RESEARCH REPORT
PUBLIC HEALTH/WORKER SAFETY

Title: Strategy to preserve efficacies of antimicrobials against important swine pathogens – **NPB #13-116**

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Industry Summary: Naturally occurring efflux pump inhibitors have been shown to significantly inhibit the activity of efflux pumps contributing to antimicrobial resistance by many bacteria, particularly against Gram-positive staphylococci and streptococci. For instance, the degradation of chlorophylls by anaerobic bacteria yields the degradation products pheophoride-a and pyropheophorbide-a and these are able to inhibit efflux pumps thereby counter-acting this resistance mechanism. Commercially reared pigs generally do not eat substantially amounts of feeds containing chlorophyll but it is not known if other naturally occurring efflux pump inhibitors may be present in their gut. Moreover, even if efflux pump inhibitors are absent in most pig diets it would be easy to supplement their diets with chlorophylls (water soluble extracts or pellets) if this were determined to be a practical way of increasing the sensitivity of otherwise antimicrobial resistant bacteria to important antimicrobials. This research was conducted to determine 1) if efflux pump inhibitors are already present and operative in the gut of grower pigs; 2) if naturally present or added efflux pump inhibitors can counteract resistance expressed by important Gram-negative and Gram-positive bacteria and 3) determine if efflux inhibitors can be developed into an inexpensive technology to preserve and enhance the efficacy of currently available antibiotics. Results from the present study provide evidence that as yet unidentified compounds affecting antibiotic resistance are present in gut contents of commercially reared pigs but further characterization of their activity is needed as the activity appears be highly specific to individual bacterial strains and with only certain antibiotics. Results further indicate that feeding chlorophyll as a source or precursor of some known efflux pump inhibitors may indeed promote a decrease in antibiotic resistance but again the activity by these potential affecter compounds appears to be highly strain and antibiotic dependent and even affecting an increase in resistance in some cases. Consequently, it is unlikely at the present time that the efflux pump inhibitors tested in the present study can be developed into an inexpensive technology to preserve and enhance the efficacy of currently available antibiotics. Results from the present study do show, however, that as yet undefined factors normally found within the gut of commercially reared pigs can have a marked impact on resistance thus providing evidence that it may be more relevant to evaluate resistance at a functional level, as it occurs in the gut environment, rather than looking solely at the genetic and metabolic capability of a microbe when assayed in pure culture.

Keywords: Antimicrobial resistance, chlorophyll metabolites, efflux pump inhibitors, medicated feed, zoonotic pathogens

Scientific Abstract: Standardized methods of determining antimicrobial resistance of microbes involves growing the test bacteria in standard medium such as Mueller Hinton broth. Presently, we compared the antimicrobial resistance of multidrug resistant *Salmonella* and select Gram-positive bacteria against a selection of antibiotics after the bacteria had been grown in Mueller Hinton broth as it is commonly done in the laboratory or during growth in Mueller Hinton broth that was modified to contain extracts of fecal contents collected from nonchlorophyll- and chlorophyll-fed pigs. The chlorophyll was added to the pig diets because evidence suggests that certain bacterial degradation products of chlorophyll may accumulate in the gut and inhibit antimicrobial efflux pumps thereby making some multidrug resistant bacteria less resistant. We found that growth of the *Salmonella* strains in Mueller Hinton broth modified to contain fecal extracts from nonchlorophyll-fed pigs had little or only modest effects on their susceptibility to chlortetracycline or norfloxacin but increased resistance of most of the strains to penicillin, carbadox and tylosin. When the Gram-positive bacteria, encompassing strains of *Staphylococcus, Streptococcus* and *Enterococcus* , were grown in Mueller Hinton broth modified to contain fecal extract from nonchlorophyll-fed pigs we found that resistance by most of the strains to chlortetracycline, penicillin, tylosin, ceftazidime and norfloxacin was increased or unaffected, the exceptions being decreased resistance to chlortetracycline by *Staphylococcus hyicus, Streptococcus agalactiae* and *Enterococcus faecium*. Conversely, resistance to carbadox by nearly all of the Gram-positive bacteria grown in the nonchlorophyll-fed extracts, the exceptions being two strains of *Staphylococcus aureus*, was decreased 4 to 16-fold compared to resistance observed in standard Mueller Hinton broth. For the *Salmonella* strains grown in Mueller Hinton broth modified to contain the fecal extracts from chlorophyll-fed pigs, appreciable decreases in resistance were observed only with norfloxacin, with resistance being increased or unchanged from that observed with cultures grown in the standard Mueller Hinton broth for all the other antibiotics tested. Resistance to chlortetracycline, penicillin, carbadox and ceftazidime was lower from that observed during growth in the standard Mueller Hinton broth by nearly all of the Gram-positive strains grown with fecal extracts from the chlorophyll-fed pigs thus supporting earlier work indicating that degradation products of chlorophyll may be more effective against Gram-positive than Gram-negative bacteria. Results from studies testing individual addition of chlorophyll or its degradation products, pheophorbide-a or pyropheophorbide-a, or with the known efflux pump inhibitor, L-phenylalanyll-arginyl-β-naphthylamide, indicated that these compounds may indeed promote a decrease in antibiotic resistance but like that observed in the studies with added fecal extracts, the activity by these potential effector compounds appears to be highly strain and antibiotic dependent; even affecting increases in resistance in some cases. Results from pig feeding studies indicated that chlorophyll-supplementation had little if any appreciable effect on endogenous populations of penicillin-insensitive, chlortetracycline-insensitive or tylosin-insensitive *E. coli* or enterococci in feces from pigs fed typical commercial grower diets containing penicillin, chlortetracycline and sulfamethazine or containing tylosin. An effect of chlorophyll-supplementation was observed on *E. coli* and enteroccal populations in separate pigs fed a commercial non-medicated diet indicating that chlorophyll itself, or its degradation products, may be inhibitory to these populations by as yet unknown mechanisms. Results indicate that feeding chlorophyll as a source or precursor of efflux pump inhibitors may promote a decrease in antibiotic resistance but the activity of these compounds appears to be highly strain and antibiotic dependent and even affect an increase in resistance in some cases. Based on these results, it is unlikely at the present time that the efflux pump inhibitors tested in the present study can be developed into an inexpensive technology to preserve and enhance the efficacy of currently available antibiotics.

Introduction:
Public health officials and many in the general public are concerned that agricultural use of antibiotics is contributing to the emergence of antimicrobial resistant bacteria and this may decrease the effectiveness of antimicrobials important in human medicine (Angulo et al., 2004; Gorbach, 2001). The acquisition of resistance by microbes on the farm may also decrease the effectiveness of the limited number of antibiotics currently available to producers (McEwen and Fedorka-Cray, 2002). In a recent highly publicized research paper, for instance, it was reported that the gut microbiome of pigs fed the antibiotic supplement ASP250 (containing chlortetracycline, sulfamethazine and penicillin), maintained higher *E. coli* concentrations than pigs fed unmedicated diets and had higher carriage of antimicrobial resistance genes as well (Looft et al., 2012). Interestingly, a high carriage of genes coding for multidrug efflux pumps was not only observed within the pig gut microbiome after but before feeding of ASP250 as well. Multidrug efflux pumps confer resistance to the bacteria by enabling them to actively excrete or pump toxic chemical agents and antibiotics out of their cytoplasm (Pagès et al., 2005). Multidrug efflux pumps can be categorized into the following five main types or families of membrane-located transport systems: 1) ATP-binding cassette transporters (ABC), 2) small multidrug resistance (SMR), 3) resistance-nodulation-division (RND), 4) major facilitator superfamily (MF) and the multiple antibiotic and toxin extrusion (MATE) systems (Stavri et al., 2007). These systems are widely distributed among numerous bacterial pathogens and can oftentimes be carried in combination within a single bacterial species. In *Salmonella* and *E. coli*, the AcrAB efflux pump which is a member of the RND family confers the ability to excrete fluoroquinolones, tetracyclines, chloramphenicol, novobiocin and can contribute to resistance to some B-lactam antibiotics and erythromycin (Nikaido et al., 1998; Poole, 2007).

Despite the rise in levels of resistant bacteria, it has long been questioned why so many oral antimicrobials are still cost effective and useful long after epidemiological evidence would suggest they should have lost their effectiveness. One hypothesis is that the phenotypic and genotypic characterization of resistance in bacteria using biased selection techniques and pure culture methods does not adequately reflect the level of resistance that may be expressed within natural habitats. For instance, it is possible that exposure to different environmental stimuli could markedly affect not only the expression but also the functionality of resistance elements. Numerous natural occurring plant compounds and anaerobic degradation products, such as the chlorophyll metabolite pheophorbide-a, inhibit efflux pumps of Gram-positive and Gram-negative bacteria similar to that by a synthetic inhibitor, L-phenylalanyll-arginyl-β-naphthylamide (PAβN)(Sáenz et al., 2004). This makes the resistant bacteria 10 to 100-fold more susceptible to a number of antibiotics (Stermiz et al., 2000; Tegos et al., 2002). Considering that pheophorbide-a and other chlorophyll degradation products accumulate in the gut of animals consuming green plant material (Barnes et al., 2012), it is reasonable to suspect that gut microbes exposed to this and similar chlorophyll degradation products may likewise be made more sensitive to important antimicrobials. Accordingly, the objective of this proposal was to test if pheophorbide-a, its related metabolites and other plant compounds can inhibit efflux pump functionality in the pig gut ecosystem. To accomplish these goals, we conducted a series of in vitro studies with a selection of multidrug resistant Gram-positive and Gram-negative bacteria as well as live animal feeding studies testing the effects of the chlorophyll and other potential efflux pump inhibitors as well as fecal extracts on the susceptibility of these antimicrobial-resistant bacteria. This research specifically addresses National Pork Board Public Health priorities as characterizes factors affecting Antibiotic Use and Resistance/Antibiotic Alternatives.

Objectives: Characterize the effect of endogenous and exogenous efflux pump inhibitors on the susceptibility of multidrug resistant, efflux pump-expressing bacteria to carbadox, chlortetracycline, sulfamethazine, and penicillin (ASP250), enrofloxacin, and tylosin during growth in porcine gut contents supplemented without or with added efflux pump inhibitors.

a. Determine the susceptibility of multidrug resistant, efflux pump-expressing bacteria to carbadox, chlortetracycline, sulfamethazine, and penicillin (ASP250), enrofloxacin, and tylosin following exposure to clarified porcine gut contents containing added efflux pump inhibitors.

b. Quantify the effect of efflux pump inhibitors on the fitness of multidrug resistant, efflux pump-expressing bacteria within the competitive pig gut environment.

Materials & Methods:

Objective 1a. Determine the susceptibility of multidrug resistant, efflux pump-expressing bacteria to carbadox, chlortetracycline, sulfamethazine, and penicillin (ASP250), enrofloxacin, and tylosin following exposure to clarified porcine gut contents containing added efflux pump inhibitors.

Preparation of fecal extracts: The first part of this objective was to determine if efflux pump inhibitors may be present in the gut of pigs fed different diets. To accomplish this objective we collected fecal material from pigs fed twice daily (07:30 and 16:30) diets containing a typical non-medicated finisher diet (Producer's CO-OP, Bryan, TX) with or without the addition of a commercially available chlorophyll product. The chlorophyll product was fed to the pigs by top-dressing and mixing into a 20% portion of each meal which was offered first to promote total consumption of the chlorophyll. Approximately 30 minutes later the remainder of the meal was offered. Each meal for pigs not receiving the chlorophyll product was fed in two portions likewise. Approximately 500 g contents collected from 3 pigs on the nonchlorophyll-supplemented diets and approximately 500 g contents collected from 3 pigs on the chlorophyll-supplemented diet were separately pooled, mixed with water (50:50 wt/vol), autoclaved and clarified by repeated centrifugations (5 times) at 10,000 x g for 15 minutes to precipitate out particulate material. The resultant supernatant fluid was filtered 3 times through a 5 µm pore size filter and an additional 2 times through a 1.2 µm pore size filter and then autoclaved for storage at 4°C. When used, the fecal extracts were substituted (20% vol/vol) in place of water to Mueller Hinton broth to make media for growth of a panel of multidrug resistant *Salmonella*, *Streptococcus*, *Staphylococcus* and *Enterococcus*.

Determination of efflux pump inhibitor activity: Determination of efflux pump inhibitory activity on the susceptibility of multidrug resistant *Salmonella*, *Streptococcus*, *Staphylococcus* and *Enterococcus* to chlortetracycline, penicillin, sulfamethazine, carbadox, tylosin as well as against ceftazidime and norfloxacin using a modulation assay (Stavri et al., 2007). Efflux pump inhibitory activity was also tested likewise on the susceptibility of the *Salmonella* to chloramphenicol, tetracycline, florfenicol, streptomycin and erythromycin. Briefly, for these tests each bacterium was grown in standard Mueller Hinton broth typically used in susceptibility tests and in Mueller Hinton broth modified to contain fecal extracts from nonchlorophyll-fed or chlorophyll-fed pigs. In other experiments, each bacterium was also tested when grown in standard Mueller Hinton broth or Mueller Hinton broth supplemented with or without a commercially available chlorophyll product (1 µg/mL) or with the potential efflux inhibitors, pheophorbide-a (1 µg/mL), pyropheophorbide-a (30 µg/mL) or PAβN (30 µg/mL). All test cultures were standardized to achieve an optical density equal to a 0.5 McFarland standard via dilution in uninoculated Mueller Hinton broth containing respective supplements. When applied to overnight cultures which contained approximately 10^8 CFU/mL this procedure resulted in a final inoculum of approximately 10^5 CFU/mL. The resulting bacterial suspensions were then transferred to 96 well microtiter plates and incubated without or with serial 2-fold dilutions of the test antibiotics. The minimum inhibitor concentration of each antibiotic was defined as the lowest concentration of antibiotics inhibiting the visible growth after 24 h of incubation at 37°C. All tests were completed at least 3 times. Chlorophyll was obtained from MP Biologicals LLC (Solon, OH, USA), pheophorbide-a and PAßN were obtained from Sigma-Aldrich (St. Louis, MO, USA) and pyropheophorbide-a was obtained from Frontier Scientific (Logan, UT, USA). Stock aqueous solutions of chlorophyll and PAßN

were prepared in water and stored at 4°C or -20°C until use. Solutions of pheophorbide-a and pyropheophorbide-a were prepared using dimethyl sulfoxide (Sigma-Aldrich) at less than 2% of final volume. The solutions were used within 24 hours of preparation. In order to determine if chlorophyll or the known efflux pump inhibitors pheophorbide-a, pyropheophorbide-a and PAβN exhibited antimicrobial activity in their own right, these compounds were tested against the multidrug resistant *Salmonella*, *Streptococcus*, *Staphylococcus* and *Enterococcus* using the assay above but in the absence of added antibiotic.

Source of bacteria: The multidrug resistant *Salmonella* strains were provided by Dr. Shaohua Zhao of the Office of Research, Center for Veterinary Medicine, Laurel, MD and have been previously characterized (Zhao et al., 2007). *Streptococcus agalactiae* and *Staphylococcus aureus* strains were provided by Dr. Max Paape (USDA, Beltsville, MD). *Enterococcus faecium and faecalis* were used by us previously to investigate persistence and dissemination of antimicrobial resistance in mixed populations of pig gut bacteria (Ramlachan et al., 2012).

Objective 1.b. Quantifying the effect of efflux pump inhibitors on the fitness of multidrug resistant, efflux pump-expressing bacteria within the competitive pig gut environment.

The objective was done in two phases to test the effect of chlorophyll supplementation on the fitness of multidrug resistant bacteria within the competitive gut environment of pigs fed medicated diets (phase 1) or non-medicated diets (phase 2). For phase 1, 12 pigs (averaging 69 ± 9.8 kg) previously grown on a non-medicated diet were randomly allocated to medicated grower diets supplemented without or with chlorophyll. Two commercially available (Producer's CO-OP, Bryan, TX) medicated diets were fed, the first was supplemented with chlortetracycline, sulfamethazine and penicillin (100, 100 and 50 g/ton, respectively) and was fed for 12 days and the second which was fed immediately after the first was supplemented with tylosin (100 µg/ton) and was also fed for 12 days. The diets were individually fed to each pig kept in separate pens twice daily (07:30 and 16:30) with or without chlorophyll treatment (6 pigs/treatment) which was fed to the pigs by top-dressing and mixing into a 20% portion of each meal which was offered first to promote total consumption of the chlorophyll (300 mg per each meal). Approximately 30 minutes later the remainder of the meal was offered. Pigs not receiving the chlorophyll product were fed each meal in two portions likewise. For phase two of the study, 9 pigs (separate from those used in phase 1 and averaging 75 ± 7.5 kg) grown the previous 3 weeks on a non-medicated diet were randomly allocated to a nonmedicated commercially available finisher diet (Producers CO-OP) that was supplemented without or with chlorophyll as described above.

In both phases, freshly fecal contents were collected after the morning feeding at 4 day intervals beginning at the start of each feeding period and continuing until the end of the study 24 days later. The fecal contents were returned to the lab and cultured on MacConkey agar and M Enterococcus (ME) agar antibiotic supplemented without or with 8 µg penicillin/mL, 16 µg chlortetracycline/mL or 100 ug tylosin/mL to quantify recovery of generic *Escherichia coli* and enterococcal bacteria. Fecal contents sampled prior to the beginning of the experiment were also qualitatively cultured for the presence of *Salmonella* via enrichment on 24 h tetrathionate broth and subsequent plating to Brilliant Green agar but *Salmonella* colonies were not observed after 24 h incubation. Consequently, fecal contents were not cultured for *Salmonella* during subsequent collections. Log_{10} transformations of *E. coli* and enterococcal counts were analyzed for main effects of dietary treatment and day of treatment using a repeated measures analysis of variance with a Tukey's Multiple Comparison of Means (Statistix 10 Analytical Software, Tallahassee, FL, USA).

Results:

Objective 1a. Determine the susceptibility of multidrug resistant, efflux pump-expressing bacteria to carbadox, chlortetracycline, sulfamethazine, and penicillin (ASP250), enrofloxacin, and tylosin following exposure to clarified porcine gut contents containing added efflux pump inhibitors.

Effects of chlorophyll and the efflux pump inhibitors on growth of test bacteria in absence of added antibiotics:

The minimum inhibitory activities of chlorophyll, pheophorbide-a, pyropheophorbide-a and PAβN against multidrug resistant *Salmonella* and select Gram-positive bacteria are presented in Table 1. Results clearly show a marked difference in sensitivity of Gram-negative and Gram-positive bacteria to pheophorbide-a and pyropheophorbide-a, with most of the Gram-positive bacteria being much more sensitive to these degradation products of chlorophyll than the *Salmonella* strains.

Effects of fecal extracts from nonchlorophyll- and chlorophyll-fed pigs on antimicrobial susceptibility:

Results from our study showed that the minimum inhibitory concentrations of the multidrug resistant *Salmonella* strains tested in this study to chlortetracycline were increased 2 to 4-fold when grown in Mueller Hinton broth modified to contain fecal extract prepared from pigs fed a non-medicated finishing diet without chlorophyll-supplementation than when grown in Mueller Hinton alone (Table 2). However, increases of 2 to 4-fold are not necessarily remarkable as these may be within the variability of the study method. There was little difference in minimum inhibitory concentrations of chlortetracycline against the multidrug resistance *Salmonella* when they were grown in Mueller Hinton broth modified to contain fecal extract prepared from pigs fed the non-medicated finishing diet with chlorophyll-supplementation or in the standard Mueller Hinton broth containing no added fecal extract. When the *Salmonella* strains were likewise tested against penicillin, minimum inhibitory concentrations were increased by 2 to 8-fold when grown in Mueller Hinton broth modified to contain fecal extracts from pigs supplemented with or without chlorophyll than when grown in the standard Mueller Hinton broth containing no added fecal extract (Table 2).

In the case of the Gram-positive bacteria, there were differential effects observed when comparisons were made between cells grown in the standard Mueller Hinton containing no added fecal extract and those grown in Mueller Hinton broth containing the with the fecal extracts (Table 2). For instance, when grown in Mueller Hinton modified to contain fecal extract from non-chlorophyll fed pigs the minimum inhibitory concentration of chlortetracycline against the *Staphylococcus aureus* strains and *Enterococcus faecalis* were either unchanged or modestly increased (by 4-fold) compared to the minimum inhibitory concentrations observed in cells grown in the standard Mueller Hinton broth but the minimum inhibitory concentrations were reduced by 8 to 16-fold in *Staphylococcus hyicus, Streptococcus agalactiae* and *Enterococcus faecium* H 1019V (Table 2). When grown in the Mueller Hinton broth modified to contain the fecal extract from chlorophyll-fed pigs, the minimum inhibitory concentration of chlortetracycline was unchanged or decreased modestly (by 4 to 16-fold) in *Enterococcus faecalis* and the *Staphylococcus aureus* strains but decreased more remarkably (128 to 256-fold) in *Streptococcus agalactiae, Staphylococcus hyicus* and *Enterococcus faecium* strain H 1019V (Table 2). The minimum inhibitory concentration of penicillin against the Gram-positive bacteria was also unaffected in most of the cultures grown in Mueller Hinton modified to contain the fecal extract from nonchlorophyll-fed pigs when compared to the cultures grown in the standard Mueller Hinton broth lacking added fecal extract, the exceptions being a modest increase (4-fold) for *Staphylococcus aureus* strain 49525 and a more impactful increase (64-fold) in *Enterococcus faecium* strain H 1019V9 (Table 2). For all of the cultures grown in Mueller Hinton broth modified to contain fecal extract from chlorophyll-fed pigs, minimum inhibitory concentrations of penicillin were decreased from those of cultures grown in the standard Mueller Hinton broth, with decreases ranging from a modest 2-fold

decrease by *Staphylococcus aureus* strain 49525 and *Enterococcus faecium* strain H 1019V9 to 253-fold decrease in the other Gram-positive bacteria (Table 2).

Comparison of the antimicrobial resistance of multidrug resistant *Salmonella* to carbadox revealed substantial increases (8 to 64-fold) in the minimum inhibitory activity of this antibiotic against when the *Salmonella* were grown in Mueller Hinton broth modified to contain fecal extracts from nonchlorophyll-fed pigs when compared to cultures grown in the standard Mueller Hinton broth (Table 3). However, results revealed modest decreases in the minimum inhibitory activity when these bacteria were grown in Mueller Hinton broth modified to contain fecal extracts from chlorophyll-fed pigs (Table 8). Minimum inhibitory concentrations of tylosin against the multidrug resistant *Salmonella* were unchanged or increased by as much as 8-fold from that of cultures grown in the standard Mueller Hinton broth by cultures grown in Mueller Hinton modified to contain fecal extracts, regardless of whether from nonchlorophyll- or chlorophyll-fed pigs (Table 3). Similarly, minimum inhibitor concentrations of tylosin against the Gram-positive bacteria tested here were unchanged or increased by as much as 512-fold in cultures grown in Mueller Hinton modified to contain fecal extracts, regardless of whether from nonchlorophyll- or chlorophyll-fed pigs (Table 3). Conversely, when compared to cultures grown in the standard Mueller Hinton broth, the minimum inhibitor concentrations of carbadox against the Gram-positive bacteria were mostly, but not entirely, decreased in cultures grown in the Mueller Hinton broth modified to contain fecal extract from the nonchlorophyll- or chlorophyll-fed pigs, with the reductions generally being more substantive in the cultures grown with the chlorophyll-fed extracts (Table 3). Exceptions where minimum inhibitor concentrations were unchanged during growth in one or both extract containing broth were with *Staphylococcus aureus* strains CP and strain 49525 and with *Enterococcus faecium* strain H 1019V (Table 3).

Comparison of the antimicrobial resistance of multidrug resistant *Salmonella* and select Gram-positive bacteria to ceftazidime or norfloxacin when grown in standard Mueller Hinton broth or Mueller Hinton modified to contain fecal extracts from nonchlorophyll- or chlorophyll-fed pigs are presented in Table 4. Briefly, minimum inhibitor concentrations of norfloxacin against the Salmonella were deceased 16 to 32-fold when grown in Mueller Hinton modified to contain extracts from chlorophyll-fed pigs but were unchanged when grown in Mueller Hinton modified to contain extracts from nonchlorophyll-fed pigs. Conversely, the minimum inhibitory concentrations of ceftazidime against the *Salmonella* were increased 16-fold when grown in the Mueller Hinton broth modified to contain extracts from either the nonchlorophyll- or chlorophyll-fed pigs when compared to those cultures grown in standard Mueller Hinton broth (Table 4). Conversely, growth of the Gram-positive bacteria in Mueller Hinton modified to contain the fecal extracts from nonchlorophyll- or chlorophyll-fed pigs resulted no change or as much as a 16-fold increase in the minimum inhibitory concentrations against norfloxacin but resulted in decreases of minimum inhibitory concentrations by as much as 32 to 256-fold for some of the Gram-positive bacteria, the exceptions being *Staphylococcus aureus* strain CP and the two *Enterococcus* strains (Table 4).

Effects of chlorophyll, pheophorbide-a, pyropheophorbide-a, or L-phenylalanyll-arginyl-ß-naphthylamide (PAβN) on antimicrobial susceptibility:

The effect of chlorophyll, pheophorbide-a, pyropheophorbide-a, or PAβN on antimicrobial susceptibility of multidrug resistant *Salmonella* and select Gram-positive bacteria are presented in Tables 5 through 9. Generally speaking the effects of added efflux pump inhibitors exhibited were highly variable across the different bacteria tested. In the case of the different *Salmonella* strains tested, the minimum inhibitory concentrations of chlortetracycline were increased 2 to 32 fold by chlorophyll, pyropheophorbide-a or PAβN but were unaffected or reduced 2 to 4-fold by pheophorbide-a (Table 5). Effects of the efflux inhibitors on minimum inhibitory concentrations of penicillin likewise were affected only modestly, being unchanged or affected by only 2 to 4-fold, the exception being an 8-fold decrease in the minimum inhibitory concentration of penicillin against *Salmonella*

Typhimurium 20731 when grown with PAβN (table 2). No effect of the efflux pump inhibitors on susceptibility of the *Salmonella* strains to sulfamethazine was observed (Table 5).

Because pheophorbide-a and pyropheophorbide-a were toxic to most of the Gram-positive bacteria at or near levels below our testing limits (1 and 30 µg/mL, respectively)(see Table 1), these efflux pump inhibitors were omitted from most of the analysis, the exception being *Staphylococcus hyicus* which was much more insensitive to these inhibitors than the other Gram-positive bacteria. The effects of chlorophyll and PAβN on minimum inhibitory concentrations of chlortetracycline were modest, with only 2 to 4-fold changes in susceptibility being observed (Table 6). In contrast, the effects of pheophorbide-a and pyropheophorbide-a on susceptibility of *Staphylococcus hyicus* to chlortetracycline were much more dramatic, with minimum inhibitory concentrations being reduced by 256 to 512-fold (Table 6). Little if any effect of chlorophyll or PAβN were observed on susceptibility of the Gram-positive bacteria to penicillin or sulfamethazine and effects of pheophorbide-a and pyropheophorbide-a on the susceptibility of *Staphylococcus* aureus to these antibiotics were also not observed (Table 6).

When carbadox was tested against the *Salmonella* strains, the efflux pump inhibitors again varied in the responses they caused, with changes in minimum inhibitory concentrations ranging from no effect to as much as 8-fold (Table 7). Similarly, when tested against the Gram-positive isolates, changes in minimum inhibitory concentrations caused by chlorophyll or PAβN ranged from no effect to as much as 32-fold (Table 7). With the Gram-positives, the only observation with pyropheophorbide-a was with *Stapylococcus hyicus* which showed a 64-fold decrease in its minimum inhibitory concentration to carbadox (Table 7). Little if any effect of chlorophyll, pheophorbide-a or pyropheophorbide-a was observed on susceptibility of the *Salmonella* strains to tylosin, however, reductions of 8 to 32-fold in the minimum inhibitory concentration of tylosin to the *Salmonella* were observed with PAβN (Table 7). Effects of chlorophyll or PAβN against the susceptibility of the Gram-positive bacteria to tylosin were not observed (Table 7).

The effects of the efflux pump inhibitors on minimum inhibitory concentrations of chloramphenicol, florfenicol, tetracycline, erythromycin, streptomycin, and ceftazidime were also against the *Salmonella* strains and the results are presented in Tables 8 and 9. Pheophorbide-a and chlorophyll were intermediate in effectiveness for most of the *Salmonella*, causing no or only modest 2 to 4-fold reductions in minimum inhibitor concentrations for only a few isolates (Tables 8 and 9). Changes of 2 to 4-fold are not necessarily remarkable, however, and may be within the variability of the study method. We did observed a more remarkable 16 fold reduction in the minimum inhibitor concentration of chloramphenicol against isolate 22430 that was affected by chlorophyll (Table 8). Chlorophyll, however, increased the minimum inhibitory concentration of florfenicol against isolate 31295 (Table 8) indicating that this compound modestly increased the sensitivity of this isolate to this antibiotic. Resistance against florfenicol was increased against another *Salmonella* isolate (#22430) by two other known efflux pump inhibitors, pyropheophorbide-a and PAßN, and this time the increase in minimum inhibitory concentration was 4 to 8-fold. However, both pyropheophorbide-and PAßN appeared to be more active in increasing the sensitivity of most of the other *Salmonella* isolates to the other tested antibiotics and in particular against ceftazidime, erythromycin and to a lesser extent against chloramphenicol, where minimum inhibitory concentrations were reduced as much as 32-fold (Tables 8 and 9).

Objective 1.b. Quantifying the effect of efflux pump inhibitors on the fitness of multidrug resistant, efflux pump-expressing bacteria within the competitive pig gut environment.

Medicated feeding trial:

Average daily feed intake did not differ between nonchlorophyll- and chlorophyll-fed pigs ($P = 0.4168$) and averaged 4.2 ± 0.22 kg feed (as feed basis) per day. Average daily feed intake did not differ ($P =$

0.6135) between the different phases of the study when in phase 1 the medicated diet contained chlortetracycline, penicillin and sulfamethazine and in phase 2 the medicated diet contained tylosin. An interaction between chlorophyll treatment and phase was not observed ($P = 0.8547$). Average daily gain did not differ between nonchlorophyll- and chlorophyll-fed pigs ($P = 0.7098$) but was affected by phase of treatment ($P < 0.0001$) and an interaction between chlorophyll treatment and phase was observed ($P = 0.0258$), with average daily gains being higher for nonchlorophyll- and chlorophyll-fed pigs (1.7 ± 0.27 and 1.4 ± 0.38 kg/d, respectively) in phase 1 than for chlorophyll- and nonchlorophyll-fed pigs in phase 2 (1.0 ± 0.14 and 0.8 ± 0.20 kg/d, respectively).

Effects of chlorophyll supplementation of E. coli and enterococcal populations during feeding of chlortetracycline, penicillin and sulfamethazine-medicated diet:

Fecal populations of generic *E. coli* were increased ($P < 0.0001$) by the fourth day on the medicated diet but did not differ ($P = 0.11$) between chlorophyll-supplemented and non-supplemented pigs (Figure 1). A significant day x supplement interaction was observed ($P = 0.03$) due mainly to a slight decrease of 0.64 log10 CFU/g in non-supplemented *E. coli* populations from day 8 to day 12 on diet that was not observed in the chlorophyll-supplemented populations. Concentrations of *E. coli* recovered on bacteriological growth medium containing 8 µg penicillin/mL or 16 µg chlortetracycline/mL did not differ medium from those recovered on medium without any selective antibiotic pressure indicating a high proportion of penicillin-insensitive and chlortetracycline-insensitive *E. coli* within the overall *E. coli* population. No other main effects or interactions were observed ($P > 0.70$).

A main effect of day on treatment was observed ($P < 0.0001$) on fecal enterococcal populations, with populations generally declining after 4 days on diet (Figure 1B). A main effect of chlorophyll supplementation was not observed ($P = 0.57$) as fecal enterococcal concentrations did not differ between chlorophyll-supplemented and non-supplemented pigs (Figure 1B). Concentrations of penicillin-insensitive enterococci were initially lower ($P < 0.05$) than the total enterococcal population recovered on bacteriological growth medium without any selective pressure or the chlortetracycline-insensitive population recovered on medium containing 16 µg chlortetracycline/mL and were generally lower when measured on subsequent days (Figure 1B). No other main effects or interactions were observed ($P > 0.11$).

Effects of chlorophyll supplementation of E. coli and enterococcal populations during feeding of tylosin-medicated diet.

A main effect of chlorophyll supplementation was observed ($P = 0.03$) on populations of generic *E. coli* but not ($P = 0.06$) on enterococcal populations (Figures 2A and 2B, respectively). The effect of chlorophyll supplementation on *E. coli* is likely only of marginal physiological impact as populations differed by less than 0.3 $\log_{10}$ CFU/g fecal material. Main effects of day on diet were observed on *E. coli* and on enterococcal populations ($P < 0.0001$) with *E. coli* populations decreasing less than 1.0 $\log_{10}$ CFU/g (Figure 2A) and enterococcal populations increasing by 1.27 to 1.80 $\log_{10}$ CFU/g feces (Figure 2B). No other main effects or interactions were observed on *E. coli* or enterococcal populations during feeding of the tylosin-medicated diet ($P > 0.26$).

Non-medicated feeding trail: Average daily feed intake did not differ between nonchlorophyll- and chlorophyll-fed pigs ($P = 0.0858$) and averaged 4.3 + 0.11 kg feed (as feed basis) per day. Average daily feed intake did not differ ($P = 0.2427$) between the different phases of the study when in phase 1 measured effects from days 1 to day 12 of feeding the nonmedicated diet and in phase 2 measured the effects from days 12 to 24 of feeding the same medicated diet. An interaction between chlorophyll treatment and phase was not observed ($P = 0.3221$). Average daily gain did not differ between

nonchlorophyll- and chlorophyll-fed pigs ($P = 0.7034$) or phase of diet (0.0528), averaging 1.2 ± 0.33 kg/d. An interaction between chlorophyll treatment and phase of study was not observed ($P = 0.5548$).

Non-medicated feeding trial: Effects of chlorophyll supplementation of E. coli and enterococcal populations during feeding of a non-medicated diet

Main effects of chlorophyll supplementation ($P = 0.001$) and day on diet ($P = 0.011$) were observed on fecal *E. coli* concentrations (Figure 3A) although the main effect means differed less than 0.6 $\log_{10}$ CFU/g. Concentrations of *E. coli* did not differ ($P = 0.98$) whether recovered on bacteriological growth medium without any selective antibiotic pressure or on medium containing 8 µg penicillin/mL or 16 µg chlortetracycline/mL. No other main effects or interactions were observed ($P > 0.86$).

Main effects of chlorophyll supplementation ($P = 0.0004$), day on diet ($P = 0.002$) and their interaction (supplement x day, $P = 0.009$) were observed on enterococcal populations during feeding of the non-medicated diet, with enterococcal populations being less in the fecal contents from chlorophyll-supplemented pigs during days 0 and 4 on the diet but not necessarily during the later days (Figures 3B and 3D). This suggests a potential adaptation of the enterococcal populations to the chlorophyll supplementation. A difference was observed ($P < 0.0001$) between the respective populations during the first 12 days on the nonmedicated diet, with enterococcal population recovered on medium containing 8 µg penicillin/mL being lower than the chlortetracycline-insensitive enterococcal populations recovered on media containing 16 ug chlortetracycline or the total population recovered on media containing no added antibiotic (Figure 3B). No difference was observed ($P = 0.27$) between enterococcal populations recovered from fecal samples cultured on medium containing either no or 100 µg added tylosin/mL (Figure 2D). Interactions between supplement x population, population x day, or supplement x population x day were not observed ($P > 0.47$).

Discussion: Explain your research results and include a summary of the results that is of immediate or future benefit to pork producers.

In this study we compared the antimicrobial resistance of multidrug resistant *Salmonella* and select Gram-positive bacteria against a selection of antibiotics after the bacteria had been grown in Mueller Hinton broth as it is commonly done in the laboratory or during growth in Mueller Hinton broth that was modified to contain extracts of fecal contents collected from nonchlorophyll- and chlorophyll-fed pigs. The chlorophyll was added to the pig diets because evidence suggests that certain bacterial degradation products of chlorophyll may accumulate in the gut and inhibit antimicrobial efflux pumps thereby making some multidrug resistant bacteria less resistant. We found that growth of the fecal extracts from nonchlorophyll and chlorophyll-fed did indeed affect resistance of some multidrug resistant *Salmonella* and Gram-positive bacteria, but these effects were highly strain and antibiotic dependent thus making it very difficult to generalize about how the extracts were affecting resistance. We had hypothesized that extract from the chorophyll-fed pigs would contain anaerobic breakdown products of chlorophyll and this may indeed be the case but further work is needed to analyze the extracts to quantify and these suspected compounds. Similarly, we observed some unexpected effects of the extracts obtained from nonchlorophyll-fed pigs and these need to be further analyzed to determine the mechanism behind their activity. Results from other studies testing individual addition of chlorophyll or its degradation products, pheophorbide-a or pyropheophorbide-a, or with the known efflux pump inhibitor, L-phenylalanyll-arginyl-β-naphthylamide, indicated that these compounds may indeed promote a decrease in antibiotic resistance but like that observed in the studies with added fecal extracts, the activity by these potential affecter compounds appears to be highly strain and antibiotic dependent; even affecting increases in resistance in some cases. Consequently, further work is needed to clarify the effects of these compounds on the multidrug resistant bacteria, particularly against many of the Gram-positive strains which were sensitive to being killed by concentrations of pheophorbide-a and pyropheophorbide-a tested in the present study.

Results from pig feeding studies indicated that pigs could be safely fed as much as 600 mg chlorophyll/pig per day without negative impacts on feed intake or performance. However, chlorophyll-supplementation had little if any appreciable effect on native populations of penicillin-insensitive, chlortetracycline-insensitive or tylosin-insensitive *E. coli* or enterococci in feces from pigs fed typical commercial grower diets containing penicillin, chlortetracycline and sulfamethazine or containing tylosin. An effect of chlorophyll-supplementation was observed on *E. coli* and enteroccal populations in separate pigs fed a commercial non-medicated diet indicating that chlorophyll itself, or its degradation products, may be inhibitory to these populations by as yet unknown mechanisms which may not necessarily involve efflux pumps.

Based on these results, it is unlikely at the present time that the efflux pump inhibitors tested in the present study can be developed into an inexpensive technology to preserve and enhance the efficacy of currently available antibiotics. Considering, however, that the fecal extracts and the as yet undefined effectors they contain can have a marked impact on resistance it seems reasonable to suspect that resistance expressed within the gut of pigs may be quite different than that determined solely when a microbe is assayed in pure culture in medium lacking these effectors.

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Table 1: Minimum inhibitory concentration (MIC) of select efflux pump inhibitors against Gram-negative and Gram-positive bacteria used in this study.

Test organism	Efflux pump inhibitor[a] (μg/mL)			
	CLP	PHEO	PYR	PAβN
	Gram-negative			
Salm. Newport 24400	128	128	240	40
Salm. Newport 30171	128	128	240	40
Salm. Newport 22489	128	128	240	80
Salm. Newport 31295	128	128	240	40
Salm. Typhimurium 22421	128	128	240	40
Salm. Typhimurium 22544	128	128	240	40
Salm. Typhimurium 20731	128	128	240	40
Salm. Typhimrium 22430	128	128	240	80
	Gram-positive			
Staph. aureus CP	>512	64	7.5	80
Staph. aureus 49521	>512	32	15	>160
Staph. aureus 49525	>512	32	30	>160
Staph. hyicus	>512	>128	>120	>160
Strep. agalactiae	>512	4	7.5	40
Ent. faecium H 1019V	>512	>128	60	>160
Ent. faecalis	>512	32	7.5	>160

[a]MIC of CLP, chlororphyll; PHEO, pheophorbide-a; PYR, pyropheophorbide-a; or PAβN, L-phenylalanyll-arginyl-β-naphthylamide.

Table 2. Minimum inhibitory concentration (MIC) of chlortetracycline and penicillin against select multidrug resistant Gram-negative and Gram-positive bacteria when grown in Mueller Hinton broth without or with addition of potential efflux pump inhibitors that may be naturally present in fecal extracts of nonchlorophyll-fed or chlorophyll-fed pigs.[a]

Isolate #	Chlortetracycline			Penicillin		
	Grown in standard MH broth alone	Grown in MH broth with fecal extracts from nonchlorophyll-fed pigs	Grown with fecal extracts from chlorophyll-fed pigs	Grown in standard MH broth alone	Grown with fecal extracts from nonchlorophyll-fed pigs	Grown with fecal extracts from chlorophyll-fed pigs
	Gram-negative					
Salm. Newport 24400	32	128	32	2344	>18750	9375
Salm. Newport 30171	32	128	32	2344	>18750	9375
Salm. Newport 22489	16	128	32	2344	>18750	9375
Salm. Newport 31295	32	128	32	4688	>18750	9375
Salm. Typhimurium 22421	64	128	32	9375	>18750	9375
Salm. Typhimurium 22544	64	128	64	4688	>18750	9375
Salm. Typhimurium 20731	64	128	64	4688	>18750	9375
Salm. Typhimurium 22430	32	128	64	9375	>18750	9375
	Gram-positive					
Staph. aureus CP	16	64	<4	9375	9375	<37
Staph. aureus 49521	64	64	8	9375	9375	<37
Staph. aureus 49525	64	64	<4	2344	9375	1172
Staph. hyicus	1024	64	<4	NA	9375	<37
Step. Agalactiae	512	64	<4	9375	9375	<37
Ent. faecium H 1019V	1024	64	<4	146	9375	74
Ent. faecalis	16	64	16	9375	9375	<37

[a]Cultures grown in standard Mueller Hinton broth alone or Mueller Hinton broth supplemented with fecal extracts from nonchlorophyll- or chlorophyll-fed pigs exhibited equivalent growth when in absence of added antibiotic.

Table 3. Minimum inhibitory concentration (MIC) of carbadox and tylosin against select multidrug resistant Gram-negative and Gram-positive bacteria when grown in Mueller Hinton broth without or with addition of potential efflux pump inhibitors that may be naturally present in fecal

Table 4. Minimum inhibitory concentration (MIC) of ceftazidime and norfloxacin against select multidrug resistant Gram-negative and Gram-positive bacteria when grown in Mueller Hinton broth without or with addition of potential efflux pump inhibitors that may be naturally present in fecal extracts of nonchlorophyll-fed or chlorophyll-fed pigs.

Isolate #	Ceftazidime			Norfloxacin		
	Grown in standard MH broth alone	Grown in MH broth with fecal extracts from nonchlorophyll-fed pigs	Grown with fecal extracts from chlorophyll-fed pigs	Grown in standard MH broth alone	Grown with fecal extracts from nonchlorophyll-fed pigs	Grown with fecal extracts from chlorophyll-fed pigs
	Gram-negative					
Salm. Newport 24400	32	>512	>512	16	16	0.5
Salm. Newport 30171	32	>512	>512	16	16	0.5
Salm. Newport 22489	32	>512	>512	2	2	1
Salm. Newport 31295	32	>512	>512	16	16	0.5
Salm. Typhimurium 22421	32	>512	>512	16	16	0.5
Salm. Typhimurium 22544	32	>512	>512	16	16	1
Salm. Typhimurium 20731	32	>512	>512	16	16	1
Salm. Typhimrium 22430	32	>512	>512	16	16	0.5
	Gram-positive					
Staph. aureus CP	>512	>512	16	1024	1024	1024
Staph. aureus 49521	>512	>512	>512	64	1024	1024
Staph. aureus 49525	>512	>512	8	1024	1024	1024
Staph. hyicus	>512	>512	2	1024	1024	1024
Strep. Agalactiae	>512	>512	2	1024	1024	1024
Ent. facium H 1019V	>512	>512	>512	1024	1024	1024
Ent. faecalis	>512	>512	>512	128	1024	1024

[a]Cultures grown in standard Mueller Hinton broth alone or Mueller Hinton broth supplemented with fecal extracts from nonchlorophyll- or chlorophyll-fed pigs exhibited equivalent growth when in absence of added antibiotic.

Table 5: Minimum inhibitory concentration (MIC) of chlortetracycline, penicillin and sulfamethazine against select multidrug resistant *Salmonella* strains when tested without or with addition of efflux pump inhibitors.

Salmonella Isolate #	Chlortetracycline (µg/mL)					Penicillin (µg/mL)					Sulfamethazine (µg/mL)				
	Alone[a]	CLP	PHEO	PYR	PaβN	Alone	CLP	PHEO	PYR	PAβN	Alone	CLP	PHEO	PYR	PAβN
24400	32	512	16	128	64	2344	4688	4688	4688	2344	>2048	>2048	>2048	>2048	>2048
30171	32	512	32	128	128	2344	4688	4688	4688	2344	>2048	>2048	>2048	>2048	>2048
22489	16	512	32	512	128	2344	4688	4688	4688	4688	>2048	>2048	>2048	>2048	>2048
31295	32	512	32	128	128	4688	4688	9375	4688	4688	>2048	>2048	>2048	>2048	>2048
22421	64	128	16	128	NA[b]	9375	2344	2344	4688	NA	>2048	>2048	>2048	>2048	NA
22544	64	1024	64	512	NA	4688	4688	4688	2344	1172	>2048	>2048	>2048	>2048	NA
20731	64	512	64	512	NA	4688	4688	4688	9375	586	>2048	>2048	>2048	>2048	NA
22430	32	512	32	512	64	9375	18750	18750	18750	9375	>2048	>2048	>2048	>2048	>2048

[a]MIC when tested alone without added efflux pump inhibitor; CLP, chlorophyll; PHEO, pheophorbide-a; PYR, pyropheophorbide-a; or PAβN, L-phenylalanyll-arginyl-β-naphthylamide .
[b]NA, not available.

Table 6. Minimum inhibitory concentration (MIC) of antibiotics chlortetracycline, penicillin and sulfamethazine against select multidrug resistant Gram-positive strains when tested without or with addition of efflux pump inhibitors.

Isolate #	Chlortetracycline					Penicillin					Sulfamethazine				
	Alone[a]	CLP	PHEO	PYR	PaβN	Alone	CLP	PHEO	PYR	PAβN	Alone	CLP	PHEO	PYR	PAβN
Staph. aureus CP	16	4	NA	NA	64	9375	9375	NA	NA	9375	>2048	>2048	NA	NA	>2048
Staph. aureus 49521	64	128	NA	NA	1024	9375	9375	NA	NA	9375	>2048	>2048	NA	NA	>2048
Staph. aureus 49525	64	8	NA	NA	128	2344	9375	NA	NA	9375	>2048	>2048	NA	NA	>2048
Staph. hyicus	1024	2048	4	4	1024	NA	NA	NA	NA	NA	>2048	>2048	NA	>2048	>2048
Strep. Agalacatiae	512	1024	NA	NA	1024	9375	9375	NA	NA	9375	>2048	>2048	NA	NA	>2048
Ent. faecium H1019V	1024	1024	NA	NA	1024	146	586	NA	NA	293	>2048	>2048	NA	NA	>2048
Ent. faecalis	16	32	NA	NA	32	9375	9375	NA	NA	9375	>2048	>2048	NA	NA	>2048

[a]MIC when tested alone without added efflux pump inhibitor; CLP, chlorophyll; PHEO, pheophorbide-a; PYR, pyropheophorbide-a; or PAβN, L-phenylalanyll-arginyl-β-naphthylamide .
[b]NA, not available.

Table 7: Minimum inhibitory concentration carbadox or tylosin against multidrug resistant Gram-negative and Gram-positive bacteria when tested without or with addition of efflux pump inhibitors.

Salmonella Isolate #	Carbadox					Tylosin				
	Alone[a]	CLP	PHEO	PYR	PAβN	Alone	CLP	PHEO	PYR	PAβN
	Gram-negative									
Salm. Newport 24400	8	1	8	1	NA	1024	1024	512	512	32
Salm. Newport 30171	4	1	2	0.50	NA	1024	1024	1024	512	64
Salm. Newport 22489	2	0.50	1	0.50	0.50	1024	1024	512	512	128
Salm. Newport 31295	2	2	4	1	1	1024	1024	512	512	128
Salm. Typhimurium 22421	2	0.25	0.25	0.25	NA	2048	2048	1024	1024	NA
Salm. Typhimurium 22544	4	1	2	1	NA	2048	2048	2048	2048	NA
Salm. Typhimurium 20731	2	0.25	8	1	NA	1024	1024	1024	1024	16
Salm. Typhimurium 22430	4	4	8	1	2	1024	1024	1024	512	64
	Gram-positive									
Staph. aureus CP	64	32	NA	NA	8	>4096	>4096	NA	NA	>4096
Staph. aureus 49521	512	256	NA	NA	512	>4096	>4096	NA	NA	>4096
Staph. aureus 49525	1024	128	NA	NA	32	>4096	>4096	NA	NA	>4096
Staph. hyicus	512	256	NA	8	512	>4096	>4096	NA	NA	>4096
Strep. Agalactiae	256	16	NA	NA	NA	>4096	>4096	NA	NA	>4096
Ent. faecium H 1019V	1024	512	NA	NA	512	>4096	>4096	NA	NA	>4096
Ent. faecalis	16	64	NA	NA	256	>4096	>4096	NA	NA	>4096

[a]MIC when tested alone without added efflux pump inhibitor; with CLP, chlorophyll; PHEO, pheophorbide-a; PYR, pyropheophorbide-a; or PAβN, L-phenylalanyll-arginyl-β-naphthylamide.
[b]NA, not available.

Table 8: Minimum inhibitory concentration (MIC) of antibiotics chloramphenicol, tetracycline and florfenicol against select multidrug resistant *Salmonella* strains when tested without or with addition of efflux pump inhibitors.

Salmonella Isolate #	Chloramphenicol					Tetracycline					Florfenicol				
	Alone	CLP	PHEO	PYR	PAβN	Alone	CLP	PHEO	PYR	PAβN	Alone	CLP	PHEO	PYR	PAβN
24400	512	256	512	64	128	128	128	128	128	64	8	8	8	2	4
30171	512	256	512	256	256	128	128	128	64	32	8	8	8	4	4
22489	512	512	512	512	256	256	256	256	128	128	16	16	16	4	8
31295	256	256	256	256	256	128	128	128	128	64	8	16	8	8	8
22421	512	512	512	512	256	256	256	256	128	64	16	16	16	4	4
22544	128	128	64	4	64	512	512	512	256	128	8	8	8	8	8
20731	512	512	512	512	512	512	512	512	128	256	1	1	0.50	0.25	4
22430	512	32	256	32	16	256	256	256	128	64	0.50	0.50	0.50	2	4

[a]MIC when tested alone without added efflux pump inhibitor; CLP, chlorophyll; PHEO, pheophorbide-a; PYR, pyropheophorbide-a; or PAβN, L-phenylalanyll-arginyl-β-naphthylamide.

Table 9: Minimum inhibitory concentration (MIC) of antibiotics chloramphenicol, tetracycline and florifenicol against se
Salmonella strains when tested without or with addition of efflux pump inhibitors.

Salmonella Isolate #	Streptomycin					Ceftazidime					
	Alone	CLP	PHEO	PYR	PAβN	Alone	CLP	PHEO	PYR	PAβN	Alone
24400	512	256	256	128	128	32	32	16	1	4	4
30171	512	512	512	256	256	32	32	32	4	4	8
22489	256	256	256	64	128	32	32	16	4	4	8
31295	512	512	512	512	512	32	32	16	4	4	32
22421	512	256	256	64	128	32	32	32	2	2	8
22544	512	512	512	256	256	32	32	16	1	4	16
20731	128	64	64	128	128	32	16	16	2	4	8
22430	512	512	512	256	256	32	32	8	8	8	4

[a]MIC when tested alone without added efflux pump inhibitor; CLP, chlorophyll; PHEO, pheophorbide-a; PYR, pyropheop
phenylalanyll-arginyl-β-naphthylamide .
[b]NA, not available.

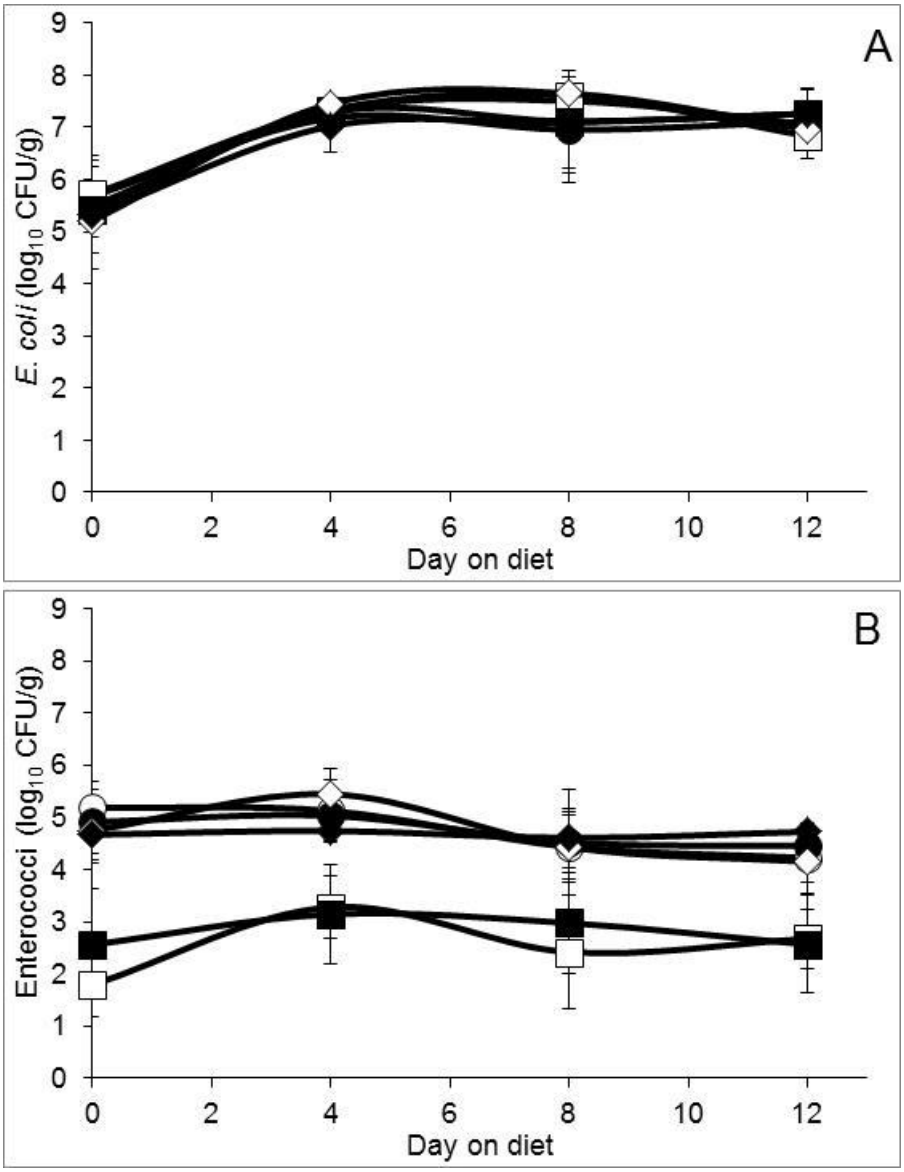

Figure 1. Effect of chlorophyll supplementation on fecal populations of generic *Escherichia coli* (Figure A) and enterococci populations (Figure B) during individually feeding (twice daily, 07;30 and 16;30) of a mediated grower diet (penicillin, 50 g/ton; chlortetracycline and sulfamethazine, each at 100 g/ton). Open symbols reflect mean bacterial counts (colony forming units, CFU/g feces) from pigs fed no chlorophyll and filled symbols reflect mean bacterial counts from pigs fed 300 mg of water soluble chlorophyll product at each feeding. Circles reflect populations recovered on bacteriological growth medium without any selective pressure, squares reflect populations recovered on growth medium containing 8 µg penicillin/mL, and diamonds reflect populations recovered on growth medium containing 16 µg chlortetracycline/mL. Each symbol represents the mean ± SD from n = 6 pigs.

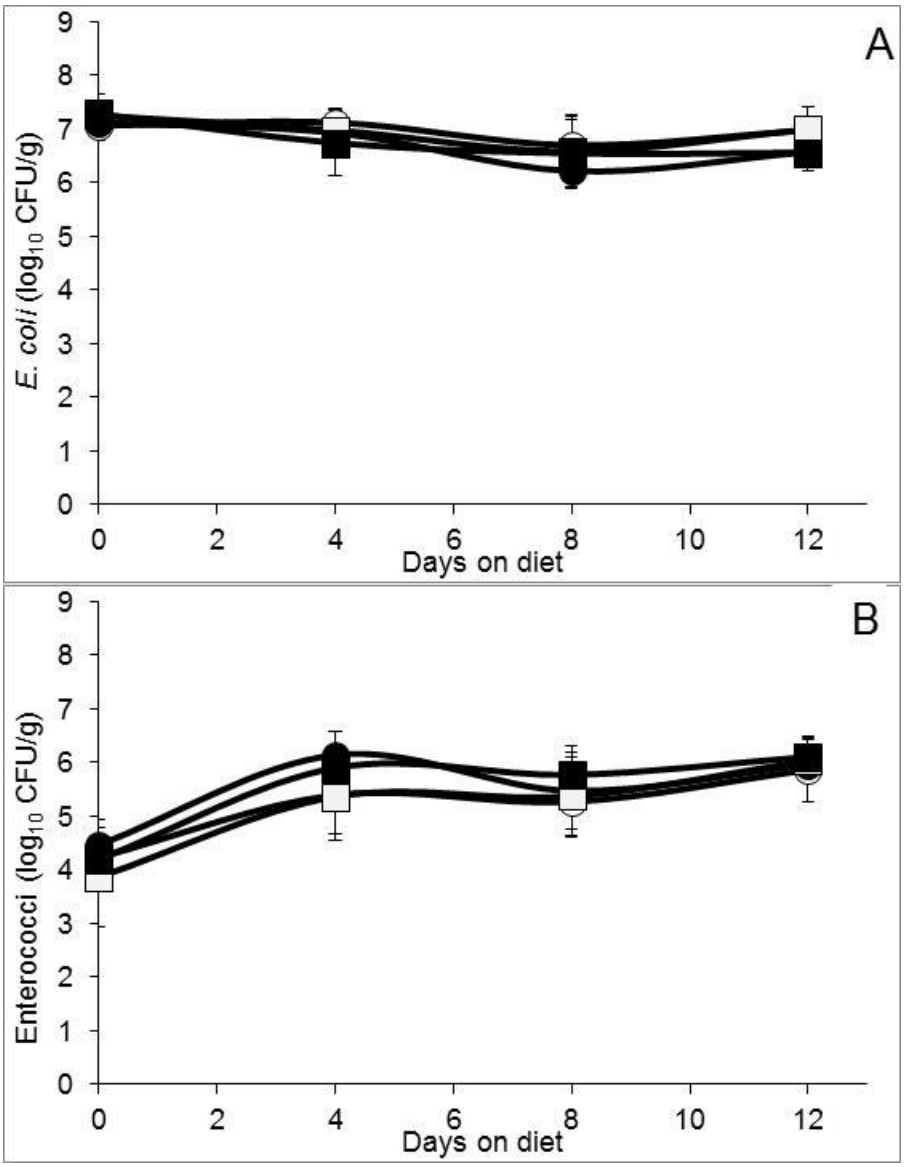

Figure 2. Effect of chlorophyll supplementation on fecal populations of generic *Escherichia coli* (Figure A) and enterococci populations (Figure B) during individually feeding (twice daily, 07;30 and 16;30) of a mediated grower diet (tylosin, 100 g/ton). Open symbols reflect mean bacterial counts (colony forming units, CFU/g feces) from pigs fed no chlorophyll and filled symbols reflect mean bacterial counts from pigs fed 300 mg of water soluble chlorophyll product at each feeding. Circles reflect populations recovered on bacteriological growth medium without any selective pressure, squares reflect populations recovered on growth medium containing 100 µg tylosin/mL. Each symbol represents the mean ± SD from n = 6 pigs.

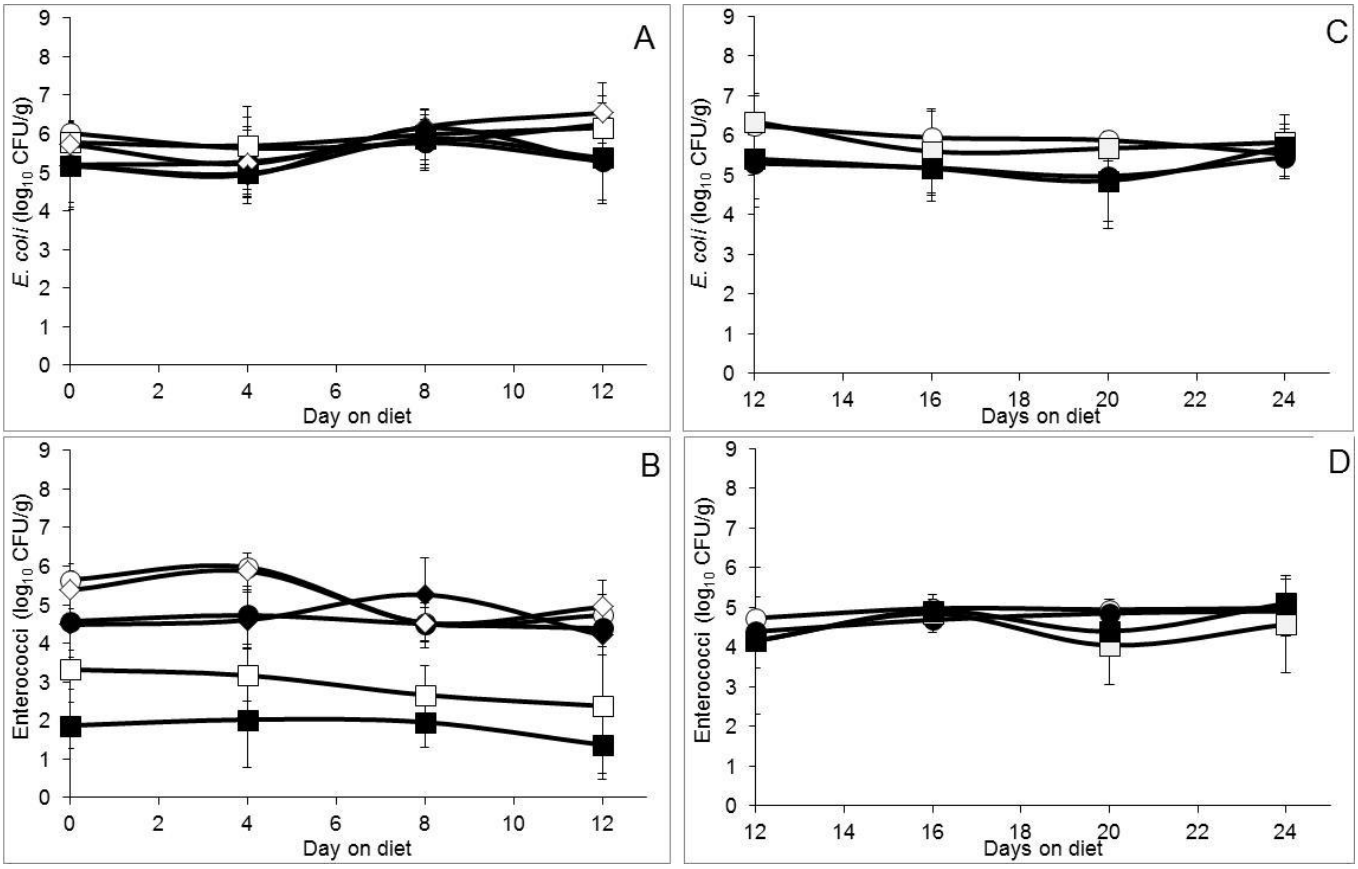

Figure 3. Effect of chlorophyll supplementation on fecal populations of generic *Escherichia coli* (Figures A and C) and enterococci populations (Figures B and D) during individually feeding (twice daily, 07;30 and 16;30) of a non-mediated diet. Open symbols reflect mean bacterial counts (colony forming units, CFU/g feces) from pigs fed no chlorophyll and filled symbols reflect mean bacterial counts from pigs fed 300 mg of water soluble chlorophyll product at each feeding. Circles reflect populations recovered on bacteriological growth medium without any selective pressure, squares and diamonds in Figures A and B reflect populations recovered on growth medium containing 8 µg penicillin/mL or containing 16 µg chlortetracycline/mL. Squares in Figures C and D represent populations recovered on medium containing 100 µg tylosin/mL. Each symbol represents the mean ± SD from n = 5 non-supplemented or n = 4 chlorophyll-supplemented pigs.