RESEARCH REPORT
PUBLIC HEALTH/WORKER SAFETY

Title: Development of a practical, cost-effective, easy to administer prebiotic intervention to reduce carriage of zoonotic pathogens on the farm and entering the abattoir – **NPB #14-077**

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Industry Summary: The gut of pigs can be colonized with important foodborne and disease causing bacteria such as *Salmonella, E. coli* and *Campylobacter*. New treatments and strategies are sought to reduce the carriage of these bacteria particularly as they are shipped to the processing plant. Thymol is an attractive candidate to be developed into an antibiotic alternative for swine because it is a natural product and thus likely to be viewed more favorably by regulatory agencies. Thymol is known to exhibit potent antimicrobial activity against *Salmonella, E. coli* and *Campylobacter* in the laboratory but its effectiveness when fed to animals is not very good. This is because thymol is very rapidly absorbed in the stomach and small intestine which consequently prevents it from arriving to the cecum and large intestine where these pathogenic bacteria primarily reside. In order to make thymol more resistant to absorption, we chemically linked thymol to glucose using a chemical bond like that used by plants to link glucose units into cellulose. The result is that the conjugated form of thymol, referred to as thymol-beta-D-glucopyranoside (which we will hereafter call beta-D-thymol for ease of use), is absorbed much more slowly than free thymol and thus has the potential to bypass absorption in the stomach and small intestine and make its way to the cecum and large intestine. Conceptually, once the beta-D-thymol arrives to the cecum and intestine, there are gut bacteria there that can degrade the protective bond and thus liberate free thymol, thereby making it available to kill *Salmonella, E. coli* and *Campylobacter.* The main objective of this project was to determine if beta-D-thymol could indeed be fed to pigs to reduce gut concentrations of *Salmonella, E. coli* and *Campylobacter*. Results from live animal studies were not successful in achieving significant reductions in cecal and rectal concentrations of *Salmonella, E. coli* or *Campylobacter,* possibly because hydrolysis and absorption of beta-D-thymol and free thymol may still have been rapid enough within the proximal small intestine to preclude their delivery to the cecum and large intestine. Additionally, it is possible also that uptake and internal compartmentalization of beta-D-thymol by gut bacteria, or its powerful chemical attraction to fats and oils, may sequester the beta-D-thymol away from hydrolytic enzymes thus preventing the release of free thymol. Comparison of antimicrobial resistance profiles between *E. coli* isolates or multidrug resistant *Salmonella* strains did not support a hypothesis that exposure to beta-D-thymol or thymol may co-select for antimicrobial resistance. Additional research is currently underway to try and learn how to overcome obstacles preventing efficacious activity of beta-D-thymol to the lower gastrointestinal tract.

Keywords: Antibiotic alternative, *Campylobacter coli, Salmonella*, *E. coli*, thymol-beta-D-glucopyranoside.

Scientific Abstract: The gut of food-producing animals is a reservoir for human foodborne pathogens. Thymol is bactericidal against pathogens including *Salmonella* and *E. coli* but its rapid absorption from the proximal gut reveals a need for protective technologies to deliver effective concentrations to the lower gut where the pathogens mainly colonize. Thymol-β-D-glucopyranoside (hereafter referred to as beta-D-thymol) is more resistant to absorption than free thymol in everted jejunal segments because of its β-glycosidic bond and thus could potentially function as a prebiotic, being undegradable in the proximal gut but hydrolysable by microbial beta-D-thymol-hydrolyzing enzymes in the distal gut. This study was conducted to determine the effective dose of beta-D-thymol against pathogenic *Salmonella* and *E. coli* and to determine if oral administration of doses intended to deliver these effective concentrations to the cecum and rectum of pigs can effectively reduce intestinal carriage of *Salmonella* and *E. coli*. Results from in vitro dose titration studies have identified efficacious doses of beta-D-thymol against *Salmonella, E. coli* and *Campylobacter* during culture with porcine gut bacteria, with concentrations of beta-D-thymol needed to achieve efficacious reductions of *Salmonella* or *E. coli* being 6 to 9 times higher than that (1 mM) needed to effectively kill *Campylobacter* species. The increased susceptibility of *Campylobacter* to beta-D-thymol may be a consequence of its dependence on amino acid fermentation as free thymol is thought to inhibit this activity. Results from live animal studies were not successful in achieving significant reductions in cecal and rectal concentrations of *Salmonella, E. coli* or *Campylobacter,* possibly because hydrolysis and absorption of beta-D-thymol and free thymol may still be sufficiently rapid within the proximal small intestine to preclude their delivery to the cecum and large intestine. Additionally, it is possible also that uptake and internal compartmentalization of beta-D-thymol by gut bacteria, or its lipophilicity, may sequester the beta-d-thymol away from hydrolytic enzymes thus preventing the release of free thymol. Comparison of antimicrobial resistance profiles between *E. coli* isolates or multidrug resistant *Salmonella* strains did not support a hypothesis that exposure to beta-D-thymol or thymol may co-select for antimicrobial resistance. Additional research is currently underway to try and learn how to overcome obstacles preventing delivery of efficacious amounts of beta-D-thymol to the lower gastrointestinal tract.

Introduction: *Campylobacter* and *Salmonella* are leading bacterial causes of human foodborne illness in the United States, with more than 1,000,000 and 800,000 human infections occurring in 2011, respectively (CDC, 2011). Poultry, swine and cattle can harbor these pathogens within their digestive tracts and risk contaminating carcasses during processing. Strategies are needed to reduce the incidence and concentration of these foodborne pathogens in animals before they arrive for processing so as to minimize risks of carcass contamination. Thymol is an essential oil that has been shown to be bactericidal against zoonotic pathogens such as *Salmonella, Campylobacter* and *Escherichia coli*, with minimum inhibitory concentrations against pure cultures ranging from 1.00 and 1.55 µmol/mL (Anderson et al., 2009; Burt, 2004). Mechanistically, the bactericidal activity of thymol is due to the insertion of this fat-loving compound into the lipid-rich bacterial cell wall and the subsequent disintegration of cell wall integrity (Burt, 2004; Varel and Miller, 2000). Results from feeding studies have shown thymol to be readily eaten but thymol is only marginally effective in reducing concentrations and shedding of these foodborne pathogens and this is because thymol is very rapidly absorbed or degraded in the stomach and small intestine (Anderson et al., 2012; Michiels et al., 2008, 2010). Thus protective technologies are clearly needed to deliver effective amounts of thymol to the lower gut where it can kill the pathogens. To address this issue, we had a company synthesize thymol-beta-D-glucopyranoside (hereafter referred to as beta-D-thymol) and we have tested this conjugated form of thymol as a potential bypass agent in monogastrics.

Conceptually, we hypothesized that beta-D-thymol would be a prebiotic in that it would be nearly undegradable in the stomach and proximal small intestine of monogastrics because higher animals do not produce the beta-glycosidase enzyme that is required to hydrolyze beta-D-thymol. For instance, the lack of beta-glycosidase enzymes in monogastrics is the reason why they cannot use cellulose, which is a polymer composed of repeating beta-glucosides. Gut microbes do express beta-glycosidase

activity however and thus we further hypothesized that when beta-D-thymol reaches microbial activity in the lower gut there should be sufficient microbial beta-glycosidase activity to liberate thymol from the glucoside thereby allowing it to be active in the desired site of the gut. We have tested these hypotheses and found that unlike free thymol, beta-D-thymol is more resistant to absorption or degradation in everted porcine small intestinal segments. We further found that microbial beta-glycosidase activity expressed by a common gut bacterium, *Parabacteroides distasonis*, did indeed liberate thymol from the beta-D-thymol which thus provides evidence that this compound can be activated by beta-glycosidase activity upon arrival to the lower tract where populations of beta-glycosidase expressing bacteria reside (Epps et al., 2015). Moreover, our results showed that beta-glycosidase activity is required to activate the beta-D-thymol as the unhydrolyzed, intact glucoside is not bactericidal to *Campylobacter*. Subsequent research has further shown that mixed populations of porcine cecal and fecal bacteria contain sufficient microbial beta-glycosidase activity to activate (hydrolyze) beta-D-thymol (Epps et al., 2015). Moreover, there are natural sources containing beta-glycosides of thymol that would theoretically provide slower release in the gut environment and could possibly be cultured to yield inexpensive sources of beta-D-thymol (Ahmed and Jakupovic, 1990).

Objectives:

1) Determine the inhibitory concentration of thymol-beta-D-glucopyranoside (called beta-D-thymol) needed to achieve efficacious reductions of *Campylobacter coli*, *Salmonella* spp. and *E. coli* in mixed populations of porcine cecal microbes.
2) Determine how much beta-D-thymol is needed in feed to reduce gut colonization of pigs by *Campylobacter coli, Salmonella* spp. and generic *E. coli*.
3) Evaluate the potential of beta-D-thymol to co-select for antibiotic resistant *Campylobacter coli, Salmonella* spp. and generic *E. coli* or to make resistant strains of these bacteria more sensitive to antibiotics when grown in mixed populations of porcine gut bacteria.

Materials & Methods:

Resistant bacterial strains for challenge studies and beta-D-thymol. The challenge *Salmonella enterica* serovar Typhimurium (NVSL 95-1776) organism, possessing natural resistance to novobiocin, had been made nalidixic acid resistant via successive cultivation in tryptic soy broth containing up to 20 µg nalidixic acid mL^{-1} (Anderson et al., 2007). Likewise, the challenge *E. coli* K88 strain (kindly furnished by Dr. Nancy Cornick, Iowa State University, Ames, IA), was made novobiocin- and nalidixic-acid resistant (NN-resistant) by successive cultivation in tryptic soy broth containing up to 25 µg/mL novobiocin and 20 µg/mL nalidixic acid. Inocula for experiments were obtained from cultures grown overnight at 37°C in tryptic soy broth (Difco, Becton Dickinson, Sparks, MD, USA) supplemented with 25 µg of novobiocin/mL and 20 µg/nalidixic acid mL. Beta-D-thymol (thymol-β-D-glucopyranoside) was from Christof Senn Laboratories, Dielsdorf, Switzerland.

Pure culture studies. The NN-resistant *Salmonella* Typhimurium or *E. coli* K88 were each inoculated (2% vol/vol) into separate 13 x 100 mm screw top culture tubes containing 10 mL full strength Mueller Hinton broth (Difco) supplemented with 0 or 0.2 ml of 600 mM stock solution of beta-D-thymol to achieve initial concentrations 0 or 12 mM. Cultures were incubated aerobically in duplicate at 37°C for 24 h.

Fecal incubation studies. Freshly voided feces was collected from approximately 25 kg conventionally-reared pigs maintained on un-medicated feed. Fecal samples were delivered to the laboratory within 1 h of collection. In studies designed to determine the effects of different doses of beta-D-thymol on antibacterial activity against the challenge NN-resistant strains of *Salmonella* Typhimurium or *E. coli* K88, aliquots of the swine feces (0.1% wt/vol each) were mixed with separate batches of anaerobically prepared half-strength Mueller Hinton Broth. Each batch was then inoculated with either the NN-resistant *Salmonella* Typhimurium or *E. coli* K88 to achieve initial concentrations of between 10^4 and 10^6 CFU/mL. The half-strength Mueller Hinton broth was

used as the medium in the fecal suspension study so as to limit excessive acid production by fermentative anaerobes. Ten milliliter volumes of the resultant mixed populations were distributed under a constant flow of 100% N_2 gas to 18 x 150 mm crimp top tubes that had been preloaded with small volumes (≤ 0.5 mL) of a 600 mM stock solution of beta-D-thymol (in 50% ethanol) to achieve upon addition of fecal-pathogen-inoculated medium, initial concentrations of 0, 3, 6 or 12 mM for NN-resistant *Salmonella* Typhimurium and 0, 3, 12, 30 mM for NN-resistant *E. coli* K88. Control tubes were preloaded with 0.2 ml of 50% ethanol. Tubes were closed with rubber stoppers, crimped and incubated anaerobically (under 100% N_2 gas) at 39°C for 24 h.

In studies examining effects of lipid or fatty acid addition without or with various emulsifies on antibacterial effect of beta-D-thymol, batches of anaerobically prepared half-strength Mueller Hinton Broth were inoculated with freshly collected porcine feces (0.5% wt/vol) and inoculated as above with NN-resistant *E. coli* K88. Ten milliliter volumes of the resultant mixed populations were distributed under a constant flow of 100% N_2 gas to 18 x 150 mm crimp top tubes that had been preloaded with small volumes beta-D-thymol as above and with our without 0.3 mL of vegetable oil, olive oil, oleic acid, linoleic acid, or glycerol. In a follow-up experiment, fecal populations prepared as above were tested without or with additions (each at 3% vol/vol) of the emulsifying agents, taurine (a component of bile acids), Tween 20, Tween 80 or Poly-oxyl-40-sorbate. Tubes were closed with rubber stoppers, crimped and incubated anaerobically (under 100% N_2 gas) at 39°C for 24 h.

Studies examining the beta-D-thymol-hydrolyzing and subsequent anti-*Salmonella* activity of digesta collected from various gut compartments of a weaned pig were conducted by mixing freshly collected jejunal, cecal and rectal contents (6.0, 15 and 15 g respectively) in separate 100 mL volumes of anaerobic half strength Mueller Hinton broth batches inoculated with approximately 2 x 10^5 CFU/mL of NN-resistant *Salmonella* Typhimurium. The suspensions were distributed to 18 x 150 mm crimp top tubes (10 mL/tube) preloaded with 0.5 mL of water or 120 mM B-thymol solutions to achieve 0 or 6 mM added beta-D-thymol and incubated anaerobically under 100% N_2 at 39°C.

Gas chromatographic analysis of beta-D-thymol and thymol. The gas chromatography (GC) standard curve and extraction method was developed to determine 1-500 ng/mL thymol in samples extracted with ethyl acetate (Petrujkić et al., 2013). Immediately prior to incubation and following incubation samples were collected from various gut segments for GC analysis (0, 6 and 24 h). To determine the presence of beta-glycosidase hydrolyzing activity of the gut microbes in various gut samples, free thymol and conjugated thymol were measured. The results obtained were compared between the jejunal, cecal, and rectal data. Conjugated thymol and free thymol were detected by analyzing each sample with and without adding a commercially beta-glucosidase enzyme. Each sample was run in triplicate. These results revealed that the maximum beta-glucopyranoside-hydrolyzing enzyme activity was in the cecal and rectal content was at 6 h. A comparable amount of enzyme activity in the jejunal content was seen at 24 h. The beta-glucopyranoside-hydrolyzing enzyme was added to the samples and incubated for 2 h prior to extraction with ethyl acetate to measure the conjugated thymol in gut samples. Previous free thymol data was subtracted from the beta-glucosidase enzyme added total thymol data to estimate the amount of conjugated thymol in the samples.

In vivo studies. Experimental care and use of all pigs were approved by the SPARC Institutional Animal Care and Use Committee. In an initial feeder pig study, 18 weaned pigs (24 ± 5 kg live body weight) were orally infected with 2 x 10^9 CFU of the NN-resistant *Salmonella* Typhimurium upon arrival (11:00) to the rearing facility and randomly allocated to 6 pens (3 pigs/pen) and twice-treated via oral gavage (2 pens/treatment) that same day (16:00 and 21:00) with 0, 6 or 12 mg beta-D-thymol/kg body weight. Pigs were euthanized 12 h after the last treatment and cecal and rectal contents collected at necropsy were cultured to enumerate the NN-resistant *Salmonella* and generic *E. coli* and *Campylobacter* species. In a following feeder pig study, 12, 11 and 12 weaned pigs, respectively, averaging 40 ± 8 kg live body weight, were randomly allocated to a single day's treatment of 0, 17 or 51 mg beta-D-thymol/kg live body weight. All except 1 of the pigs were placed

(2 per pen) to concrete-floored pens, with the remaining pig placed individually in a similar pen. Pigs were acclimated to a standard non-medicated grower grower diet (Producers CO-OP, Bryan, TX, USA) via twice a day feedings (08:00 and 16:00) for 14 days. On the evening of the 14th day, all pigs were orally inoculated with approximately 2 x 10^9 CFU of the NN-resistant *Salmonella* Typhimurium. Treatments were administered to each pen on the 15th day via top dressing half of the day's treatment into a 20% portion of each meal which was offered first to promote total consumption of the beta-D-thymol. Approximately 30 minutes later the remainder of the meal was offered. Pigs not receiving beta-D-thymol were fed each meal in two portions likewise. Sixteen h and again 24 h after administration of the last meal one pig from each pen was euthanized and necropsied for collection of jejunal, cecal and rectal contents so that n = 6, 6 and 6 pigs for 0, 17 and 51 mg/kg live body weight per day at 16 h post treatment and n = 6, 5 and 6 for 0, 17 and 51 mg/kg live body weight per day at 24 h post treatment, respectively.

Bacterial enumeration. Concentrations of NN-resistant *Salmonella* Typhimurium and NN-resistant *E. coli* K88 in fluids from in vitro suspensions sampled after 0, 6 and 24 h of incubation were enumerated via viable cell count on Oxoid Brilliant Green (Oxoid LTD, Basingstoke, Hampshire, England) agar or MacConkey (Difco) agar, each supplemented with 25 µg novobiocin/mL and 20 µg naladixic acid/mL. Samples were serially diluted into 9 mL of phosphate buffered saline (pH 6.5) from 10^{-1} out to 10^{-6}. Gut samples collected at from pigs at necropsy were diluted similarly and plated on NN-supplemented Brilliant Green agar and on MacConkey and *Campy* Cefex agars for viable cell count enumeration of the NN-resistant *Salmonella* Typhimurium and generic *E. coli* and *Campylobacter* species, respectively. *Salmonella* Typhimurium and *E. coli* were counted after 24 h aerobic incubation at 37^{o}C; *Campylobacter* were be enumerated after 48 h microaerophilic (N_2:CO_2:O_2; 85:10:5) incubation at 42^{o}C.

Antimicrobial resistance determination. Determination of potential co-selection of antimicrobial resistance by thymol, the hydrolytic product of beta-D-thymol, in randomly selected strains of generic *E. coli* selected before (6 strains from 6 different pigs) and after (6 strains from same pigs as sampled previously) beta-D-thymol administration. Additional tests with *Salmonella enterica* serovars provided by Dr. Shaohua Zhao of the Office of Research, Center for Veterinary Medicine, Laurel, MD and having been previously been characterized as possessing resistance to multiple antimicrobials (Zhao et al., 2007). Tests the antimicrobial resistant *Salmonella* were conducted with strains having no known prior exposure to thymol as well as strains adapted via three consecutive 24 h cultures in Mueller Hinton broth supplemented with sublethal amounts of thymol (0.5 mM). 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, 2003; 2004, 2005). 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 (CLSI, 2002): ampicillin, ceftiofur, chlortetracycline, clindamycin, danofloxacin, enrofloxacin, florfenicol, gentamicin, neomycin, oxytetracycline, penicillin, spectinomycin, sulphadimethoxime, tiamulin, tilmicosin, trimethoprim/sulphamethoxazole, tulathromycon, and tylosin tartrate.

Statistical analysis. In in vitro incubation studies, $\log_{10}$ transformations of NN-resistant *Salmonella* Typhimurium and *Escherichia coli* K88 colony counts enumerated at 0, 6 and 24 h were $\log_{10}$ transformed and were tested for main effects of treatment at each sampling time using a general analysis of variance and a LSD separation of means (Statistix Version 10, Tallahassee, FL, USA). Measurements of free thymol and beta-D-thymol are presented descriptively. All in vitro incubations were conducted with n = 3 experimental units.

In the first animal study, concentrations ($\log_{10}$ CFU/g gut contents) of NN-resistant *Salmonella* Typhimurium analyzed for effects of treatment using an analysis of variance. Polynomial contrasts were used to examine linear and quadratic effects of treatment level. In the second study,

concentrations (log_{10} CFU/g gut contents) of NN-resistant *Salmonella* Typhimurium, generic *E. coli* and *Campylobacter* species were analyzed for main effects of treatment and time using a repeated measures analysis of variance and a LSD separation of means.

Results and Discussion:

Objective 1: Determine the inhibitory concentration of thymol-beta-D-glucopyranoside (called beta-d-thymol) needed to achieve efficacious reductions of Campylobacter coli, Salmonella spp. and E. coli in mixed populations of porcine cecal microbes.

Results from in vitro dose titration studies revealed that beta-D-thymol had little if any bactericidal activity against pure cultures of *Salmonella* Typhimurium or *E. coli* (Fig. 1 and 2). These results suggest that these bacteria possess limited ability to hydrolyze beta-D-thymol by themselves. Likewise, studies conducted earlier revealed that *Campylobacter coli* and *Campylobacter jejuni* also lacked appreciable ability to hydrolyze beta-D-thymol when grown in pure culture (Epps et al., 2015). Bactericidal activity against *Salmonella* Typhimurium and *E. coli* K88 was observed during incubation with porcine fecal bacteria thus revealing the presence of beta-D-thymol-hydrolyzing activity in the mixed populations (Fig. 3 and 4). Evidence from earlier studies indicates that *Campylobacter* species were susceptible to levels of beta-D-thymol as low as 1 mM when incubated with mixed populations of gut bacteria (Epps et al., 2015) but results from the present studies indicate that 6 to 9 times more beta-D-thymol is needed during similar incubation to effectively kill *Salmonella* or *E. coli*. The increased susceptibility of *Campylobacter* to beta-D-thymol may be a consequence of its dependence on amino acid fermentation as free thymol is thought to inhibit this activity (Anderson et al., 2009).

Recovery of beta-D-thymol from incubation fluids of mixed populations of porcine jejunal, cecal and rectal microbes was less 15% of the 6 mM beta-D-thymol added indicating that appreciable quantities of the conjugated compound may have been internalized by certain members of the mixed population or sequestered within lipid components. Earlier research by Beier and colleagues (2012) had indicated that bacterial uptake of beta-D-thymol could occur. Thus, in the absence of cell lysis it is possible that some of the intact glucopyranoside may have been un-accessible to hydrolytic enzymes. Still, we observed the accumulation of as much as the equivalent of 2 mM free thymol after 6 h incubation of cecal population (Fig. 5A). Liberation of free thymol from beta-D-thymol was nearly as rapid in incubations of porcine rectal populations but was slower, yet still appreciable, in incubations of porcine jejunal populations, with nearly the equivalent of 2 mM accumulations observed by 24 h incubation (Fig. 5). Bactericidal activity within the mixed gut populations generally agreed with the accumulations of free thymol, with bactericidal activity against *Salmonella* and *E. coli* occurring sooner in cecal and rectal contents than in jejunal contents (Fig. 6 and 7).

To test the hypothesis that lipids may potentially influence the bactericidal activity of liberated thymol and its prebiotic precursor, beta-D-thymol (both which are lipophilic compounds albeit less so for the conjugate), treated populations of porcine fecal bacteria were incubated with additions of vegetable oil, olive oil or their predominant fatty acids, linoleic acid or oleic acid (each at 3% vol/vol). These populations were also incubated without or with additions of glycerol (3% vol/vol), the latter which can act as an emulsifying agent. Results revealed a main effect ($P < 0.0001$; SEM = 0.313) of the oil or fatty acid supplements on *E. coli* K88 concentrations, with concentrations being maximally decreased after 24 h incubation of beta-D-thymol treated cultures containing no added oil (by 4.50 log_{10} CFU/mL), intermediately decreased in beta-D-thymol-treated cultures containing added linoleic acid, oleic acid or vegetable oil (by 2.47, 1.37 and 0.37 log_{10} CFU/mL, respectively) and increased (by 0.97 log_{10} CFU/mL) in beta-D-thymol-treated cultures containing olive oil. A main effect of glycerol supplementation on the beta-D-thymol treated populations was not observed ($P = 0.9588$; SEM = 0.198), with main effect means showing 1.55 and 1.53 log_{10} CFU/mL decreases in *E. coli* K88 concentrations after 24 h incubation of populations supplemented without or with

glycerol supplementation. A trend for an interaction between glycerol supplementation and addition of the oil/fatty acid substrates was observed (Table 1). This suggests that glycerol may have helped to partially overcome the suppressive effect of the free fatty acids, but not the effects of the oil sources, on the bactericidal activity of beta-D-thymol against *E. coli* K88 (Table 1). In a follow up study designed to further assess the effects of other emulsifying agents on lipid-inhibition of beta-D-thymol's bactericidal activity, we found that additions of the major bile constituent, taurine, helped restore 21% of the vegetable oil-caused inhibition of beta-D-thymol's bactericidal activity against *E. coli* K88. Co-addition of the commercial emulsifying agents Tween 20, Tween 80 and Poly oxyl-40-stearate with taurine did not affect further restoration of activity (not shown). Further studies are needed to determine if co-feeding beta-D-thymol with appropriate emulsifying agents may promote effective bactericidal activity in the pig gut.

Objective 2: Determine how much beta-D-thymol is needed in feed to reduce gut colonization of pigs by Campylobacter coli, Salmonella spp. and generic E. coli.

Results from two separate animal studies revealed that oral administration of beta-D-thymol had little if any effect on concentrations of the NN-resistant challenge strain of *Salmonella* Typhimurium orally inoculated into the pigs and likewise had little if any effect on gut concentrations of generic *E. coli* or *Campylobacter* species (Tables 2 and 3). In the first study, for instance, cecal but not rectal concentrations of the challenge *Salmonella* were reduced in a dose dependent fashion by treatment (Table 2). Neither cecal nor rectal concentrations of wildtype *E. coli* were affected by treatment and *Campylobacter* were not recovered from any of the pigs. These doses were calculated to deliver, based on estimated 200 mL intestinal volume, a total dose of approximately 2.3 to 6.9 mM beta-D-thymol to the lumen of the pig gut. Results from a second study, calculated to deliver approximately 5.4 to 16.3 mM beta-D-thymol to the lumen of the pig gut, based on estimated 400 mL intestinal volume, and longer intervals between administration of the last dose and sample collection at necropsy, likewise failed to provide evidence of efficacious treatment. The lack of an efficacious effect of beta-D-thymol suggests that adequate amounts of beta-D-thymol or free thymol are not being delivered to the cecum or large intestine. Possible reasons for this could be that hydrolysis of beta-D-thymol and subsequent absorption of free thymol may have been sufficiently rapid within the proximal small intestine to preclude delivery of the intact glucopuranoside to the cecum and large intestine. Results from our in vitro incubations did indicate that there was indeed appreciable beta-D-thymol hydrolytic activity in populations of swine jejunal microbes. Similarly, it is possible also that despite being 3-times more resistant to absorption than free thymol (Petrujkić et al., 2013), appreciable quantities of beta-D-thymol may still be absorbable in the small intestine. It is also possible that uptake and internal compartmentalization of beta-D-thymol by gut bacteria, or the lipophilic (fat loving) properties of both thymol and, albeit to a lesser extent, beta-D-thymol, may sequester these compounds away from hydrolytic enzymes thus preventing the release of free thymol. As discussed earlier, research by Beier and colleagues (2012) had indicated that bacterial uptake of beta-D-thymol can occur. Moreover, results from our present in vitro studies indicated that fats inhibited the bactericidal activity of beta-D-thymol in mixed populations of gut bacteria thus implicating a potential role of the lipophilicity of both thymol and beta-D-thymol. Additional research is currently underway to try and overcome these possible obstacles.

Objective 3: Evaluate the potential of beta-D-thymol to co-select for antibiotic resistant *Campylobacter coli, Salmonella* spp. and generic *E. coli* or to make resistant strains of these bacteria more sensitive to antibiotics when grown in mixed populations of porcine gut bacteria.

Results from tests assessing the potential for thymol exposure to co-select for antimicrobial resistance are presented in Tables 4 through 6 and in general the results yield no definitive evidence that prior exposure to thymol may co-select for increased susceptibility or resistance to the antibiotics tested. In most cases changes in resistance profiles were not observed between isolates having been previously exposed or not to beta-D-thymol. In some cases, however, we were to detect the potential for at least

4-fold or greater increases in resistance such as increases in MIC's to ampicillin, cefitoflur and suplhadimethoxine in *E. coli* recovered from pigs fed 17 mg beta-D-thymol/kg body weight when compared to *E. coli* recovered from the same pigs before exposure to beta-D-thymol. Similarly, in comparisons between *E. coli* recovered from pigs after or before being fed 51 mg beta-D-thymol/kg body weight, we detected the potential for at least 4-fold or greater increases in resistance to ampicillin, cefitoflur and suplhadimethoxine as well as to danofloxacin, enrofloxacin, spectinomycin, tilmicosin and tulathromycin. Of the four multi-drug resistant *Salmonella* strains tested before or after repeated exposure to sublethal amounts of thymol, which is the active agent of beta-D-thymol, we were only able to detect the potential for at least 4-fold or greater increase in resistance in thymol-adapted *Salmonella* Choleraesuis 28335 and only to ampicillin and penicillin. It is important, however, to recognize that increases of 2 to 4-fold are not necessarily remarkable as these may well be within the variability of the study method.

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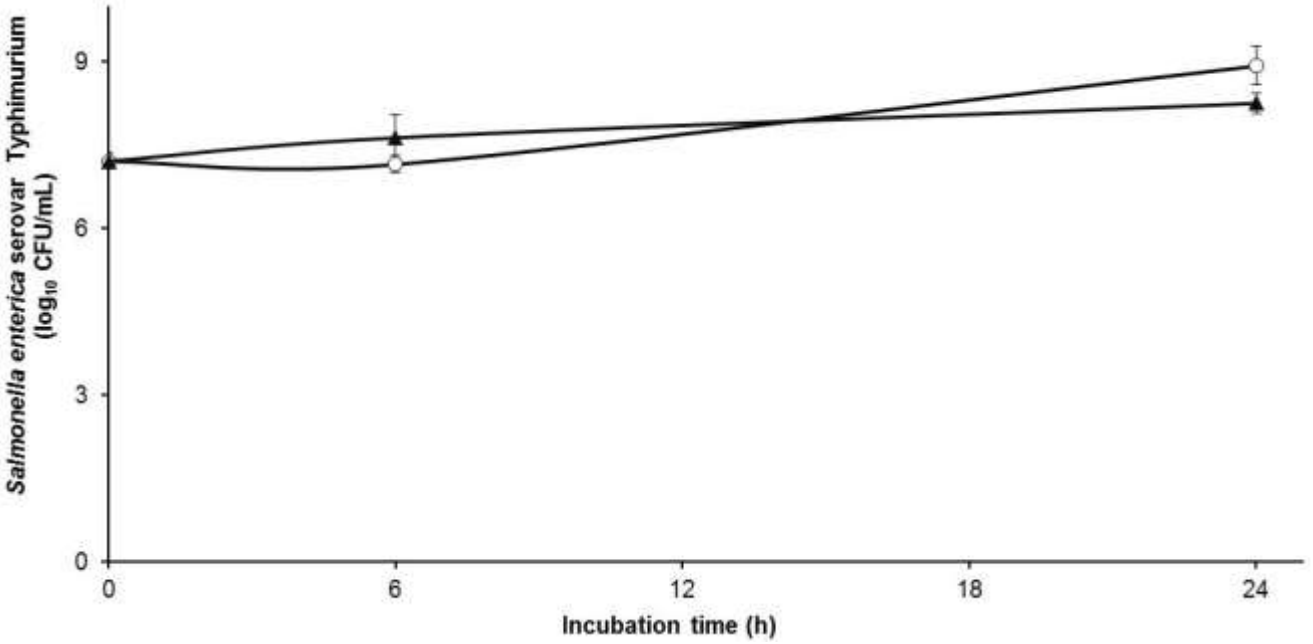

Figure 1: Concentrations of *Salmonella* Typhimurium during pure culture in Mueller Hinton broth supplemented with 0 (open circles) or 12 mM beta-D-thymol (closed triangles).

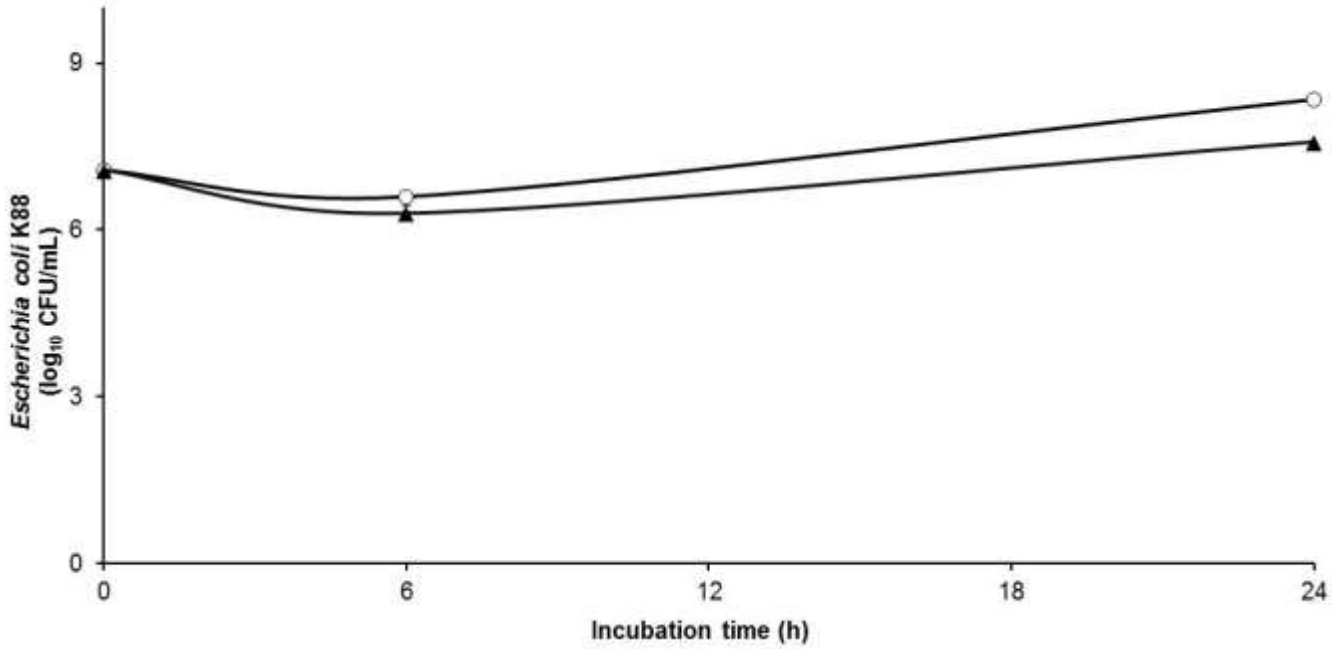

Figure 2: Concentrations of *Escherichia coli* K88 during pure culture in Mueller Hinton broth supplemented with 0 (open circles) or 12 mM beta-D-thymol (closed triangles).

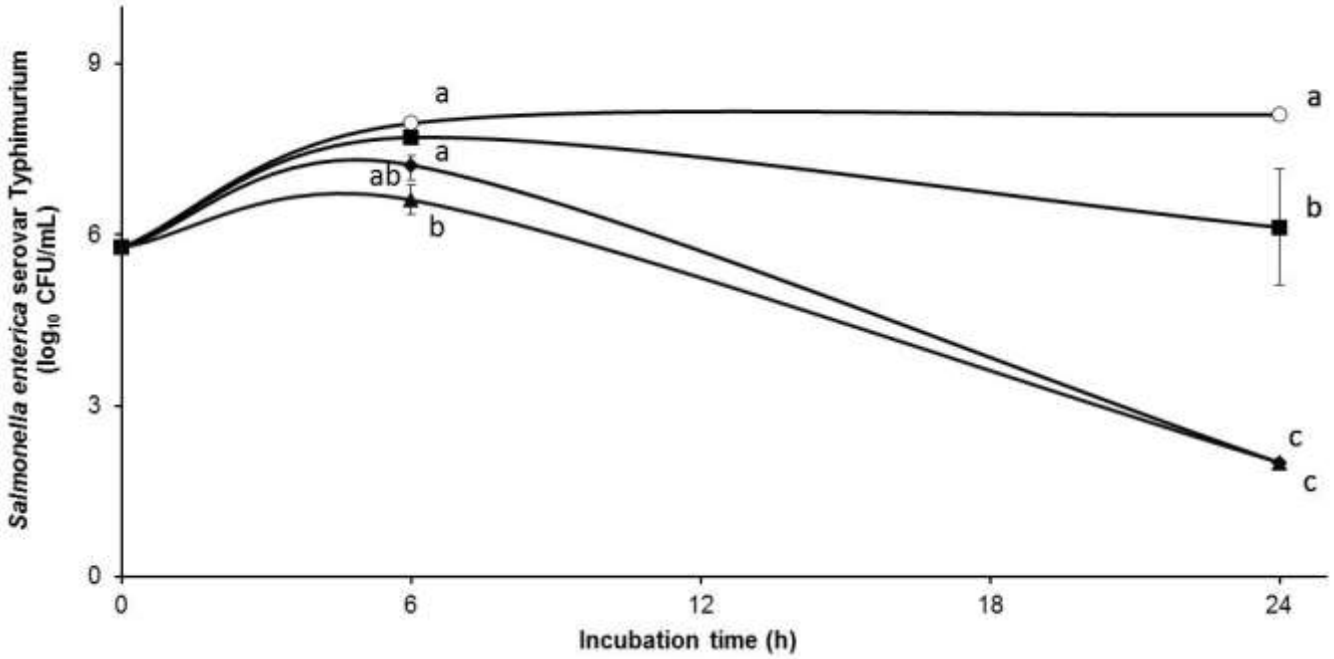

Figure 3: Concentrations of *Salmonella* Typhimurium during culture with mixed populations of porcine fecal microbes in half-strength Mueller Hinton broth supplemented with 0 (open circles), 3 (closed squares), 9 (closed diamonds) or 12 mM beta-D-thymol (closed triangles).

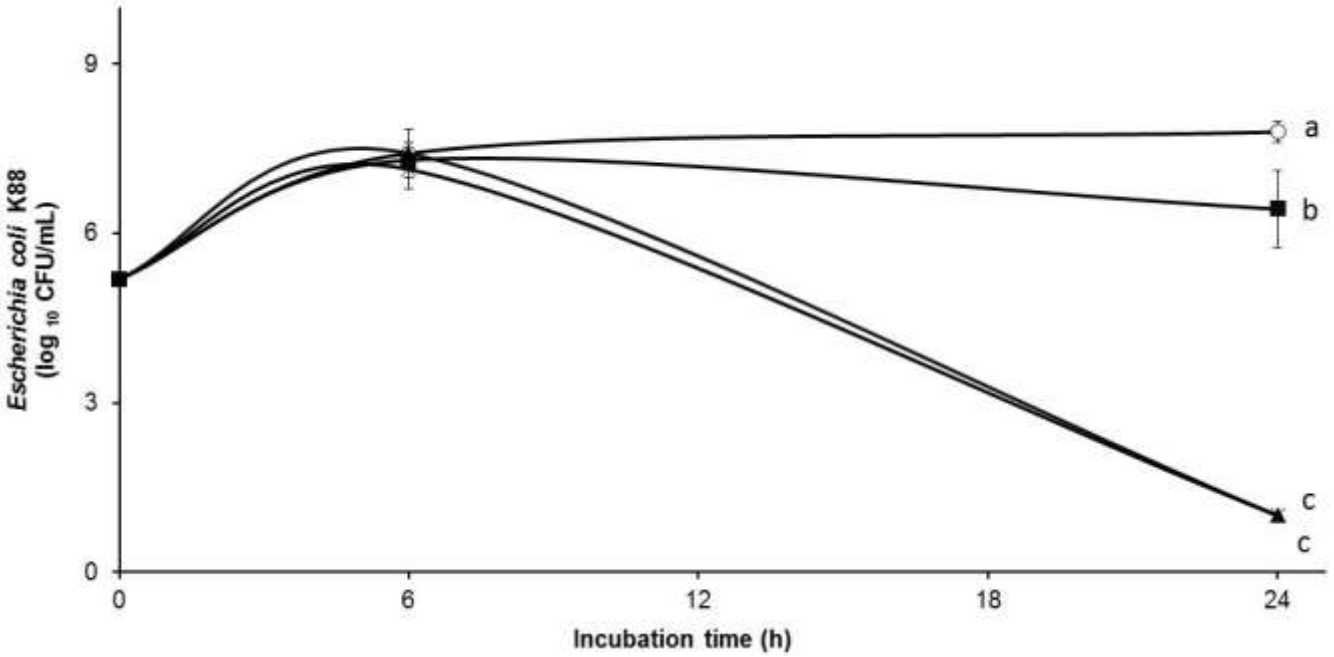

Figure 4: Concentrations of *Escherichia coli* K88 during culture with mixed populations of porcine fecal microbes in half-strength Mueller Hinton broth supplemented with 0 (open circles), 3 (closed squares), 9 (closed diamonds) or 12 mM beta-D-thymol (closed triangles).

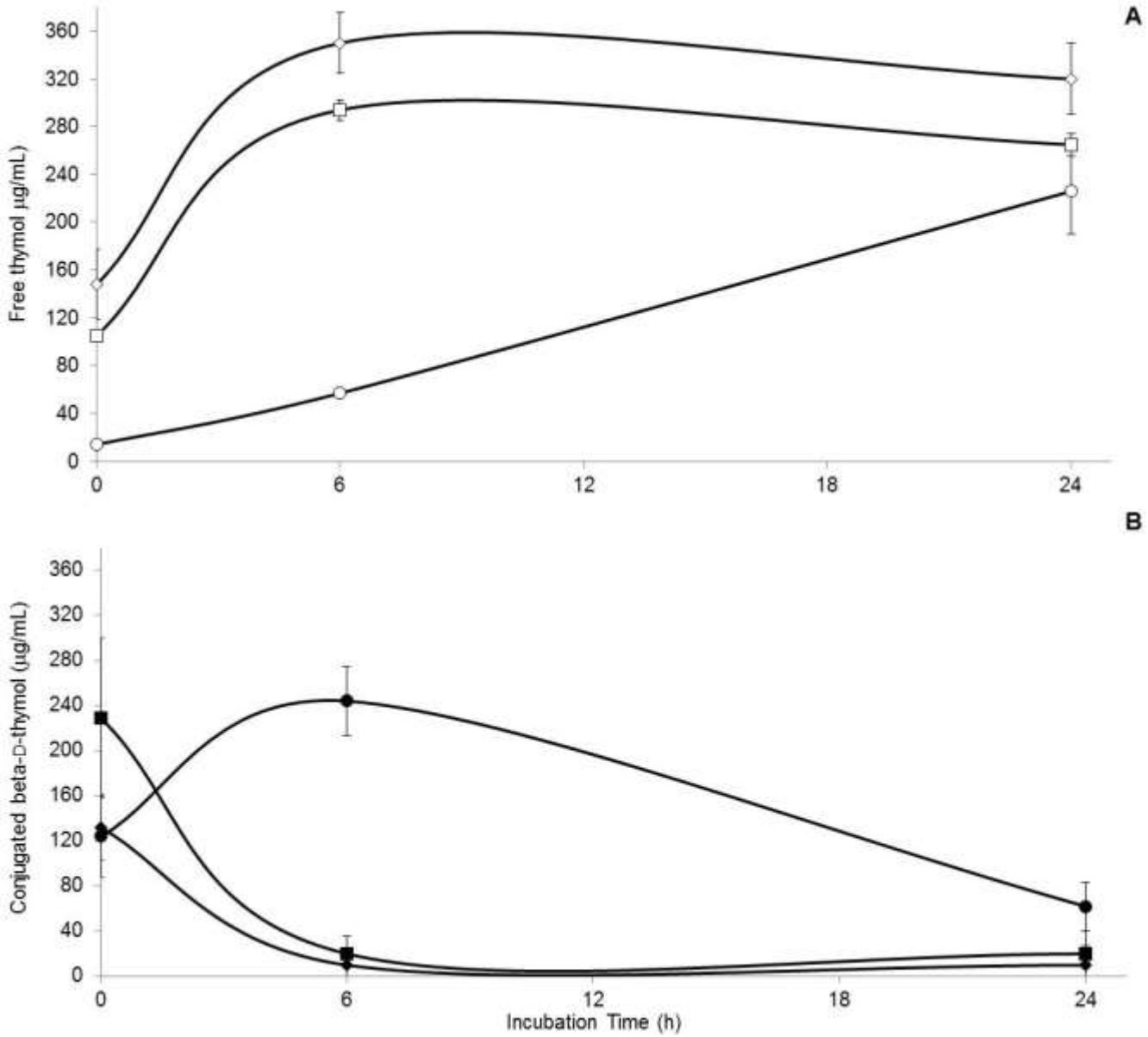

Figure 5: Accumulations of free thymol (graph A) and degradation of beta-D-thymol (graph B) during culture of mixed populations of porcine jejunal (circles), cecal (diamonds) or rectal (squares) microbes in half-strength Mueller Hinton broth supplemented with 6 mM beta-D-thymol.

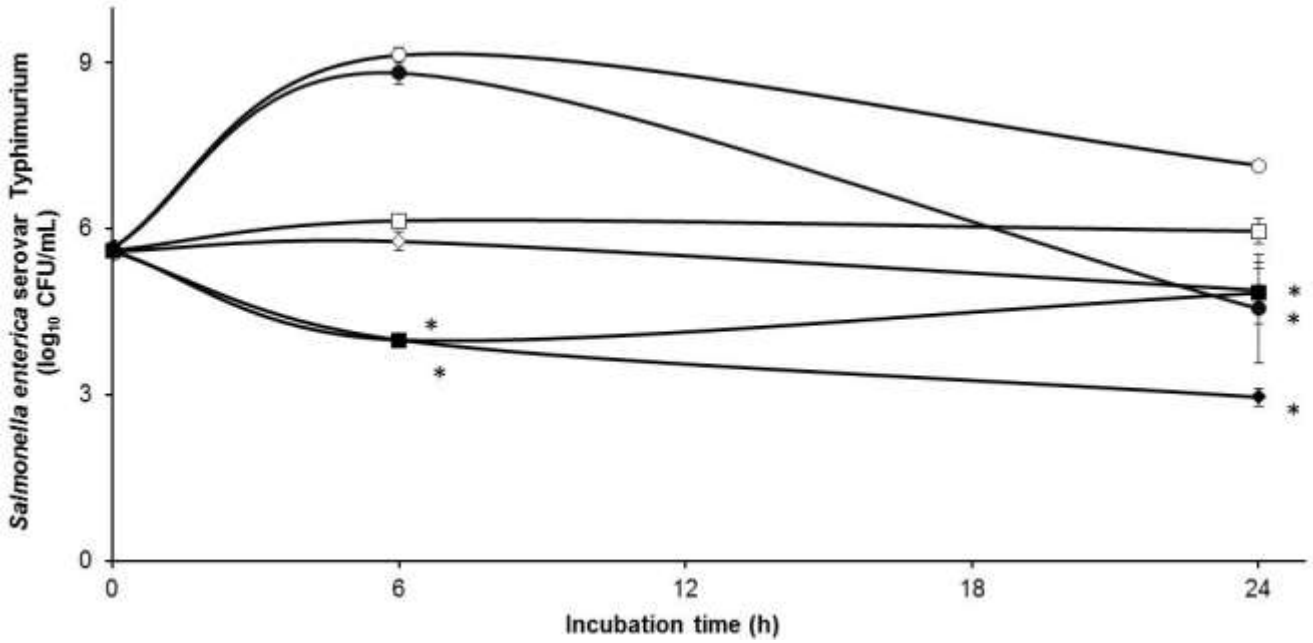

Figure 6: Concentrations of *Salmonella* Typhimurium during culture with mixed populations of porcine jejunal (circles), cecal (diamonds) or rectal (squares) microbes in half-strength Mueller Hinton broth supplemented with 0 (open symbols) or 6 mM beta-D-thymol (closed symbols). Asterisks denote significant differences ($P < 0.05$) of the treated gut populations from their untreated controls.

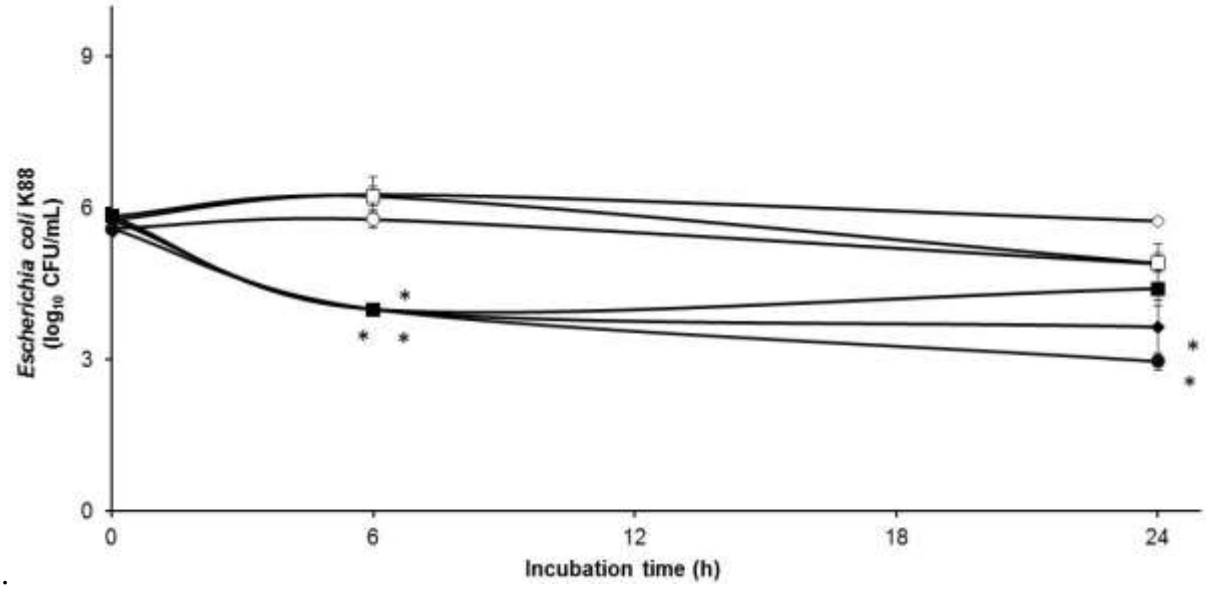

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Figure 7: Concentrations of *Escherichia coli* K88 during culture with mixed populations of porcine jejunal (circles), cecal (diamonds) or rectal (squares) microbes in half-strength Mueller Hinton broth supplemented with 0 (open symbols) or 6 mM beta-D-thymol (closed symbols). Asterisks denote significant differences ($P < 0.05$) of the treated gut populations from their untreated controls.

Table 1. Effect of different oil substrates or their predominant free fatty acid on antibacterial activity of beta-D-thymol (6 mM) against *Escherichia coli* K88 during culture in mixed populations of porcine fecal bacteria without or with co-supplemention with 3% glycerol.

	Change in *Escherichia coli* K88 concentrations (log_{10} CFU/g) after 24 h incubation	
	Without glycerol	With glycerol
None	-4.5000^{g}	-3.5767^{fg}
3% Vegetable oil	-0.3667^{bc}	0.8633^{ab}
3% Olive oil	0.9667^{a}	0.6600^{ab}
3% Oleic acid	-1.3700^{cd}	-2.2267^{de}
3% Linoleic acid	-2.4733^{def}	-3.3900^{efg}
Interaction	P = 0.0694	
SEM	0.4429	

^{a,b,c,d,e}Means with unlike superscript letters differ ($P < 0.05$).

Table 2. Effect of oral beta-D-thymol treatment on gut NN-resistant *Salmonella* Typhimurium and generic *E. coli* in weaned swine.

	Beta-D-thymol treatment (mg/kg live body weight)			P values		
	None	6	12	Linear	Quadratic	SEM
	Log_{10} CFU/g gut contents					
Salmonella Typhimurium						
Cecal	3.52	3.26	2.37	0.0287	0.3545	0.533
Rectal	3.57	2.98	2.82	0.4567	0.9988	0.501
Escherichia coli						
Cecal	6.63	6.93	7.15	0.5155	0.8281	0.267
Rectal	6.83	6.88	7.19	0.9836	0.1327	0.282

Table 3. Effect of oral beta-D-thymol treatment on gut NN-resistant *Salmonella* Typhimurium and generic *E. coli* and *Campylobacter* species in weaned swine.

	Beta-D-thymol treatment[a]								
	Treatment level (mg/kg live body weight)			Hours since last administration		Main effects			
	None	17	51	16	24	Treatment	Time	Interaction	SEM
Salmonella Typhimurium									
Cecal	2.70	2.77	2.64	2.20	3.21	0.9604	0.0290	0.8787	0.5396
Rectal	2.10	2.10	1.37	2.00	1.72	0.2696	0.4545	0.7555	0.4818
Escherichia coli									
Cecal	5.29	5.88	5.37	5.26	5.76	0.2308	0.2484	0.8594	0.5496
Rectal	4.73[bc]	5.54[b]	4.35[c]	5.01	4.73	0.0100	0.3822	0.6097	0.3977
Campylobacter species									
Cecal	4.04	4.13	3.52	3.67	4.13	0.2144	0.1232	0.2654	0.3677
Rectal	3.48	3.76	3.57	3.23	3.98	0.8103	0.0150	0.3274	0.3528

[a]n = 6, 6 and 6 pigs for 0, 17 and 51 mg/kg live body weight per day at 16 h post treatment and 6, 5 and 6 for 0, 17 and 51 mg/kg live body weight per day at 24 h post treatment, respectively.
[b,c]Values with unlike superscript letters differ based on a LSD separation of means ($P < 0.05$).

Table 4. Antimicrobial resistance profiles of generic *Escherichia coli* isolated from fecal contents collected from pigs before or after beta-D-thymol administration.

	E. coli isolates recovered from pigs assigned to 17 mg beta-D-thymol treatment/kg BW per day						*E. coli* isolates recovered from pigs assigned to 17 mg beta-D-thymol treatment/kg BW per day					
	Isolates recovered before administration of treatment			Isolates recovered 16 h after administration of treatment			Isolates recovered before administration of treatment			Isolates recovered 24 h after administration of treatment		
ANTIBIOTIC[a]	1-P1	5-P3	20-P14	1-P1	5-P3	20-P14	2-P1g	6-P3g	14-P14g	176-P1g	178-P3g	185-P14g
AMP	4	1	4	2	2	**>16**[b]	4	1	2	4	**4**	2
TIO	<=.25	<=.25	0.5	<=.25	**1**	<=.25	0.5	<=.25	<=.25	0.5	**0.5**	**0.5**
CTET	>8	>8	>8	>8	>8	>8	>8	>8	>8	>8	>8	>8
CLI	>16	>16	>16	>16	>16	>16	>16	>16	>16	>16	>16	>16
DANO	<=.12	<=.12	<=.12	<=.12	<=.12	<=.12	<=.12	<=.12	<=.12	<=.12	<=.12	<=.12
ENRO	<=.12	<=.12	<=.12	<=.12	<=.12	<=.12	<=.12	<=.12	<=.12	<=.12	<=.12	<=.12
FFN	4	8	4	8	8	4	4	8	4	4	4	8
GEN	<=1	<=1	<=1	<=1	<=1	<=1	<=1	<=1	<=1	<=1	<=1	<=1
NEO	<=4	<=4	<=4	<=4	<=4	<=4	<=4	<=4	<=4	<=4	<=4	<=4
OXY	>8	>8	>8	>8	>8	>8	>8	>8	>8	>8	>8	>8
PEN	>8	>8	>8	>8	>8	>8	>8	8	>8	>8	>8	>8
SPE	32	16	16	>64	>64	16	16	16	16	16	16	16
SDM	<=256	>256	<=256	**>256**	>256	**>256**	<=256	>256	<256	**>256**	<=256	<=256
TIA	>32	>32	>32	>32	>32	>32	>32	>32	>32	>32	>32	>32
TIL	64	64	64	64	64	>64	>64	32	64	64	64	64
SXT	<=2	<=2	<=2	<=2	<=2	>2	<=2	<=2	<=2	<=2	<=2	<=2
TUL	4	4	8	4	8	8	8	2	4	4	4	4
TYLT	>32	>32	>32	>32	>32	>32	>32	>32	>32	>32	>32	>32

[a]AMP, ampicillin; Tio, ceftioflur; CTET, chlortetracycline; CLI, clindamycin; DANO, danofloxacin; ENRO, enrofloxacin; FFN, florfenicol; GEN, gentamicin; NEO, neomycin; OXY, oxytetracycline; PEN, penicillin; SPE, spectinomycin; SDM, sulphadimethoxine; TIA, tiamulin; TIL, tilmicosin; SXT, trimethoprim/sulfamethoxazole; TUL, tulathromycin; TYLT, tylosin tartrate.

[b]MIC values bolded in red denote values that are at least 4-fold or potentially greater than observed in isolates recovered from pigs prior to beta-D-thymol administration.

Table 5. Antimicrobial resistance profiles of generic *Escherichia coli* isolated from fecal contents collected from pigs before or after beta-D-thymol administration.

	E. coli isolates recovered from pigs assigned to 51 mg beta-D-thymol treatment/kg BW per day						*E. coli* isolates recovered from pigs assigned to 51 mg beta-D-thymol treatment/kg BW per day					
	Isolates recovered before administration of treatment			Isolates recovered 16 h after administration of treatment			Isolates recovered before administration of treatment			Isolates recovered 24 h after administration of treatment		
ANTIBIOTIC[a]	43-P5	47-P8	51-P10	127-P1g	129-P8g	131-P10g	44-P5g	48-P8g	52-P10g	179-P5g	181-P8g	183-P10g
AMP	4	4	4	4	**>16**	2	4	2	2	2	1	**8**
TIO	0.5	0.5	0.5	0.5	<=.25	<=.25	0.5	0.5	<=.25	<=.25	1	**8**
CTET	>8	>8	>8	>8	>8	>8	>8	>8	>8	>8	4	4
CLI	>16	>16	>16	>16	>16	>16	>16	>16	>16	>16	>16	>16
DANO	<=.12	<=.12	<=.12	<=.12	<=.12	<=.12	<=.12	<=.12	<=.12	<=.12	**0.5**	<=.12
ENRO	<=.12	<=.12	<=.12	<=.12	<=.12	<=.12	<=.12	<=.12	<=.12	<=.12	**0.5**	<=.12
FFN	4	4	4	4	4	8	4	8	8	4	4	4
GEN	<=1	<=1	<=1	<=1	<=1	<=1	<=1	<=1	<=1	<=1	<=1	<=1
NEO	<=4	<=4	32	<=4	<=4	**<=4**[c]	<=4	<=4	<=4	<=4	<=4	<=4
OXY	>8	>8	>8	>8	>8	>8	>8	>8	>8	>8	**2**	**2**
PEN	>8	>8	>8	>8	>8	>8	>8	>8	>8	>8	8	>8
SPE	16	16	16	16	16	**>64**	16	>64	>64	16	64	**16**
SDM	<=256	<=256	>256	**>256**	<=256	>256	>256	>256	>256	<=256	>256	<=256
TIA	>32	>32	>32	>32	>32	>32	>32	>32	>32	>32	>32	>32
TIL	64	64	>64	64	64	64	64	64	64	64	>64	**>64**
SXT	<=2	<=2	<=2	<=2	<=2	<=2	<=2	<=2	<=2	<=2	<=2	<=2
TUL	4	4	16	4	4	**4**	4	8	4	4	8	**16**
TYLT	>32	>32	>32	>32	>32	>32	>32	>32	>32	>32	>32	>32

[a]AMP, ampicillin; Tio, ceftioflur; CTET, chlortetracycline; CLI, clindamycin; DANO, danofloxacin; ENRO, enrofloxacin; FFN, florfenicol; GEN, gentamicin; NEO, neomycin; OXY, oxytetracycline; PEN, penicillin; SPE, spectinomycin; SDM, sulphadimethoxine; TIA, tiamulin; TIL, tilmicosin; SXT, trimethoprim/sulfamethoxazole; TUL, tulathromycin; TYLT, tylosin tartrate.

[b]MIC values bolded in red denote values that are at least 4-fold or potentially greater than observed in isolates recovered from pigs prior to beta-D-thymol administration.

[c]MIC values bolded in green denote values that are at least 4-fold or potentially lesser than observed in isolates recovered from pigs prior to beta-d-thymol administration.

Table 6. Antimicrobial resistance profiles of multidrug resistant *Salmonella enterica* isolates before and after intentional exposure to beta-D-thymol.

ANTIBIOTIC [b]	*Salmonella enterica* serovars[a] before repeated exposure to 0.5 mM beta-D-thymol				*Salmonella enterica* serovars[a] after repeated exposure to 0.5 mM beta-D-thymol			
	Give 24349	Typhimurium 22544	Typhimurium 20731	Choleraesuis 28335	Give 24349	Typhimurium 22544	Typhimurium 20731	Choleraesuis 28335
AMP	1	>16	>16	1	1	>16	>16	**4**[c]
TIO	0.5	1	>8	1	0.5	0.5	>8	2
CTET	>8	>8	>8	8	>8	>8	>8	8
CLI	>16	>16	>16	>16	>16	>16	>16	>16
DANO	<=.12	<=.12	<=.12	<=.12	<=.12	<=.12	<=.12	<=.12
ENRO	<=.12	<=.12	<=.12	<=.12	<=.12	<=.12	<=.12	<=.12
FFN	2	4	8	2	2	4	8	**8**
GEN	<=1	<=1	<=1	<=1	<=1	<=1	<=1	<=1
NEO	<=4	<=4	32	<=4	<=4	<=4	32	<=4
OXY	>8	>8	>8	4	>8	>8	>8	4
PEN	8	>8	>8	8	8	>8	>8	**>8**
SPE	32	32	>64	32	32	32	>64	16
SDM	>256	>256	>256	>256	>256	>256	>256	>256
TIA	>32	>32	>32	>32	>32	>32	>32	>32
TIL	>64	>64	>64	>64	>64	>64	>64	>64
SXT	<=2	>2	<=2	<=2	<=2	>2	<=2	<=2
TUL	4	64	16	8	8	64	16	16
TYLT	>32	>32	>32	>32	>32	>32	>32	>32

[a]Multidrug resistant *Salmonella* serovars were provided by FDA.

[b]AMP, ampicillin; Tio, ceftioflur; CTET, chlortetracycline; CLI, clindamycin; DANO, danofloxacin; ENRO, enrofloxacin; FFN, florfenicol; GEN, gentamicin; NEO, neomycin; OXY, oxytetracycline; PEN, penicillin; SPE, spectinomycin; SDM, sulphadimethoxine; TIA, tiamulin; TIL, tilmicosin; SXT, trimethoprim/sulfamethoxazole; TUL, tulathromycin; TYLT, tylosin tartrate.

[c]MIC values bolded in red denote values that are at least 4-fold or potentially greater than observed in isolates recovered from pigs prior to beta-D-thymol administration.