RESEARCH REPORT
PUBLIC HEALTH/WORKER SAFETY

Title: Do probiotics, an antibiotic alternative, mitigate or contribute to emergence and persistence of antimicrobial resistance in gut bacteria? – NPB #15-024

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Industry Summary:

The use of antimicrobials in feed and their positive benefits on growth performance during the nursery stage of swine production system is well established. In the past, producers have widely used antimicrobials throughout the nursery stage of swine production, even in the absence of a disease challenge. With the changing perspectives on the use of in-feed antimicrobials, alternative technologies are being considered that can provide growth performance benefits. Probiotics are one such alternative that enhance gut health for improved performance benefits. Antimicrobial resistance in swine is of major public health concern. The perpetuation of antimicrobial resistance involves complex ecological and genetic factors other than the selection pressure exerted by the use of antibiotics. It is therefore important to evaluate and ensure that probiotics, an alternative to antibiotics, do not themselves contribute to AMR development, before producers can be guided as to which probiotics are effective in mitigating AMR. *We have identified probiotic products, frequently used in the swine industry, that carry or do not carry AMR*. Results from our study will help the swine industry to draw useful conclusions on potential risks for human health caused directly by the use of antibiotic alternative. Our research will significantly advance applied scientific knowledge in ways to manage levels of bacterial resistance in swine production settings that are of concern both to the swine industry and to the public. This evaluation will also help us select probiotic products that are likely to have maximum impact in the mitigation of AMR in gut bacteria. Results from this study will help to develop intervention strategies to mitigate AMR and toward maintaining the usefulness of current antimicrobial drugs in swine production system.

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Probiotics, Antimicrobial resistance, *Escherichia coli*, *Enterococcus* spp., *Campylobacter* spp., *Salmonella*

Scientific Abstract:

Probiotics are live cultures of bacteria or fungi supplemented in swine diets that can beneficially affect the host animal by improving the microbial balance in the gut. *Because probiotics favorably alter bacterial population in the gut, which includes establishment of the 'beneficial bacteria' and exclusion of 'undesirable or harmful bacteria', it is possible that they mitigate emergence and persistence of AMR in gut bacteria. An aspect of feeding probiotics that has not received any attention in the US is whether probiotics, a product generally recognized as safe (GRAS status), could be a source of AMR?* The impact of probiotics on the emergence, spread and persistence of AMR in gut bacteria is an unexplored area. Therefore, we conducted a study to answer two questions: *Do probiotics mitigate AMR in gut bacteria* and *do probiotics that carry AMR genes contribute to AMR in gut bacteria?*

Initial *in vitro* screening of eight probiotic products containing *E. faecium* revealed the presence of AMR both by phenotypic and genotypic methods. The probiotic *E. faecium* strains were phenotypically resistant to one to three antimicrobials. The pulsed-field gel electrophoresis revealed that *E. faecium* strains from two probiotic products were clonally related. The whole genome sequencing of eight *E. faecium* isolates revealed the presence of macrolide and streptogramin B resistance mediated by *msrC* gene, and tetracycline resistant determinants, *tet*(L) and *tet*(M) genes. For the field study, we selected a probiotic which possessed AMR and one that did not possess AMR to medically-important antibiotics. Study consisted of 60 pens housing 300 weaned piglets with 5 piglets per pen, which were randomly allocated to six treatment groups; control, probiotic A, probiotic B, chlortetracycline (CTC; 22 mg/kg BW), and combination of each probiotic and CTC. Fecal samples were collected from 3 piglets per pen on days 0, 7, 14, 21, 28, 35, and 42 for the isolation of *E. coli*, *Enterococcus* spp., *Campylobacter* spp., and *Salmonella enterica*. Overall prevalence of *Campylobacter* was 21.6% (273/1,260) *C. coli* (49/273) or *C. hyointestinalis* (224/273). The overall prevalence of *S. enterica* was 6.6% (84/1,260). All *E. coli* isolates were susceptible to azithromycin and sulfisoxazole and a majority of the isolates were resistant to tetracycline (93.3%), ampicillin (81.6%), streptomycin (58.5%), ceftriaxone (52.4%), and ceftiofur (36.1%). All the tested *Enterococcus* isolates were susceptible to vancomycin. Majority of the isolates were resistant to tetracycline (95%), erythromycin (86.1%), lincomycin (83.3%), ciprofloxacin (67.2%), nitrofurantoin (53.3%), streptomycin (45.2%) and kanamycin (41.5%). Based on this study, the two probiotic products tested alone or in combination with CTC, had no effects on fecal shedding of *Campylobacter* spp., and *Salmonella enterica* and on antimicrobial resistance in fecal *E. coli*, *Enterococcus* spp., *Campylobacter* spp., and *Salmonella enterica*. However, additional studies are warranted to reevaluate the impact of feeding other probiotic products on the fecal prevalence of food borne pathogens and AMR of gut commensals.

Introduction:

Antimicrobial resistance (AMR) is a significant public health issue in the US and around the world. In the swine industry, in-feed antibiotics have traditionally been used to prevent enteric infections, improve body weight gain and production efficiency. In the past two decades, there is considerable interest in the use of antibiotic alternatives to achieve growth promotion and in some cases exclude gut pathogens and reduce subclinical infections. These alternatives include probiotics, prebiotics, exogenous enzymes, bacteriophages, plant extracts, essential oils, egg yolk antibodies, and metals such as copper and zinc. The use of the growth promoting antibiotics has led to increased antibiotic resistance in gut bacteria. The use of chemical substances as antibiotic alternatives is futile because bacteria also develop resistance to these compounds. A good example of this is the use of copper for growth promotion in swine and cattle. Copper is an essential micronutrient generally included in trace amounts in the feed to meet the physiological requirements of the animal. At elevated levels, copper promotes growth and improves feed utilization, which are attributed to its antimicrobial

activities. However, bacteria exposed to copper become resistant, and the acquisition of copper resistance genes has been reported in both Gram-positive and Gram-negative bacteria. Therefore, research on strategies, particularly on alternatives to antibiotics that promote growth and mitigate AMR in swine production is timely. ***In this study, we focused on probiotics because of their wide acceptance in the swine industry, the availability of several commercial products and, more importantly, because of their potential to have a direct impact on the gut ecosystem and prevalence of AMR among gut bacteria.***

Probiotics are live cultures of microorganisms supplemented in swine diets that can beneficially affect the host animal by improving the microbial balance in the gut. Most probiotic products include one or more species of the following bacterial genera: *Lactobacillus, Bacillus, Bifidobacterium,* and *Enterococcus* and in some cases yeast (*Saccharomyces cerevesiae*) and *Aspergillus*. The proposed benefits from probiotics are growth promotion, improved digestion, stimulation of gastrointestinal immunity and increased resistance to certain infectious diseases of the gut. The effect of probiotic supplementation on growth performance has been evaluated in numerous studies. Overall, research indicates that feeding probiotics improves health and promotes growth rate in pigs in different production situations. Antimicrobial resistance in probiotic bacteria can be intrinsic, acquired by mutation or by horizontal gene transfer. The species of ***Enterococcus*** and ***Lactobacillus*** are the largest groups of bacteria in commercially available probiotic products. Several genes responsible for antibiotic resistance properties have been reported in *Lactobacillus*. The most common horizontally transferable AMR genes confer resistance to tetracyclines. Many AMR genes are carried in horizontally transferable elements in ***Bacillus*** species. For example, *ermC* and *tet*(L) have been found on plasmids, and *tet*(M) was demonstrated in a conjugative transposon Tn*5397* of *Bacillus subtilis*. Some species of ***Enterococcus*** are opportunistic pathogens, and are notorious for their ability to acquire, harbor and transfer AMR to other gut bacteria. The *tet*(M) gene is transferable among enterococci belonging to species *E. faecalis, E. faecium, E. durans, E. hirae* and *E. mundtii*. ***The impact of probiotics on the emergence, spread and persistence of AMR in gut bacteria is an unexplored area. Therefore, this research was conducted to determine whether commercially available probiotic products in the US carry AMR genes and to assess their potential to mitigate or contribute AMR to gut bacteria.***

Objectives:

Objective 1. Detect and characterize phenotypically and genotypically the AMR present in commercially available probiotics widely used in swine industry.

a) Determine phenotypic AMR profiles of the bacterial species contained in the probiotic products

b) Detection of AMR genes by whole genome sequencing

Instead of utilizing microarray technology for detecting AMR genes, we decided to use whole genome sequencing (WGS) technology for genotypic characterization of probiotic strains. The WGS analyses gives a more complete information on the prevalence of AMR genes, plasmids, transposons, virulence genes, etc. Previous studies have shown a high concordance between phenotypic susceptibility patterns and the WGS data.

Objective 2. Conduct a field study in nursery pigs fed diets supplemented with or without probiotics and CTC to assess the impact of probiotics on AMR profiles of:

a) Gut commensals (*Escherichia coli* and *Enterococcus* spp.), and
b) Foodborne pathogens (*Campylobacter* spp., and *Salmonella enterica*) that are shed in the feces.

Materials & Methods:

Animals, treatments, and study design: The animal trial was conducted from 1/11/2016 to 2/22/2016 at the segregated early weaning (SEW) facility at Kansas State University. The SEW facility has two metal

buildings (south and north barns) with 40 pens each. A total of 300 weaned piglets (21 days of age) were used in the study. Piglets were allocated into pens (5 pigs per pen) distributed in two barns so that average pen weights are relatively equal across pens. Pens were randomly assigned to six treatments in a 3*2 complete factorial design. The six treatment groups were: **a)** control: a basal diet formulation (NRC, 2012) without antibiotic or probiotic, **b)** antibiotic group: a basal diet supplemented with chlortetracycline (CTC) at 400 g/ton of feed (22 mg/kg BW), **c)** probiotic A (containing pan susceptible *E. faecium*): supplemented as per as per the label, **d)** probiotic B (containing multidrug resistant *E. faecium*), **e)** probiotic A and CTC and **f)** probiotic B and CTC. The duration of the study was 42 days. In order to comply with FDA guidelines, in the CTC-supplemented groups, the pigs were fed the control diet with no CTC on days 14 and 28 of the study period. On the next day (days 15 and 29), feeding of CTC supplemented diet was resumed. Pen-level treatments was applied to a total of 60 pens with 10 pens per treatment group. The basal diet consisted of corn, soybean meal, vitamins, amino acids, and trace mineral supplements. A common starter diet was fed to all pigs for 4 days after weaning. Trace mineral premix contained 17 ppm Cu and 110 ppm Zn. Piglets were housed in environmentally controlled nursery facilities with each pen consisting of a four-hole, dry, self-feeder and nipple water to provide *ad libitum* access to feed and water. Fecal samples were collected from 3 of 5 piglets randomly from each pen on days 0, 7, 14, 21, 28, 35, and 42. Fecal samples were transported on ice in a cooler to the Pre-harvest Food Safety laboratory at Kansas State University. Both laboratory and farm personnel were blinded to the treatment.

Growth performance: All experimental diets were fed in meal form and were prepared at the K-State O.H. Kruse Feed Technology Innovation Center. Multiple diet samples were collected, and pooled samples of each diet were submitted for analysis of DM, CP, Ca, and P. Pigs and feeders were weighed every 7 d to determine ADG, ADFI, and F/G.

Bacterial isolation and identification: Isolation of *Escherichia coli, Enterococcus* spp., and *Salmonella* isolation from fecal samples were as per our published procedures (Agga et al., 2014; Amachawadi et al. 2010; Dodd et al., 2011). For *Campylobacter,* the procedure described by Sippy et al. (2012) was used. Two isolates of each bacterial species per fecal sample was subjected for preliminary genus confirmation, followed by species identification by MALDI-TOF bacterial ID system (Bruker Corp., Fremont, CA). The confirmed isolates were stored in Cryocare-protect® beads (Key Scientific Products, Stamford, TX) at -80 ºC for future use.

Antibiotic susceptibility determinations*:* Minimum inhibitory concentrations were determined by broth-microdilution method as per Clinical Laboratory Standards Institute (CLSI) guidelines (2008). The MIC for *Enterococcus, E. coli, Campylobacter,* and *Salmonella* isolates were determined by the broth micro-dilution method using the Sensititre® automated antimicrobial system (Trek Diagnostics Systems, Cleveland, OH). National Antimicrobial Resistance Monitoring System Gram-positive panel plates (CMV3AGPF: *Enterococcus* spp.), Gram-negative (CMV3AGNF: *E. coli*) panel plates were used with the aid of the Sensititre® automated inoculation delivery system (Trek Diagnostics Systems, Cleveland, OH). Appropriate ATCC (American Type Culture Collection, Manassas, VA) quality control strains, *Escherichia coli* ATCC 25922, *Enterococcus faecalis* ATCC 29212, were used as reference standards for susceptibility testing. The MIC for each isolate was recorded and classified as resistant, intermediate or sensitive based on the CLSI guidelines.

Statistical Analysis: Growth data were analyzed as a randomized complete block design using the PROC GLIMMIX procedure of SAS (SAS Institute, Inc., Cary, NC) with pen as the experimental unit. The main effects of CTC and probiotics, as well as their interactions, were evaluated using preplanned CONTRAST statements. These contrast statements were arranged as a 2 × 3 factorial with the main effects of CTC and each of the probiotics. Differences between treatments were determined by using least squares means. A P-value $\leq$ 0.05 was considered significant and $0.05 < P \leq 0.10$ was considered marginally significant. The data were analyzed using STATA SE (v.12.1; STATA Corp., College Station, TX). Data were considered multilevel and

longitudinal in nature because pens were nested within each treatment, animals clustered within pens, and within-animal dependencies existed because of repeated sampling of the pens during the study period. A likelihood ratio chi-square test was used to compare the unadjusted effects of treatments and sampling days. A full factorial model evaluating the effects of antibiotic and two probiotics with their interaction by sampling days was used throughout the analyses. Generalized estimating equations (GEE) with binomial error distribution and logit link function was used to analyze multivariable data.

Results:

Objective 1:

Antimicrobial susceptibility testing of *E. faecium* isolates present in the probiotic products

Eight commercially available swine probiotics tested are listed in Table 1. The *E. faecium* strains from three probiotics (C, E, and H) were susceptible to all 16 antimicrobials tested. The *E. faecium* strains from four products (B, D, F, and G) were resistant to only one antimicrobial, lincomycin. The *E. faecium* strain from product B was also resistant to daptomycin. Whereas, the *E. faecium* strain from probiotic A was resistant to three antimicrobials, ciprofloxacin, daptomycin, and tetracycline (Table 2).

Clonal relatedness among *E. faecium* isolates

In total, we identified six different PFGE types among eight *E. faecium* strains. The *E. faecium* strains from product D and F were clonally related with 100% dice similarity. The strains from product B and G are also clonally related with 100% dice similarity (as shown in Fig. 1).

Whole genome sequencing of probiotic *E. faecium* isolates

The whole genome sequencing of eight *E. faecium* isolates revealed the presence of macrolide and streptogramin B resistance mediated by *msrC* gene, and tetracycline resistant determinants, *tet*(L) and *tet*(M) genes (as shown in Table 3).

Objective 2:

Growth performance:

Pigs fed diets containing CTC had higher ($P = 0.001$) BW, ADG, ADFI, and overall BW compared to those not fed CTC (table 5). From d 0 to 14, a CTC × Probiotic A interaction ($P = 0.002$) was observed for ADFI (table 5). The interaction occurred because pigs fed diets containing the combination of CTC and Probiotic A had greater ADFI compared to pigs fed the control diet or the diet with only Probiotic A, while pigs fed CTC had intermediate feed intake. From d 14 to 28, no interactions between CTC and either Probiotic A or Probiotic B were observed. Also, pigs fed diets containing Probiotic B had a tendency for greater ($P = 0.052$) ADFI than those not fed Probiotic B. From d 28 to 42, a CTC × Probiotic B interaction ($P = 0.050$) was observed for ADFI. The interaction occurred because pigs fed diets containing CTC only had greater ADFI compared to the control, while diets containing either Probiotic B or Probiotic B with CTC were intermediate. Furthermore, a tendency for a CTC × Probiotic A interaction ($P = 0.077$) was observed for F/G. The interaction occurred because pigs fed diets containing CTC in combination with Probiotic A had improved F/G comparative to pigs fed diets containing CTC or Probiotic A alone. Feeding CTC increased ($P = 0.045$) ADFI, with no impact on ADG or F/G. For the overall study (d 0 to 42), no CTC by probiotic interactions were observed.

Prevalence of *Campylobacter* spp. and *Salmonella enterica*:

The overall prevalence of *Campylobacter* spp. was 21.6% (273/1,260). Both treatment and or sampling phase had no effect on the prevalence ($P > 0.05$). However, the treatment and sampling phase interaction was significant ($P = 0.03$). The prevalence was higher in the treatment phase (31.4%; $P = 0.02$) when compared to pre and post-treatment phases. The table 5 gives overall prevalence of *Campylobacter* spp. across all treatments and sampling phases. The prevalence of *C. hyointestinalis* was 17.7% (224/1,260) when compared to *C. coli* (3.8%; 49/1260). The overall prevalence of *Salmonella* was 6.6% (84/1,260). There was no treatment effect on the prevalence ($P > 0.05$) of *Campylobacter* or *Salmonella* (Table 6).

Antimicrobial susceptibility determinations:

Escherichia coli: Table 7 shows the concentration of antimicrobials tested, breakpoints, and resistant (%).All the tested *E. coli* isolates were susceptible to azithromycin and sulfisoxazole. Majority of the *E. coli* isolates were resistant to tetracycline (93.3%), ampicillin (81.6%), streptomycin (58.5%), ceftriaxone (52.4%), and ceftiofur (36.1%). The number of multi-drug resistant isolates (resistant to 3 or >3 different classes of antimicrobials) remain constant across three testing periods and treatment groups ($P > 0.05$). The multidrug resistant phenotypes among these isolates varied anywhere from 3-8 antimicrobials with majority of them resistant to 3 different antimicrobial class (26.3%; 142/540).

Enterococcus spp.: Table 8 shows the concentration of antimicrobials tested, breakpoints, and resistant (%). Majority of the isolates were resistant to tetracycline (95%), erythromycin (86.1%), lincomycin (83.3%), ciprofloxacin (67.2%), nitrofurantoin (53.3%), streptomycin (45.2%) and kanamycin (41.5%). The number of multi-drug resistant isolates (resistant to 3 or >3 classes of antimicrobials) remain constant across three testing periods and treatment groups ($P > 0.05$). All the tested *Enterococcus* isolates were susceptible to vancomycin. The multidrug resistant phenotypes among these isolates varied anywhere from 3-14 antimicrobials with majority of them resistant to 5 different antimicrobial class (19.4%; 105/540).

Discussion:

We have employed the whole genome sequencing to detect the antimicrobial resistance genes present among *E. faecium* isolates Probiotics are a class of antimicrobial alternatives designed to enhance growth performance and digestive tract health. Feeding probiotics alone or in combination with CTC did not improve nursery pig growth performance. This main effect of CTC on growth performance throughout the study was similar to previous research with an increase in growth rate driven by increased feed intake. The addition of one of the probiotics (Probiotic B) with CTC had an additive effect on growth performance, but in later phases this benefit did not exist. In the present study, majority of the *E. coli* and *Enterococcus* spp. were resistant to tetracycline. This is in agreement with earlier studies indicating the saturation of tetracycline resistance among swine production system. Both *Salmonella* and *Campylobacter* spp. were isolated from the feces of these piglets. However, a quantitative risk assessment would need to be conducted to determine the level of risk to human safety. In conclusion, this study confirmed the positive benefits of feeding CTC during the nursery phase on pig performance. This warrants further research on whether in certain phases of nursery production it is beneficial to feed probiotics in combination with CTC to increase performance. Future research should also focus on environmental factors to help us better understand the ecology and epidemiology of both commensal and pathogenic bacteria in the swine production agriculture.

Publications / Presentations / Abstracts

1. Williams, H. E., M. D. Tokach, S. S. Dritz, J. C. Woodworth, J. M. DeRouchey, R. G. Amachawadi, T. G. Nagaraja, and R. D. Goodband. **2016**. Determination of probiotic and or chlortetracycline inclusion effects on nursery pig growth performance. **Swine Day: Kansas Agricultural Experiment Station Research Reports: 26.**
2. Williams, H. E., M. D. Tokach, S. S. Dritz, J. C. Woodworth, J. M. DeRouchey, R. G. Amachawadi, T. G. Nagaraja, and R. D. Goodband. **2017**. Effects of feeding probiotic or chlortetracycline or a combination on nursery pig growth performance. **2017 Midwest American Society of Animal Science meeting, March 13-15, Omaha, NE**.
3. Amachawadi, R. G., J. Soto, X. Shi, F. Giok, M. D. Tokach, S. K. Narayanan, J. Pluske, and T. G. Nagaraja. 2017. Antimicrobial resistance in *Enterococcus faecium* isolated from commercial probiotic products used in cattle and swine. **ASM Conference on Innovative Microbial Ecology for Mitigation of Antibiotic Resistance and Bacterial Diseases, March 22-25, Crystal City, VA**.

Table 1: List of swine probiotics with the manufacturer's information

Probiotics	Manufacturer	Bacterial species present
BioPlus	Chr. Hansen Animal Health, Milwaukee, WI	*Bacillus licheniformis, Bacillus subtilis*
Clostat	Kemin, Des Moines, Iowa	*Bacillus subtilis* strain ATCCPTA-6737 PB6,
FastTrack	Conklin Company Inc., Kansas City, MO	*Lactobacillus acidophilus, Enterococcus faecium, Bacillus subtilis, Aperigillus oryzae*
NB 600 MM Premix	Nutra Blend, NeoSho, MO	*Bacillus subtilis, Enterococcus faecium*
Primalac Livestock W/S	Star Labs, Clarksdale, MO	*Lactobacillus acidophilus, Lactobacillus casei, Bifidobacterium thermophilum, Enterococcus faecium*
ProBios	Vets Plus, Menomonie, WI	*Enterococcus faecium, Lactobacillus acidophilus, Lactobacillus casei, Lactobacillus plantarum*
Poultry Star me	Biomin, San Antonio, TX	*Enterococcus faecium, Lactobacillus reuteri, Pediococcus acidilactii, Bifidobacterium animalis*
SynGenX	Diamond V, Cedar Rapids, Iowa	*Lactobacillus acidophilus,*

Table 2: Phenotypic resistance pattern of *Enterococcus faecium* strains isolated from commercially available swine probiotics

Product code	Antimicrobials susceptible to:	Antimicrobials resistant to:
A	Chloramphenicol, Erythromycin, Gentamicin, Kanamycin, Lincomycin, Linezolid, Nitrofurantoin, Penicillin, Quinupristin/Dalfopristin, Streptomycin, Tigecycline, Tylosin, Vancomycin	Ciprofloxacin, Daptomycin, Tetracycline
B	Chloramphenicol, Ciprofloxacin, Erythromycin, Gentamicin, Kanamycin, Linezolid, Nitrofurantoin, Penicillin, Quinupristin/Dalfopristin, Streptomycin, Tetracycline, Tigecycline, Tylosin, Vancomycin	Daptomycin, Lincomycin
C	Chloramphenicol, Ciprofloxacin, Daptomycin, Erythromycin, Gentamicin, Kanamycin, Lincomycin, Linezolid, Nitrofurantoin, Penicillin, Quinupristin/Dalfopristin, Streptomycin, Tetracycline, Tigecycline, Tylosin, Vancomycin	None
D	Chloramphenicol, Ciprofloxacin, Daptomycin, Erythromycin, Gentamicin, Kanamycin, Linezolid, Nitrofurantoin, Penicillin, Quinupristin/Dalfopristin, Streptomycin, Tetracycline, Tigecycline, Tylosin, Vancomycin	Lincomycin
E	Chloramphenicol, Ciprofloxacin, Daptomycin, Erythromycin, Gentamicin, Kanamycin, Lincomycin, Linezolid, Nitrofurantoin, Penicillin, Quinupristin/Dalfopristin, Streptomycin, Tetracycline, Tigecycline, Tylosin, Vancomycin	None
F	Chloramphenicol, Ciprofloxacin, Daptomycin, Erythromycin, Gentamicin, Kanamycin, Linezolid, Nitrofurantoin, Penicillin, Quinupristin/Dalfopristin, Streptomycin, Tetracycline, Tigecycline, Tylosin, Vancomycin	Lincomycin
G	Chloramphenicol, Ciprofloxacin, Daptomycin, Erythromycin, Gentamicin, Kanamycin, Linezolid, Nitrofurantoin, Penicillin, Quinupristin/Dalfopristin, Streptomycin, Tetracycline, Tigecycline, Tylosin, Vancomycin	Lincomycin
H	Chloramphenicol, Ciprofloxacin, Daptomycin, Erythromycin, Gentamicin, Kanamycin, Lincomycin, Linezolid, Nitrofurantoin, Penicillin, Quinupristin/Dalfopristin, Streptomycin, Tetracycline, Tigecycline, Tylosin, Vancomycin	None

Table 3: Genotypic resistance patterns of *Enterococcus faecium* strains isolated from commercially available swine probiotics based on the whole genome sequencing (WGS),

Product code	Phenotypic resistance pattern	WGS based genotypic resistance pattern
A	CIP+DAP+TET	*msr*(C), *tet*(L), *tet*(M)
B	DAP+LIN	*msr*(C)
C	-	*msr*(C)
D	LIN	*msr*(C)
E	-	*msr*(C)
F	LIN	*msr*(C)
G	LIN	*msr*(C)
H	-	*msr*(C)

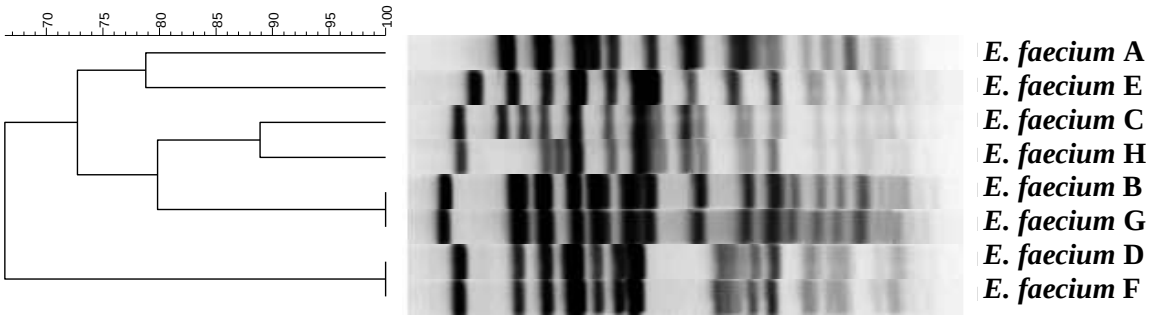

Fig. 1: Pulsed-field gel electrophoresis patterns of genomic DNA of *Enterococcus faecium* strains isolated from probiotics

Table 4: Experimental diet composition (as-fed basis)[1]

Item	Phase 1	Phase 2
Ingredient, %		
Corn	55.75	62.50
Soybean meal, 46.5% CP	25.35	33.40
Dried whey	10.00	---
HP 300[2]	5.00	---
Limestone	1.05	1.18
Monocalcium phosphate, 21%	1.20	1.20
Sodium chloride	0.30	0.35
L-Lys HCl	0.45	0.45
DL-Met	0.20	0.20
L-Thr	0.20	0.20
L-Trp	0.03	0.03
L-Val	0.10	0.10
CTC-50	---	---
Probiotic A	---	---
Probiotic B	---	---
Phytase	0.02	0.02
Trace mineral premix[2]	0.15	0.15
Vitamin premix	0.25	0.25
Total	100	100
Calculated analysis		
Standardized ileal digestible (SID) amino acids, %		
Lys	1.35	1.35
Met:Lys	36	36
Met&Cys:Lys	57	58
Thr:Lys	65	64
Trp:Lys	19.1	19.3
Val:Lys	70	70
Total Lys, %	1.49	1.50
ME, kcal/lb	1,496	1,482
CP, %	21.4	21.9
Ca, %	0.75	0.75
P, %	0.69	0.66
Available P, %	0.49	0.43

[1]Phase 1 diets were fed from d 0 to 14 (~13.0 to ~19 lb BW) and Phase 2 diets from d 14 to 42 (~19 to 55 lb BW). A common starter diet was fed to all pigs for 4 days after weaning.
[2]Trace mineral premix containing 17 ppm Cu and 110 ppm Zn.

Table 5: Effects of probiotic and/or antimicrobial on nursery pig performance[1]

CTC[2]	-	+	-	+	-	+		Probability, *P* <				
Probiotic A[3]	-	-	+	+	-	-						
Probiotic B[4]	-	-	-	-	+	+	SEM	CTC	Probiotic A	Probiotic B	CTC × Probiotic A	CTC × Probiotic B
d 0 to 14												
ADG, lb	0.35^{c}	0.43^{ab}	0.36^{c}	0.47^{a}	0.41^{bc}	0.47^{a}	0.023	0.001	0.356	0.108	0.505	0.976
ADFI, lb	0.50^{b}	0.57^{ab}	0.52^{b}	0.61^{a}	0.56^{ab}	0.61^{a}	0.022	0.001	0.124	0.018	0.002	0.938
F/G	1.46	1.30	1.48	1.30	1.36	1.31	0.165	0.001	0.788	0.362	0.861	0.252
d 14 to 28												
ADG, lb	1.00^{c}	1.12^{ab}	0.94^{c}	1.15^{a}	1.01^{cb}	1.18^{a}	0.044	0.001	0.795	0.242	0.310	0.868
ADFI, lb	1.45^{bc}	1.70^{a}	1.40^{c}	1.74^{a}	1.54^{b}	1.77^{a}	0.043	0.001	0.935	0.052	0.239	0.810
F/G	1.47	1.53	1.53	1.52	1.54	1.51	0.048	0.592	0.612	0.865	0.437	0.565
d 28 to 42												
ADG, lb	1.50^{ab}	1.55^{ab}	1.44^{b}	1.58^{a}	1.54^{ab}	1.49^{ab}	0.044	0.227	0.788	0.860	0.361	0.195
ADFI, lb	2.36^{b}	2.55^{a}	2.32^{b}	2.47^{ab}	2.49^{ab}	2.44^{ab}	0.058	0.045	0.350	0.872	0.738	0.050
F/G	1.58	1.65	1.62	1.57	1.61	1.65	0.033	0.521	0.582	0.617	0.077	0.570
d 0 to 42												
ADG, lb	0.94^{bc}	1.03^{a}	0.90^{c}	1.06^{a}	0.98^{ab}	1.04^{a}	0.029	0.001	0.808	0.340	0.214	0.545
ADFI, lb	1.42^{bc}	1.60^{a}	1.38^{c}	1.60^{a}	1.52^{ab}	1.61^{a}	0.036	0.001	0.573	0.173	0.531	0.215
F/G	1.52	1.56	1.56	1.51	1.55	1.54	0.029	0.820	0.869	0.797	0.171	0.519
BW, lb												
d 0	13.0	13.0	13.0	13.0	12.9	13.1	0.118	0.093	0.896	0.613	0.837	0.143
d 14	18.1^{c}	19.1^{ab}	18.0^{c}	19.6^{a}	18.7^{bc}	19.6^{a}	0.345	0.001	0.388	0.043	0.354	0.914
d 28	32.0^{c}	34.7^{ab}	31.3^{c}	35.7^{a}	32.8^{bc}	36.1^{a}	0.748	0.001	0.832	0.135	0.265	0.706
d 42	53.3^{bc}	56.4^{ab}	52.1^{c}	57.6^{a}	54.6^{abc}	56.9^{a}	1.152	0.001	0.988	0.438	0.289	0.728

[a,b,c] Means within the same row with different superscripts differ ($P \leq 0.05$).

Table 6: Fecal prevalence of *Campylobacter* spp. (*Campylobacter coli* and *Campylobacter hyointestinalis*) and *Salmonella* spp. isolated from piglets supplemented with probiotics alone or in combination with antibiotics

Treatments	No. tested	*Campylobacter* spp.			*Salmonella* spp.
		Total positive (%)	*C. coli*	*C. hyointestinalis*	Total positive (%)
Control	210	49 (23.3)	3	46	9 (4.3)
Chlortetracycline (CTC)	210	35 (16.6)	14	21	13 (6.2)
Probiotic A	210	54 (25.7)	5	49	17 (8.1)
Probiotic B	210	51 (24.3)	6	45	13 (6.2)
Probiotic A + CTC	210	52 (24.7)	16	36	18 (8.5)
Probiotic B + CTC	210	32 (15.2)	5	27	14 (6.6)
TOTAL	**1260**	**273 (21.6)**	**49**	**224**	**84 (6.6)**

Table 7: **Antimicrobial susceptibilities of *Escherichia coli* (n=540; 180 isolates each on days 0, 21 and 42) strains isolated from feces of from piglets supplemented with probiotics alone or in combination with antibiotics**

Antimicrobials	Concentration range (μg/ml)	Resistant breakpoint (μg/ml)	No. of isolates resistant (%)		
			Day 0	Day 21	Day 42
Amoxicillin/Clavulanic acid 2:1 ratio	1/0.5 – 32/16	≥ 32/16	6 (3.3)	11 (6.1)	17 (9.4)
Ampicillin	1 - 32	≥ 32	177 (98.3)	146 (81.1)	118 (65.5)
Azithromycin	0.12 - 16	N/A[1]	0	0	0
Cefoxitin	0.5 - 32	≥ 32	4 (2.2)	22 (12.2)	16 (8.8)
Ceftiofur	0.12 - 8	≥ 8	69 (38.3)	70 (38.8)	56 (31.1)
Ceftriaxone	0.25 - 64	≥ 4	101 (56.1)	79 (43.8)	103 (57.2)
Chloramphenicol	2 - 32	≥ 32	16 (8.8)	16 (8.8)	18 (10)
Ciprofloxacin	0.015 – 4	≥ 1	0	5 (2.7)	10 (5.5)
Gentamicin	0.25 – 16	≥ 16	0	1 (0.5)	5 (2.7)
Nalidixic Acid	0.5 – 32	≥ 32	0	15 (8.3)	6 (3.3)
Streptomycin	32 - 64	≥ 64	91 (50.5)	115 (63.8)	110 (61.1)
Sulfisoxazole	16 - 256	≥ 512	0	0	0
Tetracycline	4 – 32	≥ 16	156 (86.6)	173 (96.1)	175 (97.2)
Trimethoprim/Sulfamethoxazole	0.12/2.4 – 4/76	≥ 4/76	23 (12.7)	28 (15.5)	33 (18.3)

[1]N/A = not applicable. The National Antimicrobial Resistance Monitoring System has not established breakpoints for azithromycin interpretation; therefore, there is no Clinical and Laboratory Standards Institute resistant breakpoint.

Table 8: **Antimicrobial susceptibilities of *Enterococcus spp.* (n=540; 180 isolates each on days 0, 21 and 42) strains isolated from feces of from piglets supplemented with probiotics alone or in combination with antibiotics**

Antimicrobials	Concentration range (μg/ml)	Resistant breakpoint (μg/ml)	No. of isolates resistant (%)		
			Day 0	Day 21	Day 42
Chloramphenicol	2 – 32	≥ 32	78 (43.3)	40 (22.2)	4 (2.2)
Ciprofloxacin	0.12 – 4	≥ 4	156 (86.6)	102 (56.6)	105 (58.3)
Daptomycin	0.25 – 16	N/A[1]	180 (100)	179 (99.4)	178 (98.8)
Erythromycin	0.25 – 8	≥ 8	179 (99.4)	130 (72.2)	156 (86.6)
Gentamicin	128 – 1024	> 500	53 (29.4)	14 (7.7)	8 (4.4)
Kanamycin	128 – 1024	≥ 1024	163 (90.5)	28 (15.5)	33 (18.3)
Lincomycin	1 – 8	≥ 8	180 (100)	129 (71.6)	141 (78.3)
Linezolid	0.5 – 8	≥ 8	18 (10)	56 (31.1)	20 (11.1)
Nitrofurantoin	2 – 64	≥ 128	33 (18.3)	147 (81.6)	108 (60)
Penicillin	0.25 – 16	≥ 16	3 (1.6)	3 (1.6)	3 (1.6)
Quinupristin/Dalfopristin	0.5 – 32	≥ 4	180 (100)	137 (76.1)	134 (74.4)
Streptomycin	512 – 2048	> 1000	153 (85)	33 (18.3)	58 (32.2)
Tetracycline	1 – 32	≥ 16	174 (96.6)	176 (97.7)	163 (90.5)
Tigecycline	0.015 – 0.5	N/A[2]	6 (3.3)	2 (1.1)	10 (0.5)
Tylosin tartrate	0.25 - 32	≥ 32	175 (97.2)	55 (30.5)	93 (51.6)
Vancomycin	0.25 - 32	≥ 32	0	0	0

[1]N/A = not applicable. A susceptibility breakpoint of ≤4 μg/mL for daptomycin exists but no resistant breakpoint has been established. In this study, isolates with a minimal inhibitory concentration ≥ 8 μg/mL were categorized as resistant.
[2]N/A susceptibility breakpoint of ≤0.25 μg/mL for tigecycline exists but no resistant breakpoint has been established. In this study, isolates with a minimal inhibitory concentration ≥ 0.5 μg/mL were categorized as resistant.