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Gene signature: a novel approach to assess the effects of gut health-promoting substances

Drs. W.E. den Hertog

Supervisors

Dr. J.A. Schrickx
Prof. Dr. J. Fink-Gremmels

Dept. Veterinary Pharmacology, Pharmacy and Toxicology, Faculty of Veterinary Medicine, Utrecht University, The Netherlands

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List of abbreviations

ABCC2: ATP binding Cassette, sub-family C, member 2

B. anthracis: *Bacillus anthracis*

B. cereus: *Bacillus cereus*

B. subtilis: *Bacillus subtilis*

CAT: Catalase

C-FLIP: Cellular FLICE-like Inhibitory Protein

CFSE: Carboxyfluorescein Diacetate Succinimidyl Ester proliferation assay

CFU: Colony-Forming Unit

COX-2: Cyclooxygenase 2

E. coli: *Escherichia coli*

E. faecalis: *Enterococcus faecalis*

EPHX1: Epoxide hydrolase

ETEC: Enterotoxigenic *Escherichia coli*

GCLC: Glutamate-Cysteine Ligase, Catalytic subunit

GPX1: Glutathione Peroxidase 1

HMOX1: Heme oxygenase-1

HPRT: Hypoxanthine phosphoribosyl transferase

H. pylori: *Helicobacter pylori*

HIF1: Hypoxia Inducible Factor

HIF1α: Hypoxia Inducible Factor, α subunit

HSF1: Heat Shock Factor 1

HSP27: Heat Shock Protein 27

HSP70.2: Heat Shock Protein 70.2

IC50: Half maximal inhibitory concentration

IPEC-1: A pig intestinal cell line

IκBα: Nuclear factor of kappa light polypeptide gene enhancer in B-cells, inhibitor α

K. pneumoniae: *Klebsiella pneumoniae*

L. monocytogenes: *Listeria monocytogenes*

MIC: Minimum Inhibitory Concentration

MBC: Minimum Bactericidal Concentration

MTT assay: Cell viability assay

NF-κB: Nuclear factor of kappa light polypeptide gene enhancer in B-cells

NQO1: NAD(P)H quinone oxidoreductase 1

Nrf2: Nuclear factor (erythroid-derived)-like 2

P. vulgaris: *Proteus vulgaris*

S. aureus: *Staphylococcus aureus*

S. cholerasuis: *Salmonella cholerasuis*

SDHA: Succinate dehydrogenase

SOD2: Superoxide dismutase 2

TRX: Thioredoxin 1

XOR: Xanthine oxidoreductase

General introduction

Lately, many public debates take place in the Netherlands and beyond regarding antibiotic use in farm animals. Public concern over the contribution of dietary antibiotics used in animal production to the development of antibiotic-resistant bacteria has increased the demand for alternative approaches to in-feed antibiotics. Interest focusses on the so-called gut health-promoting substances, which comprise special nutrients, organic and anorganic acids, minerals, prebiotics and probiotics, and phytogenic compounds. The latter are frequently subject of *in vitro* assessments with promising results in terms of antimicrobial and antioxidative properties. Unfortunately, these *in vitro* experiments do not reliable predict *in vivo* effects. The objective of this research project was to contribute to standardized feeding experiments conducted with fattening pigs by measuring parameters that are indicative for a biological effect.

This honours program project was embedded in RATIA (mechanisms of toxicity and prevention) and followed a series of farm trials conducted with fattening pigs in the frame of the Fyto-V project in cooperation with the RIKILT in Raalte Practic Centre for Organic Pig Farming[1]. Three different herbal products, all of which contained *Origanum vulgare* essential oil, were used in open feeding trials with treated and non-treated groups from weaning to slaughter. Clinical observations in these farm trials showed positive effects on growth and/or feed conversion as a trend. In order to elucidate the mechanism behind these slightly positive effects, one of the aims of this research project was to review the available information and clinical evidence about the gut-health promoting effects of *Origanum vulgare* as a compound of feed additives in the healthy pig.

[1] The Fyto-V project was initiated on demand of organic farmers and funded by the Dutch Ministry of Agriculture in order to investigate the legislation, availability, effects, and effectiveness of health-promoting herbal products in farm animals. Full reports of all animal trials are available on www.fyto-v.nl.

Initially, before starting this research project, two concepts of how effects might be exerted by herbal products were hypothesized. First, oregano-containing products might stabilize the gut flora and the intestinal barrier by their antimicrobial properties and decrease the amount of toxins and microorganisms that reach the liver. Subsequently, the release of inflammatory mediators by Kupffer cells in response to activation by toxins and microorganisms and thus the expression of genes of pathways involved in oxidative stress and inflammation might decrease. Second, compounds of herbal products might exert direct effects on gene expression levels in the liver after absorption from the gastrointestinal tract. The relative expression levels of genes characteristic for pro-inflammatory reactions and cellular defence mechanisms against oxidative stress in the liver may therefore serve as markers to quantify (gut) health-promoting effects of herbal products in pigs.

To verify these hypotheses, as a 1st step, the expression levels of HMOX1 and XOR had been analyzed in samples of the liver collected from the fattening pigs at a commercial abattoir, using real-time qPCR. Both HMOX1 and XOR, which are induced in response to oxidative stress and by pro-inflammatory mediators, respectively, showed indeed decreased relative expression levels in treated compared to non-treated groups in the pilot experiments. Therefore, another main objective of this research project was the identification - on the basis of available literature - of other candidate genes associated with oxidative and inflammatory stress mechanisms, which may serve to quantify the (subclinical) effects of (gut) health-promoting substances in healthy animals.

The final aim of the research program was the measurement of relative expression levels of selected candidate genes by means of real-time qPCR, in order to evaluate the direct and indirect effects on the liver of the 3 tested herbal products in pigs, as well as the suitability of the chosen approach as an addition to traditional test strategies for herbal products.

PART 1

Review

Gut health-promoting effects of *Origanum vulgare* as a compound of feed additives in the healthy pig

Abstract

Feed additives containing *Origanum vulgare* are often used in pig husbandry as an alternative approach to in-feed antibiotics. *Origanum vulgare* and its main compounds carvacrol and thymol are thought to affect gut health on account of their antimicrobial activities, which cause a subtle shift in the divers and complex microflora composition by changes in the metabolic activity and/or amount of one or more bacterial species. MBC values for *Origanum vulgare* essential oil, carvacrol, and thymol, determined by using broth and agar dilution methods, range between 0.02-0.5, 0.1-0.5, and 0.1-5.0 mg/ml, respectively. In simulations of gut-conditions, antimicrobial activity was seen for these substances in concentrations ranging from 0.005-0.04, 0.2-1, and 0.2-1 mg/ml, respectively. Concentration that where shown to be toxic on γd T cells and intestinal cells were much higher than the concentration needed for an antibiotic effect, suggesting that at MBC concentration ranges cell proliferation and gut barrier function are not affected. Despite possible alterations of the antimicrobial activity of *Origanum vulgare*, carvacrol, and thymol by ingredients of pig diets, and variable physiologic conditions in the gut of pigs, antimicrobial activity has been denoted *in vivo*.

1. Introduction

Public concern over the contribution of dietary antibiotics used in animal food production to the development of antibiotic-resistant bacteria has increased the demand for alternative approaches to in-feed antibiotics. Interest focuses on the so-called gut health-promoting substances, which comprise special nutrients, organic and anorganic acids, minerals, prebiotics and probiotics, and phytogenic compounds. The latter include *Origanum vulgare* (oregano) essential oil and its main compounds carvacrol and thymol, which are often used as feed additives on account of their antimicrobial activity, and several non-substantial claims have been made for their effectiveness. Strategic addition of 1000 ppm oregano to diets of sows under field conditions has been claimed to improve reproductive performance of sows in terms of increased farrowing rate, increased number of liveborn piglets per litter, decreased stillbirth rate, and lower sow culling rate during lactation [40]. The authors speculated that these effects might be due to gut-health promoting effects of oregano, since stabilization of the gut microflora, diminishment of populations of undesirable microorganisms, and increase of feed digestibility might improve general health. However, there is a lack of knowledge in effective concentrations and mechanisms of action regarding antimicrobial and other gut health-promoting effects.

Gut health as such is difficult to define and is characterized by a complex network of functions involved in physiology, microbiology and immunology. It is recognized that microbial fermentation within the gastrointestinal tract is important for the pig, due to its role in water and sodium absorption, pH control, gut motility, improvement of dietary energy yield, vitamin production, stimulation of gut immunity and inhibition of pathogen adhesion (also referred to as colonization resistance) [1]. The term immune-competence is defined as the ability to generate tolerance to food and commensal bacterial antigens as well as active immune responses to pathogens. Immune-competence affects several aspects of gut health, since events involved in an early immune response are known to

influence intestinal epithelial permeability and ion transport [2]. Gastrointestinal motility, secretion of mucin, the intestinal epithelial barrier and the commensal microbiota contribute to the intestinal first-line of defence [3].

The aim of this review is to assemble all the peer-reviewed data available in PubMed and Scopus databases on *in vitro*, *ex vivo* and *in vivo* experiments conducted with *Origanum vulgare*, carvacrol and thymol on bacterial strains of porcine origin, pig tissue and pigs themselves. By doing so, an overview of the antimicrobial activity, other effects on gut-related pig tissue, effective concentrations, and evidence of *in vivo* effectiveness can be achieved. The review focuses on all subspecies of *Origanum vulgare*, since the amount of data would otherwise be very limited.

2. Oregano

Origanum vulgare is a species of *Origanum*, belonging to the Lamiacae familiy, and mainly native to the Mediterranean area. Since ancient times, oregano has been used as culinary herb and for food preservation [4]. Yield and composition of essential oils obtained from different *Origanum vulgare* plants are highly variable depending on their source. In order to illustrate this, some published analyses on yield and composition of oregano essential oils are assembled in Table 1, together with details about the geographical origin of the plant, which part of the plant the essential oil is obtained from, and the used distillation method. In several published data, the monoterpenes carvacrol and thymol and their precursors *p*-Cymene and γ-Terpinene have been reported to be the principle components of *Origanum vulgare* essential oil. Therefore, the individual as well as the sum of amounts of carvacrol, thymol, and their precursors *p*-Cymene and γ-Terpinene obtained in different studies have been listed in Table 1.

Table 1 Source, yield and composition of *Origanum vulgare* essential oils determined in different studies

Source[a]			**EO** (%)[ab]	**Chemical composition** (%)[a]					**Ref.**
Geography	Plant material	Method[c]		Carvacrol	Thymol	γ-Terpinene	*p*-Cymene	Sum of amounts[d]	
Montenegro	Aerial parts	H	1.45	61.3	13.9	3.1	0.5	78.8	4
-	-	S	-	51.83	0.71	10.75	10.98	74.27	9
Ouled Slama, Algeria	Leaves	H	2.50	2.9	31.6	11.5	23	69	10
Souhane, Algeria	Leaves	H	3.20	1.1	23.9	23.9	17.2	66.1	10
Ighzer mokrane, Algeria	Leaves	H	1.80	8	36	16.5	16.8	77.3	10
Industrial	-	H	-	57.71	3.83	7.18	10.91	79.63	11
Industrial	-	S	-	61.21	0.48	4.80	15.12	78.61	12
Industrial	-	-	-	75.71		2.08		77.79	13
Mediterranean natural sites	-	H	0.3 2.8	1.0 3.7	0.6 0.4	0.8 0.2	2.3 0.2	4.7 4.5	14 14
Kozani, continetal Greece	Stems, leaves and flowers	S	3.3	88.71	-	3.18	3.43	96.32	15
Oltu vally, Ezurum, Turkey	Aerial parts	H	2.31	0.60	0.8	0.3	0.7	2.4	16
Euboea, Greece	Aerial parts	H	-	79.58	2.45	2.07	8.76	92.86	17
Industrial	-	-	-	0.43	31.8	1.32	40.15	73.7	17
Val Mazara, Sicilia Val Demona, Sicilia Val di Noto, Sicilia	Aerial parts	H	 3.2 5.4 3.2	 0.20 1.30 0.70	 21.2 48.9 63.4	 16.2 19.7 9.3	 5.2 7.1 7.7	 42.8 77.0 81.1	18
Greenhouse, Germany	Leaves and inflorescences	H	-	77.4 74.9 70.0	0.3 3.7 0.3	8.1 8.2 9.5	5.3 4.5 4.8	91.1 91.3 84.6	19
Averaged content and chemical composition			0.3-5.4	0.20-88.71	0.3-48.9	0.3-23.90	0.2-40.15	2.4-96.32	

[a]- indicates that no data are available.
[b]EO: essential oil; In the references essential oil contents have been reported in ml per varying weight of plant material. For the ease of comparison these haven been converted into ml per 100 g.
[c]H: hydrodistillation, S: steamdistillation.
[d]Sum of amounts: sum of amounts of carvacrol, thymol, γ-Terpinene, and p-Cymene.

3. *In vitro* and *ex vivo* experiments

Results of in vitro and ex vivo experiments conducted with *Origanum vulgare*, carvacrol and thymol, are compiled in Table 2 and 3A. Concentrations of *Origanum vulgare* essential oil, carvacrol and thymol with antimicrobial activity *in vitro* determined by broth and agar dilution methods are listed in Table 2. Table 3A gives an overview of *in vitro*

and *ex vivo* experiments on effects of *Origanum vulgare*, carvacrol and thymol on parameters related to gut health in pigs. Data of both tables are discussed in more detail below.

3.1 Antimicrobial activity

There are notable differences between studies that assess antimicrobial properties of natural compounds of plant origin [5]. Factors like origin of essential oil or its components, source of bacterial isolates, size of inoculum, growth phase, culture medium used, pH of media, incubation time and temperature, choice of emulsifier and basic test method show great variation [6]. Comparison of published data is thus complicated. Furthermore, the terms used to denote antibacterial activity and their definitions differ between publications. The disk diffusion method and the agar well test are useful for screening of essential oils and their compounds for antibacterial activity, but comparison of published data is not achievable, due to variations between studies regarding volume of EO placed on paper disks, thickness of agar layer, and whether a solvent is used. To quantify antibacterial activity, the agar dilution and broth dilution methods are the most suitable methods [6]. Concentrations of *Origanum vulgare* essential oil, carvacrol, and thymol with antimicrobial activity *in vitro* determined by broth and agar dilution methods have been listed in Table 2.

MIC values (not defined) for *Origanum vulgare* essential oil, carvacrol, and thymol, determined by using broth and agar dilution methods, range between 0.00007-0.7, 0.225-2.8, and 0.15-2.5 mg/ml, respectively. MBC values range between 0.02-0.5, 0.1-0.5, and 0.1-5.0 mg/ml, respectively. These concentration ranges indicate synergism between carvacrol, thymol, and other components of *Origanum vulgare* essential oil, since MIC and MBC values of carvacrol and thymol are lower than those of the complete *Origanum vulgare* essential oil. Several authors described more specific synergistic effects between carvacrol and thymol [7], [8].

Table 2 Concentrations of *Origanum vulgare* essential oil, carvacrol and thymol with antimicrobial activity *in vitro* determined by broth and agar dilution methods

Compound	**Antimicrobial activity**[a]				**Bacterial species**		**Ref.**
	MIC (ml^{-1})	MIC (mg/ml)	MBC (ml^{-1})	MBC (mg/ml)	Gram-	Gram+	
Origanum vulgare essential oil			0.25 mg	0.25		*S. aureus*	[20]
	0.70 mg	0.7				*S. aureus*	[21]
			0.5 mg	0.5		*S. aureus*	[20]
	0.06 mg	0.06				*S. aureus*	[16]
	0.005 ml	0.005			*S. cholerasuis*		[13]
	0.01 ml	0.01			*S. typhimurium*		[13]
	0.04 ml	0.04			*E. coli*		[13]
	0.70 mg	0.70			*E.coli*		[21]
	0.5–0.7 mg	0.5-0.7			*E. coli*		[22]
			0.5 mg	0.5	*E. coli*		[20]
	0.018 ml	0.018	0.02 ml	0.02	*E. coli*		[23]
	0.03 mg	0.03			*E. coli*		[16]
	0.35 mg	0.35				*B. subtilis*	[21]
	0.03–0.06 mg	0.03-0.06				*B. subtilis*	[16]
	0.25 mg	0.25				*B. anthracis*	[20]
			0.5 mg	0.5	*K. pneumoniae*		[20]
			0.5 mg	0.5	*H. pylori*		[20]
	0.07 µl	0.00007				*L. monocytogenes*	[24]
	0.06 mg	0.06				*E. faecalis*	[16]
	0.06 mg	0.06			*P. vulgaris*		[16]
Carvacrol	0.70 mg	0.70				*B. cereus*	[21]
			0.5 mg	0.5		*S. aureus*	[20]
	0.70 mg	0.70				*S. aureus*	[21]
	0.4 mg	0.4			*E. coli*		[7]
	1.4 mg	1.4			*E. coli*		[21]
	1.5 µM	0.225			*E. coli*		[25]
	0.35 mg	0.35				*B. subtilis*	[21]
	2.8 mg	2.8				*B. cereus*	[21]
			0.1-0.283 mg	0.1-0.283	*E. coli*		[26]
			0.167 mg	0.167	*S. typhimurium*		[26]
			0.255 mg	0.255	Anaerobic bacteria		[8]
Thymol	1.0 µM	0.150			*S. typhimurium*		[25]
			0.233 mg	0.233	*S. typhimurium*		[26]
	2.5 mg	2.5	5.0 mg	5.0		*Streptococcus*	[27]
	0.4 mg	0.4			*E. coli*		[7]
	1.4 mg	1.4			*E. coli*		[21]
	1.2 µM	0.180			*E. coli*		[25]
			0.1-0.166 mg	0.1-0.166	*E. coli*		[26]
	0.70 mg	0.70				*B. subtilis*	[21]
	0.70 mg	0.70				*B. cereus*	[21]
	0.70 mg	0.70				*S. aureus*	[21]
			0.258 mg	0.258			[8]

[a]MIC: minimum inhibitory concentration,MBC: minimum bactericidal concentration
In the references MIC and MBC have been reported in varying units. For the ease of comparison these haven been converted to mg/ml. Ranges indicate different values for different strains of the same bacterial species in one study.
[b]C: carvacrol, T: thymol

Selective antimicrobial activity of carvacrol and thymol against E. coli and coliform bacteria, with little effect on lactobacilli and anaerobic bacteria, has been demonstrated [26]. This antibacterial activity of carvacrol and thymol appears to be assocoiated with their membrane permeabiliser activity [28], [6].

3.2. *In vitro* simulations of the pig gut

In order to study the degradation of compounds of essential oils in the gastrointestinal tract, Michiels et al. (2008) performed incubations with porcine gastric, jejunal and caecal contents with 100 and 1000 mg/L carvacrol and thymol. In both concentrations, carvacrol and thymol were not degraded in gastric and jejunal simulations, but significant losses up to 29% were found in caecal simulations.

Si et al. (2006) examined the efficacy of antimicrobial activity of carvacrol and thymol at low pH conditions. Incubation of these compounds in a pH 2.0 medium at 37°C for 4 h did not significantly alter the efficacy of antimicrobial activity against *E. coli* and *S. typhimurium*. The effect of pig caecal digesta on the antimicrobial activity of carvacrol and thymol was also examined. Carvacrol and thymol retained their efficacy against indigenous E. coli, coliform bacteria, and against introduced E. coli O157:H7.

Michiels et al. (2009) characterized the antimicrobial activity of carvacrol, thymol, and a mixture of both against a mixed microbial gut population by means of *in vitro* batch incubations simulating the fermentation in different sections of the pig gastrointestinal tract. The minimum concentration for carvacrol and thymol in jejunal simulations to reduce the number of total anaerobic bacteria compared to control with a propability of 99.7% was 255 and 258 mg/L, respectively. The combinations 250 mg/L carvacrol + 250 mg/L thymol and 400 mg/L carvacrol + 100 mg/L thymol were found to act synergistic against total anaerobic bacteria in jejunal simulations, however this effect was rather

small. On the contrary, the combination 100 mg/L carvacrol + 400 mg/L thymol was found to have an antagonistic effect against both coliform bacteria and E. coli. From former research, it was concluded that a concentration that gives 1 log10 CFU/mL reduction in this type of in vitro batch incubations could result in appreciable reductions of the gut flora in vivo. Michiels et al. (2009) calculated that this would equal a dose of approximately 400 mg/L of carvacrol or thymol to reduce coliform bacteria in the small intestine and 400-550 mg/L to reduce lactobacilli (-0.5 log10 CFU/mL in vitro) in the proximal gut.

3.3. Effects on gut-related pig tissue *in vitro* and *ex vivo*

Bimczok et al. (2007) studied the effects of carvacrol on porcine intestinal epithelial cells, since these cells would be the first to be exposed to carvacrol after oral application. Up to 125 µM carvacrol did not alter proliferation and metabolism of IPEC-1 cells in an MTT assay. The concentration of carvacrol found to be toxic on IPEC-1 cells (IC_{50} 525 ± 83 µM) was almost 10-fold lower than the concentration that was previously shown to disrupt epithelial barrier function in IPEC-1 cells (>5mM) [30]. In the latter study, the ability of carvacrol to exert a protective effect against ETEC-induced membrane damage in pig intestinal IPEC-1 cells was evaluated. After verification of the possible toxic effect of carvacrol on cell membrane permeability, the highest non-toxic concentration was tested by assessing the transepithelial electrical resistance and the paracellular flux of the extracellular marker phenol red. The concentration of 5 mM carvacrol did not exert any protective activity against increased membrane damage in pig intestinal cells caused by ETEC.

In addition, Bimczok et al. (2008) determined toxic levels of carvacrol on porcine γδ T cells, since it is likely that dietary phenolic compounds will cross the intestinal barrier and come into contact with immune cells. γδ T cells are thought to be involved in the

Table 3A *In vitro* and *ex vivo* experiments on effects of *Origanum vulgare*, carvacrol and thymol on parameters related to gut health in pigs

Compound	**Dose**	**(mg/ml)**	**Effect**	**Gut health[a]**	**Reference**
Origanum vulgare	0.005, 0.01 and 0.04 ml	0.005, 0.01 and 0.04	MIC against pig gastrointestinal bacteria (S. cholerasuis, S. typhimurium and ETEC, respectively)	+	[13]
Thymol	1000 mg/L	1	Significant differences in bacterial counts in jejunal and caecal simulations	+	[8]
Thymol	100 and 1000 mg/L	0.1 and 1	No degradation in jejunal simulations; significant loses in caecal simulations	±	[31]
Thymol	200 and 300 μg/ml	0.2 and 0.3	Significant inhibition when mixed with pig caecal digesta against E.coli and coliform bacteria, but little effect on total number of lactobacilli and anaerobic bacteria	+	[26]
Thymol	50 μM	7.5	Induction Cl^- and HCO_3^- secretion via activation of nervous nicotinic receptors	±	[32]
Thymol	100 μM	15	No effect on number of adherent bacteria in jejunal tissue	±	[32]
Carvacrol	1000 mg/L	1	Significant differences in bacterial counts in jejunal and caecal simulations	+	[8]
Carvacrol	5 mM	751	No protective activity against increased membrane permeability in pig intestinal cells caused by ETEC; not toxic on intestinal cells	±	[30]
Carvacrol	X – 500 μM	75	No toxic effects on γδ T cells in terms of proliferation	±	[29]
Carvacrol	200 and 300 μg/ml	0.2 and 0.3	Significant inhibition when mixed with pig caecal digesta against E.coli and coliform bacteria, but little effect on total number of lactobacilli and anaerobic bacteria	+	[26]
Carvacrol	125 μM	18.8	No alterations in proliferation and metabolism of intestinal cells	±	[29]
Carvacrol	525 ± 83 μM	78.9	Toxic (IC_{50}) on intestinal cells	-	[29]
Carvacrol	100 and 1000 mg/L	0.1 and 1	No degradation in jejunal simulations; significant loses in caecal simulations	±	[31]

[a] + indicates beneficial effect on gut health, - indicates negative effect on gut health, and ± indicates no benefical or negative effect on gut health.
In the references doses have been reported in varying units. For the ease of comparison these haven been converted to mg/ml. For details, please see text.

maintenance of the mucosal barrier and are found both in the epithelial layer and the underlying lamina propria in the porcine intestinal tract. Toxicity levels of carvacrol were assessed by using MTT and CFSE assays. Some proliferation of γδ T cells was still observed at 500 μM carvacrol.

Boudry and Perrier (2008) studied the effect of thymol on electrolyte secretion and absorption across the porcine jejunal epithelium in Ussing chambers. At a concentration of 50 μM, thymol showed to induce Cl- and HCO3- secretion via activation of nervous nicotinic receptors. Since electrolyte and water secretion by the intestinal mucosa is classically described as a means of defence to flush out pathogens and toxins, Boudry and Perrier (2008) tried to investigate if thymol-induced secretion had any effect on bacterial adhesion. Incubation of jejunal tissue with 100 μM thymol had no significant effect on the number of adherent bacteria compared to control conditions.

4. *In vivo* experiments

Several animal trials with feed additives containing a mixture of essential oils with amongst others oregano or its compounds have been published [33], [34], [35], [36], [37], [38], [39]. However, very few animal trials have been conducted with feed additives containing no other bioactive ingredients next to oregano. Used doses of oregano or its compounds in these trials and their observed effects regarding gut health are listed in Table 3B.

A pharmacokinetic study with the monoterpenes carvacrol and thymol revealed rapid absorption and excretion of both compounds. A single dose mixed with feed (13.0 and 13.2 mg/kg body weight for carvacrol and thymol, respectively) was given orally to piglets. Both monoterpenes were mainly and almost completely absorbed in the stomach and the proximal small intestine. Plasma concentrations (sum of free and conjugated compound) peaked at 1.39 and 1.35 h for carvacrol and thymol, respectively, and were accompanied by high concentrations in urine. The concentrations found in bile for specimens t = 0.5 - 6 h and 24 h ranged between 1.40 and 6.93 mg/L. The relative

amount for carvacrol and thymol found in the proximal small intestine compared to the total digestive tract nearly equaled estimated percentages of intake recovered in bile. The authors stated thus that enterohepatic recycling for carvacrol and thymol in piglets as such cannot be neglected, but more detailed studies are necessary to elucidate this phenomenon and its precise contribution to the kinetics in the gut. Taken together, one could assume that negligible concentrations of these compounds reach the intestinal sites where antimicrobial activity is desired.

Since relatively high concentrations of essential oils appear to be required for displaying beneficial effects in pigs, Si et al. (2006) hypothesized that essential oils and their components bind to fat and other hydrophobic materials in pig diets, thus becoming unavailable to target pathogenic bacteria. Indeed, they were able to demonstrate in *in vitro* assays containing 0.08% carvacrol or thymol, that pig diets are able to alter their antimicrobial activity.

A pharmacodynamic study revealed a slight effect of dietary thymol on pig jejunal microbial population. Weaned pigs were fed a diet without or with 1% thymol and remained unchallenged or were challenged with *Salmonella typhimurium*. Thymol supplementation did not affect fecal excretion of *S. typhimurium* [41]. Even though richness and diversity of the dominating bacteria were not affected, there was a visible shift in the

Table 3B *In vivo* effects of feed additives containing no other bioactive ingredients next to *Origanum vulgare*, carvacrol and thymol on parameters related to gut health in pigs.

Compound	Dose	Effect	Gut health[a]	Reference
Origanum vulgare	1000 ppm	Improved daily voluntary feed intake	±	[40]
Thymol	1%	Decreased daily voluntary feed intake	±	[41]
Thymol	1%	Shift in bacterial community	+	[42]
Thymol	1%	Fecal excretion of S. typhimurium after challenge was not changed	±	[41]

[a] + indicates beneficial effect on gut health, - indicates negative effect on gut health, and ± indicates no benefical or negative effect on gut health. For details, please see text.

bacterial community composition between thymol-treated and untreated groups. *Citrobacter freundii*, an enterobacterium that yet has not been found to be pathogenic in pigs, was more prevalent in pigs from groups fed thymol. By contrast, *Actinobacillus minor*, a low pathogenic Gram-negative rod, was reduced in those groups. Challenge with *S. typhimurium* had no major effects on pig intestinal microflora composition [42].

Regarding palatability, Jugl-Chizzola et al. (2006) reported reduced daily feed intake in pigs fed 1% thymol before and after *Salmonella typhimurium* challenge. By contrast, Allan and Bilkei (2005) found that average voluntary daily feed intake was similar for oregano-supplemented and control primiparous sows, but was higher in oregano-supplemented versus control multiparous sows.

6. Conclusion

Origanum vulgare and its main compounds carvacrol and thymol are often used as gut health-promoting feed additives on account of their antimicrobial activity. The gut flora plays an important role in maintaining gut health through diversity, stability, metabolites and cross-talk with the epithelium and the underlying immune system [43]. Improved gut health might be achieved through a subtle shift in the divers and complex microflora composition by changes in the metabolic activity and/or amount of one or more bacterial species. MBC values for *Origanum vulgare* essential oil, carvacrol, and thymol, determined by using broth and agar dilution methods, range between 0.02-0.5, 0.1-0.5, and 0.1-5.0 mg/ml, respectively. In simulations of gut-conditions, antimicrobial activity was seen for these substances in concentrations ranging from 0.005-0.04, 0.2-1, and 0.2-1 mg/ml, respectively. Concentration that where shown to be toxic on γd T cells and intestinal cells were much higher than before mentioned concentration, suggesting that at MBC concentration ranges cell proliferation and gut barrier function are not affected.

Despite possible alterations of the antimicrobial activity of *Origanum vulgare*, carvacrol, and thymol by ingredients of pig diets, and variable physiologic conditions in the gut of pigs, antimicrobial activity has been denoted *in vivo*. However, more studies on pharmacokinetics are necessary to determine, whether concentrations that have been shown to be safe and effective *in vitro* can be reached under *in vivo* conditions. Moreover, other biological effects such as the influence of *Origanum vulgare*, carvacrol, and thymol on intestinal cell turn-over, and possible post-absorptive systemic effects need to be investigated.

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PART 2

Research article

Gene signature: a novel approach to assess the effects of gut health-promoting substances

Abstract

The term gene signature refers to the analysis of a gene activity pattern, characteristic for a specific disease, measured as the amount of mRNA produced by each of these genes. In this study, the gene signature concept has been adapted to assess health-promoting effects of 3 different herbal products in pigs. The selected products had been developed by industry to be used as feed additives with the aim to improve gut health and to reduce the effect of anti-nutrients in animal feed materials. The gene signature approach focused on genes involved in oxidative stress and inflammation. Expression levels were analyzed in liver tissue samples using quantitative real-time PCR. For several molecular markers indicative for oxidative stress and inflammation, among others HO-1, XOR, IKBA and COX-2, expression levels were found to be significantly down-regulated in pigs of the experimental groups fed herbal products, compared to control groups, indicating indeed a reduction of cellular oxidative stress. These results correlated with the measured zootechnic parameters and hence suggest that the chosen approach might be a valuable addition to traditional test strategies for herbal products.

1 Introduction

Gut health has been an issue of great interest in human as well as in veterinary medicine, as demonstrated by the large number of articles and reviews (>2000) published on this topic in the last decennium. Food and feed industry endorsed this interest identifying the large market that emerges for gut health-promoting substances in Europe and beyond [1]. Public concern regarding the contribution of dietary antibiotic used in animal production to the development of antibiotic-resistant bacteria has increased the demand for alternative approaches to in-feed antibiotics. For pig husbandry, these products include special nutrients, organic and anorganic acids, minerals, growth factors, prebiotics and probiotics, and phytogenic compounds.

Although improved gut health is difficult to define and assess [2], the positive influence of gut health-promoting substances on non-intestinal sites and overall health has been observed repeatedly [3]. Of particular interest is their influence on the liver, as intestinal and liver functions are highly related. The liver serves as a 'filter' for ingested toxins and micro-organisms that passed the intestinal first-line of defence and helps to prevent the dissemination of these agents throughout the body. Kupffer cells, the resident macrophages of the liver, are the first cells exposed to gut-derived particulate materials and soluble bacterial products. Once activated by these substances, Kupffer cells release inflammatory mediators, like reactive oxygen species and cytokines [4]. High exposure of Kupffer cells to gut-derived products can lead to the intensive production of inflammatory mediators that activate in hepatocytes several pathways [5], which are involved in oxidative stress and inflammation, and finally lead to liver injury [4]. This response can be measured by the analysis of biological markers. Hence herbal products, often exerting a mild antibiotic effect, are believed to stabilize the intestinal flora and in turn reduce the exposure of the liver to toxic substances [2]. Compounds of herbal products might also exert direct effects on the liver after absorption from the gastrointestinal tract. A reduced challenge of liver cells should be reflected in an altered pattern of genes induced by cellular stressor and genes involved in cellular defence mechanisms, and these are

therefore candidates to quantify improved gut health in the liver.

Microarray-based genome wide expression analysis can be used to identify changed expression levels of genes under experimental conditions. However, since no microarrays are commercially available for pigs, the gene signature approach has been adapted in this study to assess health-promoting effects of herbal products in pigs. The term gene signature refers to the analysis of a gene activity pattern, characteristic for a specific disease or (patho)physiological conditions, measured as the amount of mRNA produced by each of these genes. Gene signatures have been developed in veterinary and human medicine to improve the understanding of the biology of diseases, and to identify novel therapeutic and diagnostic strategies [6], for example for *Staphylococcus aureus* mastitis in cows [7], osteoarthritis in horses [8], and chronic kidney disease [9], gastric cancer [10] and respiratory viral infections in humans [11]. In addition, gene activity patterns have been used in nutrigenomics, the study of the effects of bioactive compounds from food on gene expression [12].

In order to create a gene signature, genes are selected based on their function within a cellular pathway, which is assumed to be representative for a particular disease or condition. Changes in relative expression levels of a set of several genes can thus display the way a pathway is influenced under experimental conditions. As mentioned above, genes induced by cellular stressor and genes involved in cellular defence mechanisms are candidates to quantify improved gut health in the liver. Therefore target genes of the transcription factors Nrf2, NF-κB, HSF1 and HIF1 have been chosen as genes of interest.

The transcription factor nuclear factor (erythroid-derived)-like 2 (Nrf2) upregulates antioxidant and detoxifying enzymes in response to oxidative stress by activation of a redox-sensitive gene regulatory network [19]. Oxidative stress is commonly defined as an imbalance between oxidants and reductants (antioxidants) at the cellular or individual level [18]. Cells are constantly exposed to endogenously and exogenously generated reactive oxygen species (ROS) and contain multiple antioxidants and related enzymes to scavenge ROS and restore redox homeostasis. These enzymes include heme oxygenase-

1, thioredoxin 1, superoxide dismutase 2, catalase, NAD(P)H:quinone oxidoreductase 1, epoxide hydrolase, glutamate cysteine ligase, glutathione peroxidase 1, and ATP-binding cassette transporter 2. Together with their transcription factor Nrf2 itself they are part of the gene signature applied in this study. Heat shock transcription factor 1 (HSF1) regulates the induction of heat shock proteins 27 (HSP27) and 70.2 (HSP70.2) in response to several kinds of environmental and physiologic stresses, including heat, oxidative stress, inflammation, hypoxia, and exposure to toxic chemicals [37]. HSP27 and HSP70.2 have cytoprotective effects, influencing aggregation, transport, and folding of other proteins. They also act on different levels of the apoptotic-signaling pathway, thereby having anti-apoptotic properties, ensuring that stress-induced damage does not inappropriately trigger cell death [38]. Both heat shock proteins are chosen as representatives of the pathway regulated by HSF1 and pertain to before-mentioned gene signature. HIF-1α is the hypoxically inducible subunit of transcription factor hypoxia inducible factor 1 (HIF1A), which mediates the induction of a series of genes in response to hypoxia. The synthesis and transcription of HIF-1α are constitutive and seem not to be influenced by hypoxia [41]. Still, HIF-1α has been added to the gene signature in order to detect possible influences of the experimental conditions in this study on hypoxia-related events in the liver cells of pigs. The transcription factor nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) plays a central role in immune signaling, the inflammatory response, cell survival and proliferation. IκBα sequesters NF-κB in the cytoplasm and its induction by NF-κB provides a negative feedback loop against NF-κB transcriptional activity [42]. Other target genes of NF-κB include c-FLIP, COX2 and XOR, and together with IκBα they haven been chosen as representatives of the NF-κB-regulated pathway within the gene signature.

Taken together, a gene signature focusing on oxidative stress and inflammation has been set up in this study to assess direct and indirect effects of 3 different herbal products on the liver in pigs.

2 Materials and methods

2.1 Animal experiments

Pigs were housed at Raalte Practice Centre for Organic Pig Farming, belonging to the research facilities of Wageningen University. Three standardized and controlled herbal products containing *Origanum vulgare* essential oil were selected. At an age of approximately 6 weeks fattening pigs were weaned and divided at random into experimental and control groups. Either the herbal product or the same amount of wheat was added to the feed pellets. From four days after weaning, feed was fed ad lib until slaughtering. For the herbal products Ropadiar® (product A), Biomin® P.E.P. 1000 (product B) and Digestamine® (product C) the control and experimental groups consisted of 29 and 24, 15 and 20, and 24 and 30 animals, respectively. During the experimental period, weight gain and feed conversion were recorded and a disease record established. The results of these zootechnic parameters are published elsewhere (RIKILT Rapport 2008.010).

2.2 Sample collection, RNA isolation and cDNA synthesis

Liver samples were collected at the time of slaughter in a commercial abattoir. From each pig, a sample of the same region of the left lateral lobe was taken, rinsed in a beaker filled with ice-cold phosphate buffered saline, dried and then shock frozen in liquid nitrogen. Subsequently the samples were transported on dry ice to the laboratory and stored at -80°C until further processing. Total RNA was isolated with SV Total RNA Isolation System (Promega) from 30 mg tissue in 175 µl lysisbuffer according to manufacturers protocol, which includes a DNase treatment. RNA concentration and purity of individual samples were determined on a spectrophotometer (ND-1000, Isogen, IJsselstein, The Netherlands). Concentrations ranged between 78.07 and 416.36 ng/µl.

OD A260/A280 and OD A230/A260 ratios gave values between 2.00 and 2.11 and between 1.68 and 2.16, respectively. One μg RNA was converted into cDNA using the iScript^{TM}cDNA synthesis kit (Bio-Rad, Hercules, CA, USA), which contains both random hexamer and oligo dT primers. Reverse transcriptase (1 μl) was added to the final RT reaction volume of 20 μl. The complete reaction mix was incubated for 5 min at 25°C and for 45 min at 42°C, followed by heat inactivation of the enzyme for 5 min at 85°C. cDNA samples were stored at -20° until use.

2.3 Primer development

For all genes, primer sets were developed with PrimerSelect 5.07 software (DNAStar, Inc.), based on porcine NCBI database sequences or porcine EST sequences highly homologues to human genes, and commercially produced (Eurogentec, Seraing, Belgium). Specificity was assessed by blast analysis against porcine and human cDNA sequences (BLAST, NCBI). qPCR and melting curve analysis of a 5 point 4x dilution series of porcine liver cDNA and a non-template control were performed to verify efficiency and specificity and to determine the practical optimum temperature for annealing of each primer set. The real-time PCR program started with a 3 min denaturation step at 95°C, then 40 cycles of 20 s of denaturation at 95°C, 30 s of annealing, following a temperature-gradient, and 30 s of elongation at 72°C. This was followed by 1 min denaturation at 95°C and 1 min annealing/elongation at 65°C. Subsequently, a melting curve was constructed by increasing the temperature from 65°C to 95°C in sequential steps of 0.5°C for 10 s. Fluorescence was measured during annealing and elongation steps throughout the entire PCR run. Primer sets were all specific for their targets, as primer dimers were absent and melting curves showed a single melting peak in each run. A list of the final primers used and their optimum annealing temperature is given in Table 1.

Table 1. Primer sets used for the quantitative PCR analysis

Gene symbol[a]	Forward primer (5′ → 3′) Reverse primer (5′ → 3′)	Amplicon length (bp)	Ta (°C)	GenBank accession number
ABCC2	GTGGCTGTTGAGCGAATAAATGAATAC TGCTGGGCCAACCGTCTG	90	65	DQ530510
CAT	GTGCCAACGAAGATAATGTC GACCCGCAATGTTCTCAC	96	60	NM_214301
C-FLIP	GCACCCTCATCTTATTTCAGAC GCTTCGGCTTATGTGATCTC	115	60	NM_001001628
COX2	CATTGATGCCATGGAGCTGTA CTCCCCAAAGATGGCATCTG	70	60	NM_214321
EPHX1	CACATTCGACTGGAGGAAGC CACGTGGATGAAGTGGATGTC	94	60	NM_214355
GCLC	GATCCCCATCCTGAACTCTTAC TCCATCTGGCAACTGTCATC	128	60	XM_001927763
GPX1	GCTTGGCAGTTACAGTGCTAC GACATCAGGTGTTCCTCCAC	82	60	NM_214201
HIF1A	CAAATCCAGAGTCCCTGGAAC CACTGGGACTGTTAGGCTCAG	127	62	NM_001123124
HMOX1	AGACCGCCTTCCTGCTCA GGGTCTCTGGTCCTTAGTGTC	80	64	NM_001004027
HPRT	ATCATTATGCCGAGGATTTGGA CCTCCCATCTCTTTCATCACATCT	100	63	NM_001032376
HSP27	GCTGACGGTCAAGACCAAG GCAGCGTGTATTTTCGAGTG	110	60	NM_001007518
HSP70.2	GAGTCGTACGCCTTCAACAT AAATCACCTCCTGACACTTGT	109	61	NM_213766
IKBA	CTGCACTTGGCCATCATC GAGTCTGCTGCAGGTTGTTC	109	58	NM_001005150
NRF2	ACATCCCGTCAGAAACCAGTG GTAGCCGAAGAAACCTCATTGTC	145	60	AK236642
NQO1	CACTGCAGTGGTTTGGAGTC CATGGCAGCGTATGTGTAAG	89	58	AY610195
SDHA	GCAGGCCAGGAGATAAAGTTC GTTCCGTTCGCAAATCTCAG	91	60	DQ845177
SOD2	CCTCACACCGAGTACATCAAG GCCGCATTCAGAAACACTAC	97	60	XM_001926440
TRX	GATCAAGCCTTTCTTCCATTC GTTGGCATGCATTTGACTTC	117	58	NM_214313
XOR	GTTTTCGGAAGCAGATAATGTTGT GGTGCAGTGAGTCTCCAGGTAG	82	60	-

[a]Full names of the genes: ABCC2 (ATP binding cassette, sub-family, C, member 2), CAT (catalase), C-FLIP (cellular FLICE-like inhibitory protein), COX2 (cyclooxygenase 2), EPHX1 (epoxide hydrolase), GAPDH (glyceraldehyde-3-phosphate dehydrogenase), GCLC (glutamate-cysteine ligase, catalytic subunit), GPX1 (glutathione peroxidase 1), HIF1A (hypoxia inducible factor, α subunit), HMOX1 (Heme oxygenase-1), HPRT (hypoxanthine phoshoribosyl transferase), HSP27 (heat shock protein 27), HSP70.2 (heat shock protein 70.2), IKBA (nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha), NRF2 (nuclear factor (erythroid-derived)-like 2), NQO1 (NAD(P)H quinone oxidoreductase 1), SDHA (succinate dehydrogenase), XOR (xanthine dehydrogenase).

2.4 Real-time PCR analysis

Real-time qPCR was conducted on the iCycler MyIQ™ Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA) with a reaction volume of 25 μl. Each reaction mixture consisted of 12.5 μl iQ SYBR Green Supermix (Bio-Rad), 7.5 pmol forward primer, 7.5 pmol reverse primer, 1.0 μl RNAse-free water and 1 μl cDNA. The iQ SYBR Green Supermix contained 100 mM KCl, 40 mM Tris-HCl, pH 8.4, 0.4 mM each dNTP, 50 U/ml iTaq DNA polymerase, 6 mM $MgCl_2$, SYBR Green I, 20 nM fluorescein and stabilizers. The real-time PCR program was performed as described above, using the determined optimal

Ta for each specific primer (Table 1) at the 30 seconds of annealing during each cycle.

2.5 Reference gene selection and data analysis

Background-subtracted data generated by the iCycler were exported as cycle-number/fluorescence data pairs to calculate quantification cycle (Cq) values for each sample and the mean (amplicon-specific) PCR efficiencies using the LinRegPCR software [15]. Cq values were imported in the BestKeeper software tool [16] to determine whether target genes were stably expressed under control and experimental conditions. Subsequently, the Cq value of each sample was converted to relative quantity data (Q) using the measured efficiencies (E) (eq. 2) and calculated ΔCq (eq. 1). Subsequently, the most stably expressed genes among the chosen reference genes glyceraldehyde-3-phosphate dehydrogenase (GAPDH), hypoxanthine phoshoribosyl transferase (HPRT) and succinate dehydrogenase (SDHA) were identified using the GeNorm algorithm [17]. Based on the cut-off value of 0.15, the number of reference genes sufficient to calculate a reliable normalization factor was determined. The normalization factor, which is the geometric mean of the relative quantity of the most stable reference genes [17], was used to normalize the obtained relative quantity data and thus to determine the relative expression levels (rQ) (eq. 3).

The following equations were used:

$$\Delta Cq = C_q\text{mean} - C_q\text{sample} \quad (1)$$

$$Q = E^{\Delta Cq} \quad (2)$$

$$rQ\ GOI = Q_{GOI} / \text{GeoMean}(Q_{SDHA}; QR_{CAT}; Q_{HPRT}) \quad (3)$$

where C_q*mean* is the mean quantification cycle of all samples in one run, C_q*sample* is the quantification cycle of the sample and *GOI* is the gene of interest. All calculations were performed on experimental and control samples considered as 1 group. The results are presented as mean ± SEM. Probability values below 0.05 were considered statistically significant. The normality test and the unpaired t-test were performed using GraphPad Prism version 4.00 for Windows (GraphPad Software, San Diego California USA, www.graphpad.com).

3 Results

Measured relative expression levels in experimental compared to control groups are presented in Table 2 for each product. For the ease of result evaluation, target genes of the different pathways regulated by the transcription factors Nrf2, NF-κB, HSF1 and HIF1, which have been chosen as genes of interest, are clustered according to the particular pathway. Pigs fed product A showed significantly reduced relative expression levels of HMOX1, NQO1, EPXH1, GCLC, HSP70.2, IKBA, and XOR, and significantly increased relative expression levels for TRX, GPX1, and HSP27. Relative expression levels of SOD2, CAT, NRF2, ABCC2, HIF1A, COX2 and C-FLIP were not significantly altered in pigs fed product A. The experimental group fed product B showed significant decrease in the relative expression levels for HMOX1, COX2, IKBA, and C-FLIP, and a significant induction for GCLC, GPX1, ABCC2, and HSP27. Relative expression levels of TRX, SOD2, CAT, NQO1, EPXH1, NRF2, HSP70.2, HIF1A and XOR showed no significant changes in the experimental group fed product B compared to the control group. Pigs fed product C showed significantly diminished relative expression levels for HMOX1, NRF2, HSP70.2, IKBA, and C-FLIP, and significantly increased relative expression levels for GCLC. Relative expression levels of TRX, SOD2, CAT, NQO1, EPXH1, GPX1, ABCC2, HSP27, HIF1A, COX2 and XOR were not significantly influenced in the experimental group fed product C. In order to illustrate obtained results, results of measured relative expression levels for HMOX1 in experimental compared to control groups are presented in Figure 1. Significant correlations between genes in terms of relative expression levels in animals of control and experimental groups are presented in Figure 2. Genes of interest have been arranged clockwise the way they are clusterd in Table 2, and the different colors of the lines representing correlations between two genes correspond with the P-values of the analyzed correlations, which are presented in Table 3. Figure 2 presents correlations between genes belonging to the same pathway, as well as to different pathways.