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Lipid-caused antagonism of the bactericidal activity of thymol and thymol-β-D-glucopyranoside is not overcome by emulsifiers

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Introduction

Strategies are sought to reduce the carriage and dissemination of zoonotic pathogens and antimicrobial resistant microbes within food-producing animals and their production environment. Thymol is an essential oil shown to be a potent bactericide *in vitro* but demonstration of its efficacy when fed to animals has been inconsistent, due largely to its lipophilicity which limits its passage and subsequent availability in the distal gastrointestinal tract. Conjugation of thymol to glucose to form thymol-β-D-glucopyranoside can decrease absorption of the conjugate, thereby promoting passage to more distal intestinal sites where pathogens primarily reside, yet *in vivo* efficacy of the conjugate remains suboptimal. It is possible that hydrolysis and absorption of thymol-β-D-glucopyranoside and free thymol may still have been rapid enough within the proximal small intestine to preclude their delivery to the cecum and large intestine. Considering that modern swine diets often contain 5% or more fat, we hypothesized that even at 60 to 80% apparent digestibility there may be passage of enough residual undigested lipid to the distal intestinal tract to sequester free or conjugated thymol within lipidic microenvironments, thereby limiting the availability and subsequent effectiveness of these biocides.

Material and Methods

Freshly voided feces collected from 25 kg conventionally-reared pigs maintained on unmedicated feed were mixed (0.5% wt/vol) with ½-strength Mueller Hinton broth prepared under 100% N_2 gas. Fecal suspensions were then inoculated with novobiocin- and nalidixic-acid resistant (NN-resistant) challenge strains of *Salmonella enterica* serovar Typhimurium (NVSL 95-1776) or *Escherichia coli* K88 to achieve initial concentrations of approximately 10^6 colony forming units (CFU)/mL. The ½-strength broth was used to avoid excessive acid production within the fecal suspensions and the NN-resistant inocula, grown overnight at 37°C in tryptic soy broth supplemented with 25 µg of novobiocin/mL and 20 µg/nalidixic acid mL, were used to facilitate recovery and differentiation of the challenge strains from indigenous fecal microbes. The resultant suspensions were distributed (5 mL/tube) under a constant flow of 100% N_2 gas to 18 x 150 mm crimp top tubes that had been preloaded with or without 0.3 mL of vegetable oil and with or without small volumes (≤ 0.5 mL) of a 600 mM stock solution of thymol-β-D-glucopyranoside or thymol, prepared in ethanol, to achieve 6 mM upon addition of fecal suspensions. Control tubes were preloaded with 0.2 mL ethanol. In another experiment, fecal suspensions preloaded as above with oil and thymol-β-D-glucopyranoside were tested without or with additions of bile salts or taurine (0.6 or 8 mg/mL, respectively) added to assess the impact of bile acid-based micelles or their de-conjugation on pathogen survivability. The emulsifying agents Tween 20 or Tween 80 (each at 1% vol/vol) or polyoxyethylene (40) stearate (at 0.2% vol./vol) were also tested to assess their potential impact on the bactericidal activity of thymol-β-D-glucopyranoside. Tubes were closed with stoppers and incubated at 39°C for 24 h. The NN-resistant *S.* Typhimurium and *E. coli* K88 were enumerated via viable cell count on Brilliant Green or MacConkey agars supplemented with 25 µg novobiocin/mL and 20 µg naladixic acid/mL. Log_{10} CFU of NN-resistant *S.* Typhimurium and *E. coli* K88 were tested for treatment effects using a general analysis of variance and LSD separation of means. All incubations were conducted with n = 3 experimental units per treatment condition.

Results

The bactericidal effect of 6 mM free or conjugated thymol against *S.* Typhimurium and *E. coli* K88 are presented in Figure 1A and B. When expressed as log_{10}-fold reductions of CFU/mL, the addition of 3% added vegetable oil decreased (P < 0.05) the anti-*Salmonella* effects of thymol and thymol-β-D-glucopyranoside by 90 and 58%, respectively, compared to CFU reductions achieved during cultures without added oil (6.1 log_{10} CFU/mL). Addition of vegetable oil decreased (P < 0.05) the anti-*E. coli* activity of free and conjugated thymol by 86 and 84%, respectively, compared to reductions achieved in cultures incubated without added vegetable oil (5.7 log_{10} CFU/mL). Inclusion of taurine (8 mg/mL) or bile acids (0.6 mg/mL) had no effect on the antagonist-effect of vegetable oil on the bactericidal activity of thymol-β-D-glucopyranoside (not shown) and this antagonist effect was not overcome by further addition of the emulsifiers polyoxyethylene (40) stearate (0.2%), tween 20 or tween 80 (each at 1%) (Figures 2).

Discussion and Conclusion

Results from the present study are consistent with previous findings indicating that thymol-β-*D*-thymol glucopyranoside and free thymol exhibit potent bactericidal activity against *S.* Typhimurium and *E. coli* K88 when incubated with mixed populations of porcine gut bacteria. As hypothesized, the bactericidal activity of these compounds was decreased when the mixed populations were incubated with 3% added vegetable oil. Based on these results, it seems reasonable to suspect undigested lipid in the distal gut may be one of potentially several factors limiting the efficacy of free or conjugated thymol. Accordingly, additional research is warranted to learn how to overcome obstacles diminishing bactericidal activity of free and conjugated thymol in the lower gastrointestinal tract of food-producing animals.

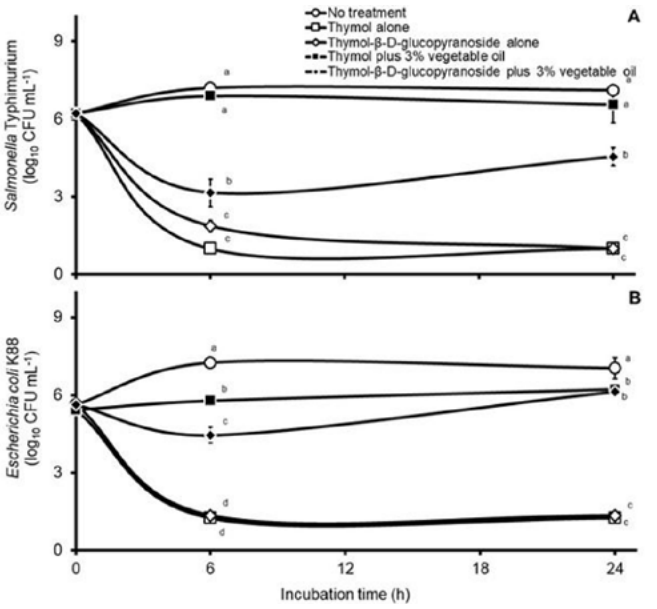

Figure 1: Concentrations of S. Typhimurium (A) or E. coli K88 (B) during incubation with mixed populations of porcine fecal bacteria treated without or with either thymol or thymol-β-D-glucopyranoside (each at 6 mM) in the absence or presence of 3% added vegetable oil. Values at each time point with unlike letters differ (P < 0.05)

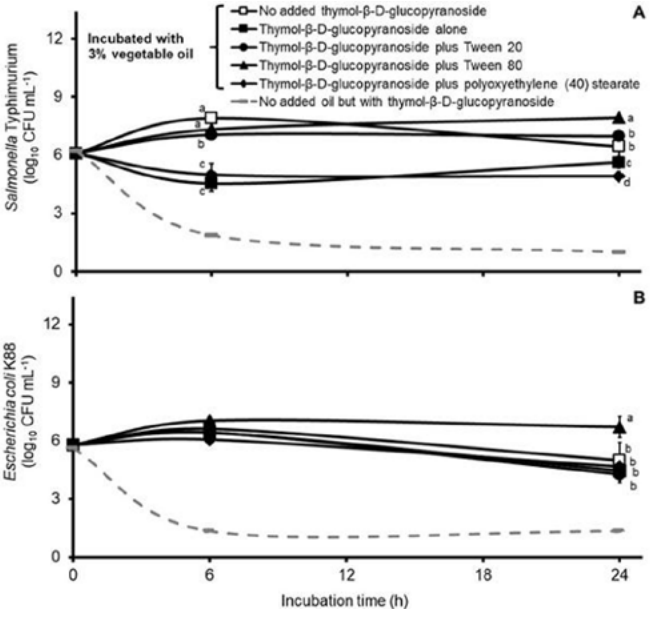

Figure 2: Concentrations of S. Typhimurium (A) and E. coli K88 (B) during culture with mixed populations of porcine fecal microbes with 3% vegetable oil, 8 mg taurine/mL and 6 mM thymol-β-D-thymol without or with added 1% tween® 20, 1% tween® 80 or 0.2% polyoxyethylene (40) stearate. Values at each time point with unlike letters differ (P < 0.05). Concentrations of populations cultured similarly except without added fat and emulsifiers are shown as shaded dashed-line for comparison