasmid in intestinal *Escherichia coli* of chickens in the absence of selective pressure. Antimicrob. Agents Chemother. **31:**784-788.

11. **Cook, R.** 1999. EU ban on four antibiotic growth promoters. Vet. Rec. **144:**158.

12. **Davis, M. A., D. D. Hancock, T. E. Besser, and D. R. Call.** 2003. Evaluation of pulsed-field gel electrophoresis as a tool for determining the degree of genetic relatedness between strains of *Escherichia coli* O157:H7. J. Clin. Microbiol. **41:**1843-1849.

13. **Desenclos, J. C., and D. Guillemot.** 2004. Consequences of bacterial resistance to antimicrobial agents. Emerg. Infect. Dis. **10:**759-760.

14. **Engelberg-Kulka, H., and G. Glaser.** 1999. Addiction modules and programmed cell death and antideath in bacterial cultures. Annu. Rev. Microbiol. **53:**43-70.

15. **Enne, V. I., P. M. Bennett, D. M. Livermore, and L. M. Hall.** 2004. Enhancement of host fitness by the sul2-coding plasmid p9123 in the absence of selective pressure. J. Antimicrob. Chemother. **53:**958-963.

16. **Enne, V. I., D. M. Livermore, P. Stephens, and L. M. Hall.** 2001. Persistence of sulphonamide resistance in *Escherichia coli* in the UK despite national prescribing restriction. Lancet **357:**1325-1328.

17. **Gerdes, K., P. B. Rasmussen, and S. Molin.** 1986. Unique type of plasmid maintenance function: postsegregational killing of plasmid-free cells. Proc. Natl. Acad. Sci. U S A **83:**3116-3120.

18. **Helms, M., P. Vastrup, P. Gerner-Smidt, and K. Molbak.** 2002. Excess mortality associated with antimicrobial drug-resistant *Salmonella typhimurium*. Emerg. Infect. Dis. **8:**490-495.

19. **Hinton, M., D. J. Hampson, E. Hampson, and A. H. Linton.** 1985. The effects of oxytetracycline on the intestinal *Escherichia coli* flora of newly weaned pigs. J. Hyg. (London) **95:**77-85.

20. **Jackson, C. R., P. J. Fedorka-Cray, J. B. Barrett, and S. R. Ladely.** 2004. Effects of tylosin use on erythromycin resistance in enterococci isolated from swine. Appl. Environ. Microbiol. **70:**4205-4210.

21. **Kehrenberg, C., and S. Schwarz.** 2001. Occurrence and linkage of genes coding for resistance to sulfonamides, streptomycin and chloramphenicol in bacteria of the genera *Pasteurella* and *Mannheimia*. FEMS Microbiol. Lett. **205:**283-290.

22. **Kelly, L., D. L. Smith, E. L. Snary, J. A. Johnson, A. D. Harris, M. Wooldridge, and J. G. Morris, Jr.** 2004. Animal growth promoters: to ban or not to ban? A risk assessment approach. Int. J. Antimicrob. Agents **24:**205-212.

23. **Khachatryan, A. R., D. D. Hancock, T. E. Besser, and D. R. Call.** 2004. Role of calf-adapted *Escherichia coli* in maintenance of antimicrobial drug resistance in dairy calves. Appl. Environ. Microbiol. **70:**752-757.

24. **Klare, I., D. Badstubner, C. Konstabel, G. Bohme, H. Claus, and W. Witte.** 1999. Decreased incidence of vanA-type vancomycin-resistant enterococci isolated from poultry meat and from fecal samples of humans in the community after discontinuation of avoparcin usage in animal husbandry. Microb. Drug Resist. **5:**45-52.

25. **Langlois, B. E., K. A. Dawson, T. S. Stahly, and G. L. Cromwell.** 1984. Antibiotic resistance of fecal coliforms from swine fed subtherapeutic and therapeutic levels of chlortetracycline. J. Anim. Sci. **58:**666-674.

26. **Lorian, V.** 1996. Antibiotics in Laboratory Medicine, 4th ed. Williams and Wilkins.

27. **Luo, N., S. Pereira, O. Sahin, J. Lin, S. Huang, L. Michel, and Q. Zhang.** 2005. Enhanced *in vivo* fitness of fluoroquinolone-resistant *Campylobacter jejuni* in the absence of antibiotic selection pressure. Proc. Natl. Acad. Sci. U S A **102:**541-546.

28. **Maloy, S. R., and W. D. Nunn.** 1981. Selection for loss of tetracycline resistance by *Escherichia coli*. J. Bacteriol. **145:**1110-1111.

29. **Mazodier, P., and J. Davies.** 1991. Gene transfer between distantly related bacteria. Annu. Rev. Genet. **25:**147-171.

30. **Moro, M. H., G. W. Beran, R. W. Griffith, and L. J. Hoffman.** 2000. Effects of heat stress on the antimicrobial drug resistance of *Escherichia coli* of the intestinal flora of swine. J. Appl. Microbiol. **88:**836-844.

31. **Moro, M. H., G. W. Beran, L. J. Hoffman, and R. W. Griffith.** 1998. Effects of cold stress on the antimicrobial drug resistance of *Escherichia coli* of the intestinal flora of swine. Lett. Appl. Microbiol. **27:**251-254.

32. **Netherwood, T., R. Bowden, P. Harrison, A. G. O'Donnell, D. S. Parker, and H. J. Gilbert.** 1999. Gene transfer in the gastrointestinal tract. Appl. Environ. Microbiol. **65:**5139-5141.

33. **Nguyen, T. N., Q. G. Phan, L. P. Duong, K. P. Bertrand, and R. E. Lenski.** 1989. Effects of carriage and expression of the Tn10 tetracycline-resistance operon on the fitness of *Escherichia coli* K12. Mol. biol. evol. **6:**213-25.

34. **Podolsky, T., S. T. Fong, and B. T. Lee.** 1996. Direct selection of tetracycline-sensitive *Escherichia coli* cells using nickel salts. Plasmid **36:**112-115.

35. **Poppe, C., L. C. Martin, C. L. Gyles, R. Reid-Smith, P. Boerlin, S. A. McEwen, J. F. Prescott, and K. R. Forward.** 2005. Acquisition of resistance to extended-spectrum cephalosporins by *Salmonella enterica* subsp. *enterica* serovar Newport and *Escherichia coli* in the turkey poult intestinal tract. Appl. Environ. Microbiol. **71:**1184-1192.

36. **Pruss, G. J., and K. Drlica.** 1986. Topoisomerase I mutants: the gene on pBR322 that encodes resistance to tetracycline affects plasmid DNA supercoiling. Proc. Natl. Acad. Sci. U S A **83:**8952-8956.

37. **Rollins, L. D., S. A. Gaines, D. W. Pocurull, H. D. Mercer, and L. T. Frobish.** 1976. Persistence of transferable drug resistance in the lactose-fermenting enteric flora of swine following antimicrobial feeding. Can. J. Compar. Med. **40:**175-183.

38. **Seppala, H., T. Klaukka, J. Vuopio-Varkila, A. Muotiala, H. Helenius, K. Lager, and P. Huovinen.** 1997. The effect of changes in the consumption of macrolide antibiotics on erythromycin resistance in group A streptococci in Finland. Finnish Study Group for Antimicrobial Resistance. N. Engl. J. Med. **337:**441-446.

39. **Shoemaker, N. B., H. Vlamakis, K. Hayes, and A. A. Salyers.** 2001. Evidence for extensive resistance gene transfer among *Bacteroides spp*. and among *Bacteroides* and other genera in the human colon. Appl. Environ. Microbiol. **67:**561-568.

40. **Singer, R. S., R. Finch, H. C. Wegener, R. Bywater, J. Walters, and M. Lipsitch.** 2003. Antibiotic resistance--the interplay between antibiotic use in animals and human beings. Lancet Infect. Dis. **3:**47-51.

41. **Smith, H. W.** 1975. Persistence of tetracycline resistance in pig *E. coli*. Nature **258:**628-30.

42. **Stavropoulos, T. A., and C. A. Strathdee.** 2000. Expression of the *tetA*(C) tetracycline efflux pump in *Escherichia coli* confers osmotic sensitivity. FEMS Microbiol. Lett. **190:**147-150.

43. **Taylor, D. J.** 1999. EU ban on four antibiotic growth promoters. Vet. Rec. **144:**158.

44. **Wierup, M.** 2001. The Swedish experience of the 1986 year ban of antimicrobial growth promoters, with special reference to animal health, disease prevention, productivity, and usage of antimicrobials. Microb. Drug Resist. **7:**183-190.

45. **Winokur, P. L., D. L. Vonstein, L. J. Hoffman, E. K. Uhlenhopp, and G. V. Doern.** 2001. Evidence for transfer of CMY-2 AmpC b-lactamase plasmids between *Escherichia coli* and *Salmonella* isolates from food animals and humans. Antimicrob. Agents Chemother. **5:**2716-2722.

46. **Witte, W.** 2000. Selective pressure by antibiotic use in livestock. Int. J. Antimicrob. Agents **16:**S19-S24.

TABLES

TABLE 1. Strains used *in vitro* and *in vivo*.

Strains	*In vitro* competition	Calf experiment in isolation	Farm experiment	Source	Resistance pattern
SSuT 22	X, X*	X*[b], X*	X [a]	Calf	SSuT
SSuT 25	X, X*	X*[b], X*	-	Calf	SSuT
SSuT 32	X	-	-	Calf	SSuT
SSuT 35	X	-	X [a]	Calf	SSuT
SSuT 45	X	-	X [a]	Calf	SSuT
SSuT 79	X	-	X [a]	Calf	SSuT
SSuT 84	X	-	X [a]	Calf	SSuT
SSuT 98	X	-	X [a]	Calf	SSuT
Pans 66	X, X*	X*	-	Calf	Susceptible wild-type
Pans 68	X, X*	X*	-	Calf	Susceptible wild-type
Pans 69	X, X*	X*	-	Calf	Susceptible wild-type
Pans 71	X, X*	X*	-	Calf	Susceptible wild-type
U3562	X	-	X	Calf	Susceptible wild-type
U3559	X	-	X	Calf	Susceptible wild-type
U3561	X	-	X	Calf	Susceptible wild-type
U3572	X	-	X	Cow	Susceptible wild-type
U3579	X	-	X	Cow	Susceptible wild-type
U3580	X	-	X	Cow	Susceptible wild-type

* Nalidixic acid resistant spontaneous mutants.

[a] Spontaneous susceptible null mutants of SSuT strains (cured-SSuT) selected in media supplemented with nickel chloride.

[b] cured-SSuT strains selected in media supplemented with fusaric acid.

TABLE 2. Results of the *in vitro* competition experiments between cured-SSuT and wild-type susceptible, or progenitor SSuT strains, or "media adapted progenitor" SSuT strains (passaged in $NiCl_2$). +, cured-SSuT strains are more competitive (competition index < 0.5); -, cured-SSuT strains are less competitive (competition index > -0.5); +/-, cured-SSuT strains are equally competitive (competition index between 0.5 and -0.5).

cured-SSuT isolates derived from (3 from each strain)	Competitiveness vs. wild-type susceptible strains	Competitiveness vs. progenitor SSuT strains	Competitiveness vs. "media adapted progenitor" SSuT strains ($NiCl_2$)
SSuT 22 (1, 2, 3)	+ + +	- +/- +	+ - +
SSuT 25 (1, 2, 3)	- - -	- - -	- - +
SSuT 32 (1, 2, 3)	+ + +	- - -	- - +
SSuT 35 (1, 2, 3)	+ + +	+ + +	- + -
SSuT 45 (1, 2, 3)	+ - +	- - -	- - -
SSuT 79 (1, 2, 3)	+/- + +	+ + +	+ - -
SSuT 84 (1, 2, 3)	- - -	+ + +	- - -
SSuT 98 (1, 2, 3)	+ + +	- - -	- - -

FIGURES

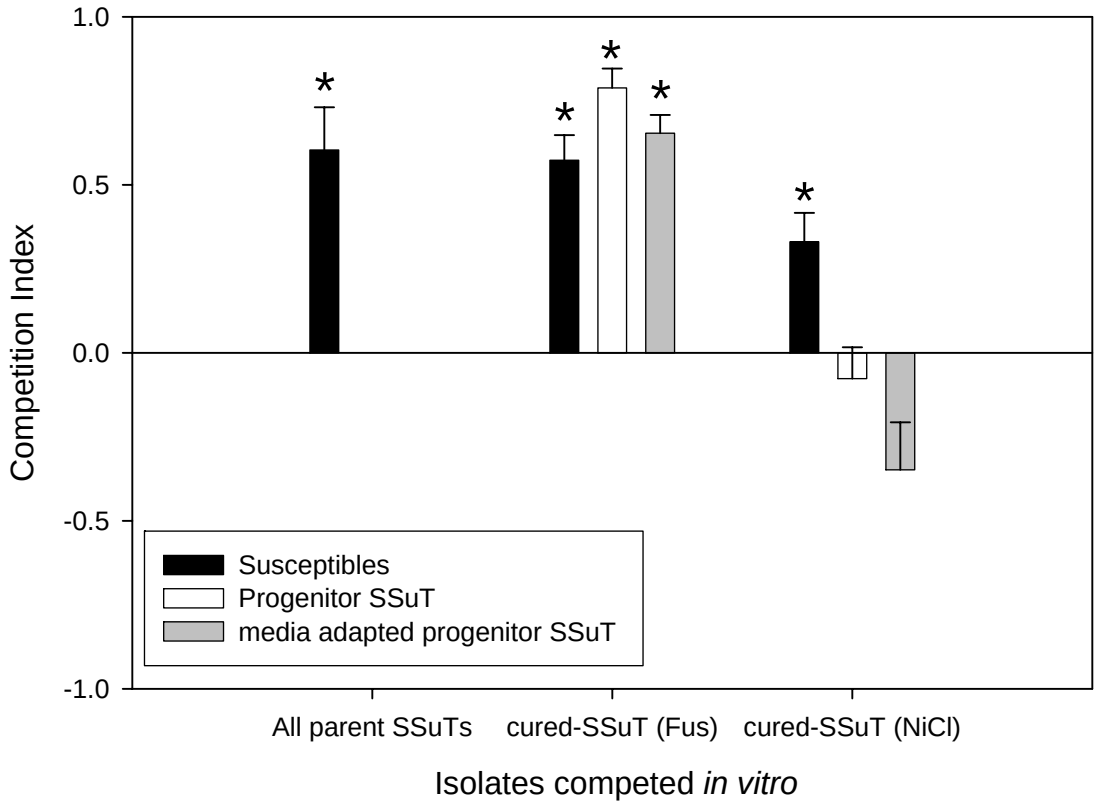


FIG. 1 *In vitro* competition experiments of SSuT and cured-SSuT strains with mixture of susceptible (black bars), progenitor SSuT (white bars) and passaged parent SSuT (grey bars) strains. Competition Index (CI) = (X – Y)/(X +Y), when values approach +1 strains listed on the x-axis dominate (* = dominant strains significantly > 0; one sample T-test, $P < 0.05$). When CI = 0, there is an equal proportion of the competing resistance patterns.

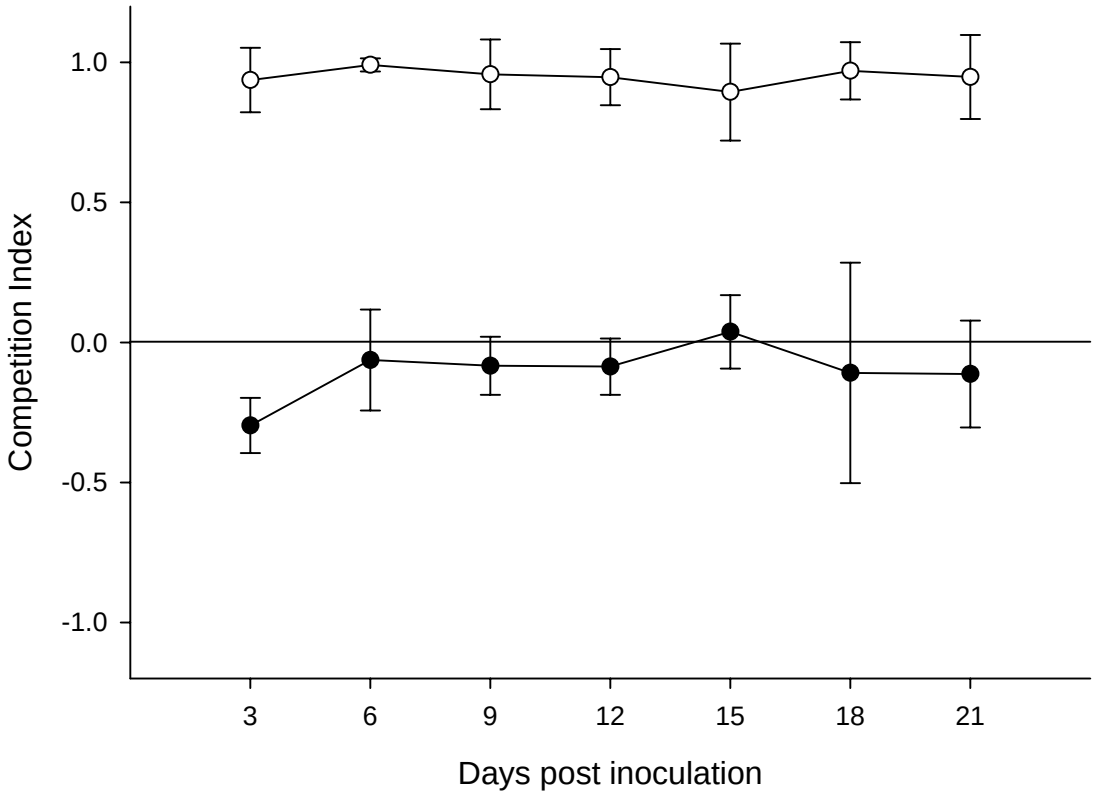


FIG. 2 Competition experiments in neonatal calves; control group (n = 3) between SSuT Nal^r and wild-type susceptible Nal^r strains (open circles), and the experimental group (n = 6) between SSuT Nal^r and cured-SSuT (susceptible) Nal^r strains (closed circles). Each circle represents the mean with the 95% confidence interval bars for time point shown on X-axis. Competition Index (CI) = (X – Y)/(X +Y) where X is number of resistant colonies and Y is number of susceptible colonies. When CI = 0, there is an equal proportion of the competing resistance patterns.

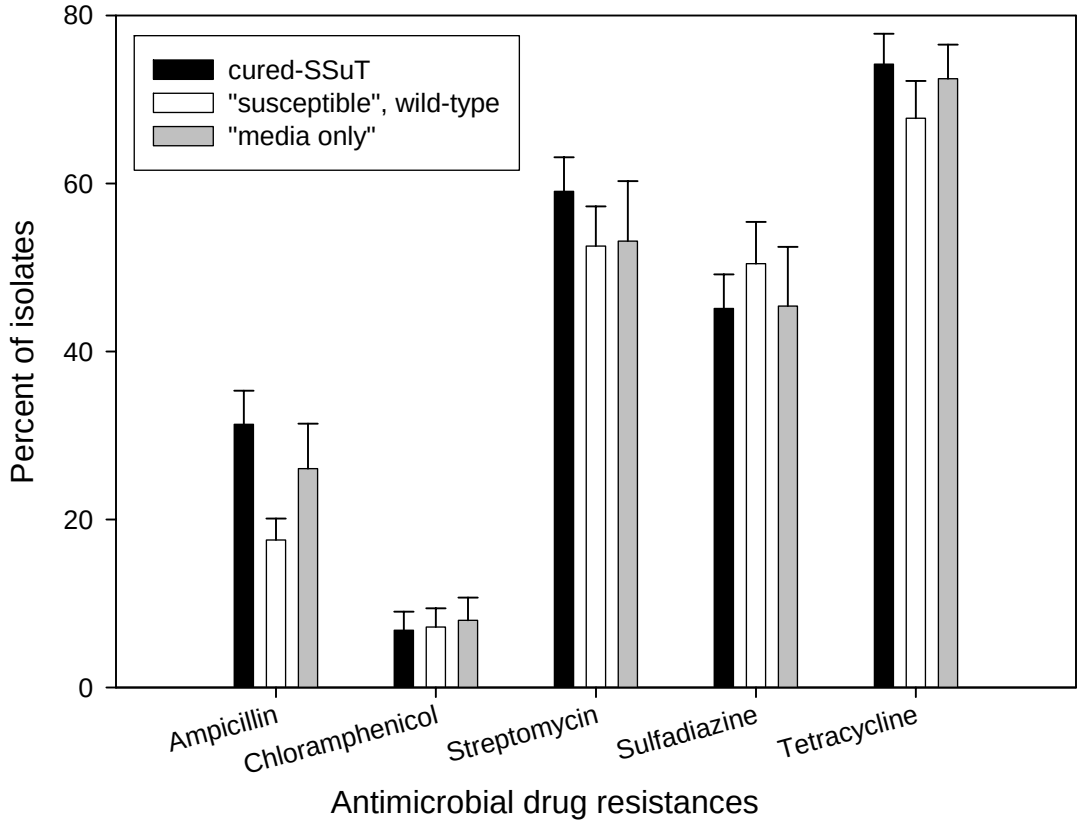


FIG. 3 Frequency of antimicrobial drug resistance for all *E. coli* shed from calves; cured-SSuT group (n = 3,415), "susceptible" wild-type group (n = 3,430) and "media only" group (n = 3,448). *, statistically significant ($P < 0.05$ with Student-Newman Keuls multiple comparison test for five tests); ns = non-significant.

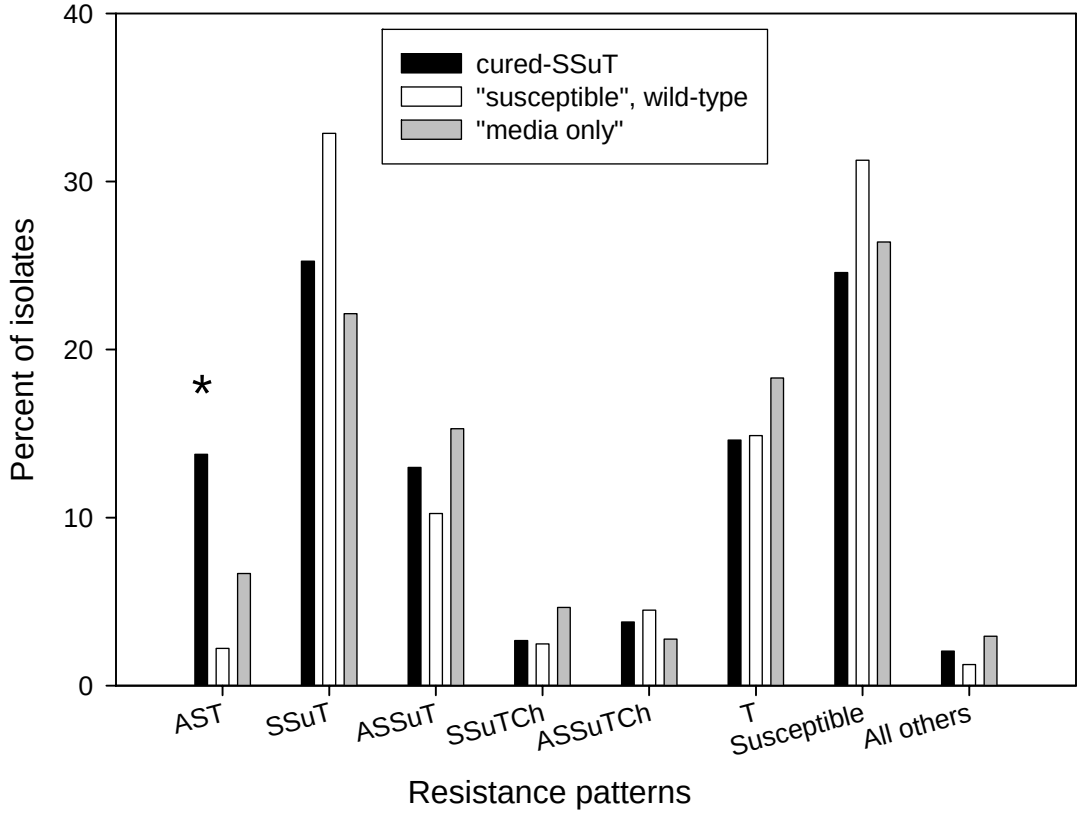


FIG. 4 Frequency of antimicrobial drug resistance patterns for all *E. coli* shed from calves; cured-SSuT group (n = 3,415), "susceptible" wild-type group (n = 3,430) and "media only" group (n = 3,448). * = statistically significant ($P < 0.05$ with Student-Newman Keuls multiple comparison test for seven tests); Resistance patterns are denoted by letters, where A = ampicillin, Ch = chloramphenicol, S = streptomycin, Su = Sulfadiazine, and T = tetracycline. Susceptible isolates were susceptible to all above mentioned antimicrobial drugs tested.

CHAPTER 3

Unmedicated Milk Supplement Provides Selection Pressure that Maintains Streptomycin-Sulfa-Tetracycline Resistant *Escherichia coli* on a Dairy Farm

ABSTRACT

Antimicrobial resistance genes can be maintained in a population (a) by direct selective pressure, (b) by secondary advantages conveyed by the resistance genes, or (c) by linkage to other selectively advantageous genes. Our previous experiments at a dairy demonstrated that (a) *E. coli* simultaneously resistant to streptomycin, sulfa and tetracycline (SSuT) are specifically selected for in neonatal calves, (b) specific antimicrobial selection does not influence the prevalence of antimicrobial resistant *E. coli* in calves, and (c) the antimicrobial resistance genes do not convey secondary advantages to SSuT *E. coli*. In this paper we examined how a milk supplement impacts the prevalence of SSuT *E. coli* by feeding oxytetracycline medicated and un-medicated milk supplement to neonatal calves. Calves receiving milk supplement had significantly higher prevalence of SSuT *E. coli* compared with the no-supplement control group (≈ 40% vs. ≈ 20% respectively). Addition of oxytetracycline to the supplement did not add any selective advantage over what was observed from the supplement alone. We compared the growth characteristics of SSuT and non-SSuT *E. coli* in LB broth enriched with milk supplement and found that the milk supplement supported a significantly higher cell density of SSuT strains. This result was consistent with SSuT *E. coli* having significant advantage *in vitro*, and thus the possibility that the milk supplement provides a direct

selective advantage to these strains *in vivo*. Our current hypothesis is that the SSuT resistance genes are linked to gene(s) conferring selective advantage in the presence of milk supplement.

INTRODUCTION

Food animal producers rely on management methods to prevent disease outbreaks and increase production. Management can include controlling the environment (temperature, humidity, etc.), providing clean living space, providing good nutrition, using passive transfer if appropriate, and implementing biosecurity measures to block transmission of infectious diseases. Producers also use antimicrobial drugs for prophylactic treatment. Besides potential benefits for preventing disease, antimicrobial drugs have also been shown to increase average daily gains and feed conversion in some food animals (6, 9, 10, 14). Consequently, the potential disease and production benefits have led to widespread use of antimicrobial drugs in food animal production.

Unfortunately, the practice of using antimicrobial drugs for growth promotion and prophylaxis has probably contributed to an increase in the prevalence of antimicrobial resistant bacteria in food animals and it may contribute to increased prevalence of resistant bacteria in humans (1, 3, 4, 12, 38, 39). A worst case scenario would occur if antimicrobial drug resistance genes can be maintained in the commensal flora from animals and that these genes may be transferred to bacteria that are pathogenic to both animals and humans (15, 18, 26, 27, 29, 30, 34, 36, 37). This concern has prompted the European Union to ban the use of several antimicrobial drugs and the World Health Organization has suggested cessation of all antimicrobial drugs for growth promotion (5,

9, 17). In theory, the cessation of non-therapeutic drug use will result in lower selection pressure for emergence and maintenance of antimicrobial resistant bacteria.

While prudent use of antimicrobial drugs has proven to be an effective way to curb rising level of antimicrobial drug resistance (23, 24, 32), there are many recorded instances where withdrawal of antimicrobial drugs has not impacted the prevalence of antimicrobial drug resistance in the population under study (13, 16, 22, 31, 35). One possible explanation for the maintenance of antimicrobial drug resistance in an apparent drug free environment is close genetic linkage between resistance genes and other genes that provide significant adaptive advantages to specific niches. It is also possible that resistance genes may convey otherwise unrecognized benefits in a complex environment. Finally, the frustrating persistence of resistance genes may be related to insignificant fitness costs. That is, if carriage of resistance genes confers little fitness cost to the host bacterium, then only sporadic selection events would be needed to maintain presence of resistance genes within a population and with no selection pressure we can expect a long half-life as resistance genes are slowly purged from the population (7).

Our research has focused on understanding the mechanisms that are responsible for maintaining antimicrobial resistance in a production dairy environment. In an earlier study we tested the hypothesis that a medicated milk supplement was necessary to maintain the most prevalent antimicrobial resistance phenotype (resistant to streptomycin, sulfadiazine, and tetracycline and susceptible to ampicillin, chloramphenicol, and nalidixic acid) in a dairy herd. We found that the oxytetracycline used in this study was not necessary to maintain a high prevalence of these strains, heretofore referred to as "SSuT strains" (22). We also demonstrated that these strains have a selective advantage

when growing in gastrointestinal tract of younger dairy calves (<3 mo) when compared with older heifers (13 mo).

If direct selection pressure is not needed to maintain resistance genes, then we hypothesized that the SSuT strains were maintained because the resistance genes themselves conveyed some unidentified but significant selective advantage for surviving in dairy calves. To test this hypothesis we developed null mutant strains and tested them using both *in vitro* and *in vivo* competition models and found that there was no apparent secondary fitness advantage attributable to the resistance genes themselves (21).

A third hypothesis is that traits closely associated (i.e., genetically linked) with the antimicrobial resistance genes confer a selective advantage in dairy calves. Results from our previous studies suggested a possibility that a milk supplement itself may trigger a selective advantage in this niche with or without the addition of oxytetracycline. The focus of the present study is to formally test this observation with the clear implication that success of SSuT strains is most likely due to phenotypic traits conferred by closely linked genes that take direct or indirect advantage of the presence of the milk supplement.

MATERIALS AND METHODS

Comparison of prevalence of antimicrobial drug resistance and patterns between years 2001 and 2004.

The Washington State University (WSU) dairy farm (Pullman, Wash.) maintains a closed dairy with all the replacement heifers raised on site. Holstein calves are housed in individual pens in a separate building 24-48 hours after birth. Calf ration includes milk

(weaned at 4-6 weeks of age) and grain supplement. A milk supplement was used until winter of 2003, and also contained oxytetracycline (5.5 g/kg). The milk supplement is composed of spray process grade A non-fat dry milk and vitamins (see below) and it is administered by adding 15-20 g directly to milk that is being fed to the calves. The practice of adding the milk supplement was used for at least 12 years prior to its cessation.

As a part of two previous studies we examined the prevalence of the antimicrobial drug resistant *E. coli* isolated from calves in years 2001 and 2004 (21, 22). To determine the effect the withdrawal of the milk supplement (containing oxytetracycline) on the prevalence of antimicrobial resistant *E. coli*, we compared the data from year 2001 (22) and data from the control group of year 2004 (21). Data was entered and analyzed with Microsoft Excel and NCSS 2001 (NCSS Statistical Software, Kaysville, UT). Fisher's LSD multiple comparison test was used to test for statistical differences in the prevalence of resistant bacteria year 2001 and 2004.

Reintroduction of milk supplement with and without oxytetracycline.

Milk supplement reintroduction experiments at the WSU dairy involved 27 neonatal calves that were consecutively assigned to one of the three groups. Group one received milk supplement without oxytetracycline (-Pennox-50); group two received milk supplement with oxytetracycline (+Pennox-50); group three did not receive any supplement. Calves were housed in individual pens, but had nose to nose contact among treatment groups. The milk supplement was prepared at the Department of Animal Sciences at WSU and contains 8.3 kg of dried skim milk (Dist. by TPI (Thomas Products

Inc.)), 156 g of vitamin D premix (Mfg. and dist. by TPI), and 241 g of vitamin A-30 premix (Mfg. and dist. by TPI). Supplement of group two (+Pennox-50) also contained 412 g of Pennox-50 (oxytetracycline hydrochloride; equivalent to 110 g/kg; Mfg. by Pennfield Animal Health, dist. by TPI). Calves received one tablespoon (15-20 g) of milk supplement per day that was added directly to the milk at the morning feeding. The supplement was not stirred in the milk. Calves received 2.3 kg of milk at each feeding (twice daily). Final concentration of the oxytetracyline hydrochloride in the milk, if properly dissolved, was 35.6 mg/L.

Fecal sampling and antimicrobial drug resistance testing

Each calf was sampled once a week for 3 months. Fecal samples were collected with sterile tongue depressors, and placed into sterile bags. Samples were returned to the lab where they were streaked for isolation on 3 separate Violet Red Bile Agar with 4-Methylumbelliferyl-β-D-glucuronide (VRB-MUG) plates (VRB from Remel, USA; MUG from Biosyth Ag, Switzerland) within 4 hr of collection and were incubated overnight at 37°C. Twenty one (21) presumptive *E. coli* colonies (pink coloration and fluorescence under UV light) per animal, 7 per sample plate were used to inoculate EC (*E. coli*) medium (Remel, USA) with MUG (EC-MUG) broth (200 µl) in a 96-well plate format, leaving 8-24 non-inoculated, negative control wells. Each 96-well plate also included two positive control isolates, Q-89 and Q-90, resistant and susceptible to all tested antimicrobial drugs respectively. The 96-well plates were then incubated at 44.5°C overnight and the MUG reaction was confirmed under UV light.

Presumptive *E. coli* were tested for antimicrobial drug susceptibility using agar dilution at breakpoint concentrations, as previously described (21, 22). Antimicrobial drug (Sigma, USA) susceptibilities were tested using Mueller Hinton agar (MH) medium (Hardy Diagnostics, USA) supplemented with ampicillin (16 μg/ml), tetracycline (10 μg/ml), chloramphenicol (16 μg/ml), streptomycin (12 μg/ml), sulfadiazine (512 μg/ml), and nalidixic acid (18 μg/ml). Replicated test plate series included a final plate of antimicrobial drug free media to confirm inoculum delivery. The results of replicator assays were recorded after overnight incubation at 37°C. Results for antimicrobial drug plates were coded as a dichotomous variable: zero for no growth and one for growth. These results were used to calculate the frequencies for different resistance patterns. Data was entered and analyzed with Microsoft Excel and NCSS 2001 (NCSS Statistical Software, Kaysville, UT). Fisher's LSD multiple comparison test was used to tests for statistical differences in the prevalence of resistant bacteria between groups.

Growth curve analysis of SSuT and susceptible strains in vitro.

Growth curves were generated for SSuT and antimicrobial drug susceptible *E. coli* in LB broth and LB broth enriched with milk supplement containing no antimicrobial drugs. Milk supplement (8.7 g of milk supplement per liter of LB) was dissolved at 55º C in a water-bath and filter sterilized (0.22 μm). A 96-well microtiter plate with appropriate medium (185 μl per well) was inoculated with 1.2 μl of LB overnight culture at 37º C for 24 h (replicated 6-12 times). The overnight cultures were comprised of a mixture of six "different" SSuT *E. coli* strains or a mixture of six "different" susceptible *E. coli* strains. Different strains were defined by unique macro-restriction patterns from PFGE (data not

shown). Some wells (16-24) were left un-inoculated to test for possible bacterial contaminants in the media. The plate was agitated before collecting absorbance values (A_{600}) every 30 min using a SPECTRAmax 384 PLUS (Molecular Devices) plate reader. Absorbance results were entered and analyzed with Sigma Plot software (SPSS Inc., Chicago, IL) and NCSS 2001 (NCSS Statistical Software, Kaysville, UT). The Mann-Whitney rank-sum test was used to test for statistical differences between growth curves.

The colony forming units (CFU) were calculated for the SSuT and susceptible *E. coli* after 24 h growth in LB and LB enriched with milk supplement. The initial culture was diluted (3 x 100-fold) in peptone buffered saline (PBS) in triplicate, and 100 ul was plated in triplicates on LB agar plates. Plates were incubated overnight at 37ºC and colony counts were determined. The counts were entered and analyzed with NCSS 2001 (NCSS Statistical Software, Kaysville, UT). The two-sample Student's *t*-test was used to tests for statistical differences between the SSuT and susceptible CFUs.

RESULTS

Comparison between years 2001 and 2004.

We compared the prevalence of antimicrobial drug resistant *E. coli* and their specific resistance patterns between years 2001 and 2004. The main objective was to assess the potential impact of removal of milk supplement containing oxytetracycline (in 2003) on the *E. coli* population in calves (< 3 mo).

The comparison of prevalence of antimicrobial drug resistant *E. coli* for the year 2001 (22) and year 2004 control group (21) revealed a statistically significant decrease in prevalence of streptomycin and sulfadiazine resistant isolates, no significant change in

the prevalence of tetracycline and chloramphenicol resistant isolates, and a significant increase in the prevalence of ampicillin resistant isolates (Fig. 1). Between years 2001 and 2004, the prevalence of the resistance patterns ASSuT, ASSuTCh, and T increased significantly, the prevalence of AST, SSuTCh and susceptible *E. coli* did not change significantly, but there was a very significant decrease in prevalence of SSuT *E. coli* (Fig. 2). The decrease in prevalence of SSuT *E. coli* for year 2004 was most marked in the first 40 days of life, a period which in year 2001 had the highest number of SSuT *E. coli* (Fig. 3). The latter observation is consistent with the close association of the SSuT *E. coli* with the presence of the dietary supplement. The difference in prevalence of ampicillin resistance between the years is difficult to account for because no ampicillin analogs are used for therapeutics at the dairy.

Results for the reintroduction of milk supplement at the dairy

Because there was a significant change in prevalence of antimicrobial drug resistant *E. coli* in calves between years 2001 and 2004, we wanted to determine whether the observed effect was due to the removal of milk supplement and whether oxytetracycline played a selective role. Consequently, we tested the impact of the supplement with and without oxytetracycline on *E. coli* population in calves. Calves were consecutively assigned to three groups and received either milk supplement without oxytetracycline, milk supplement with oxytetracycline, or no supplement at all.

Among the three groups, there was no statistically significant difference in the level of antimicrobial drug resistance to ampicillin, streptomycin, sulfadiazine and tetracycline, except that group three (no supplement) had significantly higher level of

chloramphenicol resistant *E. coli* (Fig. 1). Comparison of the resistance patterns revealed significantly higher numbers of SSuT *E. coli* in the two groups that received milk supplement regardless of presence of oxytetracycline (Fig. 2). There was no statistically significant difference among the three inoculation groups for susceptible *E. coli* although group three had significantly fewer ASSuT and significantly more ASSuTCh compared with the two calf groups receiving the dietary supplement. This experiment confirms that there is a close association between the prevalence of SSuT *E. coli* and the use of a dietary supplement with or without the presence of oxytetracycline.

Growth curves in LB enriched with milk supplement

To test the hypothesis that the milk supplement provides a direct selective growth advantage to SSuT strains, we examined the growth characteristics of SSuT and susceptible *E. coli* in LB broth that was enriched with the milk supplement used at the dairy (containing no oxytetracycline). SSuT and susceptible *E. coli* have similar growth curves in LB broth (Mann-Whitney rank-sum test; $P = 0.1$; Fig. 4a), but the SSuT *E. coli* have significantly higher optical density (OD) at stationary phase compared to susceptible *E. coli* when milk supplement is added (Mann-Whitney rank-sum test; $P = 0.001$; Fig. 4b). Similar results were found in triplicate experiments.

To verify that the OD results at 24 hr correlate with colony forming units (CFU) of SSuT and susceptible bacteria, we determined the CFUs in LB broth and LB broth enriched with milk supplement. The average CFU for SSuT and susceptible *E. coli* did not differ significantly when grown in LB broth (1.3×10^9 and 1.3×10^9, respectively; Student's *t*-test; $P = 1.0$). The average CFU for SSuT and susceptible *E. coli* when grown

in milk supplement enriched LB broth as predicted differed significantly, with susceptible *E. coli* having lower counts (9.7×10^8 and 8.1×10^8, respectively; Student's *t*-test; $P = 0.0003$).

DISCUSSION

We examined the effect of milk supplement on a population of antimicrobial resistant, commensal *E. coli* in neonatal calves. Our study site, the WSU dairy, has used a milk supplement containing oxytetracyline at least 12 years prior to the winter of 2003 after which they discontinued this practice. A comparison of prevalence data from 2001 (22) and 2004 (21) indicated a significant decline in SSuT strains between the two time points. In the current study we reintroduced the dietary supplement and found a clear increase in the prevalence of SSuT strains in the treatment groups, but not in the prevalence of SSuT strains for the no-supplement control group. The calves in each treatment group were housed in separate pens, but nose-to-nose contact was possible. Nevertheless, calves in all three treatment groups were housed in interspersed pens so differences in prevalence of SSuT strains were not due to spread from a single treatment group. Our previous work at the dairy also showed, via pulsed-field gel electrophoresis, that calves in interspersed pens maintained largely separate *E. coli* flora (21).

These data indicate that the supplement selected for SSuT strains either directly or through an indirect mechanism. We used an *in vitro* growth curve analysis to determine if there was a direct effect. Growing SSuT and non-SSuT strains in the presence of the milk supplement in LB broth conferred statistically significant advantage to the SSuT strains. Although rates of growth did not differ, SSuT strains maintained a 16.5 % higher density

of cells at stationary phase compared with non-SSuT strains. These latter strains were not inhibited by the presence of the milk supplement, but they gained no advantage in the ability to sustain a higher density of cells. We determined that the pH of the LB did not change after the addition of the milk supplement, so the effect that we observed was not due to culture acidification. Presumably, the milk supplement provided SSuT strains with the means to overcome an otherwise limiting factor in total population size. These *in vitro* results suggest that the milk supplement can provide a direct selective advantage to SSuT strains. There may be other effects *in vivo* whereby physiological or community ecology changes associated with the milk supplement make it possible for SSuT strains to increase in prevalence.

In two previous studies we determined that antimicrobials are not necessary to maintain SSuT strains (22) and we have shown that there does not appear to be any secondary advantages conferred by the presence of the *tet*(B), *sul*2 and *str*A/*str*B genes harbored by the SSuT strains (21). Consequently, the apparent relationship between prevalence of SSuT strains and the milk supplement is most likely related to close genetic linkage to other genes that confer a selective advantage in the presence of the milk supplement. Others have reported examples of genetic linkage/association to antimicrobial drug resistance genes. For example Calomiris et al. (11) found a close association between multiple antibiotic resistance traits and metal tolerance in bacteria isolated from drinking water. Kehrenberd et al. (20) confirmed physical linkage of three antimicrobial drug resistance genes, explaining simultaneous occurrence of these resistances in *Pasteurella* and *Manheimia* isolates without direct selective pressure. Aarestrup (2, 20) accounted for the persistence of glycopeptide resistance in enterococci

from broilers and pigs by genetic link between *erm*B and *van*A AR genes, resulting in co-selection as a consequence of continued use of macrolides.

While we found a close association between SSuT strains and withdrawal and introduction of the milk supplement, we also found that the overall prevalence of tetracycline resistance in fecal *E. coli* was unaffected by these treatments. For example, the prevalence of resistance to tetracycline did not change between 2001 and 2004. We attribute this lack of change to either (1) linkage to genes that confer a selective advantage to an unidentified selective pressure, and/or (2) a slow rate of "decay" attributable to minimal fitness cost of the antimicrobial resistance genes. Several authors have reported very long "decay rates" after removal of antimicrobial drug pressure (19, 25, 33) and this makes intuitive sense for tetracycline resistance efflux genes because these genes are usually associated with a repressor gene that prevents expression in the absence of a tetracycline analog (8, 40). Thus, the biological cost of harboring tetracycline resistance genes may be small.

While we expect oxytetracycline to negatively affect sensitive flora, it was interesting that the addition of oxytetracycline to the supplement had no additional effect above what was observed by the supplement alone. Under idealized conditions, the final concentration of oxytetracycline in the milk should be 35.6 μg/ml, which is much higher than the threshold concentration for susceptible *E. coli* (≤ 4 μg/ml). We have demonstrated *in vitro* that the growth of antimicrobial drug susceptible *E. coli* is suppressed in LB broth to which similar ratio of milk supplement (containing oxytetracycline) is added, but the growth of SSuT *E. coli* is uninhibited (data not shown) and given these results we would predict an additional selective advantage for the SSuT

strains in the presence of oxytetracycline. *In vivo*, however, it is possible that the effectiveness of the tetracycline is much lower due to dilution in the intestinal content, and chelation of the tetracycline by Ca^{2+} and Mg^{2+} cations present in the milk (28).

In our previous work we noted a close correspondence between the SSuT phenotype and presence of a large plasmid. Subsequent analysis (Southern blots and microarray hybridization) have demonstrated that the plasmid is not involved in the SSuT phenotype, studies are underway to determine the location and identity of the SSuT element. Analysis of this element may provide clues to the relationship between SSuT strains and the use of the dietary milk supplement.

In conclusion we have demonstrated that milk supplement directly selects for strains with a specific resistance pattern (SSuT) in the calves at a dairy. Even though the prevalence of SSuT *E. coli* was influenced by the use of the milk supplement, the overall prevalence of streptomycin, sulfadiazine and tetracycline resistances remained relatively constant indicating that the mechanism that maintains the SSuT strains is not universal for other resistance phenotypes at the dairy. Previous experiments have also demonstrated that SSuT *E. coli* are more competitive in the calf intestinal environment than susceptible ones. This supports the hypothesis of multifactor selective system that maintains a relatively constant level of antimicrobial resistant bacteria. In the case of the SSuT strains, the primary mechanism for long-term maintenance is most likely related to the presence of gene that confers direct and/or indirect selective advantage in the presence of a milk supplement and we speculate that this gene(s) is closely linked to the antimicrobial resistance genes.

REFERENCES

1. **Aarestrup, F. M.** 1999. Association between the consumption of antimicrobial agents in animal husbandry and the occurrence of resistant bacteria among food animals. Int. J. Antimicrob. Agents **12:**279-285.
2. **Aarestrup, F. M.** 2000. Characterization of glycopeptide-resistant *Enterococcus faecium* (GRE) from broilers and pigs in Denmark: genetic evidence that persistence of GRE in pig herds is associated with coselection by resistance to macrolides. J. Clin. Microbiol. **38:**2774-2777.
3. **Aarestrup, F. M., Y. Agerso, P. Gerner-Smidt, M. Madsen, and L. B. Jensen.** 2000. Comparison of antimicrobial resistance phenotypes and resistance genes in *Enterococcus faecalis* and *Enterococcus faecium* from humans in the community, broilers, and pigs in Denmark. Diagn. Microbiol. Infect. Dis. **37:**127-137.
4. **Aarestrup, F. M., H. Kruse, E. Tast, A. M. Hammerum, and L. B. Jensen.** 2000. Associations between the use of antimicrobial agents for growth promotion and the occurrence of resistance among *Enterococcus faecium* from broilers and pigs in Denmark, Finland, and Norway. Microb. Drug Resist. **6:**63-70.
5. **Aarestrup, F. M., A. M. Seyfarth, H. D. Emborg, K. Pedersen, R. S. Hendriksen, and F. Bager.** 2001. Effect of abolishment of the use of antimicrobial agents for growth promotion on occurrence of antimicrobial resistance in fecal enterococci from food animals in Denmark. Antimicrob. Agents Chemother. **45:**2054-2059.

6. **Anderson, K. L., T. G. Nagaraja, J. L. Morrill, P. G. Reddy, T. B. Avery, and N. V. Anderson.** 1988. Performance and ruminal changes of early-weaned calves fed lasalocid. J. Anim. Sci. **66:**806-813.

7. **Andersson, D. I., and B. R. Levin.** 1999. The biological cost of antibiotic resistance. Curr. Opin. Microbiol. **2:**489-493.

8. **Beck, C. F., R. Mutzel, J. Barbe, and W. Muller.** 1982. A multifunctional gene (tetR) controls Tn10-encoded tetracycline resistance. J. Bacteriol. **150:**633-642.

9. **Berge, A. C., P. Lindeque, D. A. Moore, and W. M. Sischo.** 2005. A clinical trial evaluating prophylactic and therapeutic antibiotic use on health and performance of preweaned calves. J. Dairy Sci. **88:**2166-2177.

10. **Braidwood, J. C., and N. W. Henry.** 1990. Clinical efficacy of chlortetracycline hydrochloride administered in milk replacer to calves. Vet. Rec. **127:**297-301.

11. **Calomiris, J. J., J. L. Armstrong, and R. J. Seidler.** 1984. Association of metal tolerance with multiple antibiotic resistance of bacteria isolated from drinking water. Appl. Environ. Microbiol. **47:**1238-1242.

12. **Caprioli, A., L. Busani, J. L. Martel, and R. Helmuth.** 2000. Monitoring of antibiotic resistance in bacteria of animal origin: epidemiological and microbiological methodologies. Int. J. Antimicrob. Agents **14:**295-301.

13. **Chaslus-Dancla, E., G. Gerbaud, M. Lagorce, J. P. Lafont, and P. Courvalin.** 1987. Persistence of an antibiotic resistance plasmid in intestinal *Escherichia coli* of chickens in the absence of selective pressure. Antimicrob. Agents Chemother. **31:**784-788.

14. **Donovan, D. C., S. T. Franklin, C. C. Chase, and A. R. Hippen.** 2002. Growth and health of Holstein calves fed milk replacers supplemented with antibiotics or Enteroguard. J. Dairy Sci. **85:**947-950.

15. **DuPont, H. L., and J. H. Steele.** 1987. Use of antimicrobial agents in animal feeds: implications for human health. Rev. Infect. Dis. **9:**447-460.

16. **Enne, V. I., D. M. Livermore, P. Stephens, and L. M. Hall.** 2001. Persistence of sulphonamide resistance in *Escherichia coli* in the UK despite national prescribing restriction. Lancet **357:**1325-1328.

17. **Ferber, D.** 2003. Antibiotic resistance. WHO advises kicking the livestock antibiotic habit. Science **301:**1027.

18. **Hirsh, D. C., and N. Wiger.** 1977. Effect of tetracycline upon transfer of an R plasmid from calves to human beings. Am. J. Vet. Res. **38:**1137-1139.

19. **Johnsen, P. J., J. I. Osterhus, H. Sletvold, M. Sorum, H. Kruse, K. Nielsen, G. S. Simonsen, and A. Sundsfjord.** 2005. Persistence of animal and human glycopeptide-resistant enterococci on two Norwegian poultry farms formerly exposed to avoparcin is associated with a widespread plasmid-mediated vanA element within a polyclonal *Enterococcus faecium* population. Appl. Environ. Microbiol. **71:**159-168.

20. **Kehrenberg, C., and S. Schwarz.** 2001. Occurrence and linkage of genes coding for resistance to sulfonamides, streptomycin and chloramphenicol in bacteria of the genera *Pasteurella* and *Mannheimia*. FEMS Microbiol. Lett. **205:**283-290.

21. **Khachatryan, A. R., D. D. Hancock, T. E. Besser, and D. R. Call.** In Press. Antimicrobial Drug Resistance Genes do not Convey Secondary Fitness Advantages to Calf-Adapted *Escherichia coli*. Appl. Environ. Microbiol.

22. **Khachatryan, A. R., D. D. Hancock, T. E. Besser, and D. R. Call.** 2004. Role of calf-adapted *Escherichia coli* in maintenance of antimicrobial drug resistance in dairy calves. Appl. Environ. Microbiol. **70:**752-757.

23. **Klare, I., D. Badstubner, C. Konstabel, G. Bohme, H. Claus, and W. Witte.** 1999. Decreased incidence of *van*A-type vancomycin-resistant enterococci isolated from poultry meat and from fecal samples of humans in the community after discontinuation of avoparcin usage in animal husbandry. Microb. Drug Resist. **5:**45-52.

24. **Langlois, B. E., K. A. Dawson, T. S. Stahly, and G. L. Cromwell.** 1984. Antibiotic resistance of fecal coliforms from swine fed subtherapeutic and therapeutic levels of chlortetracycline. J. Anim. Sci. **58:**666-674.

25. **Lee, C., B. E. Langlois, and K. A. Dawson.** 1993. Detection of tetracycline resistance determinants in pig isolates from three herds with different histories of antimicrobial agent exposure. Appl. Environ. Microbiol. **59:**1467-1472.

26. **Levy, S. B., G. B. FitzGerald, and A. B. Macone.** 1976. Spread of antibiotic-resistant plasmids from chicken to chicken and from chicken to man. Nature **260:**40-42.

27. **Marshall, B., D. Petrowski, and S. B. Levy.** 1990. Inter- and intraspecies spread of *Escherichia coli* in a farm environment in the absence of antibiotic usage. Proc. Natl. Acad. Sci. U S A **87:**6609-6613.

28. **Newman, E. C., and C. W. Frank.** 1976. Circular dichroism spectra of tetracycline complexes with Mg+2 and Ca+2. J Pharm Sci **65:**1728-1732.

29. **Nijsten, R., N. London, A. van den Bogaard, and E. Stobberingh.** 1996. Antibiotic resistance among *Escherichia coli* isolated from faecal samples of pig farmers and pigs. J. Antimicrob. Chemother. **37:**1131-1140.

30. **Oppegaard, H., T. M. Steinum, and Y. Wasteson.** 2001. Horizontal transfer of a multi-drug resistance plasmid between coliform bacteria of human and bovine origin in a farm environment. Appl. Environ. Microbiol. **67:**3732-3734.

31. **Rollins, L. D., S. A. Gaines, D. W. Pocurull, H. D. Mercer, and L. T. Frobish.** 1976. Persistence of transferable drug resistance in the lactose-fermenting enteric flora of swine following antimicrobial feeding. Can. J. Compar. Med. **40:**175-183.

32. **Seppala, H., T. Klaukka, J. Vuopio-Varkila, A. Muotiala, H. Helenius, K. Lager, and P. Huovinen.** 1997. The effect of changes in the consumption of macrolide antibiotics on erythromycin resistance in group A streptococci in Finland. Finnish Study Group for Antimicrobial Resistance. N. Engl. J. Med. **337:**441-446.

33. **Shimizu, M., T. Sakano, and S. Satoh.** 1988. Linkage of K99 production and STa activity in a plasmid of an *Escherichia coli* porcine isolate. Microbiol. Immunol. **32:**635-639.

34. **Shoemaker, N. B., H. Vlamakis, K. Hayes, and A. A. Salyers.** 2001. Evidence for extensive resistance gene transfer among *Bacteroides spp*. and among *Bacteroides* and other genera in the human colon. Appl. Environ. Microbiol. **67:**561-568.

35. **Smith, H. W.** 1975. Persistence of tetracycline resistance in pig *E. coli*. Nature **258:**628-630.

36. **van den Bogaard, A. E., and E. E. Stobberingh.** 2000. Epidemiology of resistance to antibiotics. Links between animals and humans. Int. J. Antimicrob. Agents **14:**327-335.

37. **Winokur, P. L., D. L. Vonstein, L. J. Hoffman, E. K. Uhlenhopp, and G. V. Doern.** 2001. Evidence for transfer of CMY-2 AmpC b-lactamase plasmids between *Escherichia coli* and *Salmonella* isolates from food animals and humans. Antimicrob. Agents Chemother. **5:**2716-2722.

38. **Witte, W.** 2000. Ecological impact of antibiotic use in animals on different complex microflora:environment. Int. J. Antimicrob. Agents **14:**321-325.

39. **Witte, W.** 2000. Selective pressure by antibiotic use in livestock. Int. J. Antimicrob. Agents **16:**S19-S24.

40. **Wray, L. V., Jr., R. A. Jorgensen, and W. S. Reznikoff.** 1981. Identification of the tetracycline resistance promoter and repressor in transposon Tn10. J. Bacteriol. **147:**297-304.

FIGURES

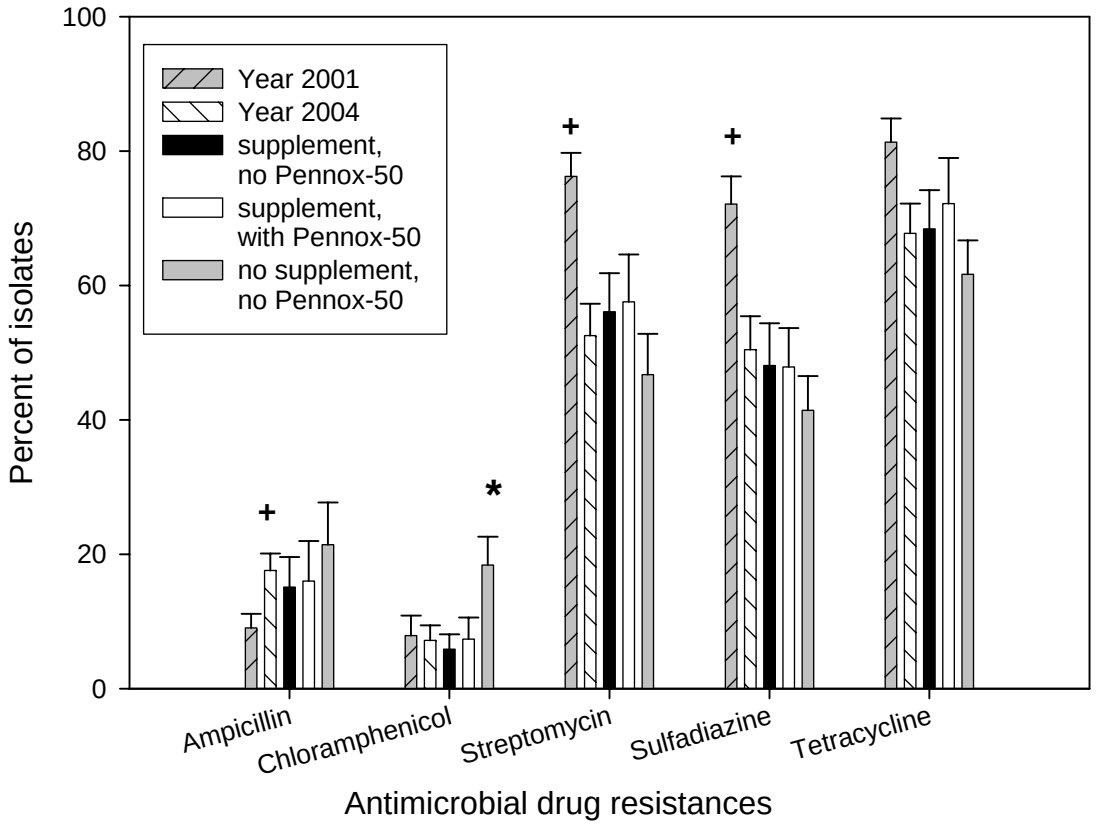


FIG. 1 Frequency of antimicrobial drug resistance for all *E. coli* shed from calves; year 2001 (n = 4,561), year 2004 (n = 3,448), group receiving supplement without Pennox-50 (n = 2,248), group receiving supplement with Pennox-50 (n = 2,239), group without supplement and without Pennox-50 (n = 1,891) . $^{+}$ and *, statistically significant ($P < 0.05$ with Fisher's LSD multiple comparison test for five tests); $^{+}$ - comparison between years and * - comparison among groups.

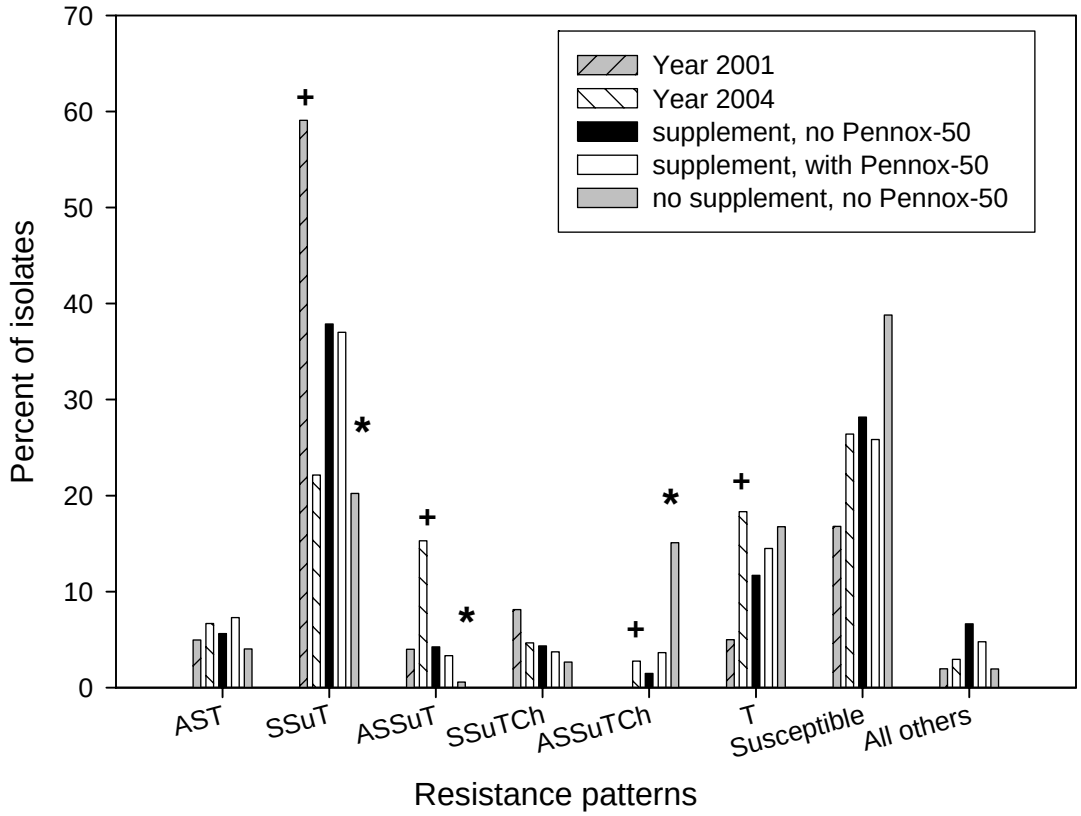


FIG. 2 Frequency of antimicrobial drug resistance patterns for all *E. coli* shed from calves; year 2001 (*n* = 4,561), year 2004 (*n* = 3,448), group receiving supplement without Pennox-50 (*n* = 2,248), group receiving supplement with Pennox-50 (*n* = 2,239), group without supplement and without Pennox-50 (*n* = 1,891) . $^{+}$ and *, statistically significant ($P < 0.05$ with Fisher's LSD multiple comparison test for five tests); $^{+}$ - comparison between years and * - comparison among groups. Resistance patterns are denoted by letters, where A = ampicillin, Ch = chloramphenicol, S = streptomycin, Su = Sulfadiazine, and T = tetracycline. Susceptible isolates were susceptible to all above mentioned antimicrobial drugs tested.

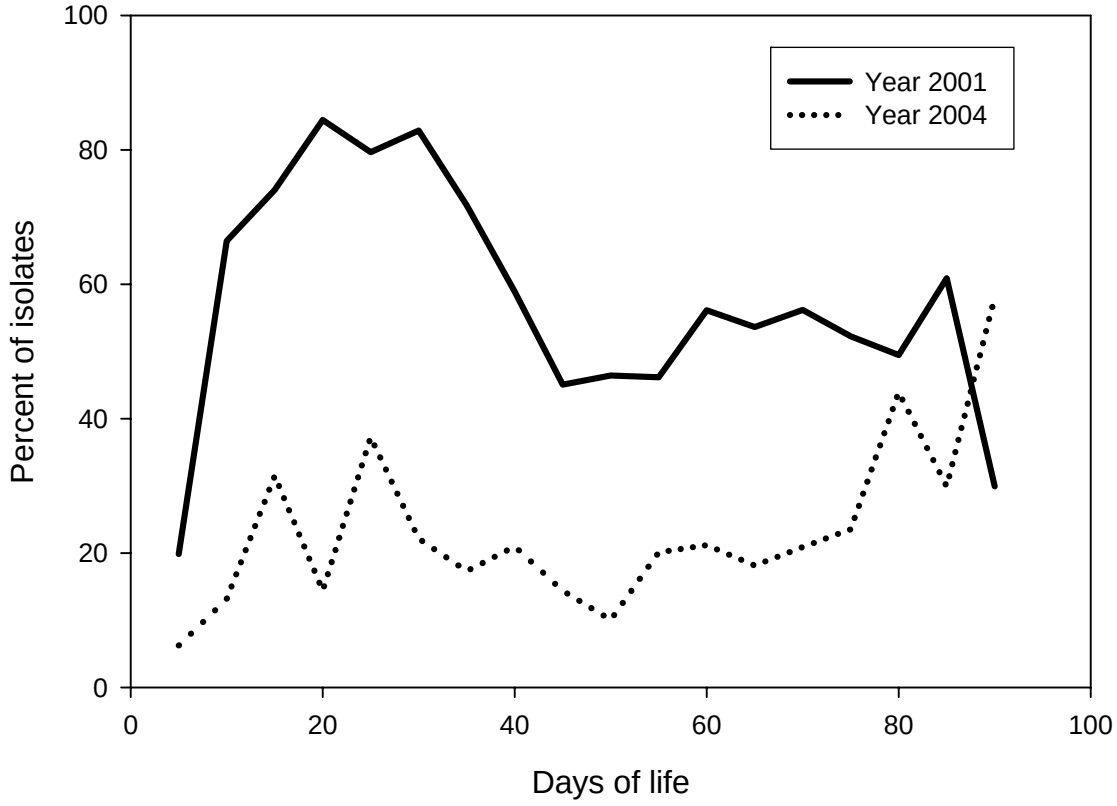
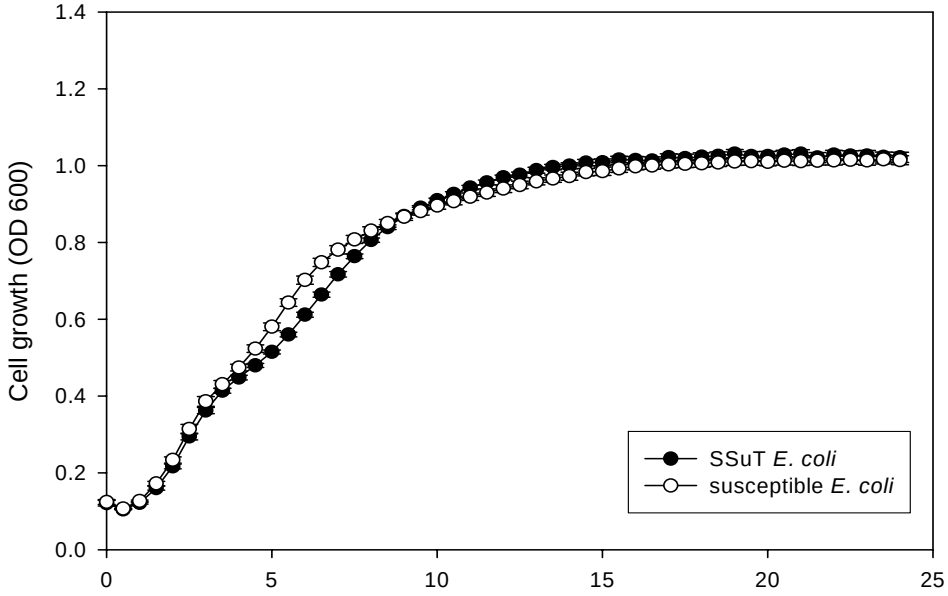


FIG. 3 Distribution of SSuT *E. coli* over days of life for all the calves by groups; year 2001 (n = 18), year 2004 (n = 10).

a



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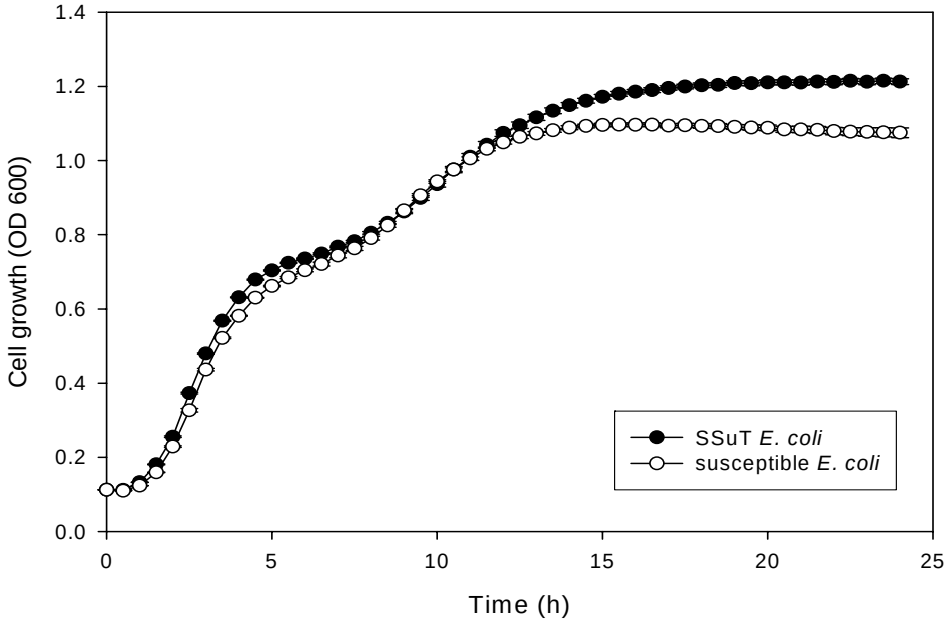

FIG. Growth curves for SSuT (open circles) and susceptible (closed circles) *E. coli* (a) in LB broth (n = 12 of each type) and (b) LB broth enriched with milk supplement (n = 6 of each type). Bars = SE. The optical density (OD) at 24 h corresponded to no difference in

the number of colony forming units (CFU) in panel (a) ($P = 1.0$), but a significant number of CFU for SSuT strains in panel (b) ($P = 0.0003$).