„Einfluss phytogener Futterzusätze auf Xenobiotika-metabolisierende und antioxidative Enzyme sowie Entzündungsparameter bei monogastrischen Nutztierspezies und bei der Ratte“

Dissertation
zur Erlangung des Doktorgrades
der Ernährungswissenschaften (Dr. troph.)

der

Naturwissenschaftlichen Fakultät III
Institut für Agrar- und Ernährungswissenschaften,
Geowissenschaften und Informatik

der Martin-Luther-Universität Halle-Wittenberg

vorgelegt von

Diplom-Ernährungswissenschaftlerin Kristin Müller
Geb. am 24.06.1983 in Wolfen

Gutachter/in: 1. Jun.-Prof. Dr. A.S. Müller
2. Prof. Dr. J. Zentek

Verteidigung am: 09.12.2013

Halle Saale (2013)

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Abkürzungsverzeichnis

AFAR	Aflatoxin B1 Aldehydreduktase
AITC	Allyl-Isothiocyanat
ARE	*Antioxidative Response Element*
bZIP	Basischer Leucin-Zipper
CNC	*Cap-N-Collar*
Cox2	Cyclooxygenase 2
DL	Deutsche Landrasse
DLG	Motiv aus Asparaginsäure, Leucin und Glycin
DNA	Desoxyribonukleinsäure
DSS	Natriumdextransulfat
ECH	*Erythroid-derived CNC Homology protein*
EHEC	Enterohämorrhagische *Escherichia coli*
EPHX	Epoxidhydrolase
ETEC	Enterotoxische *Escherichia coli*
ETGE	Motiv aus Glutaminsäure, Threonin, Glycin und Glutaminsäure
FA	Futteraufnahme
FVW	Futterverwertung
GCL	Glutamatcysteinligase
GPx	Glutathionperoxidase
GRA	Glucoraphanin
GST	Glutathion-S-Transferase
GW	Gewicht
GZ	Gewichtszunahme
H_2O_2	Wasserstoffperoxid
HO·	Hydroxyl-Radikal
HO1	Hämoxygenase 1
HOO·	Hydroperoxyl-Radikal
IBD	*Inflammatory Bowel Disease*
ICAM	*Intercellular adhesion molecule*
IkB	Inhibitorische *kappa* B-Proteine
IKK	IkB-Kinase-Komplex
IL-10	Interleukin 10

IL-1b	Interleukin 1 *beta*
IL-6	Interleukin 6
IL-8	Interleukin 8
IPEC-J2-Zellen	*Intestinal Porcine Epithelial Cells-Jejunal 2*-Zellen
ITC	Isothiocyanat
Keap1	*Kelch-like ECH associated protein 1*
LPS	Lipopolysaccharid
MHK	Minimale Hemmkonzentration
MRP	*Multi-drug resistance protein*
Neh1-Neh6	Nrf2-ECH-Homologe Domäne 1-6
NFkB	*Nuclear Factor 'kappa-light-chain-enhancer' of activated B-cells*
NO	Stickstoffmonoxid
NQO1	NADPH-abhängige Chinonoxidoreduktase 1
Nrf2	*Nuclear factor erythroid 2 related factor 2*
O_2^-	Superoxid-Anion
PGE2	Prostaglandin E2
SFN	Sulforaphan
SOD1	Superoxiddismutase 1
TBA-RS	*2-Thiobarbituric Acid-Reactive Substances*
TEAC	*TroloxÒEquivalent Antioxidant Capacity*
TNBS	2,4,6-Trinitrobenzosulfonsäure
TNFa	Tumornekrosefaktor *alpha*
UGT	UDP-Glucuronosyltransferase
UV-Strahlung	Ultraviolette Strahlung
V1-V4	Versuch 1-4
VCAM	*Vascular cell adhesion molecule 1*

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1 Einleitung

Die Gesundheit von Nutztieren stellt derzeit einen der wichtigsten Produktionsfaktoren in der modernen Tierhaltung dar. Bezüglich der Tierproduktion gelten vor allem die frühen Lebensphasen als besonders kritisch. Bis vor einigen Jahren wurden gerade in dieser Produktionsphase antibiotische Leistungsförderer eingesetzt, um die ökonomisch nötigen Lebendmassezunahmen bei möglichst geringem Futteraufwand zu erreichen. Im Hinblick auf die zunehmende Entwicklung und Verbreitung von Kreuzresistenzen gegenüber Antibiotikapräparaten mit therapeutischer Bedeutung in der Human- und in der Veterinärmedizin sprach die Europäischen Union im Rahmen des vorbeugenden Verbraucherschutzes 2006 ein endgültiges Einsatzverbot der letzen 4 bis dahin zugelassenen antibiotischen Leistungsförderer (Salinomycin, Monensin, Avilamycin, Flavomycin) als Futterzusatz aus (Verordnung (EG) Nr. 1831/2003 Artikel 11 Absatz 2). Die Nutztierernährung wird dadurch bis heute vor die Herausforderung gestellt, den Gesundheitsstatus und die erwünschte Leistung von Nutztieren auf alternativem Weg zu garantieren (Wenk 2005). In der Geflügelproduktion führen besonders in der frühen Phase nach dem Schlüpfen Durchfallerkrankungen, die durch Infektionen mit *Salmonella spp.* und mit pathogenen *Escherichia coli*-Stämmen hervorgerufen werden (Selbitz et al. 2011), zu Problemen. Bei Schweinen treten sehr häufig nach der Trennung vom Muttertier Absetzdurchfälle oder die Ödemkrankheit auf, die beide durch toxinbildende *Escherichia coli*-Stämme hervorgerufen werden (Kyriakis et al. 1998, Vögeli und Bertschinger 1999, Kamphues 1999). Diese Erkrankungen führen bei den genannten Nutztierspezies durch sinkende Futteraufnahme und verschlechterte Nährstoffabsorption zu extremen Leistungseinbußen und gehen häufig auch mit einer erhöhten Mortalität einher. Bei der Suche nach nichtantibiotischen Alternativen treten phytogene Verbindungen aufgrund ihrer Antibiotika-ähnlichen Eigenschaften mehr und mehr in den Fokus der Forschung.

1.1 Phytogene Futterzusätze

Die heterogene Gruppe der phytogenen Futterzusätze fasst verschiedenste Pflanzen, Pflanzenteile, Gewürze und daraus extrahierte oder durch Wasserdampfdestillation gewonnene Produkte wie ätherische Öle und Fettharze zusammen, die zur Erhöhung des Leistungsvermögens landwirtschaftlicher Nutztierbestände eingesetzt werden (Windisch et al. 2008).

Es existieren verschiedene Klassifizierungssysteme zur Einteilung phytogener Substanzen. Demnach werden sie:

- nach dem genutzten Pflanzenteil (ganze Pflanze, Wurzel, Sproßachse, Rinde, Blatt, Blüte, Frucht und Samen),
- nach dem Habitus (Kraut, Riedgras, Gras, Strauch, Schlingpflanze, Baum),
- nach dem Vorkommen (tropische, subtropische, gemäßigte Klimazone),
- nach der Darreichungsform und Aufbereitung (Tinktur, Absud, Tee, Sirup, Wasserdampfdestillation, Extraktion mit anderen Lösungsmitteln) oder
- nach der botanischen Systematik

eingeteilt (Wenk 2005, Windisch et al. 2008). In der Tierernährung kann des Weiteren in die vier Subklassen der Kräuter und Gewürze (einjährige, nicht verholzte Pflanzen), der Pflanzen und Pflanzenextrakte, der ätherischen Öle (Wasserdampfdestillation) sowie der Oleoresine untergliedert werden (Windisch und Kroismayr 2006).

In der Humanmedizin stellt die Phytotherapie (traditionelle chinesische Medizin) bereits seit Jahrhunderten eine wichtige Präventions- und Behandlungsstrategie dar. Während Kräuter und Gewürze in Ländern des asiatischen und afrikanischen Raumes bereits intensiv genutzt werden, steigt deren Verwendung in den Industrienationen in den letzten Jahren erst allmählich an. Dabei werden in Europa von 100% der phytotherapeutischen Maßnahmen beispielsweise 49% in Deutschland und jeweils 10% in Italien, Frankreich und Großbritannien eingesetzt (ICMR Bulletin 2003).

Da phytogene Verbindungen im Vergleich zu synthetischen Antibiotika Produkte auf Naturbasis sind, scheinen sie sich besonders gut als potenzielle Leistungsförderer im Tierfutter zu eignen (Hashemi et al. 2008). Eine Vielzahl an phytogenen Verbindungen findet bereits als Futterzusatz in der Tierernährung zur Verbesserung der Tiergesundheit Verwendung (Windisch et al. 2008).

Dabei kommen Substanzen und Extrakte verschiedenster Pflanzenfamilien zum Einsatz. Für die Schweinefütterung sind beispielsweise die Vertreter der Pflanzenfamilien der Liliaceae, der Labiatae, der Zingiberacae und der Umbelliferae besonders interessant (Tabelle 1).

Derzeit werden phytogene Futteradditive hauptsächlich auf der Basis von Kräutern und ätherischen Ölen der genannten Pflanzenfamilien angeboten. Zudem finden häufig Mischungen verschiedener Kräuter, Extrakte und ätherischer Öle Verwendung. Allerdings ist bisher wenig hinsichtlich ihrer Wirkungsmechanismen bekannt beziehungsweise wissenschaftlich belegbar.

Futtermittelrechtlich wird der Einsatz von phytogenen Verbindungen in der Tierernährung auf europäischer Ebene durch die EG-Verordnung Nr. 1831/2003 geregelt. Nach dieser Regelung des EU-Futtermittelrechts werden Futtermittelzusatzstoffe als Stoffe, Mikroorganismen oder Zubereitungen definiert, die die Beschaffenheit des Futtermittels, die Beschaffenheit der tierischen Erzeugnisse oder die Tiergesundheit positiv beeinflussen.

Je nach ihrer Funktion werden die Futtermittelzusatzstoffe einer der folgenden fünf Kategorien zugeordnet:

(1) Technologische Zusatzstoffe (Fließfähigkeit, Stabilität, Lagerfähigkeit)
(2) Sensorische Zusatzstoffe (Aroma, Schmackhaftigkeit)
(3) Ernährungsphysiologische Zusatzstoffe (Spurenelemente, Vitamine, Aminosäuren)
(4) Zootechnische Zusatzstoffe (Phosphorausscheidung, Ammoniakemmission, Stabilisierung der Darmflora, Förderung der Verdaulichkeit)
(5) Kokzidiostatika und Histomonostatika

Die Mehrzahl der aktuell zugelassenen phytogenen Addititve gehört dabei in die Kategorie 2 der sensorischen Zusatzstoffe, da die zugrunde liegenden Zulassungsvoraussetzungen im Hinblick auf Geschmacks- und Aromaverbesserung des Futters mit relativ wenig Aufwand zu erfüllen sind. Teilweise werden phytogene Futterzusätze auch den zootechnischen Zusatzstoffen zugeordnet, wobei die Anforderungen für eine Zulassung in dieser Kategorie deutlich anspruchsvoller und wissenschaftlich schwieriger belegbar sind, als für die Kategorie 2.

1.2 Diskutierte Wirkungsmechanismen phytogener Futterzusätze

Durch den Einsatz phytogener Futteradditive kann die Tiergesundheit innerhalb eines Tierbestandes gesteigert werden (Windisch et al. 2008) und möglicherweise besonders in der Jungtieraufzucht auftretenden Infektionskrankheiten, wie der Ödemkrankheit und Absetzdurchfällen, vorgebeugt werden. Diese positive Beeinflussung der Tiergesundheit scheint dabei auf den folgenden 5 Wirkungen pflanzlicher Additive zu beruhen:

(1) Verbesserung von Futteraufnahme und Leistungsparametern
(2) Direkte antioxidative Wirkung
(3) Indirekte antioxidative Wirkung
(4) Antimikrobielle/bakterizide Wirkung
(5) Anti-inflammatorische Wirkung

Die Studienergebnisse aus aktueller Literatur zeigen, dass die verschiedenen phytogenen Futterzusätze die genannten potenziellen Effekte in unterschiedlichem Maße ausüben.

Weiterhin wird auch ein Zusammenspiel zwischen den einzelnen Wirkmechanismen nicht ausgeschlossen.

1.2.1 *Verbesserung von Futteraufnahme und Leistungsparametern durch phytogene Futterzusätze*

In einigen Studien bewirkte der Einsatz von pflanzlichen Additiven eine verbesserte Schmackhaftigkeit des Futters, woraus eine erhöhte Futteraufnahme und eine Verbesserung der täglichen Gewichtszunahmen resultierte (Wald 2002, Windisch et al. 2008). In der Literatur wird diese Eigenschaft im Besonderen Kräutern und ätherischen Ölen aus der Familie der Labiatae (Oregano, Thymian, Rosmarin) zugeschrieben. So konnte gezeigt werden, dass eine Mischung von ätherischen Ölen aus Oregano und Nelke beziehungsweise aus Oregano und Zimt in der Broilermast die Futteraufnahme deutlich reduziert und den Futteraufwand signifikant verbessert (Halle 2001). Halle et al. (2004) beschrieben weiterhin, dass eine gestaffelte Supplementierung des ätherischen Öls aus Oregano (0; 0,1; 0,2; 0,5; 1 g/kg Futter) bei Broilern zu einer Verminderung der täglichen Futteraufnahme und einer gesteigerten täglichen Lebendmassezunahme führt, was sich in einer um 6 – 8 % verbesserten Futterverwertung im Vergleich zur Kontrolle äußerte. Auch für das ätherische Öl des Thymians (0,1; 0,2 g/kg Futter) konnte eine reduzierte Futteraufnahme und eine gesteigerte tägliche Gewichtszunahme belegt werden (Al-Kassie 2009). Die ätherischen Öle von Rosmarin und Oregano (0,5 g/kg Futter) führten bei Absetzferkeln zwar zu einer gesteigerten Futteraufnahme, verbesserten jedoch auch die tägliche Lebendmassezunahme (Janz et al. 2007). Kyriakis et al. (1998) beschrieben neben der Verbesserung der Mastleitung von Absetzferkeln durch den Zusatz von Oreganoöl (12,5; 25 mg/kg Futter) zusätzlich einen Rückgang der Diarrhoehäufigkeit. Auch die kombinierte Verabreichung von ätherischen Ölen aus Oregano, Zitrusschalen und Chicorée führte bei Ferkeln zu einer positiven Beeinflussung der Leistungsparameter (Zitterl-Eglseer et al. 2008).

Die leistungsfördernde Wirkung pflanzlicher Futterzusätze beruht scheinbar vor allem auf der Stabilisierung der Futterhygiene, auf der positiven Beeinflussung des gastrointestinalen mikrobiotischen Ökosystems (Roth und Kirchgessner 1998) und auf einer Entlastung des Immunsystems durch verringerten oxidativen Stress. Neben der Stabilisierung der Darmflora durch eine Reduktion von pathogenen Bakterienkolonien und von Fermentationsprodukten, wie Ammoniak und biogenen Aminen (Cross et al. 2007, Burt et al. 2007b), können phytogene Futterzusätze zu einer Verbesserung der Futtereigenschaften und zu einer verbesserten Nährstoffverdaulichkeit (Windisch et al. 2008) beitragen.

Dennoch existieren in der Literatur auch Studien mit genau gegenteiligen Ergebnissen, in denen keine oder sogar negative Effekte phytogener Additive auf die Leistungsparameter festgestellt werden konnten. So zeigten beispielsweise Basmacioğlu Malayoğlu et al. (2010), dass die Supplementation mit ätherischem Oreganoöl (250 und 500 mg/kg) bei Broilern keinen Einfluss auf die Futteraufnahme und –verwertung hatte. In einer anderen Studie mit Legehennen wurde durch den Einsatz hoher Mengen an Gelbwurzelpulver (10 g/kg) die Futteraufnahme drastisch verringert (Wenk und Messikommer 2002). Ebenso reduzierte ein Futterzusatzstoff auf Oreganobasis (2 g/kg) bei Absetzferkeln die tägliche Gewichtzunahme und verschlechterte dadurch die Futterverwertung insgesamt (Oswald und Wetscherek 2007).

Tabelle 1: Wirkungen ausgewählter Kräuter- und Gewürzpflanzen

Name (*botanische Bezeichnung)*	**Pflanzenfamilie**	**aktive Verbindung**	**Stoffklasse**	**Wirkung**
Anis *(Pimpinella anisum)*	Umbelliferaceae	Anethol	Phenylpropan-derivat	Verdauungsfördernd
Gelbwurzel *(Curcuma longa)*	Zingiberaceae	Curcumin, Turmeron	Sesquiterpen	Magensaft-fördernd, Antimikrobiell
Ingwer *(Zingiber officinale)*	Zingiberaceae	Gingerol	Phenol	Magensaft-fördernd, Antientzündlich
Knoblauch *(Allium sativum)*	Liliaceae	Allicin	Disulfid	Verdauungsfördernd, Antiseptisch
Koriander *(Coriandrum sativum)*	Umbelliferaceae	Linalol	Azyklisches Monoterpen	Verdauungsfördernd, Antimikrobiell
Oregano *(Origanum vulgure)*	Labiatae	Carvacrol	Monoterpen	Verdauungsfördernd, Antiseptisch, Antimikrobiell
Rosmarin *(Rosmarinus officinalis)*	Labiatae	Cineol	Terpenoxid	Verdauungsfördernd, Antiseptisch, Antioxidativ
Salbei *(Salvia officinalis)*	Labiatae	Cineol	Terpenoxid	Verdauungsfördernd, Antiseptisch, Antimikrobiell
Schwarzkümmel *(Nigella sativa)*	Ranunculaceae	Thymol	Monoterpen	Antiseptisch, Antioxidativ, Antimikrobiell
Thymian *(Thymus vulgaris)*	Labiatae	Thymol	Monoterpen	Verdauungsfördernd, Antiseptisch, Antioxidativ, Antimirkrobiell

Zusammengestellt nach: Sivropoulou et al. 1996, Lopez-Bote et al. 1998, Dorman et al. 1999, Kamel 2000, Friedmann et al. 2002, Wetschrek 2002, Büechi und Bolli 2004, Wald 2004

Für die beschriebenen Wirkungen phytogener Futterzusätze werden zunehmend Einzelverbindungen verantwortlich gemacht. Diese Einzelverbindungen stellen häufig den Hauptinhaltsstoff der gesamten pflanzlichen Substanz, respektive des Pflanzenextrakts, dar

und bilden chemisch betrachtet eine sehr heterogene Gruppe von Mono-, Di- und Sesquiterpenenen sowie von Terpenoxiden (Tabelle 1).

1.2.2 *Direkte antioxidative Wirkung phytogener Futterzusätze*

Vor dem Hintergrund der Gesundheit von Nutztieren spielt die antioxidative Wirkung phytogener Verbindungen eine besondere Rolle. Das antioxidative Abwehrsystem eines Organismus stellt einen wichtigen Schutz vor oxidativem Stress und den damit verbundenen Folgeschäden an DNA, Proteinen und Lipiden dar (Marnett et al. 2003). Oxidativer Stress entsteht immer dann, wenn endogen bei der Energiegewinnung produzierte reaktive Sauerstoffspezies nicht im gleichen Maße, indem sie gebildet werden, durch das antioxidative Schutzsystem abgebaut werden können. Oxidativer Stress kann aber auch durch exogene Noxen, wie UV-Strahlung und Ozon, hervorgerufen werden (Halliwell 1996, Willcox et al. 2004). Das gesamte antioxidative Potential einer Zelle beziehungsweise eines Organismus kann in 2 Hauptkomponenten unterteilt werden (Halliwell 1996, Evans und Halliwell 2001, Halliwell 2006):

(1) endogene antioxidative Enzyme (Glutathionperoxidasen, Superoxiddismutase, Katalase) und Metallionen-bindende Proteine (Ferritin, Coeruloplasmin, Metallothionein)

(2) exogene nicht-enzymatische Antioxidantien (Vitamin E, Vitamin C, Carotinoide), die vor allem über die Nahrung aufgenommen werden

In die Gruppe der nicht-enzymatischen Antioxidantien können zunächst auch die meisten phytogenen Futterzusätze eingeteilt werden. Die direkte antioxidative Wirkung beinhaltet dabei das Unschädlichmachen von freien Radikalen, insbesondere reaktiver Stickstoff- und Sauerstoffspezies, durch direkte chemische Interaktion (Halliwell 1996).

Die beschriebene direkte antioxidative Wirkung ist dabei stark von dem jeweiligen Phenolgehalt der phytogenen Substanz abhängig (Papageorgiou et al. 2008). Unter den Kräutern zeichnen sich Oregano und Thymian durch besonders hohe Gehalte an den Monoterpenen Carvacrol und Thymol aus, deren chemische Grundstruktur direkt zur Stabilisierung von freien Radikalen und damit zur Abpufferung von oxidativem Stress beiträgt (Skobot et al. 2003). Bei einer vergleichenden Analyse von 26 Gewürzextrakten konnte in vitro für Oregano eine sehr starke, für Thymian und Rosmarin eine starke antioxidative Aktivität ermittelt werden (Shan et al. 2005). Viuda-Martos et al. (2010) stellten in vitro als Folge der hohen direkten antioxidativen Wirkung von Oregano- und Thymianöl eine starke Verminderung der Lipidperoxidation fest, während Rosmarinöl einen geringeren inhibitorischen Effekt auf die Lipidperoxidation zeigte. Letzteres Resultat basiert

wahrscheinlich darauf, dass Rosmarin einen geringen Anteil an phenolischen Komponenten als Thymian und Oregano aufweist.

1.2.3 *Indirekte antioxidative Wirkung phytogener Futterzusätze*

Viele in vitro und in vivo-Studien berichteten, dass pflanzliche Verbindungen durch eine Steigerung der Enzymaktivität von der Superoxiddismutase und der Glutathionperoxidase (Youdim und Deans 2000, Wang et al. 2009) sowie durch die Erhöhung des Glutathiongehaltes (Aherne et al. 2007) zu einem verbesserten antioxidativen Status beitragen. Darüber hinaus gibt die aktuelle Literatur Hinweise auf einen weiteren bislang nahezu unbeschriebenen Mechanismus, über den phytogene Verbindungen wie Brokkoli, Gelbwurzel und Thymian, das antioxidative Schutzsystem positiv beeinflussen (Sasaki et al. 2005, Lee HS et al. 2010, Boddupalli et al. 2012). Dieser Mechanismus beruht dabei scheinbar auf der Induktion Xenobiotika-metabolisierender und antioxidativer Enzyme über die Nrf2-ARE-Signalkaskade.

Der zur Basischen Leucin-Zipper (bZIP)-Familie zählende Transkriptionsfactor Nrf2 (*Nuclear factor erythroid 2 related factor 2*) ist ein Cap-N-Collar-Protein (CNC), das für die Regulation der Genexpression von mehr als 600 zytoprotektiven Enzymsystemen verantwortlich ist. Unter anderem reguliert Nrf2 Xenobiotika-metabolisierende und antioxidative Enzyme sowie antiinflammatorische Signalwege.

In den späten 1990er Jahren wurde der Transkriptionsfaktor Nrf2 in seiner molekularen Struktur erstmals durch Itoh et al. (1999) beschrieben. Der Nrf2 beinhaltet 6 Nrf2-ECH-Homologe Domänen (Neh1-Neh6), die in vielen Spezies hoch konserviert vorliegen. Für die Funktionalität des Nrf2-Proteins sind die Domänen Neh1 und Neh2 besonders wichtig. Die Neh1-Domäne wird aus der bZIP-Region, der CNC-Region sowie einer dazwischen liegenden Region gebildet, die reich an basischen Aminosäuren ist und den eigentlichen DNA-bindenden Teil des Nrf2-Proteins darstellt (Moi et al. 1994). Über diese Neh1-Domäne bindet der Nrf2 an so genannte *Antioxidant Response Elements* (ARE) in der Promotorregion seiner Zielgene und reguliert deren Expression.

Die Neh2-Domäne des Nrf2-Proteins ist essentiell für die Wechselwirkung des Transkriptionsfaktors mit seinem Inhibitorprotein Keap1 (*Kelch-like ECH associated protein 1*). In dieser Neh2-Domäne des Nrf2 existieren 2 Bindungsstellen (ETGE und DLG), die jeweils mit der Kelch-Domäne des Keap1 interagieren. Das ETGE-Motiv hat dabei eine größere Affinität zur Bindung der Keap1-Untereinheit als das DLG-Motiv des Nrf2, sodass scheinbar erst das ETGE-Motiv an ein Keap1-Dimer bindet und danach erst die DLG-Motiv Interaktion stattfindet („*hinge and latch*“-Model).

Bei Störungen der Keap1-Nrf2-Interaktion durch endogene oder exogene Agenzien dissoziieren ETGE- und/oder DLG-Motiv des Nrf2 vom Keap1-Dimer, wodurch zum einen die kontinuierliche Ubiquitinierung und der damit verbundene proteasomale Abbau des Nrf2 vermindert werden und zum anderen eine Nrf2-Freisetzung stattfindet (Hayes et al. 2009). Nrf2 transloziert mit Hilfe von Import-Komplexen vom Zytosol in den Zellkern (Li et al. 2005, Li et al. 2006) und dimerisiert dort mit *small Maf*-Proteinen. Als Heterodimer bindet Nrf2 ARE-Motive in der Promotorregion seiner Zielgene und induziert deren Transkription (Tabelle 2).

Einen der wichtigsten endogenen Aktivatoren der Nrf2-ARE-Signalkaskade stellt oxidativer Stress dar. Der Keap1/Nrf2-ARE-Signalweg kann aber ebenso durch exogene Induktoren ausgelöst werden. Zu diesen exogenen Induktoren zählen unter anderem auch phytogene Substanzen, wie das Senföl Sulforaphan aus Brokkoli oder das Curcumin aus der Gelbwurzel. Beide Verbindungen bewirken eine Nrf2-vermittelten Aktivierung von Xenobiotika-metabolisierenden und antioxidativen Enzymen mit einem ARE-Promotor (Pugazhenthi et al. 2007, Clarke et al. 2008). Bis dato fehlen jedoch im Hinblick auf eine indirekte antioxidative Wirkung phytogener Substanzen noch aussagekräftige in vivo-Studien, insbesondere im Nutztierbereich.

Tabelle 2: Häufig untersuchte Nrf2-Zielgene und deren Funktion

Nrf2-Zielgen	Funktion
SOD1 (Superoxiddismutase 1)	antioxidativ, Umwandlung Superoxidanion in Wasserstoffperoxid
GPx (Glutathionperoxidase)	antioxidativ, Reduktion von Hydroperoxiden und Wasserstoffperoxid
NQO1 (NADPH-abhängige Chinonoxidoreduktase 1)	antioxidativ, verhindert Entstehung radikalischer Zwischenstufen
HO1 (Hämoxygenase 1)	antioxidativ, Häm-Abbau
GCL (Glutamatcysteinligase)	antioxidativ, Glutathionbiosynthese
AFAR (Aflatoxin B1 Aldehydreduktase)	Detoxifikation (Phase II), Umwandlung von Aflatoxin B_1-8,9-epoxid zum Dihydrodiol
EPHX (Epoxidhydrolase)	Detoxifikation (Phase II), Hydratisierung von Epoxiden
GST (Glutathion-S-Transferase)	Detoxifikation (Phase II), Glutathionylierung
UGT (UDP-Glucuronosyltransferase)	Detoxifikation (Phase II), Glucuronidierung
MRP (Multi-drug resistance protein)	Detoxifikation (Phase III), Ausscheidung von Konjugationsprodukten

Zusammengestellt nach: Rushmore et al. 1991, Prestera et al. 1995, Nguyen et al. 2003, Banning et al. 2005, Maher et al. 2007, Penning und Drury 2007, Wang X et al. 2007, Miao et al. 2009

1.2.4 *Antimikrobielle Wirkung phytogener Futterzusätze*

Da die gastrointestinale Mikroflora einen wichtigen Schutz vor dem Eindringen pathogener Erreger und mikrobieller Toxine für den Wirtsorganismus darstellt, sollte diese sogenannte Mikrobiota in einem stabilen Gleichgewicht gehalten werden. Die Ausbildung des als Eubiose bezeichneten Zustandes der Darmflora kann unterstützt werden, indem zum einen das

Wachstum apathogener Mikroorganismen gefördert und zum anderen pathogene Keime abgetötet bzw. in ihrem Wachstum gehemmt werden (Savage 1977, Haenel 1982).

In der Literatur werden für die verschiedensten phytogenen Verbindungen, besonders aber für die Vertreter der Labiatae (Oregano, Thymian, Rosmarin), antimikrobielle Effekte gegen *Escherichia coli* (Burt et al. 2007a) und gegen *Salmonella* Typhimurium (Kilic 2006) beschrieben. So wurden beispielsweise die ätherischen Öle aus Oregano und Thymian als hochwirksame Hemmstoffe gegen das Wachstum des Geflügel-pathogenen *Escherichia coli*-Stamm *O1:K1* eingestuft (Wald 2002). Zudem konnten Friedman et al. (2002) zeigen, dass diese beiden ätherischen Öle (Oregano, Thymian) eine bakterizide Wirkung gegen den am weitesten verbreiteten human-pathogenen enterohämorrhagischen *Escherichia coli*-Stamm (EHEC *0157:H7*) aufweisen. In diesem Zusammenhang konnte weiterhin gezeigt werden, dass auch die Hauptinhaltsstoffe von Oregano- und Thymianöl, das Carvacrol und das Thymol, eine deutliche, bakterizide Wirkung gegenüber *Escherichia coli* besitzen. Die in vitro ermittelten minimalen Hemmkonzentrationen (MHK) von Carvacrol und von Thymol lagen mit 100 und 300 µl/ml in einer ähnlichen Größenordnung wie die MHK der bisher in der Schweineproduktion eingesetzten Fütterungsantibiotika (Si et al. 2006), sodass sich eine antimikrobielle Wirkung phytogener Futteradditive auch in vivo vermuten lässt.

Darüber hinaus konnte in vitro ebenfalls für das im Brokkoli enthaltene Isothiocyanat Sulforaphan schon in einer sehr niedrigen Konzentration von 3µmol/l eine antimikrobielle Wirkung gegen *Escherichia coli* gezeigt werden (Aires et al. 2009). Neben den oben genannten Labiatae- und Brassica-Vertretern weisen auch die Pflanzen der Familie der Zingiberaceae, wie die Gelbwurzel, antimikrobielle Eigenschaften auf. Das Curcumin der Gelbwurzel bewirkte antibakterielle Effekte gegen gram-negative Bakterien (Wang Y et al. 2009).

Neben direkten antioxidativen und antimikrobiellen Eigenschaften stärken phytogene Futterzusätze wahrscheinlich zusätzlich durch eine Wachstumsförderung apathogener Mirkoorganismen (z.B. Laktobazillen) die Barrierefunktion des Darms. In diesem Kontext führte die Gabe einer Mischung aus Carvacrol (Oregano), Zimtaldehyd (Zimtbaum) und Capsicum Oleoresin (mexikanischer Pfeffer) bei Absetzferkeln zu einer vermehrten Anzahl an Laktobazillen und zu einer Abnahme der Enterobakterienzahl (Manzanilla et al. 2004; Castillo et al. 2006). Hingegen bewirkte die kombinierte Gabe ätherischer Öle des Oregano, des Anis und der Zitronenschale bei Schweinen durch eine Reduktion der Bakteriendichte und durch eine Verringerung von biogenen Aminen im Chymus eine Senkung der mikrobiellen Aktivität im terminalen Ileum und Kolon (Kroismayr et al. 2008a).

Zusammenfassend deuten die Ergebnisse bisheriger Studien daraufhin, dass die leistungsfördernde Wirkung phytogener Zusätze in der Schweine- (Manzanilla et al. 2004) und Geflügelernährung (Hernández et al. 2004) neben einer Verbesserung des antioxidativen Status der Tiere eher auf einer Stabilisierung der Darmflora als auf direkten antimikrobiellen Effekten beruht.

1.2.5 *Anti-inflammatorische Wirkung phytogener Futterzusätze*

Die Eingangs (Punkt 1, Seite 1) für junges Geflügel und Absetzferkel beschriebenen Durchfallerkrankungen gehen mit Entzündungsreaktionen in der Darmmukosa bzw. –wand einher. Der Schlüssel-Transkriptionsfaktor, der diese inflammatorischen Prozesse reguliert, ist der *Nuclear Factor 'kappa-light-chain-enhancer' of activated B-cells* (NFκB). Der aus den zwei Untereinheiten *p50* und *p65* bestehende NFκB liegt unter physiologischen (nicht inflammatorischen) Bedingungen inaktiv, an Inhibitorproteine (IκBs) gebunden, im Zytosol vor. Werden die IκBs durch Inhibitor kappa B-Kinasen (IKK) phosphoryliert, mit Ubiquitin markiert und abgebaut, kann der NFκB in den Kern translozieren und verschiedene pro-inflammatorische Zielgene, wie Adhesionsmolekühle, inflammatorische Zytokine, Wachstumsfaktoren, und anti-apoptotische Gene induzieren (Rayet und Gelinas 1999; Baldwin 2001). Die von diesen Zielgenen kodierten Proteine lösen dann die eigentliche Entzündungsreaktion aus. Durch Entzündungsmediatoren, wie den ebenfalls NFκB-regulierten Tumornekrosefaktor α (TNFα), die über das Blut im gesamten Organismus verteilt werden, liegt die Inflammation nicht unbedingt nur lokal begrenzt im Darm vor, sondern kann den gesamten Organismus betreffen. Diese Entzündungen können ebenfalls zu einer eingeschränkten Leistungsfähigkeit von Nutztieren führen.

Auch für pflanzliche Substanzen verschiedenster Herkunft konnten bereits in vitro und in wenigen in vivo Studien anti-inflammatorische Effekte gezeigt werden. Beispielsweise bewirkte das Curcumin der Gelbwurzel in vitro durch eine Inaktivierung von NFκB eine reprimierte Expression der Zielgene TNFα und IL6 (Jagetia und Aggarwal 2007). Weiterhin wurden in vitro durch Curcumin die Cox2, ICAM und VCAM in ihrer Expession gehemmt (Plummer et al. 1999, Madan et al. 2001, Colett und Campbell 2004). In einer weiteren in vitro-Studie führte das ätherische Öl des Rosmarins zu einer Hemmung der TNFα-vermittelten Monozytenadhesion (Lian et al. 2010), die eine frühe Phase in der Entzündungsreaktion darstellt. Für das Carnosol des Rosmarins wurden neben einer Verminderung der Expression pro-inflammatorischer Mediatoren (IL-1β, TNFα, IL-6, NFκB, Cox2) auch induzierende Effekte auf Nrf2-regulierte Xenobiotika-metabolisierende (Phase-II)

und antioxidative Enzyme beobachtet (Lian et al. 2010). Ähnliches wurde auch für Schweine, die 1,8-Cineol, das Leitterpen des Rosmarinöls, inhalierten, gezeigt (Bastos et al. 2010). Bei Absetzferkeln bewirkte der diätetische Zusatz einer Kombination von ätherischen Ölen aus Oregano, Anis und Zitronenschale (40 mg/kg Futter) eine verminderte Expression des NFκB und seiner Zielgene Cox-2 und TNFα (Kroismayr et al. 2008b). Dadurch wurden sowohl die Immunabwehr der Tiere entlastet als auch Nrf2-abhängige Detoxifikationsprozesse in der Leber reduziert (Kroismayr et al. 2008b).

Der im Rahmen einer jeden NFκB-vermittelten Entzündungsreaktion auftretende so genannte „*Oxidative Burst*“ (Freisetzung von reaktiven Sauerstoffspezies) beeinflusst durch den vermehrt entstehenden oxidativen Stress natürlich auch die redox-sensitive Keap1/Nrf2-ARE-Signalkaskade (Miguel 2010). Es ist daher wahrscheinlich, dass die direkten, im Besonderen aber die indirekten, antioxidativen Wirkungen phytogener Substanzen, vermittelt über den Transkriptionsfaktor Nrf2, in engem Zusammenhang mit der Reduktion von NFκB-regulierten Zielgenen stehen. Detailierte, wissenschaftlich belastbare und in die Praxis übertragbare in vivo-Studien zur Interaktion von Nrf2 und NFκB, insbesondere beim Einsatz von phytogenen Futterzusätzen in der Nutztierernährung, fehlen allerdings bis dato. Über den beschriebenen Reaktionsweg könnten phytogene Verbindungen positive Effekte gerade im Hinblick auf die Prävention und Therapie von Durchfallerkrankungen und den damit verbundenen Darmentzündungen ausüben.

2 Zielstellung

In Bezug auf den in der Einleitung geschilderten aktuellen Stand der Forschung zur Wirkung phytogener Futterzusätze ergaben sich für die vorliegende Arbeit die folgenden Fragestellungen mit verschiedenen Teilaspekten:

- Fragestellung 1:
  - In vivo-Evaluierung potenzieller leistungsfördernder Eigenschaften definierter phytogener Futterzusätze (Brokkoliextrakt, ätherische Öle aus Gelbwurzel, Oregano, Thymian und Rosmarin) in zwei verschiedenen praxisrelevanten Nutztierspezies (Broiler, Absetzferkel) unter physiologischen Bedingungen
  - In vivo-Untersuchung potenzieller direkter und indirekter Wirkungsmechanismen definierter phytogener Futterzusätze (Brokkoliextrakt, ätherische Öle aus Gelbwurzel, Oregano, Thymian und Rosmarin) in zwei verschiedenen praxisrelevanten Nutztierspezies (Broiler, Absetzferkel)
  - In vivo-Prüfung potenzieller antimikrobieller Eigenschaften definierter phytogener Futterzusätze (Brokkoliextrakt, ätherische Öle aus Gelbwurzel, Oregano, Thymian und Rosmarin) und Beeinflussung der Eubiose im Darmtrakt von Absetzferkeln

Die zur Bearbeitung der Fragestellung 1 durchgeführten Versuche (**V1** und **V2**) sind in detaillierter Form in Tabelle 3 dargestellt.

Da in der aktuellen Fütterungspraxis meist Mischungen verschiedener Kräuter, Extrakte und ätherischer Öle eingesetzt werden, welche sich nur ungenügend zur Charakterisierung der Wirksamkeit einzelner phytogener Substanzen eignen, sollte in dieser Arbeit eine vergleichende Bewertung unterschiedlicher phytogener Einzelsubstanzen getestet werden. Im Hinblick auf die zuvor beschriebenen direkten antioxidativen und antimikrobiellen Eigenschaften wurden zum einen drei ätherische Öle aus der Pflanzenfamilie der Labiatae ausgewählt: Origanum vulgare, Thymus vulgaris, Rosmarinus officinalis (Kulisic et al. 2007, Windisch et al. 2008, Burt et al. 2007b, Kilic 2006). Zum anderen wurden ein Brokkoliextrakt sowie ein ätherisches Öl der Gelbwurzel (Curcuma longa) aufgrund ihrer bereits beschriebenen Eigenschaft zur Induktion der Nrf2-ARE-Signalkaskade als Referenzsubstanzen gewählt (Mandlekar et al. 2006, Pugazhenthi et al. 2007, Hayes et al. 2008, Yoon et al. 2008; Dinkova-Kostova und Talalay 2008). In diesem Zusammenhang ist es erwähnenswert, dass auch Thymian im Nagertiermodel eine induzierende Wirkung auf Xenobiotika-metabolisierende Enzyme bewirkte (Sasaki et al. 2005).

Um die im ersten Versuch (**V1**) ermittelten direkten und indirekten Wirkmechanismen besser auf einen bestimmten Inhaltstoff des jeweiligen phytogenen Futterzusatzes zurückführen zu können, erfolgte im zweiten Versuch (**V2**) mit Absetzferkeln eine Standardisierung der eingesetzten phytogenen Testsubstanzen auf ihre jeweiligen Hauptkomponenten (150 mg/kg). Aufbauend auf den Ergebnissen der **Versuche 1** und **2**, die unter physiologischen Bedingungen mit gesunden Tieren durchgeführt wurden, ergab sich dann die zweite Fragestellung dieser Arbeit, die antiinflammatorische Wirksamkeit phytogener Futterzusätze nach Induktion einer Entzündung zu prüfen:

- Fragestellung 2:
  - Prüfung potenzieller anti-inflammatorischer Eigenschaften definierter phytogener Futterzusätze (Brokkoliextrakt, ätherische Öle aus Gelbwurzel, Oregano, Thymian und Rosmarin) in vitro an der porcinen Dünndarmzellline IPEC-J2 unter *Escherichia coli*-Infektion und in einem Rattenmodel mit milder DSS-induzierte Kolitis
  - Untersuchung potentieller Interaktionen zwischen antioxidativen und anti-inflammatorischen Wirkmechanismen phytogener Futterzusätze in vitro und in vivo unter Entzündungsstimulus

Die zur Bearbeitung der Zielstellung 2 durchgeführten Versuche (**V3** und **V4**) sind in detaillierter Form in Tabelle 3 dargestellt.

Da es sich bei dem in vitro-Versuch mit IPEC-J2-Zellen um ein reines Industrieprojekt, finanziert von der Firma DELACON Biotechnik Ges.m.b.H. handelte, sind die Ergebnisse bislang nicht publiziert. Der in vitro-Versuch (**V4**) wurde dabei in Zusammenarbeit mit der Freien Universität Berlin durchgeführt. Die Einordnung der Versuchsergebnisse in den wissenschaftlichen Kontext erfolgte zusammen mit den Ergebnissen der **Versuche 1** bis **3**, aus denen 3 Originalarbeiten mit Erstautorenschaft hervorgegangen sind, im Diskussionsteil der vorliegenden Dissertationsschrift.

Tabelle 3: Übersicht über die in der vorliegenden Arbeit durchgeführten Versuche (V1-V4)

	Getestete Substanz	Konzentration	Spezies/ Zelllinie	Untersuchte Parameter
V1	Brokkoliextrakt Gelbwurzelöl Oregano Thymian Rosmarin	3000 mg/kg Extrakt 150 mg/kg Öl 150 mg/kg Öl 150 mg/kg Öl 150 mg/kg Öl	Broiler (Ross 308)	- Futteraufnahme, Gewichtszunahme, Futterverwertung - TEAC und TBA-RS in ätherischen Ölen und Gewebe - Nrf2-regulierte xenobiotische und antioxidative Enzyme - Enzymatische Aktivität von SOD und GPx
Originalarbeit: Mueller K, Blum NM, Kluge H, Mueller AS. *Influence of broccoli extract and various essential oils on performance and expression of xenobiotic- and antioxidant enzymes in broiler chickens.* Br J Nutr. 2012 Vol.108, No. 4, 588-602				
V2	Brokkoliextrakt Gelbwurzelöl Oregano Thymian Rosmarin	150 mg/kg Sulforaphan 150 mg/kg ar-Turmeron 150 mg/kg Carvacrol 150 mg/kg Thymol 150 mg/kg 1,8-Cineol	Absetzferkel ((DL x DL) x Pietrain)	- Futteraufnahme, Gewichtszunahme, Futterverwertung - TEAC und TBA-RS in ätherischen Ölen und Gewebe - Nrf2-regulierte xenobiotische und antioxidative Enzyme - Bakterielle Untersuchung von Kot- und Mukosaproben
Originalarbeit: Mueller K, Blum NM, Kluge H, Bauerfeind R, Froehlich J, Mader A, Wendler KR, Mueller AS. *Effects of broccoli extract and various essential oils on intestinal and faecal microflora and on xenobiotic enzymes and the antioxidant system of piglets.* OJAS. 2012 Vol.2, No.2, 78-98. doi:10.4236/ojas.2012.22012				
V3	Brokkoliextrakt Gelbwurzelöl Thymian Rosmarin	8.750 mg/kg (2 mmol/kg Sulforaphan) 1.494 mg/kg (2 mmol/kg ar-Turmeron) 618 mg/kg (2 mmol/kg Thymol) 680 mg/kg (2 mmol/kg 1,8-Cineol)	Ratten (Wistar) mit und ohne milder DSS-Kolitis	- Futteraufnahme, Gewichtsentwicklung, Disease Activity Index - Histologische Untersuchung von Kolonkryoschnitten - NFκB regulierte inflammatorische Parameter - Nrf2-regulierte xenobiotische und antioxidative Enzyme
Originalarbeit: Mueller K, Blum NM, Mueller AS. *Examination of the anti-inflammatory, antioxidant, and xenobiotic-inducing potential of broccoli extract and various essential oils during a mild DSS-induced colitis in rats.* ISRN Gastroenterology. 2013 Vol. 2013, Article ID 710856, 14 pages. http://dx.doi.org/10.1155/2013/710856				
V4	Apfelextrakt 1 Apfelextrakt 2 Gelbwurzelöl Sulforaphan Glucoraphanin	2,5 – 10 µM 2,5 – 10 µM 1-100 µM 1,25 – 7,5 µM 1,25 – 7,5 µM	IPEC-J2-Zellen mit und ohne *Escherichia coli*-Infektion	- NFκB regulierte inflammatorische Parameter - Nrf2-regulierte xenobiotische und antioxidative Enzyme
Versuchsbeschreibung, Ergebnisse und Diskussion unter Punkt 3.4				

Weitere Details bezüglich der verwendeten Materialien und der angewandten Methodik sowie eine ausführliche Beschreibung und Diskussion der Ergebnisse der einzelnen Versuche (V1-V4) sind den Originalarbeiten bzw. dem Punkt 3.4 zu entnehmen. Abk.: DL, Deutsche Landrasse; DSS, Natriumdextransulfat; GPx, Glutathionperoxidase; NFκB, Nuclear factor 'kappa-light-chain-enhancer' of activated B-cells, Nrf2, Nuclear factor erythroid 2 related factor 2; SOD, Superoxiddismutase; TBA-RS, 2-Thiobarbituric Acid-Reaktive Substances; TEAC, Trolox® Equivalent Antioxidant Capacity.

3 Originalarbeiten

3.1 Mueller K, Blum NM, Kluge H, Mueller AS. *Influence of broccoli extract and various essential oils on performance and expression of xenobiotic- and antioxidant enzymes in broiler chickens.* Br J Nutr. 2012 Vol.108, No. 4, 588-602

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doi:10.1017/S0007114511005873

Influence of broccoli extract and various essential oils on performance and expression of xenobiotic- and antioxidant enzymes in broiler chickens

Kristin Mueller, Nicole M. Blum, Holger Kluge and Andreas S. Mueller*

Institute of Agricultural and Nutritional Sciences, "Preventive Nutrition Group", Martin Luther University Halle Wittenberg, Von Danckelmann Platz 2, D-06120 Halle (Saale), Germany

(Received 16 March 2011 – Revised 26 August 2011 – Accepted 3 October 2011)

Abstract

The aim of our present study was to examine the regulation of xenobiotic- and antioxidant enzymes by phytogenic feed additives in the intestine and the liver of broilers. A total of 240 male Ross-308 broiler chickens (1 d old) were fed a commercial starter diet for 2 weeks. On day 15, the birds were assigned to six treatment groups of forty birds each. The control (Con) group was fed a diet without any additive for 3 weeks. The diet of group sulforaphane (SFN) contained broccoli extract providing 0·075 g/kg SFN, whereas the diets of the other four groups contained 0·15 g/kg essential oils from turmeric (Cuo), oregano (Oo), thyme and rosemary (Ro). Weight gain and feed conversion were slightly impaired by Cuo and Oo. In the jejunum SFN, Cuo and Ro increased the expression of xenobiotic enzymes (epoxide hydrolases 1 and 2 and aflatoxin B1 aldehyde reductase) and of the antioxidant enzyme haeme oxygenase regulated by an 'antioxidant response element' (ARE) compared to group Con. In contrast to our expectations in the liver, the expression of these enzymes was decreased by all the additives. Nevertheless, all the additives increased the Trolox equivalent antioxidant capacity of the jejunum and the liver and reduced Fe-induced lipid peroxidation in the liver. We conclude that the up-regulation of *ARE* genes in the small intestine reduces oxidative stress in the organism and represents a novel mechanism by which phytogenic feed additives improve the health of farm animals.

Key words: **Chickens: Phytogenic feed additives: Broccoli extract: Essential oils: Xenobiotic enzymes: Antioxidant system**

Since the ban on antibiotic feed additives in the European Union (EU) in 2006, research in alternative substances has gained in importance. In particular for growing broilers and for weaned pigs, several feed additives have been investigated with regard to the prevention of diarrhoea and to increasing general performance and health. Besides pre- and/or probiotics and organic acids, phytogenic substances are most commonly used for this purpose. Phytogenic feed additives comprise a heterogenous group of plant-derived products including herbs, spices, essential oils or other preparations. In particular, labiatae plant oils from *Origanum vulgare*, *Thymus vulgaris* and *Rosmarinus officinalis* have been reported to promote the performance and health of poultry via three different mechanisms[1].

The first mechanism suggests that essential oils (EO) positively affect the growth and performance of chickens by improving feed palatability, secretion of digestive enzymes and nutrient digestibility. Data regarding this topic are, however, not consistent[2–5].

The second mechanism suggests that the EO of labiatae plants exert antibiotic-like bactericidal and/or bacteriostatic effects on several pathogenic intestinal micro-organisms. Most of the studies in this context have been carried out *in vitro*, frequently in the context of food safety of meat products. For instance, carvacrol, the main monoterpene compound in oregano- and thymbra spicata oil, exhibited a distinct bactericidal activity against *Escherichia coli*, including an enterohaemorrhagic strain[6,7], *Salmonella enterica*[8], *Staphylococcus epidermis* and *S. aureus*, and numerous other bacteria[9]. However, the limitation of the bactericidal effects of EO was demonstrated in an experiment with Caco-2 cells. The addition of oregano oil (Oo) and thyme oil (To) to the culture media in a concentration (0·05 %) that strongly inhibited the growth of enteroinvasive *E. coli* also

Abbreviations: ABCC2, ATP-binding cassette subfamily C member 2; AFAR, aflatoxin B1 aldehyde reductase; ARE, antioxidant response element; ARP1, acid ribosomal protein 1; Con, control; Cu/Zn SOD, cytosolic Cu/Zn superoxide dismutase; Cuo, turmeric oil; CYP1A1, cytochrome P450 family 1 subfamily A polypeptide 1; ELFA, elongation factor 1α; EO, essential oils; EPHX1, microsomal epoxide hydrolase; EPHX2, cytosolic epoxide hydrolase; EU, European Union; GAPDH, glycerine aldehyde-3-phosphate dehydrogenase; GPx, glutathione peroxidase; GPx1, cytosolic glutathione peroxidase; GPx2, gastrointestinal glutathione peroxidase; GST, glutathione-*S*-transferases; HMOX, haeme oxygenases; Keap1, Kelch-like erythroid CNC homologue-associated protein 1; Mn SOD, mitochondrial Mn superoxide dismutase; Nrf2, nuclear factor erythroid 2-related factor 2; Oo, oregano oil; Ro, rosemary oil; SFN, sulforaphane; SOD, superoxide dismutase; TBARS, thiobarbituric acid-reactive substances; TEAC, Trolox equivalent antioxidant capacity; To, thyme oil; TrxR, thioredoxin reductase.

* **Corresponding author:** Professor A. S. Mueller, fax +49 345 5527124, email andreas.mueller@landw.uni-halle.de

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was harmful to the cells, whereas lower EO concentrations (0·01 %), causing no cell damage, exhibited a drastically reduced bactericidal activity[10]. However, the *in vivo* bactericidal activity of the EO from labiatae plants *in vivo* is in doubt. Whereas two trials with growing broilers reported that even low dietary concentrations of carvacrol (100 mg/kg)[11] and thymol (15 mg/kg)[12] had a bactericidal activity against *E. coli* and Clostridiae, or influenced the growth of Lactobacillae positively[11–13], in another study an even higher concentration of Oo (1000 mg/kg) failed to influence bacterial counts in faeces and in caecal chymus[4].

The third mechanism suggests that the EO of labiatae plants positively affect animal health by direct antioxidant effects based on the high availability of the phenolic terpenes carvacrol and thymol. The TROLOX® equivalent, also referred to as Trolox equivalent antioxidant capacity (TEAC), is a common method to study the antioxidant potential of antioxidant compounds in comparison to the water-soluble vitamin E derivative TROLOX® as the reference substance. *In vitro* testing of the TEAC value (mmol TROLOX®/g DM) of ethanolic extracts of twenty-six spices showed that oregano had the highest antioxidant potential within labiatae plants (TEAC value: 100·7), followed by rosemary (38·8) and thyme (38·1)[14].

Beside the EO of labiatae plants, similar mechanisms improving the performance and health of farm animals have been reported for Zingiberaceae plant extracts, particularly for turmeric (*Curcuma longa* L.). Comparable to labiatae oils, turmeric oil (Cuo) contains a highly active terpene, the sesquiterpene ar-turmerone. Ar-turmerone has been demonstrated to evolve a strong bactericidal activity against several micro-organisms[15]. The limited direct antioxidant activity of Cuo (TEAC value: approximately 25·5)[16] compared to labiatae oils derives from the fact that ar-turmerone is not a phenolic terpene.

Broccoli extract, containing the isothiocyanate sulforaphane (SFN) in the form of its glucosinolate precursor glucoraphanin, is a phytogenic substance that has not yet been studied as a feed additive, and it is not yet permitted in the EU. In human nutrition, broccoli extract and SFN are generally accepted dietary supplements exerting a high anti-carcinogenic potential, in particular against intestinal cancers[17]. The safety of broccoli extract has been proven for humans[18]. Promising results for SFN have also been published with regard to a strong bactericidal activity against various pathogenic intestinal micro-organisms[19]. The anti-carcinogenic effects of turmeric and of SFN are based on the induction of genes with an 'antioxidant response element' (ARE) in their DNA promoter. ARE containing genes include xenobiotic- and antioxidant enzymes such as glutathione-*S*-transferases (GST), epoxide hydrolases, aflatoxin B1 aldehyde reductases (AFAR), haeme oxygenases (HMOX), thioredoxin reductase (TrxR) and cytosolic Cu/Zn-superoxide dismutase (Cu/Zn SOD) 1[20–26].

ARE gene induction depends on the transcription factor 'nuclear factor erythroid 2-related factor 2' (Nrf2). When the cells are protected sufficiently against oxidative stress, Nrf2 is associated with the Kelch-like erythroid CNC (cap-'n'-collar) homologue-associated protein 1 (Keap1) in the cytosol. Oxidative stress or electrophiles like SFN or terpenes modify Keap1 at redox-sensitive –SH-groups[27], leading to Nrf2 liberation and its nuclear translocation. Subsequently, Nrf2 binds to the ARE promoter sequence of the aforementioned xenobiotic- and antioxidant enzymes and initiates the up-regulation of their gene expression[28].

In contrast to a number of studies that have investigated the beneficial effects of phytogenic feed additives via the mechanisms (1)–(3), their indirect antioxidant potential via the induction of xenobiotic- and antioxidant enzymes has not been studied in farm animals till now. To the best of our knowledge, only one trial has reported on the induction of xenobiotic enzymes by thyme in mouse liver[29].

Consequently, we aimed to study the induction of ARE-regulated xenobiotic- and antioxidant enzymes by various EO from labiatae plants in the intestine and the liver of fast-growing broiler chickens. We used broccoli sprouts extract and Cuo as reference substances, both having a proven impact on *ARE* gene expression. Moreover, we studied the effects of broccoli extract, not yet permitted as a feed additive in the EU, on performance parameters.

Methods and materials

Bird husbandry and diets

The protocol of the broiler study was approved by the Regional Council of Halle and by the Animal Welfare Committee of the Martin Luther University Halle-Wittenberg (record token: 45.202-3-559 MLU). Further, a certificate of exemption for feeding the broccoli sprouts extract, not yet permitted to be used as a feed additive in the EU, was attested by the veterinary administrative office, Saxony Anhalt, Halle (record token: 203.2.1/22·10).

A total of 240 male Ross-308 broiler chickens (1 d old) (mean body weight: 41·9 (SE 0·57) g) were obtained from a hatchery (Geflügelhof Möckern ZN (Zucht and Nutzvieh) der Lohmann & Co. AG, Möckern, Germany) and fed a commercial starter diet (Landkornstarter, DEUKA, Könnern, Germany) without phytogenic feed additives for 14 d. On day 15, the birds were assigned to the six experimental groups of forty birds each, with a mean live weight of 442 (SE 14·7) g. Broilers were kept in a stainless-steel cage battery in groups of eight birds per cage and fed the experimental diets for 21 d. The experimental design included five cages of eight birds per diet. The broilers had free access to their diets and water. During the experiment, temperature, humidity and lighting were controlled. The temperature was gradually reduced from 34°C on day 1 to 19°C on day 35. The lighting regime consisted of a 12 h light–4 h dark–4 h light–4 h dark cycle. The light intensity of 20 lux and all other housing conditions were in accordance with the recommendations for poultry of the Society for Laboratory Animal Science[30]. During the 21 d experimental period, the control (Con) group was fed a diet, meeting the nutritional requirements of growing broilers, without a phytogenic additive. Minerals, vitamins and essential amino acids were added to all diets as recommended for broilers by the Society of Nutrition Physiology[31] and the National Research Council[32]. The diet of group SFN contained 3000 mg/kg broccoli sprouts extract providing 75·0 mg/kg SFN, whereas 150·0 mg/kg of the

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essential oils from *C. longa* (Cuo), *T. vulgaris* (To), *O. vulgare* (Oo) and rosemary oil (*R. officinalis*, Ro) were added to the diets of the other four experimental groups The active ingredients in the broccoli sprouts extract and the EO, as provided by the manufacturers, had the following concentrations (g/100 g = %): broccoli sprouts extract (SFN, 5·00), Cuo (arturmerone, 30·0), Oo (carvacrol, 65·0), To (thymol, 49·0) and Ro (1,8-cineole, 46·0). Premixes of all phytogenic feed additives were prepared in 20 g wheat bran and 10 g sunflower oil and added to 970 g of the basal diet (Table 1). All diets were pelleted with a pellet mill using a 3 mm dye. Feed intake and individual live weight were recorded once a week. Feed conversion was calculated from the ratio of feed intake (g) and weight gain (g). On day 35, the broilers were killed after stunning for organ sampling (liver, small-intestine mucosa and colon). Small-intestine mucosa samples were taken from a 15 cm jejunal segment located 10 cm distal to the duodenum. Colon tissue was taken from a 10 cm segment located distal to the caecum.

RNA preparation and real-time RT-PCR assay including stability analysis of four selected reference genes in the jejunal mucosa, colon and liver

Relative mRNA expression levels were measured for GSTα, HMOX1, TrxR1, microsomal epoxide hydrolase 1 (EPHX1), cytosolic epoxide hydrolase 2 (EPHX2), AFAR, cytochrome P450, family 1, subfamily A, polypeptide 1 (CYP1A1) and ATP-binding cassette, sub-family C (cystic fibrosis transmembrane conductance regulator multidrug resistance-associated protein; CFTR/MRP), member 2 (ABCC2). Therefore, total RNA was isolated from the liver, jejunal mucosa and colon of four birds per repetition and the experimental group (n 120, half the number of experimental birds) using Trizol® reagent (Invitrogen GmbH, Darmstadt, Germany), according to the manufacturer's protocol. RNA concentration and purity were evaluated photometrically at 260 and 280 nm. Additionally, RNA quality was controlled by checking the integrity of the 18S- and 28S-ribosomal RNA bands and by controlling the absence of genomic DNA. In brief: following the denaturation of 4·5 µl of diluted RNA (0·5 µg/µl) with 2·0 µl 5× gel running buffer, 3·5 µl formaldehyde and 10 µl formamide at 70°C for 10 min, the samples were chilled on ice. Then, 1·0 µl of a ethidium bromide solution (1·0 µg/µl) and 2·0 µl of sample loading buffer were added and the samples were run in 1·2 % agarose gels, containing 2·2 M-formaldehyde and visualised under a UV-imager (Syngene, Cambridge, UK). Subsequently, 1·5 µg of the RNA of two birds per treatment were pooled and subjected to reverse transcription using a commercial complementary DNA synthesis kit (RevertAid™ First Strand synthesis kit; Fermentas GmbH, St Leon-Rot, Germany). In this manner, n 10 complementary DNA pools per treatment were generated and could be subjected to mRNA expression analysis by real-time detection PCR (RT-PCR) using a Rotorgene 6000 apparatus (Corbett Research/QIAGEN GmbH, Hilden, Germany). The complementary DNA obtained by reverse transcription (20 µl) was diluted 2·5-fold (final volume 50 µl) with diethylpyrocarbonate-treated sterile water. The standard PCR protocol consisted of an initial denaturation step (95°C, 3 min), followed by 25–32 amplification cycles (denaturation: 95°C, 25 s, annealing: 60°C, 30 s, and elongation: 72°C, 55 s). Subsequent to the identification of the correct length of the amplification products in 1·2 % agarose gels, relative quantification of mRNA expression was performed using the $\Delta\Delta C_t$ method[33]. In accordance with the current guidelines for the proper determination of gene expression data in various tissues (MIQE guidelines)[34], a set of four reference genes was selected and their expression stability (M) was ranked according to the standard procedure[35]. Acid ribosomal protein 1 (ARP1), glycerine aldehyde-3-phosphate dehydrogenase (GAPDH), elongation factor 1α (ELFA) and β-actin were selected as capable reference genes reported in current literature[36,37], and their expression (C_t values) was measured in the jejunum, the colon and the liver. The treatment independent expression stability (M) was determined by calculating the C_t ratios of one gene with all the other genes. Subsequently, the standard deviation of the logarithmically transformed ratios was calculated and plotted[35]. According to their expression stability M, indicated by a decrease of standard deviation, a ranking of the most stable reference genes was compiled for each tissue investigated. The best set of housekeeping genes was used for normalisation of the expression data of the target genes. The expression values of the target genes were normalised using the arithmetic mean of the C_t values of ARP1 and β-actin in the jejunal mucosa, of GAPDH and ELFA in the colon, and of GAPDH and β-actin in the liver.

The primers used in PCR and their gene bank accession numbers were as follows:

Table 1. Composition of the basal diet*

Ingredient	Basal diet (g/kg)
Wheat (DEUKA GmbH und Company KG, Könnern, Germany)	500·0
Maize (DEUKA GmbH und Company KG)	165·5
Soyabean meal, 44 % CP (DEUKA GmbH und Company KG)	250·0
Soyabean oil	40·0
Calcium phosphate (Sigma-Aldrich, Taufkirchen, Germany)	10·0
Calcium carbonate (Sigma-Aldrich)	15·0
NaCl (Sigma-Aldrich)	3·00
Vitamin and mineral premix†	8·00
Lysine hydrochloride (Sigma-Aldrich)	4·00
DL-Methionine (Degussa, Duesseldorf, Germany)	3·00
L-Threonine (Sigma-Aldrich)	1·50
Total	1000
$AME_{N\text{-corrected}}$ (calculated; MJ/kg)	12·73

AME, apparent metabolisable energy; CP, crude protein

*The complete diets had the following nutrient contents according to the National Research Council recommendations for poultry[32] and did not differ between the diets: DM (analysed), 91 %; gross energy (analysed), 17·25 MJ/kg; $AME_{N\text{-corrected}}$ (calculated), 12·88 MJ/kg (the deviation in $AME_{N\text{-corrected}}$ between basal and complete diet resulted from adding the different phytogenic feed additives in terms of premixes as described in Methods and materials); crude fat (analysed), 66·4 g/kg DM; CP (analysed), 201 g/kg DM; fibre (analysed), 41·0 g/kg DM; crude ash (analysed), 59·8 g/kg DM.

†Premix supplied the following according to the supplier (BASU-Mineralfutter GmbH, Bad Sulza, Germany; per kg of complete diet): Ca, 2·3 g; vitamin A, 3·6 g (as retinyl acetate); cholecalciferol, 0·008 mg; vitamin E, 38·22 g (as DL-α-tocopheryl acetate); vitamin K_3, 2 mg; thiamine, 2 mg; riboflavin, 6·6 mg; vitamin B_6, 5 mg; vitamin B_{12}, 0·02 mg; niacin, 99 mg; folic acid, 1 mg; biotin, 0·15 mg; Ca-D-panthothenate, 15 mg; choline chloride, 0·7 g; Cu, 5 mg; Zn, 51 mg; Fe, 60 mg; Mn, 71 mg; I, 0·6 mg; Se, 0·2 mg.

ARP1 (X13876.1), primer forward (5′ → 3′): ATC GAC ATC G GA AGC CTC AT, primer reverse (5′ → 3′): GAC CAA AGC CCA TGT CAT CA; GAPDH (NM204305.1), primer forward (5′ → 3′): CCT CTC TGG CAA AGT CCA AG, primer reverse (5′ → 3′): TCT CCA TGG TGG TGA AGA CA; ELFA (L00677.1), primer forward (5′ → 3′): ACC TCT GCG TCT GCC TCT TC, primer reverse (5′ → 3′): TTC GCT AAG GGC TTC ATG GT; β-actin (LO8165), primer forward (5′ → 3′): ATG AAG CCC AGA GCA AAA GA, primer reverse (5′ → 3′): GGG GTG TTG AAG GTC TCA AA; GSTα (NM001001777), primer forward (5′ → 3′): TTC TCT CCA CCT GAG GCA AAG, primer reverse (5′ → 3′): GGC TTC CAT GAG CTG AAC ATC; HMOX1 (NM205344), primer forward (5′ → 3′): CTG GAG AAG GGT TGG CTT TCT, primer reverse (5′ → 3′): GAA GCT CTG CCT TTG GCT GTA; TrxR1 (NM001030762), primer forward (5′ → 3′): AGT CAT TTC TGG CCA CTG GAA, primer reverse (5′ → 3′): TTGGTGATGGACAGA-GTGGTG; EPHX1 (XM419497), primer forward (5′ → 3′): CAA GTG ATG CTT GGG GCT TAC, primer reverse (5′ → 3′): ACC TGC AGT GTC TGG TTT GGT; EPHX2 (NM001033645), primer forward (5′ → 3′): GAA AGC CCT TAT CCG TTC CAC, primer reverse (5′ → 3′): GGT CTC ATG TTC CGG TAC CAA; AFAR (XM417628·2), primer forward (5′ → 3′): CAA ACT GCA GGG TTC TCT TGG, primer reverse (5′ → 3′): GAA GTA GTT GGG GCA GTC GTG; CYP1A1 (NM205146), primer forward (5′ → 3′): GAAGATTCAGGCAGAGCTGGA, primer reverse (5′ → 3′): AGT AGC CAT TCA GCA CCG TGT; ABCC2 (XM421698), primer forward (5′ → 3′): CCG CAG CAT CAG TAC ACA GAG, primer reverse (5′ → 3′): GAA GGA AAA GCC CAA ACC AAC.

Differential superoxide dismutase activity in the liver

The differential measurement of SOD activity (total SOD, mitochondrial Mn SOD (Mn SOD) and Cu/Zn SOD) in the jejunum and the liver was assayed using a photometric standard procedure in which the inhibition of pyrogallol (1,2,3-trihydroxybenzol) autoxidation by the SOD activity of the samples is recorded[38]. Following this, 1:5 (w/v) crude homogenates of the liver were prepared in 0·1 M-potassium phosphate buffer (pH 6·5). The formation of purpurogallin by pyrogallol oxidation was measured for 3 min at 420 nm. Each determination included a blank without the liver homogenate. Here, one unit of SOD activity was defined as the 50 % inhibition of pyrogallol autoxidation to purpurogallin by the samples' SOD activity. The determination of Mn SOD was carried out as previously mentioned, in 50 mM-Tris succinate buffer, containing additionally 100 mM-potassium cyanide to inhibit Cu/Zn SOD. Activity of Cu/Zn SOD was calculated from the difference of total SOD and Mn SOD activity. Data were normalised to 1 mg of protein. Organ sample pools were generated in an analogous manner as described for mRNA expression. The SOD activity of each sample pool (n 10) was measured in duplicate.

Glutathione peroxidase 1 activity in the liver and combined activity of glutathione peroxidases 1 and 2 in the jejunum

The activity of liver cytosolic glutathione peroxidase (GPx1) and the combined activity of GPx1 and gastrointestinal glutathione peroxidase (GPx2) in the jejunal cytosolic supernatant were measured spectrophotometrically (Ultrospec 3300 pro; Amersham Pharmacia Biotech, Freiburg, Germany) at 340 nm using the assay protocol coupled to glutathione reductase and NADPH[39]. NADPH oxidation, which is proportional to glutathione peroxidase (GPx)-dependent peroxide reduction, was recorded for 3 min. For both enzymes, H_2O_2 was used as substrate. Here, one unit of GPx1 and of combined GPx1- and GPx2 activity was defined as 1 μmol NADPH oxidised per min and normalised to 1 mg protein. Organ sample pools were generated in an analogous manner as described for mRNA expression. GPx activity of each sample pool (n 10) was measured in duplicate.

Trolox equivalent antioxidant capacity = TROLOX® equivalent in the essential oils, the jejunal mucosa and the liver

TEAC in 1:5 (w/v) crude homogenates of the liver and of the jejunal mucosa as well as of the essential oils used in the study was measured using the method originally described by Miller *et al.*[40] with modifications of Wang *et al.*[41]. The method is based on monitoring the inhibition of 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid (ABTS) radical formation spectrophotometrically at 600 nm and 20°C for 15 min. The reaction mixture contained PBS buffer, ABTS reagent (0·15 mM), H_2O_2 (0·1 mM) and metmyoglobin (2·50 μM). Since TROLOX® inhibitory capacity decreases progressively with time, the sample TEAC values were calculated by comparison to the TEAC values of a TROLOX® standard curve (concentration range: 0–21·0 μM). The comparisons were done for the linear range of the reaction. Mean TEAC values for the liver and the jejunal mucosa were calculated as the arithmetic mean of the individual TEAC values measured after 3, 5 and 10 min. Before the determination of the TEAC values of the EO, a dilution with 70 % (v/v) ethanol was carried out. The TEAC values of the samples were expressed in μmol TROLOX® equivalent per g organ fresh matter or mmol TROLOX® equivalent per 100 g essential oil. Organ samples were pooled in an analogous manner as described for mRNA expression. The TEAC value of each sample pool (n 10) was measured in duplicate.

2-Thiobarbituric acid-reactive substances in the liver

2-Thiobarbituric acid-reactive substances (TBARS) were measured in the liver of the broilers as a parameter of lipid peroxidation according to a modified protocol from Wong *et al.*[42]. TBARS concentration was measured in liver samples subsequent to the provocation of lipid peroxidation with $FeSO_4$ in order to test their antioxidant capacity. Then, 25 μl of the 1:5 (w/v) liver crude homogenates were mixed with 375 μl H_3PO_4 (0·44 M) in sealable glass tubes. After the addition of 25 μl $FeSO_4$ solution (0·05 M), 200 μl aqua bidest and 125 μl 0·6 % 2-thiobarbituric acid, the samples were incubated in a thermo block at 100°C for 60 min. Determination of the blank was carried out, using 25 μl of potassium phosphate buffer (0·1 M) instead of the liver homogenate. Following the incubation at 100°C, the samples were chilled on ice and

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750 µl of methanolic NaOH (10 ml of 1 M-NaOH + 90 ml methanol) were added. After thorough vortexing and centrifugation at 4000 ***g*** at 4°C for 10 min, the extinction was measured spectrophotometrically at 532 nm. Sample TBARS concentrations were calculated from a calibration curve prepared with 1,1,3,3,-tetraethoxypropane in a concentration range of 0·60–1·20 µM. TBARS concentration of each sample pool (n 10) was measured in duplicate.

Protein concentration of samples

Protein concentration in the liver cytosol, jejunal mucosa, colonic tissue and in the samples for liver immunoblot analysis of Nrf2 was determined using the standard method of Bradford[43], adapted to the needs for measurement in a ninety-six-well plate reader.

Immunoblot analysis of nuclear factor erythroid 2-related factor 2 in whole liver cell lysate

For the analysis of Nrf2 protein expression in whole liver cell lysate 1:10 (w/v), liver homogenates were prepared in a non-reducing radioimmuno precipitation assay (RIPA) lysis buffer (50 mM-Tris–HCl, 150 mM-NaCl, 1 mM-phenylmethylsulphonylfluoride, 1 mM-EDTA, 1·0 % sodium desoxycholate, 0·1 % SDS and 1 % TritonX-100, pH 7·4). Then, 60 µg of protein were separated according to the standard method[44] under non-reducing conditions on 10 % SDS-polyacrylamide gels (50 mA, 4°C, 2 h). Separated proteins were transferred onto a polyvinylidene membrane (PALL Biotrace 0·45 µm™; Pall GmbH, Dreieich, Germany) by semi-dry blotting (25 min at a constant 6 V (approximately 60 mA)). After blocking the membranes overnight at 4°C in Tris-buffered saline-Tween (TBST) (20 mM-Tris–HCl, 150 mM-NaCl, 0·1 % Tween 20, pH 7·6) containing 5 % non-fat dry milk and 0·2 % bovine serum albumin, the analysis was continued by a 12 h incubation with the first antibody, a polyclonal anti-rabbit-Nrf2 antibody (Abcam, ab 31 163), in TBS (1:1000) followed by a 1 h incubation with the secondary antibody (1:3000) linked to horseradish peroxidase (Goat Anti-Rabbit IgG-h + l). Subsequent to three washes with TBST, the protein bands were detected using an ECL-kit (GE Healthcare Europe GmbH, Freiburg, Germany). Optical density of the 57 kDa band, representing active Nrf2, and of the 101 kDa band, representing ubiquitinated Nrf2 (http://www.abcam.com/index.html?pageconfig=reviews&intAbID=31 163), were evaluated with a Phoretix TotalLab TL100 imager (BioStep GmbH, Jahnsdorf, Germany) after scanning the membranes with the biostep Bio-Imaging Systems F-ChemiBIS 3·2M luminescence reader (Berthold Technologies, Bad Wildbad, Germany). The intensity of the Nrf2 bands was normalised to β-actin, carried along as the standard. Moreover, the ratio of active Nrf2:ubiquitinated Nrf2 was calculated. Whole liver tissue protein pools (n 10) were generated in an analogous manner as described for mRNA expression. Immunoblot analysis was carried out in duplicate for four selected sample pools.

Statistical analysis

Data are presented as means with their standard errors (except for Table 6 and related remarks). Following assurance of the normality of distribution (Shapiro–Wilk test and Kolmogorov–Smirnov test) and the homogeneity of variances (Levene's test), the data were analysed with SPSS 19.0 (SPSS, Inc., Chicago, IL, USA) for Windows using the one-way ANOVA procedure. If variances were homogenous, significant differences between means ($P<0·05$) were evaluated with the least significant difference-test; if not, the Games Howell test was used. Box plots for the analysis of reference gene stability were generated with SPSS 19.0 for Windows (SPSS, Inc.). Other figures were prepared with Microsoft Excel (version 2003; Microsoft Corporation, Redmond, WA, USA).

Results

Performance parameters (feed intake, body weight, weight gain, feed conversion)

All birds showed no clinical abnormalities during the whole experiment, and no broilers were lost. Both in the initial feeding period (days 1–14) on the commercial starter diet and in the experimental period on diets with the phytogenic feed additives, feed intake did not differ between groups (Table 2). Weight gain between groups was not different in the initial feeding period and in the first 2 weeks of the experimental period (Table 4).

However, in week 5 (days 29–35), broilers of the Cuo group showed a significantly reduced weight gain compared to group Con and to all the other experimental groups with phytogenic feed additives (SFN, Oo, To, Ro). The lower weight gain of group Cuo compared to group Con was also apparent when weight gain data for the experimental period (days 15–35) and the whole experiment (days 1–35) were compared. Broilers of the Oo group produced a significantly lower weight gain over the whole experimental period (days 1–35) compared to Con broilers (Tables 3 and 4). Consequently, broilers of groups Cuo and Oo had a reduced feed conversion compared to Con birds (Table 5). Although no statistical significances could be analysed, broilers of the other groups receiving diets with phytogenic feed additives (SFN, To, Ro) also showed an overall somewhat lower performance than Con broilers, as indicated by weight gain and feed conversion.

Selection of reference genes

Table 6 shows the medians of the C_t values of the four selected reference genes GAPDH, ARP1, ELFA and β-actin in the jejunal mucosa, the colon and the liver with their individual standard deviations. Calculation of the percentual standard deviation revealed the following results: In the jejunum, the C_t values of β-actin produced the highest percentual standard deviation from the median (±7·58 %), whereas that of ARP1 (±6·21 %) was the lowest. Jejunal GAPDH (7·47 %) and ELFA (±7·08 %) showed the highest C_t values, with intermediate values for the percentual standard deviation. In the colon, the following percentual standard deviations were calculated: GAPDH (±5·55 %), ARP1 (±6·46 %), ELFA (±4·67 %) and β-actin (±6·18 %). Data for the liver percentual standard deviation

Table 2. Feed intake (g) of growing broilers fed diets containing different phytogenic additives for 21 d*
(Mean values with their standard errors)

	Initial period		Experimental period									
	Days 1–14		Days 15–21		Days 22–28		Days 29–35		Days 15–35		Days 1–35	
Group	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
Feed intake (g)												
Con	36·0	1·60	107·2	0·83	149·2	0·14	191·5	3·55	149·3	1·44	104·0	1·44
SFN	36·2	2·20	106·5	2·51	147·6	1·61	194·8	1·65	150·1	1·22	104·5	1·52
Cuo	36·6	0·34	109·4	0·84	145·5	3·79	195·6	3·81	151·4	1·36	105·5	0·76
Oo	35·4	1·46	102·0	2·80	144·6	1·71	188·9	3·48	146·7	1·78	102·2	1·55
To	36·5	1·96	109·9	1·37	145·0	4·02	191·9	5·73	150·8	2·77	104·1	1·90
Ro	34·6	1·56	102·5	3·87	145·2	1·78	188·3	3·35	146·8	2·24	101·9	1·87

Con, control; SFN, sulforaphane; Cuo, turmeric oil; Oo, oregano oil; To, thyme oil; Ro, rosemary oil.
* For details of diets and procedures, see the Methods and materials section.

were: ±4·70 % for GAPDH, ±10·4 % for ARP1, ± 6·42% for ARP1 and ±3·61% for β-actin.

To select the most stable pair of reference genes, expression stability (*M*) was recalculated after repetitive exclusion of the worst performing gene. The most stable pairs of reference genes after successive exclusion were ARP1 and β-actin in the jejunal mucosa, GAPDH and ELFA in the colon, and GAPDH and β-actin in the liver (Table 6).

Expression of antioxidant response element-regulated xenobiotic-and antioxidant enzymes in the jejunum, the colon and the liver

The analysed normalised gene expression pattern of selected ARE-regulated xenobiotic-and antioxidant enzymes depended on the phytogenic feed additive and on the organ investigated. The jejunal expression of all ARE-regulated genes was up-regulated by the reference additives SFN and Cuo compared to group Con (Table 7, jejunum), with significant changes for HMOX1 (average factor: 1·73), EPHX1 (average factor: 2·10) and AFAR (average factor: 2·10). Among the labiatae oils, only Ro effected a significant up-regulation of HMOX1, EPHX1 and TrxR1 compared to group Con, whereas Oo and To produced no significant changes. In the colon, the expression pattern originating from feeding the phytogenic feed additives differed distinctly from the jejunal profile (Table 7, colon). The number of ARE-regulated genes influenced by the reference additives SFN and Cuo was distinctly lower in the colon than in the jejunum. AFAR was the only colonic gene showing a significant up-regulation by SFN addition to the diet (factor: 1·82) compared to the Con group. Cuo solely increased colonic TrxR1 expression by the factor 2·41. Ro influenced the expression of colonic ARE-regulated genes most powerful, including HMOX1 (factor: 1·98), EPHX1 (factor: 1·32) and TrxR1 (factor: 2·31). Interestingly, Oo and To that remained without a significant influence on the jejunal expression of ARE-regulated genes had the highest impact on colonic TrxR1 expression (average factor: 3·52). In addition, colonic AFAR expression also responded significantly to Oo treatment (factor: 1·96). In contrast to our expectations, both the reference additives SFN and Cuo and the labiatae oils Oo, To and Ro caused a more or less pronounced down-regulation of the mRNA expression of the ARE-regulated enzymes in the liver of the broilers (Table 7, liver). In particular, Cuo and Ro decreased the mRNA concentrations of the antioxidant enzymes HMOX1 and TrxR1, and that of the epoxide metabolising enzymes EPHX1 and 2 to a level of 40–50% of that analysed in the Con group (Table 7,

Table 3. Body weight (g) of growing broilers fed diets containing different phytogenic additives for 21 d*
(Mean values with their standard errors)

	Initial period		Experimental period							
	Day 1		Day 14		Day 21		Day 28		Day 35	
Group	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
Body weight (g)										
Con	41·0	0·57	444·9	20·8	927·5	23·1	1465·2^{a}	15·2	2350·9^{a}	26·9
SFN	42·2	0·67	443·2	16·9	917·6	21·9	1444·4^{a,b}	23·8	2301·4^{a,b}	19·7
Cuo	41·3	0·52	437·5	9·68	913·0	17·7	1427·0^{a,b}	29·6	2234·3^{b}	37·7
Oo	41·8	0·54	441·8	15·8	905·8	20·5	1401·8^{b}	12·5	2248·3^{b}	42·7
To	41·8	0·54	442·9	13·0	928·6	19·4	1446·1^{a}	8·79	2307·6^{a,b}	27·9
Ro	43·4	0·58	440·2	12·1	907·6	21·4	1421·0^{a,b}	22·8	2279·6^{a,b}	30·1

Con, control; SFN, sulforaphane; Cuo, turmeric oil; Oo, oregano oil; To, thyme oil; Ro, rosemary oil.
^{a,b} Mean values with unlike superscript letters within a column were significantly different in the least significant difference test or the Games Howell test ($P<0·05$).
* For details of diets and procedures, see the Methods and materials section.

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Table 4. Weight gain (g) of growing broilers fed diets containing different phytogenic additives for 21 d*
(Mean values with their standard errors)

	Initial period		Experimental period									
	Days 1–14		Days 15–21		Days 22–28		Days 29–35		Days 15–35		Days 1–35	
Group	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
Weight gain (g/d)												
Con	28·9	1·31	68·9	0·60	76·8	3·60	126·5^{a}	2·91	90·8^{a}	0·80	66·0^{a}	0·68
SFN	28·6	1·08	67·8	1·23	75·3	3·06	122·4^{a}	1·63	88·5^{a,b}	0·39	64·6^{a,b}	0·51
Cuo	28·3	0·62	67·9	1·40	73·4	4·30	115·3^{b}	3·27	85·6^{b}	1·45	62·6^{b}	0·95
Oo	28·6	1·00	66·3	1·36	70·9	1·46	120·9^{a}	10·1	86·0^{b}	1·16	63·0^{b}	1·09
To	28·7	0·82	69·4	1·63	73·9	1·97	123·1^{a}	2·77	88·8^{a,b}	1·30	64·7^{a,b}	0·71
Ro	28·4	1·74	66·8	1·51	73·8	1·69	122·2^{a}	3·61	87·6^{a,b}	0·89	63·9^{a,b}	0·77

Con, control; SFN, sulforaphane; Cuo, turmeric oil; Oo, oregano oil; To, thyme oil; Ro, rosemary oil.
^{a,b} Mean values with unlike superscript letters within a column were significantly different in the least significant difference test or the Games Howell test ($P<0·05$).
* For details of diets and procedures, see the Methods and materials section.

liver). Cuo additionally reduced GSTα expression significantly. In groups SFN, Oo and To, the expression of HMOX1, EPHX1 and EPHX2 was down-regulated by 15–40 % of the Con level, whereas TrxR1 expression was not influenced (Table 7, liver).

To judge the changes in the whole xenobiotic metabolising system, we have additionally measured the gene expression of the phase I cytochrome P450 enzyme CYP1A1 and that of the phase III ATP-binding-cassette exporter ABCC2, both having a proven function in mycotoxin detoxification (Table 7, liver). Cuo that caused the strongest down-regulation of all ARE-regulated enzymes also reduced the expression of CYP1A1 and ABCC2 most potently. Similarly for the other phytogenic additives (SFN, Oo, To and Ro), their efficacy on CYP1A1 and ABCC2 mRNA reduction corresponded well to their impact on the down-regulation of the xenobiotic- and anti-oxidant enzymes investigated. This specific result suggests a down-regulation of the complete liver xenobiotic metabolising machinery by the phytogenic feed additives.

Differential jejunal- and liver superoxide dismutase activity

Total SOD activity comprises the activity of the cytosolic Cu/Zn enzyme (Cu/Zn SOD) and of the mitochondrial Mn enzyme (Mn SOD). Cu/Zn SOD is an antioxidant enzyme with an ARE sequence in its DNA promoter. Total jejunal SOD activity was increased significantly by all phytogenic additives, whereas it was decreased to a greater or lesser extent in the liver (Table 8), as analogously observed for gene expression of the xenobiotic- and antioxidant enzymes. Accordingly, the raise in jejunal SOD activity was based to a higher percentage on an increased Cu/Zn SOD activity than on changes of Mn SOD. The opposite way around, total SOD activity and, in particular, that of Cu/Zn SOD activity were reduced by the phytogenic substances in the liver.

Combined cytosolic glutathione peroxidase- and gastrointestinal glutathione peroxidase activity in the jejunum and liver cytosolic glutathione peroxidase activity

In the intestine, cytosolic GPx activity comprises the activity of GPx1 and that of GPx2, whereas cytosolic GPx activity in all other organs is restricted to GPx1 action. Both GPx1 and GPx2 sensitively respond to oxidative stress with an up-regulation. Comparably to Cu/Zn SOD, GPx2 also has an ARE in its DNA promoter. As probably observed for Cu/Zn SOD, all phytogenic feed additives increased GPx activity in the jejunum to a different extent, but in contrast effected a significant reduction of liver GPx activity (Table 8).

Table 5. Feed conversion (g/g) of growing broilers fed diets containing different phytogenic additives for 21 d*
(Mean values with their standard errors)

	Initial period		Experimental period									
	Days 1–14		Days 15–21		Days 22–28		Days 29–35		Days 15–35		Days 1–35	
Group	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
Feed conversion ratio (g/g)												
Con	1·26	0·01	1·56	0·01	1·96	0·04	1·34^{a}	0·05	1·65^{a}	0·03	1·58^{a}	0·02
SFN	1·27	0·03	1·64	0·09	2·02	0·09	1·41^{a,b}	0·03	1·71^{a,b}	0·02	1·63^{a}	0·01
Cuo	1·32	0·03	1·64	0·04	2·05	0·10	1·57^{b}	0·11	1·79^{b}	0·03	1·70^{b}	0·03
Oo	1·25	0·02	1·56	0·03	2·08	0·05	1·41^{a,b}	0·06	1·73^{a,b}	0·03	1·64^{a,b}	0·02
To	1·28	0·03	1·60	0·04	2·02	0·18	1·39^{a,b}	0·03	1·71^{a}	0·02	1·63^{a,b}	0·02
Ro	1·23	0·03	1·54	0·03	2·00	0·04	1·39^{a,b}	0·05	1·68^{a}	0·03	1·60^{a}	0·03

Con, control; SFN, sulforaphane; Cuo, turmeric oil; Oo, oregano oil; To, thyme oil; Ro, rosemary oil.
^{a,b} Mean values with unlike superscript letters within a column were significantly different in the least significant difference test or the Games Howell test ($P<0·05$).
* For details of diets and procedures, see the Methods and materials section.

Table 6. Cycle threshold values of four reference genes in the jejunal mucosa, colon and liver of growing broilers fed diets containing different phytogenic additives†

(Medians and standard deviations)

	GAPDH		ARP1		ELFA		β-Actin	
Tissue	MW	SD	MW	SD	MW	SD	MW	SD
Liver	16·40*	0·77	15·42	1·61	15·54	1·00	17·02*	0·61
Jejunum	17·04*	1·27	15·77*	0·98	16·08	1·14	14·06*	1·07
Colon	13·34*	0·74	12·40	0·80	11·39*	0·53	10·58	0·65

GAPDH, glycerine aldehyde-3-phosphate dehydrogenase; ARP1, acid ribosomal protein 1; ELFA, elongation factor 1α.

*Within a line indicates the most stable reference genes for the different tissues. Average expression stability (*M*) of remaining reference genes was calculated by stepwise exclusion of the least stable pair of reference genes in the jejunal mucosa, colon and liver. Sample pools (*n* 60) for all groups, corresponding to ten sample pools per dietary treatment were analysed.

† For details of diets and procedures, see the Methods and materials section.

Immunoblot analysis of nuclear factor erythroid 2-related factor 2 regulation in the liver

All phytogenic feed additives decreased protein abundance of active Nrf2 in whole liver homogenate by 20–39% compared to group Con. In contrast, relative protein concentration of ubiquitinated Nrf2 was 2·20- to 3·60-fold higher in the liver of broilers receiving any phytogenic feed additive compared to Con broilers. As a consequence, in broilers fed phytogenic feed additives, the ratio of active Nrf2:ubiquitinated Nrf2 decreased to 28–40% of that in Con broilers (Fig. 1(A) and (B)).

Trolox equivalent antioxidant capacity = TROLOX® equivalent in the essential oils and the liver

The analysed TEAC values (mmol per 100 ml) of the essential oils were as follows: Cuo: 90·7 (SE 1·81), Oo: 120 (SE 10·1), To: 116 (SE 8·72) and Ro: 156 (SE 21·1) (Fig. 2). A TEAC value for sulforphane or its glucosinolate precursor glucoraphanin could not be analysed since these compounds possess no direct antioxidant effects. Jejunal TEAC (μmol per g of organ) was 3- to 7-fold lower than that in the liver (jejunum: Con 29·4 (SE 9·61), SFN 174 (SE 36·6), Cuo 343 (SE 22·9), Oo 392 (SE 31·0), To 446 (SE 43·1), Ro 364 (SE 59·8); liver: Con

Table 7. mRNA expression of xenobiotic- and antioxidant enzymes in the jejunal mucosa, colon and liver of growing broilers fed diets containing different phytogenic additives for 21 d relative to group control (Con) = 1*

(Mean values with their standard errors of the mRNA abundance relative to group Con = 1·0 (*n* 10 pools of two animals per experimental group))

Group…	Con		SFN		Cuo		Oo		To		Ro	
ARE gene	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
Jejunum												
GSTα	1·00^{a}	0·26	1·55^{a}	0·35	1·14^{a}	0·62	1·12^{a}	0·38	0·87^{a}	0·22	0·67^{a}	0·12
HMOX1	1·00^{a}	0·12	1·60^{b}	0·20	1·86^{b}	0·44	1·06^{a}	0·17	1·21^{a,b}	0·10	1·98^{b}	0·50
EPHX1	1·00^{a}	0·17	1·90^{b}	0·30	2·19^{b}	0·44	1·59^{a,b}	0·37	1·40^{a,b}	0·21	1·80^{b}	0·30
EPHX2	1·00^{a}	0·19	1·54^{a}	0·23	1·46^{a}	0·29	1·37^{a}	0·38	1·14^{a}	0·12	1·60^{a}	0·32
AFAR	1·00^{a}	0·06	1·87^{b,c}	0·23	2·26^{b}	0·37	1·16^{a}	0·19	1·40^{a,c}	0·26	1·37^{a,c}	0·22
TrxR1	1·00^{a}	0·12	1·29^{a}	0·23	1·62^{a,b}	0·41	1·27^{a}	0·18	1·19^{a}	0·13	2·12^{b}	0·36
Colon												
GSTα	1·00^{a}	0·14	0·79^{a}	0·10	0·99^{a}	0·09	1·11^{a}	0·12	0·99^{a}	0·14	1·10^{a}	0·17
HMOX1	1·00^{a,b}	0·23	0·87^{a}	0·15	1·38^{a,b,c}	0·25	1·59^{b,c}	0·44	1·39^{a,b,c}	0·28	1·98^{c}	0·43
EPHX1	1·00^{a}	0·12	0·99^{a}	0·10	0·86^{a}	0·06	0·95^{a}	0·09	1·00^{a}	0·07	1·32^{b}	0·10
EPHX2	1·00^{a}	0·12	0·84^{a}	0·08	0·89^{a}	0·10	0·84^{a}	0·08	1·02^{a}	0·06	0·93^{a}	0·07
AFAR	1·00^{a}	0·13	1·82^{b}	0·28	0·95^{a}	0·08	1·96^{b}	0·43	0·93^{a}	0·15	1·32^{a,b}	0·29
TrxR1	1·00^{a}	0·31	1·80^{a,b}	0·53	2·41^{b,c}	0·73	3·90^{c}	1·13	3·15^{b,c}	1·14	2·31^{b,c}	0·60
Liver												
GSTα	1·00^{a}	0·12	0·98^{a}	0·08	0·67^{b}	0·08	1·10^{a}	0·07	0·96^{a}	0·12	0·95^{a}	0·06
HMOX1	1·00^{a}	0·23	0·62^{b,c}	0·05	0·47^{b}	0·04	0·62^{b,c}	0·07	0·74^{a,c}	0·07	0·62^{b}	0·07
EPHX1	1·00^{a}	0·07	0·85^{a,c}	0·07	0·53^{b}	0·49	0·81^{c}	0·08	0·66^{b,c}	0·08	0·53^{b}	0·07
EPHX2	1·00^{a}	0·14	0·59^{b,c,d}	0·06	0·39^{b,d}	0·06	0·79^{a,c}	0·06	0·66^{c,d}	0·10	0·47^{d}	0·06
AFAR	1·00^{a}	0·12	1·01^{a}	0·11	0·96^{a}	0·09	0·95^{a}	0·14	0·79^{a}	0·09	0·88^{a}	0·14
TrxR1	1·00^{a}	0·13	0·88^{a}	0·09	0·49^{b}	0·04	1·02^{a}	0·12	0·96^{a}	0·15	0·59^{b}	0·07
CYP1A1	1·00^{a}	0·20	0·50^{b}	0·08	0·17^{c}	0·04	0·56^{b}	0·08	0·45^{b}	0·13	0·48^{b}	0·09
ABCC2	1·00^{a}	0·09	0·75^{a}	0·15	0·49^{b}	0·16	1·02^{a,c}	0·14	0·96^{a,c}	0·23	0·55^{b}	0·05

SFN, sulforaphane; Cuo, turmeric oil; Oo, oregano oil; To, thyme oil; Ro, rosemary oil; GSTα, glutathione-*S*-transferase α HMOX1, haeme oxygenase 1; EPHX1, microsomal epoxide hydrolase; EPHX2, cytosolic epoxide hydrolase; AFAR, aflatoxin B1 aldehyde reductase; TrxR1, thioredoxin reductase 1; CYP1A1, cytochrome P450 family 1 subfamily A polypeptide 1; ABCC2, ATP-binding cassette subfamily C member 2.

^{a,b,c,d} Mean values with unlike superscript letters within a row indicate significant differences in the least significant difference test or the Games Howell test ($P<0·05$). The superscript 'a' was assigned to group Con.

*For details of diets and procedures, see the Methods and materials section.

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Table 8. Differential superoxide dismutase (SOD) activity and glutathione peroxidase (GPx) activity in the jejunal mucosa and the liver of growing broilers fed diets containing different phytogenic additives for 21 d*

(Mean values with their standard errors, *n* 10 pools of two animals per experimental group)

	Con		SFN		Cuo		Oo		To		Ro	
Group	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
Jejunum												
Total SOD (U/mg protein)	4·88^{a}	0·73	6·80^{b}	0·54	7·28^{b}	0·84	7·20^{b}	0·55	7·45^{b}	0·86	7·42^{b}	0·63
Cu/Zn SOD(U/mg protein)	3·87^{a}	0·76	5·55^{b}	0·52	6·15^{b}	0·80	5·79^{b}	0·48	6·19^{b}	0·77	6·10^{b}	0·70
Mn SOD (U/mg protein)	1·00^{a}	0·07	1·25^{a,b}	0·03	1·13^{a}	0·09	1·41^{b}	0·10	1·26^{a,b}	0·10	1·32^{a,b}	0·14
GPx (mU/mg protein)	6·87^{a}	0·57	7·28^{a,b}	0·85	9·16^{b}	0·70	8·90^{b}	0·67	7·62^{a,b}	1·08	7·62^{a,b}	0·83
Liver												
Total SOD (U/mg protein)	59·6^{a}	1·90	52·5^{b,c}	2·16	57·2^{a,b}	2·37	51·2^{b,c}	1·58	56·1^{a,b,c}	1·99	49·8^{c}	3·82
Cu/Zn SOD(U/mg protein)	47·4^{a}	1·79	41·4^{b,c}	2·36	46·5^{a,b}	2·24	39·6^{c}	1·55	45·2^{a,b,c}	1·73	44·4^{b,c}	2·93
Mn SOD (U/mg protein)	12·2^{a}	0·38	11·2^{a}	0·84	10·8^{a}	0·49	11·6^{a}	0·44	10·8^{a}	0·70	9·3^{b}	1·02
GPx (mU/mg protein)	26·2^{a}	1·47	22·1^{b,c}	1·03	20·7^{b,c}	0·69	17·0^{c}	1·49	20·2^{b,c}	1·54	20·7^{b,c}	1·19

Cu/Zn SOD, cytosolic Cu–Zn SOD; Mn SOD, mitochondrial Mn SOD.

^{a,b,c} Mean values with unlike superscript letters within a row were significantly different in the least significant difference test or the Games Howell test ($P<0·05$).

*For details of diets and procedures, see the Methods and materials section.

282 (SE 28·0), SFN 680 (SE 52·2), Cuo 494 (SE 34·9), Oo 992 (SE 57·4), To 1129 (SE 87·1), Ro 764 (SE 115)). All phytogenic feed additives raised the jejunal and liver TEAC values significantly compared to group Con. The TEAC values of Oo and To directly reflected the TEAC values analysed in the jejunum and in the liver of the broilers. Cuo which had a distinctly lower TEAC value than the labiatae oils (Oo, To and Ro) had a distinctly higher influence on the jejunal TEAC than on the liver TEAC. Feeding Ro, with the highest analysed TEAC, resulted in a lower liver TEAC than Oo- and To feeding, whereas its influence on the jejunal TEAC was comparable to that of Oo and To. SFN, having no direct antioxidant activity, increased the jejunal and liver TEAC significantly less than the labiatae oils (Oo, To and Ro).

Concentration of thiobarbituric acid-reactive substances in the liver

All the phytogenic feed additives tested, effected a reduction of Fe-induced lipid peroxidation in the liver of the broilers compared to the Con group (Fig. 3). Cuo feeding thereby had the strongest effect, reducing TBARS by nearly 60% compared to group Con. To and Ro as well as SFN produced an intermediate protection against the Fe-provoked lipid peroxidation (reduction of about 40%), whereas Oo showed the smallest effect in comparison (reduction of about 20%).

Discussion

Performance parameters (feed intake, body weight, weight gain, feed conversion)

As mentioned in the introduction, the effects of phytogenic feed additives, and in particular of labiatae oils on the performance parameters of broilers are subject to a controversial discussion. Whereas one study reported on the beneficial effects of extremely high dietary concentrations of oregano leaves (up to 20 g/kg = 20 kg/tonne) and Oo (up to 1·0 g/kg = 1·0 kg/tonne) on weight gain and feed conversion[2], other studies showed no effects[3] or even opposite effects on these parameters[5]. In the last mentioned study, the influence of Oo, To and Ro on the performance of broilers was studied. In this study, Oo and Ro impaired weight gain and feed conversion compared to control broilers, whereas To influenced these parameters positively. Our results are partially in accordance with these results[5]. Although we could not confirm an improvement of performance by To addition to the diet, weight gain was reduced to a lesser extent by To compared to Oo and Ro (Table 2). In studies on the effects of turmeric on aflatoxin-metabolising enzymes, the addition of 500 g/t turmeric powder to the diets of Cobb × Cobb broilers reduced body weight gain and feed conversion of these birds compared to the Con[26,45]. Our results for Cuo confirm these results (Table 2), with the difference that the Cobb × Cobb broilers[46] had a somewhat higher weight gain and a better feed conversion than our Ross-308 broilers[45,47]. Broccoli extract (SFN), not yet permitted as a feed additive in the EU, in our trial also influenced the performance parameters of the broilers, slightly negative compared to group Con (Table 2). However, this effect reached only a magnitude comparable to that of To. Feasible negative effects of broccoli extract on performance may derive from the goitrogenic potential of isothiocyanates. However, studies with human subjects have proven that even high dietary concentrations of SFN affect thyroid metabolism only negligibly[18,48].

Weight gain and feed conversion are the two most important goals in today's animal nutrition. Frequently both parameters are associated with general animal health. Our present results and the outcome of a number of the aforementioned studies[2,3,46] suggest that phytogenic feed additives do not meet the criteria acting as mere growth promoters. However, in these studies, including our present experiment, the animals were not subjected to a challenge with pathogenic micro-organisms or toxic substances. It can be assumed that the threat of infections and the challenge with feed contaminants increase under practical feeding conditions with a high stocking rate. Experiments in which broilers were infected with *Eimeria tenella* or fed aflatoxin-containing diets, Oo or

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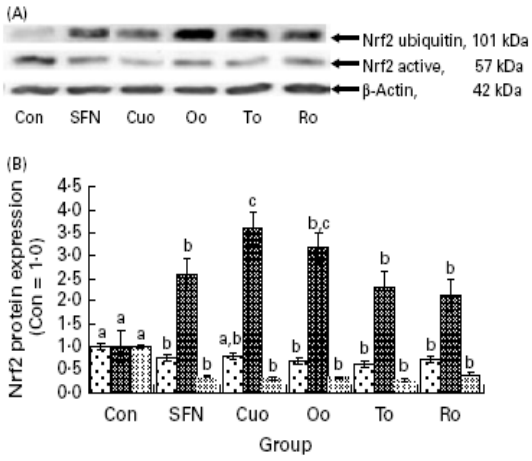

Fig. 1. Nuclear factor erythroid 2-related factor 2 (Nrf2) protein expression in whole liver lysate of growing broilers fed diets containing different phytogenic additives for 21 d. (A) Representative immunoblot of ubiquitinated Nrf2 (= Nrf2 marked with ubiquitin for proteasomal degradation, 101 kDa, upper lane), active Nrf2 (57 kDa, middle lane) and β-actin (42 kDa, lower lane), selected from four pools of whole liver tissue homogenate per experimental group. Immunoblot analysis for each protein pool was performed in duplicate. (B) Nrf2 protein expression and ratio of active:ubiquitinated Nrf2 in whole liver lysate of growing broilers fed diets containing different phytogenic additives for 21 d relative to group Con = 1·0. Values are means, with their standard errors represented by vertical bars of active and ubiquitinated Nrf2 protein abundance relative to group Con = 1·0 and the ratio of active Nrf2:ubiquitinated Nrf2 relative to group Con = 1·0 (*n* 4 pools of two animals per experimental group). ^{a,b,c}Mean values with unlike letters were significantly different in the least significant difference test or the Games Howell test ($P<0·05$). The letter 'a' was assigned to group Con. For details of diets and procedures, see the Methods and materials section. ▨, Nfr2 active; ▩, Nrf2 ubiquitinated; ▦, active:ubiquitinated. Con, control; SFN, sulforaphane; Cuo, turmeric oil; Oo, oregano oil; To, thyme oil; Ro, rosemary oil.

turmeric powder counteracted the depressed feed intake and growth[26,45,49]. Moreover, the exclusive addition of phytogenic substances as growth promoters to animal feed may be less effective than combinations with feed enzymes[1,2] or probiotics[50].

Antioxidant response element-regulated xenobiotic- and antioxidant enzymes, Trolox equivalent antioxidant capacity in the intestine

To the best of our knowledge, our data have shown for the first time that labiatae oils have differentiated and promising effects on ARE-regulated xenobiotic- and antioxidant enzymes and on antioxidant capacity in the intestine and the liver of fast-growing broilers. This assertion is also appropriate for Cuo which is permitted as a feed additive in the EU and for broccoli sprouts extract which is currently only accredited as a dietary supplement for humans. Due to their known impact on the induction of ARE-regulated genes, we have applied the aforementioned phytogenic substances as the reference additives in our study. For turmeric and SFN[51] derived from glucoraphanin cleavage by intestinal microbial β-glucosidases, our data regarding their impact on the induction of intestinal ARE-regulated xenobiotic- and antioxidant enzymes are in agreement with recent literature. A number of cell culture studies and *in vivo* studies with human subjects and laboratory animals have demonstrated the potential of turmeric and SFN on the induction of ARE-regulated genes in the intestine[22,52–54]. In humans, turmeric and SFN predominantly have been established as preventive agents against intestinal cancers. Their safety has been verified[18,48,55]. In farm animals, having a short life span and needed for food production, the application of potent inductors of ARE-regulated genes rather aims on their efficiency to strengthen the intestinal barrier against toxic feed-derived substances or endogenously produced toxic metabolites and thereby impeding their absorption into the organism. In our trial, Cuo and SFN both induced a similar pattern of ARE-regulated genes in the small intestine (Table 7, jejunum) with a particular focus on HMOX1, EPHX 1 and 2, and on AFAR[26,45].

Further, our results have shown an effect of labiatae oils (Oo, To, Ro) on the expression of intestinal ARE-regulated genes. The labiatae oils induced a differential expression pattern depending on the oil and on the intestinal segment investigated (Table 7; jejunum, colon). Within the labiatae oils, Oo and To effected a smaller increase in jejunal ARE-regulated enzymes compared to Ro. In the colon, feeding the phytogenic substances resulted in a more individual induction pattern. In the colon, all the additives induced TrxR1 possessing both, antioxidant properties, and a key role in DNA-synthesis[56] to a higher extent than in the jejunum. Whereas SFN and Oo increased the colonic AFAR expression potently, Ro had a high impact on HMOX1 expression. The exact mechanisms by which EO and SFN influence ARE-regulated genes differentially have not been studied to date. It can be speculated that the different main terpene compounds of EO and SFN modify Keap1 at sensor –SH-groups by individual chemical reactions[27]. Moreover, the number of ARE and that of other transcription-factor-regulated elements in the promoter region differ between the single xenobiotic- and antioxidant enzymes investigated[57]. That terpenes increase

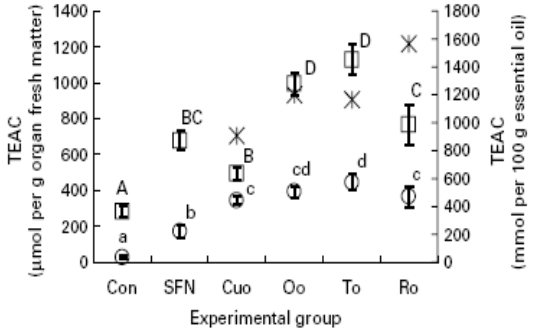

Fig. 2. Trolox equivalent antioxidant capacity (TEAC) values of essential oils (⁎) and TEAC values in jejunal (○) mucosa and the liver (□) of fast-growing broilers fed diets containing different phytogenic additives for 21 d. Values are means, with their standard errors represented by vertical bars (*n* 10 pools of two animals per experimental group). ^{a,b,c,d}Mean values with unlike letters were significantly different in the least significant difference (LSD) test or the Games Howell test for the jejunal TEAC values ($P<0·05$). ^{A,B,C,D}Mean values with unlike letters were significantly different in the LSD test or the Games Howell test for the liver TEAC values ($P<0·05$). For details of diets and procedures, see the Methods and materials section. Con, control; SFN, sulforaphane; Cuo, turmeric oil; Oo, oregano oil; To, thyme oil; Ro, rosemary oil.

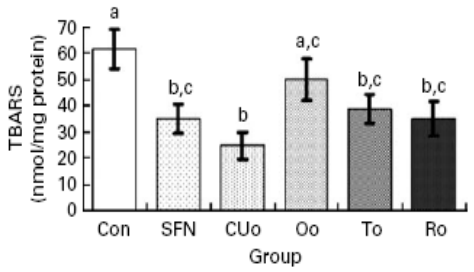

Fig. 3. Thiobarbituric acid-reactive substances (TBARS) after iron provocation in the liver of fast-growing broilers fed diets containing different phytogenic additives for 21 d. Values are means, with their standard errors represented by vertical bars (*n* 10 pools of two animals per experimental group). ^{a,b,c} Mean values with unlike letters were significantly different in the least significant difference test or the Games Howell test ($P<0{\cdot}05$). For details of diets and procedures, see the Methods and materials section.

the expression and activity of ARE-regulated enzymes has been demonstrated[23,58,59]. In contrast to carvacrol (oregano) and thymol (thyme)[14], the main terpenes of Cuo (ar-turmerone) and of Ro (1,8 cineole)[16,60], accounting for 30–50% (v/v) of these oils, have no antioxidant phenolic groups in their molecular structure. For this reason, sesquiterpenes like ar-turmerone (turmeric) or terpenes with an epoxide function like 1,8 cineole (rosemary) may induce ARE-regulated genes more powerful than phenolic monoterpenes[23]. On the other hand, the phenolic monoterpenes exert a higher direct antioxidant potential[16]. In this context, our results for the TEAC values of the oils and of jejunal mucosa are of interest (Fig. 2). The TEAC value of a tissue comprises direct antioxidant effects and that of the antioxidant enzymes increased by secondary mechanisms. In our study, the broccoli-derived isothiocyanate SFN, having no direct antioxidant properties, produced the lowest increase in jejunal TEAC compared to group Con. From this fact, it can be concluded that the increase in jejunal TEAC in group SFN mainly bases on the induction of ARE-regulated antioxidant enzymes. For Cuo showing an intermediate effect on jejunal TEAC, it can be assumed that both, its moderate direct antioxidant effect and the powerful induction of ARE-regulated antioxidant enzymes by ar-turmerone[23,58,59] have contributed to jejunal TEAC modulation (Fig. 2). Oo and To contain high concentrations of the antioxidant phenolic terpenes carvacrol and thymol[14]. In contrast, both oils have induced ARE-regulated enzymes only moderately. Therefore, the high jejunal TEAC values of Oo and To may mainly result from their high carvacrol and thymol content (Fig. 2). To complicate matters, Ro had the highest TEAC value within the labiatae oils. Additionally, Ro powerfully induced intestinal ARE-regulated genes (Table 7; jejunum, colon). However, its influence on jejunal TEAC was not stronger than that of Oo and To. This conflicting result could have two causes: on the one hand, the non-antioxidant epoxy-terpene 1,8 cineole[58] may have produced a strong induction of ARE-regulated antioxidant enzymes. On the other hand, Ro additionally contains small amounts of the phenolic diterpenes carnosic acid, carnosol and epirosmanol coming along with a high direct antioxidant activity[14,60], which may limit the further induction of ARE-regulated antioxidant enzymes. In summary, our data showed a differentiated response of intestinal ARE-regulated genes to dietary treatment with the phytogenic additives SFN, Cuo, Oo, To and Ro. Future studies with tissue cultures and other farm animal species should focus on the examination of the individual xenobiotic-and antioxidant enzyme induction pattern of EO, and in particular, on the examination of their pure main terpenes and of SFN. In those studies, the added concentration of the oils should be standardised to their main terpenes. The results of those investigations may contribute to the creation of new and optimised mixtures of phytogenic feed additives.

Nuclear factor erythroid 2-related factor 2, antioxidant response element-regulated xenobiotic- and antioxidant enzymes, Trolox equivalent antioxidant capacity and thiobarbituric acid-reactive substances in the liver

In contrast to our expectations and to data from other studies[25,61,62], SFN and Cuo, the reference substances in our experiment, as well as the labiatae oils, generally reduced the expression of ARE-regulated genes in the liver (Table 7, liver). Nevertheless, data from other trials feeding diets supplemented with curcumin, quercetin and catechin to mice and rats support our findings regarding a down-regulation of xenobiotic- and antioxidant enzymes in the liver[63,64]. This effect may have two reasons:

(1) The dietary concentration of both reference additives was too low to produce an induction of ARE-regulated genes also in the liver.
(2) The antioxidant protection achieved by the induction of ARE-regulated genes in the intestine acted as a barrier and protected the organism against the uptake of toxic xenobiotics and against oxidative stress.

However, the second hypothesis seems to be more plausible, since we found a distinct up-regulation of SOD- and GPx enzyme activity by all phytogenic feed additives compared to the control in jejunal mucosa (Table 8). Intestinal GPx activity, measured with H_2O_2 as the substrate, comprises the activity of GPx1 and GPx2. GPx2 is an unusual selenoprotein, induced by both, a sufficient selenium status and by Nrf2[65,66]. Both peroxidases underlie a coordinated regulation. In the case of lacking GPx2 activity, GPx1 is up-regulated to compensate reduced peroxide detoxification[67]. Numerous studies have demonstrated that the loss of both enzymes promotes the development of chronic inflammatory intestinal diseases. Moreover, it has been shown that GPx2 blocks the up-regulation of the proinflammatory inducible cyclo-oxygenase 2. Further, GPx2 impedes the aquaporin-mediated absorption of peroxides into the organism[68–72].

Our second hypothesis that the increased intestinal barrier against pro-oxidants and inflammatory stress protects peripheral organs like the liver from oxidative stress is further supported by our findings for Nrf2[73] (Fig. 1(A) and (B)), SOD (Table 8), GPx1 (Table 8), TEAC (Fig. 2) and TBARS (Fig. 3). The coincident down-regulation of active Nrf2 (Fig. 1(A) and (B)) and xenobiotic- and antioxidant enzymes (Table 7, liver) on the one hand, and higher TEAC values

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(Fig. 2) accompanied by a reduced, provoked lipid peroxidation (Fig. 3) of all chickens receiving a phytogenic additive on the other, suggest that the livers of supplemented broilers had an increased antioxidative capacity and reduced oxidative stress, making the further induction of antioxidant enzymes dispensable[63,64]. This particular result was also supported by the finding that protein abundance of ubiquitinated Nrf2 was significantly higher in broilers fed diets with phytogenic additives than in Con broilers (Fig. 1(A) and (B)). Contrarily it can be assumed that the livers of untreated Con broilers were exposed to higher oxidative stress, as indicated by the highest protein abundance of active Nrf2 and the lowest of ubiquitinated Nrf2. This, in turn, explains the higher expression of ARE-regulated xenobiotic-and antioxidant enzymes in Con broilers[73].

That feeding phytogenic feed additives protects peripheral organs from oxidative stress is confirmed by results from other studies reporting on reduced lipid peroxidation and increased storage stability of broiler meat and fat[74–76].

In contradiction to our results, a study with broilers found a distinct up-regulation of liver ARE-regulated genes (*AFAR*, *EPHX1*) due to feeding diets with turmeric powder. However, this response was triggered only by feeding aflatoxin simultaneously[45]. From this fact it can be concluded once again that the regulation of liver ARE genes by phytogenic substances presumably depends largely on the exposure of an organism to toxic substances and oxidative stress. In contrast, an optimum protection against oxidative stress counteracts the further induction of ARE-regulated xenobiotic- and antioxidant enzymes in peripheral organs[63,64].

An issue that should be addressed at the end of the discussion is the regulation of phase II, I and III enzymes in a unidirectional manner as analysed in our study (Table 7, liver). This aspect is very important to keep the balance between the single stages of xenobiotic metabolism[77] and to prevent the organism from damage. The simultaneous down-regulation of Cyp1A1 with an important function in mycotoxin activation[78], of the xenobiotic-and antioxidant phase II enzymes investigated[79], and of ABCC2 responsible for the excretion of conjugated mycotoxin metabolites[80], is a further indicator of a reduced exposure of the liver to toxic metabolites and oxidative stress. In summary, our results for the liver suggest that the induction of jejunal ARE-regulated genes and the increase in jejunal TEAC in the small intestine by phytogenic feed additives seem to act as a barrier against oxidative stress in the organism[71,72]. This protective function of essential oils and of broccoli extract is also of particular interest for human nutrition and with regard to inflammatory bowel disease in humans[68,69,71], since we have used chickens as a single-stomached animal species in our study.

Conclusions

(1) On the one hand, our study confirmed results from the literature that phytogenic feed additives do not merely act as growth promoters under conditions without an infectious or toxic challenge.

(2) On the other hand, our results have demonstrated for the first time that broccoli extract, Cuo and the labiatae oils of oregano, thyme and rosemary influence the expression of ARE-regulated xenobiotic- and antioxidant enzymes. The up-regulation of these genes in the intestine seems to build a barrier against oxidative stress in the organism. In particular, the up-regulation of ARE-regulated genes in the small intestine may represent an attractive and new mechanism by which phytogenic feed additives improve the intestinal and general health of farm animals.

(3) However, different regulation patterns of the single phytogenic substances demonstrate the necessity for future research with regard to this issue. Understanding the different regulation patterns of single phytogenic feed additives may contribute to the development of optimised combinations of phytogenic substances in animal nutrition and to the use of these substances as dietary supplements for humans.

(4) Moreover, we have tested for the first time a broccoli sprouts extract containing SFN as a phytogenic feed additive in animal nutrition. Compared to the other additives tested, SFN did not show significant negative effects on the performance parameters under well-controlled housing conditions without a microbial or toxic challenge. Moreover, broccoli extract acted as a modulator of the aforementioned ARE-regulated genes. For this reason, broccoli extract, currently permitted only as a dietary supplement for humans, has turned out to be an attractive phytogenic feed additive for use in the future.

Acknowledgements

The present study was supported by the H. Wilhelm Schaumann Foundation, Hamburg by the provision of a non-recurring donation for the study and by funding Dipl. troph. Kristin Mueller with a doctoral scholarship. We thank Devakumari Hiller (Jarrow Deutschland GmbH, Berlin, Germany) for arranging the contact with Jarrow L. Rogovin from Jarrow Formulas, Los Angeles, CA, USA, who provided us with the broccoli seed extract. Further thanks are addressed to Dostofarm GmbH (Westerstede, Germany) for providing us with the Oo. Moreover, we thank our stockmen Olaf Hoedel and Detlev Barth for their help with the diet preparation, animal care and organ sampling. Thanks are also addressed to our Master's degree students Anke Markert and Kathrin Sinningen for their help with the analyses within the scope of their Master's theses. The contributions of the authors were as follows: K. M. assisted in planning the trial and blending the diets. Further, she participated in the broilers' care and organ sampling. She carried out all laboratory analyses and supervised the Master's degree students who had supported her with the analyses. Besides, K. M. carried out the data evaluation, other than writing voluminous sections of the manuscript. N. M. B. assisted K. M. in the laboratory analyses and helped in the preparation of the tables. H. K. was responsible for calculating the exact composition of the diets. A. S. M. planned the trial and participated in organ sampling and

analyses. Further, he had drafted the applications for obtaining the licence for the animal study and the certificate of exemption for using the broccoli sprouts extract as a feed. A. S. M. contributed to supervising the data evaluation and manuscript writing. All authors declare that they have no conflicts of interest.

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3.2 Mueller K, Blum NM, Kluge H, Bauerfeind R, Froehlich J, Mader A, Wendler KR, Mueller AS. *Effects of broccoli extract and various essential oils on intestinal and faecal microflora and on xenobiotic enzymes and the antioxidant system of piglets.* OJAS. 2012 Vol.2, No.2, 78-98. doi:10.4236/ojas.2012.22012

Vol.2, No.2, 78-98 (2012)
doi:10.4236/ojas.2012.22012

Open Journal of Animal Sciences

Effects of broccoli extract and various essential oils on intestinal and faecal microflora and on xenobiotic enzymes and the antioxidant system of piglets

Kristin Mueller[1], Nicole M. Blum[1], Holger Kluge[1], Rolf Bauerfeind[2], Julia Froehlich[2], Anneluise Mader[3,4], Karola R. Wendler[4], Andreas S. Mueller[1*]

[1]Institute of Agricultural and Nutritional Sciences, Preventive Nutrition Group, Martin Luther University of Halle-Wittenberg, Halle, Germany; [*]Corresponding Author: andreas.mueller@landw.uni-halle.de
[2]Institute of Veterinary Hygiene and Infectious Diseases of Animals, Justus Liebig University Giessen, Giessen, Germany
[3]Institute of Animal Nutrition, Free University of Berlin, Berlin, Germany
[4]Delacon Biotechnik Ges.m.b.H., Steyregg, Austria

Received 9 February 2012; revised 15 March 2012; accepted 26 March 2012

ABSTRACT

Objective: Since the ban of antibiotics as growth promoting feed additives in the EU in 2006 research in alternatives has gained importance. Phytogenic feed additives represent a heterogenous class of different plant derived substances that are discussed to improve the health of farm animals by direct and indirect antioxidant effects and by influencing microbial eubiosis in the gastrointestinal tract. Consequently our study aimed to investigate the influence of broccoli extract and the essential oils of turmeric, oregano, thyme and rosemary, as selected individual additives, on intestinal and faecal microflora, on xenobiotic enzymes, and on the antioxidant system of piglets. Methods: 48 four weeks old male weaned piglets were assigned to 6 groups of 8. The piglets were housed individually in stainless steel pens with slatted floor. The control group (Con) was fed a diet without an additive for 4 weeks. The diet of group BE contained 0.15 g/kg sulforaphane in form of a broccoli extract. 535, 282, 373 and 476 mg/kg of the essential oils of turmeric (Cuo), oregano (Oo), thyme (To) and rosemary (Ro) were added to the diets of the remaining 4 groups to standardise supplementation to 150 mg/kg of the oils' key terpene compounds ar-turmerone, carvacrol, thymol and 1,8-cineole. The composition of bacterial microflora was examined by cultivating samples of jejeunal and colonic mucosa and of faeces under specific conditions. The mRNA expression of xenobiotic and antioxidant enzymes was determined by reversing transcriptase real time detection PCR (RT-PCR). Total antioxidant status was assayed using the Trolox Equivalent Antioxidant Capacity (TEAC), and lipid peroxidation was determined by measuring thiobarbioturic acid reactive substances (TBA-RS). Results: Compared to Con piglets all additives positively influenced weight gain and feed conversion in week 1. Over the whole trial period no significant differences in performance parameters existed between the experimental groups. Compared to group Con performance of Ro piglets was, however, slightly impaired. Compared to Con piglets Cuo, Oo and To increased the ratio of Lactobacilli:E. coli attached to the jejunal mucosa, whereas BE and Ro impaired this ratio slightly. In contrast in colonic mucosa Ro improved Lactobacilli:E. coli ratio. In faecal samples an improvement of Lactobacilli:E. coli ratio could be analysed for To and Ro. Ro was the only additive that reduced the incidence rate of piglets tested positive for enterotoxic E. coli (ETEC). All additives significantly increased jejunal TEAC and reduced TBA-RS. In the liver BE, Cuo, Oo and To increased TEAC in tendency and Ro significantly. Liver TBA-RS were slightly reduced by all additives compared to Con piglets. Whereas the influence of BE, To and Ro on jejunal TEAC mainly was derived from the induction of xenobiotic and antioxidant enzymes (indirect antioxidant effects), Cuo and Oo influenced TEAC by direct antioxidant effects. Discussion and Conclusions: Our results have shown: That within the labiatae oils Oo and To have the potential to improve performance slightly. That phytogenic substances have a small but not significant influence on intestinal microflora. That

phytogenic feed additives up-regulate the antioxidant system of piglets either by direct or by indirect antioxidant effects and that they may thereby improve health status. That within the labiatae oils Oo has a high direct antioxidant potential whereas Ro potently induces xenobiotic and antioxidant enzymes. That broccoli extract is an attractive new phytogenic additive, improving antioxidant status by indirect antioxidant effects. That defined combinations of selected phytogenic substances may produce additive effects. That health promoting effects of phytogenic additives in the future should be studied systematically under the challenge with pathogenic microorganisms or food derived toxins.

Keywords: Pigs; Phytogenic Feed Additives; Broccoli Extract; Essential Oils; Xenobiotic Enzymes; Antioxidant System

1. INTRODUCTION

Weaned pigs and fast growing broilers are frequently affected by diarrhoea in the first weeks of their lives resulting in impaired performance or the loss of animals. Antibiotics as feed additives were therefore used for a long time in order to protect the animals from this harm. The complete ban of the last three antibiotic feed additives in the European Union (EU) in 2006 bore the necessity to intensify research in alternatives. Besides the supplementation of pig diets with pre- and/or probiotics or organic acids, phytogenic feed additives have turned out as promising substances for this purpose.

In this context some studies reported that in particular the essential oils (EO) of labiatae plants (Origanum vulgare, Thymus vulgaris and Rosmarinus officinalis) influenced growth and performance of piglets positively whereas other trials found no or even contrary effects [1-5]. These contradictory results with regard to performance are extensively summarised in a recent review [6].

Antibiotic-like bactericidal and/or bacteriostatic effects on several pathogenic intestinal microorganisms may represent another mechanism by which labiatae oils influence the health of farm animals positively. However, most of the studies regarding this topic have been carried out *in vitro*, frequently in the context of food safety of meat products [7-9]. The bactericidal activity of EOs *in vivo* is also discussed controversially. Oils or herbal preparations of labiatae plants rather seem to improve microbial eubiosis [10,11] than having direct bactericidal effects against pathogenic bacterial strains [12].

A further mechanism by which labiatae oils may contribute to animal health may consist in their direct antioxidant effects. Presumably the terpene compounds of labiatae oils largely contribute to both their antioxidant potential and to their influence on bacteria. The main terpene compounds of labiatae plants include the phenollic terpenes carvacrol (oregano), thymol (thyme and rosemary) and the epoxy-terpene 1,8-cineole (rosemary). The measurement of the Trolox Equivalent Antioxidative Capacity (TEAC, mmol TROLOX equivalent®/g test substance) represents a frequently applied method to test the antioxidant potential of labiatae oils. In this context it could be demonstrated that oregano had the highest antioxidant potential within labiatae plants (TEAC value: 100.7) followed by rosemary (38.8) and thyme (38.8) [13].

Similar mechanisms improving performance and health of farm animals as reported for labiatae extracts and oils are also discussed for turmeric preparations (Curcuma longa L.). Turmeric oil contains a highly active terpene compound, the aromatic sesquiterpene ar-turmerone, possessing both a strong bactericidal activity against several microorganisms [14] and direct antioxidant effects [15].

Broccoli extract, containing glucoraphanin (GRA), the glucosinolate precursor of the isothiocyanate sulforaphane (SFN), is a phytogenic substance that has been tested as a feed additive only in one broiler study which has been carried out in our group [16]. Due to the lack of studies with other farm animals broccoli extract has no current permission as a feed additive in the EU. In contrast in humans broccoli extract and SFN are generally accepted dietary supplements with a high potential in cancer prevention [17]. *In vitro* SFN exerted a strong bactericidal activity against various microorganisms such as Enterococcus faecalis, Staphylococcus aureus, Escherichia coli S1 and S2, and Salmonella typhi [18].

The cancer protective effects of turmeric and of SFN are believed to derive from the induction of enzymes with an "Antioxidant Response Element" (ARE) in their DNA. ARE containing enzymes include a large number of xenobiotic enzymes, such as glutathione-S-transferases (GST), epoxide hydrolases (EPHX), aflatoxin aldehyde reductases (AFAR) and of antioxidant enzymes, such as heme oxygenases (HMOX1) and cytosolic copper/zinc-superoxide dismutase 1 (SOD1) [19-24]. The increased transcription of genes with an ARE sequence is initiated by translocation of the transcription factor "Nuclear factor erythroid 2-related factor 2" (Nrf2) [25] to the nucleus and its subsequent association with the ARE sequence.

Both oxidative stress and electrophiles like sulforaphane or terpenes can increase Nrf2 translocation to the nucleus by modifying of redox sensitive cysteine-SH-groups of Kelch-like ECH-associated protein 1 (Keap1)

to which Nrf2 is bound in the cytosol [25].

Consequently, in contrast to the direct antioxidant effect, the ability of phytogenic feed additives to induce ARE regulated genes is referred to as indirect antioxidant effect.

The systematical investigation of indirect antioxidant effects of phytogenic feed additives in different farm animal species, including pigs, has been carried out only in our broiler trial until today.

Another problem of a number of studies investigating the influence of phytogenic additives on performance parameters, microbial eubiosis, and on antioxidant effects consists in the use of blends of various phytogenic substances

- Consequently the first aim of our present study with piglets was to investigate systematically the impact of labiatae oils (oregano, thyme, rosemary), a zingiberaceae oil (turmeric) and of a broccoli extract on performance, microbial eubiosis and the antioxidant system.
- The second aim of our study was to standardise the addition of the different phytogenic substances to an equal dietary concentration of their key compounds.
- The third aim of our trial was to give suggestions for expedient combinations of the single additives in order to maximise the impact on performance, microbial eubiosis, and on direct and indirect antioxidant effects.

As the reference substances for the examination of indirect antioxidant effects and for the examination of xenobiotic enzyme induction we used a broccoli sprouts extract and turmeric oil, both having a proven impact on these parameters [15,26-28].

2. METHODS AND MATERIALS

2.1. Pigs Husbandry and Diets

The protocol of the piglet study was approved by the Regional Council of Halle and by the animal welfare committee of the Martin Luther University Halle Wittenberg (record token: 42502-3-559-MLU). Further a certificate of exemption for feeding the broccoli sprouts extract, which has no current permission as a feed additive in the EU was attested by the veterinary administrative office Saxony-Anhalt, Halle (record token: 203.2.1/22.10).

48 four weeks old male weaned pigs [(DL × DL) × Pietrain] were obtained from the Intitutes' own piggery (NWZ Merbitz, Germany). After a 5 day acclimatisation period the piglets (mean body weight: 9.5 ± 0.11 kg) were assigned to 6 groups of 8 animals. Piglets were housed individually in stainless steel pens with slatted floor and fed the experimental diets for 4 weeks.

The control group (Con) was fed a piglet starter diet without any additive. 3694 mg/kg broccoli sprouts extract, providing 369.4 mg/kg GRA (150.0 mg/kg SFN), were added to the diet of group BE. 535 mg turmeric oil/kg diet (group Cuo), 282 mg oregano oil/kg diet (group Oo), 373 mg thyme oil/kg diet (group To) and 476 mg rosemary oil/kg diet (group Ro) were added to the diets of the other 4 groups in order to standardise total EO addition to the amount of 150.0 mg/kg diet of the individual key terpene compound of each essential oil. The standardised main terpenes were ar-turmeron for turmeric oil, carvacrol for oregano oil, thymol for thyme oil, and 1,8-cineole for rosemary oil. The active ingredient in the broccoli sprouts extract and the EOs, as analysed by Delacon Biotechnik Ges.m.b.H. (Steyregg, Austria), had the following concentrations (g/100 g = %): broccoli sprouts extract (sulforaphane, 4.06), turmeric oil (ar-turmerone, 30.0), oregano oil (carvacrol, 65.0), thyme oil (thymol, 49.0), and rosemary oil (1,8-cineole, 45.0). Premixes of the essential oils using Sipernat® as the carrier matrix were prepared by Delacon Biotechnik Ges.m.b.H. and added to the basal diet (**Table 1**).

Piglets had free access to their respective diet and water. During the experiment temperature, humidity and lightening were controlled. The temperature was gradually reduced from 26˚C on day 1 to 21˚C on day 28. Lighting regime consisted of a 12 h light: 12 h dark circle. Minerals, vitamins and essential amino acids were added to all diets as recommended for pigs by the Society of Nutrition Physiology [29] and of the National Research Council [30].

Feed intake and individual live weight were recorded once a week. Feed conversion was calculated from the ratio of feed intake (g) and weight gain (g). On day 28 piglets were exsanguinated after stunning for organ sampling (blood, liver, jejunal mucosa, ileal mucosa and colon). Mucosa samples of small intestine were prepared from a 15 cm jejunal segment located 10 cm distal to the duodenum. Ileum mucosa was obtained from a 10 cm segment located proximal to the caecum. Colon tissue was taken from a 10 cm segment located distal to the caecum. At the beginning [first bacterial examination (BE)] and at the end (final BE) of the experiment faeces samples were collected directly from the piglets' anus using rectal swabs.

2.2. Haemogram

Blood samples of each piglet were taken at the end of the experiment using ethylenediamine tetraacetic acid (EDTA) coated tubes. The haemogram was analysed at the "Klinik für kleine Klauentiere und Forensische Medizin" of the "Stiftung Tierärztliche Hochschule Hannover" using a high throughput haemoanalyzer (Nihon Kohden Cell Tac alpha, MEK-6450K).

2.3. Microbiological Determinations in Faeces, Jejunal and Colonic Segments of Piglets

In order to determine the counts of bacteria in faeces (first and final BE) rectal swabs were cut off, put in 5 ml cold sterile PBS-buffer and mixed for 10 s. To determine bacteria attached to the jejunal and the colonic mucosa immediately after slaughter the specific gut segments mentioned under "**Pigs Husbandry and Diets**" were thoroughly rinsed with sterile physiological sodium chloride solution (0.9%w/v). Subsequently the mucosa of these jenunal and colonic segments was scraped off with a sterile glass slide. Then 1 g of the mucosa preparations were suspended in 5 ml cold sterile PBS-buffer and mixed for 10 s. Solutions were transferred into 50 ml tubes containing 4 g sterile glass beads and stored at 4˚C. For microbiological determinations log10-serial dilutions of faeces and mucosa samples were prepared. 50 µl of each serial dilution were disseminated on agar plates. Specific culture media for the single bacterial strains and incubation conditions are shown in **Table 2**. Bacterial counts are presented as CFU (colony forming units) per g mucosa or per rectal swab. To determine the count of aerobic mesophilic bioburden mucosa- and swab-preparations were plated on blood agar. For the determination of the fraction of facultative anaerobic bacteria (Enterobacteriaceae, e.g. Escherichia and Enterobacter) as a part of the aerobic mesophilic bioburden, Gassner agar was used for cultivation. In order to determine coliform bacteria (e.g. E. coli, Salmonella), representing lactose-metabolising Enterobacteriaceae, the samples were also incubated on Gassner agar with additional supplementation of the pH-indicator "soluble blue". The analysis of the samples after cultivation on Violet Red Bile agar with 4-

Table 1. Composition of the basal diet.

Ingredient	g/kg basal diet
Maize (DEUKA GmbH und Co. KG, Könnern, Germany)	289.0
Wheat (DEUKA GmbH und Co. KG, Könnern, Germany)	240.0
Barley (DEUKA GmbH und Co. KG, Könnern, Germany)	225.0
Soybean meal, 46% CP (DEUKA GmbH und Co. KG, Könnern, Germany)	205.0
Soybean oil	4.50
Calcium phosphate (Mischfutter und Landhandel GmbH, Edderitz, Germany)	6.50
Vitamin and mineral premix*	20.0
Lysine hydrochloride (Feed Grade, China)	5.20
dl-methionine (Degussa, Duesseldorf, Germany)	1.50
l-threonine (Aiinomoto Eurolysine)	1.80
l-valine (Sigma-Aldrich)	1.00
l-tryptophan (Sigma-Aldrich)	0.50
Total	1000
ME (calculated) [MJ/kg]	13.66

*Premix supplied the following according to the supplier (BASU-Mineralfutter GmbH, Bad Sulza, Germany; per kilogram of complete diet): Ca, 5.3 g; P, 0.8 g; Na, 1.2 g; Mg, 0.11 g; vitamin A, 6 mg (as retinyl acetate); cholecalciferol, 50 µg; vitamin E, 40 mg; vitamin K3, 3.0 mg; thiamine, 4.0 mg; riboflavin, 10.0 mg; vitamin B6, 6.0 mg; vitamin B12, 0.04 mg; niacin, 52.0 mg; folic acid, 0.6 mg; biotin, 0.02 mg; pantothenic acid, 25.0 mg; choline chloride, 0.4 g; Cu, 15.0 mg; Zn, 44.0 mg; Fe, 57.0 mg; Mn, 14.6 mg; I, 0.2 mg; Se, 0.2 mg; Co 0.3 mg. The complete diets had the following nutrient contents according to the NRC recommendations for swine [30] and did not differ between the diets: Dry matter (analysed): 89% Gross energy (analysed): 16.50 MJ/kg ME (calculated): 13.84 MJ/kg. **Crude fat (analysed): 57.9 g/kg dry matter. Crude protein (analysed):187 g/kg dry matter Fiber (analysed): 33.6 g/kg dry matter Crude ash (analysed): 52.1 g/kg dry matter. **The deviation in ME between basal and complete diet resulted from adding the different phytogenic feed additives in terms of premixes as described in Methods and materials.

Table 2. Bacterial specific growing conditions.

Microbiota	Culture medium	Incubation
Aerobic mesophilic bioburden	blood agar (BAP)	40 - 42 h, 37˚C, aerobic
Enterobacteriaceae	Gassner agar (GA)	16 - 18 h, 37˚C, aerob
Coliform bacteria	Gassner agar, lactose positive (GAlpo)	16 - 18 h, 37˚C, aerob
Escherichia coli	VRB-MUG agar, fluorescent (VRBflu)	16 - 18 h, 37˚C, aerob
Anaerobic mesophilic bioburden	Zeissler agar (ZSS)	40 - 42 h, 37˚C, anaerob

VRB-MUG = Violet Red Bile agar with 4-methylumbelliferyl-β-D-glucuronide.

methylumbelliferyl-β-D-glucuronide (VRB-MUG) under UV-exposure yielded the count of E. coli. Cultivation of the samples prepared from jejeunal and colonic mucosa and from rectal swabs on Zeissler agar under anaerobic conditions yielded the count of anaerobic mesophilic bioburden, including fastidious anaerobic (Lactobacilli) and facultative anaerobic bacteria.

The E. coli specific heat stable enterotoxin II (estb) was determined in faecal samples using semiquantitative PCR. Therefore bacterial colonies of selected agar plates were collected in a solution consisting of 1.5 ml sodium chloride (0.9%) and 0.5 ml glycerine. From this solution an aliquot of 3 µl was added to the PCR reaction mixture containing 1 × ammonium buffer, magnesium chloride (2 mM), estb-primer (0.5 µM each), nucleotides (133 µM each) and Taq Polymerase (0.03 U/µl). The nucleotide sequences of the estb primers were as follows: for: 5' TGCCTATGCATC-TACACAAT 3'; rev: 5' CTCCAG-CAGTACCATCTCTA 3'. Annealing temperature of the primers was 55°C. Following amplification for 30 cycles the resulting PCR products were run on 3% agarose gels and visualised under UV light in order to confirm the existence of enterotoxic estb producing E. coli in the sample.

2.4. RNA Preparation and Real Time RT-PCR Assay Including the Stability Analysis of Four Selected Reference Genes in Jejunal Mucosa, Ileal Mucosa, Colon and Liver

To determine mRNA expression levels of GPx1 (glutathione peroxidase 1), SOD1 (Cu/Zn-dependent superoxide dismutase), GSTa2 (glutathione-S-transferase alpha 2), HMOX1 (heme oxygenase 1), EPHX1 (microsomal epoxide hydrolase 1), AFAR (aflatoxin B1 aldehyde reductase) and KEAP1 (Kelch-like ECH-associated protein 1) total RNA was isolated from liver, jejunal mucosa, ileal mucosa and colon using Trizol® reagent (Invitrogen) according to the manufacturers' protocol. Following the photometrical determination of RNA concentration and purity at 260 nm and 280 nm, RNA quality was checked by testing the integrity of the 18S- and 28S-ribosomal RNA bands and by controlling the absence of genomic DNA in 1.2% agarose gels. Reverse transcription of total RNA (3 µg) was carried out using a commercial cDNA synthesis kit (RevertAid™ First Strand synthesis kit, Fermentas, Latvia). mRNA expression was analysed by real time detection polymerase chain reaction (RT-PCR) as described previously [16]. Subsequent to the identification of the correct length of the amplification products in 1.2% agarose gels, relative quantification of mRNA expression was performed using the ΔΔCt method [31]. In accordance with the current guidelines for the proper determination of gene expression data in various tissues [32], a set of four reference genes was selected and their expression stability (M) was ranked according to the standard procedure [33-35]. Beta actin (β-Actin), glycerine aldehyde-3-phosphate dehydrogenase (GAPDH), ribosomal phosphoprotein large PO subunit (RPP0) and beta-2-microglobulin (B2M) were selected as capable reference genes reported in current literature [34,35], and their expression (Ct values) was measured in jejunum, ileum, colon and liver. The treatment independent expression stability (M) was determined by calculating the Ct ratios of one gene with all the other genes. Subsequently the standard deviation of the logarithmically transformed ratios was calculated and plotted [33]. According to their expression stability M, indicated by a decrease of standard deviation, a ranking of the most stable reference genes was compiled for each tissue investigated. The best set of housekeeping genes was used for normalisation of the expression data of the target genes. The expression values of target genes were normalised using the arithmetic mean of the Ct values of RPP0 and GAPDH in jejunal and ileal mucosa, of RPP0 and B2M in colon, and of GAPDH and β-Actin in the liver. Primer sequences used in PCR and their gene bank accession numbers are shown in **Table 3**.

2.5. TROLOX® Equivalent Antioxidant Capacity (TEAC) in Jejunal Mucosa, Colon and Liver Tissue

TEAC was measured in 1:5 (w/v) crude homogenates of jejunal mucosa, colon and liver using the method originally described by Miller *et al.* [36] with modifications of Wang *et al.* [37]. The method is based on monitoring the inhibition of ABTS radical formation spectrophotometrically at 600 nm and 20°C for 15 min. The reaction mixture contained PBS buffer, ABTS reagent (0.15 mM), H_2O_2 (0.1 mM) and metmyoglobin (2.50 µM). Sample TEAC values were calculated by comparison to TEAC values of a TROLOX® standard curve (concentration range: 0 to 21.0 µM). The comparison was carried out in the linear range of the reaction (jejunal mucosa: 10 min, colon: 7 min, liver: 7 min). The TEAC values of the samples were expressed in µmol TROLOX® equivalent per g organ fresh matter and kg body weight. Samples were measured in duplicate.

2.6. 2-Thiobarbituric Acid-Reactive Substances (TBA-RS) in Jejunal Mucosa and the Liver

To analyse lipid peroxidation in intestine and liver samples 2-Thiobarbituric acid-Reactive Substances (TBA-RS) were measured in 25 µl of 1:5 (w/v) crude homogenates of jejunal mucosa and liver according to a modified protocol from Wong *et al.* [38]. After adding 375 µl

Table 3. Primer sequences of the porcine primers for PCR analyses.

Gene	Gene ID	Forward primer	Reverse primer
AFAR	NM001038626.2	ACAAGCCAGGGCTCAAGTACA	GCTCTCTTCCACCCACTTTGA
Cu/ZnSOD	AJ010339.1	TGCAGGTCCTCACTTCAATCC	GGCCAATGATGGAATGGTCT
EPHX1	NM214355.1	AAGGCCTGCACTTGAACGTAG	TGCTCTGGATGTGCATGTAGC
GPx1	NM214201.1	CAAGAATGGGGAGATCCTGA	GATAAACTTGGGGTCGGTCA
GSTA 2	NM214389.1	ATGGTTGAGATTGACGGGATG	ACAGTGGCAACAGCAAGATCA
HMOX1	NM001004027.1	CACTCACAGCCCAACAGCA	GTGGTACAAGGACGCCATCA
Keap1	NM001114671.1	TGGCTGTATCCACCACAACAG	CATTCGCCACTAATTCCTCTC
β-ACTIN	DQ845171.1	GACATCCGCAAGGACCTCTA	ACATCTGCTGGAAGGTGGAC
B2M	NM213978.1	CGGAAAGCCAAATTACCTGA	TCCACAGCGTTAGGAGTGAA
GAPDH	AF017079.1	AGGGGCTCTCCAGAACATCATCC	TCGCGTGCTCTTGCTGGGGTTGG
RPP0	NM001098598.1	CAACCCTGAAGTGCTTGACA	GCCTTGACCTTTTCAGCAAG

H_3PO_4 (0.44 M), 225 µl aqua bidest. and 125 µl 0.6% 2-thiobarbituric acid, the samples were incubated in a thermo block at 100˚C for 60 min. Blanks were determined using 25 µl of potassium phosphate buffer (0.1 M) instead of the different crude homogenates. Subsequent to heating the samples were chilled on ice and mixed with 750 µl of methanolic NaOH (10 ml of 1 M NaOH, 90 ml methanol). After vortexing and centrifugating the samples at 4000 g at 4˚C for 10 min, the extinction was measured spectrophotometrically at 532 nm. To calculate the samples' TBA-RS concentrations a calibration curve with 1,1,3,3,-tetraethoxypropane in a concentration range of 0.60 - 1.20 mM was prepared. The TBA-RS values were expressed in nmol per g organ fresh matter and kg body weight. Each sample (n = 8) was measured in duplicate.

2.7. Protein Concentration of Samples

Protein concentration in homogenates of jejunal mucosa, colon and liver was determined using the standard method of Bradford (1976) [39], adapted to the needs for measurement in a 96 well plate reader.

2.8. Statistical Analysis

Data are presented as means ± SEM or as medians ± min. and max. values for bacterial counts. Following assurance the normality of distribution (Shapiro Wilk test and Kolmogorov Smirnov test) and the homogeneity of variances (Levene's test), data were analysed with SPSS 19.0 for Windows using the one-way ANOVA procedure. If variances were homogenous, significant differences between means ($P < 0.05$) were evaluated with the Least Significant Difference (LSD) test, if not the Games Howell test was used. All tables were prepared with Microsoft Excel (Version 2003).

3. RESULTS

3.1. Performance Parameters (Feed Intake, Body Weight, Weight Gain, Feed Conversion Ratio)

With the exception of two piglets (1 from group BE and 1 from group Ro) which needed a single antibiotic treatment, all other piglets showed no clinical abnormalities during the whole experiment. The haemogram of piglets in all groups (**Table 4**) indicated a good health status, since no parameter laid outside the normal range.

During the whole experimental period no significant differences in feed intake could be registered between the experimental groups (**Table 5**). Piglets of groups Cuo, Oo and To had somewhat higher final body weights compared to Con piglets. However, these differences were not significant. Although piglets of group To had the best feed conversion (1.35:1) compared to all the other experimental groups, this parameter did not differ significantly from any group.

3.2. Microflora in Jejunal and Colonic Mucosa

Due to a high individual variation, no significant differences for aerobic mesophilic bioburden, Enterobacteriaceae, coliform bacteria, E. coli and anaerobic mesophilic bioburden in jejunal mucosa existed between the experimental groups (**Table 6**). However, it is obvious from the jejunal data, that Con pigs had the highest maximum counts for all bacterial classes investigated (50% of the animals above the median value), including coliform bacteria and E. coli. In contrast the number of Lactobacilli, calculated from the difference between anaerobic mesophilic bioburden and Enterobacteriaceae was the lowest in the jejunal mucosa of Con pigs. Nevertheless, comparison of E. coli median values revealed

Table 4. Blood parameters of piglets feeding diets containing different phytogenic feed additives for 28 days.

	Experimental group (MW ± SD)*											
blood parameter (g/l)	Con		BE		Cuo		Oo		To		Ro	
Hb	113.1	6.6	113.7	6.6	115.3	4.9	115.0	5.3	112.3	2.6	113.9	8.7
Hct	36.6	2.3	37.7	1.8	37.6	1.5	37.2	1.5	36.4	1.1	36.7	2.6
MCHC	309.0	6.4	301.5	7.4	306.9	7.1	309.5	4.8	308.6	8.4	310.1	9.8
lymphocytes	58.1	6.6	54.3	9.1	61.9	11.7	54.9	10.7	60.0	3.9	55.1	8.9
segmented granulocytes	37.8	4.7	41.2	7.4	34.3	11.1	41.8	10.9	35.8	5.0	41.1	8.7
unsegmented granulocytes	2.25	1.6	2.08	1.8	1.50	1.5	2.17	1.6	2.06	1.8	1.64	1.4
eosinophile granulocytes	0.63	0.6	0.92	0.7	0.79	1.1	0.25	0.3	0.69	0.7	0.29	0.3
basophile granulocytes	0.50	0.5	0.50	0.3	0.36	0.4	0.17	0.3	0.31	0.7	0.14	0.2
monocytes	0.81	0.9	0.67	1.2	1.00	0.7	0.33	0.4	1.19	1.5	0.50	0.8

Values are arithmetic means (MW) ± standard deviation (SD). Hb, haemoglobin; Hct, haematocrit; MCHC, mean corpuscular haemoglobin concentration.

Table 5. The effects of feeding diets containing different phytogenic feed additives for 28 days on final body weight (kg), body weight gain (kg), daily feed intake (g) and feed conversion ratio (g/g) in piglets.

	Final body weight in kg		Weight gain in kg		Feed intake in g/d		Feed conversion ratio in g/g	
Group	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
Con	20.3	0.89	10.6	0.47	535.4	27.5	1.42	0.03
BE	20.0	2.36	10.5	1.24	502.8	37.8	1.38	0.05
Cuo	21.0	1.18	11.5	0.69	576.0	35.8	1.41	0.02
Oo	21.3	0.92	11.8	0.41	589.5	25.0	1.40	0.03
To	20.9	0.58	11.5	0.47	552.0	27.3	1.35	0.04
Ro	19.9	2.36	10.4	1.27	530.2	19.3	1.45	0.06

Values represent means ± SEM. [a, b, c] Mean values with unlike superscripts within a column indicate significant differences between means ($P < 0.05$) in the LSD test or the Games Howell test.

that Cuo, Oo, and To reduced the number of E. coli bacteria compared to Con piglets, whereas BE and Ro increased their number.

As similarly observed in jejunal mucosa also in colonic mucosa no statistically significant differences regarding all bacterial classes investigated existed between the experimental groups (**Table 7**). In contrast to the results for jejunal microflora in particular Ro piglets showed a reduced E. coli number attached to colonic mucosa compared to Con piglets. Moreover in colonic mucosa Lactobacilli:E. coli ratio was better in Ro piglets than in Con piglets.

3.3. Microflora in Faecal Samples Collected with Rectal Swabs

Additionally to the determination of bacteria attached to jejunal and colonic mucosa we have determined bacterial counts in the faeces of the piglets at the beginning and at the end of the trial (**Table 8**). Although the treatment of the piglets with the different phytogenic additives caused no significant changes in faecal E. coli and Lactobacilli counts as well as in the Lactobacilli:E. coli ratio, To and Ro influenced these parameters in tendency. Whereas there existed no differences in the initial E. coli- and Lactobacilli counts between the experimental groups (E. coli: 3.73 ± 0.18; Lactobacilli: 6.54 ± 0.12; Ratio Lactobacilli:E. Coli: 1.50 ± 0.04), both To and Ro reduced final E. coli count distinctly and improved Lactobacilli:E. coli ratio compared to Con piglets and to piglets of all the other treatments.

In this context our data regarding the heat stable enterotoxin estb produced by enterotoxic E. coli strains of the serotypes O149:K91 with F4 or F6 fimbriae and O138:K81 with F18 fimbriae are of interest. At the beginning of the experiment estb was detectable with a varying incidence rate [12.5% (1 of 8) to 25.0% (2 of 8)] in faecal samples of all experimental groups. The incidence of estb detection rate remained unchanged in groups BE, Oo and To. One more piglet of the Cuo group was estb positive (+12.5%) at the end of the experiment. As the only additive Ro reduced estb incidence rate by 12.5%, whereas in Con piglets the highest increase in estb incidence rate (+25%) was analysed at the end of study (**Figure 1**).

3.4. Expression of ARE Regulated Xenobiotic and Antioxidant Enzymes in Jejunal and Ileal Mucosa and in Colon and Liver Tissue

The expression patterns of selected ARE regulated xenibiotic and antioxidant enzymes depended on the phytogenic additive and on the tissue investigated. In the jejunum BE, used as one reference substance, potently up-regulated the expression of all xenobiotic and antioxidant enzymes investigated compared to Con piglets, with significant changes for AFAR (factor: 2.14), GSTa2

Table 6. Influence of different phytogenic feed additives on bacterial microflora in jejunal mucosa of piglets.

		Experimental group (median, min., max.)*					
Jejunum	**log CFU/g**	**Con**	**BE**	**Cuo**	**Oo**	**To**	**Ro**
	median	5.28	5.98	5.44	5.34	5.67	6.22
Aerobic mesophilic bioburden	min.	4.09	4.97	4.72	4.54	4.23	4.97
	max.	9.09	6.51	7.78	6.67	7.31	7.95
	median	4.29	5.20	4.82	4.78	5.28	6.09
Enterobacteriaceae	min.	2.00	4.17	3.06	3.51	2.61	3.03
	max.	9.03	6.21	7.81	6.63	6.62	7.95
	median	4.28	5.07	4.81	4.67	5.26	6.07
Coliform bacteria	min.	2.00	4.12	3.06	2.68	2.56	3.00
	max.	9.03	6.19	7.79	6.63	6.61	7.95
	median	4.01	5.27	2.78	3.67	3.40	5.44
Escherichia coli	min.	1.70	3.31	1.70	2.22	1.70	1.70
	max.	8.96	6.09	6.85	6.63	6.61	7.26
	median	5.60	6.11	5.78	5.95	6.02	6.53
Anaerobic mesophilic bioburden	min.	4.22	5.44	5.31	4.87	4.56	5.06
	max.	9.22	6.61	7.84	7.07	7.47	7.93
Lactobacilli		5.47	6.06	5.56	5.60	5.80	6.17
Ratio Lactobacilli : E. coli		1.36	1.14	2.00	1.52	1.71	1.14

Values are medians ± minimal (min.) and maximal (max.) bacterial count in log CFU × g^{-1} jejunal mucosa. Lactobacilli were calculated by subtracting Enterobacteriaceae counts from anaerobic mesophilic bioburden counts.

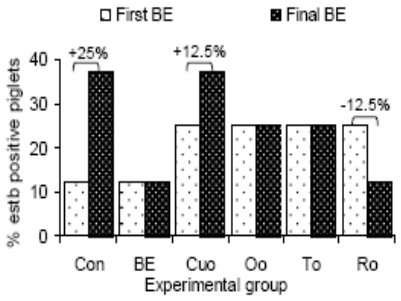


Figure 1. Influence of different phytogenic feed additives on the detection of the heat stable enterotoxin estb produced by enterotoxic E. coli strains of the serotypes O149:K91 with F4 or F6 fimbriae and O138:K81 with F18 fimbriae in faeceal samples.

(factor 2.15), GPx1 (factor 1.79) and SOD1 (factor 2.14) (**Table 9(a)**). Within the labiatae oils to and in particular Ro were most effective in the up-regulation of xenobiotic and antioxidant enzymes. Both oils increased the mRNA expression of jejunal GPx1 and EPHX1 significantly compared to Con piglets. Whereas To additionally increased SOD1 mRNA level significantly, Ro strongly influenced AFAR expression compared with Con piglets. Jejunal mRNA expression of some of the genes investigated was more potently increased by To and in particular by Ro than by BE. Jejunal HMOX1 mRNA was up-regulated by feeding the rosemary and BE diets. However, due to a high standard deviation for this enzyme the effect was not statistically significant. In contrast to our expectations, the second reference additive Cuo caused no increase in the mRNA levels of the jejunal xenobiotic and antioxidant enzymes as well as Oo, coming along with a high direct antioxidant capacity (**Table 9(a)**). A completely different induction pattern for the xenobiotic and antioxidant enzymes was analysed in the ileum. No effects of the most potent additives BE, To and Ro could be measured with regard to the induction of the antioxidant enzymes GPx1, SOD1 and HMOX1 (**Table 9(b)**). In contrast the mentioned additives positively affected the expression of the xenobiotic enzymes AFAR, EPHX1 and GSTa2, with significant effects of BE on AFAR (factor 3.80) and EPHX1 (factor 2.78), of To on GSTa2 (factor 1.93), and of Ro on AFAR (factor 3.40). More-

Table 7. Influence of different phytogenic feed additives on bacterial microflora in colonic mucosa of piglets.

		Experimental group (median, min., max.)*					
Colon	**log CFU/g**	**Con**	**BE**	**Cuo**	**Oo**	**To**	**Ro**
Aerobic mesophilic bioburden	median	8.37	8.86	8.31	8.29	8.23	8.14
	min.	8.01	7.70	7.66	7.81	5.94	7.52
	max.	9.21	9.65	8.81	8.66	9.05	9.04
Enterobacteriaceae	median	5.18	6.17	5.09	5.68	5.49	6.10
	min.	3.94	4.65	3.91	3.55	3.01	3.75
	max.	7.70	7.82	6.93	7.11	6.72	7.17
Coliform bacteria	median	5.18	6.17	4.05	5.40	5.49	6.10
	min.	3.94	4.65	3.62	1.70	2.66	3.71
	max.	7.70	7.61	6.93	7.11	6.72	7.17
Escherichia coli	median	3.98	4.66	5.45	4.98	5.33	3.76
	min.	1.70	1.70	1.70	1.70	1.70	1.70
	max.	7.57	6.77	6.81	7.00	6.59	7.07
Anaerobic mesophilic bioburden	median	8.91	8.68	8.81	8.77	8.90	8.82
	min.	8.04	8.19	8.13	8.11	7.13	8.15
	max.	9.56	9.44	9.27	9.21	9.32	9.40
Lactobacilli		8.91	8.61	8.81	8.76	8.90	8.82
Ratio Lactobacilli : E. coli		2.20	1.85	1.61	1.76	1.66	2.34

Values are medians ± minimal (min.) and maximal (max.) bacterial count in log CFU × g^{-1} colonic mucosa. Lactobacilli were calculated by subtracting Enterobacteriaceae counts from anaerobic mesophilic bioburden counts.

over the impact of BE and Ro on ileal AFAR expression (average factor: 3.60) compared to Con piglets was distinctly higher than in the jejunum.

As observed in the jejunum, the addition of Cuo and Oo caused no significant changes in the expression of xenobiotic and antioxidant genes also in the ileum.

Most interestingly in the colon a completely different regulation profile of antioxidant and xenobiotic enzymes was measured due to feeding the pyhtogenic substances. In colon rosemary was the only additive that powerfully induced both antioxidant enzymes (GPx1, factor 3.20; SOD1, factor 1.62) and xenobiotic enzymes (AFAR, factor 2.17; GSTa2, factor 1.71). The other feed additives only increased GPx1 mRNA expression to small and not significant extent, whereas nearly no changes or even a marked down-regulation could be observed for the xenobiotic enzymes (AFAR, EPHX1, GSTa2).

In the liver the reference additives BE and Cuo significantly increased the mRNA concentrations of AFAR (average factor 1.46) and EPHX1 (average factor 2.12) compared to the Con group (**Table 9(c)**). EPHX1 expression was also significantly increased in piglets of the Oo group (factor 1.97) and in the Ro group (factor 1.55). In groups BE and Ro the expression of GSTa2 was down-regulated by 39 to 43% of the Con level (**Table 9(d)**). While BE signifi- cantly increased hepatic GPx1 mRNA expression (factor 1.66), the addition of Cuo increased SOD1 mRNA levels (factor 1.47) in liver tissue.

3.5. TROLOX® Equivalent Antioxidant Capacity (TEAC) in Jejunum Mucosa, Colon and Liver Tissue

TEAC values in jejunum, colon and liver are shown in **Figure 2**. Jejunal and hepatic TEAC values showed a similar height, whereas colonic TEAC reached only about 30% to 50% of those in jejeunum and liver (**Figure 2**). All phytogenic feed additives increased jejunal TEAC by 26% to 64% compared with Con piglets. In the colon no effects of the phytogenic additives on TEAC could be measured. Although BE (20.7%), Cuo (13.5%), Oo (10.3%), To (26.1%) increased liver TEAC distinctly compared to Con piglets, only the rise produced by feeding Ro (46.4%) was significant.

Table 8. Influence of different phytogenic feed additives on bacterial microflora in faecal samples of piglets.

Group	Time of BE		Aerobic mesophilic bioburden	Enterobacteriaceae	Coliform Bacteria	E. coli	Anaerobic mesophilic bioburden	Lactobacilli	Ratio Lactobacilli: E.coli
Con	Initial BE	Median	6.08	4.50	4.50	4.18	6.84	6.80	1.51
		min	3.76	2.80	2.44	2.30	5.82		
		max	6.34	6.10	6.10	5.30	7.85		
	Final BE	Median	7.41	4.26	4.26	3.82	7.78	7.78	2.04
		min	6.82	2.00	1.70	1.70	7.47		
		max	8.05	5.48	5.48	4.32	7.97		
BE	Initial BE	Median	5.74	4.01	4.01	3.00	6.31	6.31	1.57
		min	4.37	2.48	2.48	1.70	5.33		
		max	6.47	5.43	5.40	5.07	7.41		
	Final BE	Median	7.16	5.69	5.69	5.39	7.80	7.76	1.44
		min	5.99	3.89	3.89	3.89	7.28		
		max	7.63	7.34	7.32	6.28	8.29		
Cuo	Initial BE	Median	5.70	4.78	4.76	4.03	6.62	6.57	1.37
		min	4.68	3.61	3.59	1.70	5.68		
		max	7.21	6.88	6.88	5.15	7.17		
	Final BE	Median	6.97	4.58	4.58	4.48	7.49	7.48	1.67
		min	5.37	2.00	2.00	1.70	6.35		
		max	8.34	5.71	5.71	5.61	8.25		
Oo	Initial BE	Median	5.49	3.76	3.74	3.82	6.29	6.28	1.67
		min	5.03	1.70	1.70	1.70	5.54		
		max	7.35	5.57	5.52	4.28	7.54		
	Final BE	Median	7.07	4.27	4.23	4.17	7.79	7.78	1.87
		min	6.51	1.70	1.70	1.70	6.93		
		max	7.49	6.71	6.71	5.30	8.23		
To	Initial BE	Median	6.04	4.95	4.56	3.26	7.00	7.00	1.41
		min	4.90	3.32	3.32	1.70	6.18		
		max	7.52	7.05	7.05	5.46	8.47		
	Final BE	Median	7.05	5.24	5.24	2.53	7.58	7.56	2.99
		min	5.91	1.70	1.70	1.70	6.70		
		max	7.49	6.62	6.62	5.28	8.02		
Ro	Initial BE	Median	6.46	4.32	4.31	4.07	6.30	6.26	1.45
		min	3.63	3.36	3.32	2.48	5.28		
		max	7.48	5.39	7.14	5.90	7.91		
	Final BE	Median	6.72	4.29	4.29	2.30	7.36	7.36	3.20
		min	5.89	2.60	2.60	1.70	6.78		
		max	7.34	6.16	6.15	5.25	7.83		

Values are medians ± minimal (min.) and maximal (max.) bacterial count in log CFU per rectal swab. Lactobacilli were calculated by subtracting Enterobacteriaceae counts from anaerobic mesophilic bioburden counts.

Table 9. Relative mRNA concentration of xenobiotic (GSTa2, EPHX1, AFAR) and antioxidant enzymes (GPx1, SOD1, HMOX1) and KEAP1 in jejunal and ileal mucosa, colon and liver tissue of piglets.

(a) Jejunal mucosa

Group	Con		BE		Cuo		Oo		To		Ro	
Gene	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
GPx1	1.00^{a}	0.21	1.79^{b}	0.36	0.96^{a}	0.12	0.99^{a}	0.21	1.70^{b}	0.13	2.16^{b}	0.41
SOD1	1.00^{a}	0.09	2.14^{bc}	0.22	1.36^{ab}	0.11	1.32^{abc}	0.34	1.90^{c}	0.10	2.26^{ac}	0.40
AFAR	1.00^{a}	0.18	2.14^{b}	0.27	1.33^{ab}	0.33	1.07^{a}	0.20	2.03^{ab}	0.53	2.37^{b}	0.51
EPHX1	1.00^{a}	0.23	1.56^{ab}	0.27	1.49^{ab}	0.34	1.56^{ab}	0.26	2.40^{bc}	0.61	2.86^{c}	0.55
GSTa2	1.00^{a}	0.26	2.15^{b}	0.38	1.02^{ab}	0.22	1.68^{ab}	0.59	1.72^{ab}	0.65	2.22^{ab}	0.64
HMOX1	1.00	0.25	1.42	0.41	1.07	0.33	1.01	0.26	1.18	0.24	1.73	0.24
Keap1	1.00^{a}	0.20	1.95^{b}	0.37	0.84^{a}	0.15	0.87^{a}	0.12	1.24^{ab}	0.37	1.58^{ab}	0.42

(b) Ileal mucosa

Group	Con		BE		Cuo		Oo		To		Ro	
Gene	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
GPx1	1.00^{a}	0.15	1.16^{a}	0.16	1.37^{a}	0.30	1.92^{a}	0.74	1.03^{a}	0.29	1.50^{a}	0.40
SOD1	1.00^{a}	0.34	1.00^{a}	0.38	0.75^{a}	0.39	0.59^{a}	0.16	0.95^{a}	0.46	0.61^{a}	0.14
AFAR	1.00^{a}	0.13	3.80^{b}	1.30	1.09^{a}	0.21	1.91^{a}	0.77	2.27^{a}	0.95	3.40^{b}	0.68
EPHX1	1.00^{a}	0.15	2.78^{b}	0.87	1.47^{ab}	0.37	1.17^{ab}	0.49	1.91^{ab}	0.58	1.85^{ab}	0.55
GST	1.00^{a}	0.12	1.70^{ab}	0.27	1.42^{ab}	0.18	1.49^{ab}	0.42	1.93^{b}	0.43	1.58^{ab}	0.28
HMOX1	1.00^{a}	0.33	0.90^{a}	0.17	0.93^{a}	0.39	0.68^{a}	0.19	0.83^{a}	0.27	0.80^{a}	0.24
Keap1	1.00^{a}	0.55	1.24^{a}	0.49	0.35^{a}	0.16	0.52^{a}	0.16	1.04^{a}	0.64	0.76^{a}	0.27

(c) Colon tissue

Group	Con		BE		Cuo		Oo		To		Ro	
Gene	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
GPx1	1.00^{a}	0.08	1.48^{a}	0.41	1.86^{a}	0.41	1.21^{a}	0.24	1.52^{a}	0.18	3.20^{b}	0.61
SOD1	1.00^{a}	0.05	0.92^{a}	0.24	1.00^{a}	0.11	0.78^{a}	0.14	0.92^{a}	0.05	1.62^{b}	0.22
AFAR	1.00^{a}	0.28	0.98^{a}	0.34	0.99^{a}	0.22	0.62^{a}	0.12	0.71^{a}	0.05	2.17^{b}	0.52
EPHX1	1.00^{ac}	0.08	0.55^{b}	0.05	0.69^{bc}	0.07	0.63^{b}	0.14	0.88^{c}	0.09	0.96^{ac}	0.08
GSTa2	1.00^{a}	0.13	0.91^{a}	0.21	1.01^{a}	0.18	0.88^{a}	0.13	0.79^{a}	0.06	1.71^{b}	0.37
HMOX1	1.00^{ab}	0.26	0.84^{ab}	0.16	1.63^{a}	0.45	0.59^{b}	0.16	1.39^{ab}	0.41	1.87^{a}	0.49
Keap1	1.00^{a}	0.10	1.32^{ac}	0.33	1.21^{ac}	0.18	0.65^{b}	0.12	1.08^{ac}	0.09	1.73^{c}	0.24

(d) Liver tissue

Group	Con		BE		Cuo		Oo		To		Ro	
Gene	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
GPx1	1.00^{a}	0.19	1.66^{b}	0.23	1.03^{a}	0.13	1.25^{ab}	0.18	1.06^{a}	0.24	1.47^{ab}	0.23
SOD1	1.00^{a}	0.09	0.85^{a}	0.07	1.47^{b}	0.18	1.03^{a}	0.13	0.88^{a}	0.13	0.78^{ab}	0.27
AFAR	1.00^{a}	0.10	1.40^{bc}	0.15	1.52^{b}	0.16	1.20^{ab}	0.09	1.03^{a}	0.10	1.07^{ac}	0.11
EPHX1	1.00^{a}	0.25	2.04^{b}	0.44	2.20^{b}	0.31	1.97^{b}	0.22	1.15^{ab}	0.30	1.55^{b}	0.25
GSTa2	1.00^{a}	0.10	0.61^{bc}	0.07	0.81^{ab}	0.06	0.78^{ab}	0.05	0.82^{ab}	0.10	0.57^{c}	0.10
HO1	1.00^{a}	0.75	1.29^{a}	0.72	1.99^{a}	1.09	1.42^{a}	0.72	1.34^{a}	0.65	1.73^{a}	0.62
Keap1	1.00^{a}	0.24	0.59^{a}	0.31	1.73^{a}	0.62	0.93^{a}	0.26	0.96^{a}	0.49	0.55^{a}	0.20

Values represent means ± standard error of mean (SEM) of the mRNA abundance relative to group Con = 1.0 (n = 8 animals per experimental group). $^{a, b, c}$Mean values with unlike superscripts within a row indicate significant differences ($P < 0.05$) in the LSD test or the Games Howell test. The superscript "a" was assigned to control group.

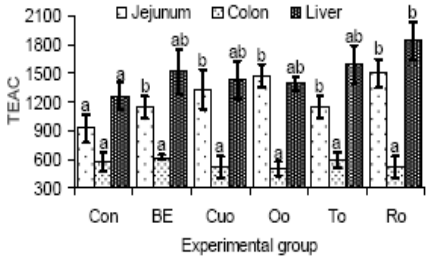
3.6. 2-Thiobarbituric Acid-Reactive Substances (TBA-RS) in Jejunal Mucosa and the Liver

In the jejunum TBA-RS concentration was distinctly ($P < 0.150$) but not significantly reduced by BE (16.1%), Cuo (14.2%), To (25.9%), and Ro (16.0%). Oo (25.7%) and To (25.9%) decreased jejunal lipid peroxidation to a significant extent (**Figure 3**). Liver TBA-RS were reduced by feeding all phytogenic additives to a greater or lesser extent (BE: 21.3%, Cuo: 8.22%, Oo: 23.8%, To: 11.5%, Ro: 23.2%). However, in the liver no significant differences resulted from these data, due to a high variation within the groups.

4. DISCUSSION

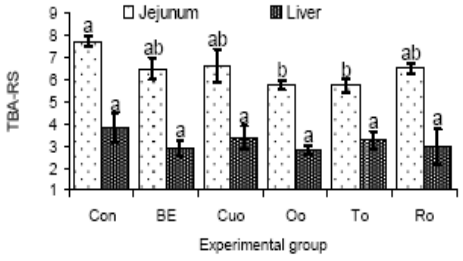
4.1. Performance Parameters (Feed Intake, Body Weight, Weight Gain, Feed Conversion Ratio)

As stated in the introduction data from the literature,



Values represent means ± SEM (n = 8 animals per experimental group). [a,b]Mean values with unlike superscripts indicate significant differences between means ($P < 0.05$) in the LSD test. The superscript "a" was assigned to the control group.

Figure 2. TROLOX® equivalent antioxidant capacity (TEAC in µmol per g organ fresh matter and kg body weight) in jejunal mucosa, colon and the liver of piglets.

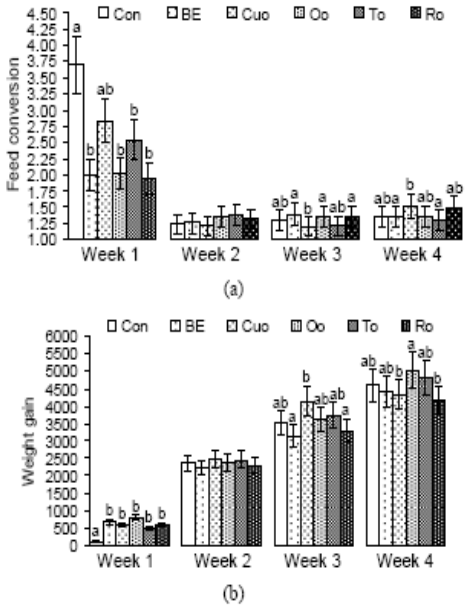


Values represent means ± SEM (n = 8 animals per experimental group). [a,b]Mean values with unlike superscripts indicate significant differences between means ($P < 0.05$) in the LSD test. The superscript "a" was assigned to the control group.

Figure 3. Thiobarbituric acid reactive substances (TBA-RS in nmol per g organ fresh matter and kg body weight) in jejunal mucosa and the liver of piglets.

justifying the declaration of phytogenic feed additives and in particular of labiatae oils as appetite stimulating substances and as growth promoters are inconsistent. Whereas some studies reported on beneficial effects of even low dietary concentrations of oregano oil (0.0125 g/kg to 0.025 g/kg diet) [1] and of blends of essential oils from oregano, anise, citrus peels, and chicory [3] on weight gain and feed efficiency ratio, other studies showed no effects [4] or even opposite effects on these parameters [5,40]. Due to the standardisation of the essential oils' concentration to that of their main terpenes (150 mg main terpene/kg diet) in our study the added oil concentrations were rather high. Thus it can be remarked as a positive result of our study that the high phytogenic concentrations in the diets caused no mentionable adverse effects on performance parameters at all. With the exception of Ro in our study all essential oils improved weight gain or feed conversion ratio in comparison to Con piglets to a greater or lesser extent (**Table 5**). However, these differences were not significant over the whole experimental period.

According to results of a prior study [1] piglets of groups Oo and To had the best feed conversion compared to the essential oil groups Cuo and Ro and to group Con. Within the labiatae oils Oo and in particular To improved weight gain during the whole experiment compared to Ro as another labiatae oil. That within the labiatae oils thyme seems to unfold the most positive effects on performance parameters has been demonstrated in a choice experiment with piglets [40]. In a study investigating the growth promoting effects of curcumin (supplementation of 200 mg curcumin/kg to the diets of Large White × Landrace × Duroc piglets), body weight gain and feed intake of supplemented piglets remained unaffected compared to the control group [41]. Our results for turmeric oil, containing mainly turmeron derivatives and only traces of curcumin, deviated from these results (**Table 5** and **Figures 4(a)**, **(b)**). Over the whole experimental period weight gain of Cuo piglets was comparable to that of Oo and To piglets. In week 3 of the experiment Cuo piglets even had the highest weight gain and the best feed conversion compared to all other experimental groups (**Table 5** and **Figures 4(a)**, **(b)**). Thus our results for Cuo are rather in coincidence with data of Wenk (2005), reporting on a dose-dependent positive effect of turmeric on performance of pigs and broilers [42]. A very interesting result with regard to the performance parameters could be observed for broccoli extract (BE), not permitted as a feed additive in the EU until today. While BE piglets had the second best feed conversion after To piglets, weight gain of BE piglets was the second lowest. Only Ro piglets had a somewhat lower total weight gain. Thus BE seems to maximise feed conversion, but to slow weight gain slightly (**Table 5** and **Figures 4(a)**, **(b)**). This



Values represent means ± SEM (n = 8 animals per experimental group). [ab]Mean values with unlike superscripts indicate significant differences between means ($P < 0.05$) in the LSD test.

Figure 4. (a) Feed conversion (g/g) and (b) Weight gain (g) of piglets fed different phytogenic feed additives for 28 days.

most interesting aspect of BE may have derived from potential goitrogenic effects of the isothiocyanate sulforaphane contained in a high concentration in the extract (4.06% w/w) and of other goitrogenic isothiocyanates contained in the extract in very low concentrations (<0.1% w/w). However, studies in humans have proven that sulforaphane, even in higher concentrations, has no or only a negligible influence on thyroid metabolism [43, 44].

Nevertheless, an important fact which should be remarked at the end of the discussion concerning performance parameters is that all phytogenic feed additives had a distinct positive effect on the performance parameters feed conversion and weight gain in the first week of the experiment (**Figures 4(a)**, **(b)**). While about half of the Con piglets gained no weight in the first week or even lost weight, all phytogenic feed additives counteracted this undesired aspect. In particular in the first week after weaning it is of importance that the piglets have a good performance in order to prevent the loss of animals and to protect them from infections. This is in accordance with other reports [45,46].

Weight gain and feed conversion are the two most important parameters in today's animal nutrition, and the feed industry frequently advertises their phytogenic additives as growth promoters. In addition, both weight gain and feed conversion are frequently associated with general animal health. In contrast the results of the entire experimental period in our present trial and the outcome of a number of other studies mentioned above [4,5,40] suggest that phytogenic feed additives frequently do not fulfil the criteria acting as mere growth promoters. To complicate matters: One explanation for the lacking or sometimes slightly negative effect of phytogenic additives on performance may surprisingly be the consequence of their beneficial antioxidant effects. In this context an *in vitro* study has shown the potent inhibition of porcine pancreatic amylase by phenolic oregano compounds [47]. However, as discussed above, in particular in the critical first week, all additives tested, exerted their potential to acting as growth promoters [45,46]. Moreover, in the majority of these studies [4,5,40], including our present experiment, the piglets were not challenged with pathogenic microorganisms or toxic substances. It can be assumed that the threat of infections and the challenge with feed contaminants increase under practical feeding conditions with a high stocking rate [48]. In this context experiments in which broilers were infected with Eimeria tenella or fed aflatoxin containing diets, the essential oil of oregano or turmeric powder counteracted the depressed feed intake and growth [49-51]. Accordingly some other studies with rats as model animals and tissue cultures have demonstrated the beneficial effects of thyme, rosemary and of sulforaphane on mycotoxin detoxification [28,52-55].

However, until today there is a lack of broadly based studies in farm animals regarding this aspect. To our opinion the verification of beneficial effects of phytogenic additives on animal performance under challenged conditions represents an important field in future research and a useful instrument to evaluate commercial feed additives.

4.2. Microflora in Jejunal Mucosa, Colonic Mucosa, Faecal Samples and Detection Rate of Estb

The analysed counts of bacteria attached to the jejunal mucosa showed differential patterns depending on the phytogenic feed additive. With regard to E. coli the maximum counts (50% of the piglets of a group) were reduced by all phytogenic feed additives. The median values for jejunal E. coli were higher in BE and Ro piglets compared to the control, whereas Cuo, Oo and To treatment produced lower counts. However, due to the high individual variation E. coli counts did not differ significantly. In contrast all phytogenic additives slightly but not significantly increased jejunal Lactobacilli compared to Con piglets. A study analysing jejunal chymus of piglets found tendentially reduced E. coli and increased Lactobacilli counts due to feeding diets contain-

ing a blend of 300 mg/kg carvacrol (oregano), cinnamaldehyde and capsicum oleoresin. In this trial the ratio of Lactobacilli:E. coli was even significantly increased [46]. Thus our results for Oo, containing noteworthy amounts of carvacrol, are in accordance with the outcome of the above mentioned trial. Moreover in our trial Oo also increased the Lactobacilli:E. coli ratio (Oo, 1.52:1) compared to Con piglets (1.37:1). Moreover in our study To also distinctly increased the Lactobacilli:E. coli ratio (1.71:1). In the literature both for To and for the essential oil of Curcuma longa (Cuo) no reports on their effects on jejunal E. coli and Lactobacilli exist. Only one study reported on the lacking effect of an essential labiatae oil mixture on microbial counts in the stomach and the ileum of pigs [10]. However, in our trial Cuo was most effective in improving the Lactobacilli:E. coli ratio (2.00:1). For sulforaphane currently only *in vitro* data on a potent bactericidal activity against several E. coli strains exist [18], which could not be confirmed *in vivo* in our present study. Similarly, for Ro the majority of studies, investigating its bactericidal effects, including those against E. coli, was carried out *in vitro* in the context of investigating the storage stability of meat [7-9]. However, due to an higher increase in jejunal E. coli compared to jejunal Lactobacilli BE and Ro had the lowest Lactobacilli:E. coli ratios of 1.14:1 and 1.13:1, respectively.

In contrast to the moderate positive effects of Cuo, Oo and To on microorganisms attached to jejunal mucosa we could not find any differences in the bacterial populations of colonic mucosa. To the best of our knowledge no studies reporting on effects of phytogenic additives on bacterial populations in the colonic mucosa exist so far. Only few studies reported on the effects of phytogenic substances on caecal and faecal microbial counts. Feeding a blend of the essential oils of gingermint (labiatae plant), anis and sage (300 mg/kg diet) had no influence on colonic digesta E. coli counts whereas Lactobacilli were increased [10]. In contrast in our trial no effects of the labiatae oils Oo and To could be analysed for both bacterial classes attached to the colonic mucosa. Only Ro slightly increased Lactobacilli: E. coli ratio compared to Con piglets. This result for Ro was further confirmed by the distinct improvement of Lactobacilli to E. coli ratio in faecal samples collected at the end of the experiment.

A further interesting and important result of our study consisted in the influence of the phytogenic additives on the incidence of estb as a marker for the existence of enterotoxic E. coli strains of the serotypes O149:K91 with F4 or F6 fimbriae and O138:K81 with F18 fimbriae in the gut of the piglets (**Figure 1**). Whereas the incidence rate of estb existence in Con piglets distinctly increased till the end of the experiment, Ro was the only additive which even reduced estb incidence. In the other labiatae oil groups (Oo and To) and in the BE group estb incidence did not increase. Only in the Cuo group a slight increase in estb incidence could be observed which however remained below the Con group. Since enterotoxic E. coli strains (ETEC) are an important risk factor for the development of diarrhoea, the reduction of these strains is of particular importance. In a trial in which piglets were inoculated with a mixture of ETEC with F4-, F5-, F6-, F18- and F41 fimbriae ETEC with F4 fimbriae caused diarrhoea preferentially in the first 2 weeks after weaning, strains with F18 fimbriae were responsible for diarrhoea in 3 to 5 week old piglets [56]. Since estb is marker for strains with both F4 and F18 fimbriae future studies should investigate the selective reduction of ETEC by phytogenic feed additives. Moreover our results demonstrate the need of future studies in which piglets are challenged with ETEC in order to evaluate the suppressive effect of phytogenic additives on these strains *in vivo*. As stated with regard to the performance parameters this topic would represent a useful tool to evaluate commercial feed additives and to optimize mixtures of phytogenic additives.

4.3. ARE Regulated Xenobiotic and Antioxidant Enzymes, TROLOX® Equivalent Antioxidant Capacity (TEAC), and Thiobarbituric Acid Reactive Substances

To the best of our knowledge our data have shown for the first time that labiatae oils (Oo, To and Ro), broccoli extract (BE), and turmeric oil (Cuo) have promising effects on a panel of ARE regulated xenobiotic enzymes and the antioxidant system in piglets. Only a trial of our group has previously demonstrated similar results for growing chicken. However, the data of the broiler trial and those of the current piglet study exert some fundamental differences with regard to the organ specific influence of the phytogenic additives. Whereas in the broiler trial ARE-regulated xenobiotic and antioxidant enzymes were mainly up-regulated in the small intestine and partially down-regulated in the large intestine and in the liver, the data of our present piglet study showed a potent up-regulation of these enzymes by Ro in the large intestine and by BE, Cuo and Ro also in the liver. This particular result may be the consequence of the higher dietary concentration of the phytogenic additives in the piglet trial due to the standardisation of the active compounds. Because of their known impact on the induction of ARE-regulated genes we have applied broccoli extract and turmeric oil [15,57] as reference additives in our study. Most interestingly, the reference substances showed a completely different induction profile of ARE-regulated genes. Whereas sulforaphane, derived by bacterial

cleavage from its glucosinolate precursor glucoraphanin (GRA) contained in BE [58], had a strong effect on the up-regulation of xenobiotic and antioxidant enzymes in the small intestine, Cuo unrolled its maximum effect on this process in the liver (**Tables 9(a), (b), (d)**). This results differs from our chicken trial in which we have observed a distinct induction of ARE regulated genes by Cuo already in the small intestine. That broccoli extract can influence the gene expression of xenobiotic and antioxidant enzymes already in the small intestine with a lower bacterial colonisation than in the large intestine is supported by results of most recent studies. In these trials the incubation of precision slices of different tissues with GRA effected a strong induction of ARE-regulated antioxidant and xenobiotic enzymes, suggesting that GRA per se can up-regulate the mentioned enzymes [26,27]. While in most other studies, investigating the effects of various turmeric extracts on the up-regulation of antioxidant and xenobiotic enzymes, dried curcumin-rich extracts were used [59,60] we have fed turmeric oil containing ar-turmerone as the primary active compound. Both curcumin and ar-turmerone have been shown to induce antioxidant and xenobiotic enzymes potently. In our study we have deliberately chosen the ar-turmerone rich turmeric oil [15], since the oil is easier to add to the diets and its price is more attractive for animal nutrition. In former studies with laboratory animals and with humans the main intention to use GRA, SFN and turmeric extracts was to study their preventive effects against intestinal cancers. Their safety has been verified [43,44,61]. In farm animals, having a short live span and needed for food production, the emphasis on application of these inducers of ARE-regulated genes rather aims on their efficiency to strengthen the intestinal barrier against oxidative stress in the organism and to activate their defence mechanisms against bacterial toxin and food borne toxins.

For the labiatae oils the results of our present study have confirmed the data of the broiler trial with regard to their differentiated effects on antioxidant processes (**Tables 9(a), (b), (d)**). Within the labiatae oils Ro and To induced jejunal ARE regulated enzymes to a much higher extent compared to Oo. In this context it is important that Ro in contrast to the broiler trial had an even higher influence on the induction of antioxidant and xenobiotic enzymes in the small intestine than the reference substance BE (**Tables 9(a), (b)**). In colon nearly all ARE-regulated enzymes showed a lowered response to feeding the phytogenic feed additives. As mentioned above only Ro maintained its strong inductive effect also in the colon.

Differences in the antioxidant mechanisms of labiatae oils can be assumed to deriving from their main terpene compounds. Whereas oregano and thyme mainly contain the phenolic terpenes carvacrol and thymol, Ro contains the epoxy-terpene 1,8-cineole. Phenolic groups again possess direct antioxidant effects and therefore have a rather low influence on the induction of ARE-regulated xenobiotic and antioxidant enzymes accounting for indirect antioxidant properties. In contrast the reactive epoxide group of 1,8 cineole (rosemary) contributes to a stronger induction of the indirect antioxidant system via activating the ARE of xenobiotic and antioxidant enzymes [62,63]. In addition to the modification of sensor -SH-groups of KEAP1 or by triggering Nrf2 phosphorylation [25,64] there may exist another mechanism for ARE-gene induction. This particular mechanism involves the metabolisation of the terpenes by phase I cytochrome P450 enzymes followed by the induction of antioxidant and xenobiotic enzymes through the activated phase I metabolites [65].

Since Keap1 per se has been shown to be induced via Nrf2, another important point to discuss is the impact of phytogenic feed additives on Keap1 de novo synthesis [64,66]. Whereas BE caused a significant and Ro a tendential increase in jejunal Keap1 expression, Cuo and Oo rather led to a decreased Keap1 mRNA concentration. Moreover the increased hepatic Keap1 mRNA level by feeding Cuo corresponded to the strongest induction of antioxidant and xenobiotic enzymes in the liver [64].

Our results for the differentiated response of the direct and the indirect antioxidant systems due to feeding phytogenic feed additives demonstrates the need of future studies with tissue cultures and model animals investigating the detailed mechanisms by which phytogenic feed additives and their key compounds influence these systems (e.g. by Keap1 modification, by Nrf 2 phosphorylation or subsequent to phase I up-regulation).

In this context our results for the TEAC values and for lipid peroxidation in jejunal mucosa, colon and the liver are of interest (**Figures 2, 3**). The TEAC value of a tissue comprises direct and indirect antioxidant effects by secondary mechanisms like ARE induction. In the jejunum the addition of all phygenic feed additives affected a significant increase in TEAC compared to Con piglets. These data are in accordance with our broiler trial. Thus it can be assumed that the rise in jejunal TEAC by BE, containing the isothiocyanate SFN, without any direct antioxidant mainly was basing on the up-regulation of antioxidant enzymes and therefore on an indirect antioxidant effect [57]. This result is in accordance with our results for the gene expression data (**Table 9(a)**). Within the oils tested in our trial Ro had the highest TEAC value of 156 mmol/100 mL, followed by Oo (120), To (116) and Cuo (90). In accordance with these data Ro increased jejunal TEAC most powerful which may be the consequence of both its high direct antioxidant potential and of the strong induction of xenobiotic and antioxidant

enzymes. A similar mechanism may exist for To [63]. In contrast Oo which had only a weak influence on the up-regulation of xenobiotic and antioxidant enzymes may have increased jejunal TEAC predominantly by its direct antioxidant effects as explained above [13]. Similarly the increase in jejunal TEAC by feeding Cuo may have derived from its main aromatic terpene ar-turmerone as a potent radical scavenger [67] (**Figure 2**). Most interestingly ar-turmerone has been shown to possess both direct antioxidant effects and strong indirect antioxidant effects [15]. Due to the contrary results for Cuo on jejunal ARE-induction in our broiler trial it can be assumed that there exists a dose-dependent effect due to the predominance of direct or indirect antioxidant effects. This particular mechanism needs intensive investigation in the future. In accordance with the decreased influence of the phytogenic additives on antioxidant and xenobiotic enzymes' expression colonic TEAC remained uninfluenced. This particular result may derive from the fact that distinctly lower concentrations of the active compounds of the phytogenic substances reach the large intestine than they are present in the small intestine. Despite an overall lower influence of the phytogenic substances on the up-regulation of liver xenobiotic and antioxidant enzymes all additives influenced liver TEAC positive in tendency or even significantly. Higher liver TEAC values in piglets fed phytogenic additives may derive from the increased antioxidant potential in the small intestine. This particular barriere hypothesis has been suggested in detail for the selenoprotein glutathione peroxidase 2 [68, 69].

In the jejunum all phytogenic feed additives reduced iron provoked lipid peroxidation to a larger or smaller extent compared to Con piglets with significant effects for Oo and To. This result confirms the important function of the small intestine as an effective barrier against oxidative stress [69].

However, in the liver the influence of the phytogenic additives on TEAC and TBA-RS was less pronounced than in the small intestine. In contrast in our broiler trial we could measure a significant increase in liver TEAC and a significant decrease in liver TBA-RS. This phenomenon may be explained by differences in feed intake between pigs and broilers. Due to the fact that feed consumption of pigs increases with body weight and the feed conversion drops it can be assumed that a longer feeding period would have increased liver TEAC significantly and concomitantly reduced TBA-RS. However, in two other pig trials feeding diets supplemented with different amounts of Oo (0.25, 0.50 and 1.00 mL/kg diet) or the essential oils of rosemary, oregano and ginger (500 mg/kg diet) for a comparable short period of 35 days also remained without an influence on meat characteristics and lipid peroxidation parameters [70,71]. In contrast, feeding a rather high amount of an oregano extract (60 mL) to finisher pigs for 2 weeks significantly improved the antioxidant potential and the storage stability of the meat [72].

A final overview of our current results regarding the influence of broccoli extract and various essential oils on xenobiotic enzymes, on the antioxidant system, and on intestinal and faecal microflora of piglets is given in **Table 10**. These summarised data may give a good basis to discuss the development of rational and expedient combinations of the single additives in order to maximise the impact on performance, microbial eubiosis, and on direct and indirect antioxidant effects.

Compared to the control group in our study To followed by Oo and Cuo showed a positive influence on the performance parameters of the piglets. In the small intestine To exerted both, direct and indirect antioxidant effects. However, its influence on the xenobiotic enzymes in the liver was rather weak. Thus the combination of To with BE or Cuo, both acting as potent inducers of xenobiotic enzymes in the liver, may contribute to the optimisation of detoxification processes in the whole organism. Similarly a combination of Oo, having a strong direct antioxidant effect, with BE may represent a further rational combination of two additives. Because of its superior direct antioxidant potential and its weak effects on xenobiotic enzymes Oo may further be a very good additive for finisher pigs in order to improve the antioxidant potential of the meat prior to slaughter. Moreover feeding Oo to finisher pigs may increase storage stability of the meat, as suggested by the above mentioned study. In our trial Ro turned out as the all-rounder additive due to its strong effects on the induction of xenobiotic enzymes in all organs investigated. Moreover Ro as the only additive even reduced the incidence rate of enterotoxic E. coli existence in the faeces. Only the performance parameters were influenced slightly negative by Ro.

Maybe replacement of a small percentage of Ro addition by To or Oo could contribute to the improvement of performance. With regard to the microbial parameters BE, Oo and To had a rather neutral influence since in the mentioned groups no increase in the number of piglets infected with enterotoxic E. coli could be detected. Only in the Cuo group the incidence rate of estb positive piglets increased. This rather negative effect should be intensively investigated in future studies and lowers the very positive impact of Cuo on performance.

In summary we have shown that the different phytogenic feed additives tested significantly and differentially influence the direct and the indirect antioxidant system (xenobiotic system) in various organs of piglets. In particular the induction of the xenobiotic system is important to improve the defense against microbial and feed derived toxic substances. Future studies are needed to

Table 10. Summary of the effects of different phytogenic feed additives on performance, the antioxidant system and on microflora in jejunal mucosa, colonic mucosa and faeces

Phytogenic additive	Feed consumption	Weight gain	Feed conversion	Overall performance
BE	–	O	+	O
Cuo	+	++	O	+
Oo	++	++	O	+
To	+	++	++	++
Ro	O	O	–	O

Phytogenic additive	Induction of ARE-regulated genes in the small intestine (jejunum /ileum)	Induction of ARE-regulated genes in the large intestine (colon)	Direct antioxidant effects in the small intestine (jejunum/ileum)	Influence on total antioxidant potential in the small intestine
BE	+++	O	O	++
Cuo	O	O	+++	++
Oo	O	–	+++	++
To	++	O	+	++
Ro	+++	+++	+	++

Phytogenic additive	Induction of ARE-regulated genes in the liver	Direct antioxidant effects in liver	Influence on total antioxidant potential in the liver	Reduction of lipid peroxidation in the liver
BE	++	O	+	+
Cuo	++	O	+	O
Oo	O	+	+	++
To	O	+	+	O
Ro	+	++	++	+

Phytogenic additive	Influence on Lactobacilli: E. coli ratio in jejunal mucosa	Influence on Lactobacilli: E. coli ratio in colonic mucosa	Influence on Lactobacilli: E. coli ratio in faeces	Influence on existence of enterotoxic E. coli in faeces
BE	O	O	–	O
Cuo	+	–	–	–
Oo	O	–	O	O
To	+	–	+	O
Ro	O	+	+	+

+++ very strong; ++ strong; + positive in tendency; O no effect; – rather negative.

investigate the impact of the different feed additives on detoxification processes even under conditions with environmental challenges. The results of these studies could further contribute to the development of optimised combinations of phytogenic feed additives.

5. ACKNOWLEDGEMENTS

This study was supported by Delacon Biotechnik Ges.m.b.H, Steyregg (Austria) to study fundamental mechanisms of phytogenic feed additives and by the H. Wilhelm Schaumann foundation, Hamburg by funding Dipl. troph. Kristin Mueller with a doctoral scholarship. We thank Devakumari Hiller (Jarrow Deutschland GmbH, Berlin, Germany) arranging the contact with Mr. Jarrow L. Rogovin from Jarrow Formulas, Los Angeles, USA, who provided us with the broccoli seed extract. Moreover, we thank our stockmen Olaf Hoedel and Detlev Barth for their help with diet preparation, animal care, and organ sampling. Thank is also addressed to Mr. Frank Schmidt for slaughtering the pigs. Further thank is addressed to our master students Jette Rahn, Annett Schulz, Marianna von Jagemann, and Bettina Hommel for their help with analyses within the scope of their master theses.

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3.3 Mueller K, Blum NM, Mueller AS. *Examination of the anti-inflammatory, antioxidant, and xenobiotic-inducing potential of broccoli extract and various essential oils during a mild DSS-induced colitis in rats.* ISRN Gastroenterology. 2013 Vol. 2013, Article ID 710856, 14 pages. http:// dx.doi.org/10.1155/2013/710856

Hindawi Publishing Corporation
ISRN Gastroenterology
Volume 2013, Article ID 710856, 14 pages
http://dx.doi.org/10.1155/2013/710856

Research Article

Examination of the Anti-Inflammatory, Antioxidant, and Xenobiotic-Inducing Potential of Broccoli Extract and Various Essential Oils during a Mild DSS-Induced Colitis in Rats

Kristin Mueller, Nicole Michaela Blum, and Andreas Stefan Mueller

Institute of Agricultural and Nutritional Sciences, Martin Luther University Halle-Wittenberg, Von-Danckelmann-Platz 2, 06120 Halle (Saale), Germany

Correspondence should be addressed to Kristin Mueller; kristinmueller@gmx.net

Received 9 December 2012; Accepted 20 January 2013

Academic Editors: A. Amedei, A. K. Rishi, and N. Senninger

Phytogenic compounds with antioxidant and anti-inflammatory properties are currently discussed as promising complementary agents in prevention and treatment of inflammatory bowel disease (IBD). Our study aimed to evaluate possible protective and curative effects of broccoli extract (BE) and of the essential oils of turmeric (Cuo), thyme (To), and rosemary (Ro) in a rat model with a mild dextran sulphate sodium- (DSS-) induced colitis. Therefore Wistar rats were fed a diet without an additive (Con) or diets with the addition of BE, Cuo, To, and Ro during the whole experiment. Pretreatment with Ro, Cuo, and To increased the expression of the tight junction protein Cldn3. All additives reduced mRNA of VCAM-1 which plays a crucial role in the first state of inflammatory response. Only Ro pretreatment affected the expression of the antioxidant enzymes HO1, GPx2, and of glutathione-S-transferases. All additives counteracted the DSS-induced rise in COX2 and VCAM-1 expression. Colonic IL-10 was increased by Cuo, To, and Ro. During the recovery phase DSS pretreatment increased NFκB, VCAM-1, and MCP-1: This response was counter-regulated by all additives. We conclude that the phytogenic additives tested have a promising anti-inflammatory potential in vivo and a particular role in the prevention of IBD.

1. Introduction

Inflammatory bowel disease, including ulcerative colitis (UC) and Crohn's disease (CD), is a multifactorial relapsing-remitting disorder, characterized by intermittent periods of acute inflammation in the small and in the large intestine. The main difference between CD and UC is the location and the nature of the inflammatory changes. CD can affect any part of the gastrointestinal tract, from mouth to anus, although the onset of the majority of cases is located in the terminal ileum. In contrast UC is restricted to the colon and the rectum. The exact pathogenic mechanisms provoking both disorders remain almost unclear. However, in a number of cases overreactions of the immune system due to inflammatory stimuli can be observed. In this context, proinflammatory immune modulators like interleukin 1 beta (IL-1β), monocyte chemoattractant protein 1 (MCP-1), and vascular cell adhesion molecule 1 (VCAM-1) play an important role in the development of the disease [1, 2]. Nuclear factor "kappa-light-chain-enhancer" of activated B cells (NFκB) represents a key transcription factor regulating the synthesis of genes involved in immune reactions and inflammatory response. In noninflamed tissues NFκB is inhibited through linkage to its cytosolic inhibitor protein kappa B (IκB). The activation of proteasomal IκB degradation via phosphorylation of critical serine residues by proinflammatory stimuli elicits NFκB translocation to the nucleus and the subsequent induction of its target genes like tumor necrosis factor (TNF)-α and other inflammatory mediators (interleukin 2, interleukin 6, interleukin 8, VCAM-1, intracellular cell adhesion molecule 1, and interferon γ) [3–5].

Dysfunction of the gut barrier accompanied by an increased intestinal permeability is another characteristic symptom in the pathophysiology of IBD [6]. As a consequence of the disordered permeability both antigenic determinants derived from food digestion and commensal

or pathogenic bacteria can overcome the mucosal barrier unimpeded and provoke a continuous intestinal immune response and tissue damage [7]. In this context the increased permeability of gastrointestinal epithelial cells frequently results from the destruction of tight junctions. This process is triggered by oxidative stress deriving from reactive oxygen species (ROS), mycotoxins (e.g., patulin), bacterial components (e.g., lipopolysaccharides, LPS), and inflammatory mediators (e.g., cytokines) [8–11].

Due to their generally accepted antioxidant and anti-inflammatory properties, the use of plant extracts, and in particular of essential oils, represents a promising approach to prevent and cure IBD. For instance, the essential oil of *Origanum vulgare*, containing high concentrations of the phenolic terpenes carvacrol and thymol, has been shown to efficiently reduce the mRNA levels of the proinflammatory cytokines TNFα, IL-1β, and IL-6 in human THP-1 macrophages. In contrast in this study the anti-inflammatory cytokine IL-10 was significantly reduced [12]. A blend of oregano, anise, and lemon peel even could be demonstrated to evolve anti-inflammatory effects in piglets in vivo [13]. Data from current literature suggest that anti-inflammatory effects of plant extracts base on their direct and indirect antioxidant properties, which again depend on the chemical compounds contained in different plant extracts. The essential oils of thyme and oregano, which mainly contain the antioxidant terpene compounds thymol and carvacrol [14, 15], could be demonstrated to impair the mRNA and the protein concentration of the pro-inflammatory cytokines IL-1β and IL-6 in mice with 2,4,6-trinitrobenzol (TNBS-) induced colitis [16]. Carnosol, a terpene of rosemary oil in vitro, showed indirect antioxidant effects via inducing nuclear factor erythroid 2-related factor 2- (Nrf2-) regulated antioxidant enzyme expression and additionally decreased pro-inflammatory mediators like NFκB, TNFα, IL-1β, IL-6, COX2, and ICAM-1 [17–19]. In another in vitro study, treatment with sulforaphane, an isothiocyanate mainly contained in broccoli, decreased the mRNA concentration of the pro-inflammatory cytokines TNFα and IL-1β in murine RAW264.7 macrophages due to a pro-inflammatory stimulus with bacterial LPS. In this experiment the reduction of inflammation was accompanied by an increase in the expression of the antioxidant enzyme HO1 via the Nrf2/Kelch-like ECH-associated protein 1 (Keap1) pathway [20]. Similar strong indirect antioxidant effects through the modification of Keap1 sulfhydryl groups have also been described for arturmeron, the main terpene of curcuma oil. In addition arturmeron possesses also direct antioxidant properties [21].

However, until today studies comparing the anti-inflammatory potential of different phytogenic substances due to a pro-inflammatory stimulus are not available. DSS is a chemical compound routinely used to induce a colitis in model animals and therefore to mimic similar inflammatory conditions as present in IBD.

Consequently our study aimed to investigate the connection between the antioxidant potential and anti-inflammatory effects of broccoli extract, turmeric oil, thyme oil, and rosemary oil in rats with a mild DSS-induced colitis.

2. Materials and Methods

2.1. Animals and Diets. The protocol of the rat study was approved by the Regional Council of Halle and by the Animal Welfare Committee of the Martin Luther University Halle-Wittenberg (record token: 42502-2-1093-MLUG). 92 four=week old male Wistar rats (mean body weight: 186.2 ± 9.45 g) were obtained from Harlan laboratories (Horst, The Netherlands). The rats were fed a standard diet without phytogenic feed additives for an acclimatisation period of 14 days.

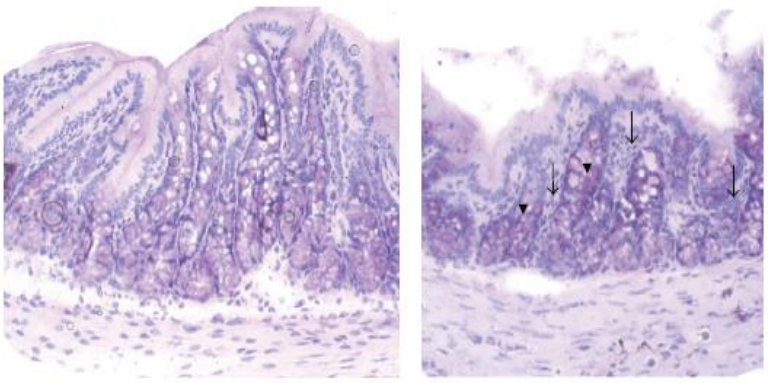
At an age of 6 weeks and a mean live weight of 231.0 ± 12.8 g the rats were assigned to 6 experimental groups of 16 rats each. During the following experimental periods the control groups (Con and DSS) were fed a basal diet that met the nutritional demands of the NRC for growing rats. This basal diet contained no phytogenic additive (Table 1).

The diet of group BE contained 8750 mg/kg broccoli sprouts extract (JARROW Formulas). 1494 mg/kg diet of *Curcuma longa* oil (Cuo), 618 mg/kg of *Thymus vulgaris* oil (To), and 680 mg/kg of *Rosmarinus officinalis* oil (Ro) were added to the diets of the other groups in order to standardize the concentration of the isothiocyanate sulforaphane (BE) and of the individual main terpenes (Cuo, To, and Ro) to a value of 2 mmol/kg diet. The main terpenes considered were ar-turmerone for Cuo, thymol for To, and 1,8-cineol for Ro. All diets were pelleted with a pellet mill using an 8 mm die and fed during the whole course of the experiment. The rats had free access to their respective diet and to tap water. Lighting, humidity, and temperature regime was in accordance with the recommendations of the Society for Laboratory Animal Science (2004) [22]. The trial consisted of 3 phases: (1) pretreatment phase (phase 1: 7 days), (2) DSS-treatment phase (phase 2: 6 days), and (3) recovery phase (phase 3: 6 days). Table 2 overviews the feeding protocol in detail.

The animal model of a DSS-induced colitis was chosen due to several histological and biochemical similarities with human IBD [23, 24]. In the DSS-treatment phase, 4% DSS (40 kDa; Sigma-Aldrich) was administered via drinking water to all rats for 6 days, with the exception of the Con group, in order to induce a mild intestinal inflammation. Feed intake and individual live weight were recorded after one week, daily during the DSS-phase and every other day during the recovery phase.

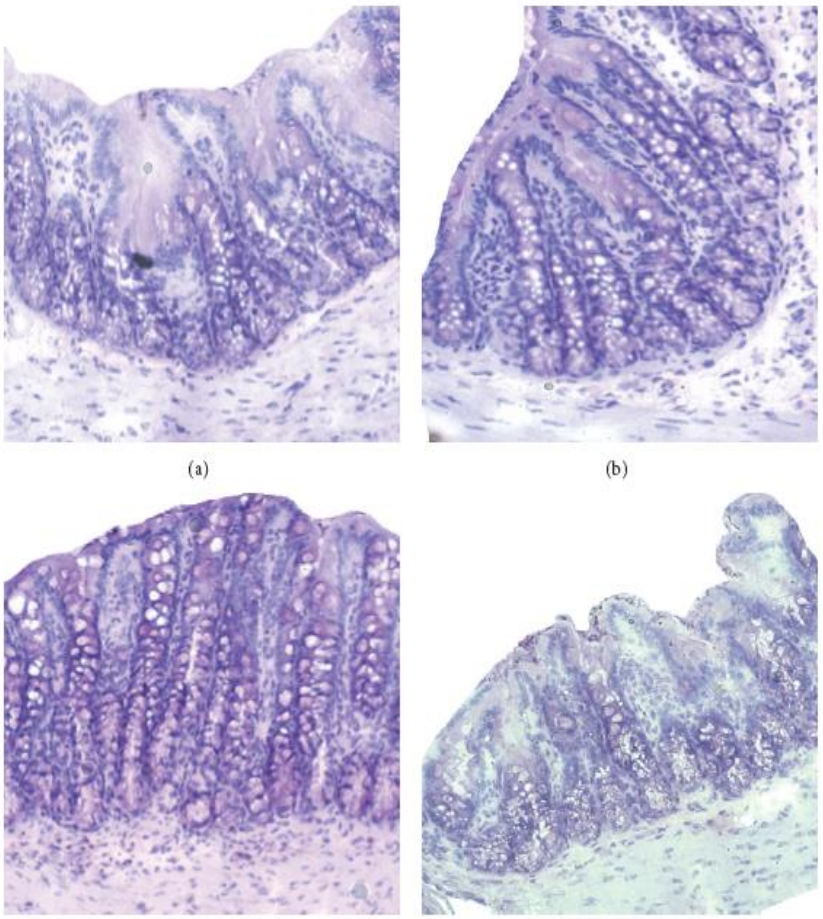
At the end of phase 1 four rats were killed for organ sampling (liver and colon), and after phases 2 and 3 six rats per group were sacrificed. For the histological examination and for the determination of relative mRNA concentrations of antioxidant and xenobiotic enzymes and of inflammation parameters, colon samples were prepared from a 10 cm segment distal to the caecum. Liver samples were excised from the middle of lobus sinister lateralis.

During treatment with 4% DSS the disease activity index (DAI) was used to assay the severity of the induced colitis. DAI was determined daily in phase 2 and every other day in phase 3 (recovery phase). The scoring system was based on body weight loss, stool consistency, and macroscopic fecal



(a) (b)

FIGURE 1: Representative pictures of proximal colon sections from healthy Con rats (a) and DSS-treated rats (b) fed the control diet. Haematoxylin and Eosin (H and E) stained colon cross-sections from phase 2 were analyzed with inversion microscopy (20x). Arrows indicate increased leucocyte infiltration, and arrowheads indicate the loss of goblet cells caused by DSS treatment (b).



(a) (b)

(c) (d)

FIGURE 2: Representative pictures of proximal colon sections from DSS-treated rats fed experimental diets with broccoli extract (a), turmeric oil (b), thyme oil (c), or rosemary oil (d). Haematoxylin and Eosin (H and E) stained colon cross-sections from phase 2 were analyzed with inversion microscopy (20x).

Table 1: Basal diet.

Ingredient	g/kg basal diet
Wheat (DEUKA GmbH und Co. KG, Könnern, Germany)	237.9
Maize (DEUKA GmbH und Co. KG, Könnern, Germany)	200.0
Barley (DEUKA GmbH und Co. KG, Könnern, Germany)	156.0
Soybean meal, 46% CP (DEUKA GmbH und Co. KG, Könnern, Germany)	220.0
Wheat bran (DEUKA GmbH und Co. KG, Könnern, Germany)	78.8
Oat (DEUKA GmbH und Co. KG, Könnern, Germany)	69.0
Sun flower oil	15.0
Lysine (Feed Grade, China)	0.3
dl-methionine (Degussa, Duesseldorf, Germany)	2.0
Vitamin and mineral premix	12.1
Calcium carbonate (Sigma-Aldrich)	2.5
Calcium phosphate (Mischfutter und Landhandel GmbH, Edderitz, Germany)	7.9

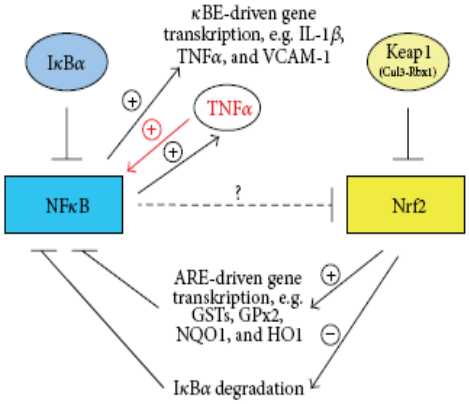


Figure 3: NFκB and Nrf2 crosstalk under balanced anti- and prooxidant conditions.

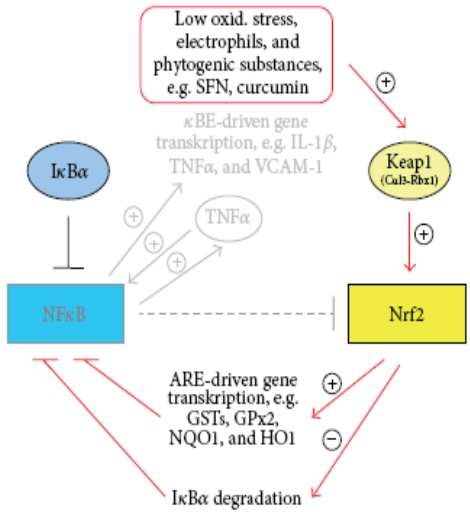


Figure 4: NFκB and Nrf2 interaction due to feeding phytogenic compounds or low oxidative stress.

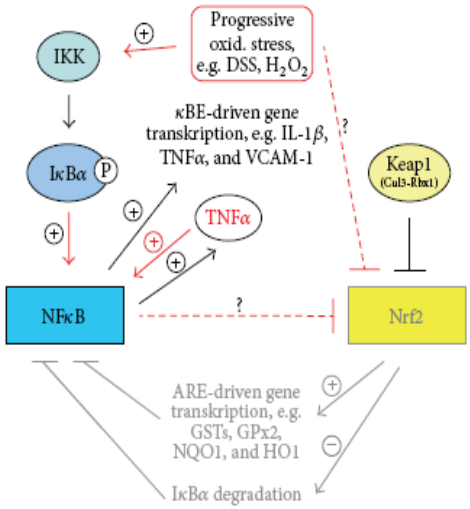


Figure 5: NFκB and Nrf2 interaction due to DSS treatment or progressive oxidative stress.

blood debris. For each mentioned parameter a scale ranging from 0 to 4 was applied as described previously [25].

2.2. Colonic Histology. For the histological examination freshly dissected colon samples were washed with 0.9 (w/v) % NaCl and cryoconserved in a freezing medium (Jung; Leica Instruments, Nussloch, Germany). Serial cross-sections (7 μm) were prepared using a microtome (CM 1850 UV microtome, Jung; Leica) and fixed on sterile usual microscope slides. After staining the samples with Haematoxylin-Eosin full-thickness slices tissue architecture, infiltration of neutrophilic granulocytes into the mucosa and into the submucosa, and the formation of crypt abscesses were examined under an inversion microscope using a blind protocol.

2.3. RNA Preparation and Real-Time RT-PCR Analysis. Total RNA from 100 mg of liver and colon tissue was isolated using the acid guanidinium thiocyanate-phenol-chloroform extraction method [26]. Most recently a strong DSS RNA interference during acute DSS-treatment resulting in the lack of signals during gene expression analysis has been described [27]. To assure the correctness of gene expression analyses in our experiment polyA+ mRNA from the colonic samples of the DSS-phase was purified using the GenElute mRNA Miniprep Kit (Sigma-Aldrich, MO, USA) according to the manufacturers' protocol. Following the photometrical determination of RNA concentration and purity at 260 nm and 280 nm, reverse transcription of 3.0 μg of total RNA or of 0.3 μg of purified mRNA and real-time RT-PCR were performed as described previously [28]. Gene bank accession

Table 2: Feeding protocol.

Group	Phytogenic additive	Koncentration per kg diet	Phase 1	Phase 2	Phase 3
Con	None	—	7 days diets and water ad libitum	6 days diets and water ad libitum	6 days diets and water ad libitum
DSS	None	—	7 days diets and water ad libitum	6 days diets ad libitum and 4% DSS	6 days diets and water ad libitum
BE	Broccoli extract	2 mmol sulforaphane	7 days diets and water ad libitum	6 days diets ad libitum and 4% DSS	6 days diets and water ad libitum
Cuo	Turmeric oil	2 mmol ar-turmerone	7 days diets and water ad libitum	6 days diets ad libitum and 4% DSS	6 days diets and water ad libitum
To	Thyme oil	2 mmol thymol	7 days diets and water ad libitum	6 days diets ad libitum and 4% DSS	6 days diets and water ad libitum
Ro	Rosemary oil	2 mmol 1,8-cineol	7 days diets and water ad libitum	6 days diets ad libitum and 4% DSS	6 days diets and water ad libitum

numbers and primer sequences ($5' \rightarrow 3'$) are shown in Table 3.

Gene specific mRNA expression was analyzed with the Rotor-Gene 6000 series software using the ΔΔCt method [29]. The amplification data of the single genes were normalized to the expression of the two most stable reference genes in each tissue (liver: β-actin, ribosomal protein L13A (Rpl13a); colon: Rpl13a, hypoxanthine phosphoribosyltransferase 1 (Hprt1)). Relative mRNA expression levels are expressed as x-fold changes relative to group Con = 1.0.

2.4. Statistical Analysis. Data are presented as means ± their standard error of the mean (SEM). Statistical differences were analyzed with SPSS 19.0 for Windows (IBM, Chicago, USA) using one-way ANOVA after verifying the normality of distribution (Shapiro Wilk test and Kolmogorov Smirnov test) and the homogeneity of variances (Levene test). The Least Significant Difference test (LSD) was used to analyze significant differences between means if variances were homogenous. If not, the Games Howell test was used. At an error probability of less than 5% ($P < 0.05$) differences between means were considered as statistically significant.

3. Results

3.1. Body Weight Development. Neither food intake (data not shown) nor final body weight (Table 4) of the rats was influenced significantly in the different experimental phases by feeding the specific diets tested. Remarkably, also DSS treatment in the second phase did not affect the above-mentioned parameters significantly.

3.2. Colitis Severity by Disease Activity Index (DAI). The course of the mild colitis, induced by the administration of 4% DSS to rats for 6 days, was controlled daily in phase 2 by measuring the DAI. A high number of rats from all DSS-treated groups had a soft stool consistency, but severe diarrhea accompanied by blood debris could not be observed. Moreover a distinct weight loss, frequently observed under DSS treatment, was present only in some cases and only for one day. Consequently the DAI in all DSS-treated groups remained below 1.0 (DSS: 0.12, BE: 0.10, Cuo: 0.10, To: 0.07, and Ro: 0.13) and did not differ significantly from Con rats receiving no DSS. In phase 3 obvious changes in stool consistency almost disappeared or were much less pronounced than in phase 2.

3.3. Histology. DSS treatment caused no significant observable macroscopic changes in colonic tissue architecture between the experimental groups, including the untreated Con rats. Furthermore microscopic analysis revealed no significant histological damage to the colonic mucosa of rats exposed to 4% DSS for 6 days. However, DSS treatment tended to accelerate initial damage to the mucosa, characterised by the loss of goblet cells and the occurrence of a more diffuse crypt architecture compared to the colon of healthy Con rats. Additionally, the accumulation of neutrophils, infiltrating the lamina propria could be observed more frequently in colonic slices of DSS-treated rats. These mentioned DSS-induced mucosal alterations are shown in Figure 1. DSS treatment in combination with the tested phytogenic extracts also caused slight mucosal damage (Figure 2), but the severity seemed to be much lower than in group DSS (Figure 1(b)).

3.4. mRNA Expression of NFκB, TNFα, and Various Inflammatory-Mediating Enzymes in Colon and Liver Tissue. The analyzed mRNA expression patterns of pro- and anti-inflammatory genes controlled by NFκB and TNFα differed among the experimental periods and the tissues investigated (Tables 5 and 6). During the 7-day pretreatment phase 1 the mRNA abundance of the colonic pro-inflammatory markers COX2 and IL-1β, of the anti-inflammatory cytokine IL-10, of the cell adhesion molecules MCP-1 and VCAM-1, and of the tight junction protein Cldn3 showed a high intraindividual variance. In contrast in phase 1 of the trial liver mRNA data of the above-mentioned genes showed a much better homogeneity. Nevertheless, feeding diets with BE-, Cuo-, To-, and Ro-addition reduced colonic VCAM-1 mRNA by 57 to 64% compared to untreated control rats. This reduction was significant for Cuo, To, and Ro and represented a trend ($P < 0.10$) for BE. Moreover in phase 1 colonic Cldn3

Table 3: Gene bank accession numbers and primer sequences of the genes investigated by real-time RT-PCR.

Gene name (abbreviation used)	Gene bank accession number	Primer sequences (5′ → 3′) for = forward; rev = reverse;
Chemokine (C-C motif) ligand 2 (Ccl2) (MCP1)	NM_031530	for: GTGCGACCCCAATAAGGAA
		rev: TGAGGTGGTTGTGGAAAAGA
Claudin 3 (Cldn3)	NM_031700	for: TATCCTACTGGCAGCCTTCG
		rev: GTTCCCATCTCTCGCTTCTG
Copper/zinc superoxide dismutase (SOD1)	NM_017050	for: CCACTGCAGGACCTCATTTT
		rev: CACCTTTGCCCAAGTCATCT
C-reactive protein (CRP)	NM_017096	for: GTCTCTATGCCCACGCTGAT
		rev: CCGTCAAGCCAAAGCTCTAC
Glutathione S-transferase K1 (GSTK1)	NM_181371	for: GAGCATGGAGCAACCAGAGAT
		rev: AGCTTGCTCTTCACCAGTTCG
Glutathione S-transferase P1 (GSTP1)	NM_012577	for: GAGGCAAAGCTTTCATTGTGG
		rev: GTTGATGGGACGGTTCAAATG
Glutathione S-transferase T2 (GSTT2)	NM_012796	for: GAGGAAAAGGTGGAACGGAAC
		rev: CGCCCCTCAAACAGATTACAG
Glutathione peroxidase 2 (GPx2)	NM_183402	for: GTGTGATGTCAATGGGCAGAA
		rev: ACGTTTGATGTCAGGCTCGAT
Heme oxygenase 1 (HO1)	NM_012580	for: AGGCACTGCTGACAGAGGAAC
		rev: AGCGGTGTCTGGGATGAACTA
Hypoxanthine phosphoribosyltransferase 1 (Hprt1)	NM_012583	for: GCAGACTTTGCTTTCCTTGG
		rev: TCCACTTTCGCTGATGACAC
Interleukin 1 beta (IL-1β)	NM_031512	for: CTGTGACTCGTGGGATGATG
		rev: GGGATTTTGTCGTTGCTTGT
Interleukin 10 (IL-10)	NM_012854	for: CTGGAGTGAAGACCAGCAAAGG
		rev: GGAGAAATCGATGACAGCGTCG
Kelch-like ECH-associated protein1 (Keap1)	NM_057152	for: GTGGCGGATGATTACACCAAT
		rev: GAAAAGTGTGGCCATCGTAGC
NAD(P)H dehydrogenase [quinone] 1 (NQO1)	NM_017000	for: CGCAGAGAGGACATCATTCA
		rev: CGCCAGAGATGACTCAACAG
Nuclear factor (erythroid-derived 2)-like 2 (Nrf2)	NM_031789	for: CCAAGGAGCAATTCAACGAAG
		rev: CTCTTGGGAACAAGGAACACG
Nuclear factor kappa B (NFκB)	L26267	for: CTTCTCGGAGTCCCTCACTG
		rev: CCAATAGCAGCTGGAAAAGC
Prostaglandin-endoperoxide synthase 2 (Ptgs2) (COX2)	NM_017232	for: GCTGTACAAGCAGTGGCAAA
		rev: CCCCAAAGACAGCATCTGGA
Ribosomal protein L13A (Rpl13a)	NM_173340	for: CCCTCCACCCTATGACAAGA
		rev: CCTTTTCCTTCCGTTTCTCC
Tumor necrosis factor alpha (TNFα)	NM_012675	for: GCCAATGGCATGGATCTCAAAG
		rev: AAATCGGCTGACGGTGTGGG
Vascular cell adhesion molecule 1 (VCAM 1)	NM_012889	for: TGACATCTCCCCTGGATCTC
		rev: CTCCAGTTTCCTTCGCTGAC
β-actin	NM_031144	for: ATCGTGCGTGACATTAAAGAGAAG
		rev: GGACAGTGAGGCCAGGATAGAG

expression was significantly higher in Ro-treated rats and tendencially higher in Cuo- and To-treated rats than in their Con littermates.

In phase 1 feeding diets containing BE, Cuo, and Ro significantly decreased liver mRNA abundance of the pro-inflammatory cytokine IL-10 by 32 to 44%.

Table 4: Body weight changes in DSS-treated rats fed with broccoli extract and various essential oils compared to an untreated control.

Period	Body weight in g											
	Con		DSS		BE		Cuo		To		Ro	
	MW	SEM	MW	SEM	MW	SEM	MW	SEM	MW	SEM	MW	SEM
start	229.4	4.05	232.3	2.45	230.9	3.20	231.2	3.10	230.7	3.58	231.8	2.85
day 7	266.0	7.80	263.4	6.48	280.0	3.75	273.5	6.90	265.0	2.13	281.0	0.70
day 13	297.7	5.72	300.0	5.92	297.3	3.26	297.5	4.21	295.4	4.19	301.1	4.13
day 19	325.2	6.74	321.1	8.16	323.4	4.04	322.1	5.92	321.1	3.96	320.5	9.14

Values are means ± SEM. n = 16 rats per group for phase 1 (start day 7), n = 12 rats per group for phase 2 (day 13), and n = 6 rats per group for phase 3 (day 19).

Table 5: Effects of feeding broccoli extract and various essential oils on relative mRNA expression of various pro- and anti-inflammatory parameters in the colon of DSS-treated rats compared to an untreated control.

Gene	Period	Experimental group compared with untreated control rats				
		DSS	BE	Cuo	To	Ro
NFκB	Phase 1	—	↔	↔	↔	↔
	Phase 2	↔	↔	↔	↔	↔
	Phase 3	↑	↔	↔	↔	↔
TNFα	Phase 1	—	↔	↔	↔	↔
	Phase 2	↔	↔	↔	↑	↔
	Phase 3	↑	↑	↑	↑	↑
COX2	Phase 1	—	↔	↔	↔	↔
	Phase 2	↑↑↑	↔	↔	↑	↔
	Phase 3	↔	↔	↔	↔	↔
IL-1β	Phase 1	—	↔	↔	↔	↔
	Phase 2	↔	↔	↔	↔	↔
	Phase 3	↑↑	↔	↔	↔	↔
IL-10	Phase 1	—	↔	↔	↔	↔
	Phase 2	↔	↔	↑	↑↑↑	↑↑
	Phase 3	n.d.	n.d.	n.d.	n.d.	n.d.
MCP-1	Phase 1	—	↔	↔	↔	↔
	Phase 2	↔	↑	↑	↑	↑
	Phase 3	↑	↑	↔	↔	↔
VCAM-1	Phase 1	—	↔	↓↓	↓↓	↓↓
	Phase 2	(↑)	(↓)	(↓)	(↓)	(↓)
	Phase 3	↑↑	↑	↔	↔	↔
Cldn3	Phase 1	—	↔	↔	↔	↑
	Phase 2	↔	↓↓	↔	↓↓	↓↓
	Phase 3	↔	↔	↔	↑	↔

Different arrows summarize the effects on gene expression: ↔ no effect, ↑: increase, ↓: decrease, ( ): significant results compared to group DSS, and n.d.: not detectable. Means ± SEM and the results of statistical analysis are presented in the appendix.

Table 6: Effects of feeding broccoli extract and various essential oils on relative mRNA expression of various pro- and anti-inflammatory parameters in the liver of DSS-treated rats compared to an untreated control.

Liver	Period	Experimental group compared with untreated control rats				
Gene		DSS	BE	Cuo	To	Ro
NFκB	Phase 1	—	↔	↔	↔	↔
	Phase 2	↔	↔	↔	↔	↔
	Phase 3	↔	↔	↔	↔	↔
TNFα	Phase 1	—	↔	↔	↔	↔
	Phase 2	↔	↔	↔	↔	↔
	Phase 3	↑	↔	↔	↔	↔
COX2	Phase 1	—	↔	↔	↔	↔
	Phase 2	↔	↔	↔	↔	↔
	Phase 3	n.d.	n.d.	n.d.	n.d.	n.d.
IL-1β	Phase 1	—	↔	↔	↔	↔
	Phase 2	↔	↔	↔	↔	↔
	Phase 3	↔	↔	↔	↓	↔
IL-10	Phase 1	—	↓	↓	↔	↓
	Phase 2	↔	↔	↔	↔	↔
	Phase 3	↓	↓	↓	↓	↓
MCP-1	Phase 1	—	↔	↔	↔	↑
	Phase 2	↔	↔	↔	↔	↔
	Phase 3	↔	↔	↔	↔	↔
VCAM-1	Phase 1	—	↓	↔	↔	↔
	Phase 2	↔	↔	↔	↔	↔
	Phase 3	↔	↔	↔	↔	↔
Cldn3	Phase 1	—	↔	↔	↔	↔
	Phase 2	↔	↔	↔	↔	↔
	Phase 3	↔	↑	↔	↔	↔

Different arrows summarize the effects on gene expression: ↔: no effect, ↑: increase, ↓: decrease, and n.d.: not detectable. Means ± SEM and the results of statistical analysis are presented in the appendix.

Following treatment with an intermediate dose of 4% DSS for 6 days (phase 2) specific effects of the phytogenic substances on inflammatory markers could be analyzed in the colon, whereas these parameters remained uninfluenced in the liver. DSS treatment had no significant influence on colonic NFκB mRNA expression. In contrast TNFα expression increased in all DSS-treated groups, including those receiving phytogenic additives, compared to untreated control rats. This pro-inflammatory response was also reflected by an 18-fold increase in colonic COX2 mRNA expression in DSS-treated rats receiving no phytogenic additive compared to untreated Con rats. Feeding diets containing BE, Cuo, and Ro reduced COX2 expression nearly to the level in untreated controls. To addition lowered COX2 expression somewhat less than the other additives. Nevertheless also DSS-treated

Table 7: Effects of feeding broccoli extract and various essential oils on relative mRNA expression of various pro- and anti-inflammatory parameters in colon of DSS-treated rats compared to an untreated control.

Colon	Period	Experimental group compared with untreated control rats				
Gene		DSS	BE	Cuo	To	Ro
	Phase 1	—	↔	↔	↔	↔
Nrf2	Phase 2	↓	↓	↔	↓	↓
	Phase 3	↔	↔	↔	↔	↔
	Phase 1	—	↔	↔	↔	↑
Keap1	Phase 2	↔	↔	↔	↔	↔
	Phase 3	↔	↔	↔	↔	↔
	Phase 1	—	↔	↔	↔	↔
HO1	Phase 2	↔	↔	↔	↔	↔
	Phase 3	↔	↔	↔	↑	↔
	Phase 1	—	↔	↔	↔	↔
NQO1	Phase 2	↔	↔	↔	↓	↔
	Phase 3	↔	↔	↔	↔	↔
	Phase 1	—	↓	↔	↔	↔
SOD1	Phase 2	↔	↔	↔	↔	↔
	Phase 3	↔	↔	↔	↔	↔
	Phase 1	—	↔	↔	↔	↑
GPx2	Phase 2	↔	↔	↑	↔	↔
	Phase 3	↓	↓	↓	↓	↓
	Phase 1	—	↓	↔	↔	↑
GSTK1	Phase 2	↔	↔	↔	↓	↓
	Phase 3	↔	↓	↓	↓	↓
	Phase 1	—	↔	↔	↔	↑
GSTP1	Phase 2	↔	↔	↔	↓	↔
	Phase 3	↓	↓	↓	↓	↓
	Phase 1	—	↔	↑	↔	↑
GSTT2	Phase 2	↓	↓	↓	↓	↓
	Phase 3	↔	↔	↔	↔	↔

Different arrows summarize the expression results: ↔: no effect, ↑: increase, and ↓: decrease. For statistically analyzed means ± SEM see Table 10.

To rats had a colonic COX2 expression, which was tendencially lower than in their DSS-treated littermates receiving no phytogenic additive. Whereas the colonic mRNA concentration of MCP-1 was not lowered by phytogenic feed additives during the DSS period, all additives tested, reduced VCAM-1 expression by 73 to 83%, and compared to DSS-treated control rats. DSS treatment generally increased the expression of the anti-inflammatory IL-10 in comparison to Con rats without DSS treatment. This effect was relatively small in DSS-treated rats receiving no phytogenic additive and in those receiving the diet containing BE. In contrast the impact of Cuo, To, and Ro addition on colonic IL-10 expression was significant. In all DSS-treated rats colonic Cldn3 mRNA decreased compared to untreated control rats. This reduction of Cldn3 expression tended to be lower in DSS-treated rats without an additive and in the DSS-treated Cuo group, and it was significant in their DSS-treated littermates receiving diets with the addition of BE, To, and Ro.

In the final recovery period (phase 3) NFκB mRNA strongly increased in the colon of DSS-treated control rats compared to their untreated littermates. All phytogenic additives lowered NFκB response considerably ($P < 0.10$). These characteristic changes in NFκB mRNA were reflected by similar changes in the expression of the pro-inflammatory cytokine IL-1β. Nevertheless, in all DSS-treated rats, including those receiving phytogenic additives, colonic TNFα mRNA levels remained significantly higher throughout the recovery period. In the recovery period also liver TNFα mRNA level was increased in DSS-treated rats without a phytogenic additive, but not in rats fed diets containing any phytogenic substance, compared to untreated controls. In the recovery period colonic COX2 mRNA levels of DSS-treated control rats dropped nearly to the level in untreated controls, and they were further decreased in DSS rats receiving phytogenic additives compared to phase 2 of the experiment. In phase 3 colonic mRNA levels of MCP-1 and VCAM-1 in DSS-treated rats without a phytogenic additive further increased compared to phase 2, and they were significantly higher than in untreated controls. Interestingly a similar effect could be observed in rats fed a BE containing diet. In contrast rats fed diets containing the other additives (Cuo, To, and Ro) showed MCP-1 and VCAM-1 mRNA levels, which were comparably low as in untreated controls and significantly reduced compared to DSS controls. However, in the liver neither DSS treatment nor combining DSS treatment with feeding phytogenic additives had an influence on MCP-1 and VCAM-1 mRNA abundance in the recovery phase. In contrast to the acute DSS-treatment period, Cldn3 levels were not lower in the DSS-treated groups than in untreated controls. In rats receiving the To diet Cldn3 mRNA was even 1.5–2.0-fold higher than in the other groups.

3.5. mRNA Expression of Nrf2, Keap1, and Various ARE-Regulated Enzymes in the Colon. The Nrf2/Keap1 system regulates the expression of antioxidant enzymes and xenobiotic enzymes. In the current experiment some characteristic changes could be observed with regard to Nrf2-, and Keap1 expression and on the expression of ARE-regulated antioxidant and phase II enzymes, depending on the experimental phase and on the treatment of the rats (Table 7). In the pretreatment phase the high impact of Ro on colonic Keap1 expression was directly reflected by an increase in the expression of several ARE-regulated antioxidant enzymes like HO1 and GPx2 and on phase II enzymes like GSTK1, P1, and T2.

In the initial phase all other additives tested had neither a distinct and directed influence on Keap1 mRNA nor on the expression of the above-mentioned target genes. During acute DSS treatment the effects on ARE-regulated enzymes were relatively small. Whereas the antioxidant enzymes HO1 and NQO1 tended to be higher in DSS-treated control rats than in untreated controls, all phytogenic additives reduced this particular DSS effect to a little extent. In contrast the expression of the ARE-regulated phase II enzymes GSTK1, P1, and T2 tended to be lowered by DSS treatment. In these cases Nrf2 mRNA abundance showed a similar alteration

Table 8: Effects of feeding broccoli extract and various essential oils on relative mRNA expression of various pro- and anti-inflammatory parameters in colon of DSS-treated rats compared to an untreated control. Values are means ± SEM and represent relative mRNA concentrations as n-fold of group Con = 1. Different small letters in a row indicate significant differences between means ($P \leq 0.05$). $n = 4$ in phase 1, $n = 6$ in phase 2 and phase 3.

Colon	Period	Experimental group											
		Con		DSS		BE		Cuo		To		Ro	
Gene		MW	SEM	MW	SEM	MW	SEM	MW	SEM	MW	SEM	MW	SEM
	Phase 1	1.00	0.53	—	—	0.56	0.10	1.04	0.10	0.56	0.21	1.17	0.70
NFκB	Phase 2	1.00	0.20	1.03	0.30	0.36	0.02	1.00	0.44	2.21	1.37	0.51	0.10
	Phase 3	1.00^{a}	0.07	3.05^{b}	1.23	1.52^{ab}	0.28	1.31^{ab}	0.30	1.09^{ab}	0.16	1.51^{ab}	0.42
	Phase 1	1.00	0.45	—	—	0.31	0.05	0.38	0.12	0.42	0.15	0.41	0.14
TNFα	Phase 2	1.00^{a}	0.15	3.96^{ab}	1.48	3.84^{ab}	1.28	1.65^{ab}	0.59	4.72^{b}	1.49	2.98^{ab}	0.76
	Phase 3	1.00^{a}	0.18	3.71^{b}	0.85	4.56^{b}	0.97	3.75^{b}	0.78	4.18^{b}	1.08	3.34^{b}	0.90
	Phase 1	1.00	0.81	—	—	0.13	0.03	0.90	0.40	0.55	0.29	0.96	0.61
COX2	Phase 2	1.00^{ac}	0.35	17.7^{b}	10.26	2.43^{ac}	1.00	1.68^{ac}	0.71	3.59^{bc}	1.10	1.25^{ac}	0.42
	Phase 3	1.00	0.17	1.31	0.55	1.41	0.34	0.87	0.27	1.10	0.33	1.00	0.21
	Phase 1	1.00	0.10	—	—	1.48	0.52	1.72	0.45	1.01	0.41	1.60	0.52
IL-1β	Phase 2	1.00	0.33	1.10	0.34	1.70	0.82	1.21	0.36	0.52	0.22	1.17	0.29
	Phase 3	1.00^{a}	0.33	4.69^{b}	1.40	2.32^{ab}	0.53	0.96^{a}	0.22	2.26^{a}	1.01	1.77^{a}	1.02
	Phase 1	1.00	0.43	—	—	2.14	1.46	1.95	0.58	2.31	1.28	2.23	1.04
IL-10	Phase 2	1.00^{a}	0.39	1.23^{ab}	0.35	1.46^{ab}	0.31	2.79^{b}	0.65	22.5^{b}	15.03	3.79^{c}	1.50
	Phase 3	n.d.		n.d.		n.d.		n.d.		n.d.		n.d.	
	Phase 1	1.00	0.12	—	—	0.88	0.09	0.94	0.04	1.46	0.45	1.04	0.23
MCP-1	Phase 2	1.00^{a}	0.22	1.51^{ab}	0.20	2.20^{b}	0.30	1.67^{b}	0.15	2.76^{b}	0.56	2.03^{b}	0.58
	Phase 3	1.00^{a}	0.27	2.60^{bc}	0.47	3.54^{b}	0.98	1.10^{a}	0.23	0.86^{a}	0.26	1.67^{ac}	0.57
	Phase 1	1.00^{a}	0.42	—	—	0.43^{ab}	0.06	0.34^{b}	0.10	0.41^{b}	0.14	0.38^{b}	0.21
VCAM-1	Phase 2	1.00^{ab}	0.46	2.40^{a}	1.18	0.39^{b}	0.19	0.39^{b}	0.13	0.62^{b}	0.25	0.66^{ab}	0.42
	Phase 3	1.00^{a}	0.35	6.21^{b}	2.20	4.56^{b}	0.53	1.97^{a}	1.14	1.61^{a}	0.79	1.07^{a}	0.53
	Phase 1	1.00^{a}	0.31	—	—	1.03^{a}	0.19	1.71^{ab}	0.20	1.63^{ab}	0.14	2.37^{b}	0.75
Cldn3	Phase 2	1.00^{a}	0.38	0.57^{ab}	0.21	0.22^{b}	0.08	0.49^{ab}	0.22	0.25^{b}	0.21	0.25^{b}	0.07
	Phase 3	1.00^{a}	0.18	1.46^{ab}	0.34	0.92^{a}	0.06	1.07^{a}	0.12	1.98^{b}	0.36	1.24^{a}	0.30

like the above-mentioned phase II enzymes. The reduction of GSTK1, P1, and T2 was slightly aggravated by the phytogenic additives. In the recovery phase, in all DSS-treated groups, GPx2 expression decreased below the expression in untreated control rats. A differential development could be analyzed for the different GST subclasses. Whereas the mRNA expression of GSTK1 and P1 in DSS-treated rats remained below the level of their untreated littermates, the mRNA concentration of GST T2 in treated rats exceeded that of untreated. In the liver no gross changes could be observed with regard to the expression of the antioxidant and phase II enzymes investigated due to the different dietary and pro-inflammatory conditions.

4. Discussion

Our current trial aimed to investigate the impact of different phytogenic substances on changes in the expression of genes related to inflammation and of ARE-regulated antioxidant and phase II enzyme genes in rats previous to (phase 1), during (phase 2) and subsequent (phase 3) to the provocation of an experimental colitis with DSS.

The use of 4% DSS was intended to induce a mild colitis and therefore to reflect the onset of an acute local gut inflammation. Interestingly, DSS application caused no significant loss of body weight, neither in the DSS-treatment phase nor in the recovery phase. These data are consistent with observations from the current literature [30, 31]. Moreover Hakansson et al. [31] also showed only small alterations in female rats' DAI due to acute treatment with 4% DSS for 7 days. Accordingly, in the mentioned study colitis symptoms completely disappeared in the recovery phase.

In contrast other studies using an equal or even a lower DSS concentration reported a high weight loss, a strongly increased DAI, and severe damage to the colonic mucosa [32–35]. The colitis inducing potential of DSS may depend on a number of factors including (1) the DSS concentration and the exact molecular weight of the DSS compound used, (2) the duration of DSS-exposition, and (3) the species and the gender of the experimental animals.

Our results for DAI confirmed the findings of the histological examination, in which only small alterations in mucosal architecture could be found. In summary these findings indicated a mild colitis as intended in our experiment. In contrast other investigations in the current literature reported

Table 9: Effects of feeding broccoli extract and various essential oils on relative mRNA expression of various pro- and anti-inflammatory parameters in the liver of DSS-treated rats compared to an untreated control. Values are means ± SEM and represent relative mRNA concentrations as n-fold of group Con = 1. Different small letters in a row indicate significant differences between means ($P \leq 0.05$). $n = 4$ in phase 1, $n = 6$ in phase 2 and phase 3.

Liver Gene	Period	Experimental group											
		Con		DSS		BE		Cuo		To		Ro	
		MW	SEM	MW	SEM	MW	SEM	MW	SEM	MW	SEM	MW	SEM
NFκB	Phase 1	1.00	0.15	—	—	1.06	0.11	0.99	0.06	1.03	0.06	0.97	0.08
	Phase 2	1.00	0.06	0.94	0.07	0.99	0.03	0.99	0.05	1.02	0.07	1.02	0.06
	Phase 3	1.00	0.04	0.96	0.03	1.00	0.03	0.99	0.04	1.07	0.05	1.06	0.06
TNFα	Phase 1	1.00	0.17	—	—	1.04	0.07	1.06	0.12	0.90	0.03	1.10	0.06
	Phase 2	1.00	0.13	1.01	0.14	0.89	0.15	1.04	0.15	0.92	0.14	0.82	0.08
	Phase 3	1.00^{a}	0.04	1.71^{b}	0.37	1.06^{ab}	0.12	1.14^{ab}	0.17	1.16^{ab}	0.11	1.55^{ab}	0.25
COX2	Phase 1	1.00^{abc}	0.15	—	—	0.81^{ab}	0.09	1.02^{ac}	0.06	0.75^{b}	0.15	1.12^{c}	0.06
	Phase 2	1.00	0.11	1.16	0.20	1.19	0.12	1.34	0.27	1.50	0.25	1.13	0.12
	Phase 3	n.d.		n.d.		n.d.		n.d.		n.d.		n.d.	
IL-1β	Phase 1	1.00	0.20	—	—	1.00	0.15	1.02	0.16	0.87	0.10	1.13	0.12
	Phase 2	1.00	0.11	1.04	0.11	0.93	0.12	1.01	0.14	1.12	0.10	0.98	0.08
	Phase 3	1.00^{a}	0.03	0.95^{a}	0.08	0.85^{ab}	0.07	0.84^{ab}	0.06	0.75^{b}	0.05	1.01^{ab}	0.06
IL-10	Phase 1	1.00^{a}	0.19	—	—	0.67^{b}	0.11	0.56^{b}	0.08	0.70^{ab}	0.15	0.68^{b}	0.08
	Phase 2	1.00	0.12	0.77	0.10	1.15	0.25	1.06	0.15	1.06	0.09	1.01	0.09
	Phase 3	1.00^{a}	0.06	0.76^{b}	0.05	0.79^{b}	0.08	0.67^{b}	0.06	0.69^{b}	0.03	0.77^{b}	0.03
MCP-1	Phase 1	1.00^{a}	0.05	—	—	1.25^{ab}	0.17	1.13^{a}	0.11	1.18^{a}	0.25	1.78^{b}	0.23
	Phase 2	1.00	0.12	1.00	0.07	1.08	0.15	1.20	0.11	1.12	0.17	1.19	0.13
	Phase 3	1.00	0.07	1.01	0.12	1.00	0.10	0.96	0.11	1.00	0.10	1.01	0.03
VCAM-1	Phase 1	1.00^{a}	0.20	—	—	0.74^{b}	0.08	0.80^{ab}	0.02	0.85^{ab}	0.07	0.91^{ab}	0.06
	Phase 2	1.00	0.07	1.06	0.12	0.85	0.10	0.98	0.02	1.09	0.07	0.89	0.05
	Phase 3	1.00	0.05	0.89	0.08	1.02	0.11	1.07	0.11	0.92	0.08	1.03	0.09
CRP	Phase 1	1.00	0.19	—	—	1.02	0.30	0.85	0.15	0.91	0.22	0.65	0.07
	Phase 2	1.00	0.20	1.07	0.21	0.74	0.05	0.93	0.20	0.85	0.10	1.14	0.15
	Phase 3	1.00^{a}	0.11	0.87^{a}	0.11	1.37^{b}	0.21	0.89^{a}	0.10	0.87^{a}	0.12	0.73^{a}	0.06

on an extensive mucosal damage accompanied by crypt loss, ulcerations and erosion by treating rats with 4% DSS [34–36]. Nevertheless, also in our trial DSS-treated rats had a higher infiltration of neutrophils in the lamina propria and small alterations in crypt architecture. However, a significant crypt loss and mucosal damage could not be observed. These differences may result from species differences due to DSS treatment, from differences in the DSS concentration used, and from differences in the DSS-application period [37, 38].

To the best of our knowledge currently there exist no comparable studies investigating the development of the above mentioned parameters under nonstimulated conditions as represented by phase 1 of our trial.

In the pretreatment phase of our experiment Ro turned out to be the most efficient phytogenic substance with regard to the upregulation of the ARE-regulated phase II enzymes GSTK1, P1, and T2 and of the ARE-regulated antioxidant enzyme GPx2. These changes were directly accompanied by a significant increase in Keap1 mRNA.

The strong effects of Ro on phase II enzymes containing an ARE promoter confirm the results of studies from our group carried out with growing chickens and growing pigs [39, 40]. In contrast, in these studies also BE, Cuo, and To effected a significant increase in the intestinal mRNA levels of the above mentioned gene family. The deviation from these results may presumably derive from the shorter prefeeding period in our current study (1 week versus 5 weeks in the chicken trial and 4 weeks in the piglet trial). Moreover also the results of a very recent rat trial of our group corroborate this "time hypothesis." In the mentioned study feeding of BE in combination with different dietary selenium concentrations to rats increased the mRNA of a broad spectrum of colonic ARE-regulated antioxidant and phase II enzymes about 3.5-fold. In the latter mentioned trial we have moreover suggested that on the basis of its mRNA expression, the cytosolic Nrf2 adapter protein Keap1 seems to be a more sensitive indicator of ARE driven gene expression than the transcription factor Nrf2 per se [41].

Table 10: Effects of feeding broccoli extract and various essential oils on relative mRNA expression of Nrf2, Keap1, and various ARE-regulated enzymes in colon of DSS-treated rats compared to an untreated control. Values are means ± SEM and represent relative mRNA concentrations as n-fold of group Con = 1. Different small letters in a row indicate significant differences between means ($P \leq 0.05$). $n = 4$ in phase 1, $n = 6$ in phase 2 and phase 3.

Colon		Experimental group											
Gene	Period	Con		DSS		BE		Cuo		To		Ro	
		MW	SEM	MW	SEM	MW	SEM	MW	SEM	MW	SEM	MW	SEM
	Phase 1	1.00	0.21	—	—	1.00	0.07	1.31	0.16	1.26	0.19	1.23	0.11
Nrf2	Phase 2	1.00^{a}	0.13	0.53^{bc}	0.09	0.51^{bc}	0.07	0.63^{ab}	0.08	0.36^{c}	0.07	0.58^{bc}	0.11
	Phase 3	1.00	0.28	0.86	0.16	0.89	0.10	1.13	0.13	1.10	0.13	0.69	0.08
	Phase 1	1.00^{ab}	0.44	—	—	0.51^{a}	0.13	1.04^{ab}	0.12	0.87^{ab}	0.21	2.44^{b}	1.29
Keap1	Phase 2	1.00	0.16	0.93	0.15	1.02	0.18	0.81	0.11	0.67	0.10	0.86	0.09
	Phase 3	1.00	0.19	1.06	0.23	0.69	0.05	1.02	0.29	0.98	0.13	1.43	0.36
	Phase 1	1.00	0.47	—	—	0.48	0.09	1.09	0.35	0.85	0.18	1.40	0.48
HO1	Phase 2	1.00^{ab}	0.23	1.71^{a}	0.33	1.46^{ab}	0.20	1.33^{ab}	0.36	0.96^{ab}	0.28	0.94^{b}	0.29
	Phase 3	1.00^{a}	0.17	1.28^{a}	0.13	0.99^{a}	0.09	1.01^{a}	0.18	1.97^{b}	0.29	1.04^{a}	0.19
	Phase 1	1.00	0.23	—	—	0.54	0.07	0.56	0.13	0.91	0.14	1.05	0.46
NQO1	Phase 2	1.00^{ab}	0.26	1.57^{a}	0.52	0.94^{ab}	0.13	0.85^{ab}	0.12	0.59^{b}	0.15	0.84^{ab}	0.15
	Phase 3	1.00	0.21	1.31	0.36	1.17	0.14	1.10	0.37	2.17	0.44	2.41	1.05
	Phase 1	1.00^{a}	0.03	—	—	0.74^{b}	0.09	0.94^{ab}	0.09	0.95^{ab}	0.08	0.78^{ab}	0.12
SOD1	Phase 2	1.00	0.12	1.08	0.19	0.80	0.06	1.05	0.12	0.76	0.17	0.92	0.17
	Phase 3	1.00	0.19	1.07	0.09	0.98	0.07	1.04	0.11	1.04	0.07	0.90	0.13
	Phase 1	1.00^{ab}	0.29	—	—	0.56^{a}	0.05	0.95^{ab}	0.23	0.61^{a}	0.11	1.43^{b}	0.34
GPx2	Phase 2	1.00^{a}	0.25	1.49^{ab}	0.40	1.16^{ab}	0.30	2.36^{b}	0.51	1.73^{ab}	0.45	1.94^{ab}	0.86
	Phase 3	1.00^{a}	0.11	0.33^{b}	0.06	0.40^{b}	0.05	0.46^{b}	0.12	0.40^{b}	0.11	0.27^{b}	0.04
	Phase 1	1.00^{a}	0.18	—	—	0.52^{b}	0.11	1.19^{ac}	0.12	0.90^{a}	0.13	1.88^{c}	0.45
GSTK1	Phase 2	1.00^{a}	0.08	0.70^{ab}	0.15	0.75^{ab}	0.11	0.90^{ab}	0.16	0.53^{b}	0.11	0.55^{b}	0.11
	Phase 3	1.00^{a}	0.05	0.79^{ab}	0.18	0.60^{b}	0.08	0.70^{b}	0.12	0.65^{b}	0.09	0.51^{b}	0.05
	Phase 1	1.00^{ab}	0.14	—	—	0.65^{a}	0.13	1.16^{bc}	0.20	0.84^{ab}	0.14	1.64^{c}	0.21
GSTP1	Phase 2	1.00^{a}	0.08	0.91^{a}	0.21	0.70^{a}	0.12	0.72^{a}	0.09	0.44^{b}	0.16	0.66^{a}	0.06
	Phase 3	1.00^{a}	0.07	0.47^{b}	0.05	0.68^{b}	0.09	0.68^{b}	0.09	0.63^{b}	0.11	0.51^{b}	0.08
	Phase 1	1.00^{ab}	0.08	—	—	0.65^{a}	0.24	1.60^{b}	0.21	0.96^{a}	0.21	1.58^{b}	0.18
GSTT2	Phase 2	1.00^{a}	0.12	0.60^{b}	0.10	0.43^{b}	0.07	0.55^{b}	0.09	0.41^{b}	0.11	0.49^{b}	0.10
	Phase 3	1.00	0.08	2.14	0.40	1.04	0.08	1.13	0.18	1.75	0.24	2.69	0.96

Even though effects on inflammation markers were not significant in all cases in our current trial, all phytogenic substances tested had an overall positive influence on inflammation related parameters. For instance, all additives reduced colonic COX2 expression, and they increased the anti-inflammatory cytokine IL-10 considerably.

Moreover VCAM-1 expression was decreased by all plant extracts tested to a high extent. This particular result is in accordance with the data of a current in vitro study [42]. Moreover in our trial the mRNA expression data for colonic Cldn3 have shown for the first time that the essential oils of turmeric, thyme, and rosemary may improve the mucosal barrier function by the upregulation of this tight junction protein. Comparable beneficial effects of plant extracts on tight junction proteins have been described only for two other plant extracts, namely, berberine and apple extracts until today [43, 44].

Treatment with 4% DSS for 6 days in our trial has caused a mild colonic inflammation as indicated by the distinct increase in the expression of pro-inflammatory parameters like COX2 and TNFα in group DSS compared to healthy Con rats. Although a rise in the expression of these pro-inflammatory mediators could also be observed in the DSS groups, receiving plant extracts, it was considerably less pronounced, suggesting anti-inflammatory effects of broccoli extract, turmeric oil, thyme oil, and rosemary oil during the acute phase of a mild colitis. With regard to this aspect our data confirm the results of current in vitro and in vivo studies describing beneficial effects of plant extracts during acute phlogistic processes via the reduction of pro-inflammatory mediators [12, 16, 20, 45–49]. However, reduced Cldn3 mRNA level in all DSS-treated rats may indicate that none of the plant extracts investigated could prevent changes in tight junctions during acute DSS exposition.

In contrast to the data of Reed et al. [50], showing the activation of NFκB subsequent to a 6-day treatment period with 5% DSS, in our trial a DSS-dependent increase in the mRNA expression of NFκB and of its targets TNFα, IL-1β,

MCP-1, and VCAM-1 did not occur until the end of phase 3 (6 days after final DSS treatment). These results may derive from the lower DSS concentration (4%) used in our trial. With the exception of group To in phase 3 no changes in Cldn3 mRNA expression could be observed in the other experimental groups compared to Con rats, suggesting that in particular To may improve intestinal barrier function [51].

Data from current literature suggest that the transcription factor Nrf2, responsible for the induction of antioxidant and xenobiotic enzymes, is of importance in the control of NFκB dependent inflammatory processes (Figure 3) [52]. Vice versa it is speculated that an increase in NFκB may inhibit Nrf2 signaling. Several well-known Nrf2 activators, for example, curcumin, resveratrol, and sulforaphane, are believed to suppress LPS- and DSS-induced NFκB activation [53–56], whereas the direct relationship between Nrf2 manipulation and NFκB-inhibition has not been proven until today. Our results for the acute inflammatory phase 2 cannot confirm an association between Nrf2 and NFκB. However, a distinct negative association exists between Nrf2 and TNFα, which again is believed to be the strongest first trigger of NFκB activation (Figures 3–5) [57]. Thus due to acute DSS exposition Nrf2 expression was distinctly decreased, whereas TNFα expression was strongly elevated. In contrast no differences in the relation between the mentioned transcription factors existed under noninflamed conditions (phase 1). Moderate oxidative stress seems to activate Nrf2 and its target genes accompanied by the simultaneous inhibition of NFκB signaling (Figure 4). This hypothesis seems to be confirmed by the general decrease of VCAM-1 mRNA (NFκB target) through application of all plant extracts and the increase in GSTK1 and T2 mRNA (Nrf2 targets) by Cuo and Ro application in phase 1 of our study (Figure 4).

In phase 2 DSS treatment alone obviously has induced considerable oxidative stress in the colon accompanied by a significant inhibition of Nrf2 expression, finally leading to an increase in NFκB mRNA in phases 2 and 3 of our trial (Figure 5). In contrast phytogenic additives seem to attenuate both NFκB induction and the upregulation of its pro-inflammatory targets. This phenomenon may be the result of the rise in Nrf2-dependent antioxidant and xenobiotic enzymes.

Our liver data indicate that DSS-dependent inflammatory processes seem to be restricted to the colon initially, but that they can also provoke a systemic inflammatory response as indicated by the increase in TNFα expression in DSS-treated rats in phase 3.

5. Conclusions

Our experimental design has allowed for the simultaneous examination of (1) preventive effects of various plant extracts on intestinal health (phase 1), of (2) their anti-inflammatory and antioxidant potential during acute DSS-induced colitis (phase 2), and of (3) their influence on parameters related to inflammation and the antioxidant system during the recovery process (phase 3).

We conclude the following.

(i) The treatment of rats under noninflamed conditions with broccoli extract and the essential oils of turmeric, thyme, and rosemary promotes intestinal health by reducing the pro-inflammatory adhesion molecule VCAM-1 and by increasing the tight junction marker Cldn3 leading to an improved gut barrier.

(ii) The phytogenic additives investigated have anti-inflammatory properties as indicated by the reduction of the DSS-induced increase in pro-inflammatory mediators like NFκB, VCAM-1, MCP-1, and COX2 to a greater or lesser extent.

Further research is needed to evaluate the role of phytogenic additives in modulating NFκB-Nrf2 interactions in more detail. Moreover detailed investigations with regard to the time-dependent regulation of inflammatory and antioxidant responses during an acute gut inflammation are urgently needed. Nevertheless, plant extracts and in particular essential oils may represent promising substances in the complementary therapy of IBD with a particular focus in the prevention.

Appendix

The complete results (means ± SEM) of the mRNA expression analyses, including statistical evaluation, are shown in Tables 8, 9, and 10.

Conflict of Interests

All authors declare that they have no conflict of interests.

Acknowledgments

This study was partially funded by Delacon Biotechnik Ges.m.b.H, Steyregg (Austria) to investigate fundamental mechanisms of phytogenic feed additives under inflammatory conditions. The authors thank Devakumari Hiller (Jarrow Deutschland GmbH, Berlin, Germany) for arranging the contact with Mr. Jarrow L. Rogovin from Jarrow Formulas, Los Angeles, USA, who provided us with the broccoli seed extract. Thank is also addressed to our master students Nina Wodrich, Lisa Schering and Anne Lehmann for their help with analyses.

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3.4 Versuch 4 (IPEC-J2-Zellen)

Für diese in vitro-Untersuchungen wurde die Zelllinie IPEC-J2 (Intestinal Porcine Epithelial Cells-Jejunal 2), eine jejunale nicht-transformierte Epithelzelllinie neugeborener, abgesetzter Ferkel (Schierack et al. 2006) genutzt. Die Zellen wurden bis zur Verwendung in 75 cm^2 Zellkulturflaschen (Cellstar® Tissue Culture Flasks, Greiner bio-one, Deutschland) in einem Zellnährmedium (DMEM/HAM´s F-12 (1:1), Biochrom AG, Deutschland), supplementiert mit 10% Schweineserum und mit 1% Penicillin, bei 37% in 5%iger CO_2-Atmosphäre kultiviert. Der Versuch gliederte sich in 2 Teilversuche (A und B). Für beide Teilversuche wurden in jede Kavität einer 24er Zellkulturplatte (Cellstar®24 Well Cell Culture Plate sterile, Greiner bio-one, Deutschland) Zellen mit einer Zelldichte von 10^5/ml ausgesät und für 24 Stunden bei 37% in 5%iger CO_2-Atmosphäre inkubiert.

Teilversuch A:

24 Stunden nach der Aussaat erfolgte die Behandlung der IPEC-J2-Zellen mit den jeweiligen Extrakten für weitere 24 Stunden bei 37% in 5%iger CO_2-Atmosphäre. Anschließend wurden die Inkubationsmedien entfernt, die Zellen mit PBS gewaschen und für die RNA-Extraktion in Guanidinthiocyanate-Puffer (4M) aufgenommen.

Teilversuch B:

Die für 24 Stunden mit den jeweiligen Extrakten vorinkubierten IPEC-J2-Zellen (Teilversuch A) wurden anschließend mit einem enteropathogenen *Escherichia coli*-Stamm Abbotstown (Escherichia coli: *O149:K91:K88ac*) für 90 Minuten bei 37% in 5%iger CO_2-Atmosphäre infiziert. Anschließend erfolgte die Probengewinnung für die RNA-Extraktion wie in Teilversuch A.

Tabelle 4: Getestete phytogene Verbindungen und Konzentrationen

	Konzentrationsstufe		
phytogene Substanz	1	2	3
Apfelextrakt 1	10 µM	5 µM	2.5 µM
Apfelextrakt 2	10 µM	5 µM	2.5 µM
Gelbwurzel (Öl)	100 µM	10 µM	1 µM
Glucoraphanin	7.5 µM	3.75 µM	1.25 µM
Sulforaphan	7.5 µM	3.75 µM	1.25 µM

Die Konzentration der Apfelextrakte wurde auf den Phenolgehalt (75%) eingestellt, wobei Phloridzin als Polyphenol-Äquivalent zur Berechnung herangezogen wurde.

Die beschriebenen Zellkulturversuche wurden im Rahmen eines Gemeinschaftsprojektes von unserem Kooperationspartner, der Freien Universität Berlin, am Institut für Tierernährung durchgeführt. Die Analyse der mRNA-Expression mittels RT-PCR erfolgte dann am Institut

für Ernährungswissenschaften der Martin-Luther-Universität Halle-Wittenberg. In Tabelle 4 sind alle im Versuch 4 verwendeten phytogenen Verbindungen sowie die in beiden Teilversuchen eingesetzten Konzentrationen aufgeführt.

Da bei der ursprünglichen Auswertung der in vitro-Daten keine Konzentrations-abhängigen Effekte der einzelnen Substanzen, weder unter nicht infizierten (Teilversuch A) noch unter mit *Escherichia coli* infizierten Bedingungen (Teilversuch B), ermittelt werden konnten, wurden jeweils alle Konzentrationsstufen einer Testsubstanz zusammengefasst und erneut statistisch ausgewertet. Die Ergebnisse der RT-PCR Analyse wurden bislang nicht publiziert und sind zusammengefasst in den folgenden Abbildungen 1-3 sowie in Tabelle 5 dargestellt.

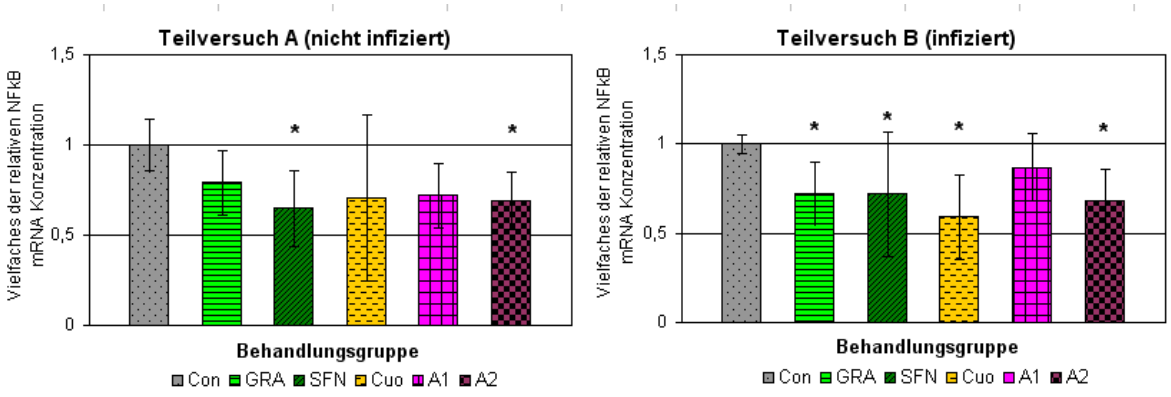

Abbildung 1: Einfluss der getesteten phytogenen Substanzen auf die NFκB mRNA-Expression von IPEC-J2-Zellen unter physiologischen (Teilversuch A) sowie unter stimulierten (Teilversuch B) Bedingungen.
Dargestellt sind Mittelwerte ± Standardabweichung (n=3) der NFκB mRNA Konzentration in Relation zur Expression in den Kontrollzellen (Con=1). * kennzeichnet signifikante Unterschiede zur Kontrolle (p≤0,05). Abk.: NFκB, Nuclear Factor 'kappa-light-chain-enhancer' of activated B-cells; Con, Kontrolle; GRA, Glucoraphanin; SFN, Sulforaphan; Cuo, Gelbwurzel; A1, Apfelextrakt 1; A2, Apfelextrakt 2.

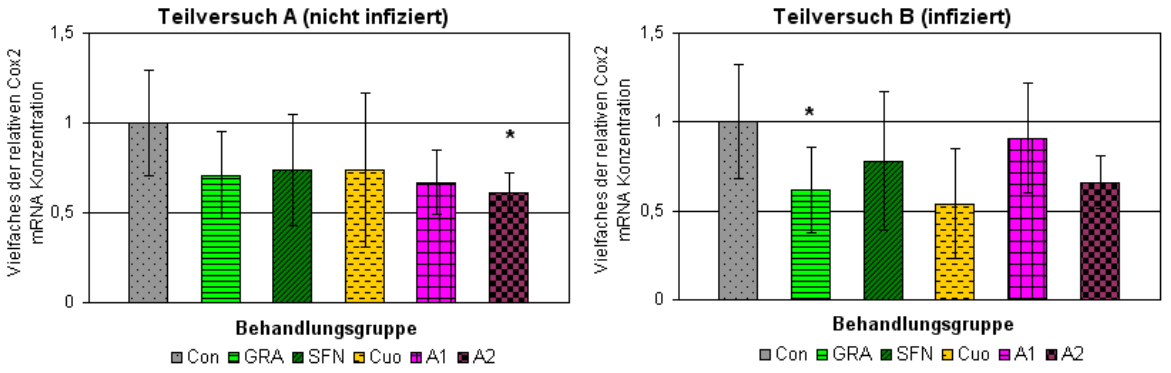

Abbildung 2: Einfluss der getesteten phytogenen Substanzen auf die Cox2 mRNA-Expression von IPEC-J2-Zellen unter physiologischen (Teilversuch A) sowie unter stimulierten (Teilversuch B) Bedingungen.
Dargestellt sind Mittelwerte ± Standardabweichung (n=3) der Cox2 mRNA Konzentration in Relation zur Expression in den Kontrollzellen (Con=1). * kennzeichnet signifikante Unterschiede zur Kontrolle (p≤0,05). Abk.: Cox2, Cyclooxygenase 2; Con, Kontrolle; GRA, Glucoraphanin; SFN, Sulforaphan; Cuo, Gelbwurzel; A1, Apfelextrakt 1; A2, Apfelextrakt 2.

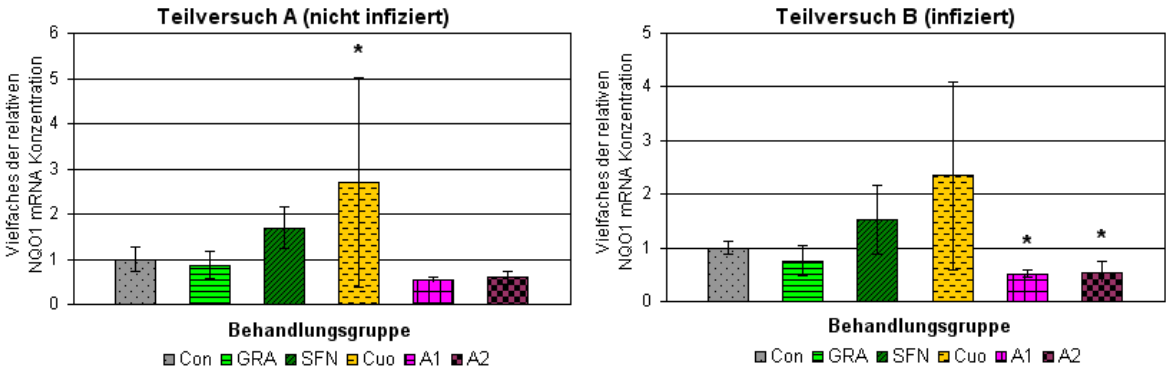

Abbildung 3: Einfluss der getesteten phytogenen Substanzen auf die NQO1 mRNA-Expression von IPEC-J2-Zellen unter physiologischen (Teilversuch A) sowie unter stimulierten (Teilversuch B) Bedingungen.
Dargestellt sind Mittelwerte ± Standardabweichung (n=3) der NQO1 mRNA Konzentration in Relation zur Expression in den Kontrollzellen (Con=1). * kennzeichnet signifikante Unterschiede zur Kontrolle ($p \leq 0{,}05$). Abk.: NQO1, NADPH-abhängige Chinonoxidoreduktase 1; Con, Kontrolle; GRA, Glucoraphanin; SFN, Sulforaphan; Cuo, Gelbwurzel; A1, Apfelextrakt 1; A2, Apfelextrakt 2.

Tabelle 5: Einfluss der getesteten phytogenen Substanzen auf die mRNA-Expression der inflammatorischen Mediatoren TNFα, IL-8, IL-1β sowie dem antioxidativen Enzym GPx2 in IPEC-J2-Zellen unter physiologischen (Teilversuch A) und unter stimulierten (Teilversuch B) Bedingungen.

Teilversuch A (nicht infiziert)					Teilversuch B (infiziert)				
Parameter	Gruppe	Vielfaches der relativen mRNA Konzentration		Signifikanz zur Kontrolle	Parameter	Gruppe	Vielfaches der relativen mRNA Konzentration		Signifikanz zur Kontrolle
		MW	SD	p			MW	SD	p
TNFα	Kontrolle	1.00	0.25		TNFα	Kontrolle	1.00	0.29	
	Glucoraphanin	1.01	0.28	0.988		Glucoraphanin	2.28	1.88	0.178
	Sulforaphan	0.92	0.72	0.496		Sulforaphan	2.13	0.68	0.096
	Gelbwurzel	0.72	0.47	0.339		Gelbwurzel	1.26	0.57	0.685
	Apfelextrakt 1	0.37	0.23	0.012		Apfelextrakt 1	1.27	0.93	0.885
	Apfelextrakt 2	1.23	0.81	0.887		Apfelextrakt 2	2.32	1.09	0.081
IL-8	Kontrolle	1.00	0.33		IL-8	Kontrolle	1.00	0.56	
	Glucoraphanin	0.68	0.35	0.173		Glucoraphanin	0.59	0.37	0.089
	Sulforaphan	0.76	0.44	0.302		Sulforaphan	0.58	0.24	0.127
	Gelbwurzel	0.76	0.41	0.325		Gelbwurzel	0.40	0.19	0.005
	Apfelextrakt 1	0.70	0.31	0.203		Apfelextrakt 1	0.90	0.11	0.956
	Apfelextrakt 2	0.70	0.28	0.202		Apfelextrakt 2	0.99	0.51	0.951
IL-1β	Kontrolle	1.00	0.43		IL-1β	Kontrolle	1.00	0.20	
	Glucoraphanin	1.23	0.14	0.534		Glucoraphanin	1.89	1.38	0.288
	Sulforaphan	0.75	0.52	0.507		Sulforaphan	1.92	0.70	0.293
	Gelbwurzel	0.70	0.41	0.457		Gelbwurzel	0.76	0.26	0.786
	Apfelextrakt 1	0.78	0.98	0.554		Apfelextrakt 1	1.59	1.52	0.499
	Apfelextrakt 2	0.81	0.78	0.647		Apfelextrakt 2	2.01	1.78	0.248
GPx2	Kontrolle	1.00	0.63		GPx2	Kontrolle	1.00	0.33	
	Glucoraphanin	0.73	0.44	0.425		Glucoraphanin	2.03	1.05	0.176
	Sulforaphan	0.72	0.40	0.458		Sulforaphan	1.71	0.85	0.348
	Gelbwurzel	0.49	0.20	0.209		Gelbwurzel	1.48	1.12	0.526
	Apfelextrakt 1	0.55	0.73	0.049		Apfelextrakt 1	0.60	0.41	0.591
	Apfelextrakt 2	0.50	0.29	0.146		Apfelextrakt 2	1.56	0.70	0.452

Abk.: GPx2, Glutathionperoxidase 2; IL-1β, Interleukin 1 *beta*; IL-8, Interleukin 8; MW, Mittelwert; SD, Standardabweichung; TNFα, Tumornekrosefaktor *alpha*.

4 Diskussion

In der landwirtschaftlichen Praxis, besonders in der Nutztierhaltung ist die Leistung einer der wichtigsten wirtschaftlichen Faktoren. Das EU-weite Einsatzverbot von antibiotischen Leistungsförderern in der Tierernährung als Maßnahme des gesundheitlichen Verbraucherschutzes und zu der Reduktion des Antibiotikaeinsatzes beim Menschen soll das Auftreten von Antibiotikaresistenzen vermindern. Um den durch das Einsatzverbot entstehenden Leistungseinbußen in der Nutztierproduktion entgegenzuwirken, fehlen in der Tierernährung allerdings geeignete Alternativen.

Die bislang fast einzige Alternative in der Nutztierernährung stellt der Einsatz phytogener Additive dar. Über ihre verschiedenen diskutierten Wirkungsmechanismen könnten pflanzliche Futterzusätze leistungs- und gesundheitsfördernde Effekte bei Nutztieren erzielen. Dabei scheinen einzelne Inhaltsstoffe (zum Beispiel: Carvacrol aus Oregano, Thymol aus Thymian, 1,8-Cineol aus Rosmarin, Sulforaphan aus Brokkoli, ar-Turmeron aus Gelbwurzel) maßgeblich diese positiven Effekte zu verursachen. Die bisherige kontroverse Datenlage und das Fehlen aussagekräftiger in vivo-Studien zu dieser Thematik, insbesondere mit Nutztieren, bildeten die Grundlage für die vorliegende Arbeit.

4.1 Einfluss von Brokkoliextrakt und ätherischen Ölen auf Leistungsparameter bei Nutztieren

Die **Versuche 1** und **2** der vorliegenden Arbeit zeigten, dass die Fütterung von Brokkoliextrakt und der ätherischen Öle aus Gelbwurzel, Oregano, Thymian und Rosmarin keine nennenswerte Verbesserung der Leistungsparameter bewirkte. Nach den Ergebnissen dieser beiden Untersuchungen stellen die in der vorliegenden Arbeit getesteten phytogenen Futterzusätze im Hinblick auf einen leistungsfördernden Effekt weder für Broiler noch für Absetzferkel eine echte Alternative zu den seit 2006 in der EU verbotenen leistungsfördernden Antibiotika (Salinomycin, Avilamycin, Flavomycin) dar.

Durch den Verzicht auf antibiotische Leistungsförderer verringerte sich der Tageszuwachs bei Absetzferkeln um 8% und bei Broilern um 3% (Pfirter 2003). Die Auswertung von publizierten Versuchsergebnissen der Jahre 1974 bis 1997 zeigte beispielsweise, dass der antibiotische Leistungsförderer Avilamycin im Durchschnitt zu einer Steigerung der täglichen Zunahme um 12,2% und Futteraufnahme um 4,8% führte (Freitag et al. 1999). Daraus resultierte durch den Einsatz von Avilamycin in der Ferkelaufzucht eine Verbesserung der Futterverwertung um durchschnittlich 8,4% (Freitag et al. 1999). Ähnliche leistungssteigernde

Effekte wurden für die Verabreichung von Salinomycin in der Schweinemast ermittelt (Freitag et al. 1999).

Zusammenfassend betrachtet wird die Wirkung phytogener Substanzen auf Leistungsparameter in der Nutztierernährung wie Futteraufnahme, tägliche Gewichtszunahme, Mastendgewichte und Futterverwertung (Tabelle 6) derzeit allerdings kontrovers diskutiert. In diesem Kontext zeigten Studien von Hernández et al. (2004), Muhl und Liebert (2006) und Mares et al. (2007) übereinstimmend mit den Ergebnissen aus den **Versuchen 1** und **2** der vorliegenden Arbeit (**V1:Tab. 2-5; V2:Tab. 5**) ebenfalls keine leistungsfördernden Effekte pflanzlicher Additive. Mindestens ebenso viele in vivo-Studien weisen eine deutliche Steigerung von Leistungsparameter durch phytogene Futterzusätze nach (Wenk 2005, Blödner und Thieme 2006, Kyriakis et al. 1998, Zitterl-Eglseer et al. 2008). Im Gegensatz zu einem mangelnden Effekt auf Leistungsparameter existieren auch Studien, welche sogar negative Wirkungen pflanzlicher Verbindungen belegen (Wenk 2005, Jugl-Chizzola et al. 2006, Oswald und Wetscherek 2007). In Tabelle 6 sind zu dieser kontroversen Datenlage einige repräsentative Studien für den Geflügel- und Ferkelbereich aus den letzten 10 Jahren im Überblick dargestellt.

Bei näherer Betrachtung der aktuellen Literatur sowie der **Versuche 1** und **2** fällt auf, dass sich phytogene Additive bei Broilern besonders in den ersten 21 Tagen nach dem Schlüpfen (**V1:Tab. 2-5,** Bozkurt et al. 2009, Basmacioğlu Malayoğlu et al. 2010, Tabelle 6A) beziehungsweise in der Ferkelaufzucht in der ersten Woche nach dem Absetzen vom Muttertier günstig auf die Leistungsparameter auswirken (**V2:Abb. 4,** Wetscherek 2005, Zitterl-Eglseer et al. 2008, Tabelle 6B). Während eine längere Fütterung der phytogenen Additive, zum Beispiel Oregano und Gelbwurzel, beim Broiler eher zu einer Verschlechterung der Leistungsparameter, hervorgerufen durch eine verminderte Futteraufnahme und vor allem durch eine reduzierte Gewichtszunahme führte (**V1:Tab. 2-5**), scheint eine längere Verabreichung pflanzlicher Futterzusätze sich beim Ferkel nicht nachteilig auszuwirken (**V2: Tab. 5, Abb. 4**), was jedoch aus Kostengründen vermieden werden sollte. Hieraus kann gefolgert werden, dass in zukünftigen Studien über eine kürzere, gezieltere und eventuell auch über eine alternierende Verabreichungsdauer phytogener Additive nachzudenken ist.

Tabelle 6: Aktuelle Datenlage zur Wirkung phytogener Futteradditive als Leistungsförderer

A. Geflügel

ätherisches Öl/ Extrakte	**Konzentration**	**FA**	**GW/GZ**	**FVW**	**Literatur**
Oregano	150 mg/kg	±	±	±	Basmacioglu et al. 2004
Oregano	300 mg/kg	±	±	±	Basmacioglu et al. 2004
Oregano	100-1000 mg/kg			+/+	Halle et al. 2004
Thymian	100-200 mg/kg	-/-	+/+	+/+	Al-Kassie 2009
Thymian	200 mg/kg	±	±	±	Najafi und Torki 2010
Zimtrinde	100-200 mg/kg	-/-	+/+	+/+	Al-Kassie 2009
Zimtrinde	200 mg/kg	±	±	±	Najafi und Torki 2010
Anis	40 ml eines 6%-Konzentrats		+	+	Durrani et al. 2007
Thymol	100 mg/kg	±	±		Lee KW et al. 2003
Zimtaldehyd	100 mg/kg	±	±		Lee KW et al. 2003
Curcuma longa	500 mg/kg		±	±	Rangsaz und Ahangaran 2011
Knoblauch (Propylpropan-Thiosulfonat)	45-90 mg/kg		+/+	+/+	Peinado et al. 2012
Beifuß	125-500 mg/kg	-/-	±/-		Engberg et al. 2012
Sonnenhutextrakt	1000 mg/kg	-	-	-	Rahimi et al. 2011
Nelke	200 mg/kg	±	±	±	Najafi und Torki 2010
gesamte Pflanze/ Pflanzenteil					
Rosmarin	25.000 mg/kg	±	±		Loetscher et al. 2012
Hagebutte	25.000 mg/kg	±	±		Loetscher et al. 2012
Apfelbeerentrester (Aronia)	25.000 mg/kg	±	±		Loetscher et al. 2012
Brennessel	25.000 mg/kg	±	±		Loetscher et al. 2012
Beifuß	5.000-20.000 mg/kg	-/-	-/-	-/-	Engberg et al. 2012
Schwarzkümmelsamen	10.000 mg/kg		+	+	Khalaji et al 2011
Gelbwurzel	2.500 mg/kg	+			Wenk und Messikommer 2002
Gelbwurzel	10.000 mg/kg	-	±	±	Wenk und Messikommer 2002
Thymian	2.000 mg/kg	±		±	Ocak et al. 2008
Pfefferminze	2.000 mg/kg	±	+	±	Ocak et al. 2008
Mischungen und komerzielle Produkte					
Digestarom®	150 mg/kg	-		+	Blödner und Thieme 2006
5% Carvacrol, 3% Zimtaldehyd, 2% Capsicum oleoresin	50-500 mg/kg	±/±	±/±	±/±	Muhl und Liebert 2006
Herb-Mos Oregano®	1.000 mg/kg	±	±	±	Bozkurt et al. 2009
Oregano, Zimt, Pfeffer	200 mg/kg	±		±	Hernández et al 2004
Salbei, Thymian, Rosmarin	5.000 mg/kg	±	±	±	Hernández et al 2004

B. Ferkel

ätherisches Öl/ Extrakte	**Konzentration**	**FA**	**GW/GZ**	**FVW**	**Literatur**
Thymol	67 mg/kg	±	±		Anderson et al. 2012
Thymol	201 mg/kg	±	±		Anderson et al. 2012
Rosmarin	500 mg/kg	+	+	±	Janz et al. 2007
Knoblauch	500 mg/kg	+	+	±	Janz et al. 2007
Oregano	500 mg/kg	+	+	±	Janz et al. 2007
Ingwer	500 mg/kg	+	+	±	Janz et al. 2007
Curcumin	200 mg/kg	±	±	±	Isley et al. 2005
Schwarzer Teeextrakt	4.000 mg/kg	-	-	-	Bruins et al. 2011
Schwarzer Teeextrakt	8.000 mg/kg	-	-	-	Bruins et al. 2011
gesamte Pflanze/ Pflanzenteil					
Oregano	2.000 mg/kg	-	-	-	Oswald und Wetscherek 2007
Mischungen und komerzielle Produkte					
5% Carvacrol, 3% Zimtaldehyd, 2% Capsicum oleorein	200 mg/kg	±	±	±	Manzanilla et al. 2009
20% Salbei, 30% Zitrone, 30% Brennessel, 20% Sonnenhut	500 mg/kg		+	+	Hanczakowska und Swiatkiewicz 2012
AROMEX®-*ME* (Thymian, Rosmarin, Oregano, Kaolin)	100 mg/kg	±	+	±	Yan et al. 2010
Biomin® P.E.P. 1000 (ätherisches Öl aus Oregano, Anis und Zitrusfruchtschalen)	40 mg/kg	±	±	±	Kroismayr et al. 2008a
5% Carvacrol, 3% Zimtaldehyd, 2% Capsicum oleorein	300 mg/kg	±	±	±	Manzanilla et al. 2006
traditionelle Chinesische Medizin (10% Glycyrrhiza glabra, 25% Salvia officinalis, 15% Rosmarinus officinalis, 25% Ocimum bacilicum, 25% Lavandula angustifolia)	3.000 mg/kg	+	±	±	Yeh et al. 2011

Abk.: FA, tägliche Futteraufnahme; GW, Körpergewicht; GZ, tägliche Gewichtszunahme; FVW, Futterverwertung. Symbole: +, Steigerung/Verbesserung; ±, kein Einfluss; -, Verminderung/Verschlechterung.

Die Tabelle 6 verdeutlicht zudem, dass die Ergebnisse aller bislang durchgeführten Studien bedauerlicherweise nur schlecht miteinander vergleichbar sind. In allen Untersuchungen unterscheiden sich Art (Kraut, Extrakt, ätherisches Öl) und Höhe der verabreichten Konzentration (45-25.000 mg/kg) der Futterzusätze extrem stark voneinander. Hinzu kommt die unterschiedliche und zum Teil ungeklärte, beziehungsweise nicht analysierte Zusammensetzung der verwendeten phytogenen Additive. Eine Standardisierung auf wichtige Hauptinhaltsstoffe, wie sie beispielsweise in dem vorliegenden **Versuch 2** erfolgte, beziehungsweise eine genauere Analytik der verabreichten Verbindungen ist daher für

zukünftige Studien unabdingbar, um eine bessere Vergleichbarkeit der Ergebnisse zu erzielen und damit aussagekräftige Einschätzungen zur Wirksamkeit phytogener Futterzusätze zu gewährleisten.

Weitere wichtige Faktoren im Hinblick auf die leistungsfördernden Eigenschaften von pflanzlichen Verbindungen stellen die Haltungs- und Hygienebedingungen dar (Wenk 2003). So werden in Versuchen mit hohen hygienischen Bedingungen (Versuchstation) oder in kleineren Mastbetrieben mit optimalen Fütterungs- und Haltungsbedingungen deutlich geringere positive beziehungsweise keine Effekte phytogener Futterzusätze auf die Leistungsparameter beobachtet (Weiß und Quanz 2000) als unter ungünstigen Hygiene- und Haltungsbedingungen oder in größeren Mastbetrieben mit schlechtem Management und Umweltbedingungen (Wenk 2003, Wald 2004). Die jeweilige mikrobielle Last (pathogenes Erregermilieu), der die Nutztiere ausgesetzt sind, scheint also ebenfalls eine wichtige Rolle bezüglich der Wirksamkeit phytogener Futterzusätze zu spielen.

Zusammenfassend kann anhand der Ergebnisse aus den **Versuchen 1** und **2** geschlussfolgert werden, dass es neben substanzspezifischen Unterschieden auch speziesspezifische Effekte hinsichtlich des Verabreichungszeitraumes phytogener Futterzusätze auf die geprüften Leistungsparameter zu geben scheint. So deutete sich für Broiler eine nur auf die ersten 3 Lebenswochen beschränkte Fütterung phytogener Additive als vorteilhaft an, während die Daueranwendung ätherischer Öle aus Oregano und Gelbwurzel möglicherweise zu Leistungseinbußen führt (**V1:Tab. 2-5**). Beim Ferkel scheinen sowohl die längere Gabe als auch eine relativ hohe Dosierung (150 mg Leitsubstanz/kg Diät, entsprechend mindestens 300 mg ätherisches Öl/kg Diät) pflanzlicher Verbindungen hingegen zumindest nicht negativ auf die Leistungsparameter auszuwirken. Aber auch hier traten eher zu Beginn der Fütterung (1. Woche nach dem Absetzen) positive Wirkungen auf, die sich schließlich über den Versuchzeitraum verloren (**V2:Tab. 5, Abb. 4**).

Weiter kann resümiert werden, dass die getesteten phytogenen Futterzusätze aus Brokkoli, Gelbwurzel, Oregano, Thymian und Rosmarin nicht in die Kategorie der klassischen Leistungsförderer einzuordnen sind und sie somit bezüglich einer leistungsfördernden Wirkung keine Alternative zu den ehemals verwendeten Antibiotika, mit denen eine signifikante Leistungssteigerung von durchschnittlich 3-4% je nach Tierart und Altersgruppe erzielt wurden (Zentek 2005), darstellen.

Zudem ist hervorzuheben, dass phytogene Verbindungen auch in hohen Dosen beim Absetzferkel keine gegenteiligen Wirkungen zu entfalten scheinen (**V2:Tab. 5**), sodass

potenzielle gesundheitsfördernde Wirkungen gerade im Hinblick auf die weitere Mast zum Tragen kommen könnten.

4.2 Einfluss von Brokkoliextrakt und ätherischen Ölen auf die Darmgesundheit von Nutztieren

Eine intakte Darmbarriere ist nicht nur zum Schutz vor Infektionen mit pathogenen Erregern, wie ETEC, essentiell (Pluske et al. 2002), sondern ist auch für eine optimale Nährstoffabsorption entscheidend. Störungen dieser Barrierefunktion führen folglich meist zu einer erhöhten Infektionsgefahr gegenüber pathogenen Mikroorganismen und zu einer verringerten Nährtstoffaufnahme (Kampues 1997, Freitag und Hensche 1998). Insgesamt kann die daraus resultierende Beeinträchtigung der Darmgesundheit zu Leistungseinbußen bis hin zu einer gesteigerten Mortalität von Nutztieren führen (Kyriakis et al. 1998).

Endogener und exogener oxidativer Stress, wie er beispielsweise durch die Nahrung per se oder im Besonderen durch die bei der Nahrungsumstellung beim Absetzen sowie durch Medikamente zu Stande kommen kann, steht im Zusammenhang mit verschiedenen Erkrankungen (*Inflammatory Bowel Disease*, Krebs) (Halliwell 1994, Willcox et al. 2004). Zudem scheint oxidativer Stress auch für die Darmgesundheit insgesamt eine wichtige Rolle zu spielen. Daher erscheint es vorteilhaft, das antioxidative Abwehrsystem von Nutztieren zu stärken.

Phytogene Futteradditive scheinen bezüglich ihrer antioxidativen Wirkung beide Bereiche des antioxidativen Abwehrsystems zu bedienen. Zum einen konnte in zahlreichen in vitro-Studien gezeigt werden, dass besonders ätherische Öle aus Oregano, Thymian und Rosmarin, eine starke antioxidative Aktivität aufweisen (Sacchetti et al. 2005; Bozin et al. 2006; Bozin et al. 2007; Viuda-Martos et al. 2010). Gleichzeitig besitzen pflanzliche Verbindungen eine große antioxidative Kapazität, reaktive Sauerstoffradikale abzufangen (Shan et al. 2005). Zum anderen zeigen die Ergebnisse der vorliegenden Arbeit (**V1:Tab. 8, Abb. 2; V2:Abb. 2**), dass auch das antioxidative Potential in den Geweben (Darm und Leber) von Nutztieren durch die Verabreichung phytogener Futterzusätze gesteigert werden kann.

Zusätzlich lässt sich vermuten, dass pflanzliche Verbindungen über ihre in vitro vielfach beschriebenen antimikrobiellen und bakteriziden Effekte einen positiven Einfluss auf die Darmgesundheit von Nutztieren ausüben. Die antimikrobielle Wirkung richtet sich möglicherweise zum einen direkt gegen Nutztier-pathogene Mikroorganismen, wie *Escherichia coli* (ETEC, *Escherichia coli O1K1*) und Salmonellen (*Salmonella enterica*, *Salmonella* Typhimurium) (Burt et al. 2007a, Burt et al. 2007b). Diese Bakterienspezies lösen die typischen Erkrankungsbilder der Coliruhr beim Absetzferkel, der Coliseptikämie beim

Geflügel und von Salmonellosen aus (Rolle und Mayr 2007). Zum anderen wird scheinbar das Wachstum apathogener Mirkroorganismen, wie das von Laktobazillen, durch den Einsatz von phytogenen Futterzusätzen verbessert, wodurch eine Stabilisierung der Darmflora und der Barrierefunktion der Darmmukosa erreicht wird (Manzanilla et al. 2009, Maenner et al. 2011). Die hieraus resultierende verbesserte Nährstoffabsorption und die gestärkte Abwehr gegenüber pathogenen Erregern könnte in der Folge die Leistung von Nutztieren stabilisieren oder sogar verbessern (Kroismayr et al. 2008b, Windisch et al. 2008).

Neben der Beeinflussung des antioxidativen Status und der Eubiose im Darmtrakt durch phytogene Futteradditive spielen auch ihre potenziellen anti-inflammatorischen Eigenschaften über die Hemmung der NFκB-Signalkaskade im Hinblick auf die Darmgesundheit von Nutztieren eine wichtige Rolle. Besondere Aufmerksamkeit kommt dabei Polyphenol-haltigen pflanzlichen Verbindungen zu. Da die in der landwirtschaftlichen Praxis häufig auftretenden Durchfallerkrankungen meist mit Entzündungsprozessen im Darm der Nutztiere einhergehen, die häufig nicht auf den Darmtrakt beschränkt bleiben und zu einer eingeschränkten Leitungsfähigkeit von Nutztieren beitragen können (Kyriakis et al. 1998, Miguel 2010), sind phytogene Futterzusätze möglicherweise potente präventive Substanzen zur Vermeidung von entzündlichen Darmerkrankungen.

4.2.1 *Direkte antioxidative Wirkung von Brokkoliextrakt und ätherischen Ölen*

In **Versuch 1** deuteten sich eindeutige Substanz-spezifische Unterschiede bezüglich der direkten antioxidativen Wirkung phytogener Futterzusätze an. Dabei zeigten sich beispielsweise für die ätherischen Öle aus Oregano und Thymian durch eine Steigerung der TEAC-Werte deutlich ausgeprägtere direkte antioxidative Effekte als für Brokkoliextrakt, Gelbwurzel- und Rosmarinöl (**V1:Abb. 2**). Darüber hinaus konnte für beide Nutztierspezies (Broiler, Absetzferkel) nachgewiesen werden (**Versuch 1** und **2**), dass das direkte antioxidative Potential der getesteten phytogenen Verbindungen nicht nur in den ätherischen Ölen selbst vorliegt (**V1:Abb. 2**) und zu einer geringeren Lipidperoxidation führt (**V1:Abb. 3, V2:Abb. 3,** Sacchetti et al. 2005, Viuda-Martos et al. 2010), sondern auch im Gewebe über eine Steigerung der TEAC-Werte zu einem verbesserten antioxidativen Status beitragen kann (**V1:Abb. 2; V2:Abb. 2)**.

Dabei ist eine direkte antioxidative Wirkung durch die Fähigkeit einer Verbindung gekennzeichnet, oxidativen Stress unmittelbar, d.h. ohne Sekundärreaktionen, zu vermindern (Fahey und Talalay 1999). Das unterschiedlich starke direkte antioxidative Potential von phytogenen Substanzen wird durch ihre chemische Struktur, respektive der ihres jeweiligen

Hauptinhaltsstoffes, determiniert. Zudem sind mögliche additive Effekte durch das Vorhandensein von wirksamen Minorkomponenten vorstellbar.

Carvacrol und Thymol, die Hauptinhaltstoffe der eingestzten ätherischen Öle aus Oregano und Thymian, weisen als Phenolderivate chemisch gesehen ein mit einer Hydroxylgruppe substituiertes aromatisches Grundgerüst auf, wodurch freie Radikale stabilisiert werden können. Darüber hinaus kann durch die Oxidation dieser Hydroxylgruppen unter Ausbildung einer Chinonstruktur zusätzlich oxidativer Stress vermindert werden (Skrobot et al. 2003, Abbildung 4).

Dieser Sachverhalt ist vermutlich ursächlich dafür, dass in **Versuch 1** ein hohes direktes antioxidatives Potential für die ätherischen Öle des Oregano und Thymians im Vergleich zu den übrigen verwendeten Pflanzenextrakten ermittelt werden konnte (**V1:Abb. 2**). Einhergehend führte die Verabreichung von Oregano- und Thymianöl sowohl beim Broiler als auch beim Absetzferkel zu einer hohen antioxidative Kapazität (TEAC) im Gewebe (Darm, Leber) und zu einer Verminderung der Lipidperoxidation (**V1:Abb. 2, 3; V2:Abb. 2, 3**).

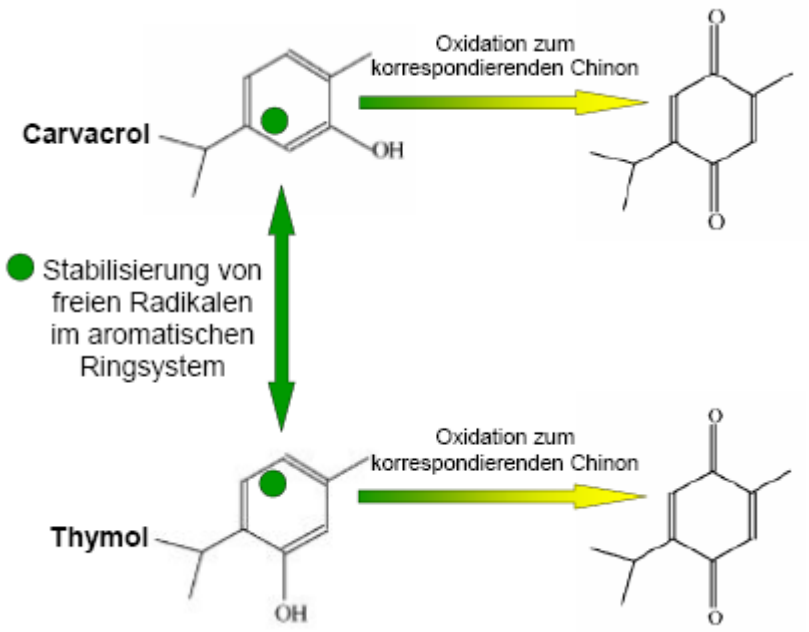

Abbildung 4: Chemische Struktur der Leitterpene Carvacrol (Oregano) und Thymol (Thymian)

Das 1,8-Cineol des Rosmarinöls ist ebenfalls eine terpenoide Substanz. Im Gegensatz zu Carvacrol und Thymol verfügt das 1,8-Cineol aber nicht über eine Hydroxylgruppe, wodurch die deutlich geringere direkte antioxidative Wirkung von Rosmarinöl zu erklären ist (**V1: Abb. 2,** Abbildung 5). Ähnliches trifft auch auf das ar-Turmeron der Gelbwurzel zu, dessen ätherisches Öl nur ein geringes direktes antioxidatives Potential aufweist (**V1: Abb. 2,** Abbildung 5).

Dennoch bewirkte die Fütterung der ätherischen Öle aus Rosmarin und Gelbwurzel sowohl beim Broiler als auch beim Absetzferkel eine Erhöhung der antioxidativen Kapazität im Gewebe (**V1: Abb. 2; V2:Abb. 2**). Ursächlich für diese TEAC-steigernde Wirkung scheint dabei für beide Substanzen, die Aktivierung von indirekten antioxidativen Signalwegen zu sein (siehe Punkt 4.2.2., Abbildung 5).

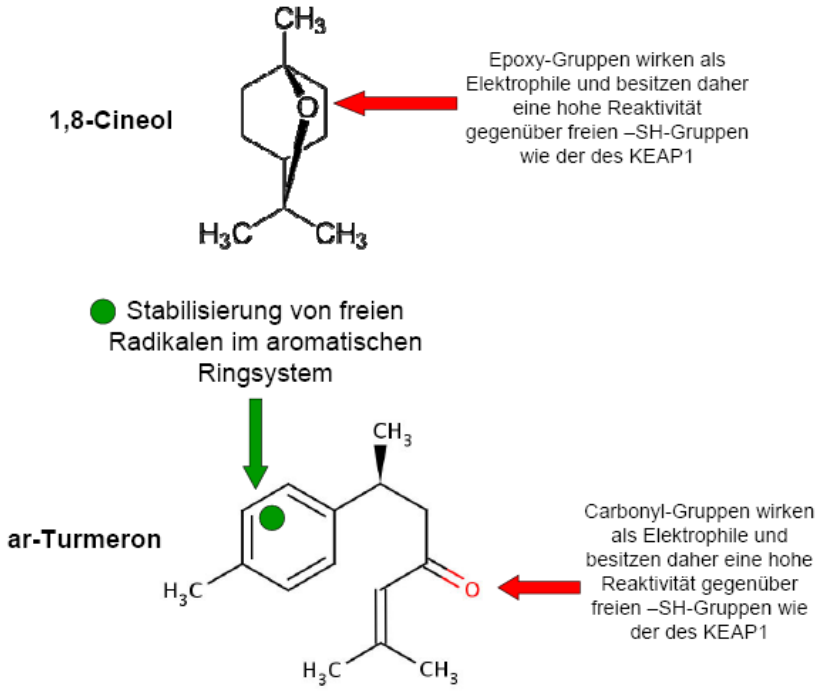

Abbildung 5: Chemische Struktur der Leitterpene 1,8-Cineol (Rosmarin) und ar-Turmeron (Gelbwurzel)

Das Sulforaphan stellt die Leitsubstanz des verwendeten Brokkoliextrakts dar und gehört zu den Isothiocyanaten. Aufgrund der chemischen Struktur weist das Sulforaphan keine direkten antioxidativen Eigenschaften auf, wodurch die fehlende direkte antioxidative Wirkung von Brokkoliextrakt in vivo zu begründen ist (**V1:Abb. 2; V2: Abb. 2,** Abbildung 6).

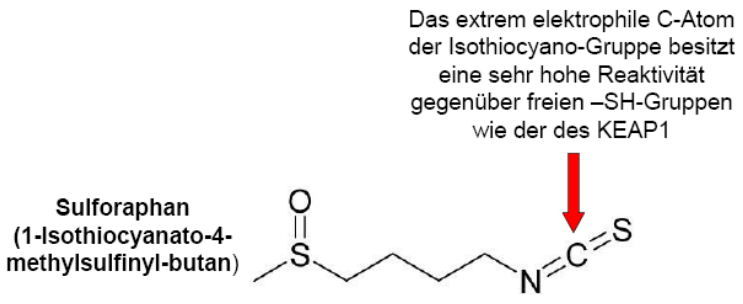

Abbildung 6: Chemische Struktur des Isothiocyanats Sulforaphan (Brokkoli)

4.2.2 *Indirekte antioxidative Wirkung von Brokkoliextrakt und ätherischen Ölen über die Induktion Xenobiotika-metabolisierender Enzyme*

In den **Versuchen 1** und **2** der vorliegenden Arbeit konnte erstmals im Nutztierbereich sowohl für den Broiler als auch für das Ansetzferkel festgestellt werden, dass der als Futterzusatzstoff verwendete Brokkoliextrakt eine indirekte antioxidative Wirkung über die Modulation der Keap1/Nrf2-ARE-Signalkaskade aufweist (**V1:Tab. 7A;** Tabelle 7). Allerdings blieb, entgegen den Erwartungen, in **Versuch 3** die Verabreichung von Brokkoliextrakt für 7 Tage im Darm von gesunden Ratten ohne Wirkung auf indirekte antioxidative Systeme (**V3:Tab. 7, 10**). Diese fehlende indirekte antioxidative Wirkung von Brokkoliextrakt in **Versuch 3** lässt sich wahrscheinlich auf die deutlich kürzere Verabreichungsdauer zurückführen. Im Vergleich zu den 7 Tagen in **Versuch 3** wurde der Brokkoliextrakt in **Versuch 1** (Broiler) für 3 Wochen und in **Versuch 2** (Absetzferkel) für 4 Wochen gefüttert und eine Induktion antioxidativer Nrf2-abhängiger Enzyme nachgewiesen.

Tabelle 7: Indirekte antioxidative Wirkung von Brokkoliextrakt und ätherischen Ölen im Darm von Nutztieren (siehe auch V1; V2)

A. Broiler (entspricht Tabelle 7A aus Mueller K et al. (2012), Br. J. Nutr., Vol.108, No. 4, 588-602)

Group…	Con		SFN		Cuo		Oo		To		Ro	
ARE gene	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
Jejunum												
GSTα	1·00^{a}	0·26	1·55^{a}	0·35	1·14^{a}	0·62	1·12^{a}	0·38	0·87^{a}	0·22	0·67^{a}	0·12
HMOX1	1·00^{a}	0·12	1·60^{b}	0·20	1·86^{b}	0·44	1·06^{a}	0·17	1·21^{a,b}	0·10	1·98^{b}	0·50
EPHX1	1·00^{a}	0·17	1·90^{b}	0·30	2·19^{b}	0·44	1·59^{a,b}	0·37	1·40^{a,b}	0·21	1·80^{b}	0·30
EPHX2	1·00^{a}	0·19	1·54^{a}	0·23	1·46^{a}	0·29	1·37^{a}	0·38	1·14^{a}	0·12	1·60^{a}	0·32
AFAR	1·00^{a}	0·06	1·87^{b,c}	0·23	2·26^{b}	0·37	1·16^{a}	0·19	1·40^{a,c}	0·26	1·37^{a,c}	0·22
TrxR1	1·00^{a}	0·12	1·29^{a}	0·23	1·62^{a,b}	0·41	1·27^{a}	0·18	1·19^{a}	0·13	2·12^{b}	0·36

B. Absetzferkel (entspricht Tabelle 9A aus Mueller K et al. (2012), Open J. Anim. Sci. 2:78-98)

(a) Jejunal mucosa

Group	Con		BE		Cuo		Oo		To		Ro	
Gene	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
GPx1	1.00^{a}	0.21	1.79^{b}	0.36	0.96^{a}	0.12	0.99^{a}	0.21	1.70^{b}	0.13	2.16^{b}	0.41
SOD1	1.00^{a}	0.09	2.14^{bc}	0.22	1.36^{ab}	0.11	1.32^{abc}	0.34	1.90^{c}	0.10	2.26^{ac}	0.40
AFAR	1.00^{a}	0.18	2.14^{b}	0.27	1.33^{ab}	0.33	1.07^{a}	0.20	2.03^{ab}	0.53	2.37^{b}	0.51
EPHX1	1.00^{a}	0.23	1.56^{ab}	0.27	1.49^{ab}	0.34	1.56^{ab}	0.26	2.40^{bc}	0.61	2.86^{c}	0.55
GSTa2	1.00^{a}	0.26	2.15^{b}	0.38	1.02^{ab}	0.22	1.68^{ab}	0.59	1.72^{ab}	0.65	2.22^{ab}	0.64
HMOX1	1.00	0.25	1.42	0.41	1.07	0.33	1.01	0.26	1.18	0.24	1.73	0.24
Keap1	1.00^{a}	0.20	1.95^{b}	0.37	0.84^{a}	0.15	0.87^{a}	0.12	1.24^{ab}	0.37	1.58^{ab}	0.42

In der aktuellen Literatur werden für die indirekte antioxidative Wirkung von Brokkoliextrakt verschiedenste Inhaltsstoffe, wie Isothiocyanate (ITC) und deren Glucosinolatvorstufen

(Glucoraphanin, Sinigrin), verantwortlich gemacht. Beide führen zu einer Nrf2-vermittelten Steigerung der Aktivität von antioxidativen Enzymen, wie der NAD(P)H-abhängigen Quinonoxidoreduktase 1 (NQO1) und der katalytischen Untereinheit der Glutamat-Cystein-Ligase sowie von Xenobiotika-metabolisierenden Enzymen, wie der Glutathion-S-Transferase, führen (McWalter et al. 2004).

ITC, wie Sulforaphan und Allyl-ITC, kommen in Form von schwefelhaltigen Glucosinolaten besonders häufig in Pflanzen der Familie der Cruciferae vor und sorgen für den charakteristischen Geschmack von Senf, Kresse, Brokkoli und verschiedenen anderen Kohlgemüsearten. Freie ITC werden letztlich durch pflanzliche oder bakterielle β-Thioglucosidasen enzymatisch gespalten und folglich beim Verzehr von Glucosinolat-haltigen Gemüsearten über die Nahrung aufgenommen. Aufgrund ihrer Lipophilie weisen ITC im Darmtrakt von Menschen und Säugetieren eine hohe Bioverfügbarkeit auf, die im Falle von Sulforaphan bei circa 74% liegt (Petri et al. 2003).

Aus den bisherigen Literaturdaten kann geschlossen werden, dass in den **Versuchen 1** und **2** zum einen die Glucosinolate, wie das Glucoraphanin (Perocco et al. 2006), für die Nrf2-abhängige Induktion antioxidativer und Xenobiotika-metabolisierender Enzyme durch den verabreichten Brokkoliextrakt verantwortlich sein können. Zum anderen kann diese indirekte antioxidative Wirkung auch auf hydrolysierte Isothiocyanate, wie das Sulforaphan und seine Analoga, (Thimmulappa et al. 2002, Gross-Steinmeyer et al. 2004, Banning et al. 2005, Dinkova-Kostova und Talalay 2008, Riedl et al. 2009) zurückgeführt werden. Eine derartige Steigerung der mRNA Expression Nrf2-abhängiger antioxidativ und xenobiotisch wirksamer Enzyme konnte auch im Kolon von Ratten, die einen Brokkoliextrakt in Kombination mit verschiedenen Selenstufen in der Diät erhielten, beobachtet werden (Blum et al. 2012).

In Gegensatz hierzu zeigten die Ergebnisse der in vitro-Studie mit porcinen Dünndarmzellen (IPEC-J2), dass nur reines Sulforaphan, nicht aber Glucoraphanin, die mRNA-Expression der antioxidativ wirksamen NQO1 induziert (**V4:**Abbildung 3). Hieraus lässt sich schließen, dass die in vivo ermittelte indirekte antioxidative Wirkung von Brokkoliextrakt beim Broiler und beim Absetzferkel vermutlich durch das beim Verzehr gebildete Sulforaphan und nicht durch dessen Glucosinolatvorstufe Glucoraphanin hervorgerufen wurde. Für diese Hypothese spricht auch die chemische Struktur des Sulforaphans. Das Kohlenstoffatom der ITC-Gruppe des Sulforaphans stellt ein sehr starkes Elektrophil dar, das eine hohe Reaktivität gegenüber freien Sulfhydrilgruppen (SH-Gruppen) aufweist (Abbildung 6). Bei Interaktion mit den freien SH-Gruppen des Keap1-Proteins kann es in der Folge zur Freisetzung von Nrf2 und zur Induktion der Genexpression seiner Zielgene kommen (Abbildung 7).

Für das ätherische Öl der Gelbwurzel zeigte sich ein entgegengesetzter Effekt bezüglich der antioxidativen Wirkung zwischen den beiden untersuchten Spezies. Während im Broilerversuch eher die indirekten antioxidativen Effekte des Zusatzes von Gelbwurzelöl belegt werden konnten (**V1:Tab. 7A,** Tabelle 7), verursachte die Verabreichung dieses Zusatzes bei Ferkeln eher direkte antioxidative Effekte (**V2:Abb. 2, Tab. 9A,** Tabelle 7). Die unterschiedlichen Ergebnisse zwischen Broiler (**V1**) und Absetzferkel (**V2**) basieren wahrscheinlich in erster Linie auf dem Einsatz unterschiedlicher Konzentrationen des ätherischen Öls der Gelbwurzel (Broiler: 150 mg ätherisches Öl/kg Diät; Ferkel: 150 mg ar-Turmeron/kg Diät ≈ 535 mg ätherisches Öl/kg Diät). Im Nagermodell (**V3:Tab. 7, 10**) hatte die Supplementierung mit Gelbwurzelöl, ebenso wie die mit Brokkoliextrakt, keine Wirkung hinsichtlich der Induktion indirekter antioxidativer Enzyme, was ebenfalls auf die kürzere Verabreichungsdauer zurückzuführen sein könnte.

Die Ergebnisse des in vitro-Versuches mit IPEC-J2-Zellen deuten allerdings auf eine GPx2 und NQO1 induzierende Wirkung von ätherischem Gelbwurzelöl hin (**V4:**Abbildung 3**,** Tabelle 5) und stimmen daher mit der aktuellen Literatur überein, die Hinweise auf eine indirekte antioxidative Wirkung von Gelbwurzelextrakten liefert (Lee et al. 2010, TEGO 2011). Dabei ist allerdings unklar, ob die Nrf2-abhängige Induktion von antioxidativen und Xenobiotika-metabolisierenden (Phase-II) Enzymen möglicherweise durch Curcuminreste oder durch die Leitsubstanz des ätherischen Gelbwurzelöls, dem ar-Turmeron, verursacht wird. Viele in vitro- und in vivo-Studien bescheinigen dem Farbstoff Curcumin eine indirekte antioxidative Wirkung (McNally et al. 2007, Nishinaka et al. 2007, Garg et al. 2008, Farombi et al. 2008). Das ar-Turmeron weist aufgrund seiner chemischen Struktur eine hohe Reaktivität gegenüber freien SH-Gruppen in Proteinen auf und kann ebenfalls den Keap1/Nrf2-ARE-Signalweg modulieren und Nrf2-abhängige Zielgene induzieren (TEGO 2011, Abbildung 5, Abbildung 7).

Die Curcuminoide der Gelbwurzel und isolierte Isothiocyanate wie das Sulforaphan (SFN) des Brokkolis sind in vitro und in Nagermodellen bezüglich ihrer indirekten antioxidativen Effekte bereits sehr gut charakterisierte phytogene Substanzen. Als indirekte antioxidative Wirkung werden alle durch eine Substanz ausgelösten sekundären Reaktionen bezeichnet, die zu einer Verminderung von oxidativem Stress beziehungsweise zu einer Erhöhung des antioxidativen Potentials im Organismus führen (Fahey und Talalay 1999). So besitzen aerobe Organismen die Möglichkeit, die Genexpression einer Vielzahl zellschützender Enzyme, z.B. die von antioxidativen und Xenobiotika-metabolisierenden (Phase-II) Enzymen, zu induzieren (Xu et al. 2005). In der Literatur finden sich neben endogenen Nrf2-Induktoren (H_2O_2, NO)

viele Daten zu verschiedensten exogenen Induktoren wie pflanzlichen Substanzen. Als elektrophile Reagenzien (Abbildung 5, Abbildung 6) modifizieren sie den Keap1-Nrf2-Komplex. In der Folge kommt es zu einer Induktion der Genexpression zytoprotektiver Enzyme und zu einer Verminderung von oxidativem Stress innerhalb einer Zelle (Abbildung 7).

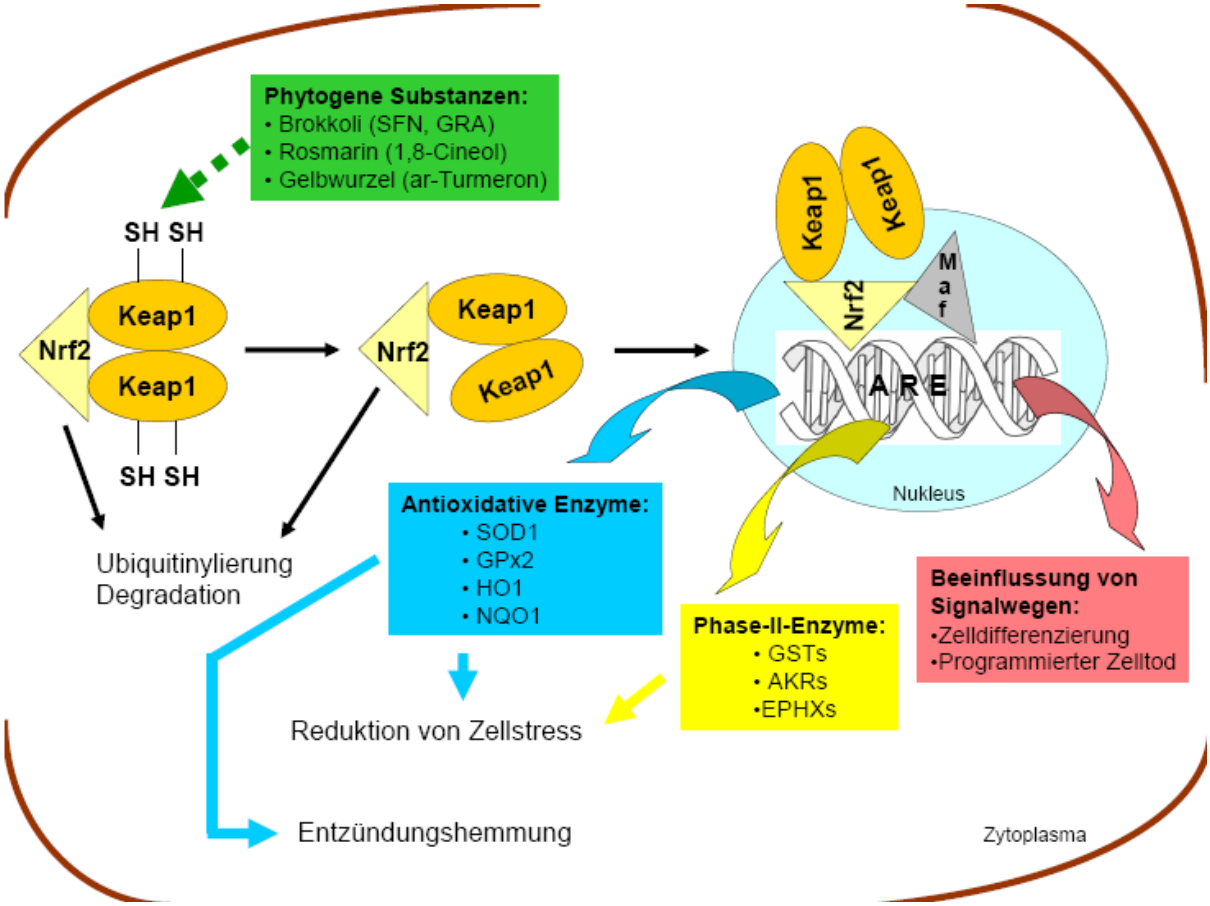

Abbildung 7: Keap1/Nrf2-ARE-Signaltransduktion
Abk.: SFN, Sulforaphan; GRA, Glucoraphanin; SOD1, Superoxididsmutase 1; GPx2, Glutathionperoxidase 2; HO1, Hämoxygenase 1; NQO1, NADPH-abhängige Chinonoxidoreduktase 1; GSTs, Glutathion-S-Transferasen; AKRs, Aldo-Keto-Reduktasen; EPHXs, Epoxidhydrolasen

Innerhalb der getesteten Labiatae-Öle (Oregano, Thymian und Rosmarin) besaß in beiden Versuchen mit Nutztieren das ätherische Öl aus Rosmarin die stärkste indirekte antioxidative Wirkung (**V1:Tab. 7A**; **V2: Tab. 9A**, Tabelle 7). Beide Versuche zeigten darüber hinaus deutlich, dass das ätherische Öl des Rosmarins eine starke Induktion Nrf2-regulierter Zielgene (AFAR, EPHX1, HO1, GPx1/2, SOD1) bewirkt und damit eine strake indirekte antioxidative Wirkung im Darmgewebe von Nutztieren entfalten kann (**V1:Tab. 7A**, **V2:Tab. 9A,** Tabelle 7). Die in beiden Studien ermittelte TEAC-steigernde Wirkung von ätherischem Rosmarinöl (**V1:Abb. 2**; **V2:Abb. 2**) beruht dabei vermutlich sowohl beim Broiler als auch beim Absetzferkel vor allem auf einer Nrf2-vermittelten Induktion von antioxidativen und Xenobiotika-metabolisierenden (Phase-II) Enzymen (**V1:Tab. 7A**; **V2:Tab. 9A**). Die hohe

indirekte antioxidative Wirkung von Rosmarinöl basiert vermutlich auf dem Vorhandensein des 1,8-Cineols, der Leitsubstanz des verwendeten Rosmarinöls. Die Epoxygruppe von 1,8-Cineol kann als Elektrophil wirken und interagiert mit freien Sulfhydrilgruppen, wie sie im Nrf2-Inhibitorprotein Keap1 vorliegen. In der Folge kann es zur Induktion Nrf2-regulierter Enzyme kommen (Abbildung 5, Abbildung 7). Des Weiteren konnte diese indirekte antioxidative Wirkung auch in der ersten Phase des **Versuches 3** im Nagetiermodell gezeigt werden (**V3:Tab. 7, 10**).

Während das ätherische Öl aus Thymian noch zu einer moderaten Induktion Nrf2-abhängiger Zielgene beim Absetzferkel führte (**V2:Tab. 9A,** Tabelle 7), konnte für das ätherische Oreganoöl kaum eine indirekte antioxidative Wirkung, weder beim Broiler noch beim Absetzferkel, nachgewiesen werden (**V1:Tab. 7A**; V**2:Tab. 9A**, Tabelle 7). Daraus lässt sich schlussfolgern, dass die TEAC-steigernde Wirkung von Oreganoöl vorrangig durch direkte antioxidative Mechanismen (siehe 4.2.1), vermutlich durch den hohen Gehalt an Carvacrol hervorgerufen, vermittelt wird, während Thymianöl seine Wirkung eher über indirekte antioxidative Signalwege erzielt.

Zusätzlich deutete sich für Thymianöl zunächst eine speziesspezifische Wirkung an, da im Broilerversuch die direkten antioxidativen Eigenschaften des ätherischen Öls überwogen (**V1:Abb. 2**, **Tab. 7A**) und beim Absetzferkel eher indirekte antioxidative Mechanismen beeinflusst wurden (**V2: Tab. 9A**). Da in beiden Studien allerdings unterschiedliche Konzentrationen an phytogenen Futterzusätzen verabreicht wurden (Broilerversuch: 150 mg ätherisches Öl/kg Diät; Ferkelversuch: 150 mg Thymol/kg Diät ≈ 373 mg ätherisches Öl/kg Diät), resultieren die unterschiedlichen Ergebnisse der beiden Versuche mit hoher Wahrscheinlichkeit daher. Möglicherweise sind auch andere Komponenten des Thymianöls, wie Borneol oder Luteolin, für die in **Versuch 2** beobachtete indirekte antioxidative Wirkung ursächlich (Horváthová et al. 2012, Sun et al. 2012, Hur et al. 2013). Zudem können aufgrund von anatomischen Unterschieden, insbesondere des Gastrointestinaltraktes, speziesspezifische Wirkungen von Thymianöl nicht gänzlich ausgeschlossen werden.

Die Verabreichung unterschiedlicher Konzentrationen an ätherischem Öl in **Versuch 1** und **2** trifft letztlich für alle verwendeten phytogenen Substanzen zu, scheint aber nur beim Thymian- und beim Gelbwurzelöl eine größere Rolle im Hinblick auf das unterschiedliche Vorherrschen direkter und indirekter antioxidativer Wirkungen zu spielen.

Die übrigen in **Versuch 4** (**Tab. 5**) getesteten zwei Polyphenol-reichen Apfelextrakte bewirkten in IPEC-J2-Zellen eher eine Abnahme der mRNA-Konzentration der antioxidativ wirksamen Enzyme GPx2 und NQO1, obwohl die aktuelle Literatur eine gegenteilige

Wirkung erwarten lässt. So induzieren Polyphenole, wie Quercetin und Resveratrol, die Expression von NQO1, GPx und anderen Nrf2-Zielgenen (Tanigawa et al. 2007, Kluth et al. 2007). Zudem konnten Soyalan et al. (2011) zeigen, dass Polyphenol-reiche Apfelsäfte im Gegensatz zu Polyphenol-freien Apfelextrakten zu einer gesteigerten Expression von Nrf2 und Nrf2-abhängiger Zielgene (GPx2) im Kolon von Ratten führten.

4.2.3 *Antimikrobielle Wirkung von Brokkoliextrakt und ätherischen Ölen*

In **Versuch 2** der vorliegenden Arbeit konnte für keinen der verabreichten phytogenen Futterzusätze eine signifikante direkte antimikrobielle Wirkung in vivo nachgewiesen werden (**V2:Tab. 6-8**). Dennoch zeigten sich tendenzielle substanzspezifische Effekte bei der bakteriologischen Untersuchung von Jejunum- und Kolonmukosa sowie von faecalen Proben (**V2:Tab. 6-8**). Während die ätherischen Öle des Oregano, des Thymians und der Gelbwurzel in den Jejunumproben zu einer Verminderung der *Escherichia coli*-Zahlen führten, hatte die Supplementation von Brokkoliextrakt und Rosmarinöl beim Absetzferkel eher gegenteilige Effekte (**V2:Tab. 6**). Im Gegensatz zu den Jejunumproben veränderten das ätherischen Öle des Rosmarins und des Thymians die bakterielle Zusammensetzung der Fäzesproben tendenziell positiv, indem sie zu einer reduzierten finalen Anzahl an *Escherichia coli* und einem verbesserten Verhältnis von Laktobazillen zu *Escherichia coli*-Bakterien beitragen (**V2:Tab. 8**).

Somit reihen sich die Ergebnisse der vorliegenden Arbeit in die bis dato sehr kontroverse Datenlage hinsichtlich der antimikrobiellen Wirkung phytogener Futterzusätze ein und lassen gleichzeitig vermuten, dass die positiven Effekte pflanzlicher Verbindungen auf die Darmgesundheit weniger über eine direkte bakterizide Wirkung gegen pathogene Mikroorganismen erzielt werden, als vielmehr durch die Förderung der Eubiose. Hierunter kann eine Erhöhung des Anteils an Laktobazillen im Darm verstanden werden.

In einer Studie mit Absetzferkeln konnte gezeigt werden, dass die Fütterung von 200 mg/kg eines Pflanzenextrakts aus 5% Carvacrol (Oregano), 3% Zimtaldehyd (Zimtbaum) und 2% Capsicum Oleoresin (spanischer Pfeffer) durch eine signifikante Steigerung der Laktobazillen-Anzahl im distalen Jejunum ein verbessertes Laktobazillen/*Escherichia coli*-Verhältnis bewirkt (Manzanilla et al. 2009). Auch Kroismayr et al. (2008a) konnten bei Absetzferkeln eine Reduktion der anaeroben und aeroben Mikroorganismen durch die Verabreichung eines ätherischen Ölgemisches aus Oregano, Anis und Zitrusfruchtschalen feststellen. Des Weiteren konnten Maenner et al. (2011) zeigen, dass ätherische Ölgemische auf Mentholbasis zwar die Gesamtkeimzahl im Kolon von Absetzferkeln nicht verändern, allerdings zu einer signifikanten Erhöhung der Laktobazillen-Anzahl beitragen.

Dem gegenüber stehen in vivo-Studien, in denen beispielsweise für Thymian in einer Dosis von 10 g/kg keine bakterizide Wirkung gegen hämolysierende *Escherichia coli* nachgewiesen werden konnte (Jugl-Chizzola et al. 2005, Hagmüller et al. 2006). Auch die Untersuchungen von Muhl und Liebert (2007) zeigten keine signifikanten Veränderungen in der Zusammensetzung der faecalen Mikroflora von Absetzferkeln durch die Verabreichung von 1 g/kg eines pflanzlichen Zusatzes, bestehend aus 53% Inulin, 8% ätherisches Ölgemisch (Carvacrol, Thymol) und 3% Tannine.

Auch für Broiler ist die Datenlage zu den antimikrobiellen und zu den die Eubiose förderden Eigenschaften phytogener Futterzusätze derzeit gegensätzlich. Einerseits konnten Rahimi et al. (2011) zeigen, dass die Supplementation von 1 g/kg Thymian im Darm von Ross-308 Broilern eine signifikante Reduktion an *Escherichia coli* und gleichzeitig eine gesteigerte Laktobazillen-Zahl bewirkt. Ähnliches wurde auch für eine sehr geringe Thymolkonzentrationen (15 mg/kg), die ebenso eine deutliche Reduktion von *Escherichia coli* und Clostridien bewirkte (Tiihonen et al. 2010), gezeigt. Andererseits konnten Westendarp et al. (2006) keine Beeinflussung der fäkalen Mikroflora bei Masthähnchen durch Carvacrol (52,4 mg/kg) nachweisen.

Hingegen existiert eine Vielzahl von in vitro-Studien (Hammer et al. 1999, Olasupo et al. 2003, Burt et al. 2005, Si et al. 2006), die eine eindeutige antimikrobielle Wirkung phytogener Substanzen belegt. So wurde beispielsweise für Oreganoöl eine minimale Hemmkonzentration (MHK) von ≥ 1% (v/v) gegen Geflügel-pathogene *Escherichia coli* (*O1*) und eine MHK ≥ 2% (v/v) gegen enterotoxische *Escherichia coli* (ETEC) des Schweins ermittelt (Penalver et al. 2005, Mathlouthi et al. 2011). Aber auch für Thymian- und Rosmarinöl lassen sich in vitro bakterizide Wirkungen unter anderem gegen enterohämorrhagische *Escherichia coli* (EHEC) O157:H7 und *Salmonella*-Spezies feststellen (Friedman et al. 2002, Mathlouthi et al. 2011). Einen ebenfalls sehr starken antimikrobiellen Effekt wie die Labiatae-Öle besitzt das Gelbwurzelöl, für das in vitro eine MHK im Bereich von 0,005-0,02% gegen verschiedenste Erreger (*Escherichia coli*, *Bacillus cereus*, *Staphylococcus aureus*) ermittelt werden konnte (Norajit et al. 2007). Zudem belegten Aires et al. (2009) in vitro auch für das Sulforaphan, das Haupt-Isothiocyanat des Brokkoli, eine eindeutige bakterizide Wirkung gegen enterohaemorrhagische *Escherichia coli* (68% Wachstumsinhibierung) und gegen *Salmonella* Typhimurium (50% Wachstumsinhibierung).

Desweiteren sind im Zusammenhang mit der antimikrobiellen Wirkung beziehungsweise mit der Stabilisierung der mikrobiellen Eubiose im Darm indirekte Effekte phytogener Futterzusätze wie gesteigerte Nährstoffverdaulichkeit und veränderte Passagerate zu nennen.

Beispielsweise führten Thymian- und Rosmarinextrakte zu einer verbesserten Nährstoffverdaulichkeit bei Broilern, die scheinbar auf verdauungsfördernden und antimikrobiellen Eigenschaften pflanzlicher Verbindungen beruhte (Hernández et al. 2004). Der phytogene Futterzusatz Biomin®P.E.P 1000 (Oregano, Anis, Zitrusschale) ermöglichte eine gesteigerte Nährstoffverwertung bei Absetzferkeln, wofür die Autoren ein verringertes Mikrobenwachstum als ursächlich ansahen (Zitterl-Eglseer et al. 2008). Zudem zeigten Manzanilla et al. (2004), dass die Gabe einer Mischung aus Carvacrol (Oregano), Zimtaldehyd (Zimtbaum) und Capsicum Oleoresin (mexikanischer Pfeffer) bei Absetzferkeln eine verlangsamte Magenentleerung beziehungsweise eine verminderte Passagerate bewirkte.
Insgesamt zeigten die vorliegenden Ergebnisse aus **Versuch 2** und anderen in vivo-Studien, dass die Übertragbarkeit der in vitro-Experimente in den in vivo-Bereich gerade in Bezug auf die antimikrobielle Wirkung phytogener Futterzusätze nicht beziehungsweise nur unzureichend gegeben ist. In vivo müssen meist sehr hohe Konzentrationen der phytogenen Substanzen verabreicht werden, um den in vitro-Bedingungen gerecht zu werden. Dies führt häufig zu veränderten Geschmackseigenschaften des Futters, worauf die Tiere meist mit einer verringerten Futteraufnahme reagieren (Wenk und Messikommer 2002, Wenk 2005).
Daher sind derzeit nur wenige in vivo-Studien zu dieser Thematik verfügbar, von denen die meisten allerdings keine antimikrobielle Wirkung pflanzlicher Verbindungen, ähnlich wie in dem vorliegenden **Versuch 2**, feststellen konnten. Des Weiteren variierten unter anderem die jeweils verabreichte Dosis und die Zusammensetzung der phytogenen Verbindungen, die Zeitpunkte der Probenahme sowie die untersuchten Darmabschnitte, was einen Vergleich der bisher durchgeführten Studien erschwert. Dennoch deuteten die vorliegenden Ergebnisse aus **Versuch 2** an, dass besonders die ätherischen Öle der Labiatae-Pflanzen (Oregano, Thymian, Rosmarin) einen günstigen Einfluss auf die intestinale Mikroflora ausüben könnten und so möglicherweise die Darmgesundheit von Nutztieren fördern.

4.2.4 *Einfluss von Brokkoliextrakt und ätherischen Ölen auf verschiedene Entzündungsparameter*

Die Ergebnisse der **Versuche 3** und **4** wiesen auf eine anti-inflammatorische Wirkung der verwendeten phytogenen Verbindungen hin. Der positive Einfluss auf die untersuchten Entzündungsparameter zeigte sich in vivo (**V3:Tab. 5, 6, 8, 9**) und in vitro (**V4:**Abbildungen 1-3, Tabelle 5) sowohl unter physiologischen Bedingungen als auch unter Entzündungs- bzw. Infektionsstimulus.
So wurden bei gesunden Ratten zunächst nur numerische Veränderungen hinsichtlich der Verminderung der NFκB und Cox2 mRNA-Expression durch die Verabreichung von

phytogenen Futterzusätzen, im Besonderen durch Brokkoliextrakt und Thymianöl, beobachtet (**V3:Tab. 5, 6, 8, 9**). Im Zellkulturversuch mit nicht infizierten IPEC-J2-Zellen bestätigte sich dieser Sachverhalt in Form einer signifikanten Reduktion der NFκB mRNA-Konzentration um rund 30% bei allen getesteten phytogenen Substanzen (**V4:**Abbildung 1). Ebenso konnte eine grundsätzliche Verminderung der Cox2 mRNA-Expression, besonders durch die Inkubation mit den Apfelextrakten um rund 36%, festgestellt werden (**V4:**Abbildung 2). Des Weiteren deuteten die signifikante Verminderung der VCAM-1 mRNA-Expression sowie die geseigerte Konzentration des anti-inflammatorisch wirksamen IL-10 darauf hin (**V3:Tab. 5, 9**), dass die getesteten phytogenen Futterzusätze bereits unter physiologischen Bedingungen anti-inflammatorische Effekte entfalten und damit potenzielle präventive Eigenschaften im Hinblick auf die Darmgesundheit von Nutztieren ausüben könnten.

Neben NFκB, dem Hauptregulator inflammatorischer Prozesse auf molekularer Ebene, spielt die von ihm abhängige Cyclooxygenase 2 (Cox2), besonders bei der Entstehung einer Entzündung sowie bei der Auslösung von typischen klinischen Entzündungssymptomen, wie Fieber und Schmerz, eine wichtige Rolle (Simmons et al. 2004). Die induzierbare Cox2 katalysiert dabei unter anderem in verschiedenen Geweben und Zelltypen die Biosynthese von Prostaglandin E2 (PGE2), ein pro-inflammatorisches lokal wirkendes Gewebehormon, aus Arachidonsäure (Vane 1998). Zusammen mit pro-inflammatorischen Zytokinen (IL-1β, TNFα, IL-6) scheinen solche Lipidmediatoren, wie PGE2, das Entzündungsgeschehen zu stimulieren (Simmons et al. 2004).

In der Literatur finden sich bislang keine vergleichbaren in vivo-Untersuchungen zur anti-inflammatorischen Wirksamkeit phytogener Verbindungen unter physiologischen, d.h. nicht stimulierten, Bedingungen, die ähnlich den Ergebnissen der vorliegenden Arbeit auf eine günstige Beeinflussung von Entzündungsparametern (NFκB, Cox2) durch phytogene Futterzusätze hindeuten.

Anhand der Ergebnisse dieser Arbeit konnte unter Belastungsbedingungen, ebenso wie unter physiologischen Maßgaben, ein grundsätzlich positiver Einfluss phytogener Futteradditive auf die untersuchten Entzündungsparameter festgestellt werden (**V3:Tab. 5, 6, 8, 9;** V**4:**Abbildungen 1-3**,** Tabelle 5). In **Versuch 3** führte die durch Natriumdextransulfat (DSS) induzierte Kolitis, wie erhofft, zu einer Steigerung der mRNA-Expression von Entzündungsparametern im Vergleich zu gesunden Ratten (Abbildung 8). Zudem konnte gezeigt werden, dass dieser Effekt in den experimentellen Gruppen, die zusätzlich zum DSS einen phytogenen Futterzusatz erhielten, weniger stark ausgeprägt war als in der DSS-Gruppe (**V3:Tab. 5, 8**). Die Cox2 und die VCAM-1 mRNA-Expression wurden sehr deutlich durch

alle getesteten phytogenen Verbindungen vermindert und im Gegensatz die des anti-inflammatorisch wirkenden IL-10 erhöht (**V3:Tab. 5, 8**). Insgesamt betrachtet, deuteten diese Ergebnisse auf eine potenzielle anti-inflammatorische Wirkung phytogener Futterzusätze in vivo unter Belastungsbedingungen hin.

Die in der vorliegenden Arbeit erhobenen Daten belegten und unterstützten dabei Hinweise und Ergebnisse aus der aktuellen Literatur. So konnten Bukovská et al. (2007) zeigen, dass die gleichzeitige Verfütterung von ätherischen Ölen aus Oregano und Thymian bei Mäusen mit einer 2,4,6-Trinitrobenzosulfonsäure (TNBS)-induzierten Kolitis zu einer verminderten mRNA-Konzentration pro-inflammatorischer Mediatoren, wie von IL-1β, IL-6 und TNFα, führte. Auch das Borneol, ein Monoterpen des Rosmarinöls, entfaltete bei Mäusen mit TNBS-induzierter Kolitis eine anti-inflammatorische Wirkung in Form einer Reduktion von pro-inflammatorischen Zytokinen (IL-1β, IL-6) (Juhás et al. 2008b). Die Autoren wiesen auch für die ätherischen Öle des Thymians und des Rosmarins eine Hemmung der IL-1β und IL-6 Expression im Kolon von Mäusen mit TNBS-induzierter Kolitis nach, wobei diese Effekte zum Großteil numerischer Natur waren (Juhás et al. 2008a, Juhás et al. 2009). Des Weiteren konnte im Kolon von Ratten mit TNBS-induzierter Kolitis gezeigt werden, dass die Verabreichung von Pflanzenextrakten aus Gingko oder Flieder die Protein- und mRNA-Expression von NFκB, TNFα und IL-6 verminderte (Zhou et al. 2006, Liu und Wang 2011). In einer weiteren in vivo Studie wiesen Márquez et al. (2010) bei Ratten mit DSS-induzierter Kolitis eine anti-inflammatorische Wirkung für einen Mangoextrakt nach, die sich in einer verminderten Cox2 und TNFα mRNA-Konzentration im Kolon und in gesenkten IL-6- und TNFα-Serumspiegeln äußerte. Ebenso verringerte auch das Polyphenol Resveratrol systemische Entzündungsmarker wie IL-1β und die Cox2 mRNA-Expression im Rattenkolon während einer DSS-induzierten Kolitis (Larrosa et al. 2009). Überdies führten Resveratrol und auch das Curcumin der Gelbwurzel im Ileum von Mäusen mit einer durch *Toxoplasma gondii* ausgelösten Ileitis zu einer gesteigerten IL-10 Expression und verminderten gleichzeitig die Produktion pro-inflammatorischer Zytokine (TNFα, IL-6, MCP-1) (Bereswill et al. 2010).

Auch die in **Versuch 4** ermittelten in vitro Ergebnisse für IPEC-J2-Zellen unterstützen die in **Versuch 3** erhobenen Daten und Schlussfolgerungen im Hinblick auf eine positive Beeinflussung von Entzündungsparametern durch pflanzliche Substanzen unter Belastungsbedingungen. So konnte im durchgeführten in vitro Infektionsmodell gezeigt werden, dass alle getesteten Substanzen zu einer Verminderung der NFκB mRNA-Expression um 10% bis 41% führten beziehungsweise der durch die Infektion mit *Escherichia coli*

bedingten NFκB Aktivierung in den IPEC-J2-Zellen entgegenwirkten (**V4:**Abbildung 1). Auch die IL-8 mRNA-Konzentrationen wurden insgesamt betrachtet durch die getesteten phytogenen Verbindungen gesenkt, wobei das ätherische Öl der Gelbwurzel mit einer Reduktion um 52% die stärksten IL-8 vermindernden Effekte aufwies (**V4:**Tabelle 5). Einen weiteren Beweis für das anti-inflammatorische Potenzial der untersuchten pflanzlichen Substanzen lieferte die reduzierte Cox2 mRNA-Expression (9% bis 46%), während der Apfelextrakt 2, das Gelbwurzelöl und das Glucoraphanin am effektivsten wirkten (**V4:**Abbildung 2).

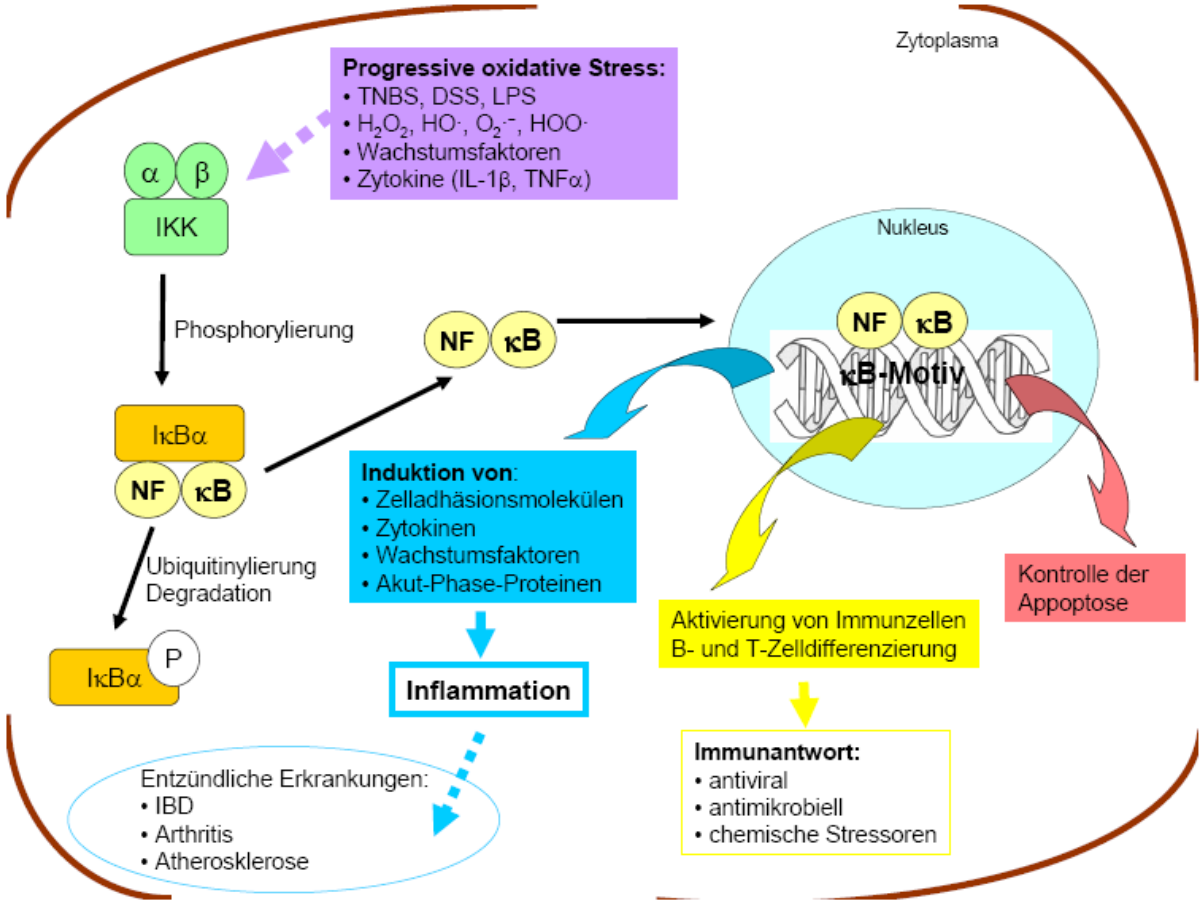

Abbildung 8: NFκB-Signaltransduktion
Abk.: IKK, Inhibitor kappa B-Kinase; IκB, Inhibitorprotein kappa B; TNBS, 2,4,6-Trinitrobenzosulfonsäure; DSS, Natriumdextransulfat; LPS, Lipopolysaccharid, IL-1β, Interleukin 1β; TNFα, Tumornekrosefaktor α; IBD, Inflammatory Bowel Disease.

Diese ermittelten in vitro-Daten stehen dabei nicht nur im Einklang mit den in vivo-Ergebnissen aus **Versuch 3** (**V3:Tab. 5, 6, 8, 9**), sondern gehen auch mit bisherigen in vitro Studien zur anti- inflammatorischen Wirkung von phytogenen Substanzen unter Belastungsbedingungen einher.

In diesem Zusammenhang konnte gezeigt werden, dass Curcumin die Strahlungs-induzierte NFκB-Aktivierung sowie die Induktion seiner Zielgene, wie Cox2 und TNFα, in humanen Kolonkarzinomzellen hemmte (Sandur et al. 2009, Hanai und Sugimoto 2009). Neben dem

Curcumin scheint auch das ar-Turmeron der Gelbwurzel ein anti-inflammatorisches Potential aufzuweisen, da es in vitro die Cox2 und die induzierbare Stickstoffmonooxidsynthase inhibierte (Lee et al. 2002). Lee et al. (2010) beobachteten hingegen in HD11-Makrophagen eine Erhöhung der mRNA-Expression von verschiedenen pro-inflammatorischen Zytokinen (IL-1β, IL-6, IL-12, IL-18) durch einen Gelbwurzelextrakt und werteten dies als Verbesserung der angeborenen Immunität. In weiteren in vitro-Studien wurden auch für die phytogenen Verbindungen, Allyl-Isothiocyanate (AITC) und Sulforaphan, die vor allem in Brokkoli und anderen Brassicaceae-Vertretern enthalten sind, anti-inflammatorische Eigenschaften ermittelt. Dabei konnte gezeigt werden, dass AITC und Sulforaphan die NFκB-Aktivierung, die Induktion pro-inflammatorischer Zytokine (IL-1β, IL-6) sowie die VCAM1 und Cox2 Proteinexpression hemmten (Shibata et al. 2010, Wagner et al. 2011, Kim et al. 2012, Sharma et al. 2011).

Ähnliche in vitro-Untersuchungen existieren auch für die ätherischen Öle der Labiatae-Pflanzen sowie deren Inhaltsstoffe. So stellten Ocaña-Fuentes et al. (2010) fest, dass der von ihnen verwendete Oreganoextrakt mit hohem Carvacrol- und Thymolgehalt sowohl die Synthese von TNFα, IL-1β und IL-6 verminderte als auch die Produktion des anti-inflammatorisch wirkenden Zytokins IL-10 steigerte. Auch Rosmain-, Salbei- und Thymianöl wiesen ein anti-inflammatorisches Potenzial auf, indem sie die IL-8-Freisetzung und die Cox2 Expression hemmten (Hotta et al. 2010, Chohan et al. 2012). In diesem Zusammenhang scheinen spezifische Inhaltsstoffe der Öle, wie das 1,8-Cineol des Rosmarins sowie das Carvacrol des Oregano und des Thymian, ihre anti-inflammatorische Wirkung über eine Inhibierung der Cox2- und Zytokinexpression (TNFα, IL-1β, IL-6, IL-8) zu vermitteln (Juergens et al. 2004, Landa et al. 2009, Hotta et al. 2010).

4.2.5 *Zusammenhang zwischen antioxidativen und anti-inflammatorischen Effekten phytogener Futterzusätze (Nrf2-NFκB-Interaktion)*

Aktuelle in vitro- und in vivo-Studien weisen darauf hin, dass ein enger Zusammenhang zwischen einem hohen antioxidativen Status eines Gewebes und dem verminderten Auftreten von Entzündungsreaktionen besteht. Die durch den Transkriptionsfaktor NFκB vermittelte Inflammation, die in der landwirtschaftlichen Praxis bei Nutztieren beispielsweise durch pathogene Mikroorganismen (*Escherichia coli, Salmonella spp.*) hervorgerufen werden kann, führt zu einer vermehrten Freisetzung von reaktiven Sauerstoffspezies („*oxidative burst*“) in Monozyten, Makrophagen und verschiedenen anderen Immunzellen (Miguel 2010, Abbildung 8). Der Organismus ist dabei bestrebt, dem erhöhten oxidativen Stress entgegenzuwirken und

aktiviert redox-sensitive Signalkaskaden wie den Keap1/Nrf2-ARE-Weg (Abbildung 7). Derzeit ist allerdings unklar, ob diese Reaktionen durch eine direkte Interaktion der beiden Transkriptionsfaktoren Nrf2 und NFκB vermittelt werden, oder ob die jeweilig regulierten Zielgene eine Rolle spielen.

So konnten Khor et al. (2006) in Nrf2-Kockout-Mäusen zeigen, dass die hier fehlende Nrf2-Aktivierung unter DSS-Gabe zu einer Verstärkung der inflammatorischen Reaktion im Vergleich zu Wildtyp-Tieren mit aktivem Nrf2 führte. Entsprechend wurde in den Wildtyp-Mäusen eine gesteigerte Expression Nrf2-abhängiger Zielgene (NQO1, HO-1, GST) festgestellt, während in den Nrf2-Knockout-Tieren eine Steigerung von Entzündungsmarkern (Cox2, TNFα, IL-1β, IL-6) beobachtet wurde (Khor et al. 2006). Des Weiteren konnte bei Mäusen mit TNBS-induzierter Kolitis gezeigt werden, dass die Induktion der HO-1 zu einer Hemmung des NFκB-Signalweges führte (Jun et al. 2006).

Die in der Literatur angedeutete und in den **Versuchen 1-4** belegte indirekte antioxidative Wirkung phytogener Verbindungen via Nrf2 scheint also auch in die Ausprägung von NFκB-vermittelten Entzündungsreaktionen involviert zu sein. So gelten das Resveratrol der Weintraube, das Curcumin der Gelbwurzel und das Sulforaphan des Brokkolis als potente Nrf2-Aktivatoren und werden gleichzeitig als Inhibitoren der LPS- und DSS-induzierten NFκB-Aktivierung diskutiert (Larossa et al. 2009, Brandenburg et al. 2010, Nishida et al. 2010, Blum et al. 2012). Eine inverse Korrelation Nrf2-regulierter Xenobiotika-metabolisierender (Phase-II) und antioxidativer Enzyme sowie pro-inflammatorischer Mediatoren (IL-1β, TNFα, IL-6, NFκB, Cox2) konnte auch für das Carnosol und das 1,8-Cineol des Rosmarins gezeigt werden (Lian et al. 2010, Bastos et al. 2010).

Die Ergebnisse der vorliegenden Arbeit, insbesondere die aus **Versuch 3**, lassen allerdings keinen direkten Zusammenhang zwischen Nrf2 und NFκB zu, weder unter physiologischen noch unter Belastungsbedingungen. Vielmehr deuteten die Daten aus **Versuch 3** darauf hin, dass eine negative Korrelation zwischen Nrf2 und TNFα, der wiederum einen der stärksten NFκB-Aktivatoren während einer beginnenden Entzündung darstellt (Zhou et al. 2006), besteht.

Im Rahmen der vorliegenden Arbeit konnte keine generelle Interaktion zwischen den beiden Transkriptionsfaktoren Nrf2 und NFκB nachgewiesen werden.

4.2.6 *Schlussfolgerungen und Ausblick*

Die Ergebnisse der vorliegenden Arbeit zum Einfluss phytogener Futterzusätze auf Xenobiotika-metabolisierende und antioxidative Enzyme sowie auf Entzündungsparameter

stellen einen hochaktuellen Forschungsschwerpunkt in der Nutztierernährung dar. Trotz der Vielzahl an bereits durchgeführten Studien, besonders im Hinblick auf potenzielle leistungs- und gesundheitsfördernde Eigenschaften phytogener Futterzusätze, besteht ein besonderes Interesse an der weiterführenden Aufklärung der zu Grunde liegenden Wirkungsmechanismen.

Gegenstand der vorliegenden Arbeit war es daher, zu untersuchen, ob und über welche Mechanismen phytogene Futterzusätze eine günstige Wirkung auf Leistungsparameter und die Tiergesundheit ausüben. Die kontroverse Datenlage bezüglich leistungssteigernder Effekte phytogener Additive und das Fehlen funktioneller und praxisbezogener in vivo-Studien zur Wirkungsweise phytogener Futterzusätze bildete die wesentliche Basis dieser Arbeit.

Zur weiterführenden Klärung der Thematik wurden 3 in vivo-Studien (**V1-3**) und eine in vitro-Untersuchung (**V4**) durchgeführt. Die hieraus gewonnenen Ergebnisse lieferten neue Erkenntnisse zur Wirkungsweise phytogener Futterzusätze im Nutztierbereich. So konnten beim Broiler und beim Absetzferkel wichtige Hypothesen aufgestellt werden, über welche Reaktionsmechanismen phytogene Verbindungen in vivo eine gesundheitsfördernde Wirkung entfalten können (**V1, 2**). Die in vivo und in vitro-Untersuchungen an Ratten und der porcinen Dünndarmzelllinie IPEC-J2 wiesen auf ein anti-inflammatorisches Potenzial der verwendeten phytogenen Substanzen hin und untersuchten einen möglichen Zusammenhang zwischen antioxidativen und pro-inflammatorischen Prozessen (**V3**, **4**). Anhand der vorliegenden Daten und unter Einbeziehung der aktuellen Literatur kann Folgendes geschlussfolgert werden:

(1) Die getesteten phytogenen Futterzusätze aus Brokkoli, Gelbwurzel, Oregano, Thymian und Rosmarin sind keine Leistungsförderer im klassischen Sinn.

Dennoch liessen sich insbesondere zu Beginn der Fütterung der phytogenen Substanzen sowohl beim Broiler als auch beim Absetzferkel zunächst positive Effekte auf die Leistungsparameter feststellen, die sich allerdings bei längerer Gabe der Futterzusätze eher verloren oder bei Oregano- und Gelbwurzelöl gegenteilige Effekte zeigten (**V1, 2**). Daher ist zukünftig, gerade im Hinblick auf die Produktionskosten, eher eine kürzere und eventuell auch eine alternierende Verabreichungsdauer phytogener Substanzen in Betracht zu ziehen.

(2) Die getesteten phytogenen Futterzusätze aus Brokkoli, Gelbwurzel, Oregano, Thymian und Rosmarin weisen auf Grund ihrer Hauptinhaltsstoffe große substanzspezifische Unterschiede hinsichtlich der Beeinflussung direkter und indirekter antioxidativer Schutzmechanismen auf.

Während das ätherische Öl des Oregano in vivo seine antioxidative Wirkung vorrangig über direkte Effekte (Stabilisierung von freien Radikalen, Abpufferung von oxidativem Stress) vermittelte und nahezu keine indirekten antioxidativen Eigenschaften in Form einer Aktivierung der Keap1/Nrf2-ARE-Signalkaskade besaß, wirkten der Brokkoliextrakt und das Rosmarinöl stärker über den indirekten antioxidativen Signalweg. Die ätherischen Öle des Thymians und der Gelbwurzel nahmen in vivo im Vergleich zu den übrigen getesteten phytogenen Substanzen eine Zwischenstellung ein und wiesen einerseits eine direkte antioxidative Wirkung auf, aktivierten andererseits aber auch die Keap1/Nrf2-ARE-Signalkaskade (**V1, 2**). Insgesamt betrachtet führte die Fütterung der getesteten phytogenen Futterzusätze zu einer Steigerung des antioxidativen Status der Gewebe, was besonders im Hinblick auf die Aufnahme von Fremdstoffen wie Futtermittelkontaminanten (Aflatoxin, Desoxynivalenol), Medikamenten (Antibiotika) und bezüglich der Abwehr und der Entgiftung von Bakterientoxinen (Enterotoxin, Shigatoxin), die gerade in den frühen Lebensphasen von Nutztieren ein zusätzliches Problem darstellen, wichtig sein könnte. Da diese Stoffe in erster Linie von den Nrf2-regulierten Xenobiotika-metabolisierenden Enzymen verstoffwechselt werden, stellt die Modulation des Xenobiotika-Metabolismus durch phytogene Verbindungen insbesondere im Darmtrakt von Nutztieren einen potenziell gesundheitsfördernden Mechanismus dar. Zusammen mit ihren direkten antioxidativen Eigenschaften könnten phytogene Futterzusätze einen positiven Einfluss auf die Tiergesundheit ausüben und somit einen wichtigen Produktionsfaktor günstig beeinflussen.

(3) Die in anderen Studien belegte antimikrobielle in vitro-Wirkung lässt sich für die getesteten phytogenen Futterzusätze aus Brokkoli, Gelbwurzel, Oregano, Thymian und Rosmarin unter den gewählten Bedingungen in vivo nicht übertragen.

Die in vivo-Ergebnisse bezüglich der antimikrobiellen Wirkung der getesten phytogenen Substanzen, insbesondere im Hinblick auf die ätherischen Öle der Labiatae-Pflanzen (Oregano, Thymian, Rosmarin) deuteten jedoch einen günstigen Einfluss auf die intestinale Mikroflora an (**V2**), sodass möglicherweise eine höhere oder eine längere verabreichte Dosis an phytogenen Futterzusätzen die Darmgesundheit von Nutztieren fördern könnte.

(4) Die getesteten phytogenen Futterzusätze und Einzelverbindungen aus Brokkoli, Gelbwurzel, Thymian, Rosmarin und Apfelextrakt besitzen offensichtlich sowohl unter physiologischen als auch unter stimulierten Versuchbedingungen (DSS-induzierte Kolitis, *Escherichia coli*-Infektion) eine anti-inflammatorische Wirkung.

Gerade diese in den **Versuchen 3** und **4** ermittelte anti-inflammatorische Wirkung der getesteten phytogenen Verbindungen könnte in der landwirtschaftlichen Praxis im Hinblick

auf die Tiergesundheit zukünftig eine wichtige Rolle spielen. Die getesteten pflanzlichen Verbindungen stellen damit möglicherweise eine potenzielle Alternative zum präventiven Antibiotikaeinsatz hinsichtlich der Reduktion von Durchfallerkrankungen, welche in frühen Phasen der Nutztieraufzucht besonders häufig auftreten (Kükenruhr bei Geflügel, Absetzdurchfälle und Ödemkrankheit beim Schwein) dar und könnten so Leistungseinbußen und Tod der Nutztiere entgegenwirken. Dazu müssen allerdings weitere in vivo-Untersuchungen mit Nutztieren unter Belastungsbedingungen (Infektion mit pathogenen Erregern oder deren Lipopolysacchariden) durchgeführt werden.

(5) Es besteht keine direkte Interaktion zwischen den Transkriptionsfaktoren Nrf2 und NFκB.

Hinsichtlich dieses Sachverhalts ist jedoch eine überlappende beziehungsweise gegenseitige Regulation über die entsprechenden Zielgene nicht auszuschließen. Derzeit ist weiter unklar, welche Rolle der oxidative Status einer Zelle hinsichtlich der Aktivierung antioxidativer Nrf2-abhängiger beziehungsweise Hemmung prooxidativer NFκB-regulierter Reaktionswege spielt. Zur Aufklärung dieses Mechanismus sind weitere grundlegende Studien nötig.

Die in dieser Arbeit durchgeführten Untersuchungen weisen durch Auswahl der Zellkultur- (IPEC-J2) und Tiermodelle zur Prüfung der Zielstellung einen hohen Praxisbezug auf. Sowohl Broiler als auch Absetzferkel stellen als landwirtschaftliche Nutztiere geeignete und in der wissenschaftlichen Literatur etablierte Modeltiere dar und eigenen sich hervorragend als in vivo-Model zur Untersuchung leistungs- und gesundheitsfördernder Eigenschaften phytogener Futterzusätze. Zudem hat sich die wissenschaftlich anerkannte porcine Zellline IPEC-J2 in der Erforschung wichtiger intestinaler Prozesse wie der Nährstoffabsorption und der Inflammation bewährt und lässt gleichzeitig Rückschlüsse auf regulatorische Mechanismen beim Schwein zu. Als ebenso ideales und günstiges in vivo-Modeltier wird die Ratte in der wissenschaftlichen Forschung angesehen und eignet sich daher zusammen mit den IPEC-J2-Zellen sehr gut zur Untersuchung antioxidativer, anti-inflammatorischer und Xenobiotika-metabolisierender Stoffwechselwege unter physiologischen Bedingungen sowie unter einem Entzündungsstimulus.

Zum anderen unterstützen die vergleichende Untersuchung phytogener Futterzusätze und vor allem die Standardisierung der verabreichten Additive auf ihre jeweiligen Leitsubstanzen die Originalität der im Rahmen dieser Arbeit gewonnenen Ergebnisse.

Bisher wurden in den meisten Studien zur Wirkung phytogener Futterzusätze Mischungen verschiedener Kräuter, Extrakte und ätherischer Öle mit unterschiedlicher und zum Teil ungeklärter beziehungsweise nicht analysierter Zusammensetzung verwendet, wodurch kaum

Rückschlüsse auf die eigentlich wirksamen Einzelverbindungen möglich waren. Eine Standardisierung auf wichtige Hauptinhaltsstoffe ist daher zukünftig nicht nur für die Forschung, sondern insbesondere auch für die Anwendung in der landwirtschaftlichen Praxis von größter Bedeutung. Ebenso wird sich die Futtermittelindustrie in der Zukunft besonderen Herausforderungen bezüglich der Zusammensetzung der pflanzlichen Futterzusätze, insbesondere im Hinblick auf die Gewährleistung standardisierter Ausgangsmaterialien für die Produktion sowie die anschließende adäquate Verpackung und Lagerung phytogener Futteradditive, stellen müssen.

Denn aus der Literatur ist bekannt, dass sich gerade die Gehalte an charakteristischen Hauptinhaltsstoffen pflanzlicher Produkte in Abhängigkeit von der Herkunft, dem verwendeten Pflanzenteil, dem Erntezeitpunkt und der Verarbeitung (Trocknung, Extraktion) unterscheiden, wodurch die Wirksamkeit der daraus hergestellten Produkte ebenfalls enorm variiert.

Um in der Zukunft die Vergleichbarkeit der Ergebnisse und Studien zur Wirksamkeit phytogener Futterzusätze zu gewährleisten, ist es essenziell, dass ausschließlich analysierte und in der Zusammensetzung bekannte, am besten standardisierte phytogene Produkte zum Einsatz kommen. Zudem sollte auf eine genaue und praxisnahe Dosierung sowie auf die Dauer der Verabreichung der verwendeten phytogenen Futterzusätze geachtet werden.

Neben den originären Ergebnissen für den Nutztierbereich weisen die in dieser Arbeit erhobenen Daten auch eine Übertragbarkeit auf die Humanernährung auf beziehungsweise wirken sich möglicherweise positiv auf diese aus. Die antimikrobielle Wirkung phytogener Verbindungen findet derzeit bereits große Anwendung im Lebensmittelbereich im Hinblick auf die Haltbarkeit von tierischen Produkten, wie Wurst- und Fleischwaren. Der zukünftige Einsatz von pflanzlichen Substanzen könnte zusätzlich eine wichtige Rolle hinsichtlich Verminderung der Übertragung humanpathogener Erreger über Lebensmittel spielen. Weitaus bedeutender für den Humanbereich sind allerdings die in vivo ermittelten antioxidativen und anti-inflammatorischen Wirkungen phytogener Substanzen als mögliche Präventions- und Behandlungsstrategie für entzündliche Darmerkrankungen, wie Colitis ulcerosa und Morbus Crohn. Die in vitro und in vivo ermittelten Wirkungsmechanismen der getesteten phytogenen Verbindungen könnten zudem die Grundlage für Humanstudien hinsichtlich der Entwicklung geeigneter Medikamente auf Basis phytogener Wirkstoffe zur Behandlung chronisch entzündlicher Darmerkrankungen und anderer entzündlicher Erkrankungen bilden.

5 Zusammenfassung

Seit dem endgültigen Einsatzverbot von antibiotischen Leistungsförderern als Futterzusatz in der Tierernährung 2006 erweisen sich besonders die in frühen Phasen der Geflügel- und Schweineproduktion durch *Salmonella spp.* und pathogene *Escherichia coli* hervorgerufenen Durchfallerkrankungen (Coliruhr, Coliseptikämie, Salmonellosen) als problematisch. Um die Produktionskosten trotz Leistungseinbußen und steigenden Mortalitätsraten in der modernen Tierhaltung ohne antibiotische Leistungsförderer im Futter gering zu halten, wird die Suche und die Entwicklung nichtantibiotischer Alternativen, zu denen unter anderem die phytogenen Futterzusätze gezählt werden können, forciert.

Anhand von aktuellen Daten aus der Literatur scheinen phytogene Futterzusätze die Tiergesundheit über 5 verschiedene Wirkungsweisen positiv zu beeinflussen. Dazu zählen (1) eine Steigerung der Nutztierleistung, (2) direkte und (3) indirekte antioxidative Effekte, (4) antimikrobielle sowie (5) anti-inflammatorische Wirkungen pflanzlicher Verbindungen. Aktuell finden bereits eine große Zahl an phytogenen Verbindungen, besonders auf der Basis von Kräutern und ätherischen Ölen, als Futteradditiv in der Tierernährung Verwendung, allerdings ohne die zugrunde liegenden biochemischen und molekularbiologischen Mechanismen im Nutztierorganismus zu kennen.

Ziel der vorliegenden Arbeit war es daher, diese 5 diskutierten potenziellen Mechanismen unter möglichst praxisnahen Bedingungen zu untersuchen. In einer ersten Studie mit Broilern (**V1**) wurde der Effekt von Brokkoliextrakt (3000 mg Extrakt/kg Futter) und von ätherischen Ölen aus Gelbwurzel, Oregano, Thymian und Rosmarin (150 mg Öl/kg Futter) auf verschiedene Leistungsparameter untersucht. Aufgrund der in vitro beschriebenen direkten und indirekten antioxidativen Wirkung verschiedener Inhaltsstoffe aus Brokkoli (GRA, SFN) und den getesteten ätherischen Ölen (Gelbwurzel: ar-Turmeron; Oregano: Carvacrol; Thymian: Thymol; Rosmarin: 1,8-Cineol) selbst wurde die antioxidative Kapazität (TEAC, TBA-RS) der phytogenen Substanzen sowie deren Wirkung auf Nrf2-regulierte xenobiotische und antioxidative Enzyme in verschiedenen Geweben der Broiler ermittelt. Im Anschluss erfolgte in einer zweiten Studie mit Absetzferkeln (**V2**) eine Standardisierung der eingesetzten phytogenen Testsubstanzen auf ihre jeweiligen Hauptinhaltsstoffe (150 mg Hauptinhaltsstsoff/kg Futter), um die ermittelten Effekte besser auf einen bestimmten Inhaltsstoff des jeweiligen phytogenen Futterzusatzes zurückführen zu können. Zusammenfassend zeigten beide Versuche, dass die getesteten phytogenen Futterzusätze aus Brokkoli, Gelbwurzel, Oregano, Thymian und Rosmarin keine klassischen Leistungsförderer

sind, zumindest aber zu Beginn der Fütterung bei beiden untersuchten Spezies zu einer positiven Beeinflussung der Leistungsparameter führten (**V1**, **V2**).

Zudem konnte in der vorliegenden Arbeit erstmals eine substanzspezifische Beeinflussung direkter und indirekter antioxidativer Schutzmechanismen durch phytogene Additive in verschiedenen monogastrischen Nutztierspezies nachgewiesen werden (**V1**, **V2**). Dabei ließen sich für das ätherische Öl des Oregano vorrangig direkte antioxidative Effekte feststellen, während der Brokkoliextrakt und das ätherische Rosmarinöl in vivo ihre antioxidativen Eigenschaften eher über die Induktion von xenobiotischen und antioxidativen Enzymen wie AFAR, EPHX1, GST, SOD1, GPx1 ausüben (**V1**, **V2**). Des Weiteren konnten für die ätherischen Öle aus Thymian und Gelbwurzel sowohl direkte als auch indirekte Nrf2-vermittelte antioxidative Effekte in beiden Versuchen belegt werden. Die Ergebnisse dieser Versuche liefern wichtige und interessante Hinweise bezüglich molekularer Regulationsmechanismen und deuten einen potenziellen Zusammenhang der 5 diskutierten Wirkungswege phytogener Verbindungen an.

Die Ergebnisse des zweiten Versuchs mit Absetzferkeln (**V2**) konnten diesbezüglich die in der aktuellen Literatur beschriebenen antimikrobiellen Eigenschaften phytogener Futterzusätze nicht bestätigen, wobei sich eine marginale, positive Beeinflussung der intestinalen Mikroflora insbesondere durch die ätherischen Labiatae-Öle abzeichnete.

Ein weiteres Ziel der vorliegenden Arbeit war es, potentielle anti-inflammatorische Effekte der getesten phytogenen Futterzusätze und von Einzelverbindungen aus Brokkoli, Gelbwurzel, Thymian, Rosmarin und Apfel zu untersuchen. Die Durchführung eines in vivo-Versuchs mit Wistar-Ratten (**V3**) und eines in vitro-Versuchs mit IPEC-J2-Zellen (**V4**) sollten zur weiteren Aufklärung dieses Sachverhalts dienen. Zusammenfassend belegten beide Versuche sowohl unter physiologischen als auch unter stimulierten Versuchsbedingungen (DSS-induzierte Kolitis bei Ratten, *Escherichia coli*-Infektion der IPEC-J2-Zellen) eine anti-inflammatorische Wirkung der getesteten phytogenen Substanzen in Form einer Reduktion der NFκB und Cox2 mRNA-Konzentration (**V3**, **V4**). Weiterhin konnte keine direkte Interaktion zwischen dem redox-sensitiven Transkriptionsfaktor Nrf2 und dem die Inflammation regulierenden Transkriptionsfaktor NFκB festgestellt werden. Dennoch liefern die Daten dieser Versuche erste wichtige Anhaltspunkte bezüglich der komplexen Regulation oxidativer und inflammatorischer Signalwege sowie deren positive Beeinflussung durch phytogene Verbindungen. Zur weiteren Aufklärung bis hin zur Identifikation der kompletten Signalkaskaden, insbesondere deren Wechselwirkung untereinander, sind weiterführende Studien notwendig.

Zusammenfassend belegt die vorliegende Arbeit, dass die getesteten phytogenen Verbindungen aus Brokkoli, Gelbwurzel, Oregano, Thymian und Rosmarin über ein Zusammenspiel von direkten und indirekten antioxidativen Eigenschaften ihrer Inhaltsstoffe den Gesundheitsstatus von Nutztieren steigern und in der entsprechenden Dosis und Verabreichungsdauer die Produktionsfaktoren in der Nutztierernährung günstig beeinflussen können. Außerdem stellen phytogene Substanzen besonders im Hinblick auf ihre anti-inflammatorische Wirkung eine mögliche Alternative zum präventiven Antibiotikaeinsatz hinsichtlich der Reduktion von Durchfallerkrankungen in der Nutztierhaltung sowie eine potenzielle Präventions- und Behandlungsstrategie für entzündliche Darmerkrankungen beim Menschen dar.

6 Summary

Gastroenteritis, caused by *Salmonella spp.* and pathogenic *Escherichia coli*, often appears in early stages of poultry and swine production. Since the ban on antibiotic feed additives in the European Union in 2006 gastroenteritis has become a major problem in animal nutrition. Consequently, research in non-antibiotic alternative compounds, like phytogenic feed additives, is forced.

On the basis of recent data phytogenic feed additives affect animal health positively by five different modes of action: (1) increase in animal performance, (2) direct antioxidant effects, (3) indirect antioxidant effects, (4) anti-microbiotic, and (5) anti-inflammatory properties of phytogenic substances. Currently, a high number of plant compounds, especially based on herbs and essential oils, are used as feed additives in animal nutrition, although in farm animals the knowledge on basic biochemical and molecular mechanisms is very limited.

The recent studies aimed to analyse the mentioned modes of action of phytogenic feed additives using practical conditions. The first study with broiler chickens (**V1**) investigated the effects of broccoli extract (3.000 mg extract/kg diet) and of essential oils from turmeric, oregano, thyme, and rosemary (150 mg oil/kg diet) on different performance parameters. The antioxidative capacity (TEAC, TBA-RS) of the phytogenic compounds and their impact on Nrf2-regulated xenobiotic and antioxidant enzymes in various broiler tissues was analysed due to the in vitro described direct and indirect antioxidant effects of various ingredients of broccoli (GRA, SFN) and the essential oils tested (turmeric: ar-turmerone; oregano: carvacrol; thyme: thymol; rosemary: 1,8-cineol). In a second trial with piglets (**V2**), the phytogenic feed additives tested, were standardised to their main ingredient (150 mg main compound/kg diet) to ascribe the detected effects to a certain compound of the particular phytogenic feed additive. In summary, both studies prove that the tested phytogenic feed additives from broccoli, turmeric, oregano, thyme, and rosemary do not improve performance parameters of farm animals, but seem to be beneficial in early feeding stages of both investigated species (**V1**, **V2**). For the first time, the recent data approve that phytogenic additives affect direct and indirect antioxidant protection mechanisms in various monogastric farm animals in a substance-specific manner (**V1**, **V2**). The essential oil from oregano primarily possessed direct antioxidant effects, whereas broccoli extract and the essential oil from rosemary rather developed their antioxidant properties in vivo by induction of xenobiotic and antioxidant enzymes, like AFAR, EPHX1, GST, SOD1, GPx1 (**V1**, **V2**). Furthermore, the first two trials demonstrated direct and indirect antioxidant effects via Nrf2 for the essential oils from thyme and turmeric (**V1**, **V2**). Altogether, the data of this recent work provide important details

regarding molecular mechanisms and indicate a potential correlation between the five discussed modes of action of phytogenic compounds.

The results of the second study with piglets (**V2**) could not confirm the recent literature data regarding the anti-microbiotic effects of phytogenic feed additives. In contrast the intestinal microbiota was affected marginally positive by the phytogenic compounds.

Another aim of the present work was to analyse potential anti-inflammatory effects of phytogenic feed additives and main compounds of broccoli, turmeric, thyme, rosemary, and apple. Therefore, an in vivo study with wistar rats (**V3**) and an in vitro experiment with IPEC-J2 cells (**V4**) were carried out. In summary, both trials indicate anti-inflammatory properties of the phytogenic substances tested due to the reduction of NFκB and Cox2 mRNA concentration under both physiological and stimulated conditions (**V3**: DSS-colitis, **V4**: *Escherichia coli*-infection). Additionally, there was no direct interaction between the redox-sensitive transcription factor Nrf2 and the inflammation-associated transcription factor NFκB detectible. However, the present data provide important initial evidence regarding the extensive regulation of the oxidant and inflammatory process. They also show a positive impact with regard to phytogenic substances. To identify the entire modes of action of phytogenic substances and interactions among the pathways further studies are required in the future.

In summary, the present work proves that the phytogenic compounds from broccoli, turmeric, oregano, thyme, and rosemary could increase health status of farm animals by an interaction of direct and indirect antioxidant effects of their ingredients. The phytogenic substances tested, could also positively influence production factors in animal nutrition due to the appropriate concentration and feeding period. Moreover, selected phytogenic compounds with strong anti-inflammatory effects may have the potential to further replace the use of antibiotics in the treatment of intestinal problems in farm animals. Due to their positive effects on intestinal health phytogenic substances may also represent hopeful alternatives in the prevention and the therapy of inflammatory bowel diseases in humans.

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8 Danksagung

An dieser Stelle bedanke ich mich bei allen Personen, die mich im Laufe meiner Promotionszeit begleitet haben und damit wesentlich zum Gelingen dieser Arbeit beigetragen haben.

Ein besonderer Dank gilt *Juniorprof. Dr. oec. troph. habil. Andreas S. Müller* für die Überlassung des interessanten Forschungsthemas und die Möglichkeit, meine Dissertation am Institut für Agrar- und Ernährungswissenschaften innerhalb seiner Arbeitsgruppe „Präventive Ernährung“ anzufertigen. Vor allem bedanke ich mich für die fortwährend freundliche und konstruktive Unterstützung sowie für die Bereitstellung der nötigen Forschungsmöglichkeiten während meiner Promotionszeit.

Des Weiteren bedanke ich mich herzlichst bei *Frau M. Sc. troph. Nicole Blum* für die hervorragende Zusammenarbeit und freundschaftliche Arbeitsatmosphäre innerhalb der Arbeitsgruppe sowie für die tatkräftige Unterstützung hinsichtlich methodischer Fragen und bei den zum Teil sehr aufwendigen Versuchsdurchführungen. Besonders danke ich auch *Frau Dipl. Ernährungswissenschaftlerin Nicole Wege* für die stete Diskussionsbereitschaft und die tolle unkomplizierte Zusammenarbeit in Labor und Büro.

Ein herzliches Dankeschön gilt allen Mitarbeitern, Doktoranden und Diplomanden des Instituts für Agrar- und Ernährungswissenschaften sowie allen Mitarbeitern des Nutztierwissenschaftlichen Zentrums Merbitz für die stets freundliche Unterstützung im Labor und im Tierstall während meiner Zeit am Institut.

Außerdem bedanke ich mich bei allen Koautoren der im Rahmen dieser Arbeit entstandenen Publikationen für ihre Mitarbeit sowie bei bei allen Personen, die bei der Durchsicht und Korrektur dieser Dissertationsschrift behilflich waren.

Der *H. Wilhelm Schaumann Stiftung* danke ich für die Gewährung von Sachmitteln und meines Promotionsstipendiums. Weiterer Dank gilt der Firma *DELACON Biotechnik Ges.m.b.H.* sowie Herrn Rogovin von der Firma *Jarrow Formulas Inc*.

Meiner Familie und meinen Freunden, die stets an das Gelingen dieser Arbeit und an mich geglaubt haben, danke ich an dieser Stelle besonders für die moralische und mentale Unterstützung in der Vergangenheit.

Abschließend bedanke ich mich besonders herzlich bei meinem Freund Michael Felke, der mir in der ganzen Zeit das nötige Verständnis und eine bemerkenswerte Geduld entgegengebracht hat. Vielen Dank auch für deine mentale und tatkräftige Unterstützung!

9 Lebenslauf

Persönliche Daten

Name:	Müller
Vorname:	Kristin
Geburtsdatum:	24.06.1983
Geburtsort:	Wolfen
Familienstand:	ledig (1 Kind)

Schulbildung

09/1990-06/2003	Besuch allgemein bildender Schulen, Grundschule Gossa und Europagymnasium Bitterfeld Abschluss: Abitur

Studium

10/2003-09/2008	Ernährungswissenschaften, Martin-Luther-Universität Halle-Wittenberg
Abschluss	Diplom-Ernährungswissenschaftlerin

Beruflicher Werdegang

01/2009-11/2012	Promotionsstipendium der H. Wilhelm Schaumann Stiftung (01/2009-09/2011) und wissenschaftliche Mitarbeit in der Arbeitsgruppe „Präventive Ernährung“ am Institut für Agrar- und Ernährungswissenschaften
Seit 05/2013	Elternzeit

Halle/Saale, Unterschrift

Weitere Publikationen, veröffentlichte Kurzfassungen und Vorträge

***Mueller K.**, Blum N.M., Mueller A.S. (2013):* Influence of dextran sulfate sodium (DSS) on real-time polymerase chain reaction amplification in the colon of rats exposed to DSS. Proceedings of the Society of Nutrition Physiology Vol. 22, 72, ISBN: 978-3-7690-4106-4, Seite 102

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*Blum N.M., **Mueller K.**, Hirche F., Lippmann D., Most E., Pallauf J., Linn T. and Mueller A.S. (2011):* Glucoraphanin does not reduce plasma homocysteine in rats with sufficient Se supply via the induction of liver ARE-regulated glutathione biosynthesis enzymes. Food & Function Vol. 2 (11): 654-64

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*Blum N.M., **Mueller K.**, Mueller A.S. (2011):* Influence of Selenium and sulforaphane on homocysteine metabolism in growing rats. International Oskar Kellner Symposium 9th-11th Sept. 2011, ISSN: 0946-1981, Seite 107

*Wolf N.M., **Mueller K.**, Hirche F., Most E., Pallauf J., Mueller A.S. (2010):* Study of molecular targets influencing homocysteine and cholesterol metabolism in growing rats by manipulation of dietary selenium and methionine concentration. British Journal of Nutrition Vol. 104: 520-532

***Mueller K.**, Blum N.M., Kluge H., Mueller A.S. (2010):* Einfluss verschiedener phytogener Futterzusätze auf die Expression von Phase II Enzymen und weiterer Parameter des antioxidativen Schutzsystems in unterschiedlichen Darmabschnitten sowie in der Leber wachsender Ross 308 Broiler. Tagungsband 11. Tagung Schweine- und Geflügelernährung, 2010, ISBN: 978-3-86829-250-3, Seiten 25-27

***Mueller K.**, Markert A., Sinningen K., Kluge H., Mueller A.S. (2010):* Effects of broccoli extract and various essential oils on the induction of phase II enzymes in the intestine of Ross 308 broilers. Proceedings of the Society of Nutrition Physiology Vol.19, 45, ISBN: 978-3-7690-4102-6

*Wolf N.M., **Mueller K.**, Pallauf J., Mueller A.S. (2009):* Selenium and methionine affect homocysteine and cholesterol metabolism in growing rats. Proceedings of the 25th GMS conference 1st - 3rd Oct. 2009; Herbert Utz Verlag GmbH München, 2011, ISBN: 978-3-8316-4090-4, Seiten: 81-94

*Mueller A.S., **Mueller K.**, Wolf N.M., Pallauf J. (2009):* Selenium and diabetes: An enigma? Invited Review. Free Radical Research Vol. 43 (11): 1029-59

10 Eidesstattliche Erklärung / *Declaration under Oath*

Ich erkläre an Eides statt, dass ich die Arbeit mit dem Titel: **„Einfluss phytogener Futterzusätze auf Xenobiotika-metaboliesierende und antioxidative Enzyme sowie Entzündungsparameter bei monogastrischen Nutztierspezies und bei der Ratte“** selbstständig und ohne fremde Hilfe verfasst, keine anderen als die von mir angegebenen Quellen und Hilfsmittel benutzt und die den benutzten Werken wörtlich oder inhaltlich entnommenen Stellen als solche kenntlich gemacht habe.

I declare under penalty of perjury that this thesis is my own work entirely and has been written without any help from other people. I used only the sources mentioned and included all the citations correctly both in word or content.

Ich erkläre weiterhin, bisher keine vergeblichen Promotionsversuche unternommen und die wissenschaftliche Arbeit an keiner anderen wissenschaftlichen Einrichtung zur Erlangung eines akademischen Grades eingereicht zu haben.

I declare that the thesis has not been used previously at this or any other university for reasons of graduation.

Halle/Saale, Unterschrift