

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

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Mission of the USDA/ARS Research Team in College Station:

**Develop interventions to reduce epizootic
pathogenic bacteria in swine and cattle**

Need for interventions



Byrd et al. 2002. Poultry Sci. 81: 70-74

- 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

PEER REVIEWED

Feed additives for swine: Fact sheets – prebiotics and probiotics, and phytonics

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

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.¹ Also referred to as phytobiotics or botanicals, phytonics are plant-derived products used in feed to potentially improve pig performance. Aside from having antimicrobial activity, these products potentially provide antioxidative effects, enhance palatability, improve gut functions, or promote growth.¹ 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 intensive 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 roots; and oleoresins, which are extracts derived by non-aqueous solvents from plant material.¹ Two of the most common phytonic substances evaluated in swine include the spices oregano and thyme.¹⁻⁵

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

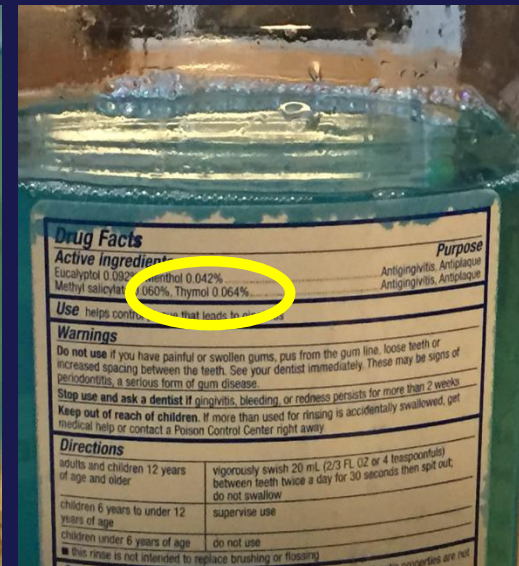
Phytonic feed additives are substances derived from plants.

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.

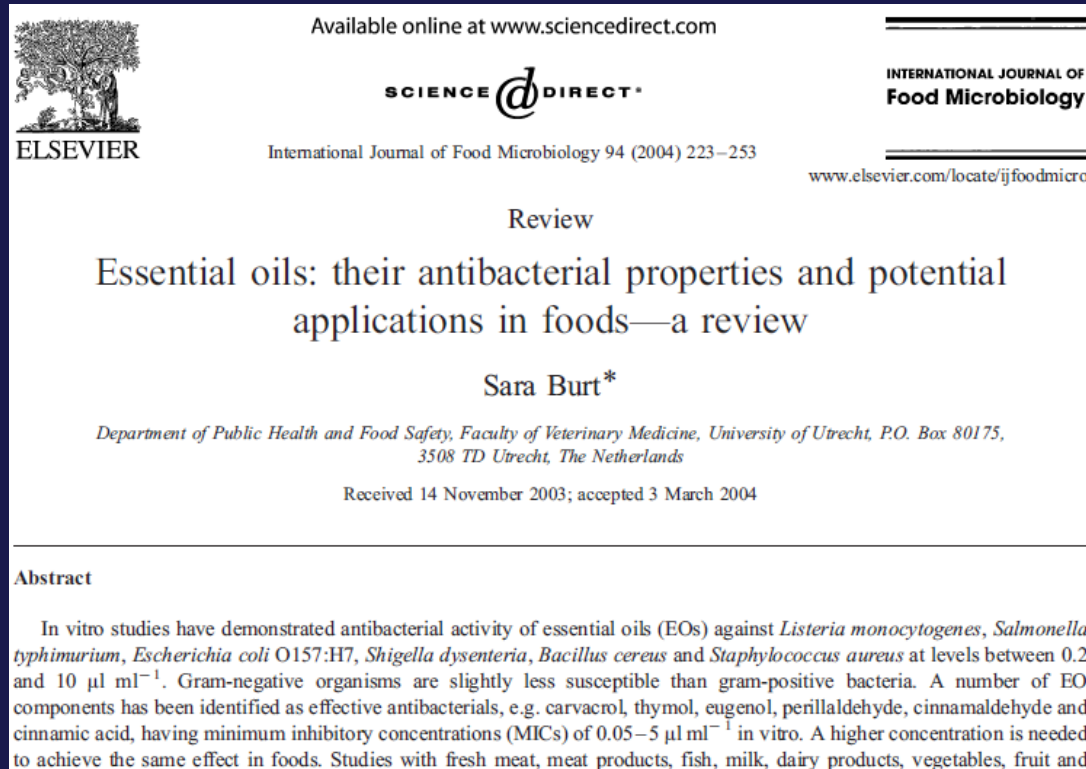
Anti-oxidative effects. Anti-oxidative properties of some phytonic substances have been attributed to the phenolic terpenes in the essential oils.^{17,18} Essential oils of plants belonging to the Labiatae family have been widely used as antioxidants in human and pet foods with high fat content.¹⁰ Plants high in terpenes include rosemary, oregano, and thyme.^{1,10} However, whether they can be added in amounts sufficient to replace the effects of antioxidants commonly used in pig diets, such as ethoxyquin and butylated hydroxytoluene, remains to be seen.

Antimicrobial effect. The medicinal or antimicrobial properties of plant-derived substances have been well known for centuries.^{19,20} This property is mainly attributed to the essential oils of these plants. Oregano and thyme are among those which have received a great deal of interest. These plants contain the monoterpenes carvacrol and thymol, respectively, and have demonstrated high efficacy *in vitro* against several pathogens found in the intesti-



Consequently may be more near to market than strategies requiring FDA approval

Mechanism of thymol and other essential oils

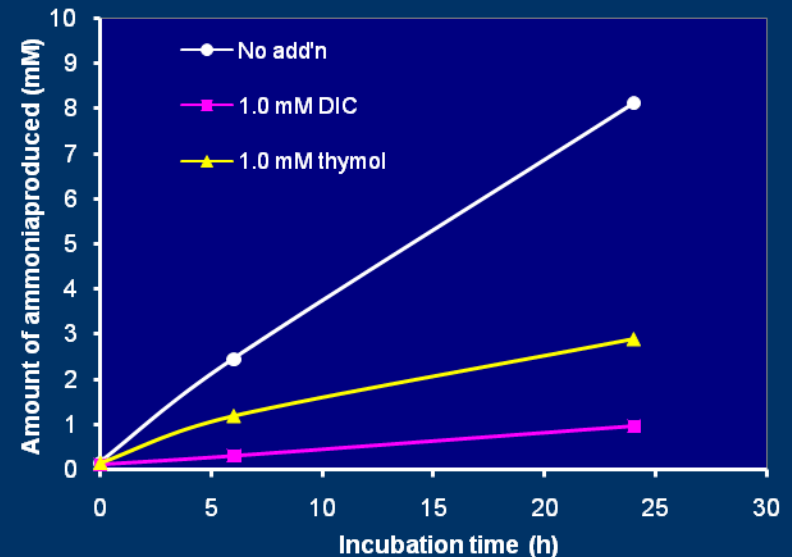
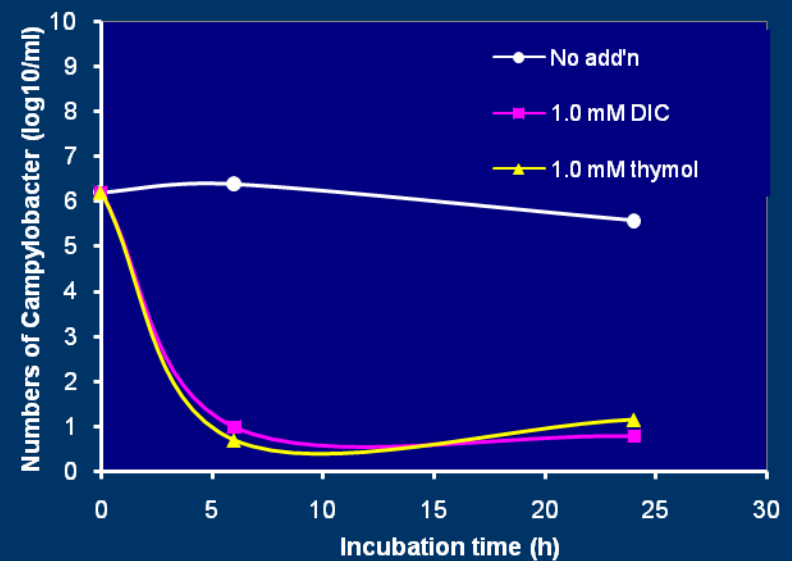


- Thymol
- Carvacrol
- Eugenol
- Perillaldehyde
- Cinnamaldehyde
- Cinnamic acid

MIC's of 0.05 to 5 $\mu\text{l/mL}$ against pure cultures
-higher concentrations needed when applied to foods
-mechanistically, thought to disrupt cell walls

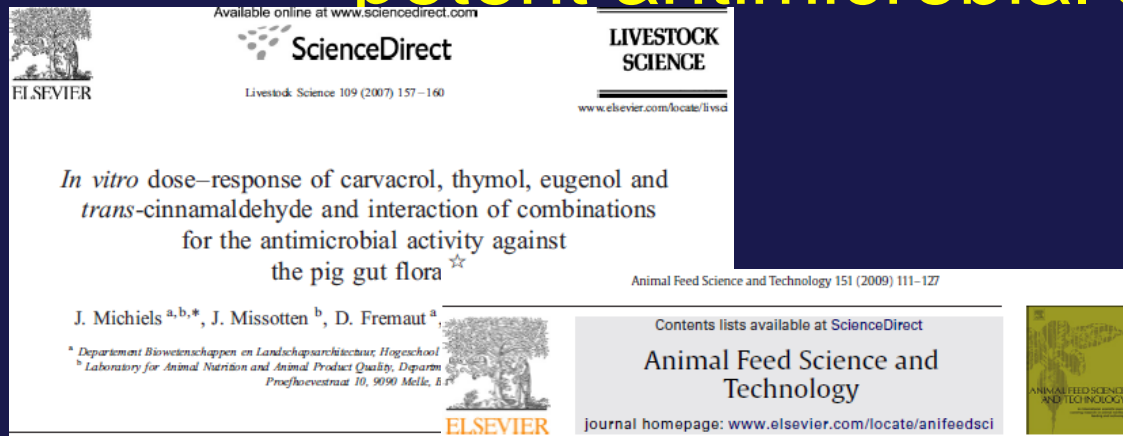
Early evidence testing inhibitors of amino acid metabolism revealed that thymol can reduce survivability of *Campylobacter*

- 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)



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

Numerous in vitro (bench top) studies have shown essential oils such as thymol to exhibit potent antimicrobial activity



Abstract

In vitro simulations of the gastric and small intestinal fermentation with various essential oils and binary combinations thereof were carried out to calculate the antimicrobial activity against the main pig gut flora components. Interaction effects were evaluated.

In vitro characterisation of the antimicrobial activity of selected essential oil components and binary combinations against the pig gut flora

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ABSTRACT

The antimicrobial activity of selected essential oil components against the major culturable components of the pig gut flora has been characterised by means of an *in vitro* incubation simulating the fermentation in different sections of the gastrointestinal tract (GIT). In a first study 7 components were tested for their antimicrobial properties. Dose-response equations were established for the 4 components with the highest

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ORIGINAL ARTICLE

Effects of thymol and diphenyliodonium chloride against *Campylobacter* spp. during pure and mixed culture *in vitro*

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Keywords

biocides, *Campylobacter*, cell injury/sub-lethal injury, mechanism of action, metabolism.

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Abstract

Aims: To determine if the purported deaminase inhibitors diphenyliodonium chloride (DIC) and thymol reduce the growth and survivability of *Campylobacter*.

Methods and Results: Growth rates of *Campylobacter jejuni* and *Camp. coli* were reduced compared to unsupplemented controls during culture in Mueller-Hinton broth supplemented with 0.25 μmol DIC or thymol ml^{-1} but not with 0.01 μmol monensin ml^{-1} or 1% ethanol. Recovery of *Camp. jejuni* and *Camp. coli* was reduced $>5 \log_{10}$ CFU from controls after 24 h pure culture in Bolton broth supplemented with 0.25 or 1.0 μmol DIC ml^{-1} or with 1.0 μmol thymol ml^{-1} . Similarly, each test *Campylobacter* strain was reduced $>3 \log_{10}$ CFU from controls after 24 h mixed culture with porcine faecal microbes in Bolton broth supplemented with 0.25 or 1.0 μmol DIC ml^{-1} or with 1.0 μmol

The work in this bottom paper was funded in part by the National Pork Board.

Limitations of essential oils like thymol as feed additives is that they are extensively degrade or absorbed in proximal gastrointestinal tract

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In vitro degradation and *in vivo* passage kinetics of carvacrol, thymol, eugenol and *trans*-cinnamaldehyde along the gastrointestinal tract of piglets



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Abstract

BACKGROUND: The essential oils (EO) carvacrol, thymol, documented antimicrobial properties and offer therefore an alternative for antibiotics in pig feeds. The aim of this work was to determine the degradation and passage kinetics of these EO in the gastrointestinal tract (GIT) of piglets, which is necessary information for correct

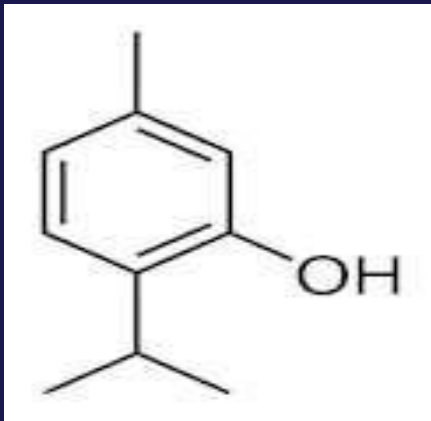
Effects of dose and formulation of carvacrol and thymol on bacteria and some functional traits of the gut in piglets after weaning

RESULTS: None of these compounds was significantly degraded in *in vitro* simulations. Carvacrol and thymol were not degraded in jejunal simulations. Eugenol and *trans*-cinnamaldehyde showed caecal simulations. A single dose mixed with feed (13.0, 13.2, 13.4, 13.6, 13.8, 14.0, 14.2, 14.4, 14.6, 14.8, 15.0, 15.2, 15.4, 15.6, 15.8, 16.0, 16.2, 16.4, 16.6, 16.8, 17.0, 17.2, 17.4, 17.6, 17.8, 18.0, 18.2, 18.4, 18.6, 18.8, 19.0, 19.2, 19.4, 19.6, 19.8, 20.0, 20.2, 20.4, 20.6, 20.8, 21.0, 21.2, 21.4, 21.6, 21.8, 22.0, 22.2, 22.4, 22.6, 22.8, 23.0, 23.2, 23.4, 23.6, 23.8, 24.0, 24.2, 24.4, 24.6, 24.8, 25.0, 25.2, 25.4, 25.6, 25.8, 26.0, 26.2, 26.4, 26.6, 26.8, 27.0, 27.2, 27.4, 27.6, 27.8, 28.0, 28.2, 28.4, 28.6, 28.8, 29.0, 29.2, 29.4, 29.6, 29.8, 30.0, 30.2, 30.4, 30.6, 30.8, 31.0, 31.2, 31.4, 31.6, 31.8, 32.0, 32.2, 32.4, 32.6, 32.8, 33.0, 33.2, 33.4, 33.6, 33.8, 34.0, 34.2, 34.4, 34.6, 34.8, 35.0, 35.2, 35.4, 35.6, 35.8, 36.0, 36.2, 36.4, 36.6, 36.8, 37.0, 37.2, 37.4, 37.6, 37.8, 38.0, 38.2, 38.4, 38.6, 38.8, 39.0, 39.2, 39.4, 39.6, 39.8, 40.0, 40.2, 40.4, 40.6, 40.8, 41.0, 41.2, 41.4, 41.6, 41.8, 42.0, 42.2, 42.4, 42.6, 42.8, 43.0, 43.2, 43.4, 43.6, 43.8, 44.0, 44.2, 44.4, 44.6, 44.8, 45.0, 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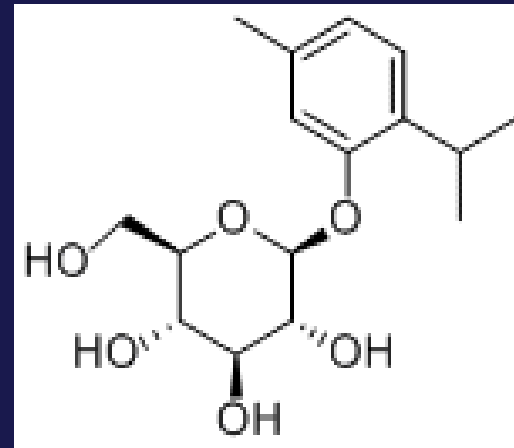
So we thought we could perhaps protect thymol from rapid absorption by conjugating to glucose with a β -glycosidic bond



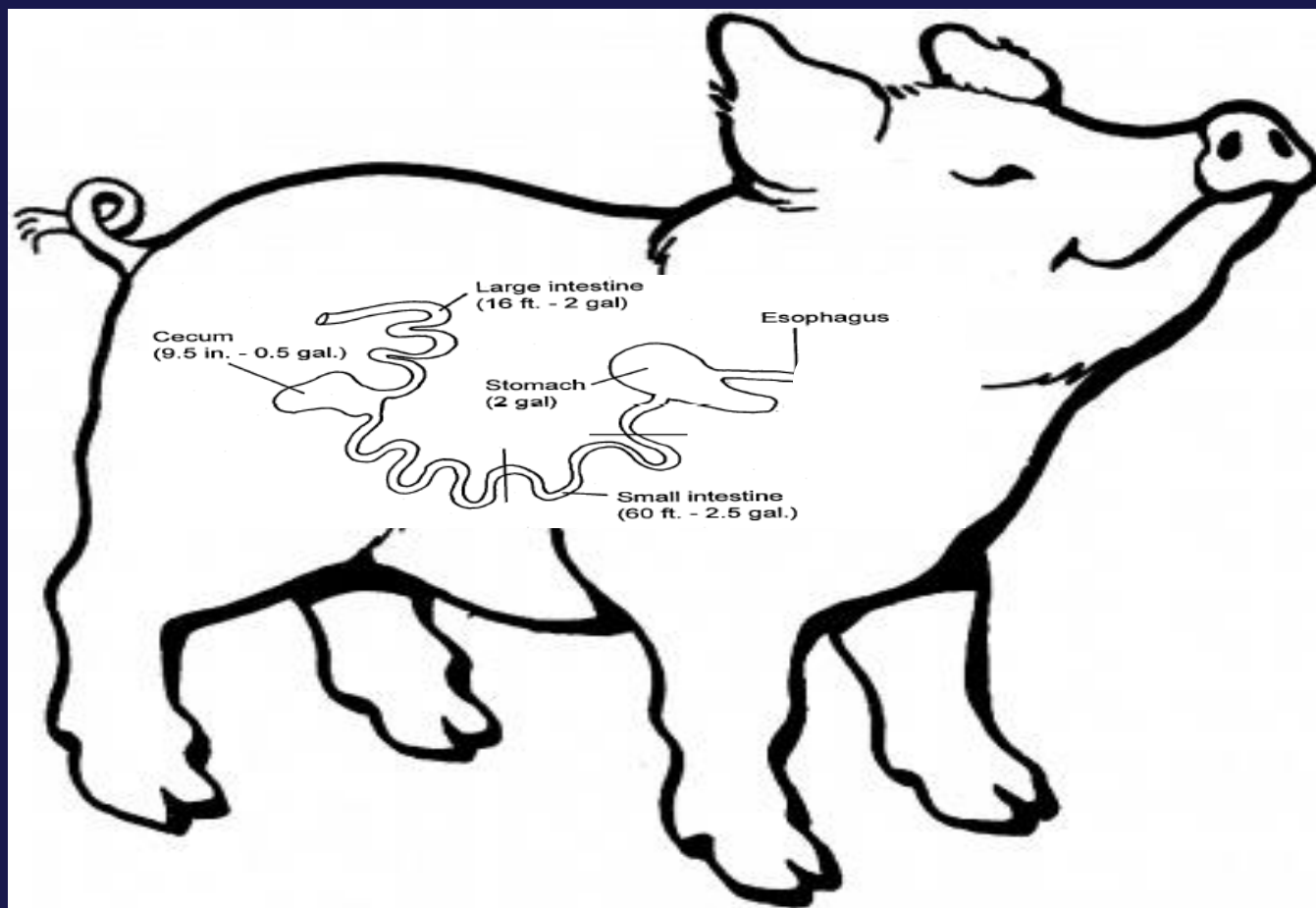
Thymol



Thymol- β -D-glucopyranoside (β -D-thymol)

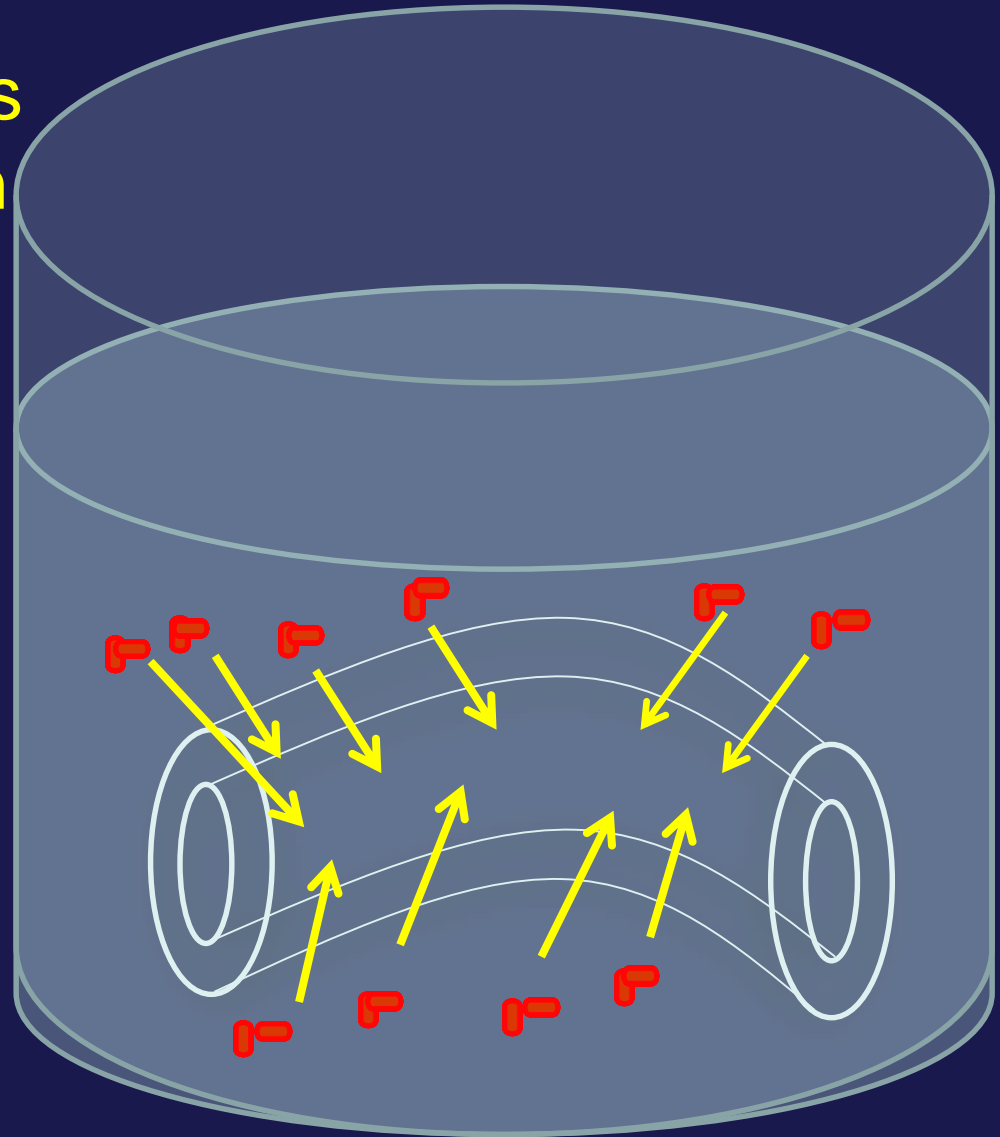


Thymol- β -D-glucopyranoside (β -D-thymol) as a potential bypass additive

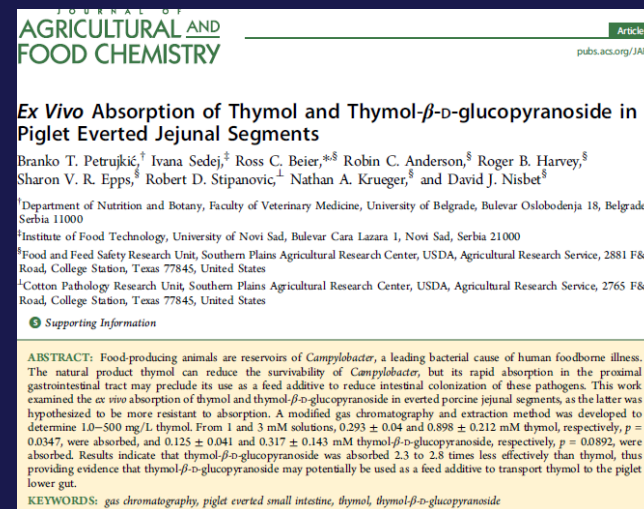
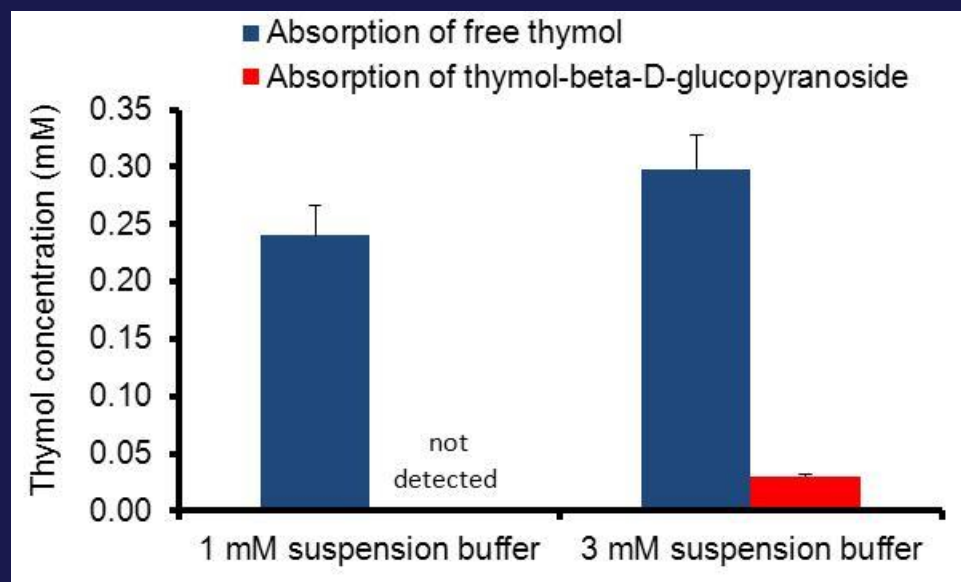


Everted jejunal segments to investigate absorption

- 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

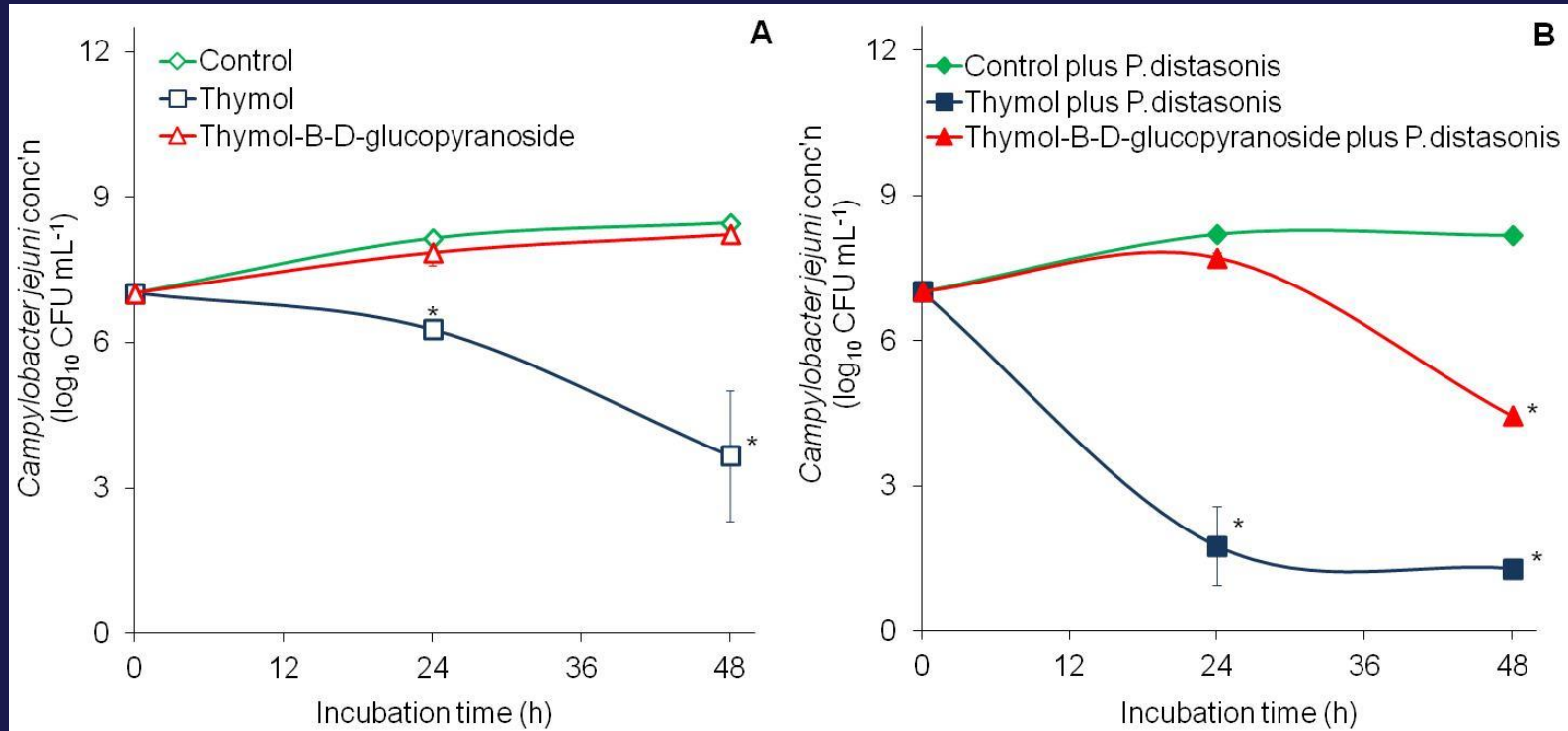


The work in this paper was funded in part by the National Pork Board.

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

Petrujkic et al. 2014. J. Agric. Food Chem.

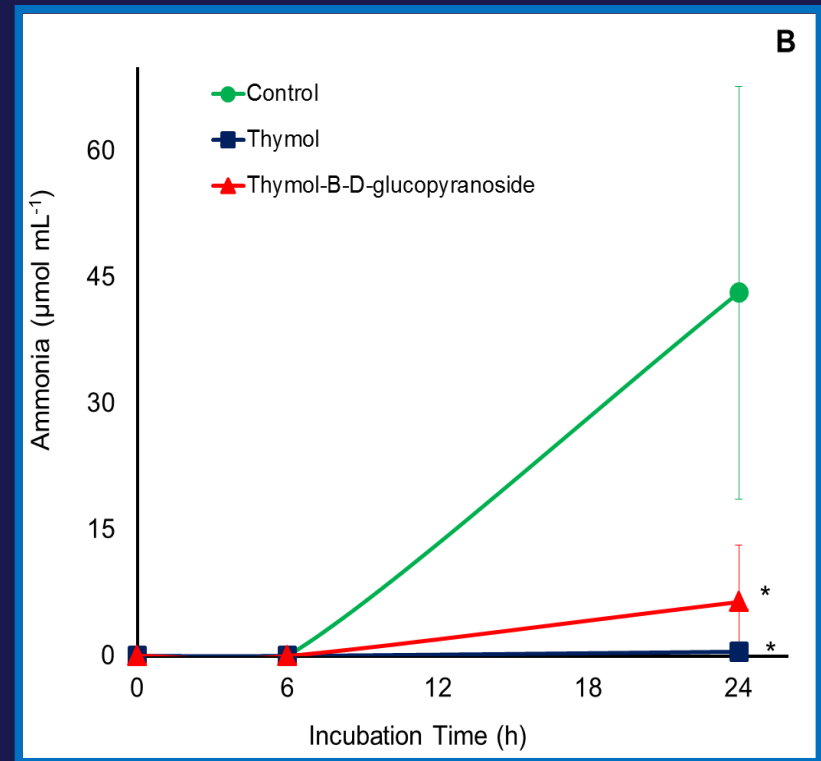
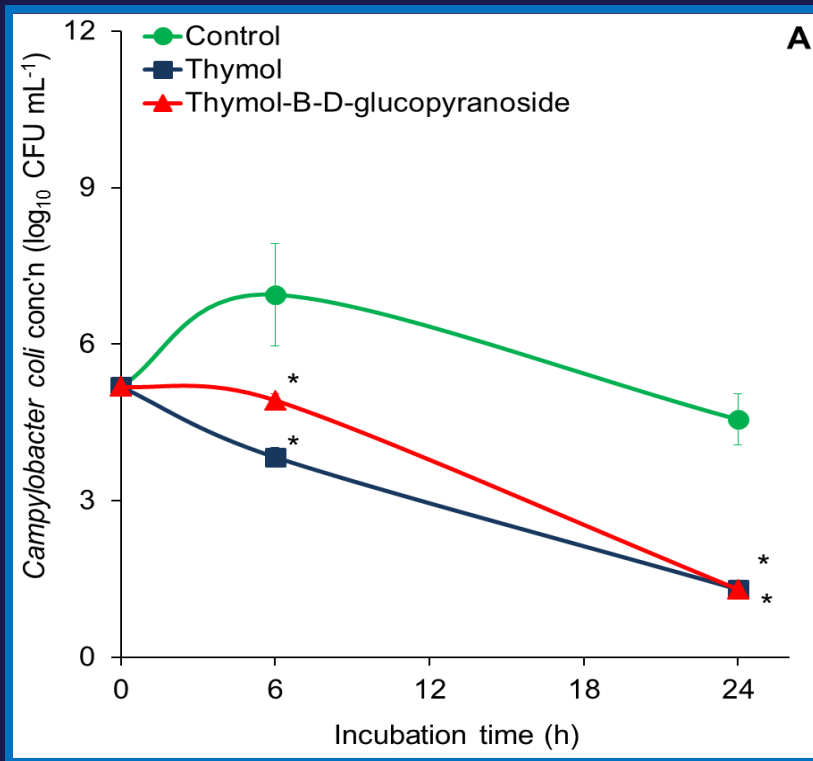
Thymol- β -D-glucopyranoside has little bactericidal activity unless activated by β -glycosidase expressing bacteria



-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.

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

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 ^a (mg/kg live body weight)			P values		
	None	6	12	Linear	Quadratic	SEM
Log ₁₀ CFU/g gut contents						
<i>Salmonella</i> 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
<i>Escherichia coli</i>						
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

^a6 pigs per treatment were twice-treated via oral gavage (approximately 5 hours apart) and euthanized 12 h after receiving last treatment.

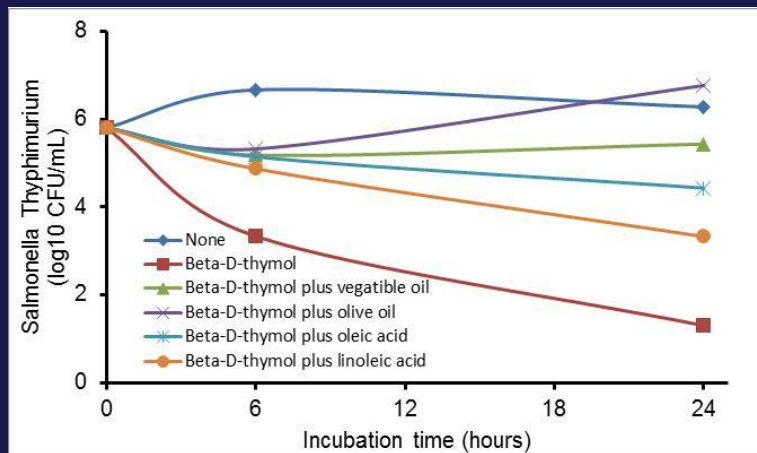
Table 3. Effect of oral beta-D-thymol treatment on gut NN-resistant *Salmonella* Typhimurium and generic *E. coli* and *Campylobacter* species in weaned swine.

	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
<i>Salmonella</i> 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
<i>Escherichia coli</i>									
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
<i>Campylobacter</i> 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.

Investigating three hypotheses as to why the β -D-thymol did not work

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

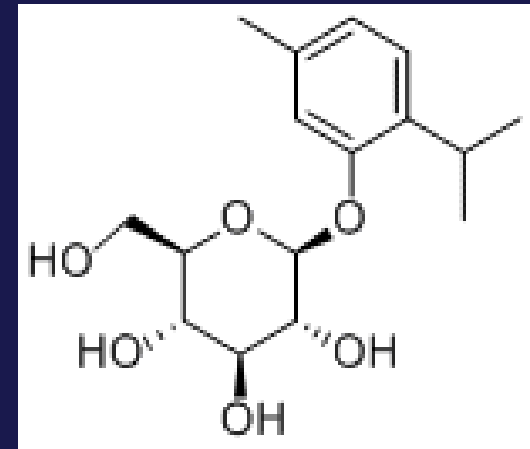


Evidence in support of this hypothesis is that lipids markedly decrease the bactericidal activity of free thymol.

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

Second hypotheses, β -D-thymol may still be too 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

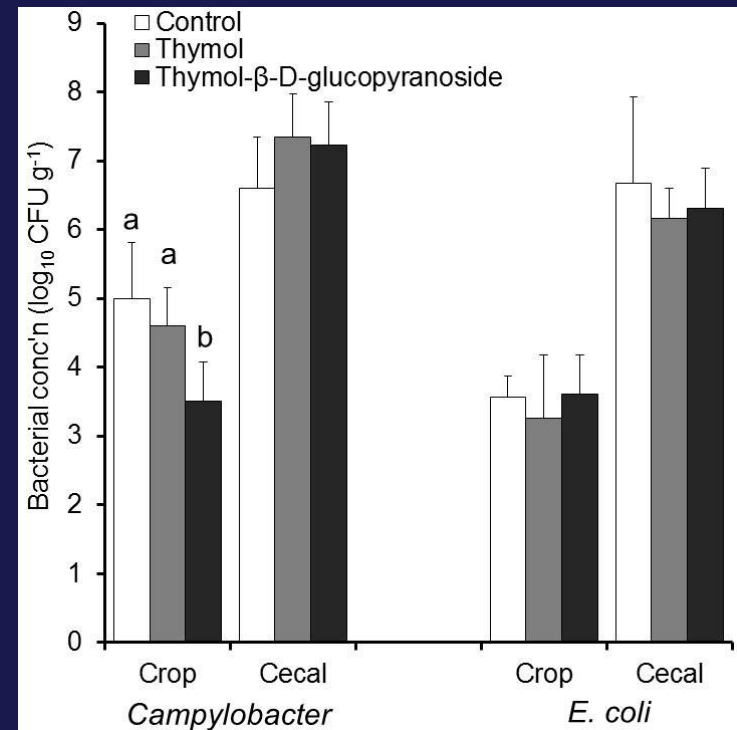


**Thymol- β -D-glucopyranoside
(β -D-thymol)**

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.

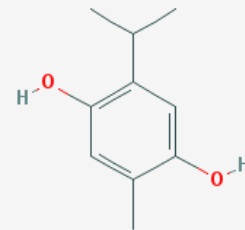


Another approach we are investigating is to use natural sources of thymol as these may contain thymol in naturally-conjugated or protected forms

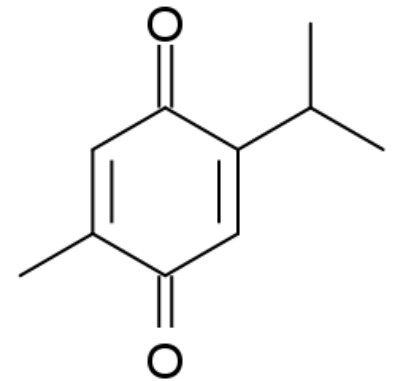
Nigella sativa
Black cumin seed



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



Thymohydroquinone



Thymoquinone

https://en.wikipedia.org/wiki/Nigella_sativa

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!

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Effect of Adding Different Dietary Levels of Black Cumin (*Nigella sativa* L.) Seed on Productive Performance of Laying Hens

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Journal of Ethnopharmacology 141 (1991) 275–278
Elsevier Scientific Publishers Ireland Ltd.

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Studies on the antimicrobial activity of *Nigella sativa* seed (black cumin)

M.S.M. Hanafy and M.E. Hatem

The Journal of Animal & Plant Sciences, 25(2): 2015, Page: 372-379
ISSN: 1018-7081

University, Cairo, Giza (Egypt)

EFFECT OF DIETARY SUPPLEMENTATION OF ACETONE EXTRACTS OF *NIGELLA SATIVA* L. SEEDS ON SERUM CHOLESTEROL AND PATHOGENIC INTESTINAL BACTERIAL COUNT IN BROILERS

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author's Email address: tofazzalislam@yahoo.com

g extract/disc) caused concentration-
m-negative bacteria represented by
c yeast *Candida albicans*. The extract
bacterial action with spectinomycin,
nd sulphamethoxazole-trimethoprim
tion in mice when injected at the site

The Journal of Animal & Plant Sciences, 25(4): 2015, Page: 921-934
ISSN: 1018-7081

Review paper

NUTRITIONAL AND ZOOTECHNICAL ASPECTS OF *NIGELLA SATIVA*: A REVIEW

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ABSTRACT

This review outlines the knowledge on the nutritional and zootechnical aspects of *Nigella sativa* (NS), which is an annual herbaceous plant native to Turkey, Pakistan and Iran. The popularity of this plant is due to its beneficial actions. NS is considered one of the most important medicinal plants in the world. Its seeds have many therapeutic effects, including antimicrobial, anticoccidial and anthelmintic activities, most of which are due to the presence of thymoquinone, which is the major bioactive component. NS seeds are also a significant source of proteins, carbohydrates and fatty acids, and thus could be added as an ingredient to formulate balance rations for farm animals. NS had positive effects on productive and reproductive performances, mortality rate, digestibility, blood chemistry parameters, milk yield and composition, compositional characteristics of eggs and carcass traits.

ABSTRACT

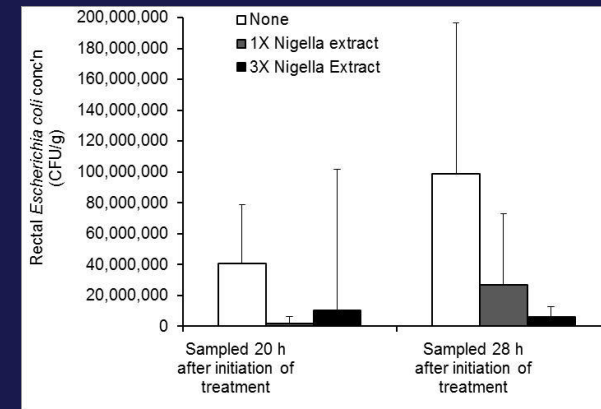
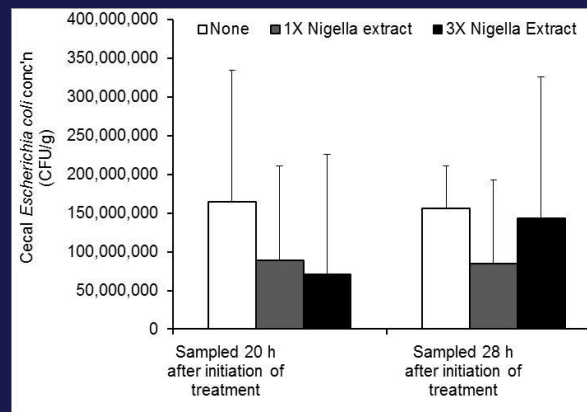
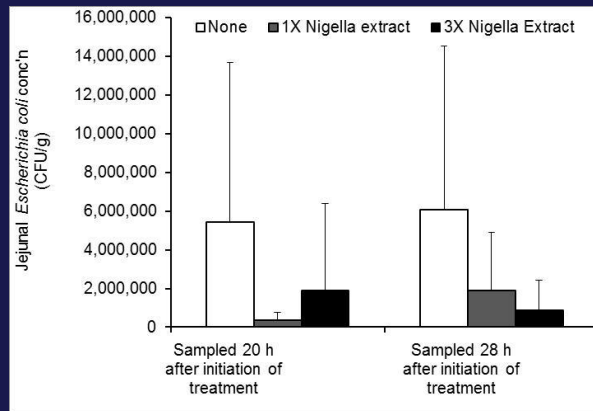
arying doses of *Nigella sativa* seed powder or acetone extracts in diet on feed population of intestinal microflora of broilers. A total 168, day-old broiler ally prepared feeds supplemented with 0, 1.5, 2.5, 3.0% seed powder or 0, ed for 4 weeks. The experiment was conducted in a complete randomized three replications. *N. sativa* supplemented feed had no significant effects on e of broiler. However, supplementation of either 3.0% seed powder or 0.4% (p<0.05) decreased serum cholesterol and triglycerides contents in broiler. nd extract supplemented feed also suppressed harmful bacterial (*Escherichia* suggest that *N. sativa* seed might have potential as an alternative to hazardous ulate low cost and environment-friendly diet for the broiler.

Results from just completed studies in our laboratory

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

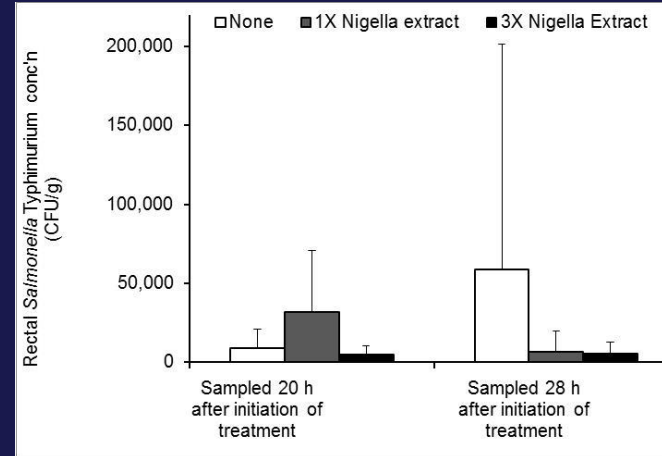
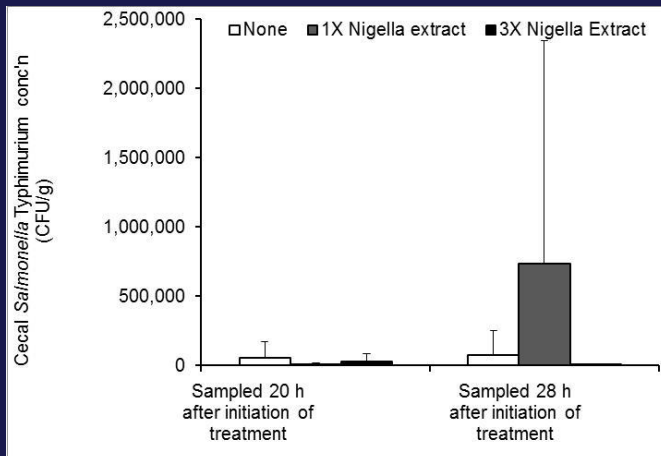
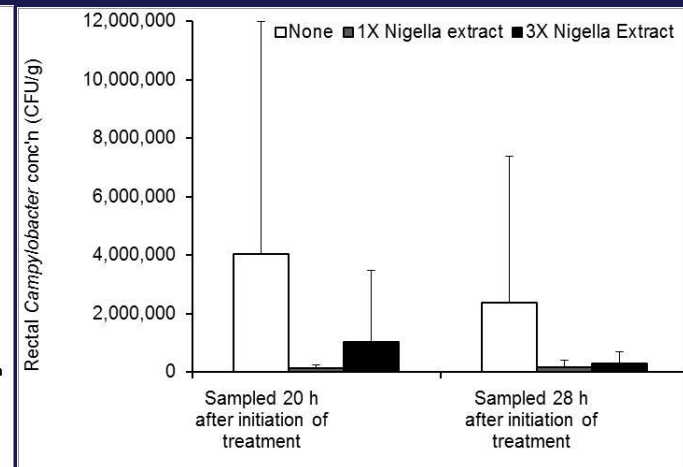
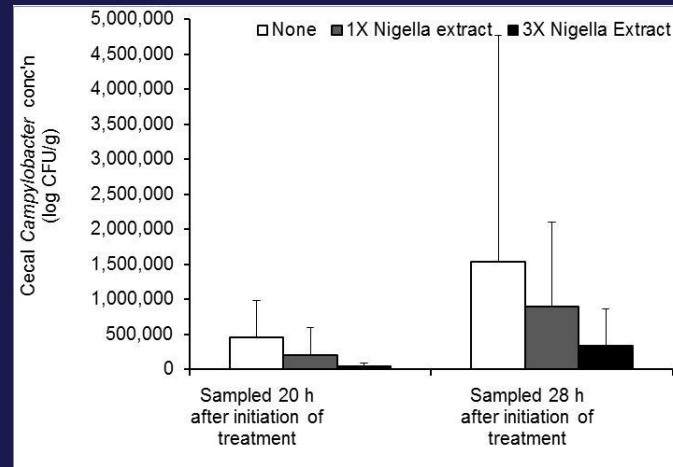
- Higher doses needed for *Salmonella*

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

Evidence for positive effects against *Campylobacter* were also observed



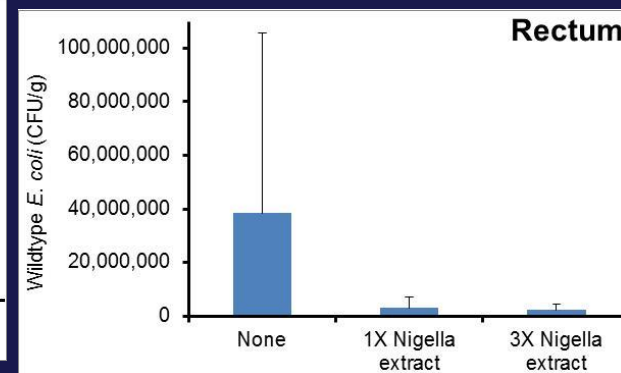
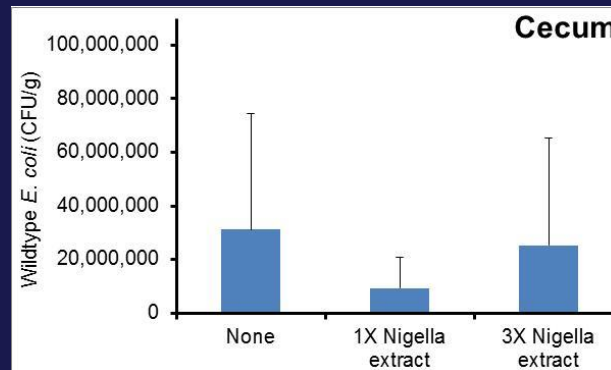
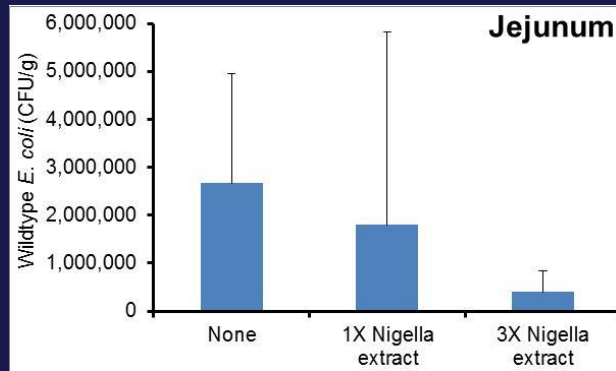
But not against *Salmonella* at the doses tested

1X dose equivalent to 0.15% diet DM

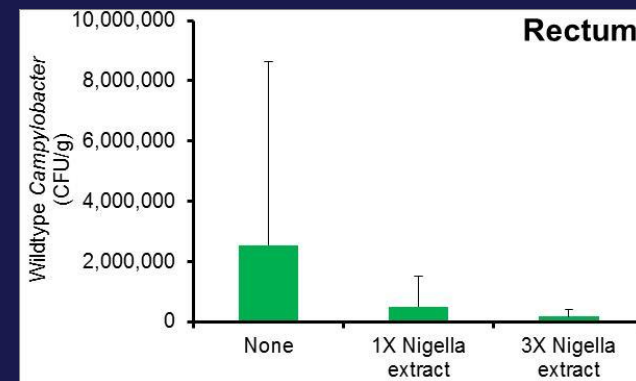
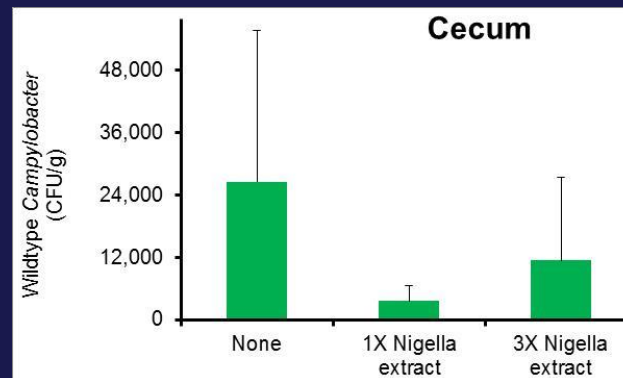
Negative effects against total culturable anaerobes (good fermenting bacteria) were not observed.

Results from a follow up animal feeding study with weaned pigs

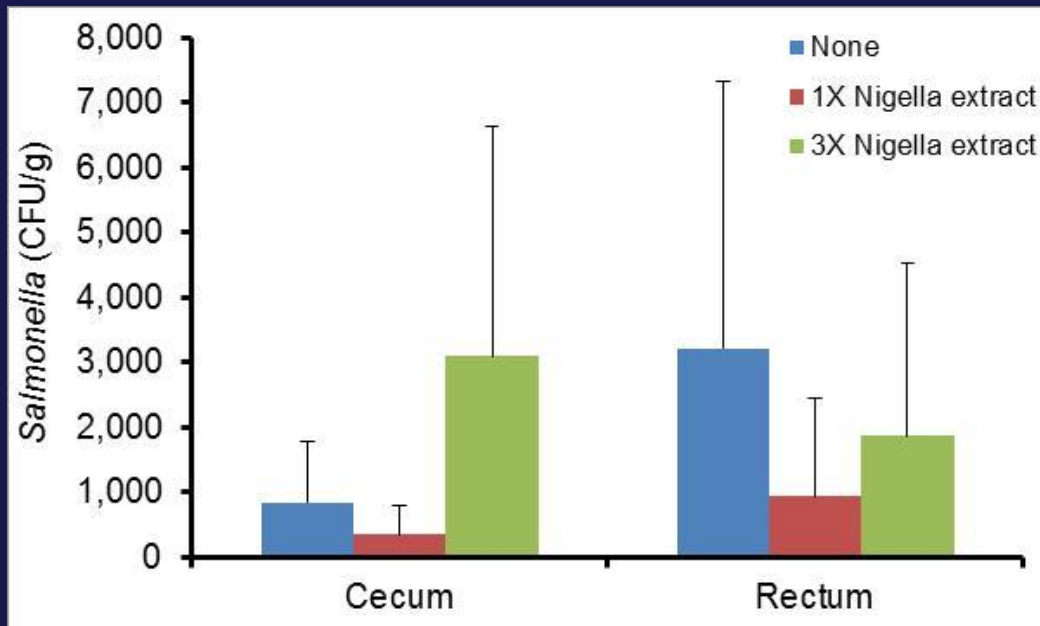
Again showed evidence for a positive treatment effect in reducing gut concentrations of wildtype *E. coli* and *Campylobacter*



1X dose in feeding study
= 0.15% diet DM

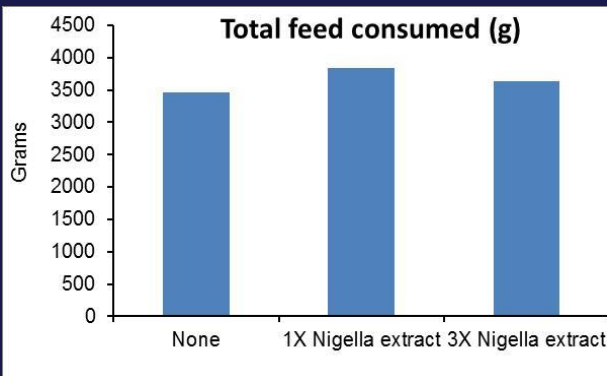


But not against our challenge strain of *Salmonella* Typhimurium

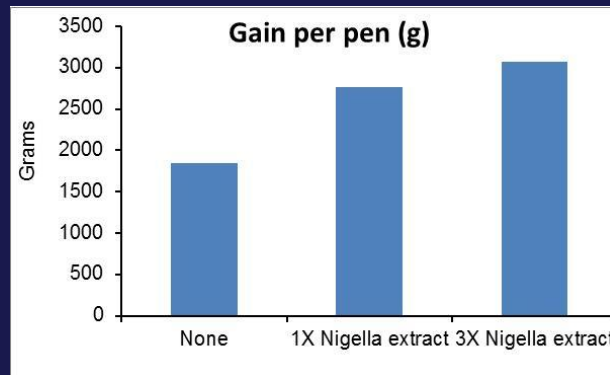


1X dose in feeding study
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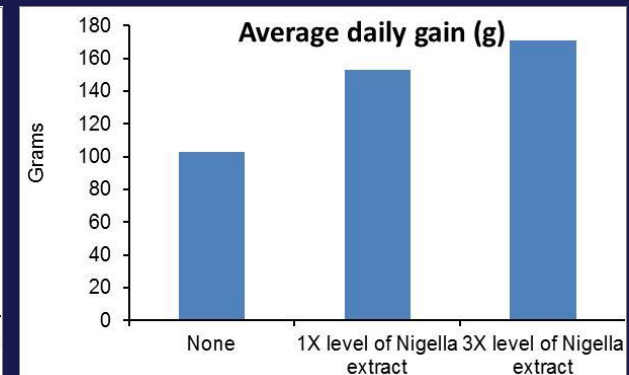
Positive effects of feeding Nigella extract were observed on piglet performance



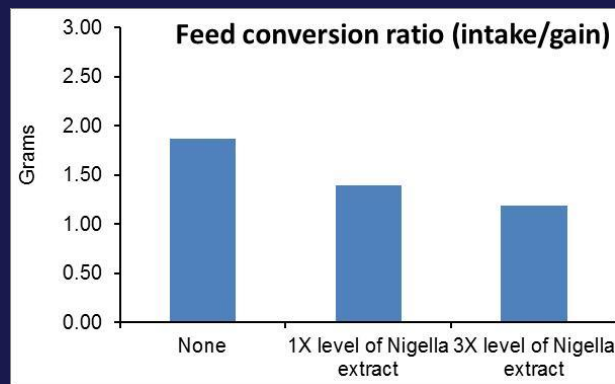
Treated pigs ate slightly more feed



Treated pigs gained more weight



Treated pigs had higher average daily gain



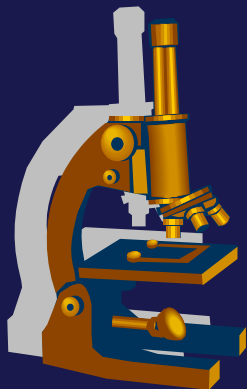
Treated pigs had a better feed conversion

1X dose in feeding study
= 0.15% diet DM

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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