RESEARCH REPORT
PORK SAFETY

Title: Quantitative evaluation of selective factors impacting the development, amplification, persistence and transfer of macrolide resistance elements within mixed populations of commensal and pathogenic gut bacteria. **NPB #05-198**

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Abstract

Continuous flow cultures of feral (culture FC) and domesticated (culture RPCF) pig gut microflora were established in steady state. Cultures, in duplicate, were continuously infused with low (25 µg/ml) or high (100 µg/ml) levels of tylosin and sampled at intervals to assess effects on total culturable anaerobes, *Bacteroides* spp. and *Enterococcus* spp. via plating of serial 10-fold dilutions to anaerobic Brucella blood (BRUC) agar, Bacteroides bile esculin (BBE) agar, and M Enterococcus (ME) agar supplemented without or with 100 µg tylosin/ml, the later to assess bacterial sensitivity to tylosin. Concentrations of endogenous tylosin-insensitive anaerobes within the FC and RPCF cultures prior to tylosin administration ranged from 8.8 to 9.2 log10 CFU/ml and were likely composed with different prominent endogenous tylosin-resistant bacteria, likely a *Clostridium hathawayi* in culture FC and tylosin-resistant *Bacteroides uniformis* and *B. stercoris* in culture RPCF. Consequently, these populations responded differently during the experiments, with tylosin-insensitive concentrations in RPCF cultures becoming enriched more than those in FC cultures during administration of either 25 or 100 µg tylosin/ml to the continuous flow cultures. Moreover, tylosin-insensitive anaerobes persisted at their increased concentrations after cessation of tylosin administration whereas concentrations from FC cultures decreased. Concentrations of *Bacteroides* and endogenous *Enterococcus* spp. decreased to near or below detectable levels (1.0 $\log_{10}$ CFU/ml) in culture FC following administration of 25 or 100 µg tylosin/ml although tylosin-insensitive populations were present (> 1.0 $\log_{10}$ CFU/ml) before initiation of tylosin administration. Concentrations of endogenous tylosin-insensitive *Bacteroides* spp. were not enriched in FC cultures during 25 µg tylosin/ml treatment but were enriched to > 5 $\log_{10}$ CFU/ml in RPCF cultures after 4 days of this treatment. While present at > 2.0 $\log_{10}$ CFU/ml prior to initiation of tylosin administration, tylosin-insensitive *Bacteroides* spp. were not enriched in one experiment and transiently enriched to > 7.6 CFU/ml in another when 100 µg tylosin/ml was administered to FC and RPCF cultures, with the enriched populations rapidly diminishing upon cessation of tylosin treatment. When administered at 25 µg tylosin/ml, populations of endogenous tylosin-insensitive *Enterococcus* sp. were enriched in RPCF cultures but not in FC cultures. For cultures administered 100 µg tylosin/ml, tylosin-insensitive *Enterococcus* spp. exhibiting two types of colony morphology were recovered at increasing concentrations from two RPCF cultures and from one FC culture, thus

suggesting the enrichment of the experimentally inoculated tylosin-resistant *Enterococcus faecium* as well as endogenous *Enterococcus*. Further tests of representative isolates confirmed tylosin-resistance in *Enterococcus faecalis* from cultures RPCF and *Enterococcus hirea* from the FC culture and that this resistance was genotypically related to that of *Enterococcus faecium* thus suggesting the occurrence of gene transfer. The results of these studies revealed that tylosin-resistant bacteria, although of different type, were endogenous to gut microbial populations from both a feral and domestically reared pig. These results further illustrate that under the conditions of these tests, administration of tylosin promoted the enrichment of tylosin-insensitive bacterial populations (capable of growing on media supplemented with 100 µg tylosin/ml) within RPCF cultures (originating from a traditionally reared domesticated pig) and FC cultures (originating from a feral pig) and that transfer of resistance between *Enterococcus* spp. likely occurred.

III. Introduction

Macrolide antibiotics are commonly used in human and veterinary medicine, primarily to treat infections caused by Gram-positive bacteria and also as a feed additive to improve production efficiency in swine (Gaynor and Mankin, 2003). Resistance can occur via acquisition of *erm* methyltransferases, which catalytically inactivate the macrolide's targeted binding site, via acquisition of multidrug efflux pumps or even, albeit infrequently, via point mutations in the microbe's genome (Chopra and Roberts, 2001; Gaynor and Mankin, 2003; Karlsson et al., 2004; Poole, 2005). Recovery of bacteria harbouring *erm* genes from domestic swine and swine production habitats is not uncommon (Chee-Sanford et al., 2001; Wang et al., 2005) but less is known regarding the quantitative acquisition and selection of resistance in bacteria, particularly in swine not reared traditionally (Stanton and Humphrey, 2004). Continuous flow culture of intestinal microorganisms has been used to study competitive interactions between commensal and pathogenic microflora (Harvey *et al.*, 2002; Hume *et al.*, 2001; Nisbet *et al.*, 2000) as well as to investigate potential factors affecting spontaneous acquisition of antibiotic resistance (Kim *et al.*, 2005). Presently, continuous flow cultures established with mixed populations of gut bacteria originating from feral and domesticated swine were used to assess the effects of low (25 µg/ml) and high (100 µg/ml) amounts of tylosin administration on select populations of resident bacteria.

IV. Objectives of Research Project:

The objectives of this study were to quantitatively model the effects of low and high level tylosin administration on the development, amplification, persistence and transfer of genetic elements conferring macrolide resistance within mixed populations of commensal and pathogenic gut bacteria grown in continuous flow culture.

V. Materials and Methods

Continuous flow culture establishment. Two separate mixed populations of porcine gut bacteria were established in continuous flow culture as previously described (Harvey et al., 2002; Hume et al., 2001). The RPCF had been previously established with cecal contents obtained from a traditionally reared pig and its initial characterization has been reported previously (Harvey et al., 2002). The other culture, defined as FC, was established under similar conditions except with cecal contents from an adolescent feral boar killed near Caldwell, Texas, USA, approximately 2 to 4 h prior to necropsy. The cecal contents of this feral boar were stored anaerobically under nitrogen in 20% glycerol until the next morning at which time approximately 100 ml suspension was used as inoculum. Both parent cultures of RPCF and FC were established and maintained in BioFlo chemostats (New Brunswick Scientific Company, Edison, NJ) with a culture volume of 550 ml. The culture medium was Viande Levure broth which was prepared and maintained anaerobically under a stream of carbon dioxide and infused at 0.40 ml/min which corresponds to a 24 h vessel turnover. Cultures were incubated at 39°C and agitated at 100 rpm.

Experimental design. Once established in steady state, initial characterization of culture FC was accomplished using traditional bacteriological culture methodologies and antibiotic susceptibility testing was performed as described in the National Committee for Clinical Laboratory Standards (now known as the Clinical and Laboratory Standards Institute [CLSI]) (NCCLS, 2004). Both parent cultures were used to provide inoculum to

establish separate RPCF and FC test cultures, in duplicate, which after at least 14 vessel turnovers, were continually infused with culture medium containing 25 (Experiment 1) or 100 (Experiments 2 and 3) µg tylosin/ml for 6 to 8 further vessel turnovers. Fluid samples collected immediately before and during tylosin infusion were quantitatively cultured, via plating of 10-fold serial dilutions, to the following media, each prepared with or without 100 µg tylosin/ml: anaerobic Brucella blood (BRUC) agar and Bacteroides bile esculin (BBE) agar (Anaerobe Systems, Morgan Hill, CA), for detection of total anaerobes and *Bacteroides* spp., respectively, and M Enterococcus (ME) agar (Becton Dickinson and Company, Sparks, MD) for detection of *Enterococcus* spp. Inoculated agar media were incubated 48-72 h at 37°C and colonies enumerated on agar medium without tylosin selection include those in the population that are both sensitive and insensitive to tylosin; colonies enumerated on agar medium with 100 µg tylosin/ml are only those in the population that are tylosin-insensitive. Specific identification of bacteria from select colonies was achieved using rapid ID 32 STREP, rapid 20E, 20NE, 20A, and rapid ID 32 A identification strips (bioMérieux, Hazelwood, MO). Indole spot tests (Anaerobe Systems, Morgan Hill, CA), E-test™ (AB Biodisk, Piscataway, NJ) and gas chromatography were also used in this analysis. Samples containing no detectable colonies of bacteria were given a value of 1.0 $\log_{10}$ CFU/ml. Tylosin was withdrawn from all test chemostats after 6-8 days of initial application of the antibiotic. In experiments 2 and 3, samples collected for an additional 6 to 7 vessel turnovers after cessation of tylosin infusion were also cultured as above. In experiment 3, reinoculation of each FC and RPCF test culture with 10% (vol/vol) of culture fluid from the parent cultures occurred on 7 days after cessation of tylosin treatment and sampling continued as above for an dditional 7 days. After the test cultures in experiments 2 and 3 had been established for at least 1 week, a tylosin-resistant *E. faecium* strain demonstrating multi-drug resistance, including to tylosin (kindly donated by Roger Harvey, USDA ARS/SPARC and Morgan Scott at Texas A&M University, College Station TX), was inoculated (5 ml of 10^7 CFU/ml and 10^9 CFU/ml for experiments 2 and 3, respectively) as a potential gene donor into all FC and RPCF test cultures. This inoculation time corresponds to day -9 as presented in our figures.

Antibiotic resistance confirmation. A representative group of select colonies from Experiment 3 were tested for confirmation of antibiotic resistance. Sensititre microdilution plates (Trek Diagnostics System, UK) were used according to CLSI guidelines to determine minimum inhibitory concentrations (MIC) of the following antibiotics at NCCLS breakpoints (NCCLS, 2002): Ampicillin, Ceftiofur, Chlortetracycline, Clindamycin, Danofloxacin, Enrofloxacin, Erythromycin, Florfenicol, Gentamicin, Neomycin, Oxytetracycline, Penicillin, Spectinomycin, Sulphacholoropyridazine, Sulphadimethoxime, Sulphathiazole, Tiamulin, Tilmicosin, Trimethoprim/Sulphamethoxazole. The Etests® (AB Biodisk, Piscataway, NJ) were performed for *Bacteroides* as described by the manufacturer with BRUC and interpreted at 24-48 h. The MIC was defined as the lowest concentration of each antibiotic that inhibited visible growth.

PCR to confirm transfer of resistance genes

Bacteria were lysed with equal volume of 0.2% (w/v) of Triton X-100 and heated to boiling at 100°C for 5 mins in water bath, then allowed to cool and used as templates for PCR. Specific primers were used for amplifications as previously published (Bendle et al., 2004; Frye et al., 2006 for the following genes: *erm A*, *erm B*, *erm C*, *erm F* and *erm Q* (see Table 1). DNA amplification was carried out on a MJ thermocycler Model PTC200 (Fisher Scientific, Hampton, NH) using the following conditions: 5 min at 94°C; 35 cycles of 94°C for 1 min, 55 °C for 1 min and 72 °C for 2 min. A multiplex for detection of detection of genes determining streptogramin resistance as previously published (Perrin-Guyomard et al., 2005) using the following PCR conditions: 10 min 94°C, 35 cycles of 30s at 95°C, 30 s at 50°C and 30 s at 72°C followed by 10 min at 72°C. All PCR products were analyzed by gel electrophoresis (1% agarose in 1x TAE buffer), stained with ethidium bromide and visualized by UV light. Sizes of products were determined by comparing them with 100 bp ladder (New England Biolabs, #N3271S).

Statistical analysis. Log_{10} transformations of bacterial concentrations obtained from duplicate cultures were analyzed for main effects of day, culture type (i.e., RPCF or FC culture) and the possible interaction using a

repeated measures analysis of variance (Statistix®8 Analytical Software, Tallahassee, FL, USA). Multiple comparison of means was accomplished using a Tukeys procedure.

VI. Results and Discussion

Characterization of microflora in culture FC. Culture FC achieved steady state after 14 days continuous flow culture and the bacteriological composition was found to include *Streptococcus bovis*, *Proteus mirabilis*, *Staphylococcus epidermidis*, *Alcaligenes denitrificans*, and members of *Bacteroides*, *Lactobacillus*, *Enterococcus*, and *Clostridium.* Many of these bacteria were found to be in culture RPCF (Harvey et al., 2002) and are typical members of the normal pig gut flora (Robinson et al., 1981). *Campylobacter and Salmonella* were never detected in the feral culture and *E. coli* that was initially isolated from the cecal contents was never recovered once the culture had achieved steady state. In culture FC, *Enterococcus hirae*, *Streptococcus bovis*, *Proteus mirabilis*, *Staphylococcus epidermidis*, *Alcaligenes denitrificans* were susceptible to tylosin and erythromycin; *Bacteroides uniformis* and *Bacteroides stercoris* were resistant to gentamicin, ciprofloxacin, ceftriaxone and ampicillin. In culture RPCF, *Bacteroides uniformis* and *Bacteroides stercoris* were resistant to erythromycin, gentamicin, ciprofloxacin, azithromycin, clindamycin, ceftriaxone and ampicillin. *Bacteroides uniformis*, *B. stercoris* from culture RPCF and *Clostridium hathewayi* from culture FC showed resistance to tylosin at MIC >512 µg/ml; the former two *Bacteriodes* spp. being prominent within culture RPCF and *Clostridium hathewayi* being the predominant anaerobe recovered from culture FC. Volatile fatty acid and pH profiles varied slightly for FC and RPCF (Table 2). The FC produced higher amounts of butyric acid but a similar pH to the RPCF with all treatments (Table 2).

Experiment 1, effect of 25 µg tylosin/ml on select bacterial populations. Recovery of anaerobes from non-selective BRUC agar was not affected ($P > 0.05$) by culture; however, main effects of day and day by culture interaction were observed ($P < 0.01$) on recovery of anaerobes from nonselective BRUC agar due to a decrease in anaerobes in the FC and an increase in RPCF cultures (Figure 1A). Main effects of culture, day and a day by culture interaction were observed ($P < 0.01$) on recovery of anaerobes from tylosin-selective BRUC agar due to a temporary increase in tylosin-insensitive anaerobes from the FC culture and a gradual enrichment of tylosin-insensitive anaerobes from the RPCF culture (Figure 1A). Tylosin-insensitive anaerobes were prominent even before tylosin administration in both culture RPCF and culture FC. In the FC culture, the prominent tylosin-insensitive anaerobe was *Clostridium hathewayi*. Main effects of culture, day and a day by culture interaction were observed ($P < 0.05$) on recovery of *Bacteroides* from BBE agar supplemented with or without tylosin (Figure 1B) due to higher *Bacteroides* concentrations in the RPCF cultures prior to administration of tylosin and to an enrichment of tylosin-insensitive *Bacteroides* spp. beginning by day 3 of tylosin administration (Figure 1B). In the case of total anaerobes and *Bacteroides* spp., tylosin-insensitive populations were prominent in RPCF cultures even before initiation of tylosin administration and were quite probably were propagated from either or both of the tylosin-resistant *B. uniformis* and *B. stercoris* endogenous to culture RPCF. Conversely, tylosin-insensitive *Enterococcus* spp., were not apparent prior to tylosin administration; however, upon initiation of treatment recovery was highly variable between the two RPCF cultures regardless of culturing on ME agar supplemented with or without tylosin. This indicates that the two RPCF cultures contained markedly different enterococcal populations. For instance, even though mean concentrations of tylosin-insensitive *Enterococcus* recovered from the RPCF cultures began to increase markedly beginning 4 days after initiation of tylosin administration (Figure 1C) this increase occurred in only one of the cultures. As a consequence, main effects of culture, day or day by culture interactions on quantitative recoveries on ME agar supplemented with or without tylosin were not observed ($P > 0.05$) (Figure 1C).

Experiment 2, effect of 100 µg tylosin/ml on select bacterial populations. Main effects of culture and day were not observed ($P > 0.05$) on recovery of total culturable anaerobes neither from BRUC agar supplemented without or with of tylosin (Figure 2A). Day by culture interactions were observed ($P < 0.01$) on recoveries from both BRUC agars due to a gradual increase in concentrations of culturable tylosin-insensitive anaerobes in culture RPCF that began after tylosin administration was ceased and to concurrent decreases in culturable anaerobes in culture FC (Figure 2A). Main effects of culture were not observed ($P > 0.05$) on recovery of

Bacteroides spp. or *Enterococcus* spp. from tylosin-supplemented or non-supplemented BBE agar or ME agar, respectively (Figure 2B-C, Figure 3). Main effects of day and day by culture interactions were observed ($P < 0.05$); however, with concentrations of *Bacteroides* spp. decreasing markedly after initiation of tylosin administration and remaining at undetectable levels for the rest of the study, even after tylosin administration was ceased (Figure 2B). Thus, in this case the 100 µg tylosin/ml treatment was sufficient to rid *Bacteroides* spp., even the tylosin-resistant *Bacteroides* spp. endogenous to culture RPCF. There was a day effect ($P < 0.05$) on *Enterococcus* spp. recovered on non-tylosin supplemented ME agar in both cultures RPCF and FC, due to transient decreases in concentrations during the tylosin-administration period, more so within culture FC than culture RPCF, and a resurgence after cessation of treatment (Figure 2C). An evident decrease in concentrations of *Enterococcus* spp. from tylosin-supplemented ME agar recovered from culture FC was observed (Figure 2C); however, there was not a main effect of day ($P > 0.05$) on these cells, primarily because of an absence of a culture effect or a day by culture interaction. Main effect of day was not observed ($P > 0.05$) on concentrations of *Enterococcus* spp. from tylosin-supplemented ME agar, which in the case of culture RPCF were only transiently reduced (Figure 2C).

Tylosin-resistant *Enterococcus faecium* were rapidly depleted immediately following inoculation (on day -9 relative to tylosin administration) into both FC and RPCF test cultures (data not shown), with concentrations being at or below our level of detection by day 0 (Figure 3). However, concentrations of this bacterium increased rapidly following initiation of tylosin administration but then began to decrease after cessation of treatment as evidenced by a main effect ($P < 0.05$) of day on cells recovered from either unsupplemented or tylosin-supplemented ME agar (Figure 3). These results suggests that the tylosin-resistant *Enterococcus faecium* was not very competitive in the mixed microbial cultures when in the absence of the selective effect of tylosin. Main effects of day or day by culture interactions were not observed ($P > 0.05$); however, thus indicating that this tylosin-resistant *Enterococcus faecium* behaved similary within populations of both FC and RPCF (Figure 3).

Experiment 3, confirmation of transfer of antibiotic resistance elements from added Enterococcus faecium to the endogenous Enterococcus spp. in both feral and domestic pig cultures. The endogenous *Enterococcus* spp. in both FC and RPCF cultures were confirmed to be susceptible to tylosin/erythromycin prior to the start of this experiment. Main effects of day and day by culture interactions were observed ($P < 0.05$) on recoveries of total culturable anaerobes on BRUC agar supplemented with or without tylosin (Figure 4A) due mainly to an enrichment in concentrations after initiation of tylosin administration and a marked decrease in tylosin-insensitive anaerobes from culture FC upon cessation of treatment (Figure 4A). Main effects of culture were not observed ($P > 0.05$). Main effects of culture, day and day by culture interactions were observed on recoveries of *Bacteroides* spp. on non-supplemented and tylosin-supplemented BBE agar (Figure 4B). In this case, tylosin administration markedly and rapidly selected for tylosin-insensitive *Bacteroides* populations in both FC and RPCF cultures but recovery of these bacteria after tylosin administration stopped decreased dramatically and only began to rebound within RPCF cultures upon reinoculation with populations from untreated parent cultures (Figure 4B). This suggests that in this case the tylosin-insensitive *Bacteroides* spp. endogenous to the cultures may have become habituated to tylosin. Such habituation could possibly arise via linkage of the resistance gene to essential housekeeping gene(s) that may have been repressed upon removal of the antibiotic (Martínez and Baquero, 2002). Alternatively, removal of tylosin in this study may have unleashed the suppressive effect of the antibiotic on competing bacteria. Whether these or other possible mechanisms were operative remain speculative at best in the absence of supporting evidence. A main effect of culture was not observed ($P > 0.05$) on recovery of endogenous *Enterococcus* spp. recovered on ME agar without tylosin, however, there was an effect of day ($P < 0.05$) on these populations due mainly to a gradual increase in *Enterococcus* spp. in culture FC during and immediately after tylosin administration (Figure 4C). A day by culture interaction was not observed ($P > 0.05$) on endogenous *Enterococcus* spp. recovered on ME agar without tylosin. Main effects of culture and day were observed ($P < 0.05$) on recovery of tylosin-insensitive *Enterococcus* spp. with more rapid and approximately 10,000-fold (4 $\log_{10}$ units) greater increases in concentrations observed in cultures RPCF than cultures FC. Concentrations of tylosin-insensitive *Enterococcus* spp. in both FC and RPCF cultures began

to decline upon cessation of tylosin treatment but the decline was more rapid and was accentuated upon reinoculation with the population from the untreated FC parent culture (Figure 4C). Tests of select tylosin-insensitive colonies recovered from ME agar supplemented with 100 µg tylosin/ml confirmed resistance to tylosin and erythromycin within endogenous *Enterococcus* spp. isolated from both RPCF replicate cultures and one of the FC cultures. The pattern of resistance matched that of the phenotypically distinguishible *Enterococcus faecium* donor strain that was added to the cultures on day -9 thus suggesting that transfer of the *erm B* gene from this donor organsm to endogenous enterococci had occurred. Transfer of the *erm B* gene was confirmed by PCR (Figure 6) which shows the band of the expected size and sequence (data not shown) being obtained for the *Enterococcus faecium* and endogenous tylosin-resistant *Entercoccus* spp. from both the FC and RPCF cultures.

In conclusion, results from this study revealed that tylosin-resistant bacteria were present in the mixed gut populations obtained from both the feral and domestically reared pig but that these resistant populations were different, with that from the feral pig (culture FC) being a *Clostridium hathewayi* and those from the domestically reared pig (culture RPCF) being *Bacteroides uniformis* and *Bacteroides stercoris*. Results also showed that low tylosin administration promoted the enrichment of tylosin-insensitive bacterial populations (capable of growing on media supplemented with 100 µg tylosin/ml) within RPCF cultures (originating from a traditionally reared domesticated pig) but not from FC cultures (originating from a feral pig). We were also able to confirm transfer of resistance elements (specifically an *erm B* gene conferring tylosin/erythromycin resistance) from the previously characterized *Enterococcus faecium* challenge strain to endogenous *Enterococcus* spp. in both replicates of the domestic pig and one replicate of the feral pig competitive exclusion cultures.

VIII. Lay Interpretation

A series of laboratory experiments were conducted to assess the effects of low (25 ug/ml) or high (100 ug/ml) tylosin treatment on potential development, amplification, persistance and transfer of tylosin resistance within a mixed population of gut bacteria derived from gut contents of a feral or a domestic pig. Results showed that tylosin-resistant bacteria such as *Clostridium* and *Bacteroides*, were differentially prominant in the mixed bacterial populations from the pigs, with tylosin-resistant *Clostridium* being recovered from the feral pig and the tylosin-resistant *Bacteroides* being recovered from the domestically reared pig. Results further showed that under the experimental conditions used here, these populations could certainly be amplified or increased more easily by administering a low rather than a high level of tylosin and that resistant populations amplified in mixed populations from the feral pig did not persist as long as those from the domestic pig. Results also demonstrated that tylosin-insensitive *Enterococcus* spp., while initially absent or at very low concentrations from both of the mixed populations, were enriched and amplified in the population from the domestic pig but not from the feral pig. Experimental infection of the populations with a known tylosin-resistant *Enterococcus faecium* bacterium provided evidence that at 100 µg tylosin/ml, genetic transfer occurred between this challenge organism and the naturally present *Enterococcus* spp. in the mixed populations, as the resistance gene, known as *ermB*, was found in the naturally present *Enterococcus* after they had acquired resistance but not before. These results will help antibiotic users to appropriately manage all antibiotics at their disposal and ultimately will help provide farmers, scientists and U.S. public health officials with important information to make sound, science-based decisions for the good of public health and animal production.

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References

BENDLE, J.S., JAMES P.A., BENNETT P.M., AVISON M.B., MACGOWAN A.P., AL-SHAFI, K.M. 2004. Resistance determinants in strains of *Clostridium difficile* from two geographically distinct populations. *International Journal of Antimicrobial Agents* 24**:**619-21.

CHEE-SANFORD, J.C., AMINOV, R.I., KRAPAC, I.J., GARRIGUES-JEANJEAN, N., MACKIE, R.I., 2001. Occurrence and diversity of tetracycline resistance genes in lagoons and groundwater underlying two swine production facilities. *Applied and Environmental Microbiology,* 67,1494-1502.
CHOPRA, I., ROBERTS, M., 2001. Tetracycline resistance: mode of action, applications, molecular biology, and epidemiology of resistance. *Microbiology and Molecular Biology Reviews,* 65,232-260.
FRYE J.G., JESSE, T., LONG, F., RONDEAU, G., PORWOLLICK, S., MCCLELLAND, M., et al. 2006. DNA microarray detection of antimicrobial resistance genes in diverse bacteria. *International Journal of Antimicrobial Agents* 27**:**138-51.
GAYNOR, M., MANKIN, A.S., 2003. Macrolide antibiotics: binding site, mechanism of action, resistance. *Current Topics in Medicinal Chemistry,* 3,949-961.
HARVEY, R.B., DROLESKEY, R.E., HUME, M.E., ANDERSON, R.C., GENOVESE, K.J., ANDREWS, K., NISBET, D.J., 2002. *In vitro* inhibition of *Salmonella enterica* serovars Choleraesuis and Typhimurium, *Escherichia coli* F-18, and *Escherichia coli* O157:H7 by a porcine continuous-flow competitive exclusion culture. *Current Microbiology,* 45:226-229.
HUME, M.E., NISBET, D.J., BUCKLEY, S.A., ZIPRIN, R.L., ANDERSON, R.C., STANKER, L.H., 2001. Inhibition of in vitro *Salmonella* Typhimurium colonization in porcine cecal bacteria continuous-flow competitive exclusion cultures. *Journal of Food Protection,* 64,17-22.
KARLSSON, M., FELLSTRÖM, C., JOHANSSON, K.-E., FRANKLIN, A., 2004. Antimicrobial resistance in *Brachyspiira pilosicoli* with special reference to point mutations in the 23S rRNA gene associated with macrolide and lincosamide resistance. *Microbial Drug Resistance,* 10,204-208.
KIM, W.K., KARABASIL, N., BULAJIC, S., DUNKLEY, K.D., CALLAWAY, T.R., POOLE, T.L., RICKE, S.C., ANDERSON, R.C., NISBET, D.J., 2005. Comparison of spontaneous antibiotic frequency of *Salmonella* Typhimurium growth in glucose amended continuous culture at slow and fast dilution rates. *Journal of Environmental Science and Health Part B,* 40,475-484.
MARTÍNEZ, J.L., BAQUERO, F., 2002. Interactions among strategies associated with bacterial infection: pathogenicity, epidemicity, and antibiotic resistance. *Clinical Microbiology Reviews,* 15,647-679.
NCCLS, 2002. Performance Standards for Antimicrobial Susceptibility Testing; Twelfth Informational Supplement M100-S12. Wayne, PA: NCCLS; 2002.
NCCLS, 2004. Methods for Antimicrobial Susceptibility Testing of Anaerobic Bacteria; Approved Standard, 6th Ed., NCCLS document M11-A6, Wayne, PA: National Committee for Clinical Laboratory Standards.
NISBET, D.J., ANDERSON, R.C., CORRIER, D.E., HARVEY, R.B., STANKER, L.H., 2000. Modeling the survivability of *Salmonella typhimurium* in the chicken cecae using an anaerobic continuous-culture of chicken cecal bacteria. *Microbial Ecology in Health and Disease,* 12,42-47.
PERRIN-GUYOMARD, A., SOUMET, C., LECLERCQ, R., DOUCET-POPULAIRE, F., SANDERS, P. 2005. Antibiotic susceptibility of bacteria isolated from pasteurized milk and characterization of macrolide-lincosamide-streptogramin resistance genes. *Journal of Food Protection* 68**:**347-52
POOLE, K., 2005. Efflux-mediated antimicrobial resistance. *Journal of Antimicrobial Chemotherapy,* 56,20-51.
ROBINSON, I.M., ALLISON, M.J., BUCKLIN, J.A. 1981. Characterization of the cecal bacteria of normal pigs. *Applied and Environmental Microbiology* 41,950-955.
STANTON, T.B., HUMPHREY, S.B., 2004. Tetracycline resistant bacteria in organically raised and feral swine. *Abstract of the 104th American Society for Microbiology General Meeting,* Z-029.
WANG, Y., WANG, G., SHOEMAKER, N.B., WHITEHEAD, T.R., SALYERS, A.A., 2005. Distribution of the *ermG* gene among bacterial isolates from porcine intestinal contents. *Applied and Environmental Microbiology,* 71,4930-4934.

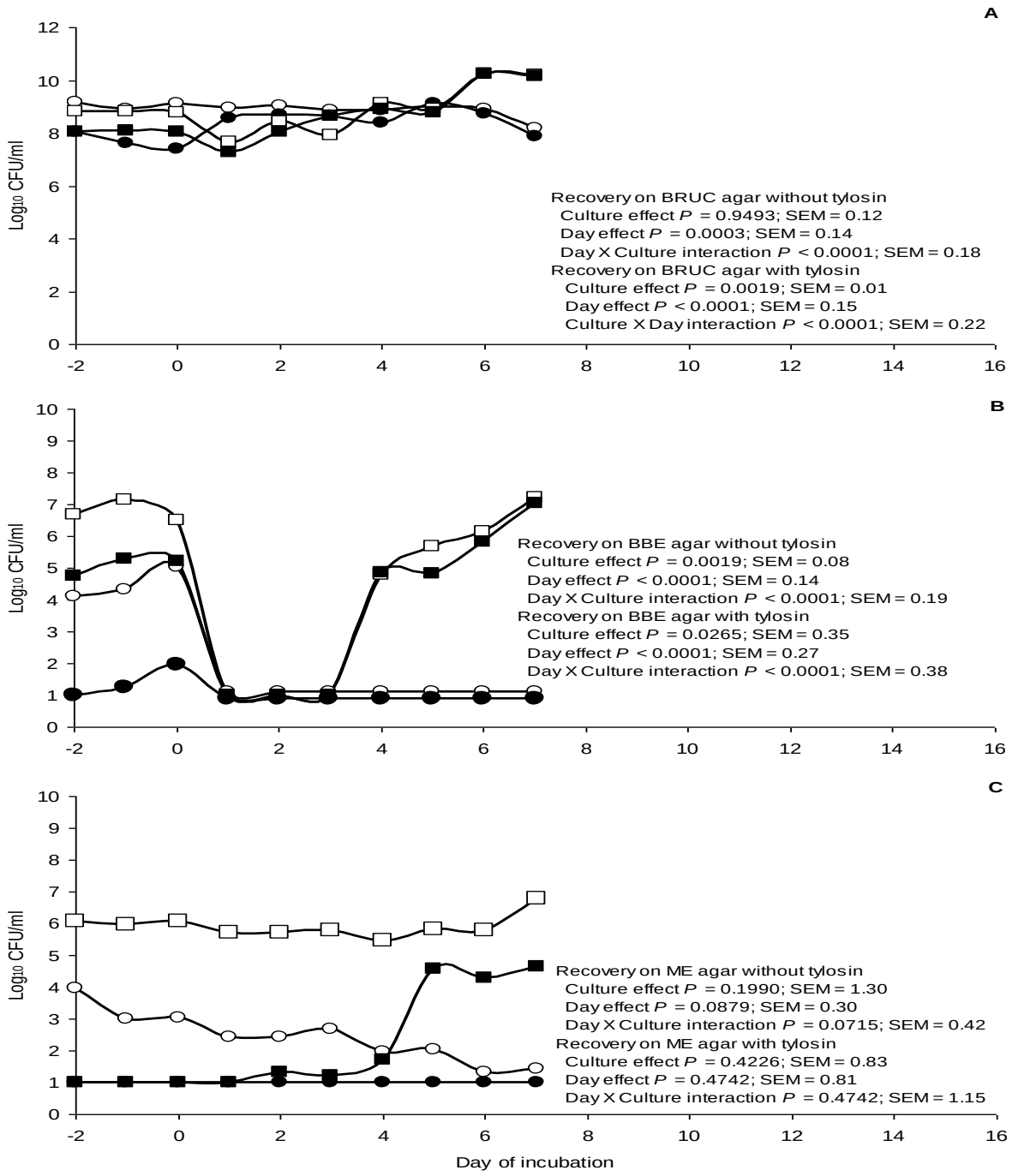


Figure 1. Effects of low level (25 µg/ml) tylosin administration (at day 0) on populations of anaerobes, *Bacteroides* spp., and *Enterococcus* spp. from continuous flow cultures of mixed populations of porcine gut bacteria obtained from feral (culture FC, circles) or domestic (culture RPCF, squares) swine. Bacteria were quantitatively recovered on anaerobic Brucella blood (BRUC) agar, Fig. A), Bacteroides bile esculin (BBE) agar (Fig. B) and M Enterococcus (ME) agar (Fig. C) each supplemented without (open symbols; representing those in the population that are both sensitive and insensitive to tylosin) or with (closed symbols, representing those in the population that are tylosin-insensitive) 100 µg tylosin/ml. Cultures were incubated in duplicate and *P* values and standard error measurements are indicated.

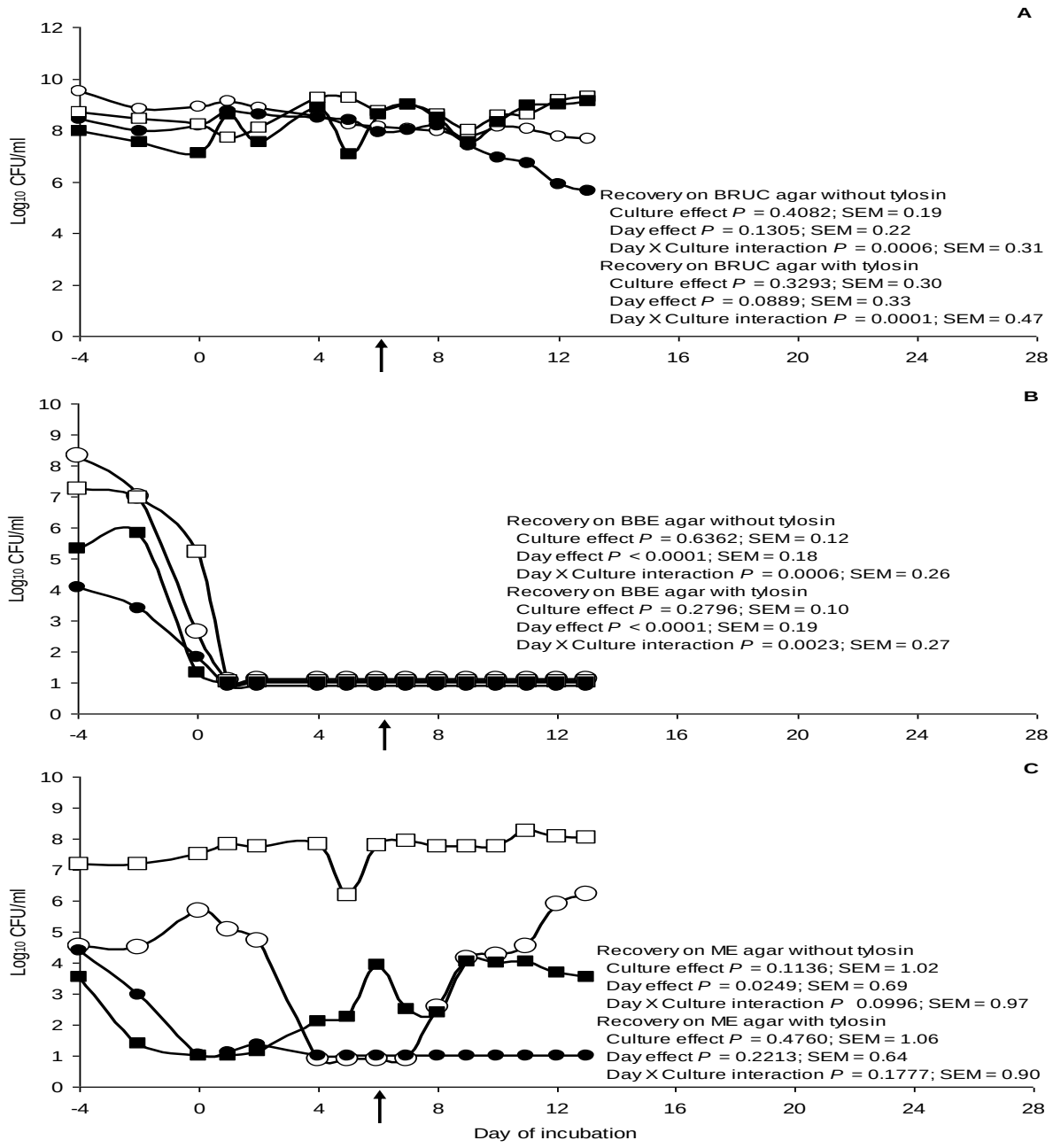


Figure 2. Effects of high level (100 µg/ml) tylosin administration (at day 0) and subsequent cessation of treatment (at day 6 as indicated by arrow) on populations of anaerobes, *Bacteroides* spp., and *Enterococcus* spp. from continuous flow cultures of mixed populations of porcine gut bacteria obtained from feral (culture FC, circles) or domestic (culture RPCF, squares) swine. Bacteria were quantitatively recovered on anaerobic Brucella blood (BRUC) agar (Fig. A), Bacteroides bile esculin (BBE) agar (Fig. B) and M Enterococcus (ME) agar (Fig. C) each supplemented without (open symbols; representing those in the population that are both sensitive and insensitive to tylosin) or with (closed symbols, representing those in the population that are tylosin-insensitive) 100 µg tylosin/ml. Cultures were incubated in duplicate and *P* values and standard error measurements are indicated.

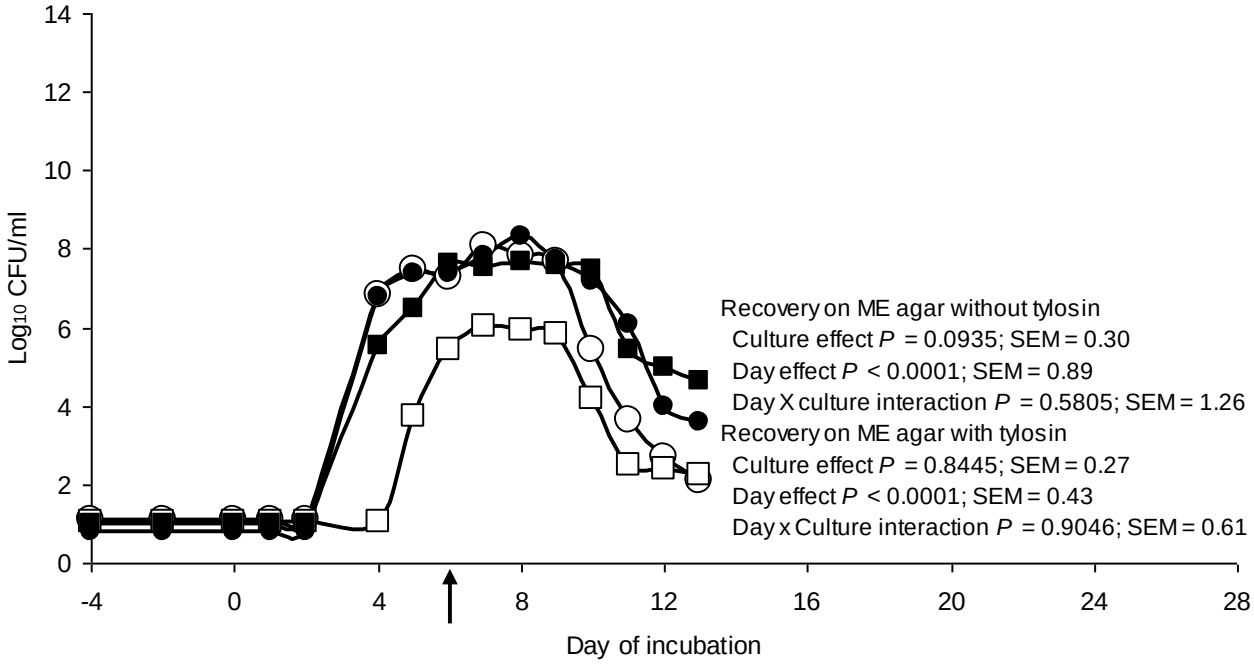


Figure 3. Effects of high level (100 µg/ml) tylosin administration (at day 0) and subsequent cessation of treatment (at day 6 as indicated by arrow) on experimentally inoculated (5 ml of 10^5 CFU/ml on day -7) tylosin-resistant *Enterococcus faecium* in continuous flow cultures of mixed populations of porcine gut bacteria obtained from feral (culture FC, circles) or domestic (culture RPCF, squares) swine. *Enterococcus faecium* were quantitatively recovered on M Enterococcus (ME) agar supplemented without (open symbols; representing those in the population that are both sensitive and insensitive to tylosin) or with (closed symbols, representing those in the population that are tylosin-insensitive) 100 µg tylosin/ml. Cultures were incubated in duplicate and *P* values and standard error measurements are indicated.

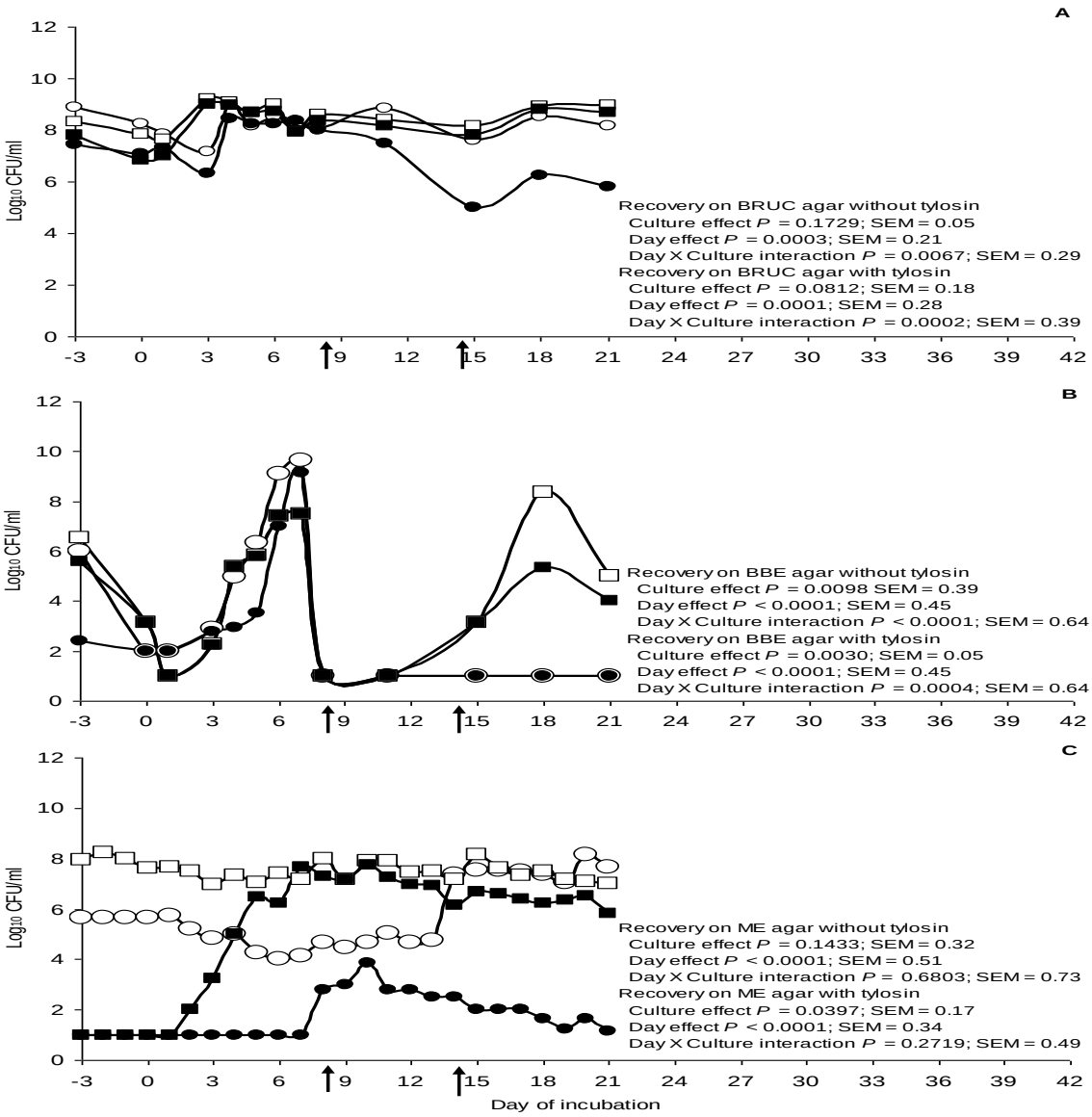


Figure 4. Effects of high level (100 µg/ml) tylosin administration (at day 0), its subsequent cessation of treatment (at day 8 as indicated by arrow) and reinoculation with 10% vol/vol of original non-tylosin treated culture (on day 14 as indicated by arrow) on populations of anaerobes, *Bacteroides* spp., and *Enterococcus* spp. from continuous flow cultures of mixed populations of porcine gut bacteria obtained from feral (culture FC, circles) or domestic (culture RPCF, squares) swine. Bacteria were quantitatively recovered on anaerobic Brucella blood (BRUC) agar (Fig. A), Bacteroides bile esculin (BBE) agar (Fig. B) and M Enterococcus (ME) agar (Fig. C) each supplemented without (open symbols; representing those in the population that are both sensitive and insensitive to tylosin) or with (closed symbols, representing those in the population that are tylosin-insensitive) 100 µg tylosin/ml. Cultures were incubated in duplicate and *P* values and standard error measurements are indicated.

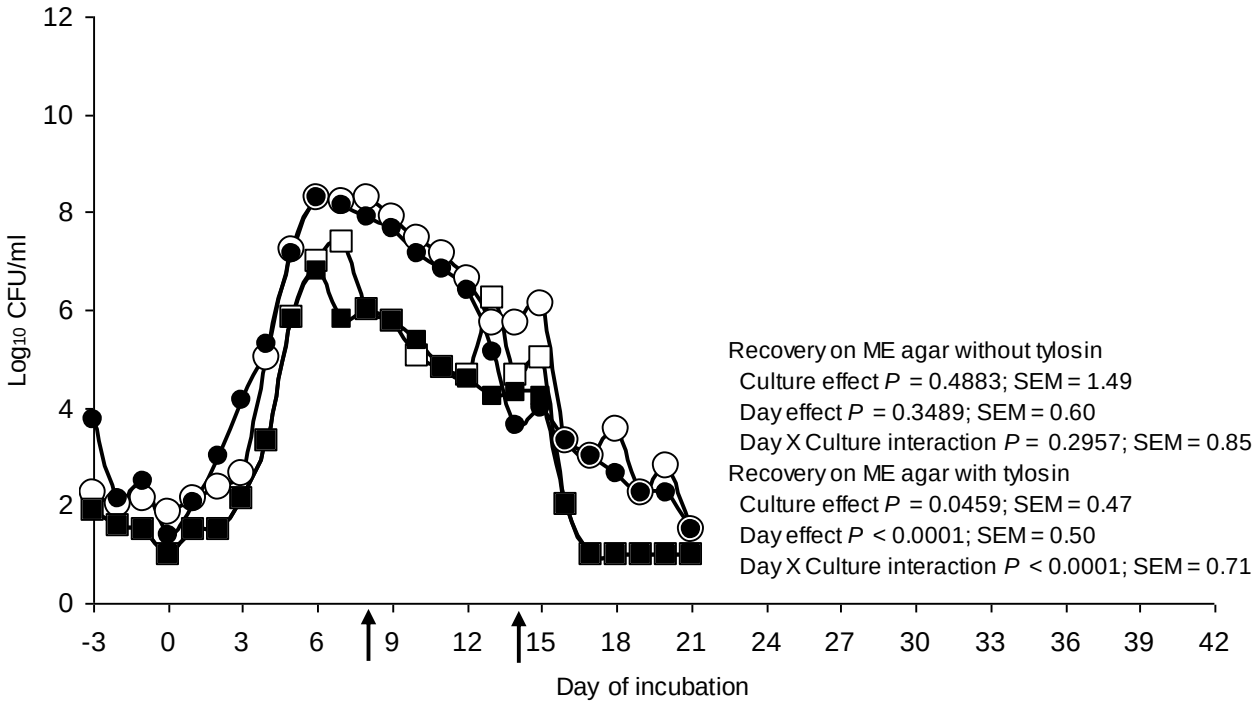
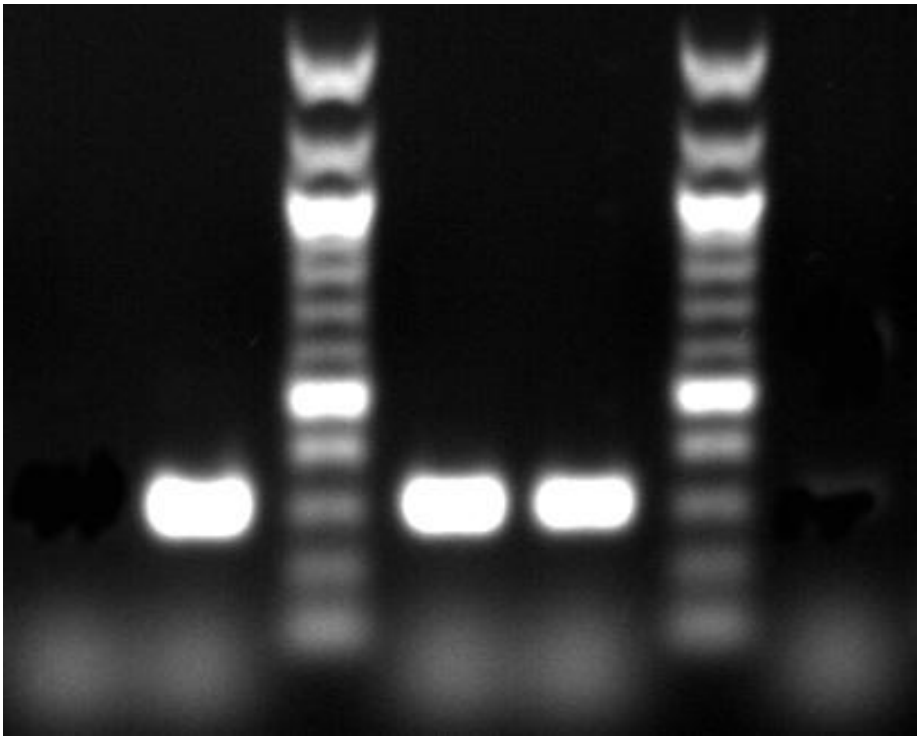


Figure 5. Effects of high level (100 µg/ml) tylosin administration (at day 0), subsequent cessation of treatment (at day 8 as indicated by arrow) and reinoculation with 10% vol/vol of original non-tylosin treated culture (on day 14 as indicated by arrow) on experimentally inoculated (5 ml of 10^9 CFU/ml on day -7) tylosin-resistant *Enterococcus faecium*, in continuous flow cultures of mixed populations of porcine gut bacteria obtained from feral (culture FC, circles) or domestic (culture RPCF, squares) swine. *Enterococcus faecium* were quantitatively recovered on M Enterococcus (ME) agar supplemented without (open symbols; representing those in the population that are both sensitive and insensitive to tylosin) or with (closed symbols, representing those in the population that are tylosin-insensitive) 100 µg tylosin/ml. Cultures were incubated in duplicate and *P* values and standard error measurements are indicated.



1 2 3 4 5 6 7

Figure 6. A PCR product was obtained for ermB (311bp) at the expected size for select colonies of *Enterococcus faecium* (lane 2), *Enterococcus* spp. from FC (lane 4) and *Enterococcus* spp. from RPCF (lane 5) all grown on tylosin plates (100ug/ml). Colonies tested at the start of the experiment showed that neither the *Enterococcus* spp. from the FC (lane 1) or the RPCF (lane 7) showed evidence of having the ermB gene. Lanes 3 and 6 are the marker lanes illustrating the product obtained to be approximately 311 bp.

Tables

Table 1. Oligonucleotide primers used in PCR amplification of *Enterococcus* spp. isolate.

Gene	Primer Sequence (5'-3')	Accession Number	Reference
ermB	F: TAACGACGAACCTGGCTAAAAT R: ATCTGTGGTATGGCGGGTAAG	AJ243541	Frye et al., 2006
ermC	F: AGTACAGAGGTGTAATTTCG R: AATTCCTGCATGTTTTAAGG	NC001386	Frye et al., 2006
ermF	F: GCCAACAATGTTGTTGTT R: CGAAATTGTCCTGACCTG	N/A	Bendle et al., 2004
ermQ	F: CACCAACTGATATGTGGC R: CAATCTACACTAGGCATG	N/A	Bendle et al., 2004
vatA	F: ATAATGAATGGAGCAAACCATAGGATG R: ACCAATCCAAACATCATTACC	N/A	Perrin-Guyomard et al., 2005

Table 2 - Competitive exclusion cultures pH and volatile fatty acid profiles

Culture	Treatment	pH	Acetic µmol/ml	Propionic µmol/ml	Butyric µmol/ml
RPCF	None[a]	6.29	23.91	10.27	10.44
	E. faecium[b]	6.13	27.95	11.33	13.09
	tylosin[c]	6.32	21.33	7.64	8.92
	no tylosin[d]	6.25	28.31	10.92	10.53
FC	None[a]	5.98	30.64	9.68	15.84
	E. faecium[b]	5.79	32.32	11.68	20.38
	tylosin[c]	5.99	25.11	8.13	14.32
	no tylosin[d]	5.88	31.10	10.97	20.11

[a] Cultures were tested after 7 days after inoculation (7 vessel turnovers)
[b] *Enterococcus faecium* added at 10^5 CFU/ml total concentration to cultures
[c] Tylosin added at concentration of 100µg/ml to medium of cultures
[d] After removal of medium containing 100µg/ml of tylosin