Efficacy of thymol as a practical, cost-effective, easy administer prebiotic

Robin C. Anderson
USDA/ARS
Food and Feed Safety Research Unit
Southern Plains Agricultural Research Unit
College Station, Texas
Mission of the USDA/ARS Research Team in College Station:

Develop interventions to reduce epizootic pathogenic bacteria in swine and cattle
Need for interventions

- The gut of food producing animals and their production environment can be reservoirs for foodborne pathogens and antimicrobial resistant microbes

- Foodborne pathogens and resistant bacteria can contaminate the carcass and can be disseminated in environment

- Postharvest interventions are running against a wall (i.e., hard to get cleaner and better)

- Consumer demand for safe, high quality product

- INCREASING PRESSURE TO REMOVE ACCESS TO ANTIBIOTICS
Preferred Qualities of Preharvest Interventions and Antibiotic Alternatives

- Low cost and practical
- Easy to handle and administer
- Efficacious and safe
- Mechanistic and specific
- Amendable to varied production practices
- Cost recoverable
Practical challenges to development of usable interventions

• interventions should, where possible, provide incentives for implementation by reducing costs associated with production inefficiencies and environmental emissions (increase production of high quality products with less environmental footprint).

• research should be balanced to include work with technologies near to market such as those Generally Recognized As Safe (GRAS), as well as fundamental research to elucidate mechanistically-defined interventions that can ultimately be exploited to provide efficacy over the long-term.
Research with Essential Oils

Attractiveness of Essential Oils

**Practice tip**

Feed additives for swine: Fact sheets – prebiotics and probiotics

Jay V. Jacala, DVM, PhD; Joel M. DeRouchey, PhD; Mike D. Tokach, PhD; Robert D. Goodband, PhD; Jim L. Nelsen, PhD; David G. Hunter, DVM, PhD; Steve S. Dritz, DVM, PhD

**FACT Sheet: Phytonic feed additives (phytobiotics or botanicals)**

Restriction on the use of in-feed antibiotics in many countries has fueled the interest in alternative products. A group of natural products known as phytonics has been the focus of several studies in recent years. Also referred to as prebiotics or probiotics, phytonics are plant-derived products used in feed to potentially improve pig performance. Aside from having antimicrobial activity, these products potentially provide antioxidant effects, enhance palatability, improve gut functions, or promote growth. However, there is limited research validating their potential benefits for pigs.

What products are being used as phytonic feed additives?

Phytonics comprise a wide range of substances and thus have been further classified according to botanical origin, processing, and composition. Phytonic feed additives include herbs, which are non-woody flowering plants known to have medicinal properties, spices, which are herbs with intense smell or taste, commonly added to human food; essential oils, which are aromatic oily liquids derived from plant materials such as flowers, leaves, fruits, and more; and eleutherosides, which are extracts derived by non-aqueous solvents from plant material.

How do phytonic feed additives exert their claimed effects?

The mode of action of most phytonic feed additives is still not fully understood. However, the following are some of the potential mechanisms by which they may improve performance:

- **Increased feed intake**: The stimulatory effect of phytonics on

**Fast facts**

- Phytonic feed additives are substances derived from plants.
- The potential benefits of phytonics in pig diets have not been fully substantiated.
- Current research data show that growth responses to phytonic feed additives are still inadequate compared to responses obtained with the use of in-feed antimicrobials.

**Natural compounds that have history of use and may be viewed favorably by FDA**

Consequently may be more near to market than strategies requiring FDA approval
Mechanism of thymol and other essentials oils

- Thymol
- Carvacrol
- Eugenol
- Perillaldehyde
- Cinnamaldehyde
- Cinnamic acid

MIC’s of 0.05 to 5 µl/mL against pure cultures

-higer concentrations needed when applied to foods

-mechanistically, thought to disrupt cell walls
Thymol and Diphenyliodonium chloride (DIC) have been studied extensively for ability to reduce the inefficiency of ruminal amino acid catabolism (Chalupa et al., 1980’s).

Early evidence testing inhibitors of amino acid metabolism revealed that thymol can reduce survivability of *Campylobacter*.

Results with swine gut populations confirmed that thymol and DIC knock the “heck” out of *Campylobacter* and ammonia production in mixed culture.

This Project was funded by the National Pork Board.
Numerous in vitro (bench top) studies have shown essential oils such as thymol to exhibit potent antimicrobial activity.
Limitations of essential oils like thymol as feed additives is that they are extensively degrade or absorbed in proximal gastrointestinal tract. So bypass technologies are needed to deliver thymol to the lower gut.
So we thought we could perhaps protect thymol from rapid absorption by conjugating to glucose with a $\beta$-glycosidic bond.

Thymol

\[ \text{Thymol-}$\beta$-D-glucopyranoside (\(\beta$-D-thymol) \]
Thymol-β-D-glucopyranoside (β-D-thymol) as a potential bypass additive
Everted jejunal segments to investigate absorption

- Turn jejunal segments inside out and tie off.
- So measures absorption of thymol or thymol-β-D-glucopyranoside from buffer to inside of tied-off segments.
Thymol-β-D-glucopyranoside was translocated across everted porcine jejunal segments less efficiently than free thymol. These results indicate that the conjugated form of thymol may resist absorption in the proximal alimentary tract and thus pass intact to the lower gut where it can be activated (hydrolyzed) by microbial expressed β-glycosidase.

Thymol-β-D-glucopyranoside has little bactericidal activity unless activated by β-glycosidase expressing bacteria.

In pure culture, viable cell counts of *Campylobacter jejuni* were reduced in cultures treated with thymol but not in cultures treated with thymol-β-D-glucopyranoside (Fig. A).

Conversely, when co-cultured with a β-glycosidase expressing *Parabacteroides distasonis* (Fig. B), growth of *Campylobacter jejuni* was inhibited in cultures treated with thymol and in cultures treated with thymol-β-D-glucopyranoside.

This work was funded in part by the National Pork Board.
Mixed populations of pig gut microbes also possess sufficient β-glycosidase activity to activate (hydrolyze) the conjugated form of thymol.

These results indicate that there is significant ammonia inhibition with thymol and thymol-β-D-glucopyranoside.

This work was funded in part by the National Pork Board.
Results from two separate animal studies were not particularly encouraging.

This work was funded in part by the National Pork Board.

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

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Beta-o-thymol treatment (mg/kg live body weight)</th>
<th>P values</th>
<th>Log_{10} CFU/g gut contents</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>6</td>
<td>12</td>
<td>Linear</td>
</tr>
<tr>
<td>salmonella Typhimurium</td>
<td>3.52</td>
<td>3.26</td>
<td>2.37</td>
</tr>
<tr>
<td>Cecal</td>
<td>3.57</td>
<td>2.98</td>
<td>2.82</td>
</tr>
<tr>
<td>Rectal</td>
<td>6.63</td>
<td>6.93</td>
<td>7.15</td>
</tr>
<tr>
<td>Escherichia coli</td>
<td>6.83</td>
<td>6.88</td>
<td>7.19</td>
</tr>
</tbody>
</table>

*6 pigs per treatment were twice-treated via oral gavage (approximately 8 hours apart) and euthanized 12 h after receiving last treatment.

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

<table>
<thead>
<tr>
<th>Treatment level</th>
<th>Beta-o-thymol treatment (mg/kg live body weight)</th>
<th>Hours since last administration</th>
<th>Main effects</th>
<th>Log_{10} CFU/g gut contents</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>6</td>
<td>12</td>
<td>18</td>
<td>24</td>
</tr>
<tr>
<td>Salmonella Typhimurium</td>
<td>2.70</td>
<td>2.77</td>
<td>2.64</td>
<td>2.20</td>
</tr>
<tr>
<td>Cecal</td>
<td>2.10</td>
<td>2.00</td>
<td>1.37</td>
<td>0.2696</td>
</tr>
<tr>
<td>Rectal</td>
<td>5.26</td>
<td>5.78</td>
<td>5.26</td>
<td>0.2308</td>
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<tr>
<td>Escherichia coli</td>
<td>4.73</td>
<td>5.54</td>
<td>4.35</td>
<td>0.0100</td>
</tr>
<tr>
<td>Campylobacter species</td>
<td>4.64</td>
<td>4.13</td>
<td>3.52</td>
<td>0.2144</td>
</tr>
<tr>
<td>Rectal</td>
<td>3.48</td>
<td>3.76</td>
<td>3.57</td>
<td>0.0103</td>
</tr>
</tbody>
</table>
Investigating three hypotheses as to why the $\beta$-D-thymol did not work

The first hypothesis is that thymol may be sequestered within the gut environment such as in fat complexes or within microbial cells.

Evidence in support of this hypothesis is that lipids markedly decrease the bactericidal activity of free thymol.

Presently looking at ways to potentially overcome hurdle by adding emulsifiers to break up fats or extra calcium which might bind to the fat.

This work was funded in part by the National Pork Board.
Second hypotheses, β-D-thymol may still be to small and even though absorbed more slowly than free thymol it not make it all the way to the cecum and large intestine.

We are looking at making conjugates with larger side chains and more binding sites for thymol to see if this may promote passage to the lower gut.

This work was funded in part by the National Pork Board.
The third hypothesis is that the β-D-thymol hydrolyzing activity may be too rapid or too slow to achieve optimal liberation of free thymol.

Evidence in support of this hypothesis is that the thymol is liberated very rapidly in the poultry crop which has a relatively shorter retention time than the pig gut.

Potential ways to overcome this may be to employ additional encapsulation or protection technologies.
Another approach we are investigating is to use natural sources of thymol as these may contain thymol in naturally-conjugated or protected forms.

*Nigella sativa*
Black cumin seed

- A natural source of thymol derivatives as well as a number of other phytochemicals

[Link to Wikipedia page](https://en.wikipedia.org/wiki/Nigella_sativa)

![Thymohydroquinone](image1)

![Thymoquinone](image2)
Investigated in the Middle East as a feed additive poultry and small ruminants

Generally finding benefits in intake, performance and some microbiological measurements

Little, if any, prior work has been done with pigs!
Results from just completed studies in our laboratory

Bench top studies confirmed dose-dependent antimicrobial activity against *E. coli*, *Campylobacter* and *Salmonella*

- Higher doses needed for *Salmonella*

Preliminary animal study with nursery pigs administered Nigella extract via oral gavage provided supporting evidence of a positive effect against *E. coli*

1X dose in gavage study is equivalent to a dose that would be expected if mixed 0.15% with diet DM

This work was funded in part by the National Pork Board.
Evidence for positive effects against *Campylobacter* were also observed. Negative effects against total culturable anaerobes (good fermenting bacteria) were not observed.

But not against *Salmonella* at the doses tested.

1X dose equivalent to 0.15% diet DM

This work was funded in part by the National Pork Board.
Results from a follow up animal feeding study with weaned pigs
Again showed evidence for a positive treatment effect in reducing gut concentrations of wildtype *E. coli* and *Campylobacter*.

1X dose in feeding study = 0.15% diet DM

This work was funded in part by the National Pork Board.
But not against our challenge strain of *Salmonella Typhimurium*

1X dose in feeding study = 0.15% diet DM
Positive effects of feeding Nigella extract were observed on piglet performance

Treated pigs ate slightly more feed

Treated pigs gained more weight

Treated pigs had higher average daily gain

Treated pigs had a better feed conversion

1X dose in feeding study = 0.15\% diet DM

This work was funded in part by the National Pork Board.
Summary

• Essential oil compounds have demonstrated antimicrobial activity but their rapid adsorption and high solubility in lipid limits their efficacy in animals

• Attempts to protect free thymol using glucose conjugates have not overcome these limitations

• Use of extracts of Nigella sativa have shown promise in reducing *E. coli* and *Campylobacter* but not *Salmonella*
  • Greater range of concentrations and terminal application may be needed to achieve efficacious reductions in *Salmonella*

• Positive effects of Nigella extracts on animal performance may allow recovery of costs of use
  • Longer term studies with different extract preparations are warranted to more clearly define potential benefits
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Branko Petrujkić
University of Belgrade
Faculty of Veterinary Medicine
petrujkic@yahoo.com

Ross Beier
USDA/ARS
ross.beier@ars.usda.gov

and the contribution of Ross Beier & Branko Petrujkić