Institut für Tierernährung
des Bundesforschungsinstitutes für Tiergesundheit
des Friedrich-Loeffler-Institutes
&
Institut für Agrar- und Ernährungswissenschaften
der Naturwissenschaftlichen Fakultät III
der Martin-Luther-Universität Halle-Wittenberg

Origanum vulgare L. and *Tropaeolum majus* L. in feeding of young pigs- Effects on health and performance

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Master of Science (agr.) Katrin Stelter
Geb. am 19.11.1983 in Sangerhausen

Gutachter: Prof. Dr. Dr. Sven Dänicke
Prof. Dr. Annette Zeyner
Prof. Dr. Werner Zollitsch

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Abbreviations

(used in Introduction, Background, General Discussion and Conclusion)

ABW	average body weight
ADFI	average daily feed intake
ADG	average daily gain
AGP	antibiotic growth promoter
AUC	area under the time cure
Bac	Bacteroidaceae and Prevotellaceae
Bif	Bifidobacterium spp.
BITC	benzyl-isothiocyanates
BW	body weight
CD	cluster of differentiation
CFU	colony forming units
CON	control
DLG	Deutsche Landwirtschaftsgesellschaft
Ebac	Enterobacteriacae
EC	European Community
EU	European Union
FCR	feed conversion ratio
FDA	U.S. Food and Drug Administration
FISH	Fluorescence in situ hybridisation
FNR	Fachagentur für Nachwachsende Rohstoffe
Fpr	Faecalibacterium prausnitzii group
GALT	gut-associated lymphoid tissue
GIT	gastro intestinal tract
GTL	glucotropaeolin
HEM	herbal extract mixture
ITC	isothiocyanate
LPS	lipopolysaccharide
MHC	major histocompatibility complex
MIC	minimal inhibitory concentration
ORE	oregano
PCR	polymerase chain reaction
RFLP	Restriction fragment length polymorphism

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1 Introduction

Until the year 2050, the world's population is supposed to increase from 7 to between 8.8 and 10 billion (Cleland, 2013). Therefore, the growing demand for agricultural products and foodstuffs will become an important issue during the next decades. The global meat consumption is expected to expand by an overall of 19 percent (FAO, 2009). The most important meat supplier is the pig with a global average per capita consumption of 15.8 kg/year (FAO, 2014). In Germany, the per capita consumption of pork amounted to 37.9 kg in 2012, which is considerably higher compared to poultry meat (11 kg/year) and beef (8.9 kg/year) (Anonymous, 2013).

The consumer's awareness concerning the health and welfare of farm animals is steadily increasing. Especially, the use of antibiotics as feed additives since the 1950s (Freitag and Hensche, 1998) and the ensuing development of resistant bacterial strains (Mcdermott et al., 2002, Aarestrup, 1999) resulted in an increasing feeling of insecurity of the consumer. In addition, the importance of the association of the health of the intestine and the innate immune system is becoming an important issue in animal nutrition steadily. The mucosal immune system is known as one of the most important defence systems against pathogens (Bauer et al., 2006b). Considering the different needs of the consumer and the global market and supporting the development of the intestinal microbiota in animals, the animal nutritionists are faced with seeking for alternatives to antibiotic growth promoters (AGP), whose approval was withdrawn from the EU Council of Agricultural Ministers on 1. January 2006.

Organic acids, pro- and prebiotics, feed enzymes, herbs and spices were considered as potential alternatives to AGP (Wenk, 2003, Vondruskova et al., 2010, Close, 2000). A number of experiments were carried out with these replacement products in the last decade, but the results varied markedly (Freitag and Hensche, 1998). Huyghebaert et al. (2011) reviewed some alternative strategies for broilers and suggested that there were some products with a clear potential within each product class while others efficacy was still unclear. In accordance to these researchers, Close (2000) postulated that in-feed antibiotics could be replaced by alternative strategies considering the combination of the alternatives (herbs, organic acids, enzymes etc.), the husbandry and the management. Nowadays, the use of herbs or essential oils will be of rising interest in the future. In the meantime, more than 73% of the German population older than 16

years use naturopathic remedies (Bodeker and Ong, 2005) and the mentioned awareness concerning animal health and antibiotic resistances is growing continuously. Hence, taking an advantage of these human interests, the animal nutrition also focused on natural based feed additives like medicinal plants and spices. Although the use of these materials is widely known in alternative medicine, the results of most investigations with animals showed that there is a need to describe the mechanism of action, to clarify a possible synergy between the biological active constituents and to set standards regarding dosage, stability of the active substance and the processing methods like harvesting, storage and the final manufacturing process to the form which is used as feed in livestock.

2 Background

2.1 Historical background of antibiotic growth promoters

Since the 1940s the production of antibiotics has increased steadily with an associated rising amount of by-products (Gustafson and Bowen, 1997, Freitag and Hensche, 1998). The mycelium, as a by-product, was considered as a possible supplement in animal feeding (Freitag and Hensche, 1998). Subsequently, the first experiments with antibiotics as feed additives were carried out at that time. Moore et al. (1946) observed that the supplementation of streptomycin improved the growth performance of the animals in an investigation with chicken, but they noticed this effect only as positive side-effect. A few years later, Stokstad et al. (1949) appreciated the importance of this discovery. They used small amounts of the fermented mesh out of the antibiotic production of aureomycin (chlortetracycline) in chicken and also showed a rapid increase of weight gain and a better feed efficiency. The use of antibiotics as feed additive has been sealed since those investigations. The feed industry recognized the unambiguous effects of the in-feed antibiotics compared to unsupplemented feed and consequently, antibiotic substances have been widely used in livestock farming to improve the performance. Table 1 shows the feed conversion ratio for selected studies of the different animal species with in-feed antibiotics. This performance parameter is mainly influenced due to the linking of feed intake and body weight gain.

Table 1: Efficacy of antibiotics as growth promoters in livestock animals

	Pig (25-120 kg)	**Ruminants** (146-555 kg)	**Poultry** (1-42 d)
Control			
Feed conversion ratio	2.97[a]	5.49	1.79
Antibiotic supplemented group	Avilamycin (20-40 mg/kg feed)	Monensin (30 mg/kg feed)	Flavomycin (1 g/kg feed)
Feed conversion ratio	2.88[b]	5.35	1.74
Change compared to control (%)	-3.0%	-2.6%	-2.8%
Reference	Kampf et al. (1998)	Daenicke et al. (1982)	Sarica et al. (2005)

*Av., average; [ab] means with different superscripts within the same column are significantly different ($p<0.05$)

BACKGROUND

In 1969, scientists began evaluating the effect of long-term use of antibiotics in food-producing livestock and formed the Swann Committee in the United Kingdom. One of their recommendations was the division of antibiotics into feed and therapeutic classes and that feed antibiotic class should not include drugs used therapeutically in humans or animals (Swann et al., 1969). A task force of the U.S. Food and Drug Administration (FDA) recognized that the use of antimicrobials in animal feeding resulted in the development of resistant pathogens in 1970 (Tollefson and Miller, 2000). Since that time, the debate about the ban of antibiotics as feed additive has been opened. Many investigations with animals and humans have been carried out to clarify the association of feeding antimicrobials to food-producing animals and development of resistant bacteria strains in humans, but there is still no consensus. In Table 2 the published reports and committees employed with this issue are listed.

Table 2: Reports on the use of antimicrobials in animals and associated public health implications (from McDermott et al. (2002), modified)

Year	Report
1969	Swann Committee Report-Joint Committee on the Use of Antibiotics in Animal Husbandry and Veterinary Medicine
1969	National Academy of Sciences - The Use of Drugs in Feed Animals
1977	U.S. General Accounting Office Report- Need to Establish Safety and Effectiveness of Antibiotics Used in Animal Feeds
1980	Institute of Medicine Report - The Effects on Human Health of Subtherapeutic Use of Antimicrobials in Animal Feeds
1981	Council for Agricultural Science and Technology - Antibiotics in Animal Feeds
1989	Institute of Medicine Report - Human Health Risks with the Subtherapeutic Use of Penicillin or Tetracylines in Animal Feed
1995	American Society for Microbiology Task Force Report
1997	World Health Organization- The Medicinal Impact of The Use of Antimicrobials in Food Animals
1998	World Health Organization- Fluoroquinolone Use in Food Animals
1998	Ministry of Agriculture, Fisheries, and Food - A Review of Antimicrobial Resistance in the Food Chain
1998	National Research Council - Use of Drugs in Food Animal: benefits and Risk
1999	U.S. General Accounting Office Report - The Agricultural Use of Antibiotics and Its Implications for Human Health
2001	EU SCAN Report - Opinion of the Scientific Committee on Animal Nutrition on the Criteria for Assessing the Safety of Microorganisms Resistant to Antibiotics of Human Clinical and Veterinary Importance
2002	The FAAIR Report - The Need to Improve Antimicrobial Use in Agriculture: Ecological and Human Health Consequences
2006	EU Legislation - Ban of Antibiotics as Feed Additive
2008	ECDC organised the 1st annual European Antibiotic Awareness Day
2010	EFSA – Summary Report on Antimicrobial Resistance in Zoonotic and Indicator Bacteria from Humans, Animals and Food

2.2 Herbs and spices – a natural alternative to antibiotic growth promoters

The use of herbs and botanicals for the treatment of ailments has been known for thousands of years. While herbs were defined as flowering plant valued for its medical properties, botanicals were specified as an extract made from parts of a plant (Anonymous, 1989). About 2600 BC the first records of this kind of medicine system were found in Mesopotamia (Newman et al., 2000) and the interest in it is still present. Herbal medicine is the most popular form of traditional medicine and includes herbs, herbal materials, herbal preparations, and finished herbal products (WHO, 2008). The World Health Organization reported that 70 – 80% of the population in developing countries uses forms of alternative medicines. The trend of the consumers towards "natural" based food and the thinking about agriculture and animal production systems in the future has increased the market for phytogenic performance enhancers since the 1990s (Wenk, 2003, Greathead, 2003). Herbs and botanicals are versatile and therefore interesting in both humans and animals. The broad range of effectiveness is shown in Figure 1. It is obvious that most of the listed herbs and spices influence the gastro intestinal tract and therefore they possibly contribute to the intestinal health. Some herbs have an antimicrobial effect against pathogen bacteria and therefore possible measures in case of a bacterial infection (Table 3).

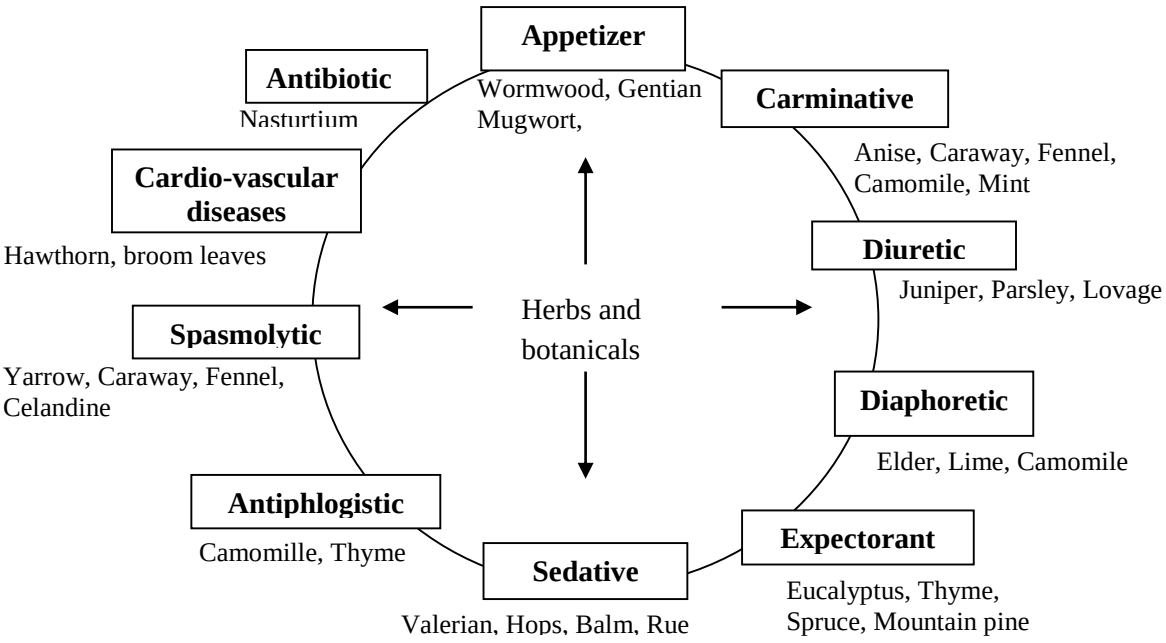

Figure 1: Utilisation of herbs and botanicals as medicinal plants (from Kaemmerer (1978), modified)

Many investigations with herbs and herbal products like essential oils in particular have been carried out both *in vivo* and *in vitro*, but the results leave some questions open. Wenk (2003) postulated the beneficial effects of herbs and botanicals in animal nutrition. He emphasized the regulation of the feed intake and the stimulation of digestive secretion in response to the use of herbs in animal feed. In contrast, some researchers investigated various mixtures of herbs or essential oils as feed additive with no satisfactory results in performance data (Untea et al., 2011, Neill et al., 2006, Namkung et al., 2004).

Table 3: Minimum inhibitory concentrations (MIC) of different antibiotics and natural antimicrobials (from Palaniappan and Holley (2010), modified)

	S. typhimurium	**E.coli**	**S.aureus**	**S. pyogenes**
Antibiotics *MIC (µg/ml)*				
Ampicillin	>512	>512	32	sensitive
Penicillin	>512	>512	128	sensitive
Tetracycline	64	128	sensitive	sensitive
Erythromycin	1024	512	sensitive	>512
Bacitracin	>512	>512	32	sensitive
Novobiocin	256	64	sensitive	sensitive
Antimicrobials *MIC (mM)*				
Eugenol (clove)	2.5	2.5	2.5	0.63
Thymol (thyme)	2.5	2.5	2.5	0.63
Cinnamaldehyd (cinnamon)	2.5	2.5	2.5	0.63
Carvacrol (oregano)	2.5	2.5	1.25	0.31
Allyl-isothiocyanat (horseradish)	0.31	0.31	0.15	0.63

The efficacy of experiments with herbs and herbal mixtures in livestock husbandry are listed in Table 4. Verstegen and Williams (2002) suggested that the growth promoting effect of antibiotics mainly results from their antimicrobial activity in the gastrointestinal tract (GIT). Namkung et al. (2004) proposed a reduction of harmful bacteria in response to herbs, while antibiotics reduced both harmful and beneficial bacteria in the pigs' gut. Furthermore, additional effects on the intestinal morphology were observed. In animals fed with herb supplemented diet the numerically higher proliferation of goblet cells and the lower crypt depths was observed. These changes in the gut due to the dietary composition were the reason for altering intestinal function

(Namkung et al., 2004). Nevertheless, there are a lot of gaps in knowledge regarding the mode of action of herbal substances due to the high variety of secondary plant substances in these natural products (Windisch et al., 2008). These substances vary quantitatively and qualitatively and are also important for the interaction of the plant with its environment (Greathead, 2003), for example, to protect the plant against herbivores and pathogens (Briskin, 2000).

Table 4: Efficacy of specific herbs in livestock animals

	Pig	**Ruminants**	**Poultry** (broiler chicken)
Age	0-35 d	5-60 d	1-42 d
Control			
Feed conversion ratio (kg/kg or g/g)	1.68	5.55[a]	2.19
Herb supplemented group	Oregano oil 1.5 g/kg diet	Garlic extract 250 mg/kg BW	Fennel seed 3 g/kg diet
Feed conversion ratio (kg/kg or g/g)	1.63	2.46[b]	1.88
Change compared to control (%)	-2.9%	-55.7%	-14.2%
Reference	Ragland et al. (2007)	Ghosh et al. (2010)	Mohammed and Abbas (2009)

[a,b] means with different superscripts within the same column are significantly different ($p<0.05$)

Some of these secondary substances are of interest in the herbal medicine because of their additive and/or synergistic actions (Greathead, 2003). The glucotropaeolin of *Tropaeolum majus* L., for example, is inactive in the raw plant material and even metabolized into the active form isothiocyanat under specific conditions like chewing or tissue damage (Fahey et al., 2001, Brüsewitz et al., 1977). Therefore, the knowledge about the bioavailability and the biological activity of the active substances is highly important when using herbal substances (Greathead, 2003). A further problem in the utilisation of herbs is the imbalance of the content of the active substances due to the different cultivation conditions, the harvesting season, and the geographical origin as well as the processing of the final product (Windisch et al., 2008). The final product is

used in animal nutrition and should have some defined qualities regarding the practicability, in particular, the standardisation of the active substance. Identifying the most suitable productive form investigations with various galenic preparations of herbs, for example, essential oil or dried plant material, were tested. Essential oil which is easy to handle both in processing by the feed manufacturer and the farmer himself is mostly used as feed additive. Unfortunately, the production costs are very high at present and the results of the investigations regarding performance and preventive health care are not satisfying up to now. A consequence of this fact is the alternative use of dried or fresh plant material, which is cheaper in processing and also easy to handle, but there are hardly experiences with this pharmaceutical form at present.

2.3 The use of herbs and botanicals in pigs

Most of the pigs in Germany are shielded from the natural environment because of the housing in closed systems with high hygienic standards. Due to this issue pigs are very sensitive to changes in their environmental conditions. In piglet rearing, the time of weaning, which is associated with the change in feed, is a critical period characterized by an increased susceptibility to illness. Especially, the incidence of digestive disorders such as oedema disease (Imberechts et al., 1992) or post-weaning diarrhoea (Vondruskova et al., 2010) usually increases in that time period. In the reproductive performance of sows diseases of the urogenital tract are often the main reason for a decreased farrowing rate and sow mortality (Glock and Bilkei, 2005). Further problems in pig farming are the respiratory diseases (Reeth and Nauwynck, 2000) which cause high economic losses. Before 2006, the use of in-feed antibiotics in pig nutrition had been found to reduce the incidence of the mentioned diseases (Cromwell, 2002), but since that time alternative strategies like pro- and prebiotics, organic acids, enzymes and herbs have been examined. Herbs, in particular, used in pig nutrition should especially address the intestinal microbiota to stabilise the intestinal immune system, and the urinary and the respiratory tract to prevent infections in these areas associated with an immunmodulating effect. Additionally, the used herbs should act as appetizer and immune stimulants. In Table 5 there are some herbs listed which have been used in pig nutrition.

Table 5: Plant extracts in pig nutrition (from Vondruskova et al. (2010), modified)

Plant	**The effect observed**	**Reference**
Oregano (*Origanum spp.*), cinnamon (*Cinnamomum spp.*) and Mexican Pepper (*Capsicum annum*)	decreased total ileal microbial mass, increased the lactobacilli : enterobacteria ratio	Manzanilla et al. (2004)
Sangrovit (alkaloids of *Macleaya cordata*)	increased body weight gain and feed conversion by growing pigs	Borovan (2004)
Cinnamon (*Cinnamomum spp.*), thyme (*Thymus spp.*), oregano (*Origanum spp.*)	inhibited pathogenic *E. coli* in piglet intestine	Namkung et al. (2004)
Dried leaf and flower of oregano (*Origanum spp.*) enriched with essential oil of oregano	Higher proportion of MHC class II antigen, $CD4^+$ and $CD8^+$ T-lymphocytes and double positive $CD4^+CD8^+$ T-lymphocytes	Walter and Bilkei (2004)
Thyme (*Thymus spp.*), clove (*Syzygium aromaticum*), oregano (*Origanum spp.*), eugenol (*Syzygium aromaticum*) and carvacrol (*Origanum spp.*)	improved pig performance	Oetting et al. (2006)
Clove (*Syzygium aromaticum*), oregano (*Origanum spp.*)	growth performance of pigs close to pigs fed antimicrobials	Costa et al. (2007)
Nasturtium (*Tropaeolum majus* L.)	ingestion yielded antibacterial isothiocyanat concentrations in urine	Bloem et al. (2008)
AstragalusMembranaceus Bunge, Lycium barbarum L., *Atractylodes macrocephala Koidz, Shenqu and Glycyrrhiza uralensis Fiseh*	increase ADG, decrease feed conversion ratio in finishing pigs	Liu et al. (2008)
Aged garlic extract (*Allium sativum*), allicin (*Allium sativum*)	improved body weight, morphological properties of intestine villi and non-specific defense mechanisms of piglets	Tatara et al. (2008)
Camellia (*Camellia sinensis*)	decrease of clostridia and enterococci counts in the faeces of piglets	Zanchi et al. (2008)
Buckwheat (*Fagopyrum esculentum*), thyme (*Thymus spp.*), ginger (*Zingiber off.*), curcuma (*Curcuma*), black pepper (*Capsicum spp.*)	beneficial effect on nutrient digestibility, increase of lymphocytes and decrease of fecal E. coli concentration	Yan et al. (2012)

2.3.1 Level of feed intake in response of herbal supplementation

In young pigs the declined feed intake following weaning and change in feed from milk to solid feed is a major problem. Young animals need a regular intake of feed and water to build up their immune system and to develop the intestinal microbiota (Wenk, 2003). If piglets refuse the feed, they will become growth-retarded and will be more sensitive to diseases ensued by a financial loss for the livestock owner. Therefore, the supplementation of feed with aromatic substances is regarded as a measure to avoid this effect in piglet rearing.

Plants containing essential oils like oregano are suitable to act as appetizer in feed. Ragland et al. (2007) used 1.5 kg oregano oil/tonne feed in 2 trials with weaned piglets and recognized a different eating pattern of the animals. While piglets of trial 1 showed a reduced feed intake due to the prominent odour of the oregano associated with a negative influence on the growth, animals of trial 2 fed with oregano oil supplemented feed achieved the highest average daily feed intake (ADFI) and average daily gain (ADG). In investigations with growing-finishing pigs the utilisation of 3 g Oregpig® (a mixture of dried oregano and oregano oil) led to an improved effect on ADG and feed conversion ratio (FCR) compared to the control animals (Walter and Bilkei, 2004). The mentioned facts suggested that oregano, as representative of essential oil containing plants, could influence the eating pattern of pigs at each stage of development. Nevertheless, when drawing conclusions from the investigations mentioned above, it can be stated that the feed intake was not only influenced by the odour, but also by the change in intestinal microbiota and/or immune reactions in response to oregano.

2.3.2 The effect on the gastro intestinal tract and the intestinal microbiota

The gastro intestinal tract of newly born mammals is accustomed to milk and its functionality is not yet fully developed for feed of plant origin (Wenk, 2003). With the natural rearing of the animals the nutrient digestion changes slowly from milk to solid feed associated with an alteration in intestinal microbiota (Konstantinov et al., 2004). Especially at weaning, the abrupt separation of the piglets from the sow and the complete change to solid feed connected with the transport and the regrouping (Blecha et al., 1985) are possible facts of the increase of the susceptibility to digestive disturbances (Manzanilla et al., 2004). Different investigations have shown that herbs as feed additives may influence the intestinal microbiota due to their

antimicrobial activity or its beneficial stimulation on the microbial eubiosis (Wenk, 2003, Frankič et al., 2009, Namkung et al., 2004). Further, the utilisation of herbs and botanicals, for example oregano and garlic, against bacterial diseases like proliferative enteropathies or infections with enterotoxic *Escherichia coli* is used habitually in practice (Frankič et al., 2009, Papatsiros et al., 2009). Piglets are often susceptible to diarrhoea and post-weaning anorexia (Lallès et al., 2007) during critical situations, like weaning (Windisch et al., 2008). It is well known that a stable and complex intestinal microbiota serves as a basis for an increased nutrient digestion and consequently for a good development of the animal (Windisch et al., 2008, Wenk, 2003). In addition to the intestinal microbiota, the mucosal immune system with its innate immunity and the gut-associated lymphoid tissue (GALT) are also important for protecting the host against the penetration of pathogens resulting in a good health status and an appropriate increase in performance (Bauer et al., 2006b). The final development of the lymphoid tissue and the bacterial community is not finished until the piglet is approximately 6 weeks old. In modern livestock systems piglets were weaned at 3-5 weeks (Lallès et al., 2007) and therefore subjected to stress. Consequently, the intestinal microbiota and the GALT do not have enough time for full development with the result that the piglets are more susceptible to pathogens (Bauer et al., 2006b, Lallès et al., 2007).

2.3.3 The immunmodulating abilities of herbs

Regarding the immune system *in vitro* and *in vivo* investigations showed that herbs and botanicals have immune modulating abilities (Walter and Bilkei, 2004, Bimczok et al., 2008, Liu et al., 2012, Yan et al., 2012). Critical situations like change in feed, castration, weaning and transport imply stress for the animal which is mostly associated with a decline in growth and an increased susceptibility to diseases due to a weak immune defence. In the investigation of Yan et al. (2012) a herbal extract mixture (HEM) including buckwheat, thyme, ginger, curcuma and black pepper was administered to 21 day old pigs over a period of six weeks. The results showed a higher lymphocyte concentration in the pigs fed the HEM compared to the non-HEM animals which might have an effect on the immune defence. Furthermore, Walter and Bilkei (2004) examined the proportion of $CD4^+$ and $CD8^+$ T-lymphocytes in response to oregano and found significantly higher T-lymphocyte counts in the oregano-fed pigs than in the control group. Additionally, the proportions of double positive $CD4^+CD8^+$ T-lymphocytes in peripheral blood

and in mesenteric lymph nodes were also significantly higher compared to the control animals. Especially, the high frequency of these double positive expressing T-lymphocytes in pigs are of interest for immune related questions. It has been known that these T-lymphocytes take part in the adaptive immune response against pathogens (Nascimbeni et al., 2004) and that they belong to the virus antigen-specific memory cells (Saalmüller et al., 1999), but little is known about their biological function and significance (Nascimbeni et al., 2004).

Other researchers reported on an enhanced production of immunoglobulins after feeding thymol (Trevisi et al., 2007) or a stimulating effect of an ethanolic tumeric extract on murine lymphocytes in general (Chakravarty and Yasmin, 2005). Finally, the supplementations of herbs seem to have immuno-modulating effects.

2.3.4 The utilisation of herbs in piglets, growing-pigs and sows

The use of herbs in piglets, in particular, might be more effective than using it in growing-finishing pigs, because of the undeveloped microbial intestinal ecosystem. Piglets are more sensitive to environmental changeover and therefore the susceptibility to bacterial infections and other diseases is higher. As postulated by Wenk (2003) a balanced intestinal microbiota is optimal for an effective defence against pathogens and an intact immune system. Nevertheless, the use of herbs in pregnant sows as a possible prevention measure for the newly born piglets or as a phytogenic mediated defence against environmental impacts could also be useful. Additionally, the supplementation of herbs to the diet of the sow also contributes to stabilisation of her own health. Some reports about the reasons of sow mortality suggested that infections of the urogenital tract have been the most common cause of death of sows (Karg and Bilkei, 2002, Glock and Bilkei, 2005, Bilkei et al., 1995). Avoiding these economic losses for the livestock owner without using antibiotics it is important to find a feasible solution. Bloem et al. (2008) examined the influence of dried *Tropaeolum majus* L. (nasturtium) in piglets and growing pigs resulting that the concentration of the active substance, the isothiocyanat, in urine after intake of recommended amounts (2,2 mg GTL/ kg body weight) of nasturtium might be effective against bacterial pathogens. Therefore, inflammatory changes in the urinary bladder and urethra (Glock and Bilkei, 2005) could be avoided when feeding nasturtium to sows.

The facts mentioned above underlined the wide possibility of using herbs and botanicals in pig nutrition, but the gap of knowledge is enormous at present. Particularly, the lack of using oregano as a terpenic plant and nasturtium, which is well known in human medicine, is enormous in pig nutrition.

3 Scope of the thesis

Considering the literature, it is obvious that there is an ongoing need for recommendations for using herbs as feed additives in animal nutrition. The gap of knowledge does not only exist in cultivation and harvesting, but also in the effect of the active substances *in vivo*. Among the broad range of herbs *Origanum vulgare* L. and *Tropaeolum majus* L. are chosen as the plants with the potential to support the immune system in general. Oregano as terpenic plant is most important because of its antimicrobial and immune modulating effects resulting in a stable immune status and a good performance, but there has been little research on feeding dried oregano to weaning piglets and its effect on immune related parameters and performance as well as on the colonisation of the intestinal microbiota so far. Nasturtium contains mustard oil and is supposed to be enriched in the urinary bladder and the lungs, the active sites to develop their antimicrobial effectiveness. Moreover, the bioavailability and the metabolism in growing pigs are less examined. Therefore, several animal experiments with the focus on either oregano or nasturtium have been carried out.

- The effect of *Origanum vulgare* L. as probable reason for changes in performance and immunological parameter like lymphocyte proportion and granulocyte activity by means of a piglet trial is the first aim of the present thesis **(Paper I)**.
- A further aim was to test the effect of *Origanum vulgare* L. on the colonisation of the intestinal microbiota using piglets fed with oregano for five weeks. The data was verified after slaughtering using restriction fragment length polymorphism (RFLP) and fluorescence *in situ* hybridization (FISH) **(Paper II)**.
- Another aim was to determine the effect of *Tropaeolum majus* L. on the metabolism of glucotropaeolin and the bioavailability of isothiocyanat using catheterized growing pigs **(Paper III)**.

In order to test the hypotheses a five weeks lasting experiment with piglets was carried out to clarify the influence of oregano on the performance and to examine its immunmodulating properties. A sub-set of these piglets were used for investigating the oregano effects on the intestinal microbiota. Clarifying the metabolism of ingested nasturtium and its bioavailability

catheterised pigs were utilized over a time period of 24 h. The results of these three publications are presented and discussed in the context of the literature in the General Discussion.

4 Paper I

Effects of oregano on performance and immunmodulating factors in weaned piglets

Katrin Stelter[1], Jana Frahm[1], Jana Paulsen[2], Andreas Berk[1], Maik Kleinwächter[2], Dirk Selmar[2] and Sven Dänicke[1]

[1]Institute of Animal Nutrition, Friedrich-Loeffler-Institute (FLI), Federal Research Institute for Animal Health, Braunschweig

[2]Institute for Plant Biology, Technische Universität Braunschweig, Braunschweig

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Abstract

Many health effects can be attributed to the Mediterranean herb oregano (*Origanum vulgare* L.) and several studies demonstrated the improving effect on performance, changes in blood count, antibacterial, antifungal and immunmodulating abilities. The majority of these investigations were carried out with processed essential oil, while whole plant material was only used in a few studies. Thus, the aim of the present experiment was to test the effect of increasing proportions of dried oregano in piglet feed on health and performance, with a special focus on immune modulation. A total of 80 male castrated weaned piglets (body weight [BW] 7.9 kg±1.0 kg) were used in a feeding experiment lasting 5 weeks. They were assigned to 4 experimental groups: a control diet, and 3 diets with an oregano supplementation at 2 g, 4 g and 8 g per kg feed, respectively, corresponding to 23.5 mg, 46.9 mg and 93.9 mg carvacrol/kg DM. After 3 weeks, half of each group was challenged with 5 µg lipopolysaccharides (LPS) per kg BW. Blood samples were collected 2 h after LPS stimulation and analysed for T-cell phenotypes, granulocyte activity, clinical-chemistry as well as white and red blood count. The results indicate no effects of oregano on performance. In contrast, oregano altered the lymphocyte proportion and the ratio of $CD4^+$ and $CD8^+$ T-cells as well as the triglyceride concentration in the serum of non-stimulated and in LPS-stimulated piglets.

In conclusion, whole plant supplementation of oregano to piglet feed altered immune-related parameters, but did not modulate the acute inflammatory response induced by LPS stimulation.

Keywords: $CD4^+$ lymphocytes; $CD8^+$ lymphocytes; granulocytes; lipopolysaccharides; *Origanum vulgare* L.; performance; piglets

Introduction

Essential oils, fresh or dried herbs and spices supplemented to feed could influence feed intake and the metabolism in the gastrointestinal tract (GIT) of animals. Therefore, the latter means that the phytochemical ingredients could affect the microbiota (Wenk, 2003) and the morphology of the gut endothelium (Manzanilla et al., 2004) followed by a better nutrient utilisation. Additionally, the combination of consumer awareness of antibiotics in food and cross-resistance

as well as the raising interest in phytomedicine (Who, 2008) leads to investigations with herbs and spices not only in human medicine but also in animal nutrition.

The Mediterranean herb oregano containing the monoterpenes carvacrol and thymol was studied in several *in vitro* investigations concerning its antimicrobial ability with the result that these terpenes act against lactobacilli and coliform bacteria (Şahin et al., 2004, Michiels et al., 2007, 2009). Some researchers suggested an improved health status due to antimicrobial mode of action (Frankič et al., 2009, Wenk, 2003). Carvacrol is an essential oil component of oregano and was suggested to have functional properties. Bimczok et al. (2008) suggested that dietary carvacrol crosses the intestinal barrier and contacts epithelial immune cells. Furthermore, the same researchers also showed a dose-dependent decrease in cell proliferation of lymphocytes and a higher rate of apoptosis in different leukocyte populations in response to carvacrol. Leukocytes are cellular components of the innate immune system and one of their functions is to recognise endogenous and exogenous substances like toxins or bacteria. Most bacteria, especially gram negative bacteria, have intrinsic components on the outer membrane, the lipopolysaccharides (LPS). These components interfere with the immune system by stimulating the signalling cascade in mononuclear phagocytes (Kullik et al., 2013) and stimulate the expression of pro-inflammatory cytokines, such as tumor necrosis factor alpha (TNF-α) and interferon-β (IFN-β) (Pålsson-Mcdermott and O'neill, 2004). As a result of the LPS-induced acute phase reaction, secondary changes in clinical-chemical parameter might occur; especially an increased aspartate aminotransferase (ASAT) activity and bilirubin concentration have been reported (Dänicke et al., 2013, Stanek et al., 2012). Taken together, LPS belongs to the pathogen associated molecular patterns, which are recognised by immune cells and which induce acute phase reaction, which includes immediate effects of releasing soluble mediators by immune-component cells into the circulation and secondary inflammatory effects resulting in, for example, tissue and cellular lesions with consequences on measurable parameters of blood chemistry and hematology. In addition to the mentioned property of oregano compounds, it is also known to have antioxidative (Capecka et al., 2005, Şahin et al., 2004), antifungal (Akgül and Kivanç, 1988, Şahin et al., 2004) and antiparasitic (Anthony et al., 2005) abilities.

Due to the broad range of effectiveness of oregano, the aim of the present study was to investigate whether the supplementation of this herb improves the performance data as well as

the clinical chemistry and some chosen immune-related parameters like white and red blood cells either in the absence or presence of a LPS-induced systemic inflammation.

Materials and methods

Plant material

The current investigation is part of a collaborative project supported by the Fachagentur für Nachwachsende Rohstoffe (FNR, Gülzow, Germany). The aim of the project is to promote cultivation of medicinal and spice plants in Germany. Several medicinal plants were cultivated and harvested in another part of the project and then used for feeding experiments in the current study to develop and verify phytogenic growth promoters. Therefore, dried plant material as the simplest pharmaceutical formulation and not essential oil is the basis for the following animal investigation.

Origanum vulgare L. was cultivated and harvested on the experimental plots of the Institute for Plant Cultivation, Schnega, Germany. After the flowering stage (mid of September), the aerial parts were harvested by manual cutting. Subsequently, the plant material was dried down to a final moisture content of approximately 9% at 40°C in tray driers. Using a hammer mill (Joachim Kreyenborg & Co, type 10, Münster-Kinderhaus, Germany), the dried material was ground to 4 mm. The monoterpenes content of the plant material was determined as follows: p-cymol (0.18 g/kg), *β*-caryophyllene (0.38 g/kg), γ-terpinene (0.39 g/kg), thymol (0.94 g/kg), carvacrol (11.7 g/kg). The analytical method applied is described in detail in Section "Oregano and feed".

Animals and experimental design

Eighty male castrated weaned piglets of a commercial hybrid line ((German Landrace × Large White) × Piétrain) with an average BW of 7.9±1.0 kg were housed at the research station of the Friedrich-Loeffler-Institute in Braunschweig, Germany. All animals were assigned to pens to allow the adaptation to the new environmental conditions for 5 days prior to the experiment. On the first day of the investigation, the BW of all piglets was measured, followed by an allocation to 20 pens according to their individual weight. Henceforth, all piglets were divided into 4 different feeding groups with 20 piglets in each. They were assigned to the 4 experimental diets: a control diet without supplementation of oregano, and 3 diets with an oregano supplementation at 2 g, 4 g and 8 g per kg feed, respectively, corresponding to 23.5 mg, 46.9 mg and 93.9 mg

carvacrol/kg DM. Before adding to the basal diet, oregano supplement was dried and milled. The basal diet consisted mainly of cereals and soy feedstuffs (Table 1); to meet the recommendations of the Society of Nutrition Physiology (Gfe, 2006) also minerals, vitamins, amino acids and phytase were supplemented. All diets were offered *ad libitum* in mesh form. Every piglet was weighed weekly during the feeding experiment. Furthermore, the feed consumption per box was also registered every week.

After feeding the experimental diets for 3 weeks, half of each feeding group was challenged with LPS (*Escherichia coli* serotype O55:B5, Sigma-Aldrich) while the other half remained unchallenged in order to examine the effects of oregano on immune-related parameters in the presence and absence of an acute systemic inflammation. All animals were administered the injections intraperitoneally in the lower abdominal region, either 5 µg LPS/kg BW dissolved in physiological saline (0.125 ml/kg BW) or with pure physiological saline (0.125 ml/kg BW). Blood samples (EDTA, heparin and serum) were collected from the cranial vena cava region of all animals 2 h after LPS and saline injection. The serum and heparin samples were centrifuged (2000 *g* for 15 min, 15°C, Heraeus Varifuge® 3.0R) and frozen in aliquots (-80°C) until analysis, while EDTA blood was used in total on the experimental day.

Analyses

Oregano and feed

Representative samples of each diet were collected and analysed for crude nutrients according to the methods of the Association of German Agricultural Analysis and Research Centres (Verband Deutscher Landwirtschaftlicher Untersuchungs- und Forschungsanstalten, VDLUFA). Therefore, dry matter, crude ash, crude protein (N x 6.25), ether extract (HCl digestion), crude fiber, sugar (Luff-Schoorl) and starch were analysed according to the methods of Naumann and Bassler (1993). Before mixing the feed with oregano, the monoterpenes content was examined. In brief, 50 mg of dried plant material spiked with tetradecane as internal standard was extracted with 1 ml n-hexane in the ultrasonic bath (1 h, 15°C). After centrifugation (10 min at about 10,000 *g*) and filtration, the extract was subjected to gas chromatography (Perkin Elmer Auto System, equipped with flame ionization detector). The extract was chromatographed on a DB1-like Zebron ZB-AAA capillary column (10 m, 0.25 mm). The injection volume was set to 1.5 µl and

the flow rate to 450 ml He/min. The oven temperature program was as follows: 2 min at 70°C, 140°C at 8°C/min followed by 220°C at 20°C/min and held for 2 min at 220°C.

Table 1. Composition of experimental diets (g/kg)

	Group			
	Con*	1*	2*	3*
Components:				
Barley	339.35	337.35	335.35	331.35
Wheat	300.0	300.0	300.0	300.0
Maize, extruded	100.0	100.0	100.0	100.0
Soybean meal, extracted	150.0	150.0	150.0	150.0
Soy protein concentrate	50.0	50.0	50.0	50.0
Soybean oil	30.0	30.0	30.0	30.0
Mineral and vitamin premix [1]	19.0	19.0	19.0	19.0
Amino acids[2]	11.5	11.5	11.5	11.5
Phytase	0.15	0.15	0.15	0.15
Oregano	-	2.0	4.0	8.0
Calculated terpenes (mg/kg DM):				
Carvacrol	-	23.5	46.9	93.9
Thymol	-	1.9	3.8	7.5
y-Terpinen	-	0.8	1.6	3.1
ß-Caryophyllen	-	0.8	1.5	3.1
p-Cymol	-	0.4	0.7	1.4
Analysed composition (g/kg DM):				
Dry matter	899	901	901	896
Crude protein	213	214	211	203
Ether extract	61	57	58	55
Crude fiber	34	37	37	39
Starch	479	471	468	486
Sugar	52	54	54	51
ME [MJ/kg DM][3]	15.87	15.76	15.75	15.73

Note: :*Con, control group; 1, feeding group with 2 g oregano/kg feed; 2, feeding group with 4 g oregano/kg feed; 3, feeding group with 8 g oregano/kg feed

[1] Ingredients per kg complete diet: Ca 4.5 g, P 1.4 g, Na 0.9 g, Mg 0.08 g, Fe 75 mg, Cu 15 mg, Mn 40 mg, Zn 50 mg, I 1 mg, Se 0.2 mg, Co 0.4 mg, Vit A 10.000 IU, Vit D3 1.000 IU, Vit E 50 mg, Vit B1 1 mg, Vit B2 3.1 mg, Vit B6 2.5 mg, Vit B12 20 µg, Vit K3 2 mg, Vit C 50 mg, Nicotinic acid 12.5 mg, Folic acid 0.5 mg, Choline chloride 125 mg, Biotin 50 µg.

[2] Ingredients per kg complete diet: lysin-HCl 6 g, DL-methionine 3 g, L-threonine 2 g, L-tryptophan 0.5 g.

[3]Calculated on base of digestible (table values of the used compounds) crude nutrients (as analysed) according to the formula of the GfE (2008): ME [MJ] = 0.0205 •DCP [g] + 0.0398 • DCL [g] + 0.0173 • St [g] + 0.0160 •Su [g] + 0.0147 • (DOM – DCP – DCL – St - Su) [g] ; where: OM = organic matter, CP = crude protein, CL = crude fat, St = starch, Su = sugar and D = digestible

Hematology and biochemical analyses

Whole blood was used for red blood cell count and analysed using the hematology analyser Celltac-α (MEK 6450, Nihon Kohden Corporation, Tokyo, Japan). Additionally, blood smears were prepared using glass slides and stained with Pappenheim solution to differentiate the white blood cells. At least 200 cells were identified and counted based on morphological characteristics. Parameters of clinical chemistry including albumin, glucose, cholesterol, aspartate aminotransferase (ASAT), γ-glutamyltransferase (γ-GT), bilirubin, total protein, triglycerides and urea were measured in serum samples by an automatic clinical chemistry analyser (Eurolyser CCA180, Eurolab, Austria). Hemolytic samples were ignored while analysing ASAT. Heparinised plasma samples were analysed for cytokines by commercially available ELISA (Quantikine® ELISA, R&D systems). The used immunoassay kits are specific for porcine TNF-α and interleukin-6 (IL-6).

Flow cytometry

Whole EDTA blood was used to determine the production of reactive oxygen species (ROS) in granulocytes and also to phenotype the T-lymphocytes by flow cytometry.

The non-fluorescent dye dihydrorhodamine 123 (DHR) was used for analysing the intracellular radical production of granulocytes. DHR was converted to the fluorescent rhodamine 123 by oxidation of reactive oxidant species (Rothe et al., 1988, Gomes et al., 2005).

The protein kinase C activation agent Phorbol 12-myristate 13-acetate (PMA) induces a maximum of NADPH-oxidase activity and served as positive control. Diphenyleneiodonium chloride (DPI) was used as substance control as it inhibits the activity of the NADPH-oxidase. All samples were incubated with DHR (40 µM) for 15 min at 37°C with or without PMA (20 nM) and/or DPI (10 µM). After the addition of lysis buffer (BD Pharm Lyse™, BD Bioscience San Jose, USA) to the cell suspension to lyse red blood cells for 10 min at room temperature in the dark, all samples were centrifuged (250 *g*, 5 min, 4°C). The cells were resuspended in HEPES buffered saline (HBS), pipetted in a U-bottomed 96-well microplate, and analysed by flow cytometry using a FACS Canto II (BD Sciences, San Jose, USA). The population of granulocytes was identified based on their size and granularity using forward and side scatter measurements and at least 10,000 granulocytes were examined. The results of ROS

production are shown as stimulation index, calculated as the ratio between the percentages of PMA-stimulated cells and percentages of basal-activated granulocytes.

In order to phenotype T cells, whole blood samples were double stained with monoclonal antibodies (mAbs) for $CD4^+$ (Mouse Anti Pig CD4a: FITC, AbD seroTEC, Oxford, United Kingdom) and $CD8^+$ (Mouse Anti Pig wCD8a: RPE, AbD seroTEC, Oxford, United Kingdom) or the corresponding isotype controls (Mouse IgG2a negative Control: RPE and Mouse IgG2b negative Control: FITC, AbD seroTEC, Oxford, United Kingdom) for 30 min at room temperature. After red blood cells were lysed in the lysis buffer (BD Pharm Lyse™), samples were washed by centrifugation in HBS and analysed by flow cytometry. An acquisition gate was set for the lymphoid population according to their side- and forward-scattering properties. At least 10,000 lymphocytes were stored in list mode data files and the spillover of both fluorochromes (FITC and PE) was compensated by the BD FACSDiva™ Software (BD Bioscience, San Jose, CA, USA). An estimated number of cells of each phenotype were further calculated using percentages obtained by flow cytometer. The ratio of $CD4^+$ and $CD8^+$ cells were calculated as the percentage of $CD4^+$ cells divided by the percentage of $CD8^+$ cells.

Calculations and statistics

The performance data were calculated as follows:

Average daily gain (ADG) [g/d] = BW_{final} [g] - $BW_{initial}$ [g]/Time of feeding period [d]; Daily feed intake (DFI) [g] = Feed intake [g]/(Time of feeding [d] · Number of piglets per box); Feed conversion ratio, FCR [kg/kg]= Feed intake [kg]/Gain [kg]; efficiency of metabolizable energy, (MEe)= FCR [kg/kg] · Energy content [MJ/kg feed].

The ME concentration of the diet was calculated by the equation given by the GfE, which is based on digestible nutrients (GfE, 2006). BW was registered for each individual animal (4 animals per box and 5 boxes per group; n=20), while data of feed intake could only be registered per box (5 boxes per treatment; n=5). The statistics of the zootechnical data were performed by using the SAS software package (SAS Enterprise Guide 4.3) and the procedure GLM to evaluate the data. Feeding group was considered as fixed effect in one way analysis of variance (ANOVA).

Data involving the LPS challenge were evaluated according to a complete 2 by 4 factorial design of ANOVA using the procedure GLM of the SAS-software package (SAS Enterprise Guide 4.3).

Oregano supply, challenge (NaCl vs. LPS) and interactions between oregano supply and challenge were considered as fixed effects in the multifactorial ANOVA. When effects were considered as significant in the F test (p <0.05), the means were compared using the Tukey test. Values presented in the paper below are shown as means±standard error (SE). For flow cytometry analysis the FACS Diva software (BD Bioscience) was used. The results are expressed as the percentage of granulocytes and lymphocytes subpopulation, respectively.

Results

Diet composition

The analytical results of the chemical compositions of the various diets are shown in Table 1. The values achieved were according to the recommendations of the Society of Nutrition Physiology (Gfe, 2006).

Performance data

The different diets showed no significant effects on the performance parameters during the whole experimental period (Table 2). All animals accepted the offered feed. On average, all piglets had an ADG of 473±80 g/d and a daily feed intake of 796±79 g/animal during 5 experimental weeks. Accordingly, the values for FCR and the efficiency of the metabolizable energy were on average 1.7±0.3 kg feed/kg gain and 24.2±4.2 MJ/kg, respectively, for all piglets throughout the entire test. No acute pathologies were seen in any of the piglets, all animals appeared to be in good health.

Hematology and blood chemistry

In general, LPS injection induced an acute systemic inflammatory response as indicated by significantly elevated blood levels of the pro-inflammatory cytokines TNF-α and IL-6 (Table 3). Red blood count was characterised by an increased erythrocyte (NaCl, $5.3 \cdot 10^6$ µl; LPS, $5.7 \cdot 10^6$ µl) and haemoglobin concentration (NaCl, 10.3 g/dl; LPS, 11.3 g/dl) and an elevated hematocrit (NaCl, 29.5%; LPS, 32.5%, Table 4) while the derived indices remained unaffected. LPS further induced a general leucopenia caused by a lymphopenia, an associated decrease in $CD8^+$ cells, a neutropenia and a monocytopenia (Tables 5 and 6).

Table 2. Growth performance parameter of piglets fed with oregano supplemented feed

	Oregano supplementation per kg feed					
	0 g (Control)	2 g	4 g	8 g	PSEM[†]	*p*
Average daily gain [g/d](n=20)						
Day 1-14	297	308	314	328	19	*0.705*
Day 15-35	594	568	570	588	22	*0.786*
Day 1-35	475	464	468	484	18	*0.868*
Daily feed intake [g/animal d] (n=5)						
Day 1-14	487	518	509	514	17	*0.919*
Day 15-35	1017	936	984	997	22	*0.599*
Day 1-35	817	769	794	804	19	*0.823*
Feed conversion ratio [kg/kg] (n=5)						
Day 1-14	1.64	1.68	1.64	1.57	0.03	*0.575*
Day 15-35	1.73	1.66	1.73	1.69	0.03	*0.816*
Day 1-35	1.74	1.67	1.71	1.66	0.03	*0.709*
ME efficiency [MJ/kg BWG[‡]] (n=5)						
Day 1-14	23.45	23.89	23.34	22.10	0.4	*0.483*
Day 15-35	24.63	23.64	24.61	23.85	0.4	*0.765*
Day 1-35	24.76	23.67	24.25	23.35	0.4	*0.596*

Note: [†]PSEM, pooled standard error of means, [‡]BWG, Body weight gain

Blood chemistry was not altered due to LPS injection except triglycerides, which were elevated (NaCl, 43.5 mg/dl; LPS, 56.7 mg/dl, Table 7). The LPS-treated piglets showed one or more of the clinical signs: elevated rectal temperature (Table 3), tremor, dyspnoea and lethargy. These signs became obvious within 30 min after injection and lasted for approximately 3 to 4 h.

Table 3. Effects of oregano and lipopolysaccharide (LPS) challenge on cytokine production

Oregano [g/kg]	LPS [µg/kg]	TNF-*α** [pg/ml]	IL-6[†] [pg/ml]	Body temperature [°C]
—	0	93	—	39.0
—	5	2896	258	39.4
0	—	1845	268	39.1
2	—	1532	233	39.1
4	—	1280	447	39.4
8	—	1154	209	39.3
PSEM[‡]		515	154	0.13
ANOVA (p-value)				
Oregano		0.794	0.603	0.333
LPS		<0.001		0.010
Oregano×LPS		0.800		0.488

Notes: *TNF- α, tumor necrosis factor- α; IL-6[†], interleukin-6; [‡]PSEM, pooled standard error of means

There were no effects of oregano on red blood cell count parameters, neither in non-stimulated- nor in LPS-stimulated piglets (Table 4). In contrast, white blood cell count tended to be higher ($p < 0.1$) and that of lymphocytes increased significantly by oregano supplementation ($p = 0.044$; Table 5). These effects occurred in non-stimulated- and LPS-stimulated piglets and seemed to be independent of oregano dose (Table 6).

Table 4. Effects of oregano and lipopolysaccharide (LPS) challenge on parameters of red blood cell count

Oregano [g/kg]	LPS [µg/kg]	†RBC [$\cdot 10^6$/µl]	‡HGB [g/dL]	*HCT [%]	°MCV [fL]	$MCH [pg]	§MCHC [g/dL]	#RDW [%]
—	0	5.3	10.3	29.5	56.3	19.8	35.1	15.6
—	5	5.7	11.3	32.5	56.6	19.8	34.9	15.6
0	—	5.4	10.5	30.3	56.0	19.5	34.8	15.7
2	—	5.5	11.0	31.4	56.7	20.1	35.4	15.6
4	—	5.4	10.6	30.6	56.7	20.0	35.2	15.6
8	—	5.6	11.0	31.8	56.4	19.6	34.6	15.5
PSEM⬩		0.2	0.3	1.0	0.6	0.3	0.3	0.2
ANOVA (p-value)								
Oregano		0.794	0.579	0.689	0.808	0.315	0.167	0.857
LPS		0.009	0.003	0.004	0.500	0.932	0.350	0.940
Oregano × LPS		0.593	0.680	0.693	0.744	0.795	0.877	0.906

Notes: †RBC, red blood cells, ‡HGB, hemoglobin, *HCT, hematocrit, °MCV, mean cell volume, $MCH, mean corpuscular hemoglobin, §MCHC, mean corpuscular hemoglobin concentration, #RDW, red cell distribution width; ⬩PSEM, pooled standard error of means

More particularly, $CD4^+$ and $CD8^+$ T-cell subpopulation contents seemed to be smaller in oregano-fed animals. These effects emerge both in stimulated- and non-stimulated piglets with no clear oregano dosing effect neither in the $CD4^+$ subpopulation nor in $CD8^+$ cells (Table 6). The significant feeding effect of oregano was, however, recognized in the ratio of both subpopulations ($p = 0.011$) in LPS-stimulated as well as in non-stimulated animals. $CD4^+CD8^+$ double positive cells were significantly lower in LPS-stimulated animals ($p < 0.05$, Table 6), but independent of oregano supplementation. The relative density of CD8 expression of $CD8^+$ cells and $CD4^+CD8^+$ double positive cells was determined by the mean fluorescence intensity and did not show significant changes between feeding group or differences between LPS-stimulated or non-stimulated group (data not shown).

Table 5. Effects of oregano and lipopolysaccharide (LPS) challenge on parameters of white blood cell count [$\cdot 10^3/\mu l$].

Oregano [g/kg]	LPS [µg/kg]	White blood cells	Lympho-cytes	Segmented neutrophils	Banded neutrophils	Mono-cytes	Eosino-phils	Baso-phils	Normoblasts
—	0	21.2	10.1	9.1	0.5	1.2	0.12	0.02	0.20
—	5	14.0	6.5	5.7	1.1	0.5	0.08	0.03	0.14
0	—	14.9	6.6	6.6	0.71	0.71	0.1	0.02	0.09
2	—	20.1	9.1	8.9	0.69	1.16	0.1	0.02	0.15
4	—	17.5	8.4	7.3	0.80	0.79	0.06	0.02	0.21
8	—	17.6	9.1	6.7	0.86	0.64	0.13	0.04	0.22
PSEM[†]		1.5	0.7	0.8	0.2	0.1	0.03	0.01	0.05
ANOVA (p-value)									
Oregano		0.093	0.044	0.172	0.943	0.065	0.455	0.468	0.202
LPS		<0.001	<0.001	<0.001	0.003	<0.001	0.266	0.435	0.174
Oregano × LPS		0.462	0.313	0.325	0.298	0.350	0.367	0.053	0.930

Notes: [†]PSEM, pooled standard error of means

Table 6. Effects of oregano and lipopolysaccharide (LPS) challenge on relative numbers of $CD4^+$ and $CD8^+$ T-lymphocytes

Oregano [g/kg]	LPS [µg/kg]	$CD4^+$ [%]	$CD8^+$ [%]	$CD4^+CD8^{+\dagger}$ [%]	Ratio $CD4^+$ to $CD8^+$
—	0	27.2	28.8	11.6	0.98
—	5	34.3	22.3	9.6	1.67
0	—	32.5	29.0	11.7	1.18^{ab}
2	—	28.1	28.6	11.1	1.04^{b}
4	—	31.5	28.0	10.5	1.20^{ab}
8	—	30.9	24.1	8.8	1.45^{a}
PSEM[1]		1.36	1.53	0.92	0.08
ANOVA (p-value)					
Oregano		0.134	0.092	0.144	0.011
Challenge		<0.001	<0.001	0.045	<0.001
Oregano x challenge		0.193	0.966	0.835	0.255

Notes: † $CD4^+CD8^+$, Double positive T-lymphocytes; [‡]PSEM, pooled standard error of means

[ab] Means with different superscripts within the same column are significantly different ($p < 0.05$)

The granulocytes stimulation index was not altered by oregano supplementation, neither in LPS-stimulated nor in non-stimulated animals. Nevertheless, the basal activity was not affected by LPS stimulus but it tended to be influenced by oregano supplementation ($p < 0.1$, Table 8). The inhibition of the NADPH-oxidase by the inhibitor DPI leads to no change in basal radical formation in granulocytes (data not shown).

There were no effects of oregano on clinical-chemistry parameters, neither in stimulated nor in non-stimulated animals with an exception of triglycerides and γ-glutamyltransferase. Triglycerides were both affected by feeding of oregano ($p < 0.03$) and by LPS stimulus ($p = 0.001$), whereas γ-glutamyltransferase tended to be higher in LPS-stimulated animals independent of oregano dose (Table 7).

The cytokine production of TNF-α and IL-6 was not affected by oregano feeding in LPS-stimulated or in non-stimulated animals (Table 3).

Table 8. Effects of oregano and lipopolysaccharide (LPS) challenge on the activity of granulocytes

Oregano [g/kg]	LPS [µg/kg]	Basal activated granulocytes (CON) [%]	PMA[#] [%]	Stimulation index (CON/PMA)
…	0	1.5	95.7	104.2
…	5	2.6	97.1	102.3
0	–	2.5	94.1	67.8
2	–	1.9	96.5	97.8
4	–	1.4	97.4	138.7
8	–	1.4	98.6	121.0
PSEM[†]		0.5	1.3	23.7
ANOVA (p-value)				
Oregano		0.073	0.105	0.175
LPS		0.102	0.159	0.912
Oregano × LPS		0.284	0.862	0.721

Notes: [#]PMA, cells stimulated with Phorbol 12-myristate 13-acetate,
[†]PSEM, pooled standard error of means

Table 7. Effects of oregano and lipopolysaccharide (LPS) challenge on parameters of clinical chemistry

Oregano [g/kg]	LPS [µg/kg]	albumin [g/l]	glucose [mg/dl]	cholesterol [mg/dl]	ASAT* [U/l]	γ-GT† [U/l]	bilirubin [mg/dl]	total protein [g/l]	triglyceride [mg/dl]	urea [mg/dl]
—	0	29.4	102.9	67.5	44.9	30.0	1.6	47.4	43.5	22.4
—	5	29.0	106.7	65.9	51.8	34.0	1.6	45.4	56.7	21.2
0	—	28.8	112.1	65.0	49.3	30.2	1.5	44.3	43.9	20.4
2	—	28.8	102.5	63.5	39.4	32.3	1.6	46.4	44.2	24.2
4	—	29.0	100.4	69.8	51.6	30.5	1.7	46.8	58.2	20.6
8	—	30.3	104.3	68.6	51.5	35.2	1.6	48.0	54.1	21.8
PSEM[#]		1.1	4.5	2.9	5.5	2.4	0.1	1.4	4.0	1.6
ANOVA (p-value)										
Oregano		0.713	0.286	0.396	0.322	0.441	0.177	0.300	0.026	0.340
LPS		0.734	0.401	0.580	0.318	0.103	0.333	0.136	0.001	0.458
Oregano × LPS		0.370	0.953	0.283	0.014	0.323	0.108	0.618	0.209	0.385

Notes:***** ASAT, Aspartate aminotransferase (haemolytic samples were excluded, $n = 69$); γ-GT, γ-glutamyltransferase; [#]PSEM, pooled standard error of means

Discussion

The present experiment has aimed to answer the question if oregano supplementation has an effect on performance data, like feed intake and daily gain. Furthermore, the immunmodulating aspects in both non-stimulated and LPS-stimulated piglets were determined. Consequently, immune-related cells like T-cell subpopulations ($CD4^+$ and $CD8^+$) and granulocytes were phenotyped and analysed for the release of reactive oxygen radicals. Taken together, the mentioned parameters were chosen as indicators for the effects of oregano on the immune system.

Influence of oregano supplementation on performance data of weaning piglets

In the present study, the supplementation of dried oregano plants did not influence the performance of the piglets according to other investigations (Hebeler et al., 2000, Henn et al., 2010, Neill et al., 2006). With regard to the feed intake, our findings indicate slightly reduced values in oregano-supplemented animals compared to the control group (Table 2). Investigations of Walter and Bilkei (2004) with growth-retarded, growing-finishing pigs showed improved ADG and FCR with only 3 g oregano/kg which results in 180 mg carvacrol per kg feed. The period of oregano supplementation was in accordance with the current study, but the carvacrol content was in our trial at the highest inclusion rate, with 94 g/kg almost half compared to the trial of Walter and Bilkei (2004). Therefore, dose-dependent and age-related effects could be important when using oregano in feed. The dosage of our investigation was based on data of different studies which were already conducted with weaned piglets or other animal species like lambs and chicken (Schuhmacher et al., 2002, Bampidis et al., 2005, Docic and Bilkei, 2003, Ragland et al., 2007).

Influence of oregano and LPS-challenge on immunological parameters

LPS is recognised by specific receptors on macrophages and other immune cells and activates a cascade of different acute-phase and inflammatory reactions (Pålsson-Mcdermott and O'neill, 2004). In accordance with Ceciliani et al. (2002) the application of LPS induced a significantly higher body temperature (Table 3, LPS-stimulated animals, 39.4°C; non-stimulated, 39.0°C) of animals in the present study ($p < 0.05$) after 2 h, followed by diminished feed intake and changes in plasma concentration of acute phase proteins (Wright et al., 2000). Our results showed

significantly higher TNF-α values in LPS-treated piglets compared to non-stimulated animals ($p < 0.001$) similar to Webel et al. (1997) and Liu et al. (2012). Consequently, the implementation of the LPS-challenge to provoke a systemic inflammation was successful.

Immunmodulating effects of oregano were shown in the investigations of Major et al. (2011). These researchers demonstrated an effect of oregano on intestinal mucin dynamics in chickens, which acts as a barrier for pathogens and harmful intraluminal components (Smirnov et al., 2005). Oregano was supposed to have a beneficial effect on the mucin quantity while initiating the mucin production resulting in an increased thickness. Consequently, the translocation of pathogens could become more difficult, noteworthy especially with regard to the fact that an increased mucin layer corresponds to increasing proportions of IgM+ cells and the activity of phagocytic cells (Major et al., 2011). In vitro investigations of Liu et al. (2012) with peripheral blood cells showed that a 24-h LPS stimulation inhibited cell viability of macrophages in a significant way. In contrast to that, they showed that the viability of LPS-stimulated macrophages treated with carvacrol was similar to that without LPS. Hotta et al. (2010) also showed the beneficial effect of carvacrol on LPS-stimulated macrophages. The result of their investigation was that carvacrol influenced the prostaglandin biosynthesis in that it supressed the enzyme cyclooxygenase-2. Major et al. (2011) and Ramadan et al. (2013) also investigated the relationship between oregano supplementation and an artificial infection *in vivo*. The researchers also recognised a tendency of increased proportions of WBC in oregano-fed chickens or albino rats, too. Our results indicate an influence of oregano supplementation on immune cells, especially on lymphocytes. Piglets receiving oregano-supplemented feed had higher proportions of lymphocytes compared to control animals (Table 5). Major et al. (2011) suggested that oregano-supplemented feeding of infected chickens led to a higher leukocyte content, but they did not differentiate if B or T-lymphocytes were the reason for this increased number. It is possible that pigs receiving oregano were able to mobilise more lymphocytes. Our results showed an increased concentration of lymphocytes in non-stimulated animals by oregano supplementation, but only a tendency of oregano on $CD4^+$ and $CD8^+$ T-cell subpopulations in stimulated and non-stimulated animals, which may suggest that the increased proportion of lymphocytes were caused by B-lymphocytes. The content of $CD4^+$ T-cells in the current study decreased not significantly while the oregano concentration increased, whereas $CD8^+$ T-

lymphocytes decreased from 31.5%±3.1 to 24.9%±1.5. Finally, the ratio between both subpopulations differed significantly (Table 6). These results differ from the investigation of Walter and Bilkei (2004) who demonstrated higher proportions of both $CD4^+$ and $CD8^+$ cells in oregano-fed animals. This contradiction to the present investigation could be the result of the age-related increase of the $CD4^+$ and $CD8^+$ T-cells (Joling et al., 1994) as we used 5-10 week-old piglets, whereas Walter and Bilkei (2004) used fattening pigs. Under the conditions of an artificial infection Major et al. (2011) ascribed the decreased number of $CD8^+$ cells with its migrating to the infected area. In addition, bacterial infection-like conditions as stimulated by the LPS challenge in our study, enhanced the activity of T-cells by increased migration and infiltration of $CD4^+$ and $CD8^+$ T-lymphocytes in the infected area (Saalmüller, 1998). With regard to the present experiment it seems to be possible that $CD4^+$ and $CD8^+$ cells were attracted to the peritoneal cavity where LPS was injected. It is suggested that the induced endotoxin shock results in a decrease in circulating WBC (Al-Sagair et al., 2009, Opdahl et al., 1993, Smedegard et al., 1989). Further investigations with LPS stimulation and oregano treatment demonstrated, with special regard to the function of macrophages, that LPS-activated cells blended with oregano present significant nitric oxide (NO) suppressing activity (Tsai et al., 2007). Nitric oxides play an important role as a second messenger in case of an inflammation. Next to nitrosative radicals, oxygen radicals are also important for bacterial defence. Our evaluations of granulocyte radical activity demonstrated no effect of oregano whereas other researchers demonstrated that carvacrol leads to enhanced ROS release (Huang et al., 2010). These researchers showed that carvacrol pre-treated glioma cells caused an increased release of ROS by simultaneous stimulation of LPS and PMA *in vitro*. According to the present study the proportions of granulocyte activity tended to be higher in LPS-stimulated animals (Table 8, $p < 0.1$). Therefore, it is suggested that animals had higher proportions of ROS producing granulocytes after LPS stimulus than non-stimulated animals independent of oregano dose. Furthermore, it was possible that the released radicals were independent of NADPH-oxidase enzyme system.

Investigations of Basmacioğlu Malayoğlu et al. (2010) with broilers showed altered antibody titer of IgG and IgM by oregano feeding. Furthermore, these researchers noted no significant effect of oregano on clinical-chemistry parameter serum triglyceride, which contrasts with our findings.

Several studies reported about an increased serum triglyceride concentration induced by LPS stimulation, which is caused by an increased lipoprotein production as well as a decrease in lipoprotein clearance (Uchiumi et al., 2004, Feingold et al., 1992). Considering the effect of oregano on the lipid metabolism alone, investigations with mice fed a high-fat diet demonstrated a decreased BW and lower visceral fat-pad weights when the diet was supplemented with 0.1% carvacrol and additionally a slightly lower triglyceride content compared to the high-fat group (Cho et al., 2012). The researchers concluded that carvacrol inhibits visceral lipogenesis by suppressing different signalling cascades. With regard to ASAT, Ramadan et al. (2013) demonstrated no significant changes in rats treated with sweet majoram (*Origanum majorana* L.), but high levels of this herb (100 mg/kg body weight) mitigated the effects of isoproterenol treatment. Our findings demonstrated an interaction between the feeding group and LPS treatment for ASAT, which was caused by a marked increase in ASAT activity in piglets stimulated with LPS and fed the highest level of oregano. It needs to be considered that this effect was characterised by a high inter-individual variation. Such effects were not observed for the control group and the other two lower oregano supplementation levels. The nature and the relevance of these findings need to be clarified further.

In conclusion, the results of the current study did not show an improved performance by oregano supplementation, whereas alterations on immunological parameters, especially on lymphocytes, were recognised. In combination with an induced systemic inflammation, higher proportions of lymphocytes, but not higher proportions of $CD4^+$ and $CD8^+$ T-cells were demonstrated. Further studies are necessary to investigate effects on lymphocytes with a focus on further subpopulations, such as B-cells or not yet primed T-cells and other immunological parameter like phagocytic activity. With regard to the mode of action of essential oils versus dried plant material, higher concentrations on carvacrol and thymol in oregano material could be of interest. Further investigations of the comparison of both pharmaceutical formulations concerning the absorption of the active substances are necessary.

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5 Paper II

The use of restriction fragment length polymorphism and fluorescence *in situ* hybridization to investigate the microbiota of piglets after feeding oregano

Katrin Stelter[1], Andreas Berk[1], Lutz Geue[2], Stefanie Barth[2], Petra Schlien[3], Alexander Swidsinski[3] and Sven Dänicke[1]

[1]Institute of Animal Nutrition, Friedrich-Loeffler-Institute (FLI), Federal Research Institute for Animal Health, Braunschweig

[2]Institute of Molecular Pathogenesis, Friedrich-Loeffler-Institute (FLI), Federal Research Institute for Animal Health, Jena

[3]Charité Hospital, CCM, Laboratory for Molecular Genetics, Polymicrobial Infections and Bacterial Biofilms and Department of Medicine, Gastroenterology, Universitätsmedizin Berlin, Berlin

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PAPER II

Abstract

A total of 80 piglets (7.9 ± 1.0 kg) were used in a feeding experiment with dried oregano. The diets differed in their oregano content: 0 g, 2 g, 4 g and 8 g oregano/kg feed, corresponding to 0, 23.5, 46.9 and 93.9 mg carvacrol/kg DM. After the experimental period of 5 weeks 20 piglets of both extreme feeding groups were slaughtered: 10 animals of the control group and 10 animals of the group that received 8 g oregano/kg. Ingesta samples of jejunum, caecum and colon were collected and analyzed by FISH and PCR-RFLP to compare the diversity of microbiota. The results showed no significant changes in microbiota in response to oregano. The patterns of the PCR-RFLP showed a similarity of 61.8 - 91.8% in both feeding groups. In conclusion, an effect of oregano on the intestinal microbiota could not be shown under the methods used.

Keywords: Piglets; *Origanum vulgare* L.; Fluorescence in situ hybridization; Restriction fragment length polymorphism; Intestinal microorganisms

Introduction

Weaning with the separation of the sow and the new environmental conditions is a very stressful situation for piglets. Especially the change from suckling to solid feed is a major challenge for the pig and is associated with major changes in the microbiota of the gastrointestinal tract (GIT). The GIT represents a dynamic ecosystem which is very complex and consists of micro-aerophilic and anaerobic microbes (Konstantinov et al., 2004) that are important for nutrient digestion, the support of the mucosal immunity and the development of the gut-associated-lymphoid tissue (Bauer et al., 2006b). Stress during weaning often caused an altered composition of the microbiota in the gut (Wallgren and Melin, 2001, review of Lallès et al., 2007). Piglets with a low diversity of the enteric flora were more susceptible to diarrhea than animals with a high diversity (Kühn et al., 1993). Konstantinov et al. (2004) postulated that a stable and complex microbiota is a prerequisite for healthy bowel function. To support the mucosal immunity and the microbiota in this critical period of life, the Mediterranean herb O*riganum vulgare* L. with its antimicrobial abilities was focused on (Şahin et al., 2004, Burt, 2004). The main components of its essential oil are carvacrol and thymol which were used in several *in vitro* investigations to evaluate the antimicrobial activity (Henn et al., 2010, Mathlouthi et al., 2012, Çetin et al., 2011).

For example, in vitro investigations of Michiels et al. (2009) with essential oil components of oregano (carvacrol and thymol) showed antimicrobial opportunities in gastric, jejunal and ceacal simulations. The researchers recognized a reducing effect on total anaerobic bacteria in all three types of simulations. In vivo investigations with oregano supplementation to the feed could lead to alterations in microbial counts, for example lactobacilli (Manzanilla et al., 2004, Castillo et al., 2006). Herb supplementations, in general, are suggested to reduce only harmful coliform bacteria in the gut while in-feed antibiotics presumably inhibit also the proliferation of beneficial bacteria like lactobacilli (Namkung et al., 2004). In previous studies oregano was commonly administered as extract or essential oil, whereas the aim of the current investigation was to examine the influence of dried oregano plant material on the gut microbiota of weaning piglets.

Material and methods

Plant material

The utilized *Origanum vulgare* L., belonging to the labiate family, was cultivated and harvested on the experimental plots of the Institute for Plant Cultivation, Schnega, Germany. The aerial parts were harvested by manual cutting after the flowering stage in mid-September 2011. Thereafter, the plant material was dried down to a final moisture content of app. 9% at 40°C in tray driers. Finally, the dried oregano was ground to 4 mm using a hammer mill (Joachim Kreyenborg & Co, type 10, Münster-Kinderhaus, Germany). Analysis of the monoterpenes content ensued and was determined on dry matter (DM) basis.

Experimental design

Animal experiment

The experiment was conducted at the Institute of Animal Nutrition, Federal Research Institute for Animal Health, Braunschweig, Germany in March 2012 according to the European Community regulations concerning the protection of experimental animals and the guidelines of the regional council of Oldenburg, Lower Saxony, Germany.

The present investigations were performed within the frame of a more comprehensive piglet experiment examining the effects of increasing proportions of dried oregano plant material in piglet feed on performance and health. The experiment is described in detail elsewhere (Stelter et al., 2013).

PAPER II

For the investigation 80 male castrated piglets with an average body weight of 7.9 ± 1.0 kg were housed in floor pens of the piglet compartment. Piglets were allowed five days of adaptation prior to the onset of the experimental period. On day one of the study all piglets were weighed and allocated to four feeding groups according to their individual weight (n=20). The allocation into four groups resulted in a number of four animals per box (n=5). The diet without oregano supplementation served as control diet (CON) while the other three diets included 2 g oregano/kg feed (group 1), 4 g oregano/kg feed (group 2) or 8 g oregano/kg feed (group 3), corresponding to calculated dietary carvacrol concentrations of 23.5 mg, 46.9 mg and 93.9 mg/kg DM, respectively. The dried and milled oregano was admixed to the basic diet to obtain the desired concentrations. The diets consisted mainly of cereals, soy feedstuff, amino acids, vitamins and minerals to meet the recommendations of the Society of Nutrition Physiology (Gfe, 2006). The compositions of the diets are shown in Table 1. Feed, in mesh form, and water were offered *ad libitum* during the experimental period of five weeks. For the current experiment, only 20 animals of the extreme feeding groups (CON and 8 g oregano/kg feed) were focused on.

Collection procedures

After terminating the feeding experiment of five weeks, 10 piglets of the control group and 10 animals of group 3 (8 g oregano/kg) with an average body weight of 19.9 ± 2.7 kg were slaughtered by electrical stunning followed by exsanguination to evaluate intestinal microbiota. The piglets were allowed feed intake prior to slaughter to obtain well-filled intestines. The gastrointestinal tract (GIT) was removed carefully to avoid digesta mixing and samples taken from jejunum, cecum and colon. For analysis the entire ingesta of the jejunum was collected by carefully squeezing and mixed thoroughly while samples of cecum and colon were collected at a defined location. All samples for PCR-RFLP analysis were frozen immediately after collection at -20°C, while samples for FISH were fixed in Carnoy's solution (Matsuo et al., 1997) until analysis.

Table 1. Composition and characteristics of experimental diets (g/kg)

	Group	
	CON	ORE
Components:		
Barley	339.35	331.35
Wheat	300.00	300.00
Maize. extruded	100.00	100.00
Soybean meal. extracted	150.00	150.00
Soy protein concentrate	50.00	50.00
Soybean oil	30.00	30.00
Mineral and vitamin premix [1]	19.00	19.00
Amino acids[2]	11.50	11.50
Phytase	0.15	0.15
Oregano	-	8.00
Calculated terpenes (mg/kg)		
Carvacrol	-	93.9
Thymol	-	7.5
y-Terpinen	-	3.1
ß-Caryophyllen	-	3.1
p-Cymol	-	1.4
Analyzed composition (g/kg DM):		
Dry matter	899	896
Crude protein	213	203
Ether extract	61	55
Crude fiber	34	39
Starch	479	486
Sugar	52	51
ME [MJ/kg DM][3]	15.87	15.73

Notes: CON, control group; ORE, feeding group with 8 g oregano/kg feed

[1] Ingredients per kg complete diet: Ca 4.5 g, P 1.4 g, Na 0.9 g, Mg 0.08 g, Fe 75 mg, Cu 15 mg, Mn 40 mg, Zn 50 mg, I 1 mg, Se 0.2 mg, Co 0.4 mg, Vit A 10.000 IU, Vit D_3 1.000 IU, Vit E 50 mg, Vit B_1 1 mg, Vit B_2 3.1 mg, Vit B_6 2.5 mg, Vit B_{12} 20 µg, Vit K_3 2 mg, Vit C 50 mg, Nicotinic acid 12.5 mg, Folic acid 0.5 mg, Choline chloride 125 mg, Biotin 50 µg.

[2] Ingredients per kg complete diet: lysin-HCl 6 g, DL-methionine 3 g, L-threonine 2 g, L-tryptophan 0.5 g.

[3]Calculated on base of digestible (table values of the used compounds) crude nutrients (as analysed) according to the formula of the GfE (2008): ME [MJ] = 0.0205 •DCP [g] + 0.0398 • DCL [g] + 0.0173 • St [g] + 0.0160 •Su [g] + 0.0147 • (DOM – DCP – DCL – St - Su) [g]; where: OM = organic matter, CP = crude protein, CL = crude fat, St = starch, Su = sugar and D = digestible

Analyses

Oregano and feed

Representative samples of each diet were collected and analyzed for dry matter, crude ash, crude protein (N x 6.25), ether extract (HCl digestion), crude fiber, sugar (Luff-Schoorl) and starch according to the methods of the Association of German Agricultural Analysis and Research Centres (Verband Deutscher Landwirtschaftlicher Untersuchungs- und Forschungsanstalten, VDLUFA, Naumann and Bassler (1993)). Prior to mixing the feed with oregano, the monoterpenes content was examined. For this, 50 mg dried plant material spiked with tetradecane as internal standard was extracted with 1 ml n-hexane in the ultrasonic bath (1 hour, 15°C). After centrifugation (10 minutes at about 10,000 x g) and filtration the extract was subjected to gas chromatography (Perkin Elmer Auto System, equipped with flame ionization detector). The extract was chromatographed on a DB1-like Zebron ZB-AAA capillary column (10 m, 0.25 mm). The injection volume was set to 1.5 µl and the flow rate to 450 ml He/minute. The oven temperature program was as follows: 2 minutes at 70°C, 140°C at 8°C/minute followed by 220°C at 20°C/minute and held for 2 minute at 220°C. Analysis of the monoterpenes revealed the following composition: p-cymene (0.18 g/kg), β-caryophyllene (0.38 g/kg), γ-terpinene (0.39 g/kg), thymol (0.94 g/kg), carvacrol (11.7 g/kg).

PCR-RFLP analysis

For DNA analysis, samples of about 1 g of ingesta of the proximal colon were kept in sterile Falcon tubes and frozen immediately (-20°C) until PCR-RFLP analysis. Prior to the analysis DNA of the ingesta samples was extracted with the commercial QIAamp® DNA Stool Mini Kit (QIAGEN®, Hilden, Germany) as recommended by the manufacturer. The total bacteria of the ingesta was analyzed according to Castillo et al. (2006). A 580-bp fragment of 16S rRNA gene was amplified from DNA extracts by PCR. For these purpose primers specific to conserved sequences flanking variable regions V3, V4 and V5 were utilized: 5'-CTACGGGAGGCAGCAGT-3' (forward) and 5'-CCGTCWATTCMTTTGAGTTT-3' (reverse). The DNA was amplified using a FlexCycler (Analytik Jena AG, Jena, Germany) with the following conditions: One denaturation step of 4 minutes at 94°C, followed by 35 cycles of denaturation at 94°C (1 minute), annealing at 45°C (1 minute) associated with an increasing

temperature of 0.1°C per cycle, extension at 72°C (1 minute 15 seconds) and a final extension at 72°C (15 minutes). After amplification the PCR products were visualized with agarose gel electrophoresis. The next step was the enzymatic restriction with three independent restriction enzymes (*Rsa*I, *Hpa*II and *Hha*I (New England Biolabs GmbH, Frankfurt/M., Germany). The digestion was carried out with the appropriate restriction buffer at 37°C for 3 hours as recommended by the manufacturer. Using a 2% high-resolution agarose gel, the fragments were separated. Bands between 100 and 580 bp length were used to generate restriction profiles for each piglet. For pairwise comparisons of restriction profiles and construction of dendrograms, similarity matrices were generated with Hierarchical Clustering Explorer 3.0 (Seo and Shneiderman, 2003) using Manhattan distance.

Fluorescence in situ hybridization (FISH)

Ingesta samples of about 1 ml from jejunum, cecum and colon for fluorescence in situ hybridization (FISH) were immediately fixed in Carnoy's solution (Matsuo et al., 1997) for at least 12 hours. Similar conditions were used for gram positive and gram negative bacteria. The ingesta samples were assessed with group specific 16S rRNA gene-targeted oligonucleotide probes (Table 2). FISH method was performed according to Swidsinski (2011). The soft chyme sediment from the fixed samples was directly pipetted on a slide (50 µl) and then dried at 50°C for 30 minutes. Next, the hybridization step followed. The utilized oligonucleotide probes were synthesized with the reactive fluorescent dye carbocyanite 3 (Cy3) (*Bifidobacterium* group, *Faecalibacterium prausnitzii* group) or Cy5 (*Enterobacteriaceae* group, *Bacteroides* group, MWG Biotech, Ebersberg, Germany) at the 5'-end. Detecting all relevant bacteria the universal probe Eub338 was used as well as the nonsense probe Non338 to test nonspecific binding of oligonucleotide probes. Hybridization temperature was 50°C and formamide concentration was 1%. The quantification of the fecal bacteria was performed under a Nikon e600 fluorescence microscope (Nikon, Tokyo, Japan). The documentation of the bacteria was conducted using a Nikon DXM 1200F color camera and software (Nikon, Tokyo, Japan).

Table 2: Sequences of oligonucleotide probes

Probe	**Probe sequence (5'→3')**	**Targeted species**	**Fluorescent dye**	**Reference**
Bac303	CCA ATG TGG GGG ACC TT	most Bacteroidaceae and Prevotellaceae, some Porphyromonadaceae	Cy5	Manz et al. (1996)
Bif164	CAT CCG GCA TTA CCA CCC	*Bifidobacterium* spp.	Cy3	Langendijk et al. (1995)
Fprau645	CCT CTG CAC TAC TCA AGA AAA AC	*Fusobacterium prausnitzii* and relatives	Cy3	Suau et al. (2001)
Ebac1790	CGT GTT TGC ACA GTG CTG	Enterobacteriaceae	Cy5	Bohnert et al. (2000)
Eub338	GCT GCC TCC CGT AGG AGT	most Bacteria	FITC	Amann et al. (1990)
NonEub338	ACT CCT ACG GGA GGC AGC	control probe		Wallner et al. (1993)

Notes: Bac, Bacteriodes and Prevotella group; Bif, Bifidobacterium ssp; Fprau, Faecalibacterium prausnitzii group; Ebac, Enterobacteriaceae; Eub, Eubacteria

Statistical analysis

The results of the FISH analysis were tested with a multifactorial ANOVA using the GLM procedure of the SAS-software (SAS Enterprise Guide 4.3). The intestinal segment and the feeding group were defined as fixed effects. Values presented in the paper below are shown as mean ± standard deviation (SD).

Analysis of RFLP pattern was performed with Bionumerics (version 6.6, applied Maths, Belgium) and Hierarchical Clustering Explorer 3.0 (Human-Computer Interaction Lab, University of Maryland).

Results

In general, all animals accepted the oregano supplemented feed and there were no acute pathologies in any of the piglets, all animals appeared to be in good health. An effect on the performance data has failed during the whole experimental period. The piglets had an average daily gain of 473 ± 80 g/d and a daily feed intake of 796 ± 79 g/animal during the whole experimental time (Stelter et al., 2013).

Diet composition

The analytical results of the chemical compositions of the various experimental diets are shown in Table 1. The values were attained according to the recommendations of the Society of Nutrition Physiology (GfE, 2006).

Distribution and morphology of bacteria analyzed by FISH

The microorganisms in the investigated intestinal segments were mainly concentrated in the caecum and colon independent of oregano supplementation. Small amounts of microorganisms were counted in the jejunum in contrast to caecum and colon, except for B*ifidobacteria* (Bif) which were the major genus in this segment. In Figure 1 the distribution of the microorganisms is summarized. It was notable that the bacteria have populated the majority of the caecum and colon. While higher counts of enterobacteria were detected in colon, more amounts of *Bifidobacteria* colonized the caecum. The results of the FISH analysis are not documented in writing, because there are, partially, an insufficient number of bacterial counts in the different

parts of the intestine and due to the high individual variations and the ensued high standard deviation, no significant differences are recognized (Figure 1). Additionally, the results showed no influence on the microorganisms of the examined intestinal segments in response to the oregano supplementation.

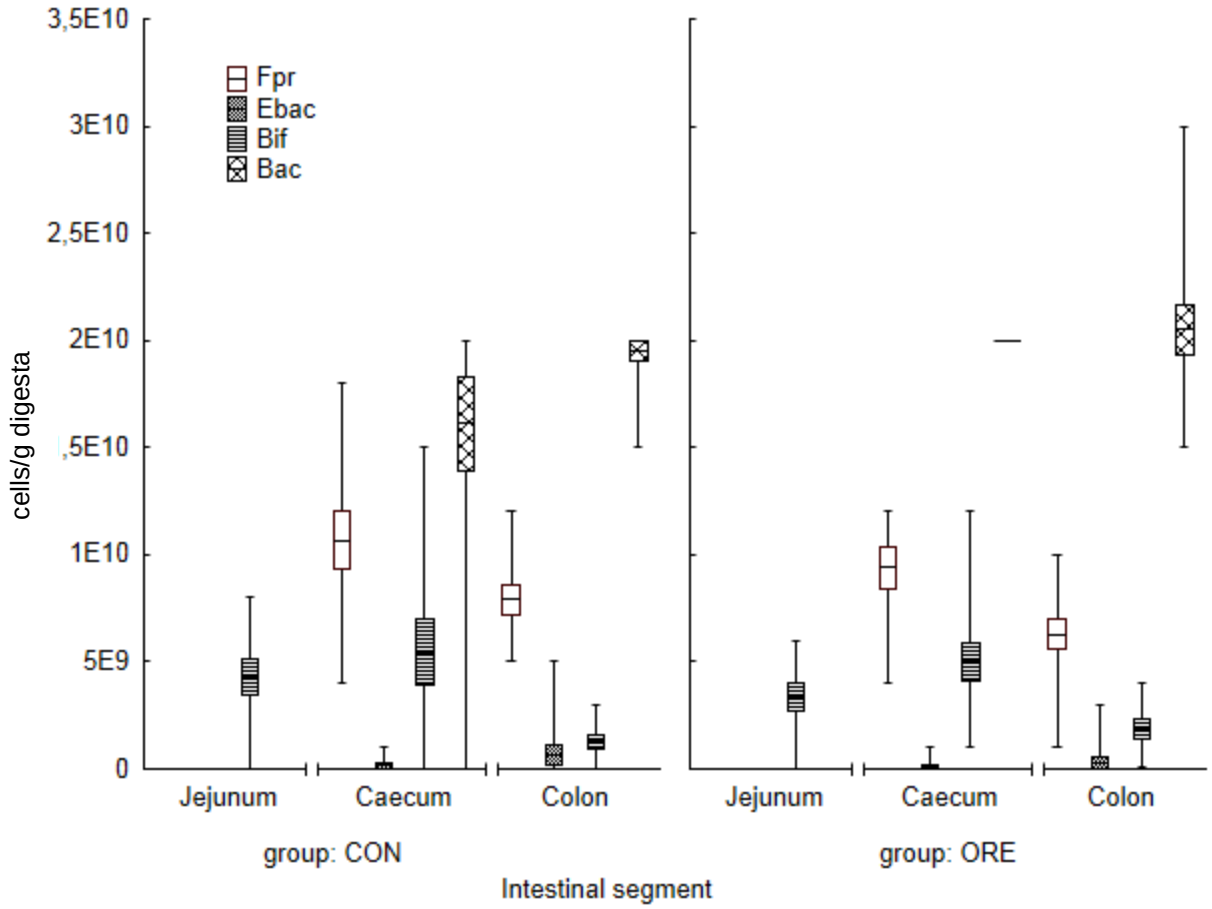

Figure 1: Microbiological biodiversity in the intestine as measured by FISH

Notes: Comparison of the microflora of the chyme collected from different intestinal segments of control (CON) and oregano group (ORE, 8 g oregano/ kg diet). Shown are mean±SD (box) and minimum-maximum (whisker)

Morphology of the microorganisms in the jejunum was inconspicuously in the control group and oregano supplemented animals of group 3. Noteworthy in the caecum was the formation of isles of Fpr in oregano and control group. Further, there were small and chain coccus-type species as

well as rod bacteria. Similarly to the morphology of the Fpr the Bif were also accumulated in isle formation independent of oregano supplementation. The morphology of the microorganisms of the colon showed no difference in control animals and oregano group.

Effect of oregano on microbial composition as estimated by RFLP

The used three enzymatic restrictions resulted in a specific number of bands to express the bacterial richness in the collected ingesta of the three different intestinal parts. The analysis of the *Hha*I restriction profiles included the presence or absence of 16, for *Hpa*II of 14 and for *Rsa*I of 12 clearly differentiable bands. By combining the results for all three enzymes for each piglet was an individual pattern created characterized by 17 through 29 bands per profile. The cluster analysis of patterns by the example of three animals per group is shown in Figure 2.

The patterns clustered with 61.8 to 91.8% similarity between piglets from control group and oregano fed animals and up to 97.2% similarity between piglets within one of the both groups. Thereby no clear assignment of the patterns to the groups of the piglets according to their feeding background was possible.

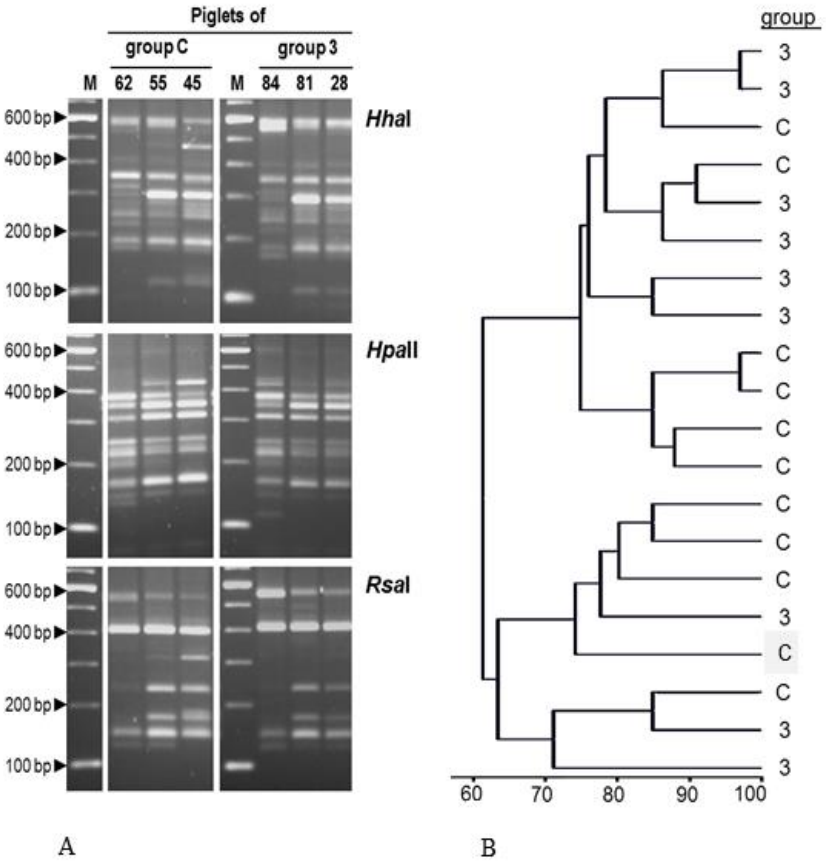

Figure 2: Microbiological biodiversity in the proximal colon measured by PCR-RFLP.

Notes: **A,** Agarose gels after ethidium bromide staining with representative restriction profiles of 16S rRNS fragments with the three enzymes used (shown by the example of 3 animals of each group). **B,** The dendrogram represents the percentage of similarity of the 16S rRNA fragment restriction profiles for the oregano fed animals (group 3, fed with 8 g/kg oregano) and the control animals (group C, without oregano) (UPGMA, Manhattan distance)

Discussion

The present experiment is aimed at examining the effect of an oregano supplementation on the microorganisms of jejunum, caecum and colon as representative segments and supposed action sites of oregano within the intestinal tract. Therefore, piglets were slaughtered and ingesta of the mentioned intestinal parts was collected and analyzed with regard to the spatial organization of the gut microbiota.

The microbial biodiversity in the gastrointestinal tract (GIT) of the piglets is a very complex system of several anaerobic and micro-aerophilic microbes. Several researchers have been working on the microbial communities in the GIT (Konstantinov et al., 2004, Castillo et al., 2007). Most of the microbiota located in the intestine belongs to the genera *Streptococcus, Lactobacillus, Fusobacterium,* and *Peptostreptococcus*. Additionally, there is the *Bacteroides* and *Prevotella* group which are the gram-negative organisms (Konstantinov et al., 2004).

In the present study the genera *Faecalibacterium*, *Enterobacteria*, *Bifidobacteria*, *Bacteroides* and *Prevotella* group in piglet's ingesta of jejunum, caecum and colon were examined by FISH analysis and PCR-RFLP.

No significant effects of oregano supplementation on microbial composition of the control group compared to oregano fed animals were recognized with the methods used in the current study. Accordingly to investigations of Muhl and Liebert (2007) in which a phytogenic feed additive (PFA) with carvacrol and thymol was utilized no significant influences by dietary treatment were achieved. In contrast to the current investigation, the mentioned researchers examined the populations of total culturable anaerobic bacteria while in the present study the detection of the microbiota based on molecular biology techniques. Manzanilla et al. (2004) also used the traditional cultivation technique but recognized a significant increase of lactobacilli and a tended decrease of enterobacteria under the stimulation of a PFA consisting of 5% carvacrol, 3% cinnamaldehyd and 2% capsicum oleoresin. As a consequence of the supplementation of the plant extract mixture the ratio between lactobacilli and enterobacteria showed a significant increase. This ratio was suggested as a parameter to allow evaluating the intestinal health. The enterobacteria in the current study were not influenced by dried oregano supplementation, therefore it is possible that the effects mentioned by Manzanilla et al. (2004) were due to the use of a plant extracts or the mixture of the three plant materials. Castillo et al. (2006) also used a

PFA with the same concentration of plant extracts like the working group of Manzanilla et al. (2004) did. In their investigation no influence of the PFA on the enterobacteria in jejunum and caecum was recognized, which is in accordance with the current study.

Carvacrol and thymol as main components of the essential oil of oregano did not influence any bacterial group when administered as pure compound (2000 mg/kg) in different formulation types (soybean oil, inert carrier and microencapsulation) in the investigation of Michiels et al. (2010). They suggested that the high dry matter content of the gastric ingesta and the fast absorption in the small intestine were the reason for the non-existent influence on the bacterial load. Hence, the question arises, whether a higher amount of dried oregano plant material resulted in a slower absorption rate in the small intestine followed by an increased availability of the active substances in the gut.

The PCR-RFLP analysis of 16S rRNS genes in DNA extracts of the ingesta was chosen to mirror the whole bacterial diversity. In contrast to Castillo et al. (2006, 2007) we were not able to detect specific ecological changes in feeding groups fed with or without oregano. Castillo et al. (2006) pointed out that different treatments leads to different clusters. The differences were the result of the increasing biodiversity in the microbial ecosystem due to the use of feed additives. Investigations in humans showed a high similarity in intestinal microbiota in twins (Palmer et al., 2007). Therefore it is noteworthy that no information about the litter, the dam or the sire was available in the current investigation which could also be possible factors for non-detectable ecological changes by oregano supplementation.

Conclusions

The supplementation of dried oregano did not influence the microbiota of the intestinal tract of piglets. Therefore, the question arises whether a higher amount of dried oregano influences the microbiota and if other genera like *Lactobacillus* were more affected by oregano than the analyzed ones. The PCR-RFLP pattern showed no influences of oregano supplementation in the piglets. Maybe there is an influence of the parentage on the microbiota, which could be recognized in further investigations, especially with siblings by using molecular biology techniques.

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6 Paper III

Metabolism of glucotropaeolin from *Tropaeolum majus* L. (Nasturtium) and the bioavailability of benzyl-isothiocyanates in growing pigs

Katrin Stelter[1], Elke Bloem[2], Andreas Berk[1] and Sven Dänicke[1]

[1]Institute of Animal Nutrition, Friedrich-Loeffler Institute (FLI, Federal Research Institute for Animal Health, Braunschweig

[2]Institute for Crop and Soil Science, Federal Research Centre for Cultivated Plants (JKI), Braunschweig

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Abstract

Tropaeolum majus L. is widely known as a medicinal plant in human medicine. It belongs to the Tropaeolaceae which contain mustard oil glycosides like cruciferous plants. In the case of *T. majus*, the intact glucosinolate glucotropaeolin showed no biological activity, but their degradation products, the isothiocyanates, did. The substances are thought to be enriched in the urinary bladder and the lungs, the active sites to develop their antimicrobial effectiveness. In animal nutrition these effects are of interest in sow management and piglet rearing. Therefore, the kinetics of benzyl-isothiocyanat (BITC) in plasma and the excretion with pig urine in response to nasturtium supplementation at different dosing regimens and galenic forms were examined. Four different groups with catheterized pigs were studied. The animals received the *T. majus* in different dosages (2.3, 6.9 and 13.4 mg GTL/kg BW) and different galenic forms (enteric coated tablets, pulverized tablets and powder) admixed to the feed ration as single bolus to the morning feeding. Blood and urine samples were collected within 24 h after nasturtium intake and analysed for free BITC.

The results indicated that the enteric coating was not effective in animal feeding. The concentration of BITC in tablet fed pigs was lower compared to animals fed powder or pulverized tablets. The bioavailability of the tablets was only 45% within 24 h relative to pulverized tablets. Nevertheless, the tablets could have some advantages in terms of the handling, the stability of the active substance and the dosing. Furthermore, it could be shown that all dosages yielded concentrations in urine and plasma, which can be considered to have an antimicrobial effect.

Keywords: *Tropaeolum majus* L.; Benzyl-Isothiocyanate; Glucotropaeolin; Pig, Metabolism, Urine, Plasma, Kinetics

Introduction

Glucosinolates are mainly present in cruciferous plants, but also in Tropaeolaceae such as *Tropaeolum majus* L. which is well known for its healing effects since the time of the Incas (Anonymous, 1985, Fahey et al., 2001). Glucosinolates can be found in all plant parts. In the case

of *T. majus,* they enriched leaves, flowers and seeds. Intact glucosinolates show no biological activity, but their degradation products, the isothiocyanates (ITC) which are said to be highly active against a broad range of organisms, do. ITC are released when the plant material is physically damaged by external influences, such as chewing or cutting. This physical damage activates the enzyme myrosinase which hydrolyses the glucosinolates to the ITC (Fahey et al., 2001, Vermeulen et al., 2006, Kumar and Sabbioni, 2010, Pintão et al., 1995). The wide spectrum of the medicinal activity of *T. majus* ranges from antimicrobial, antiviral, antifungal to cancer preventing properties (Shapiro et al., 2001, Bazylko et al., 2013, Halbeisen, 1954b, Pintão et al., 1995). Hence, in human medicine *T. majus* is especially applied in diseases of the respiratory and the urinary tract (Gasparotto Junior et al., 2012). Additionally, the antiviral activity to influenza infections in human is of interest (Winter and Rings-Willeke, 1958).

The broad range of effectiveness of *T. majus* is also of interest in animal nutrition, especially as phytogenic alternative to antibiotic growth promoters. Previous investigations in the 1950s with mustard oil of *T. majus* have shown improving effects on the performance of pigs and poultry (Boeger et al., 1955). In particular, the healing effect on respiratory diseases, which is a major problem in pig production and causes high economic losses, is of interest (Reeth and Nauwynck, 2000). Another problem in modern swine production is lactational insufficiency in sows (Martineau et al., 1992). High amounts of bacteria in urine and faeces cause Mastitis-Metritis-Agalactiae-complex (MMA-complex). The active form of glucotropaeolin (GTL), the benzyl-isothiocyanat (BITC), is renally eliminated and therefore excreted with the urine (Winter, 1954), resulting in a decreased bacterial load in the lower urinary tract and the liquid manure (Bergmann et al., 1966) and consequently in a reduced risk of coming down with mastitis (Gerjets and Kemper, 2009). Hence, the use of *T. majus* might be a useful tool to improve the health status in pig production.

In various experiments with catheterized fattening pigs the influence of enteric coated tablets and non-capsulated *Tropaeolum majus* L. at different dosages was examined to clarify if it is necessary to protect ITC by encapsulated tablets in order to increase their concentration in urine, to elucidate a relationship between the GTL-intake and the ITC concentration in blood and urine. Finally, the investigations were used to demonstrate how much dried plant material of *T. majus*

could be ingested by the pigs and identify the maximum ITC concentrations that can be found in plasma and urine.

Material and methods

Animals and housing

The experiment was conducted at the Institute of Animal Nutrition, Federal Research Institute for Animal Health, Braunschweig, Germany according to the European Community regulations concerning the protection of experimental animals and the guidelines of the regional council of Oldenburg, Lower Saxony, Germany.

The pigs of the current investigations were divided into four different groups with a total of 26 male castrated pigs, crossbred German Landrace x Piétrain, with an initial average body weight (BW) of 47.6±4.0 kg. The pigs were individually housed in floor pens and fed two equal portions (750 g) restrictively for adapting to the feeding regimen applied during the stay in the balance cages. The feed was given in the morning and in the afternoon and mixed with tap water for a wet feeding. Animals were surgically equipped with *Vena jugularis externa* catheters after the adaptation to the balance cages according to Goyarts and Dänicke (2006). Briefly, pigs were pre-medicated intramuscularly with azaperone (Stresnil®, Janssen, Animal Health, Neuss, Germany) and ketamine (Ursotamin®, Serumwerk Bernburg, Germany) for sedation, with Atropinum sulfuricum 0.5 mg (Eifelfango®, Bad Neuenahr-Ahrweiler, Germany) to reduce salivation. Directly after sedation, deep level of anaesthesia was achieved by inhalation narcosis with isoflurane (1.5%). Sterile human silicone hoses (1.57 x 3.18 mm and 1.98 x 3.18 mm, 1.50 m, Amt Aromando, Düsseldorf) were used as catheters. After the surgery the pigs were replaced into balance cages until the start of the test protocol. The catheters were cleaned twice a day using heparinized physiological saline (25.000 IE/ 5 ml, Braun Melsungen AG) to keep the functionality.

Tropaeolum majus L. *batches*

Tropaeolum majus L. 1: Plants were grown in the years 2005-2007 on a field at the experimental station of the Julius Kühn-Institut (JKI) in Braunschweig. Vegetative plant material was

harvested from main vegetative growth until flowering by sickle, delivering plant material with a great proportion of leaf material. The plant material was dried in a ventilated oven at 40°C until constancy of weight and finely ground (<0.12 mm) using an ultra-centrifugal mill (RETSCH, ZM-1000, Haan, Germany). The plant material from the different years was thoroughly mixed and provided the raw material for enteric coated tablets produced by Schaper and Brümmer in Salzgitter, Germany. Before the kernel of the tablet was pressed, the plant material was mixed with microcrystalline cellulose, magnesium stearate, polyvinylpyrrolidone and fine-particle silica as additives. 95 mg plant material was pressed into each kernel. These kernels were enteric coated with eudragit, talcum and trietylcitrate and had a final weight of 190 mg.

Tropaeolum majus L. 2: Plants were grown in 2011 and 2012 and not used for tablet production. The plants were cultivated and harvested on the experimental plots of the Institute for Plant Cultivation, Schnega, Germany. At the flowering stage, the aerial parts (mainly flowers and leaves) were harvested by manual cutting. Subsequently, the plant material was dried down to a final moisture content of app. 9% at 40°C in tray driers. The dried material was ground with a 4 mm screen using a hammer mill (Joachim Kreyenborg & Co, type 10, Münster-Kinderhaus, Germany).

Dosing scheme and experimental design

Two days after the surgery of the pigs, pig feed was supplemented with nasturtium on top of a basal diet as a single bolus in the morning feeding according to the plan shown in Table 1. The experimental diets meet the recommendations by the German Society of Nutrition Physiology (Gfe, 2006). The diet contained mainly wheat (379 g/kg), barley (350 g/kg) and soy bean meal (220 g/kg). The distributions of the pigs in the different groups are also shown in Table 1. In general, the applied dosages of nasturtium were based on the recommendations of the commission E for humans, which describes a daily amount of 130 mg GTL as a suitable dosage for a person of 60 kg BW (equivalent to 2.17 mg GTL/kg BW) (Blumenthal et al., 1998). The applied dosage in the current investigation ranged on average from 2.3 to 13.4 mg GTL/kg BW of the pigs (Table 1).

Table 1. Distribution of *Tropaeolum majus* L. as a single bolus in different galenic forms

Group	Dosage	Form	Average GTL-Dose [mg/kg BW]	Dosage range [mg/kg BW]	n
1	1	Tablets	2.3	2.1 – 2.6	5
2	1	Pulverized tablet	2.4	2.2 – 3.1	9
3	2	Pulverized tablet	6.9	6.6 – 7.2	4
4	3	Powder	13.4	8.2 – 22.6	8

Nasturtium of Batch 1 was supplemented as tablets (Group 1) and pulverized tablets (Group 2) with an average dosage of 2.3 mg or 2.4 mg GTL/kg BW to investigate the effect of different galenic forms. The pigs of Group 3 also received nasturtium of Batch 1, but only as pulverized tablets in an average dose of 6.9 mg GTL/kg BW. To investigate the dose response animals of Group 2 and 3 were either fed with pulverized nasturtium of dosage level 1 or 2. The animals of Group 4 were fed with finely ground nasturtium powder of Batch 2 and an applied amount of GTL which was 5 to 10-fold (8.2-22.6 mg GTL/kg BW) higher than the dosage level of Group 1. The aim of this investigation was to examine the impact of a high GTL level on acceptance by the pigs and BITC excretion under different feeding regimens. Half of the animals (n=4) received the nasturtium in one portion while the other half (n=4) received the powder evenly distributed over the entire feeding time of approximately 10-15 min.

The unequal distribution of the number of pigs fed with powder and tablets was caused by different factors, especially the acceptance of the tablets and the high dosage of nasturtium powder.

Blood and urine sampling

Blood samples (EDTA) were drawn in the morning (control) prior to feeding the nasturtium supplemented meal and 0.25 h, 0.5 h, 0.75 h, 1 h, 1.5 h, 2 h, 3 h, 4 h, 6 h, 8 h, 10 h, 12 h, 24 h after the bolus. The tubes were centrifuged (2,000 x g for 15 min, 15°C, Heraeus Varifuge® 3.0R) and the plasma was frozen in aliquots (- 80°C) until analysis of BITC.

The non-acidified urine samples were collected in an interval of 12 h until 24 h after the bolus. The urine was filtered and frozen (-20°C) prior to analysis.

Analyses

Nasturtium and feed

Representative samples of the diets were collected and analysed for dry matter, crude ash, crude protein (N x 6.25), ether extract (HCl digestion), crude fiber, sugar (Luff-Schoorl) and starch according to the methods of the Association of German Agricultural Analysis and Research Centres (Naumann and Bassler, 1993).

The GTL content of each Batch and galenic form was determined before mixing the *T. majus* to the feed of the pigs. The determination was performed by HPLC according to the EU official method (ISO 9167-1) for desulfoglucosinolates (Anonymous, 1990). The GTL content was calculated by reference to a calibration curve using purified GTL (Calbiochem-Novabiochem 347358). Desulfoglucosinolates were measured by HPLC (Merck Hitachi D6000 Interface, L6200A Intelligent Pump, L4200 UV-VIS Detector, L7200 Autosampler and a column oven which kept the column at 30 °C) with UV detection at 229 nm using acetonitrile (20%) and water as eluents and a Lichrospher RP-8 column (Merck, Darmstadt, Germany) (100 x 4 mm, 5 µm) for separation.

Quantification of Isothiocyanate in urine and plasma

Urine (filtered) and plasma samples were thoroughly mixed before analysis. The content of total ITC in urine and plasma was determined according to the method of Ye et al. (2002). Briefly, a cyclocondensation-assay was prepared with benzene-1, 2-dithiol building a complex with isothiocyanates that can be determined at 365 nm. Blood samples were treated with 6% Polyetylene glycol (PEG) on ice for 10 min prior to analysis to precipitate the proteins followed by centrifugation for 5 min at 17,986 x g and 4°C. Urine samples were only centrifuged. For the cyclocondensation-assay 400 µL of the supernatant (from plasma or urine) was mixed with 400 µL potassiumphosphate buffer (100 mmol/L, pH 8.5) and 800 µL 1, 2-benzenedithiol (20 mmol prepared in acetonitril) and were incubated for 2 h in a water bath at 65°C. After incubation the samples were centrifuged at 1,466 x g for 5 min at 4°C. The supernatant can be

measured directly by HPLC (Merck Hitachi D6000 Interface, L6200A Intelligent Pump, L4200 UV-VIS Detector, L7200 Autosampler and a column oven which kept the column at 25°C) with UV detection at 365 nm. An isocratic eluent consisting of 85% methanol and 15% water was used with a flow rate of 0.3 mL/min. 25 µL of the cyclocondensation-assay were injected and for separation a Supelcosil LC-18 HPLC column (150 x 2.1 mm, 5 µm, Sigma-Aldrich) was used. The BITC content was calculated by reference to a calibration curve using phenyl isothiocyanate (PITC) as standard (Fluka 78781).

Calculations and statistics

The data of the urine samples and plasma analysis were evaluated using the GLM procedure of the SAS-software package (SAS Enterprise Guide 4.3). Group was considered as fixed effects in one way analysis of variance (ANOVA). When effects were considered as significant in the F-test ($p<0.05$), the means were compared using Tukey test. Values in the current paper are shown as means. The non-linear regression model of the application STATISTICA (StatSoft, version 10) was used to fit the data according to Mercer et al. (1989), but modified to force the regression through the origin:

$$\text{ITC } (\mu g/L) = \frac{R_{max} \times t^{o}}{(K_{0.5})^{o} + t^{o} + t^{2 \times o} / (K_s)^{o}} \qquad (1)$$

where R_{max} is the maximum theoretical BITC concentration, o is the apparent kinetic order, $K_{0.5}$ is the time for ½ of R_{max} and K_s is the time indicative for the decreasing part of the regression. The estimated parameters were used to calculate the area under the plasma concentration time curve (AUC) numerically by applying the trapezoidal method. The parameter I_{max}, the time corresponding to R_{max}, was calculated as follows:

$$I_{max} = (K_s \times K_{0.5})^{0.5} \qquad (2)$$

Results

Diet composition and feed intake

The analysed composition of the diet meets the recommendations of the Society of Nutrition Physiology (Gfe, 2006). The analysis of the dried plant material of *T. majus* revealed 26.9 mg GTL/g DM for Batch 1 and 10.4 mg GTL/g DM for Batch 2. The GTL content for the tablets were determined to be 2 mg GTL/tablet. All pigs accepted the supplemented *T. majus* as tablets (Dose level 1) and pulverized tablets (Dose level 1 and 2) without any problems. The animals dosed with level 3 (Group 4) showed a variation in their acceptance of the nasturtium-containing meal. The administered amount of dried nasturtium was 80 or 160 g per portion, corresponding to 800 and 1600 mg GTL/portion. In both dosages the acceptance by the animals ranged from restrained feed intake to refusal of feed. Consequently, the actual amount of GTL-intake was calculated by backweighing of not accepted feed.

Kinetic of benzyl isothiocyanate in plasma

In general, the free BITC was detected in all plasma samples. The maximum theoretical concentration (R_{max}) of the tablet-fed animals was 94.4 µg BITC/L and approximately 2.5-fold lower compared to the values of the pigs that received the pulverized tablets at the same dosage (227.7 µg BITC/L). The values of the area under the time curve (AUC) confirmed these data (Table 2). The AUC ranged from 886 to 2027 µg BITC/L plasma·h at dosage 1. In general, AUC values in tablet-fed animals were lower than the values of pigs fed with pulverized tablets or powder. Significant differences in the AUC were recognized between animals received dosage level 1 (Group 1 and 2) and dosage level 2 (Group 3) as well as between animals of Group 1 and 4 ($p<0.001$). The calculated AUC increased with increasing GTL-intake as well. Therefore, the AUC of animals receiving dosage level 2 and 3 was nearly twice as high as the AUC of animals fed dosage 1 (Table 2). Time-dependent parameters $K_{0.5}$, K_s and I_{max} showed no significant differences between the groups, galenic forms or the various dosages. In Figure 1 the time-dependent course of BITC concentration in plasma is shown for the different galenic forms. The curve had an exponential increase followed by a slow decline. The curve progression of animals

fed dosage 1 was nearly the same for the different galenic forms, tablets and pulverized tablets. Nevertheless, the level of BITC concentration was higher in animals of Group 1 which received the pulverized form of nasturtium. The concentrations at the point in time of I_{max}, $K_{0.5}$, K_s and after 24h (Table 2) reflected the fact that the values of animals fed pulverized tablets tended to be higher than in the tablet supplemented animals ($p<0.1$, Table 2). With regard to the other dosages the curve resulted a similar progression, but on a higher level. Animals fed Doses 2 and 3 showed higher concentrations of BITC in plasma over the entire experimental period than animals fed Dose 1. While tablet-fed animals (Group 1) revealed a C_{Imax} of 71.77 µg/L, animals of Group 4 (powder) received values of 371.09 µg/L, which were more than 5-fold higher. This effect could be also recognized with regard to the concentrations corresponding to $K_{0.5}$ and after 24 h. After a period of approximately 1.93 h the maximum BITC concentration was reached in all experiments independent of the dose and galenic form.

Table 2. Estimated kinetic parameters of pigs supplemented with *Tropaeolum majus* L. as single bolus in different galenic forms and dosages

Group	Form	Dosage	AUC_{24} [µg/L·h]	k_{05} [h]	R_{max} [µg/L]	k_s [h]	I_{max} [h]	$C_{K0.5}$ C_{Ks} [µg/L]	C_{Imax} [µg/L]	C_{24h} [µg/L]
1	Tablets	1	886^{c}	0.53	94.4^{b}	7.07	1.73	51.51^{b}	71.77^{b}	19.94^{b}
2	Pulverized tablet	1	2027^{bc}	0.60	227.7^{b}	7.60	1.89	121.89^{b}	181.04^{b}	40.54^{ab}
3	Pulverized tablet	2	3958^{a}	0.66	421.3^{a}	5.87	1.96	250.33^{a}	403.73^{a}	83.24^{a}
4	Powder	3	3720^{ab}	0.77	412.6^{a}	5.77	2.07	240.55^{a}	371.09^{a}	77.62^{a}
	PSEM		442	0.15	46.9	1.49	0.30	26.28	40.60	11.93
	p-Value		<0.001	0.639	<0.001	0.717	0.864	<0.001	<0.001	0.003

^{abc} Means with different superscripts within the same column are significantly different ($p<0.05$)
AUC_{24}, Area under the time curve after 24 h; $K_{0.5}$, time for ½ R_{max}; R_{max}, the maximum theoretical BITC concentration; K_s,time indicative for the decreasing part of R_{max}; $C_{K0.5}$, BITC concentration to $K_{0.5}$; C_{KS}, BITC concentration to K_S. C_{Imax}, BITC concentration to I_{max}; C_{24h}, BITC concentration after 24 h; PSEM, pooled standard error of means

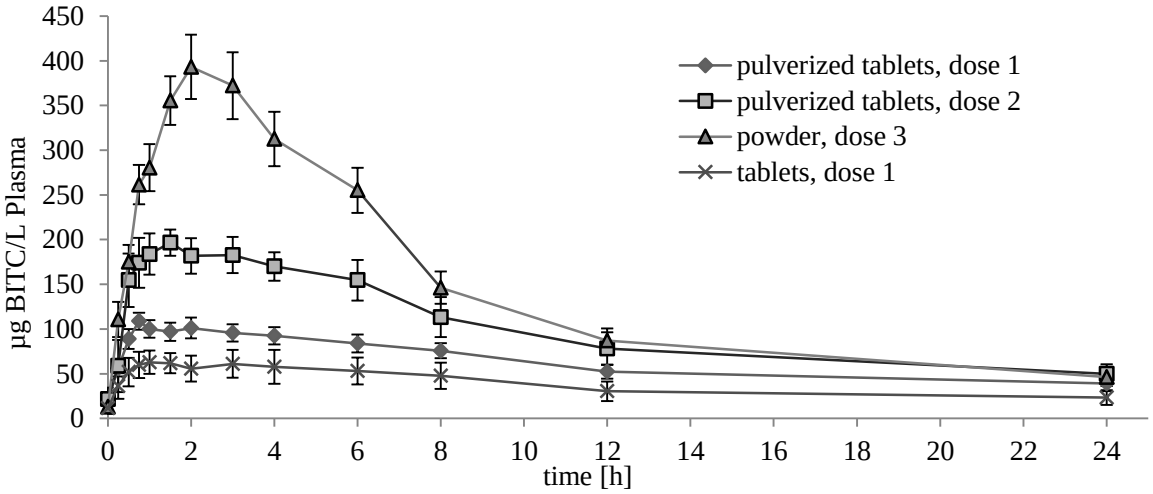

Figure 1. Benzylisothiocyanat concentration in plasma of piglets dosed with *Tropaeolum majus* L. in different concentrations

Excretion of glucotropaeolin in urine

The analysed metabolite of glucotropaeolin in urine was the free form of BITC as in the plasma analysis. In general, the BITC content in the urine reached its maximum within the first 12 h (1-12 h) after intake of *T. majus* independent of the galenic form or dosage. Within the next 12 h (13-24 h) the amount of BITC was already 6-times lower independent of dosage and form (Table 3). With regard to the various dosages there were significant differences in the BITC values between the low Doses 1 (Group 1 and 2) and the highest Dose 3 (Group 4) within 24 h. These observation indicated that the relation of BITC in urine and the GTL-intake tended to rise as well ($p=0.056$). The amounts ranged from 0.19 (Dose 1, tablets) to 10.22 mg/animal (Dose 3) within the complete 24 hours. No significant differences in the galenic forms (Groups 1 and 2) in animals fed Dosage 1 were recognized. Additionally, there were also no significant differences in the excretion of BITC in response to the different GTL-levels in the feed.

In Group 3, in which the highest dosage of nasturtium was applied, the supplementation of the nasturtium was also examined. Half of the animals (n=4) received the grounded powder as single

portion while the other half (n=4) got the nasturtium powder in little portions distributed over the entire feeding time of approximately 10-15 minutes. The results of this administration trial showed that the feeding regimen had a strong impact on ITC excretion with the urine, whereas no effect could be recognized for the transfer into the plasma. The animals which received the nasturtium powder as single portion excreted only 12.2% of the BITC compared to those animals with the gradually added nasturtium (2.22 vs. 18.22 mg/animal) within 24 hours (Table 4). Accordingly, the excretion rate of BITC in animals that received the powder as single portion yielded 0.92% compared to 9.08% in the pigs receiving the nasturtium in small portions over the feeding time. Therefore, a nearly 10-fold higher BITC excretion in urine can be achieved when the nasturtium is administered gradually to the diet.

Table 3. Isothiocyanate (ITC) excretion in urine of pigs fed with *Tropaeolum majus* L. as single dose and balance of GTL intake and ITC excretion in relation to the daily urine volume

Group	**Form**	**Dosage**	**Average GTL intake** [mg/animal]	**0-12 h** [mg/animal]	**13-24 h** [mg/animal]	**0-24 h** [mg/animal]	**Excretion** [% of intake]
1	Tablets	1	100	0.13	0.05	0.19	0.57
2	Pulverized tablet	1	113	0.20	0.10	0.30	0.78
3	Pulverized tablet	2	307	1.73	0.55	2.28	2.23
4	Powder	3	668	8.78	1.44	10.22	5.00
	PSEM*			2.21	0.41	2.52	1.49
	p-Value			0.013	0.043	0.012	0.056

Table 4. Isothiocyanate (ITC) excretion in urine and area under the plasma time curve of pigs fed with *Tropaeolum majus* L. as powder (dose 3) in different feeding regimens and ITC excretion in relation to the daily urine volume and the GTL intake

Group	**Form-feeding regimen**	**Dosage**	**Average GTL intake** [mg/animal]	**0-12 h** [mg/animal]	**13-24 h** [mg/animal]	**0-24 h** [mg/animal]	**Excretion** [% of intake]	**AUC_{24}** [µg/l·h]
4	Powder-all in one	3	726	1.93	0.29	2.22	0.92	3356
4	Powder-little by little	3	610	15.63	2.58	18.22	9.08	4084
	PSEM*			3.13	0.65	3.47	5.77	417
	p-Value (form-feeding regimen)			0.021	0.048	0.017	0.021	0.264

*PSEM, pooled standard error of means, AUC_{24}, area under the curve after 24 h

Discussion

Glucotropaeolin, the only glucosinolate of *Tropaeolum majus* L., is relatively non-reactive. Tissue damage releases the enzyme myrosinase which converts the non-reactive form into the active form, the BITC (Brüsewitz et al., 1977, Fahey et al., 2001, Lamy et al., 2011). The bioavailability of this active metabolite is not well established in animals, especially in pigs. Therefore, the current investigation was performed to examine different dosages of GTL to demonstrate the dose response and further to figure out the level of intake. Moreover, different galenic forms were examined: enteric coated tablets were compared with pulverized tablets to reproduce the metabolism of GTL and the passage into the systemic blood circulation as well as in the urinary pathway over an experimental period of 24 hours.

Effect of Tropaeolum majus L. *on the plasma kinetic*

Despite an intensive literature search no investigations comparable to the present animal experiment could be found. Some human studies or investigations with rats were conducted with *Tropaeolum majus* L. or other plants containing mustard oil glucosinolates, but there were no kinetic studies using pigs. In investigations of Brüsewitz et al. (1977) rats were administered orally with radioactive labelled cysteine conjugates of benzyl isothiocyanate to examine the excretion of BITC in urine and the kinetic of radioactivity in plasma. The results showed the maximum plasma concentration of radioactivity within 45 min. These results differ from those of the current investigation in which the maximum plasma values were reached within 1.93 h. However, Brüsewitz et al. (1977) applied the isothiocyanate directly as substance, while animals in the present investigation were fed with the dried plant material. Interestingly, the same researchers also investigated the application of radioactive labelled cysteine conjugates to dogs and recognized a slower absorption than in rats. The maximum plasma concentration was achieved after 1.5 hours and remained until 6 hours. These results were more comparable to our curve progression of plasma BITC. In consequence, the researchers concluded a species-specific, and in addition gender specific, kinetics of the cysteine conjugate of [^{14}C] benzyl isothiocyanate. The AUC as determined for male rats was significantly higher than that for female rats. Within

the current investigation there was no possibility to make a statement about gender related differences, as only male pigs were used. In a human study of Platz et al. (2013) a dosage of 10 mg freeze-dried nasturtium was administered to male and female volunteers to determine metabolites of BITC in plasma and urine. The results showed that the maximum of BITC metabolites in plasma were detected within 1.5 h, which is in accordance to the findings of the present investigation. A further similarity of the current investigation and Platz et al. (2013) was the time dependent decrease of BITC in urine within the experimental period of 24 h. The majority of the metabolites were excreted after that time. In all previously published investigations the applied forms of glucosinolates containing material differed from that of the current study. In human studies vegetables in raw or cooked form, like broccoli or garden cress, were used to examine the pharmacokinetics of glucosinolates after ingestion (Ye et al., 2002, Kumar and Sabbioni, 2010). Brüsewitz et al. (1977) used benzyl isothiocyanate and its conjugates in the direct form dissolved in ethanol, water or pyridine by different administrations (orally, intravenously). In the current investigation there were enteric coated tablets, pulverized tablets and nasturtium powder administered orally to the animals with the diet. It was found that glucosinolates were already degraded to a great proportion during chewing of the material (Fahey et al., 2001). With regard to the ratio of $AUC_{pulverized\ tablets}$ and $AUC_{tablets}$ it was noticeable that the bioavailability of tablets was only 45% of that of pulverized tablets within 24 hours after ingestion. Nevertheless, the different galenic forms do not lead to significant differences in the analysed BITC concentrations in plasma in animals fed Dose 1. The reason for this effect was the high animal individual variability and the small number of animals. These effects were possibly caused by the feeding behaviour of the pigs. Some of the pigs fed very slowly while others ate very fast or they chewed the feed more extensively. Nevertheless, regarding the time curves of BITC concentration after consuming tablets and pulverized tablets, it was obvious that the GTL of the pulverized form was metabolised faster than the tablets. Consequently, the active substance in pulverized tablets was detected faster in the systemic blood circulation and showed a higher bioavailability compared to enteric coated tablets. Hence, the encapsulation of nasturtium did not lead to a significantly better bioavailability within 24 h.

The blood analysis showed a clear dose response independent of the galenic form. Animals fed the enteric coated tablets corresponding to the lowest GTL dose showed the smallest BITC

concentration in plasma at every time point (Table 2), whereas animals administered to Dosage 2 and 3 with a higher application of GTL also revealed higher BITC concentration over the entire investigation.

BITC excretion with urine

Benzyl-isothiocyanate is absorbed in the gastrointestinal tract and metabolized sequentially by various enzymes like glutathione S-transferase (GST) and γ-glutamyltranspeptidase (GTP) to mercapturic acid and finally excreted in urine (Zhang et al., 1995, Brüsewitz et al., 1977).

In the current investigation a dose dependent excretion of BITC was observed while other degradation products were not monitored. The pigs which received the lowest level of nasturtium also excreted much smaller BITC proportions via urine compared to animals fed the higher dosage. This effect was in accordance with Bollard et al. (1997) who administered allyl isothiocyanate (AITC), the ITC of sinigrin, in different ways (intravenous and peros) to rats. They also revealed a dose dependent excretion of thiocyanates. Shapiro et al. (1998) recognized a linear correlation between the dosage and the metabolites excreted with the urine as well. The higher the dosage, the higher was the BITC excretion with the urine. Bollard et al. (1997) identified metabolites of nearly 80% of the applied oral dosage of AITC in the urine after 4 days, whereas in the current study only 0.5 to 9.1% of the oral dose could be detected in the urine within 24 h. The dosage Bollard et al. (1997) applied was comparable to the Dosage 2 (2.4 mg GTL/kg) used in the current investigation. While the pigs of the present study achieved GTL-containing plant material, Bollard et al. (1997) administered pure dissolved AITC solution to the rats. Consequently, it can be suggested that the metabolism of the pure substance in rats differed from that of the plant material. Shapiro et al. (1998), (2006) postulated the important influence of the gastrointestinal microflora on the metabolism of glucosinolates in human. They recognized that the conversion to isothiocyanate was mediated by the microbiota in the gut. These, in turn, could be affected by diet, host genetic factors, gastrointestinal transit time and the enterohepatic circulation (Shapiro et al., 2006).

The low recovery rate of BITC in urine in the current investigation can be attributed to the administration of the nasturtium plant material. Bloem et al. (2007) demonstrated that fresh plant material from *T. majus* which was crushed with water lost 96.5% of GTL within 1.25 min and

only traces were left. Therefore, it was expected that with an enteric coated encapsulation of the plant material, higher proportions of BITC could be yielded in urine. However, it was not possible to prevent the destruction of the tablets by chewing of the pigs which caused the early degradation of the GTL in the mouth and an obvious complete loss prior to absorption. The release of isothiocyanate is dependent on mechanical factors like chewing, damage of the plant tissue (Bloem et al., 2008, Fahey et al., 2001), moistening of the feed and of other factors like the pH value (Gil and Macleod, 1980, Halkier and Gershenzon, 2006). Nasturtium supplemented feed of Doses 1 and 2 was consumed very quickly and chewing was not very distinctive, while animals fed Dose 3 were restrained in their feeding and ate very slowly. The feeding behaviour notably influenced the concentration in urine. Feeding the nasturtium in little portions over the feeding time caused a 10-fold higher BITC excretion via the urine compared to animals administered the nasturtium as a single portion. Possibly the longer soaking of the plant material of nasturtium in the water-mixed feed or the slower feed intake were responsible for the lower excretion rate. It can be assumed that the pigs ingested BITC and metabolites of GTL as soon as the feed was soaked for a longer time period.

A further effect for the low recovery rate in the urine could also be due to a release of BITC with the lungs or an excretion of non-absorbed BITC with the faeces. BITC can also be eliminated by pulmonary exhalation (Anonymous, 1985) which is the rationale of the action of *T. majus* against infections of the respiratory tract. Brüsewitz et al. (1977) detected 0.4% of the administered radioactive labelled cysteine conjugate of BITC in the expired air of rats while Bollard et al. (1997) recovered 10-18% of the applied proportion in faeces and in the expired air. In the current study it was not possible to estimate the release of BITC via other pathways, but the data indicated that the feeding regimen influenced the recovery rate conspicuously.

Nevertheless, the analysed concentrations of BITC in the urine, independent of the dosage and the galenic form of *T. majus*, were sufficiently high to yield a bactericide or bacteriostatic effect. Concentrations of benzyl mustard oil of 0.5 to 50 µg/mL were shown to have a high antimicrobial effectiveness (Anonymous, 1985). The values of the current investigation ranged from 0.19 to 10.22 mg BITC/animal with an average amount of urine of 764±345 g within 24 h. Therefore, pathogenic bacteria in the gastrointestinal tract of the pigs and in the liquid manure

could probably be effectively inhibited by the supplemented nasturtium independent of the galenic form, because of the high concentration of active BITC in the urine.

Conclusion

The current investigation in which pigs were supplemented with *Tropaeolum majus* L. in different dosages and galenic forms showed that the encapsulation of the plant material was not necessary to increase BITC concentrations in the urine. The active substance reached the systemic blood circulation faster when administered in pulverized form or as nasturtium powder. The bioavailability of the tablets was only 45% within 24h relative to the pulverized tablets. Therefore, the expensive encapsulation is not necessary in animal feeding. Nevertheless, encapsulated tablets could have some benefits in practice. On the one hand, the defined dosage could be more easily administered to the animals, and on the other hand the encapsulation ensured a longer stability of the active substance compared to dried plant material. Due to the results of the BITC in urine it can concluded that the feeding regime has a strong influence on the urinary excretion of BITC, despite the fact of the ingested amount of nasturtium. Even with the intake of the lowest dosage of GTL, independent of the galenic form, the BITC concentration in the urine was high enough to potentially reduce the bacterial load in the lower urinary tract of pigs. To sum up, even small amounts of nasturtium powder are able to inhibit pathogen bacteria in urine and further improve the health status of pigs, especially in sow management and piglet rearing.

Acknowledgements

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7 General Discussion

The present examinations aimed at answering the questions if a supplementation of *Origanum vulgare* L., as a representative of terpenic compounds containing plants, to piglets had an influence on the performance, some immune parameters and the intestinal microbiota and if the supplementation of *Tropaeolum majus* L. (nasturtium), as example plant containing mustard oil, to growing pigs had an effect on the metabolism and the bioavailability of isothiocyanat. The herbs used in the current investigations have been cultivated and harvested in a collaborative project supported by the Fachagentur für Nachwachsende Rohstoffe (FNR, Gülzow, Germany) with the aim to promote cultivation of medicinal and spice plants in Germany. Moreover, the aerial parts (flowers, leaves and stems) of these herbs were used in dried form as simplest pharmaceutical formulation in feeding experiments to develop and verify phytogenic additives. The core of this study was the examination of the mentioned herbs with the focus on the decisive stages of pig's life like weaning and rearing.

Taking into account the different active sites of oregano and nasturtium, oregano effects are discussed according to the intestinal microbiota and immune modulating abilities, while effects of nasturtium are discussed in compliance with its antimicrobial potential in respiratory and urinary tract. The impact of dried oregano on the host animal and its possible effects on the actives sites (**Paper I, II**) as well as the importance of dried nasturtium on its bioavailability (**Paper III**) in pig are considered here in detail. Finally, the results of the current study allow conclusions about the suitability of the mentioned herbs in pig nutrition which are also discussed.

7.1 The use of oregano in piglet nutrition

7.1.1 The effect on performance and feed intake

The use of herbs and botanicals in human nutrition has been known since thousands of years. Today, as well as then, the main benefit of using herbs is the flavouring of the food (Jugl-Chizzola et al., 2003). In the focus of animal nutrition flavouring additives are declared as sensory additives that are mixed in the feed to improve or change the organoleptic qualities of the feed according to the EU Regulation No. 1831/2003. The flavour of the herbs is primarily caused by the aromatic ingredients in their essential oils and oleoresins (Craig, 1999). The Mediterranean

herb O*riganum vulgare* L. contains mainly carvacrol and thymol as essential oil components and has often been used in several feeding experiments with various animal species like poultry and pigs (Neill et al., 2006, for example Florou-Paneri et al., 2006, Namkung et al., 2004, Radford et al., 2002, Henn et al., 2010). The expectations regarding the use of oregano in pig nutrition were a higher feed intake and an improved performance (Windisch et al., 2008). In spite of the numerous studies, most of the results were controversial. Some researchers recognized an improving effect on the performance, whereas other results did not show any effects. For example, Ragland et al. (2007) administered various dosages (0.5, 1 and 1.5 kg/tonne) of oregano oil to the feed of early weaned piglets (5.6±0.03 kg) and noticed a significantly improving effect on the highest dosage on the ADG, the ADFI and the FCR in comparison to the control animals. While piglets of the control group had an ADG of 271 g/day, animals fed the oregano supplemented diet showed an ADG of 300 g/day over the experimental period of 35 days. In contrast to these results, the investigations of Untea et al. (2011) and Neill et al. (2006) showed no effects on the performance of the piglets in accordance with the findings of the current study (**Paper I**). The ADG of the piglets fed with oregano of the present investigation were similar to those of the control animals. Piglets of the control group had an ADG of 475±18 g/d, whereas piglets fed the highest concentration of dried oregano (8 g) achieved 484±16 g/d. The body weight of the piglets at the onset of the trial and the experimental period, however, varied between the present study and those mentioned above. While piglets of the current investigation had an average body weight (ABW) of 7.9±1.0 kg, the animals of Untea et al. (2011) showed higher weights (13.5±2.8 kg) and those of Neill et al. (2006) were with an ABW of 5.9 kg the smallest. Consequently, it should be recognized that Ragland et al. (2007) used early weaned piglets and observed improving effects on the performance. Therefore, it can be suggested that the age of the piglets is not the main effect regarding the improvement of the performance in response to oregano neither in dried form nor as essential oil, but it should be considered that the various BW of the piglets of the experiments mentioned above are caused by different suckling periods associated with a dependency on digestive capacity for solid feed. The course of the ADG of the four feeding groups, which is shown in Figure 2, illustrates this statement. The curve progression is similar in every feeding group disregarding oregano supplementation, except for week 4. The differences in this week are maybe the result of the lipopolysaccharide (LPS)-

challenge at the beginning of experimental week 4. In contrast to our assumptions the LPS-stimulated animals had a significantly higher ADG (535.7±128.4 g/d) than the non-stimulated piglets (450.0±120.2 g/d) regardless of oregano supplementation in this week (p=0.003; Figure 3). However, it should be noticed that animals administered saline had an ABW of 15.3±2.3 kg, while LPS stimulated animals showed an ABW of 16.2±2.3 kg on the challenge day.

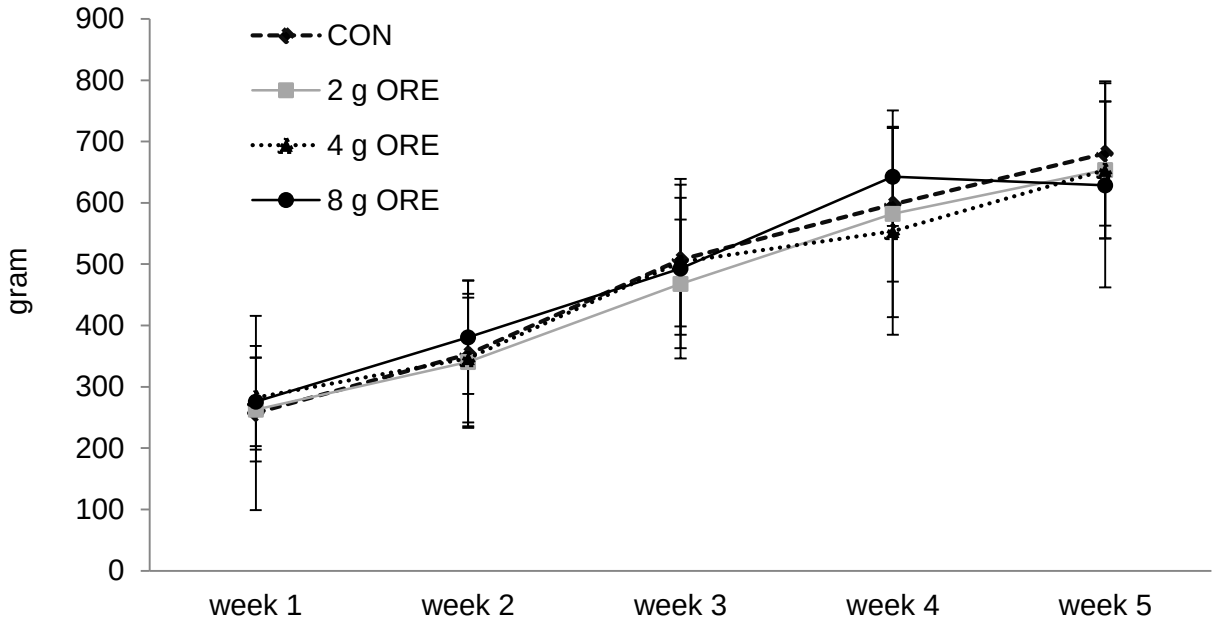

Figure 2: Average daily gain of oregano supplemented piglets over the entire experimental period (mean±SD)

CON, control group; ORE, oregano

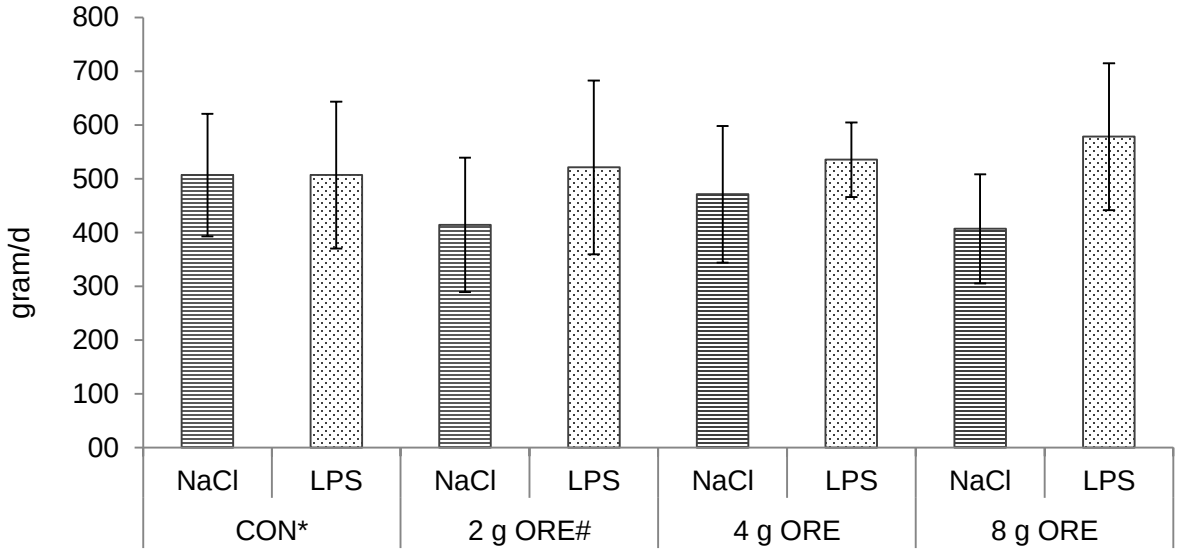

Figure 3: The effect of oregano and lipopolysaccharide challenge (mean±SD) on the daily weight gain on experimental day 21

* CON, control group; #ORE, oregano; NaCl, piglets with physiological saline injection; LPS, piglets with injection of 5 µg lipopolysaccharide of E.coli (O55:B5)/kg BW

With regard to the feed intake, nearly the same effect was observed after week 4 (Figure 4). In contrast to investigations of Chaves et al. (2008) in which LPS-stimulated pigs showed a decrease in feed intake, the resultant data of the current study (**Paper I**) suggested that animals fed 8 g oregano/kg showed a short-lived increase in feed intake between week 3 and 4. Unfortunately, the recording of individual feed consumption data was not possible in the present investigation due to the group-housing conditions in the stable. Nevertheless, LPS injection induced an acute systemic inflammatory response as indicated by significantly elevated blood levels of the cytokines tumor necrosis factor- α and interleukin-6 (**Paper I**) and therefore it can be assumed that feeding oregano in case of a systemic inflammation may have a positive impact on feed intake.

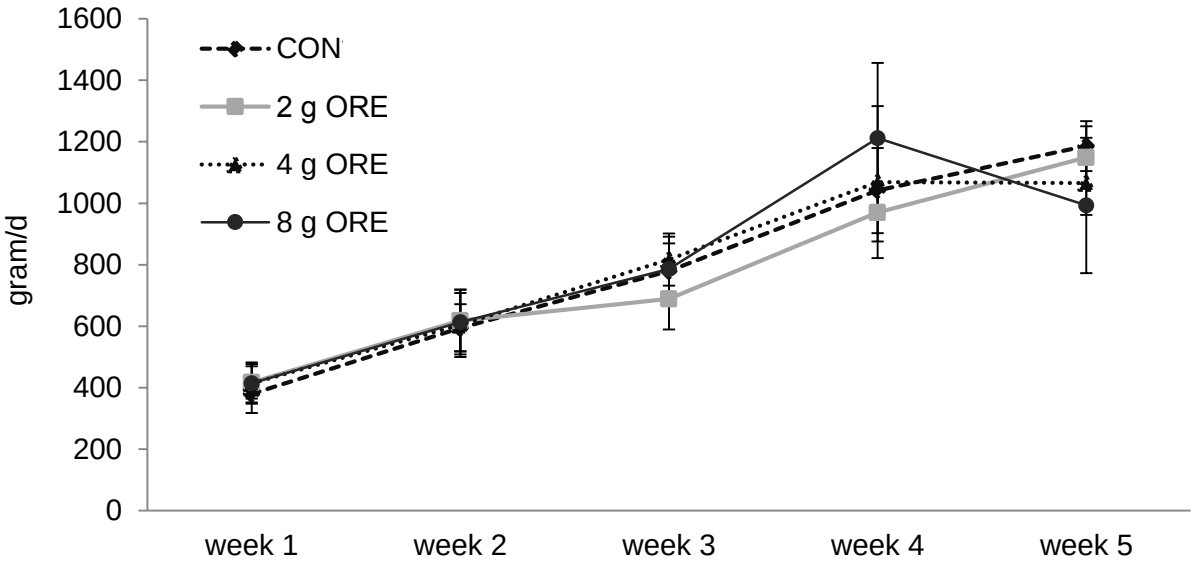

Figure 4: Feed intake of oregano supplemented piglets over the entire experimental period (mean±SD)

CON, control group; ORE, oregano

Most researchers used oregano oil or an extract of the plant, only a small proportion of investigations were conducted with dried oregano plant material like in the present study. Untea et al. (2011) used dried oregano, but they also altered the concentration of copper (Cu) and zinc (Zn) in the diet. The results of these examinations revealed no significant influence on the ADG, but on the FCR. Animals receiving the oregano supplemented diet, enriched with Cu or reduced of Cu, had a significantly improved FCR compared to the control animals (2.27 and 2.22 vs. 2.30 kg/kg). In contrast, Jugl-Chizzola et al. (2006) examined the palatability of oregano and found that it was not readily accepted by the weaned piglets. These researchers recognized that a supplementation of 0.02 or 0.002% oregano essential oil influenced the weaned piglets feeding pattern in the direction to feed without oregano or a mixture with other herbs. Apparently, the high concentration of terpenes (1.8%) was the supposed cause for this eating pattern. The terpene content of the oregano used in the present study was 1.3%. The piglets of Jugl-Chizzola et al. (2006) preferred the combination of both oregano and thyme next to the thyme mixture. The

avoidance of the oregano diets do not correspond to the findings of the present study (**Paper I**) and other researchers (for example Neill et al., 2006, Untea et al., 2011) in which higher amounts of dried oregano or essential oil were used and the feed intake was not adversely influenced. Nonetheless, the variety of the used oregano plant material needs to consider.

In general, the supplementation of oregano, regardless of form, does not influence feed intake of piglets in an adverse way. Admittedly, based on **Paper I** and other literature, an improving effect of feeding oregano to weaned piglets concerning the performance cannot be confirmed. As mentioned in Paper I there is considerable variety in concentrations of the compounds of oregano essential oil used in the previous studies which underlines the necessity of guidelines in view of lower and upper limits and the chemical composition of herbal additives in pig nutrition.

7.1.2 The effect on the intestinal microbiota

The use of herbal substances as antimicrobial agents is of common interest regarding scientists, industry and consumer. Both in food preservation and in feed industry the research for phytogenic antimicrobials has increased. Since the ban of antimicrobial growth promoters in 2006 (regulation EC/1831/2003) the animal nutritionists have examined various herbal plants, extracts and essential oils in reference to their antimicrobial ability. Commonly, the time after weaning is associated with health problems due to the altered environmental conditions. Post-weaning diarrhoea and reduced feed intake is not only caused by environmental changes, but also by modulations of the intestinal microbiota as cited by Lallès et al. (2004), (2007). They emphasized the importance of a homeostasis of the composition of the intestinal microbiota contributing to a balanced gut health. Particularly, the essential oil components carvacrol and thymol of the Mediterranean herb oregano have been examined in several investigations due to their known antimicrobial abilities (Michiels et al., 2008, Namkung et al., 2004). Michiels et al. (2007) demonstrated that carvacrol and thymol were potent inhibitors of *E.coli* and other coliform bacteria in their *in vitro* simulation of the pig gut flora. These researchers stressed the effectiveness of carvacrol and thymol to reduce the bacterial load particularly in the acidic parts of the GIT. Moreover, it is well established the highest microbial density is localised in the colon (Manzanilla et al., 2004). Based on this knowledge the alteration of the intestinal microbiota in response to ingested dried oregano was examined in the current study (**Paper II**). Digesta

samples of three parts of the small intestine and the proximal colon were investigated with two molecular biological methods: RFLP and FISH. The methods used in the present investigation to establish the microbiota along the GIT correspond to those of Castillo et al. (2006) who pointed out that using FISH has the advantage to avoid potential PCR-bias. Both methods are commonly used in human medicine to quantify microbial communities in the gut.

However, the results of both methods disagree with the current literature based on the distribution of the microbiota between control group and animals fed with oregano. But, it should be considered that piglets were used for microbial investigations just once after five experimental weeks. Therefore our results are limited to these specific conditions. As shown in Figure 5, the total count of the bacterial load did not differ between both feeding groups. The investigated microbial community was not altered in response to ingested dried oregano plant material.

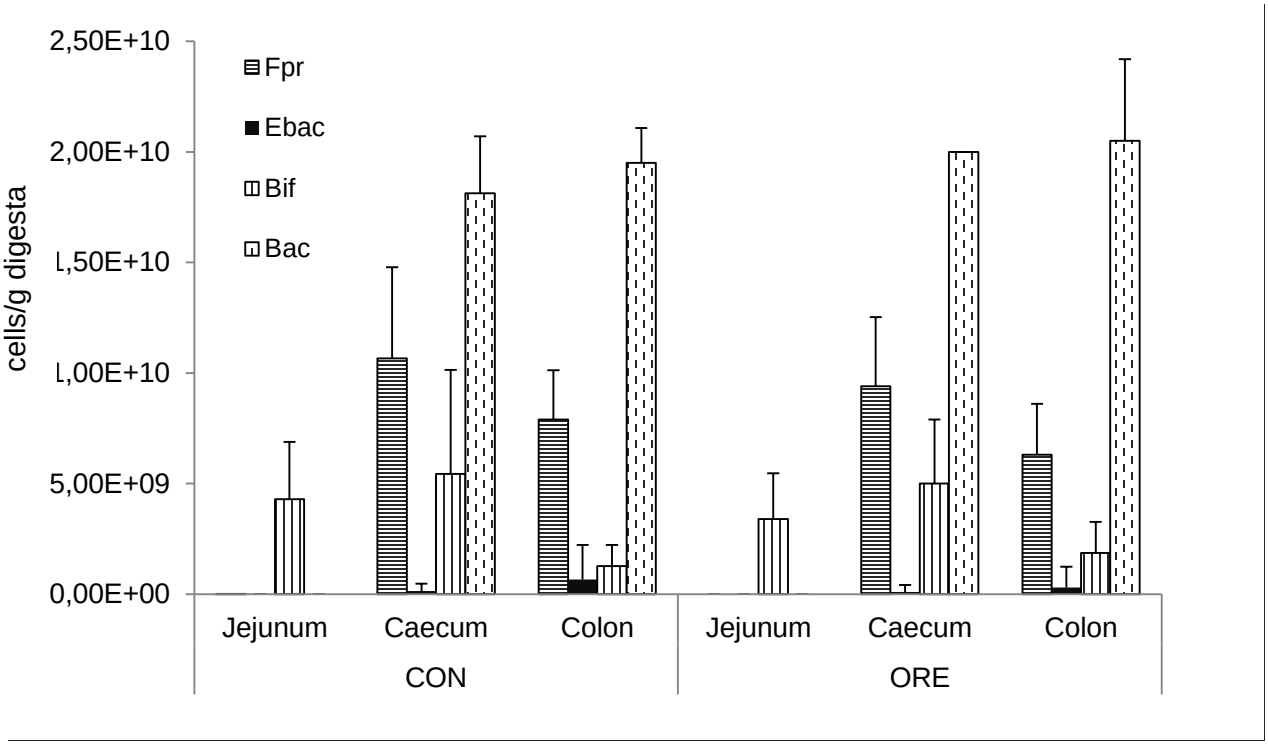

Figure 5: Spatial organisation of the intestinal microbiota along the gastrointestinal tract based on FISH (mean±SD)

CON, control group; ORG, feeding group with 8 oregano/kg diet; Fpr, Faecalibacterium prausnitzii group; Ebac, Enterobacteriacae; Bif, Bifidobacterium spp.; Bac, Bacteroidaceae and Prevotellaceae

Castillo et al. (2006) likewise observed that the supplementation of 0.03% plant extracts (5% carvacrol, 3% cinnamaldehyd, 2% capsicum oleoresin) did not lead to alterations in total bacterial loads. Equally, Muhl and Liebert (2007) used a phytogenic feed additive in three levels (0.5, 1.0, 1.5 g/kg) consisting of 6% carvacrol and 0.14% thymol and did not detect a significant modification of the intestinal microbiota. Indeed, the mentioned researchers used firstly an extract of various herbal plants, and secondly oil based products, while in the current study (**Paper II**) dried oregano plant material was supplemented to the diets. The analysed total terpene content of the used oregano was 1.36%, of which 86% was carvacrol and 7% thymol. This underlines the diversity of the oregano used in the mentioned investigations and limited the comparability with literature data. Consequently, the necessity to know about the standardisation of essential oil concentrations used in animal nutrition is highly appreciated.

7.1.3 The immunomodulation effect

The strengthening of the immune system of neonatal animals will only work if a good management exists, which takes particular care of a variety of factors, including housing conditions like temperature and, especially, the ensured intake of colostrum. Shortly after birth, the colonisation of the GIT by anaerobic and facultative anaerobic bacteria takes place (Bauer et al., 2006a). The literature stressed the importance of a complex bacterial community in view of the development of a functional mucosal immune system (Bauer et al., 2006a, Konstantinov et al., 2004, Castillo et al., 2007) and the general health (Bauer et al., 2006a, Wenk, 2003). After weaning the piglets still have an immature immune system due to the dependency on the sow's milk compounds like immunoglobulin A (Edwards and Parrett, 2002) which is known for its biological activity against viruses and bacteria, and as barrier preventing foreign antigens and pathogens to penetrate the intestinal mucosa (Cunningham-Rundles, 2001). After weaning the immunoregulatory and immuno protective compounds of maternal milk are declining and the piglet is more sensitive to diseases. Weaning is also associated with an induced antigenic challenge due to fed foreign proteins resulting in antibody formation (Lallès et al., 2007). Consequently, it is important to support the immune system of piglets during the weaning period. There is evidence that the use of oregano in diets of different animal species has the potential of stimulating the immune system (Walter and Bilkei, 2004, Revajová et al., 2010, Frankič et al.,

2009, Major et al., 2011). The essential oil components of this herbal plant, carvacrol and thymol, were evaluated regarding their leukocyte response and their antioxidant properties in previous studies. Similarly, the results of the present study (**Paper I**) showed immune modulating effects in response to ingested oregano. Strikingly, the total number of lymphocytes increased in response to oregano. Figure 6 represents the lymphocyte content of the two extreme groups (control and highest level of oregano) under the stimulation of a lipopolysaccharide challenge. The significant increase of the lymphocytes in the non-stimulated oregano fed animals is obvious. Regarding the LPS-stimulated animals there is no difference in the proportion of lymphocytes observed in control- and oregano group. Nevertheless, the proportions of lymphocytes in the LPS-stimulated oregano-fed animals were nearly half of the proportion in the oregano-fed unstimulated animals. Consequently, the LPS challenge enhanced the activity of immune related cells, like T-lymphocytes, by increased migration and infiltration in the infected area (Saalmüller, 1998).

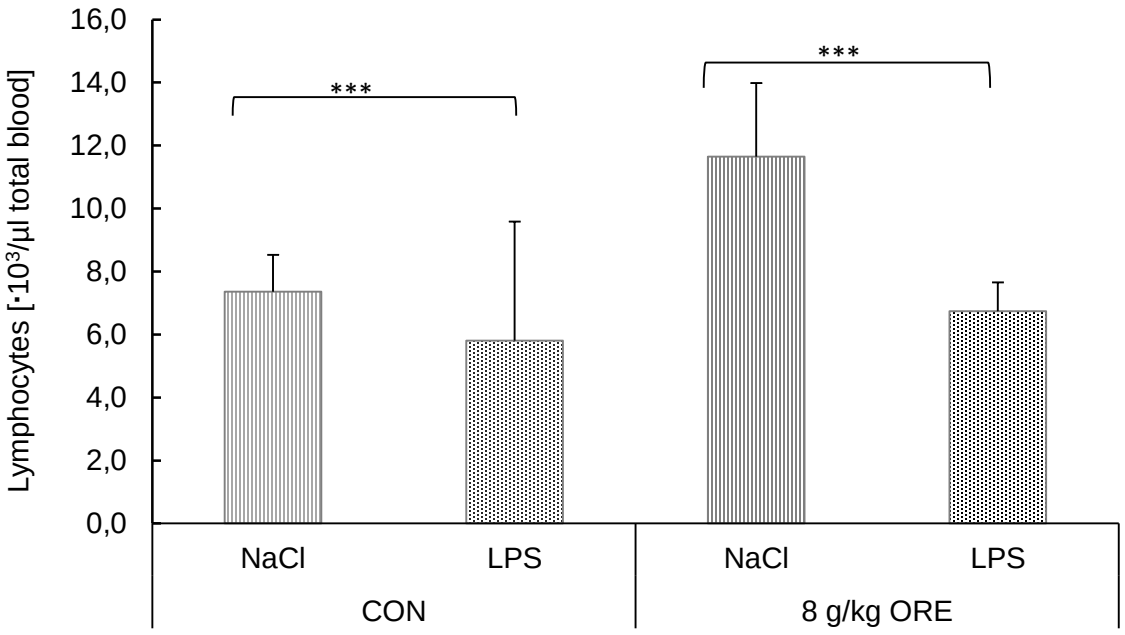

Figure 6: Effects of oregano and lipopolysaccharide challenge on lymphocytes (mean±SD), $p<0.05$

According to Major et al. (2011) the supplementation of oregano oil causes a significant increase of leukocytes after 3 days. Indeed, the examined animal species of the mentioned investigations belong to poultry and can hardly be compared with the results of the current study with pigs. Walter and Bilkei (2004) examined the immunostimulatory effect of oregano in pigs. They determined an increased proportion of peripheral blood lymphocytes, in particular, $CD4^+$, $CD8^+$, MHC class II antigens, and non-T/non-B-cells in animals fed with oregano compared to control animals. In contrast, the supplementation of dried oregano in the current study (**Paper I**) did not result in higher proportions of $CD4^+$ or $CD8^+$ T-lymphocytes compared to the control group independent of the LPS stimulation. As an illustration and because of the similar behaviour of $CD4^+$ and $CD8^+$ T-cells under the LPS-stimulation and in response to oregano, Figure 7 shows the ratio between both phenotypes of T-lymphocytes. The significant differences only occur under the LPS stimulation within each group ($p<0.01$).

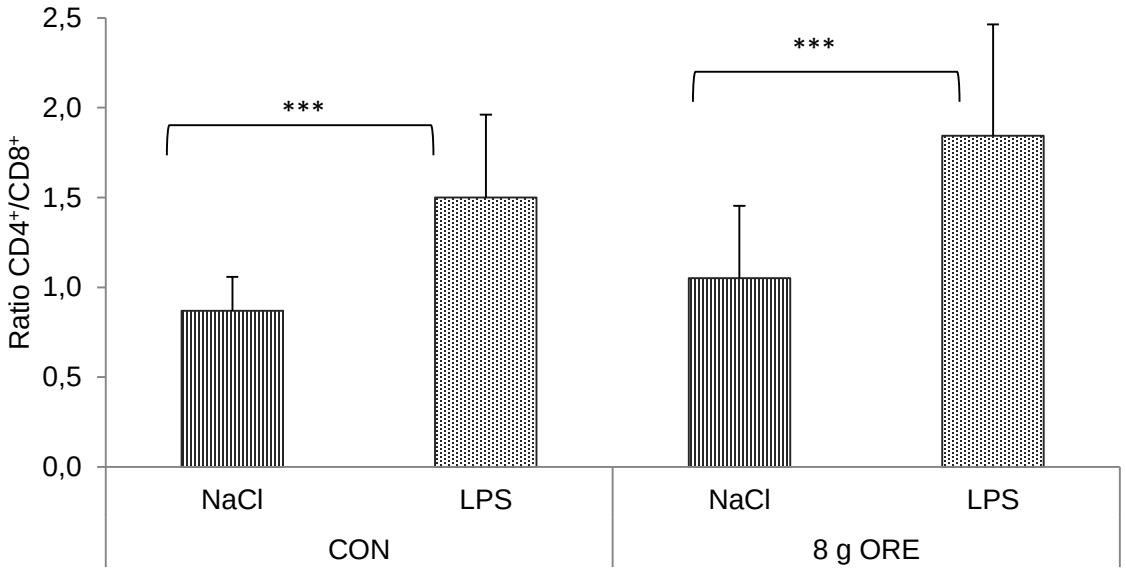

Figure 7: Effects of oregano and lipopolysaccharide challenge on the ratio of $CD4^+/CD8^+$ T-lymphocytes (mean±SD), $p<0.05$

Besides, Walter and Bilkei (2004) observed significantly higher proportions of double positive $CD4^+CD8^+$ T-lymphocytes in peripheral blood and mesenteric lymph nodes of oregano-fed animals. In contrast, the results of **Paper I** did not show a significant effect of oregano on the proportion of double positive $CD4^+CD8^+$ T-lymphocytes. Consequently, we assumed that B-cells or not yet primed T-cells were the reason for the high lymphocyte proportion. These assumptions were complemented by the study of Nofrarías et al. (2006) who showed an increased percentage of blood macrophages and lymphocytes density in the lamina propria in response to 0.03% of a plant extract mixture (containing carvacrol, cinnamaldehyd, and capsicum oleoresin). Whereas, Walter and Bilkei (2004) used growth-retarded pigs with an average weight of 58.2±2.4 kg, the piglets used in the current study (**Paper I**) had an average weight of 15.8±2.3 kg on the challenge day. This underlines that the proportion of lymphocytes in pigs is age-dependent (Bauer et al., 2006b, Saalmüller and Bryant, 1994). The age dependency is not the only reason for alterations in lymphocytes, but also the oregano supplementation. While Walter and Bilkei (2004) added a mixture of dried oregano and oregano oil to the diet, dried oregano plant material was admixed to the pig's diet in the current study resulting in a different content of carvacrol as active substance (180 mg carvacrol/kg diet vs. 94 mg carvacrol/kg diet). Michiels et al. (2008) suggested that the kinetic of essential oil depends on the adsorption of organic matter. Therefore, it is possible that the essential oil compounds of the ingested dried oregano interact more intensively with the organic matter than a pure oregano oil preparation. Consequently, the active substances pass the stomach and escape the solubilisation and absorption therein and the release of the active substances can occur in the small intestine. Michiels et al. (2008) showed high concentrations of carvacrol, thymol and eugenol in plasma within 1.4 h, which might reflect the rapid absorption of essential oil in the stomach in their investigation. For this reason, it is important to know in which part of the GIT the active compound will be absorbed because the formulation of effective inclusion levels in the diet can be examined more precisely.

Therefore, the overall effects of essential oils, including their immuno-modulatory effects, depend on pharmacokinetic properties of the herb preparation, such as the galenic form. Furthermore, there is still lack of knowledge regarding the mode of action of oregano. In conclusion, a standardisation of the oregano supplements is necessary. Moreover, investigations

with pigs on the effects of oregano on functional immune parameters are required besides a deeper insight into the properties and responsiveness of the gut associated lymphoid tissue.

7.2 The use of *Tropaeolum majus* L. (nasturtium) in growing pigs

7.2.1 The effect on feed intake

Tropaeolum majus L. belongs to the Tropaeolaceae and is commonly used in human medicine against respiratory diseases and urinary tract infections. Its efficacy is based on the mustard oil glucosinolates enriched in leaves, flowers and seeds. Although, the glucosinolates in their natural state are inactive, their degradation products, the isothiocyanates, are highly active. In view of palatability and edibility, both nasturtium leaves and flowers are edible and the taste of it is spicy and slightly bitter (Anonymous, 1985). Pigs are known for their sensitivity to alterations in the diet and therefore, the expectations regarding an improving feed intake due to the supplementation of nasturtium were correspondingly low. In contrast to this, the literature evidenced an uncomplicated feed intake in response to nasturtium-added diets in animals (Bloem et al., 2008, Bahramikia and Yazdanparast, 2008); independent of age, species or galenic form. Whereas Bloem et al. (2008) used piglets and ground seeds, Bahramikia and Yazdanparast (2008) examined an extract of nasturtium, but in rats. No one of the mentioned researchers reported on an adverse effect on feed intake although a very high dosage was used in the study with rats (500 mg nasturtium extract/kg BW). In the current experiment (**Paper III**) various dosages and galenic forms were tested regarding the level of feed intake. The dosages used in Bloem et al. (2008) complied with the recommendations for a person of 60 kg BW (equivalent to 2.17 mg glucotropaeolin (GTL)/ kg BW) (Blumenthal et al., 1998). Consequently, the dosages of the experiment in **Paper III** were also based on this reference. However, the dose levels ranged from 2.1 mg GTL/kg BW up to 22.6 mg GTL/kg BW in the various feeding groups to determine the level of intake. The animals accepted the diet with nasturtium tablets, and pulverized tablets without problems, but the dried powder only to a certain degree. Obviously, the administered amount of 80 or 160 g dried nasturtium per portion (corresponding to 800 and 1600 mg GTL/ portion) was not accepted by the pigs. The feeding pattern ranged from a strongly declined feed intake to complete refusal. Boeger et al. (1955), likewise, examined an extract of nasturtium in growing pigs and recognized smaller weight gains, but they also noticed a smaller protein

turnover in those animals. The control animals consumed 14.3% more feed protein compared to animals fed the nasturtium diet, concluding in a higher protein turnover compared to nasturtium-fed animals.

Apparently, nasturtium has the potential to influence the protein metabolism in pigs, but further research is needed to clarify this result. Based on the results, the intake of nasturtium is limited up to a level of 80 g dried powder per feed ration of 750 g in growing pigs. Apart from that, the recommended dosage of 2.2 mg GTL/kg BW, which was ingested without any problems, is completely sufficient to achieve an antimicrobial effect in animals which is described in detail in the following part.

7.2.2 The effect on the metabolism and the bioavailability

Tropaeolum majus L. contains glucosinolates in order to protect them from natural predators. The glucosinolates are natural components deriving from glucose and amino acids, and they are chemically stable and biologically inactive within sub-cellular vacuoles distributed in the plant (Johnson, 2002, Mithen et al., 2000). Due to tissue damage of the plant the glucosinolates get in contact with the enzyme myrosinase (EC 3.2.1.147) followed by a rapid hydrolysis to the active breakdown product, isothiocyanate (ITC) (Fenwick et al., 1982, Fahey et al., 2001, Vermeulen et al., 2006). These ITCs are widely known for their antimicrobial, antiviral and cancer preventing abilities in human medicine (Pintão et al., 1995, Shapiro et al., 2001, Bazylko et al., 2013, Halbeisen, 1954b), but little is known about their use as alternative for antibiotics against respiratory or urinary diseases in pigs.

Based on the experiment of Bloem et al., 2008 who found no adverse effects on feed intake by the piglets fed nasturtium and further determined urinary ITC concentrations which were high enough to be effective against pathogens, the current study (**Paper III**) confirms these effects and additionally extended the urinary parameters with the concentration of ITC in plasma. Nasturtium was fed in different galenic forms: enteric coated tablets, pulverized tablets and powder of the dried plant material. Johnson (2002) postulated that ingested glucosinolates are broken down in the small intestine due to the plant myrosinase or by bacterial myrosinase in the colon. Consequently, the active ITC can be absorbed from the small- and large intestine and can exert its systemic effects. The results of **Paper III** showed a bioavailability in the following order: tablets

(dose 1) > pulverized tablets (dose 1) > powder (dose 3) > pulverized tablets (dose 2). Figure 8 shows the area under the curve of the different galenic forms after 24 h in relation to the dosage. Interestingly, the highest bioavailability in relation to the dosage was determined in animals fed the pulverized form (dose 1 and 2). This effect is probably caused by an immediate absorption by oral or gastric mucosa. Quite unclear is the value of powder fed animals which were administered the highest dosage, but showed the smallest bioavailability. We assumed that this value is caused by the different feeding regimens within this feeding group (gradually feeding vs. all in one), and the slow feed intake and should therefore not be over-interpreted. Mithen et al. (2000) specified an unambiguous effect of plant and microbial myrosinase activity in view of the digestive conversion of glucosinolates in their breakdown products. Processing such as cooking and freezing are able to influence the activity of myrosinase (Bianchini and Vainio, 2004). Investigations with raw and cooked broccoli sprouts showed a 1.7 times higher AUC in consumers eating raw broccoli than in those ingested cooked broccoli. The peak plasma concentration of the breakdown product sulforaphane was 20 times higher compared to that of volunteers consuming cooked broccoli, relative to intake (Vermeulen et al., 2008). For this reason, the researchers concluded that the conversion from glucoraphanin, the main glucosinolate of broccoli, into sulforaphane was markedly declined due to cooking and inactivating myrosinase.

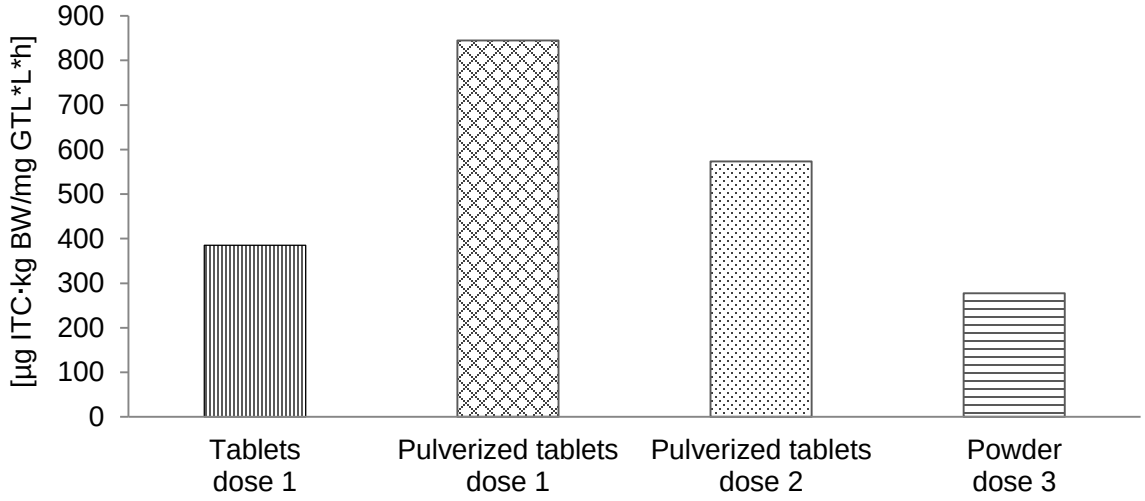

Figure 8: The area under the curve after 24 h in relation to the dosage of glucotropaeolin of the ingested *Tropaeolum majus* L.

All in all, many investigations with raw or cooked glucosinolates containing vegetables in humans are available, but no comparable with studies with animals. It is known that cooking these vegetables inactivates myrosinase, consequently, the hydrolysis of glucosinolates to the active breakdown product cannot takes place (Johnson, 2002). Not only cooking, but also the water solubility of the breakdown products affects the bioavailability in the host. ITC are known to be soluble in water and if the water is not ingested, the active substances will get lost (Bianchini and Vainio, 2004). In addition, Bloem et al. (2007) discovered that the GTL content was lost up to 96.5% within 1.25 min due to crushing with water. In the current study pigs' feed was mixed with tap water for a wet feeding and some water was still left in the trough. The urinary excretion rates of the current investigation (**Paper III**) illustrated these findings. Pigs receiving the nasturtium (80 or 160 g/portion) all at once in the feeding trough showed a slower feed intake and lower excretion rates than pigs fed with the nasturtium in little portions in the ration. Urinary data showed a 10 times higher excretion of ITC in animals fed with the nasturtium powder gradually than in pigs receiving the powder as all in one portion, whereas the AUC_{24}-value was similar in both feeding regimens. It might be possible that the conversion from glucotropaeolin into isothiocyanate was incomplete because of the longer soaking in the wet mash or because another way of excretion, like exhalation, was used (Vermeulen et al., 2008). In contrast to the present study, Getahun and Chung (1999) administered cooked and uncooked watercress to human volunteers and they detected a recovery of ITC from 150g uncooked watercress which ranged from 17.2-77.7% and, was markedly higher than in the current investigation. In spite of the high GTL-concentration (13.4 mg/kg BW) used in the pig experiment, the urinary recovery ranged only from 0.57 to 5.0%, relative to intake. Interestingly, the total amount of ITC in the uncooked watercress diet of Getahun and Chung (1999) was 972 µmol, while pigs of the present study (**Paper III**) ingested 1481 µmol GTL/portion. It is obvious that the dosage form influences the metabolism and the excretion.

Notwithstanding, the aim of feeding nasturtium was to reach an antimicrobial effect. Concentrations of about 0.5-50 µg of benzyl mustard oil/ml urine are known to be effective against bacteria (Anonymous, 1985). The results of the current investigation (**Paper III**) showed ITC concentrations up to 11.1 µg ITC/ml urine. Previous studies with nasturtium demonstrated minimal inhibitory concentrations of 1.75-3.5 µg/ml against Salmonella paratyphi B and 0.75-2.5

µg/ml against Streptococcus pyogenes (Halbeisen, 1954a). This researcher underlined the broad effectiveness of nasturtium against gram positive and gram negative bacteria as well as against acid-proof rod bacteria and fungi. In view of this, it is obvious that the enteric coating did not achieve the necessary antimicrobial concentration in urine which is effective against the most important pathogen bacteria strains. In consequence, the recommended dosage in pulverized form has the potential of an antimicrobial effect, however, more effective would be a higher concentration.

8 Conclusions

The use of dried *Origanum vulgare* L. as representative of terpenic plants did not affect the performance of weaned piglets in the present investigations. Feed intake was not adversely influenced up to the highest concentration of 8 g oregano/ kg diet.

Feeding dried oregano to weaned piglets resulted in an alteration of lymphocytes in the present study. The proportion of lymphocytes in unstimulated oregano-fed animals was significantly higher than in immunostimulated control animals, but no alterations were found in the subpopulations $CD4^+$ and $CD8^+$ T-cells. It was assumed that B-cells, not yet primed T-cells or monocytes were responsible for this effect. For further research the mentioned leukocytic cells and the leukocytic infiltration into the mucosal lamina propria should be considered.

Feeding oregano to piglets over a period of five weeks did not alter the examined intestinal microbiota at that time. In control animals and in oregano-fed animals, both bacterial colonisations were similar when RFLP and FISH was used. From this study it has been concluded that the administered dosage and galenic form need to be considered carefully when evaluating the treatment effects. Furthermore, other important bacteria, such as *Lactobacillus* should be focused. Moreover, future studies should examine treatment effects on the intestinal microbiota more intensively at critical periods, such as weaning.

Based on the results of the current investigation it can be confirmed that *Tropaeolum majus* L. can be used as a preventive measure regarding urinary infections in pigs. The reached BITC concentration in plasma and urine is potentially efficient to affect pathogen bacteria after the ingestion of the pulverized form and the powder. Additionally, the investigation of the different galenic forms resulted in not advising the enteric coating, because of its very low bioavailability and the ineffective concentration in urine within 24 hours.

In conclusion both feeding *Origanum vulgare* L. and *Tropaeolum majus* L. to pigs affect immunmodulating parameters. Furthermore, they are adapted as preventive measures regarding the stabilisation of the immune system. In view of the growth promoting advantage of antibiotics, the supplementation of dried oregano in the used dosage has failed. Additionally, due to the

volatile components of oregano the dosage form should be reconsidered in further studies. However, not only the administered form of oregano or the dosage level led to the unsatisfactory results, but the good health status of the used animals during the time of sampling could also be a reason.

Regarding the supplementation of nasturtium there is a need for tests in sows due to the antimicrobial potential in urine, and further research on the minimal inhibitory concentration of the measured plasma and urinary BITC concentrations. In addition, the results showed a markedly influence on the urinary excretion due to the feeding regime. It should be examined which way of supplementation aimed at the desired effect.

9 Summary

Herbs and botanicals have been widely known as remedies since thousands of years. At present, not only the interest regarding antibiotic resistances, but also the conscious awareness about the nutrition rises every day. Combining these two facts at the beginning of the supply of nutrients in animals is an important starting-point in this respect. Pigs, as one of the most sensitive animals, were fed with in-feed antibiotics until 2006 and nowadays, after the ban of antibiotic growth promoters, still belong to the animal species with the highest use of antibiotics. In this context, some herbs and botanicals show antimicrobial activities in different investigations both *in vitro* and *in vivo* and therefore, have the potential to act as a phytogenic antibiotic. Especially plants containing essential oils and mustard oil glycosides are of interest in alternative medicine. Oregano essential oil compounds like carvacrol and thymol have been widely examined regarding their cancer-preventing, antioxidative and antimicrobial abilities in human and nasturtium, as a representative of plants containing mustard oil, is widely used against respiratory and urinary diseases in human medicine. Most researchers used plant extracts or essential oils, but in the current study the simplest pharmaceutical form was used, because the knowledge about immuno-modulatory potential of dried oregano and the metabolism of dried nasturtium powder in pig is either limited or non-existent.

For this reason the aim of the first part of the current investigation was to examine the effect of oregano on the eating pattern of the piglets, immunological parameters in unstimulated or lipopolysaccharides challenged piglets, and finally, the alteration of the intestinal microbiota.

In the second part of the study the effect of nasturtium on the metabolism, the bioavailability and the urinary excretion of glucotropaeolin, the main glucosinolate of nasturtium, was focused on.

In order to clarify the mentioned issues several experiments with weaned piglets and growing pigs were conducted and published in three scientific articles. In the first experiment, a total of 80 male castrated piglets were assigned to four different diets with various concentrations of oregano: 0 g, 2 g, 4 g and 8 g oregano/ kg diet. After an experimental time of three weeks, half of each group was challenged with 5 µg lipopolysaccharide/kg body weight to induce a systemic inflammation. Whereas performance was not affected after the five experimental weeks, blood parameters like lymphocytes and triglyceride concentration in serum was influenced by oregano

supplementation in stimulated and non-stimulated animals. In view of the lymphocytes, animals fed with oregano showed more than 38% of lymphocytes compared to the control group. Nevertheless, the detailed analysis of the $CD4^+$ and $CD8^+$ subpopulation did not clarify this phenomenon. There were no significant changes in dependence of oregano supplementation in these T-lymphocytes. The results of the clinical-chemical parameter serum triglyceride indicated a dose dependency. The values increased up to a concentration of 4 g oregano and declined in the animals fed with 8 g oregano. Actually, triglyceride increased up to 33% in relation to the control animals. Obviously, oregano compounds, like carvacrol, are able to affect lipogenesis. Generally, dried oregano as feed additive does not influence the feed intake in an adverse way and is able to stimulate immune-related parameter, but does not modulate the immune response induced by LPS stimulation in the current experiment.

The second scientific article described the alteration of intestinal microbiota in response to oregano. For this purpose, 20 animals of Experiment 1 (10 animals of the control group and 10 of the group fed with 8 g oregano) were slaughtered after the experimental time of five weeks and intestinal contents of jejunum, caecum and colon were analysed with RFLP and FISH. The following groups of microorganisms were investigated: *Faecalibacterium prausnitzii* group (Fac), *Enterobacteriaceae* (Ebac), *Bifidobacterium spp* (Bif), *Bacteriodaceae* and *Prevotellaceae* group (Bac). The results indicated that the used dosage of dried oregano did not affect the microbial pattern and concentration in the different intestinal segments. Both used methods showed similar results in oregano-fed animals and control animals. Mostly Fac and Bif were detected in the caecum, whereas Fac and Ebac were the main parts of the microbiota in the colon independent of oregano supplementation.

In paper three, a total of 26 male castrated pigs were surgically equipped with vena jugularis externa catheters and administered to different galenic forms (enteric coated tablets, pulverized tablets and powder) and dosage levels (2.3, 6.9 and 13.4 mg GTL/kg body weight) in form of dried nasturtium. To elucidate the kinetic of the active substance, isothiocyanate, blood and urine samples were collected within 24 h after nasturtium intake. As a result, the enteric coating showed the smallest bioavailability compared to the other galenic forms, especially in the case pulverized tablets with the same dosage level. Additionally, the plasma concentration of BITC increased with raising GTL-intake which emphasised a dose dependency. Animals fed with

pulverized tablets and the powder in the concentration which was about 3 or 6 times higher than the recommended dosage showed nearly 2 till 4 times higher AUC_{24}-values than animals with the lowest dosage. Regarding the urinary excretion, a dependence on the feeding regimen was noticed. Animals given the nasturtium at even one portion showed a 10 times lower excretion rate than animals receiving the nasturtium gradually ($p<0.05$) in relation to GTL intake/kg BW. Notably, is also the higher bioavailability of ITC in animals fed gradually compared to the animals receiving the single portion in relation to the intake (335 vs. 230 µg ITC·kg BW/mg GTL·L·h). Finally it was recognized that the BITC concentration in urine can be considered to have an antimicrobial effect in the different dosage forms, but it can only be realised in a limited manner in enteric coated tablets.

In general, the results of the current experiment showed that the use of oregano and nasturtium as phytogenic feed additives can be regarded as preventive measures in pig farming. In particular, oregano as a representative of terpenic plants can be useful in the time after weaning to modulate the immune system, while feeding nasturtium is more relevant to reduce pathogen bacteria in urine.

10 Zusammenfassung

Die Heilwirkung von Kräutern und pflanzlichen Stoffen ist seit Jahrtausenden bekannt. Die zunehmende Besorgnis hinsichtlich der steigenden Resistenzbildung durch den Einsatz von Antibiotika in der Nutztierhaltung und das Umdenken der Verbraucher in Richtung bewusster Nahrungsaufnahme ist in der heutigen Zeit von wachsendem Interesse. Um diese Punkte unter einem wissenschaftlichen Hintergrund zu vereinen, ist es notwendig bei der Nährstoffversorgung der Tiere anzufangen.

Bis zum Jahr 2006 war der Einsatz von Fütterungsantibiotika in der Nutztierhaltung offiziell erlaubt. Vor allem im Bereich Schwein, welches mit zu den empfindlichsten Tieren unter unseren Nutztieren gehört, wurden antibiotische Leistungsförderer in großem Maße eingesetzt. Heutzutage sind die Leistungsförderer zwar verboten, jedoch ist der Antibiotikaverbrauch in erheblichen Teilen in Richtung der behandelnden Tierärzte umgelagert worden. In diesem Zusammenhang ist die Suche nach Alternativen zu erwähnen. Besonders Kräuter und pflanzliche Substanzen konnten in verschiedenen *in vitro* und *in vivo* Versuchen durch ihre antimikrobiellen Fähigkeiten überzeugen. Ätherische Öle, wie auch Senföle sind von großem Interesse in der Alternativen Medizin. Als Vertreter der terpenhaltigen Pflanzen ist vor allem Oregano mit seinen Komponenten Carvacrol und Thymol und seiner antikanzerogenen, antioxidativen und antimikrobiellen Wirkung von großer Bedeutung. Die Kapuzinerkresse, als bekannte Heilpflanze mit ihren Senfölen, ist in der Humanmedizin bei Atemwegs- und Harnwegsinfektionen weit verbreitet. In den meisten bisher durchgeführten Studien erfolgte die Verwendung von ätherischen Ölen oder Pflanzenextrakten. In der vorliegenden Arbeit wurden dagegen die oberirdischen Teile von in Deutschland kultivierten und geernteten Kräutern in getrockneter und gemahlener Form als einfachste pharmazeutische Darreichungsform genutzt.

Weitreichende Kenntnisse bezüglich der immunmodulierenden Wirkung von getrocknetem Oregano und bezüglich der Kinetik von Isothiocyanat im Schwein sind nur unzureichend bis gar nicht vorhanden. Aus diesem Grund wurden in der vorliegenden Untersuchung im ersten Teil sowohl der Einfluss von Oregano auf die Futteraufnahme und immunologische Parameter unter Einfluss einer systemischen Infektion als auch auf die Mikrobenpopulation im Darm näher

betrachtet. Der zweite Teil der Arbeit beschäftigte sich mit den Auswirkungen der Kapuzinerkresseaufnahme hinsichtlich Bioverfügbarkeit und Ausscheidung über die Harnwege.

Um die aufgeführten Fragestellungen zu beantworten, wurden verschiedene Versuche mit Absetzferkeln und wachsenden Schweinen durchgeführt und die Ergebnisse in drei wissenschaftlichen Publikationen veröffentlicht.

Im ersten Versuch wurden 80 männlich, kastrierte Absetzferkel mit vier verschiedenen Futterrationen, die sich in der Konzentration des zugesetzten Oregano unterschieden, gefüttert. Der Zusatz von getrocknetem Oregano war wie folgt: 0 g, 2 g, 4 g und 8 g/ kg. Die Hälfte der Tiere wurde nach einer Versuchslaufzeit von drei Wochen mit Lipopolysacchariden (LPS) (5 µg/kg KM) behandelt, um eine systemische Inflammation auszulösen. Nach der gesamten Versuchsperiode (5 Wochen) konnten keine signifikanten Unterschiede hinsichtlich der Leistung erfasst werden. Allerdings, war ein Einfluss des zugesetzten Oregano auf die Anzahl der Lymphozyten und den Triglyceridwert, unabhängig von der Stimulation mit LPS, zu erkennen. Die mit Oregano gefütterten Tiere zeigten einen um 38% höheren Gehalt an Lymphozyten als die Kontrolltiere. Dieser erhöhte Gehalt konnte allerdings nicht durch die Untersuchung der $CD4^+$ und $CD8^+$ T-Zell Subpopulation abgeklärt werden. Es gab keine signifikanten Unterschiede in diesen Subpopulationen hinsichtlich der Oreganofütterung. Die Ergebnisse der Triglyceride deuten auf eine Dosisabhängigkeit hin, denn mit steigender Oreganokonzentration bis zu 4 g, erhöhen sich die Triglyceride um bei einer Konzentration von 8 g Oregano wieder abzufallen. Der Triglyceridgehalt der Oreganotiere war insgesamt bis zu 33% höher als bei den Kontrolltieren. Im Allgemeinen ist zu erwähnen, dass Oregano als Futterzusatzstoff die Futteraufnahme nicht nachteilig beeinflusst, jedoch das Immunsystem unspezifisch stimulieren kann.

In der zweiten Veröffentlichung wurde der Einfluss von Oregano auf die Darmmikrobiota untersucht. Um diesen Sachverhalt zu klären, wurden 20 Tiere (10 Kontrolltiere und 10 Tiere aus Gruppe 3, mit der höchsten Oreganozulage von 8 g/kg) aus Experiment 1 nach der fünfwöchigen Versuchszeit geschlachtet und Proben vom Darminhalt aus Jejunum, Caecum und Colon genommen und mittels RFLP und FISH analysiert. Folgende Bakterienpopulationen/-gruppen wurden untersucht: *Faecalibacterium prausnitzii* (Fac), *Enterobacteriaceae* (Ebac), *Bifidobacterium spp* (Bif) und *Bacteriodaceae* und *Prevotellaceae* (Bac). Die Ergebnisse zeigen,

dass die Fütterung von Oregano in der gewählten Konzentration zu keinen signifikanten Änderungen in den untersuchten Populationen geführt hat bzw. die Tiere über einen sehr guten Allgemeinzustand verfügten, so dass keine positive Wirkung mehr erzielt werden konnte. Sowohl die RFLP-Analyse wie auch die FISH führten zu ähnlichen Ergebnissen. Im Allgemeinen konnte festgestellt werden, dass vor allem das Caecum mit Fac und Bif besiedelt war, während im Colon vorwiegend Fac und Ebac detektiert wurden.

Grundlage der dritten Veröffentlichung stellten 26 kastrierte wachsende Schweine dar, die Venenverweilkatheter eingesetzt bekommen haben. Den Tieren wurde die gemahlene Kapuzinerkresse in verschiedenen Darreichungsformen (magensaftresistent-verkapselte Tabletten, zermahlene Tabletten und Pulver) und Dosierungen (2,3, 6,9 und 13,4 mg GTL/kg LM) vorgelegt. Um Aussagen bezüglich des Metabolismus und der Bioverfügbarkeit treffen zu können, erfolgte die Sammlung von Harn und Blut über einen Zeitraum von 24 h. Die Ergebnisse lassen darauf schließen, dass eine magensaft-resistente Verkapselung nicht zum gewünschten Erfolg führt. Die Bioverfügbarkeit der verkapselten Tabletten lag zu 55% unter der der zermahlenen Tabletten gleicher Dosierung. Weiterhin zeigten die Ergebnisse, dass mit steigender Dosierung eine Steigerung der Isothiocyanatkonzentration im Plasma verbunden ist. In den Tieren, denen eine drei- bis sechsfach höhere GTL-konzentration im Vergleich zu der empfohlenen Dosierung vorgelegt wurde, zeigten sich zwischen zwei- bis vierfach höhere AUC_{24}-Werte. Die Auswertung der Harnwerte deutete darauf hin, dass ein Zusammenhang zwischen der Art der Fütterung und dem Gehalt an ITC im Harn besteht. In den Tieren, die die Kresse als Einmalportion bekommen haben, konnten 10-fach geringere Konzentrationen der wirksamen Substanz (ITC), in Relation zur Aufnahme, gemessen werden. In diesem Zusammenhang war vor allem die höhere Bioverfügbarkeit des ITC der Tiere, die die Kresse portionsweise verabreicht bekommen haben im Vergleich zu den Tieren mit der Einmalportion Kresse, in Relation zur aufgenommenen Menge an GTL, auffällig (335 vs. 230 µg ITC·kg LM/ mg GTL·L·h). Abschließend kann gesagt werden, dass mit der Verabreichung von Kapuzinerkresse in getrockneter, gemahlener Form antimikrobielle Effekte im Harn ausgelöst werden. Allerdings ist zu beachten, dass diese Wirkung bei Verwendung von magensaft-resistenten Tabletten nur begrenzt erzielt wird.

Zusammenfassend ist zu erwähnen, dass die hier untersuchten Heilpflanzen Oregano und Kapuzinerkresse als therapeutische Maßnahme in der Schweinehaltung förderlich sein können. Oregano, als Vertreter der terpenhaltigen Pflanzen, kann besonders im Zeitraum um das Absetzen eingesetzt werden, um das Immunsystem zu modulieren, während die Kapuzinerkresse vor allem im Hinblick auf die Keimreduzierung im Harn Anwendung finden könnte.

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CURRICULUM VITAE

Katrin Stelter
Born on 19 November 1983
in Sangerhausen, Germany

1994-2003	Johann-Wolfgang von Goethe Gymnasium, Kelbra Degree: Abitur
2003-2006	Training as biologic laboratory technician at the Landwirtschaftliche Untersuchungs- und Forschungsanstalt Nord-West, Oldenburg
2006-2009	Bachelor studies of Agricultural Sciences at the Martin-Luther-Universität Halle-Wittenberg Degree: Bachelor of Science (agr.)
2009-2011	Master studies of Agricultural Sciences at the Martin-Luther-Universität Halle-Wittenberg Degree: Master of Science (agr.)
2011- 2014	Guest scientist at the Institute of Animal Nutrition, Federal Research Institute for Animal Health, Friedrich-Loeffler-Institute, Braunschweig

Eidesstattliche Erklärung

Ich erkläre an Eides Statt, dass ich die Arbeit selbstständig und ohne fremde Hilfe verfasst, keine andere als die von mir angegebenen Quellen und Hilfsmittel benutzt und die den benutzten Werken wörtlich und inhaltlich entnommenen Stellen als solche kenntlich gemacht habe.

Südharz, 20.09.2014 _____

Katrin Stelter

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