

Effects of herbal feed additives and processing methods on the concentration of skatole in the adipose tissue of pigs

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LIST OF ABBREVIATIONS

CYP	Cytochrome
HepG2	Human Hepatoblastoma Cell Line
IGF-I	Insulin-like growth factor 1
Nrf-2	Nuclear Factor Erythroid 2-related Factor 2
P(450)CYP	Cytochrom P450

CHAPTER 1

GENERAL INTRODUCTION

1 GENERAL INTRODUCTION

For a long time, male piglets were allowed to be surgically castrated without anesthesia up to 7 days after birth, as meat from male pigs can, in specific conditions, develop an undesirable odor. However, surgical castration without anesthesia or analgesia is painful and compromises the physical integrity these animals (Von Borell et al., 2009). In the last decade, and notably in recent years, a noticeable transformation occurred in consumer purchasing behavior, placing greater emphasis on the quality of meat and considerations for animal welfare (Heid & Hamm, 2013). Therefore, the EU member states agreed to prohibit the non-anesthetized castration of piglets by the end of 2020. Since the beginning of 2021, castration in Germany is only allowed under general anesthesia and pain relief (§ 21 Abs. 1 Tierschutzgesetz).

Surgical castration or immunocastration is a method that is commonly used to prevent the occurrence of boar taint in the fattening of pigs. Boar taint is mainly caused by two compounds, androstenone and skatole (Bonneau et al., 2000). Androstenone is a steroidal pheromone produced in the testes. Skatole is the result of microbial degradation of typtophan, which is mainly produced in the hindgut of pigs. These two compounds differ in their origin, physiological significance and how they are manipulated by management factors, but they have in common that they accumulate in the adipose tissue of pigs due to their lipophilic properties (Claus et al., 1994; Neupert et al., 1995; Robic et al., 2008).

The incorporation of these substances into the tissues of pigs plays an important role in the production of pork and its processed products (Meier-Dinkel et al., 2013). Boar taint poses a risk to consumer acceptance (Font-i-Furnols, 2012a). Androstenone and skatole are associated with an unpleasant fecal or urine-like odor which develops when cooking pork and can lead to a negative perception. However, its intensity is not perceived in the same way by all consumers and has been shown to depend not only on gender but also on culture and region and probably on age (Blanch et al., 2012; Bonneau et al., 2000; Meier-Dinkel et al., 2016). In order to reduce its presence in the final product, various methods have been developed for screening carcasses before entering the processing chain (Aluwé et al., 2012; Font-i-Furnols et al., 2020). These techniques help to determine the intended use of the carcasses, aligning with specific final products or manufacturing processes based on the boar taint threshold (Škrlep et al., 2020). To ensure the marketability of the end meat products, several established methods are available for cases with elevated taint thresholds, ensuring consumer palatability through approaches such as masking, diluting or smoking (Aaslyng & Koch, 2018; Martínez et al., 2016a; J. Mörlein et al., 2019a)

For this reason, it is important to prevent whether the formation or the presence of both substances in the animal before it is slaughtered.

Androstenone is produced in the Leydig cells of entire male pigs and is regulated by the hypothalamic-pituitary-gonadal axis (Bonneau & Weiler, 2019). Besides other synthesised steroids in the testis, androstenone is stored in much higher concentrations and increases with puberty (Babol et al., 1999). Castration before sexual maturity is therefore one of the few ways to prevent storage.

Whereas skatole originates from the microbial degradation of the amino acid tryptophan and is synthesized through intermediates such as indole pyrovate and indoleacetic acid. The latter is considered a precursor to skatole and undergoes further decarboxylation to form 3-methylindole (skatole) by specialized bacteria (Deslandes et al., 2001; Whitehead et al., 2008). However, only a small number of bacteria from the *Clostridium* and *Lactobacillus* families are able to synthesise skatole from indoleacetic acid via a decarboxylase reaction (Whitehead et al. 2008). An exception is the strain *Clostridium scatologenes*. This strain can synthesise skatole directly from tryptophan without the intermediates described above (Jensen et al. 1995). The primary sources of tryptophan for this microbial process are found in the pig's diet, particularly from components with inadequate pre-cecal digestibility, and cellular debris from intestinal mucosal apoptosis (Claus & Raab, 1999; Laue et al., 1998; Leong et al., 2011; Neupert et al., 1995). *In vivo* and *in vitro* studies have indicated that supplementing L-tryptophan to the diet has no effect on total skatole concentrations, likely due to absorption in the small intestine. Consequently, the caecum has been identified as the beginning site of skatole synthesis (Bernal-Barragan, 1992; B. B. Jensen & Jorgensen, 1994; M. T. Jensen et al., 1995; Knarreborg et al., 2002). The highest concentration of skatole is found in the hindgut, increasing along the intestinal tract, peaking in the rectum. While a significant portion of the produced skatole is excreted, the remaining amount is absorbed by the intestinal mucosa, entering the bloodstream through passive diffusion and transported to the liver. The liver is considered the critical site for skatole metabolism, as demonstrated by differential measurements of skatole concentrations from the vena portae to the vena hepatica (Knarreborg et al., 2002; Laue et al., 1998). Skatole metabolism occurs in two consecutive phases, one oxidative and one conjugative. Key enzymes involved in phase I belong to the Cytochrome P(450) subfamilies CYP1A, CYP2A, CYP2E1, and CYP3A. In phase II, seven resulting intermediates are conjugated to a glycuronyl or sulphate residue by uridine diphosphate glucuronosyltransferase (UGT) or sulfotransferase SULTA1, respectively, facilitating urinary excretion by increasing their hydrophilicity (Wesoly & Weiler, 2012b). Although skatole production is independent of sex,

studies have shown that enzymatic P(450)CYP degradation is not, since the presence of androstenone leads to decreased phase I CYP enzyme activity, significantly influencing its degradation and subsequently on deposition of skatole in adipose tissue (Rasmussen et al., 2011a).

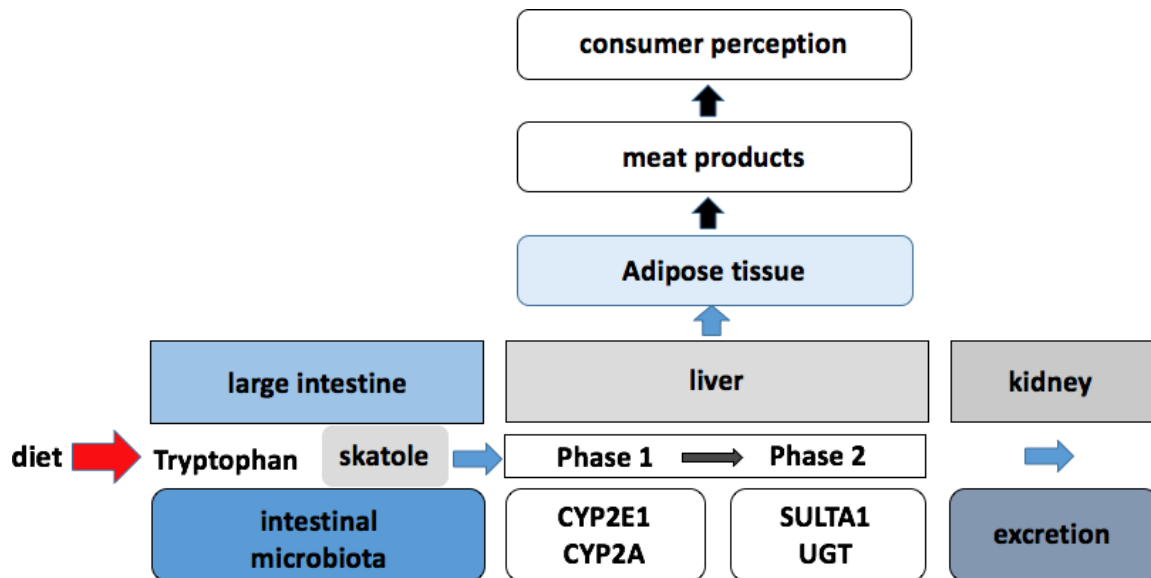


Figure 1: Schematic overview of the skatole pathway

It can be concluded that the formation of skatole is depended on microbial composition and the amount of available of tryptophane. The latter can be attributed to the cellular debris of the intestinal epithelium. Feed additives such as purines have been demonstrated to increase IGF-I, leading to an elevated mitotic rate and subsequent cell debris. One effective way to counteract the apoptosis rate in the colon is by incorporating raw potato starch into the diet. These prebiotic substances undergo bacterial conversion, releasing short-chain fatty acids that inhibit apoptosis and, consequently, the availability of tryptophan necessary for skatole formation (Claus & Raab, 1999; Lacorn, 2009; L. Zhou et al., 2017). Similarly, the use of chicory root and inulin has proven effective in reducing skatole concentrations. Intestinal studies have indicated that feeding varying concentrations of inulin enhances the production of short-chain fatty acids, particularly apoptosis-preventing butyrate (Aluwé et al., 2017; X. Li et al., 2019a). Additionally, an observed impact of inulin on hepatic pathways involved in skatole metabolism further supports its potential efficacy (Rasmussen et al., 2011b).

In addition to preventing intestinal cell debris, manipulation of the bacteria's energy management is a key strategy. The most effective approaches to date involve shifting microbial

metabolism from proteolytic to saccharolytic pathways to inhibit skatole formation. The residual energy in the colon plays a pivotal role in influencing bacterial activity during this metabolic shift. Studies have demonstrated the effects of feed additives such as sugar beet pulp supplementation, high amylase supplementation, and fructo-oligosaccharide supplementation, influencing bacterial energy production in the hindgut (Kjos et al., 2010; Lösel & Claus, 2005; Pieper et al., 2014; Pinto et al., 2023; Salmon & Edwards, 2015). A different but effective approach to reduce skatole was the utilization of antibiotics by influencing the microbial composition in disfavor of skatole (Hansen et al., 1997; Hawe et al., 1992), but was discontinued due to the prohibition of its use as growth promoters in the EU.

Recently Essential oils/plant extracts are under discussion to be used in the animal nutrition. The abandonment of antibiotics as antimicrobial growth promoters for livestock requires alternative solutions to maintain the performance of current livestock production (Osaili et al., 2023). Essential oils have great potential and are considered to be natural, safe and free of any residual chemicals. Studies showed that these plant compounds have beneficial effects on nutrient digestibility, microbiota and gut function (Franz et al., 2010; Windisch et al., 2008; Zhai et al., 2018). In addition, studies have shown that some essential oils have strong antimicrobial properties, making them safe alternatives to antibiotics (Cui et al., 2019a; Dorman & Deans, 2000a; Smith-Palmer et al., 1998). Another property of some essential oils or plant extracts is that they have been shown to interact with hepatic metabolism (Awortwe et al., 2015; Nguyen et al., 2014). Specifically with the hepatic P(450)CYP enzymes and therefore a potential target site for interacting with the skatole pathway through interference with hepatic clearance (Robic et al., 2008).

One aim of the study was to investigate whether the selected essential oils / plant extracts were able to reduce the production of skatole or influence its metabolism. For this purpose, the effect on liver enzymes was examined. At the same time, it was investigated to what extent an influence was exerted on the formation of skatole in the large intestine. As the first part of the study dealt with the prevention of skatole in adipose tissue, the second part of this study investigated the extent to which manufacturing processes of sausage can eliminate residual concentrations of skatole and indole in sausage.

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CHAPTER 2

OVERVIEW AND OBJECTIVES

2 OVERVIEW AND OBJECTIVES

Skatole, along with androstenone, is a major component of the unpleasant off-odor boar taint and therefore its accumulation in the fat tissues of pigs is to be prevented. The accumulation of skatole in adipose tissue is mainly influenced by two mechanisms: the extent of its formation in the large intestine and the degree of hepatic degradation. Given that plant extracts are known to significantly impact microbial populations in the gut, there has been scientific interest in their application in animal nutrition. In addition to implementing management strategies, it is anticipated that a certain number of carcasses will still exhibit this unwanted taint. Hence, it is also essential to implement effective strategies in meat processing to produce marketable meat products.

The aim of this doctoral thesis was to investigate the influence of various plant extracts and essential oils on the formation, degradation and accumulation of skatole, as well as to assess the efficiency of meat processing techniques on the concentrations of skatole and androstenone for different types of sausages.

An *in vivo* study was conducted to assess the impact of dietary plant extracts, specifically garlic essential oil, *Origanum vulgare* essential oil, and *Schisandra chinensis* extract, on the activity of the hepatic cytochrome P450 enzyme complex. This complex is a critical regulator of skatole plasma concentrations, influencing its degradation in the liver and, consequently, its accumulation in adipose tissue (MANUSCRIPT I). Additionally, the effects of these plant extracts on skatole and indole formation throughout the large intestine were evaluated (Manuscript II). Moreover, it was assessed whether techniques for processing meat and adipose tissue impact skatole and androstenone concentration in three different types of sausages (MANUSCRIPT III).

MANUSCRIPT 1: Influence of different plant extracts on the CYP-mediated skatole and indole degradation

Although skatole is produced by bacteria in the hindgut of pigs, the most important regulator of plasma concentrations and hence adipose tissue accumulation is the hepatic cytochrome P450 complex. This complex represents one of the most important drug-metabolising enzyme families, the CYPs, which play a key role in hepatic oxidative phase I and thus in the degradation of skatole. In recent years, bioactive plant compounds have come to the focus of attention as a component of the diet and have been shown to affect this family of enzymes in both humans and animals. The aim of the study was to evaluate whether skatole concentrations in blood plasma and adipose tissue

can be affected by the modulating effects of plant extracts and whether such effects can be attributed to changes in hepatic P(450)CYP2E1 and CYP2A-activity. Therefore, an *in vivo* study was performed in pigs to investigate Schisandra chinensis extract (SC) and Oregano essential oil (OEO) as dietary supplements for their potential to reduce skatole accumulation in fat, while (GEO) was tested for its potential to increase skatole in fat.

The Manuscript has been submitted to *Animals*

MANUSCRIPT 2: Extracts of oregano, garlic and *Schisandra chinensis* are not effective to influence skatole formation along the large intestine of pigs

Based on the same experiment as Manuscript 1, this work dealt with the impact of the plant extracts of OEO, SC and GEO on the formation of skatole in the hindgut of pigs. It aimed on the suppressing of skatole forming bacteria and thus on the amount of available skatole for accumulation into adipose tissue. Samples were taken from seven different sectors to determine whether the supplemented extracts had an effect on skatole-producing bacteria throughout the hindgut.

The Manuscript has been submitted to the *Journal of Animal Physiology and Animal Nutrition*

MANUSCRIPT 3: Effect of processing on the concentrations of boar taint compounds skatole and androstenone in different types of sausage

The accumulation of boar taint, mainly consisting of skatole and androstenone, in carcasses poses a threat to the production of various meat products due to partial or complete loss of consumer palatability. This investigation focused on the efficiency of different production processes in eliminating androstenone and skatole concentrations. The study was based on boar carcasses with known taint levels, which were used to produce model sausages. Three different German sausage types were investigated as raw fermented (salami), cooked (wiener) and cooked meat sausage (liver sausage).

This Manuscript has been published in the *Journal of Food Processing and Preservation*

CHAPTER 3

INFLUENCE OF DIFFERENT PLANT EXTRACTS ON CYP-MEDIATED SKATOLE AND INDOLE DEGRADATION IN PIGS

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Article

Influence of different plant extracts on CYP-mediated skatole and indole degradation in pigs

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+ This study was co-planned and supported by Prof. Dr. Ulrike Weiler, who unfortunately passed away before this manuscript could be completed.

Simple Summary: One of the main substances responsible for the unpleasant odor in boar meat is skatole, which is created by the breakdown of tryptophan by bacteria in the hindgut of pigs. This study aimed to assess the impact of three different plant extracts on skatole levels in the blood and adipose tissue of pigs, while elucidating their relationship with the hepatic skatole metabolism. *Origanum vulgare* essential oil and *Schisandra chinensis* extracts were chosen for their potential to mitigate intestinal skatole formation or enhance hepatic skatole degradation, thereby reducing its accumulation in adipose tissue. Garlic essential oil was investigated due to its established capacity to elevate skatole concentrations in adipose tissue. The findings revealed that garlic essential oil significantly influenced skatole accumulation by impeding hepatic degradation, whereas both oregano essential oil and *Schisandra chinensis* extracts had no discernible impact on skatole metabolism or its adipose tissue concentrations.

Abstract: One of the primary substances responsible for the unpleasant odor in boar meat is skatole. Enzymes belonging to the cytochrome P450 (CYP) family play a pivotal role in the hepatic clearance of skatole. This study aimed to investigate the impact of Oregano Essential Oil (OEO), *Schisandra chinensis* extract (SC), and Garlic Essential Oil (GEO) on hepatic CYP2E1 and CYP2A activity in pigs. In three consecutive trials, cannulated castrated male pigs were provided with a diet containing 0.2 - 0.3% of one of these plant extracts. Following a 14-day feeding period, the animals were slaughtered and liver and fat samples were collected. The findings indicate that the activities of CYP2E1 were unaffected by any treatment. However, GEO treatment demonstrated a significant reduction in CYP2A activity ($P < 0.05$). Pigs treated with GEO also exhibited a notable increase in skatole concentrations in both plasma and adipose tissue. In contrast, animals fed SC displayed elevated skatole concentrations in plasma, but not in fat tissue.

OEO did not influence skatole concentrations in either blood or fat. Furthermore, the study revealed that a supplementation of 6 g GEO per animal per day induced a significant increase in skatole concentrations in blood plasma within 24 hours.

Keywords: Pig, CYP2E1, CYP2A, garlic, oregano, *Schisandra chinensis*

1. Introduction

Surgical castration of pigs without pain relief is at present regarded unacceptable in many countries. Traditionally, the castration of entire male pigs has been conducted to prevent boar taint, which has been primarily attributed to the accumulation of androstenone, skatole and other minor contributing indoles in the adipose tissue of intact male pigs [1]–[3]. Alternative methods to traditional surgical castration in order to mitigate boar taint, in particular androstenone, encompass procedures such as surgical castration with anesthesia and analgesia, or the fattening of boar. Androstenone is a testicular steroid and serves as a pheromone in porcine species. Its synthesis is intricately linked to the production of testicular hormones and, consequently, to pubertal development [4]. Skatole (3-methylindole) and other indoles are generated in the large intestine of pigs and various other species through microbial degradation of the amino acid L-tryptophan [5].

Both skatole and indole are absorbed by the gut, with most of it undergoing rapid metabolism in the liver. However, due the lipophilic nature of skatole, a certain percentage is also deposited and accumulates in adipose tissue [6]. Several studies describe the involvement of enzymes of the cytochrome P(450) CYP family in skatole degradation, demonstrating that the hepatic clearance of skatole is strongly dependent on the activity of the key enzymes CYP2E1 and CYP2A [7-9]. Prior investigations into boar taint and its correlation with porcine CYPs have highlighted the significance of endocrine status and gender in influencing the expression of CYP2E1 [10]. Further studies [11, 12] have demonstrated that the presence of androstenone, along with high testosterone levels, contributes to the down-regulation of CYP2E1 expression, leading to diminished skatole degradation. In contrast, surgical castration and immunocastration have been found to result in higher activities of CYP2E1, CYP2A and CYP1A [13].

Given the important role of P(450) CYP in pharmacology, especially in drug clearance, extensive research has been conducted to uncover potential implications of substances affecting this enzyme complex on drug metabolism. Numerous studies have reported that phytochemicals present in dietary compounds can influence various CYPs [14]. Consequently, there is a growing interest in considering a specific diet as an access point for CYP-mediated degradation. Rasmussen et al. (2014) [13] and Čandek-Potokar et al. [15] have suggested that diet could be a potential factor in enhancing hepatic clearance by supplementing plant extracts and their respective bioactive constituents. Several studies have explored the role of phytochemicals, such as varying concentrations of polyphenols like tannins, flavonoids, and lignins (ranging from 1% to 10% per meal), when used as dietary additives. [16].

Schisandra chinensis is commonly used in traditional Chinese medicine (TCM), owing to its well-known hepatoprotective properties [17]. The fruits, leaves and stems of *Schisandra chinensis* contain dibenzo[a,c]cyclooctadiene lignans and are thus suggested to have bioactive properties [17]. An *in vitro* study examining the hepatic effects of *Schisandra chinensis* confirmed its role in activating Nrf-2 on HepG2 cell lines [18]. Nrf-2 activation is known to mediate the activation of target genes involved in phase I and phase II liver metabolism [19]. Further studies have reported an induction of CYP2E1 expression following the oral administration of *Schisandra chinensis* in mice and rat models [20,21].

In contrast, garlic essential oil (GEO) has the opposite effect on CYP2E1. Studies conducted in rats have demonstrated that garlic can provoke a decline in P450(CYP)2E1 activity, attributed to the interaction of diallyl sulphide with the protein structure of CYP [22, 23]. In order to obtain garlic-flavored pork meat, Leong et al. (2011) [24] introduced GEO into the diet of growing pigs. This intervention resulted in higher concentrations of skatole in adipose tissue, assuming a significant reduction of CYP-activity. Such considerations become crucial when defining a standardized challenge for testing different genotypes for their susceptibility to skatole accumulation in adipose tissue [25].

Origanum vulgare comprises the bioactive compounds thymol and carvacrol, both recognized for their antimicrobial properties [26]. Studies conducted on human liver microsomes have revealed inhibitory effects against CYP3A4. However, information regarding potential effects on porcine CYP2E1 and CYP2A is currently limited.

The primary objective of the present study was to investigate whether concentrations of skatole and indole in blood plasma and adipose tissue can be modulated by the addition of plant extracts and oils in the diet, and whether such effects could be explained by alterations in hepatic P(450) CYP2E1 and CYP2A activity. Specifically, *Schisandra chinensis* extracts (SC) and oregano essential oil (OEO) were assessed as dietary additives in order to examine their potential in reducing skatole accumulation in adipose tissue, while GEO was investigated for its potential to increase skatole levels in adipose tissue. Our hypothesis posited that a supplementation of SC and OEO in the diet would enhance hepatic enzyme activity, thus preventing skatole deposition in adipose tissue. Conversely, in the case of GEO, we hypothesized that CYP2E1 in pigs would be similarly affected as observed in rats and mice, and thus aimed to evaluate its actual influence on skatole degradation. Our study intended to clarify whether these plant extracts can be used as a rapid and efficient tool for *in vivo* identification of genotypes that exhibit a lack of responsiveness to GEO. Such non-responsiveness may potentially indicate the presence of polymorphisms in the coding genes of CYP2E1 and CYP2A, attributable to elevated expression rates.

2. Materials and Methods

2.1. Animals, experimental design and sampling

Our study was performed at the experimental unit of the Department of Behavioral Physiology of Livestock at the University of Hohenheim (Stuttgart, Germany). All experiments were approved by the ethical committee for animal experiments by the regional authority (Regional Council, Stuttgart, Germany; approval number V307/13TH). All data presented here is part of a comprehensive experiment examining the impact of plant additives on both skatole formation and degradation, using the same animals, experimental design and methodology (Marro, Wesoly & Stefanski, submitted).

A total of 36 castrated male pigs (German Landrace x Piètrain) were studied in three consecutive trials. Animals (initial body weight: 90 ± 5 kg) were housed individually in pens of 5.3 m² that enabled visual and tactile contact with other pigs, cushioned with dust-free wood shavings. They were kept under a light regime of 12 h/12 h (light/dark), with free access to water. A concentrated standard feeding (1.5 kg/meal, metabolizable energy 14 MJ/kg; Appendix 1A) was provided twice per day.

Each trial consisted of two periods: In period 1 (experimental days 1-14), all pigs were provided a standard diet (Appendix 1A). Given that only castrates, which intrinsically produce low levels of skatole, could be used for the experiment, 12.1% of dried brewer's yeast was given in accordance with the standard ratio to assure high availability of tryptophan for skatole-producing bacteria and, consequently, to stimulate natural skatole production. During period 2 (experimental days 15-29), all pigs excluding the control group (CON), were fed the standard diet along with one of each plant additives as a top dressing. This resulted in four groups: CON (n = 12), SC (n = 8), OEO (n = 10), and GEO (n = 6). Due to the intense odor of the plant additives, the animals were habituated to the smell. Moreover, to avoid potential side effects due to the intense and different scents, the study was conducted in successive trials, each involving the supplementation of a single plant additive.

For stress-free and frequent blood sampling, indwelling jugular vein catheters were implanted in all pigs three weeks prior to the start of the experiment. Blood samples were collected daily at 08:00 h from the catheter for analysis of skatole and indole concentrations. During the first experimental period (days 1-14, without plant additives in the diet), there were 9 catheter failures. In the second experimental period (day 15-29, with plant additives in the diet), one catheter failure occurred (in the SC group), rendering daily blood sampling unfeasible. This resulted in an altered number of experimental animals available for evaluating the trajectory of skatole and indole

concentrations over the entire experimental period. The actual number of animals used for analysis therefore was the following: CON (n=3), SC (n=7), OEO (n = 10), GEO (n = 6).

In order to monitor skatole and indole concentrations in adipose tissue during the experimental periods, punch biopsies of back fat were conducted at weekly intervals (day 0, 7, 14, 21), in accordance with [27] and [28]. Due to a high stress response, fat samples could not be obtained from 3 animals at one respective sampling date.

At the end of the experiment, all pigs were slaughtered in order to obtain a final sample of liver and adipose tissue from the back.

2.2. Dietary additives

Oregano essential oil (OEO) (DOSTO®Konzentrat 500, powder, Dostofarm® (Westerstede, Germany) and *Schisandra chinensis* extract (SC) (Xian Yuensun Biological Technology Co., Ltd, China) were each added in a final concentration of 0.3% to the diet. Garlic essential oil (GEO, 80X, NS; KALSEC® Europe LTD) was added to a concentration of 0.2 %. (Table A1).

2.3. Analytical methods

2.3.1. Skatole and indole determination in adipose tissue

Measurements of skatole and indole were carried out with UHPLC (Dionex Ultimate 3000 RS and Dionex Ultimate 3000 RS pump and Fluorescence Detector based on the protocol of Wesoly et al. [29]. In brief, 100µl melted fat was dissolved in 1ml hexane. Thereafter a solvent distribution was carried out with acetonitrile:water (4:1). After mixing and centrifugation, the hexane phase was removed and the remaining sample was measured on the UHPLC with the fluorescence detector at emission wavelength 275 nm and extinction wavelength of 352 nm.

Precision was determined by measuring skatole- and indole-spiked samples. The mean recovery rate for skatole and indole was between 93 % and 99 %, respectively. Intra-assay and inter-assay variabilities were determined with biological samples for skatole and indole and were below 10 % each.

2.3.2. Skatole and indole determination in blood plasma

The concentrations of skatole and indole were determined according to the protocol by Wesoly et al. (2015) [29]. Briefly, skatole and indole were extracted from blood plasma (500 µl) with diethyl ether (2 ml). After mixing and centrifugation of the samples, the aqueous phase was frozen and the liquid supernatant was transferred into a vial with 500 µl eluent. After the

evaporation of diethyl ether at 50°C in a heating block, the concentrations in the remaining eluent were determined by UPLC.

Precision was determined by measuring skatole- and indole-spiked blood samples. The mean recovery rate for skatole and indole was between 92 % and 102 % for samples spiked with 125 ng/ml and 250 ng/ml, respectively. Intra-assay and inter-assay variabilities were determined with biological samples for skatole and indole, and were below 10% each.

2.3.3. CYP-Assays

The preparation of microsomes from liver samples was based on the protocol of [30]. Aliquots of the microsomal samples were stored at -80°C until analysis. The quantification of microsomal protein was carried out with a commercial kit using bicinchoninic acid (Applichem GmbH, Darmstadt). Bovine serum albumin was used for the calibration curve.

CYP2E1-activity was determined photometrically by measuring the formation of p-nitrocatechol according to Gao et al., [31] with slight modifications of Chang et al. [32]. The final reaction mixture of 400 µl contained 0.4 mg/ml of microsomal protein, 0.5 mM 4-nitrophenol, 1mM NADPH, and an incubation buffer (0.1 M PBS). After incubation for 60 min in a 37°C water bath, the reaction was stopped by adding 100 µl 20 % trichloroacetic acid.

After mixing vigorously and subsequent centrifugation (10 000 x g for 5 min), 500 µl of the supernatant was transferred into 250 µl of 2 M NaOH. After a further mixing step, the samples were transferred onto a 96-well plate (NUNC, Thermo Fischer). Measurements were carried out at 530 nm on a plate reader (X8000, BioTrek). The calibration curve covered the range of 0.625 to 20 nmol nitrocatechol, and was prepared in buffer after the addition of heat had inactivated the microsomal protein (0.4 mg/ml). The enzymatic activity was expressed in pmol/min*mg protein. Precision was determined by measuring nitrocatechol-spiked microsomal protein samples. However, due to technical limitations, two samples could not be analyzed. The mean recovery rate was 93%. Intra-assay variability was lower than 10%, and inter-assay variability was below 15%.

CYP2A-activity was measured fluorimetrically by measuring coumarin 7-hydroxylation formation according to the protocol of Zamaratskaia et al [33] with slight modifications. The final reaction mixture of 400 µl contained 0.4 mg/ml microsomal protein, 0.2 mM coumarin, 0.5 mM NADPH and an incubation buffer. 4-methylumbelliferone (50 nmol) was used as an internal standard. The 60 min incubation in a 37°C water bath was stopped by adding 100 µl 20% trichloroacetic acid. After mixing vigorously and a subsequent centrifugation step (10 000 x g for 5 min), the supernatant was transferred to a glass vial and measured by UHPLC. The enzymatic activity was expressed in pmol/min*mg protein. Precision was determined by measuring 7-

hydroxycoumarin-spiked microsomal protein samples. The mean recovery rate was between 94% and 97%. Intra-assay and inter-assay variabilities were below 10%.

2.4. Statistical Analysis

Statistical analyses were carried out using the IBM SPSS Statistics Version 23 software (Armonc, NY). All data were normalized. If residuals failed to pass the normality test (Shapiro-Wilk), *logarithm* and *square root transformations* were performed. In order to evaluate the effect of the different treatments on skatole and indole accumulation in adipose tissue on the day of slaughter (day 29), one-way ANOVAs (fixed effect: treatment and CYP-activity) for each compound were used. In order to detect differences in the accumulation of skatole and indole in adipose tissue over the entire study, statistical evaluation was performed for each day with included and excluded fat samples: 0, 7, 14, 21 and 29 by one-way ANOVAs (fixed effect: treatment). In the case of missing normal distribution of residuals, a non-parametric Kruskal-Wallis test was used. In order to evaluate whether a change of skatole and indole concentrations in plasma occurred between the control period (day1-14) and treatment period (day 15-28), a paired t-test was performed. In order to clarify if the feed additives led to a significant short-term response in plasma 24 h after the first feeding of herbal additives, the respective concentrations of skatole and indole were compared in the last sample before feeding the additive (day 15) and 24 h later (day 16; paired t-test).

Last square (LS) means were calculated for ANOVAs and considered significant when $P < 0.05$. Differences between groups were identified using Games-Howell or GT-2 (Hochberg) post hoc tests.

Correlations between CYP2E1 or CYP2A and skatole or indole in adipose tissue and plasma, respectively, were evaluated by the Spearman's rank correlation coefficient. This evaluation was performed with the complete dataset, irrespective of the treatment group, (r); $P < 0.05$ was considered as significant.

3. Results

3.1. Effects of the plant extracts on skatole concentrations in blood plasma

Skatole and indole concentrations in plasma throughout the experimental period are depicted in Figure 1. The supplementation of GEO for 14 days resulted in a significant 83% increase of skatole levels in blood plasma (by 2 ng/ml on average; $P < 0.05$) compared to the control period (day 1-14). Administration of SC led to a modest 11% increase ($P < 0.05$), while

the addition of OEO had no discernible effect on plasma skatole concentrations. One animal, however, exhibited no response at all (mean of period 1: 1.63 ng/mL, mean of period 2: 1.57 ng/mL, termed as a "non-responder animal" in the following discussion) (Figure 1a). Nevertheless, animals receiving GEO as a dietary supplement displayed a notable increase in skatole concentrations within 8 h in plasma and within 36 h in back fat (Table 1) ($P < 0.05$). None of the plant additives had an impact on indole concentrations in the plasma (Figure 1b).

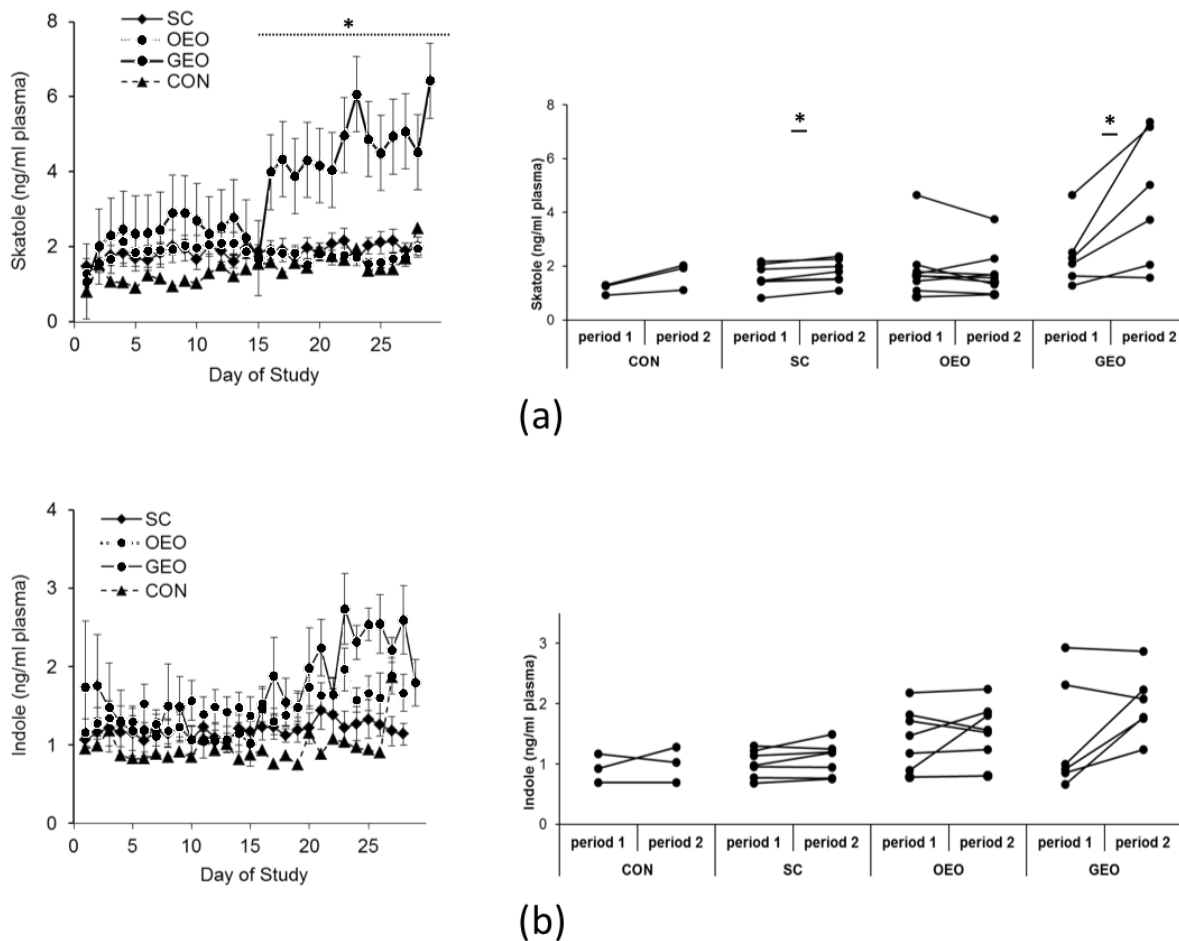


Figure 1: Mean \pm SEM of plasma concentrations of skatole (a) and indole (b) throughout the experimental period. From day 1 to day 14 (period 1), all animals received the same standard diet. From day 15 to day 29 (period 2), the animals received one of the plant additives, *Schisandra chinensis* (SC; $n = 7$), oregano essential oil (OEO; $n = 9$), garlic essential oil (GEO; $n = 6$) in addition to their standard diet, or received food without additives (CON; $n = 3$). ANOVA and paired t-test, * $P < 0.05$ is considered as significant in comparison to CON.

Table 1: Skatole concentrations in plasma (ng/ml) and back fat (ng/g) at different time points after garlic essential oil (GEO) supplementation (n = 6). Skatole analysis at the different time points coincide with the feeding and correspond to a feeding regimen of twice a day; blood sampling was conducted during the feeding; 0 h = blood sampling occurred immediately before the supplementation of GEO. Different superscripts indicate significant differences ($P < 0.05$ is considered as significant in comparison to CON; paired t-test).

Animal	Skatole concentration						
	Blood plasma (ng/ml)					Adipose tissue (mg/g)	
	0h	8h	24h	32h	48h	0h	360h
1	1.60	1.97	4.14	3.89	5.27	75.5	313.3
2	1.46	2.22	5.89	3.55	5.19	38.3	164.3
3	0.65	1.08	1.82	1.49	1.96	14.0	52.4
4	0.98	1.76	3.74	2.91	4.10	30.4	100.4
5	0.88	1.33	2.20	2.04	2.72	14.7	39.8
6	4.61	4.83	6.17	5.85	6.79	92.3	197.2
mean	1.69 ^a	2.20 ^b	3.99 ^c	3.29 ^d	4.34 ^c	44.2 ^a	144.4 ^b

3.2. Effects of the plant extracts on hepatic CYP-activity and accumulation in back fat

Statistical analysis revealed that the level of CYP2E1 has a significant effect ($P < 0.05$) on skatole accumulation in the back fat on day 29. Only GEO had an effect on CYP2A activity, resulting in a notable 48% reduction ($P < 0.05$) (with non-responders excluded) (Figure 2), indicating a clear impact on porcine hepatic enzyme activity. CYP2E1 activity was not affected by any of the plant additives.

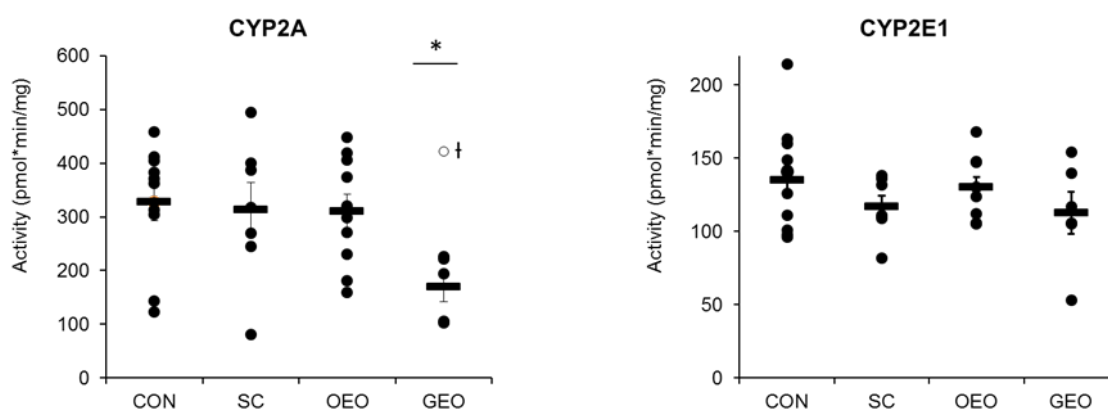


Figure 2: CYP-activity (mean \pm SEM) of the control (CON; n = 10), *Schisandra chinensis* extract (SC; n = 7), oregano essential oil (OEO; n = 9), and garlic essential oil (GEO; n = 5) after 14 days of supplementation; one-way ANOVA, * $P < 0.05$ is considered as significant in comparison to CON; I = non-responder and excluded from statistical evaluation.

The skatole measurements in back fat are presented in Figure 3. Comparing the different sampling days (0, 7, 14, 21, 29), a significant increase ($P < 0.05$) on day 29 was evident in the GEO group. The SC and OEO groups did not differ from the CON group with regard to accumulated skatole concentrations in adipose tissue. Moreover, no treatment-related effect on indole could be observed.

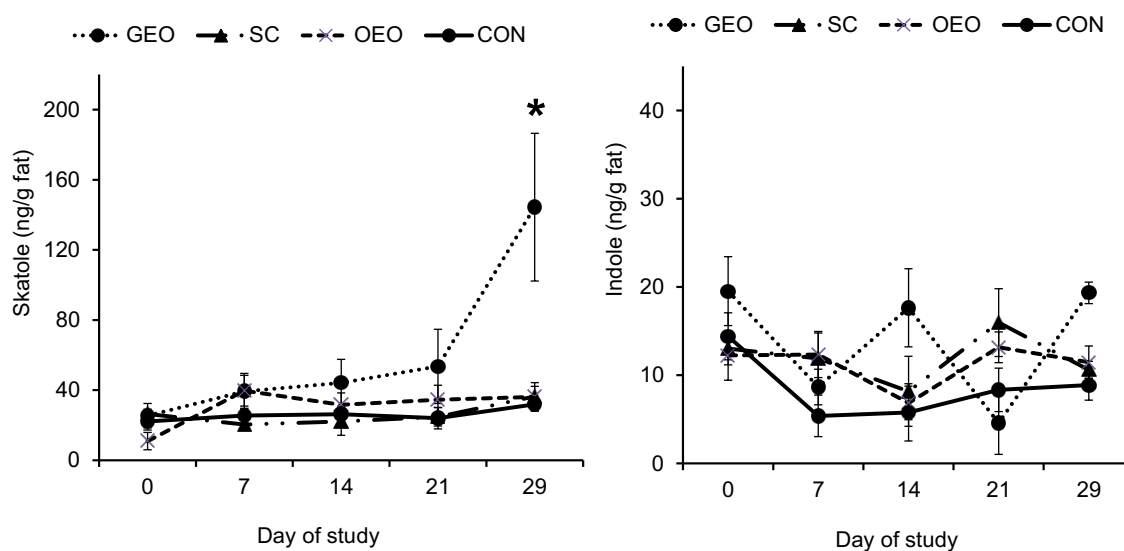


Figure 3: Skatole and indole concentrations in back fat (mean \pm SEM) of control animals (CON; $n = 10$) and animals fed either *Schisandra chinensis* extract (SC; $n = 7$), oregano essential oil (OEO; $n = 9$), or garlic essential oil (GEO; $n = 6$); one-way ANOVA, * $P < 0.05$ is considered as significant in comparison to CON.

3.3. Relationship between CYP-activities, skatole and indole in plasma and back fat

3.3.1. Relationship between CYP-activity, skatole and indole concentrations in plasma and back fat at the end of the study

The associations between skatole concentrations in plasma and back fat and the activity of CYP2E1 and CYP2A were examined independently for each treatment group and collectively for all animals within a unified dataset. Skatole concentrations in the back fat exhibited negative correlations with both CYP2E1 ($r = -0.45$, $P < 0.01$, $n = 34$) and CYP2A ($r = -0.43$, $P < 0.01$, $n = 36$). Consequently, the correlation of skatole in plasma on day 29 with CYP2E1 was negative ($r = -0.48$, $P < 0.05$, $n = 20$), as was the case for CYP2A ($r = -0.41$, NS; $P = 0.06$, $n = 22$), thereby affirming the significance of both CYP enzymes for plasma and adipose tissue concentrations of skatole. In contrast, indole demonstrated no significant association with either of the two CYP enzymes ($P < 0.69$).

3.3.2. Relationship between CYP-activity, skatole- and indole concentrations in plasma back fat throughout the experimental period

The relationship of CYP-activity, skatole- and indole concentrations in back fat and plasma was examined utilizing data obtained from the weekly performed punch biopsies on days 7, 14, 21, 29. Notably, the correlations for skatole were consistently significant ($P < 0.05$) and positive, except for the correlation observed on day 7 ($P = 0.14$). In contrast, no significant correlations were observed for indole.

4. Discussion

One of the most important drug-metabolizing enzyme family in humans and animals comprises a group of hemoproteins, known as cytochrome P450 (CYP), playing a pivotal role in the oxidative metabolism of phase I [34]. Previous investigations have explored the potential of various compounds, aiming to discern their capacity to influence CYP gene expression. Distributed across various tissues, notably in the liver, small intestine, skin and lungs, these enzymes play a pivotal role in the conversion of both endogenous and exogenous substances, encompassing compounds such as skatole and indole [10], [35]. The intricate relationship between skatole production in the hindgut and its subsequent accumulation in adipose tissue remains not fully understood, despite well-documented evidence highlighting the crucial role of hepatic clearance through P(450) CYP in diminishing skatole concentrations in the bloodstream [36]. This hepatic clearance mechanism thereby restricts the accumulation of skatole in adipose tissue. As skatole significantly contributes to boar taint, it is important in the production of meat products and consequently influences their palatability for consumers. Effective strategies to enhance the activity of CYP2E1 and CYP2A enzymes through feed compounds are currently under intensive investigation. The pharmaceutical and chemical property of dried chicory root [10], as well as of hydrolysable tannins [15], have demonstrated significant potential to enhance hepatic clearance, characterized by increased enzymatic activity and a reduced tissue deposition of skatole.

This study specifically investigates the influence of GEO, OEO, and SC supplementation, focusing on their impact on the CYP2 family, primarily on CYP2E1 and CYP2A.

Oregano is a commonly used dietary additive in various animal production systems [37] due to its known antimicrobial effect and the resulting improved health and performance of growing animals. It is assumed that its chemical properties enable modulations of the gut microbiome and the immune system in piglets [38]. However, there is relatively limited knowledge regarding its effects on hepatic P(450) CYP. Nguyen et al. (2014) [14] have demonstrated the effects of functional foods on human P450(CYP) and revealed an inhibitory role of oregano on CYP3A5 and CYP3A7 [39], aligning with the observations by Foster et al. (2003) [39]. However, neither

of these studies investigated the effects of oregano on CYP2E1 or CYP2A [38]. The present study found no discernible effect of OEO on hepatic CYP-activity, nor on skatole or indole concentrations in blood plasma. Consequently, we conclude that OEO does not exert an influence on porcine CYP2E1 or CYP2A.

As reviewed by Nowak et al. (2019) [17], a considerable amount of literature has illustrated the health-promoting and beneficial effects of *Schisandra chinensis* on health parameters [40]. The effect of different *Schisandra chinensis* lignans (either singly or in combination) on hepatic enzyme activity has been widely investigated in rodent models [20, 40-43], yet not in adult pigs until now.

Our study is focused on enzyme activity, which results from both gene expression and post-translational modifications. The efficiency of these enzymes can be partially estimated by changes in skatole concentrations in blood plasma. Hepatic CYP2E1 and CYP2A activities are important determinants of skatole clearance in pigs and represent major targets for intervention. Our study demonstrated that a long-term treatment of pigs with SC does not lead to reduced skatole accumulation in adipose tissue or enhanced enzymatic activities. Instead, it results in an increase of skatole in blood.

In contrast with studies in rodents, our results suggest that SC has no conclusive impact on porcine CYP2E1 and CYP2A. Investigations of Su et al. [22] in rats reported an enhanced CYP2E1 expression after one single dose of SC extract, whereas multiple doses (14 day supplementation) led to diminished gene expression [43]. A comparable biphasic response to SC lignans has been described by Lai et al. for CYP3A in rats [43], contrasting with the lack of significant changes in hepatic CYP2E1 and CYP2A activity observed in the present study. Nevertheless, we cannot rule out that other factors, particularly complex regulatory processes, might have impeded the translation of higher CYP-mRNA expression into active enzymes [44-45]. Alternatively, disparities between rodent and pig models, such as differences in administered amounts or a potential lack of susceptibility, might contribute to the observed distinctions.

Notably, researchers utilizing rodent models employed considerable variation in administered concentrations: 75 mg/kg/d [21]; 400 mg/kg/d [46]; 800 mg/kg/d [21] and 1500 mg/kg/d [20]. This study employed SC concentrations comparable to the investigation of Jin et al. [47]. Given our results and the absence of an increase in CYP-activity, we cannot exclude the possibility that the detected rise in skatole in plasma might in part be attributed to the long-term feed with high purine content in the standard diet, a known factor favoring skatole production in pigs. Since SC extracts are produced from raw plant material, many factors are involved in the reproduction of the desired bioactive activity [48]. Nevertheless, the majority of studies have not specified the parts of the

plant utilized or included. This further complicates the replication and extrapolation of results from rodent models to our study in pigs.

The consequences of garlic consumption on CYP2E1 have been intensively studied in rodents and humans [23, 47, 49]. In these species, it has been demonstrated that garlic induces an irreversible inhibition of the CYP2E1 enzyme, mediated by diallyl sulphide (DAS). Previous studies on the influence of garlic on pigs focused only on growth improvement [50], but not on an impact on P(450) CYP's breakdown after the administration of garlic or garlic derivatives. Leong et al. (2008) investigated whether a 57-day supplementation of GEO leads to garlic-flavored meat and reported skatole levels exceeding 1000 ng/g fat. The authors suggested that this was due to an inhibition of CYP2E1 by garlic ingredients. The current study used the same concentration of GEO (0.2 %) in the feed and also observed an enhanced skatole accumulation in adipose tissue, but was unable to determine a significant decline in CYP2E1 activity.

Zheng et al., [23] performed a similar experiment with mice and concluded an adaption of the CYP2E1 activity to a prolonged treatment. The authors could provoke a substantial reduction after just one single dose of garlic oil and observed a less strong inhibition of CYP2E1 activity after 60 days. Our study did not assess the explicit CYP activity but observed congruent effects by monitoring the strong CYP2E1-dependent skatole levels in plasma. 8 h after the first single dose of GEO, the blood levels of skatole showed a substantial increase. Surprisingly, and in contrast to the previously mentioned authors, we were not able to detect a significant decline in CYP2E1 activity except for CYP2A at the end of the experiment. In 5 out of 6 animals of the GEO treatment group, the CYP2A activity was lower than in 80% of all other animals, irrespective of treatment group. However, one animal with a high CYP2A activity and nearly unchanged skatole levels in plasma at the end of the treatment period masked the results.

The significant long-term reduction in CYP2A activity, as well as the short-term reaction of skatole levels in the blood to GEO, may strengthen the assumption of a modified hepatic metabolism due to GEO supplementation in pigs. This is further supported by the significant increase of accumulated skatole in adipose tissue and additionally may be derived from the long-time course of skatole and indole in the plasma, indicating irregular periodical oscillations.

Most of the studies investigating CYP-mediated influences on boar taint compounds focus on 3-methylindole in pigs, but not on indole. Although the degradation of indole seemed not be affected by the different treatments in this study, indole may also be sensitive to porcine CYP-activity [35] (Appendix B, Figure B1). This becomes particularly noticeable as indole in the plasma seemed to be more vulnerable to CYP interference. This strengthens the hypothesis of endogenous adaption, evident from its absence in other treatments. It may signify hepatic compensation in response to exogenous influences affecting CYP, potentially explaining the

observed absence of breakdown. Considering the high variability in enzymatic activity and peripheral skatole concentrations, the question arises whether GEO can be used as tool to characterize the susceptibility of the hepatic metabolism in a genotype, resulting in high skatole values in blood and fat following adverse environmental conditions such as stress [29].

The heterogeneous reaction of hepatic enzymes may be partly explained by genetic variations among individuals, giving the impression that certain animals appear to be non-responders. However, the non-responder animal clearly reacted with increasing skatole levels in its blood during the first 48 h of GEO treatment but returned to pre-treatment values during the two-week treatment period. This anomaly might be caused by genetic polymorphisms within the gene coding for CYP enzymes as described by Mörlein et al. [51] and Cederbaum [52]. Although genetic variations within or between different breeds of pigs are well documented, the present study showed a considerably high range of skatole concentrations both in blood and back fat, suggesting the importance of differences in CYP-coding genes. This assumption is supported by the investigations of Rowe et al. [53], showing that variations in skatole levels have a highly significant association with SNPs of CYP2E1, and consequently might explain the varying accumulation of skatole in adipose tissue even within a close relationship status. The present data may indicate the genetic difference which, according to Turner et al. [54], is required to establish molecular markers for breeding programs to reduce skatole accumulation in fat and, consequently, influence the levels of boar taint. Moreover, this study highlights the potential to intervene in CYP-mediated skatole degradation, providing an opportunity to conduct challenge tests with short-term GEO application to screen for genotypes with a favored hepatic metabolism at a relatively low cost.

5. Conclusions

Our findings suggest that GEO influences the hepatic metabolism of skatole in pigs, while SC and OEO exhibit no significant effects. In the case of GEO, the data clearly demonstrate that a time frame of 24 h is sufficient to increase skatole concentrations in the plasma, indicating the time frame required for the plant extract to interact with hepatic enzymes. However, dietary additives SC and OEO were unable to induce higher CYP-activity in pigs during a 14-day treatment, consequently lacking the capacity to influence the accumulation of skatole in adipose tissue of the back.

Furthermore, our investigation underscores the potential and efficiency of plant additives due to their rapid mode of action. This study emphasizes the importance of further research on potential dietary additives that might interfere in CYP regulation and, thus, modulate the degradation of skatole.

Appendix A

Table A1: Analysis of the standard diet

Analysis of the standard diet	g/kg DM
Dry matter	87.5 - 88.5
Crude ash	3.9 - 4.1
Crude protein	14.9 - 16.0
Crude fiber	3.3 - 3.4
Crude fat	1.9 - 2.0
Starch	51.2- 53.1
MJ/kg	14.0 -14.2
Composition of the standard diet	%
Barley	23.6 - 23.8
Soy meal	9.3
Triticale	11.9 - 12.0
Wheat	12.3
Mineral premix	2.7
Soy oil	1.6
Corn starch	26.2 -26.3
Brewer's yeast*	12.1
Dietary additives	%
DOSTO® Konzentrat 500	0.3
<i>Schisandra</i> extract	0.3
Garlic essential oil	0.2

MNr. 20102, Leiber GmbH, Bramsche, Germany

*yeast,

Appendix B

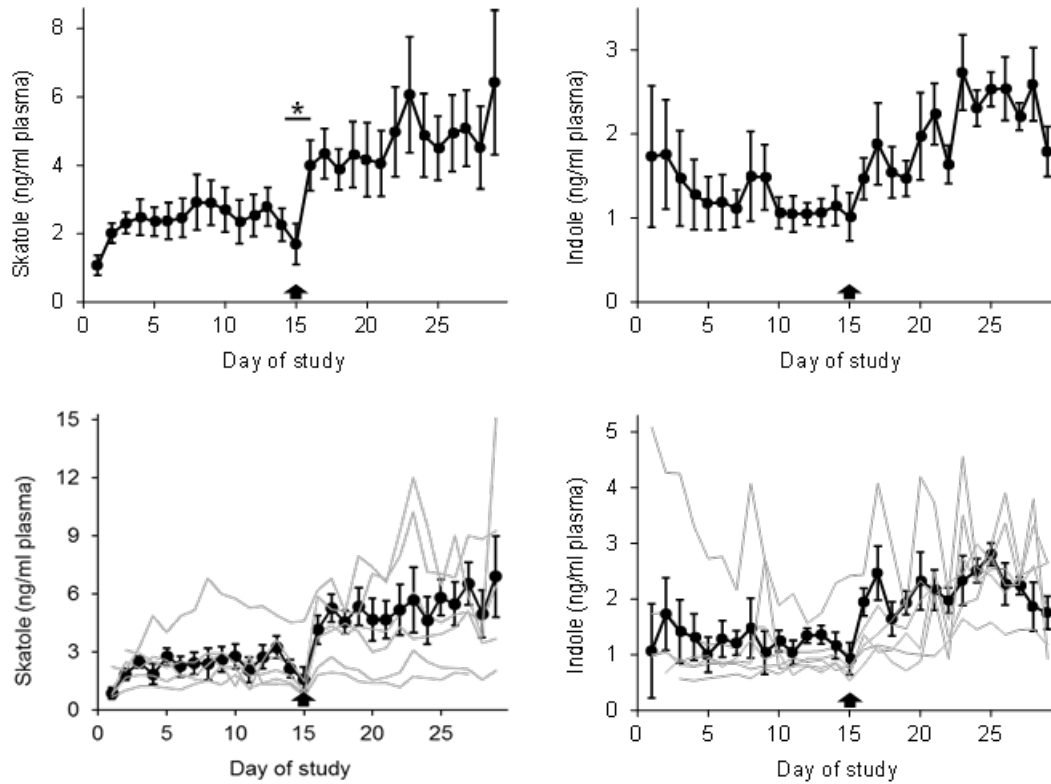


Figure B1: Mean \pm SEM pf plasma skatole and indole concentrations of animals treated with garlic essential oil (GEO) (n = 6); the arrow indicates the starting point of treatment with GEO; the grey lines are the individual plasma concentrations of the animals; statistics: day 15 and 16: paired t test, * P < 0.05 is donated as significant in comparison to CON.

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CHAPTER 4

EXTRACTS OF OREGANO, GARLIC AND SCHISANDRA CHINENSIS ARE NOT EFFECTIVE TO INFLUENCE SKATOLE FORMATION ALONG THE LARGE INTESTINE

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Extracts of oregano, garlic and *Schisandra chinensis* are not effective to influence skatole formation along the large intestine of pigs

running title: Influence of essential oils on skatole formation in pigs

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ABSTRACT

Skatole is a product of microbial degradation of tryptophan in the hindgut of pigs and contributes to boar taint. The accumulation of skatole in fat tissue can be influenced by feeding strategies that may affect the production and/or resorption in the gut, or the hepatic degradation of skatole resorbed from the gut. This study aimed to assess the impact of three plant additives in the diet on the skatole concentrations in different parts of the large intestine of pigs: oregano essential oil, *Schisandra chinensis* extract, and garlic oil. No significant differences in skatole concentrations could be observed among the three pig treatment groups across seven sampling points in the large intestine. Therefore, the bioactive compounds of our plant additives do not exhibit effectiveness in reducing skatole production in the large intestine. Furthermore, no specific sector along the large intestine could be identified as the predominant site for skatole production and accumulation in adipose tissue.

Keywords: pig; skatole; oregano; *Schisandra chinensis*; garlic

INTRODUCTION

Skatole and indole are by-products of the microbial degradation of the amino acid tryptophan and are produced in the hindgut of pigs (Wesoly & Weiler, 2012b). Due to its lipophilic nature, skatole accumulates in adipose tissue. With a faecal-like odour, skatole is considered a major component of boar taint, whereas indole plays a less important role (Heyrman et al. 2021; Zamaratskaia and Squires 2009). Skatole accumulation varies between animals and is influenced by two mechanisms: the extent of formation in the large intestine and the degree of hepatic degradation. Skatole-producing bacteria, comprising only approximately 0.01% of the total bacterial population (Deslandes et al., 2001), are considered to be highly specialized and distributed throughout the large intestine. In contrast, many bacteria species in the colon are able to produce indole.

Considering the recognized impact of some plant extracts on gut microbial populations (Mo et al., 2022; Omonijo et al., 2018; Tiihonen et al., 2010; C. Yang et al., 2019; Zhai et al., 2018), their use as dietary additives is a topic of interest for animal nutrition. With regard to the current study, therefore we suggested that plant essential oils (EOs) and extracts modulate the composition of the skatole-producing microbiota in the large intestine.

Extracts of the Mediterranean herb oregano (*Origanum vulgare*) are known to have beneficial effects on growth performance as well as on gut health in pigs and are widely used as additives in poultry diets (Friedman, 2014; Ognik et al., 2016). Oregano extracts comprise a range of compounds, including the bioactive monoterpenes phenols carvacrol and thymol. Both possess antimicrobial properties, enhancing the permeability of the cytoplasmic membrane and thus leading to the depletion of ATP in gram-negative bacteria (Can Baser 2009). *In vitro* studies (Michiels et al., 2009; Şahin et al., 2004) similarly suggest the potential of oregano to unfavourably influence skatole-producing bacteria of the gastrointestinal tract. To date, however, most studies in pigs that incorporated diets supplemented with oregano or oregano essential oils (OEO) have been conducted in piglets and primarily focused on immune parameters or microbial composition after weaning, rather than on skatole formation in fattening pigs (Manzanilla et al. 2004; Pellikaan et al. 2010; Stelter et al. 2013).

Schisandra chinensis (SC) is extensively used in traditional Chinese medicine and has gained increased attention in European phytotherapy. In addition to its antibacterial, antiviral, anticarcinogenic and immunomodulatory properties hepatoprotective effects have also been demonstrated (Zhang et al., 2013a). Nevertheless, the impact of SC on the microbial composition, including skatole-producing bacteria, is not known.

Garlic (*Allium sativum*) is also reported to have antimicrobial properties, with allicin considered its most potent compound. Allicin is formed by alliin lyase upon crushing parts of the plant. *In vitro* studies (Cutler and Wilson 2004; Koçkar, et al. 2001) have confirmed the antimicrobial activity of garlic and its potential to affect the microbial composition of the gastrointestinal tract.

Research on the impact of plant extracts like oregano, SC, and garlic on skatole formation in pigs has not been conducted to date. Due to the antimicrobial properties associated with these herbs, we assessed the effects of OEO, SC and garlic essential oil (GEO) on skatole formation along the large intestine. We hypothesized that these substances influence microbial skatole formation along the large intestine, subsequently affecting the accumulation of skatole in plasma and adipose tissue. The hindgut was divided into seven sectors in order to identify the primary site of action of the plant additives.

Animals, Housing and Experimental Design

All experiments were conducted in accordance with the guidelines of the local authority's animal ethics committee (regional council (*Regierungspräsidium*) Stuttgart, Germany, approval number V307/13TH). Animals were kept at the experimental unit of the Department of Behavioral Physiology of Livestock, University of Hohenheim, Stuttgart, Germany. All data presented here are part of a comprehensive experiment analyzing the impact of plant additives on both skatole formation and degradation, using the same animals, experimental design and methodology (Marro, Wesoly & Stefanski, submitted). A total of 36 castrated male pigs (German Landrace x Piètrain, initial body weight of 90 ± 5 kg) were used, obtained from Hohenheim University's Agricultural Experimental Station (Unterer Lindenhof, Eningen, Germany). Pigs were housed individually in 5.3 m² pens that enabled visual and tactile contact to other pigs. All animals were surgically equipped with two indwelling vein catheters by cannulation of the cephalic vein to ensure stress-free blood sampling for skatole analysis (Marro, Wesoly & Stefanski, submitted). Due to catheter failure in some animals, an unequal number of experimental animals were left for the evaluation blood levels for skatole and indole (CON =3, SC =7). A concentrate standard feeding (1.5 kg/meal, metabolizable energy 14 MJ/kg; Table 1) was provided twice a day, with water *ad libitum*. The entire experiment consisted of three consecutive feeding trials. In each feeding trial, one of the three plant supplements added to the standard diet was tested. Each trial consisted of two periods: In period 1 (experimental days 1-14), all animals were provided a standard diet (Table 1). Since castrates were used for the experiment, 12.1% of dried brewer's yeast was added to the standard ration to ensure high availability of tryptophan for skatole-producing bacteria and to stimulate

natural skatole production. During period 2 (experimental days 15-29), all pigs (excluding the control group), were fed the standard diet along with one of each plant additives as a top dressing. This results in four groups, comprising the control (n = 12), SC (n = 8), OEO (n = 10), and GEO group (n = 6). Due to the intense odor of the plant additives, the animals were pre-exposed to the smells, and the pigs were not fed the diets with all three additives at the same time. Due to laboratory technical issues, some of the samples obtained from the animals could not be reliably analyzed. The resulting exact sample numbers are specified in the legends.

Dietary additives

Oregano essential oil (DOSTO®Konzentrat 500, powder, Dostofarm® (Westerstede, Germany) and *Schisandra chinensis* extract (Xian Yuensun Biological Technology Co., Ltd, China) were each added in a final concentration of 0.3% to the diet. Garlic essential oil (80X, NS; KALSEC® Europe LTD) was added in a concentration of 0.2 %. (Table 1).

Table 1: Composition of the standard and treatment diets

Analysis of the standard diet	g/kg DM
Dry matter	87.5 - 88.5
Crude ash	3.9 - 4.1
Crude protein	14.9 - 16.0
Crude fibre	3.3 - 3.4
Crude fat	1.9 - 2.0
Starch	51.2- 53.1
MJ/kg	14.0 -14.2
Composition of the standard diet	%
Barley	23.6 - 23.8
Soy meal	9.3
Triticale	11.9 - 12.0
Wheat	12.3
Mineral premix	2.7
Soy oil	1.6
Mais starch	26.2 -26.3
Breyer's yeast*	12.1
Dietary additives	%
DOSTO® Konzentrat 500	0.3
Schisandra Extract	0.3
Garlic Essential Oil	0.2

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Sampling

At the end of the experiment, pigs were slaughtered for digesta and fat tissue sampling. The large intestine was excised, and digesta was sampled at seven positions (Figure 1) according to the methods devised in studies by Bernal-Barragan, (1992), Kjos et al. (2010) and Vhile et al. (2012). Digesta samples from each segment were collected into sterile 50 ml containers (Sarstedt, Wexford, Ireland), immediately frozen in liquid nitrogen, and further stored at -80°C until analysis. Samples of adipose tissue were collected from back fat and stored at -20°C until further analysis.

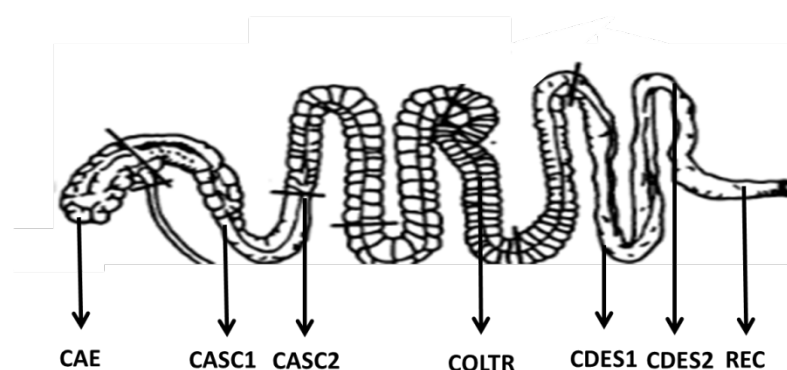


Fig.1 Schematic overview of the sampling points. (Jensen and Jorgensen (1994) modified). Arrows indicate sampling points: **CAE** (caecum); **CASC1** (20 cm distal to the ileocaecal valve); **CASC2** (40 cm distal to the ileocaecal valve); **COLTR** (colon transversum); **CDES1** (50 cm proximal to the rectum); **CDES2** (10 cm proximal to the colon) and **REC** (rectum)

Analysis of Skatole and Indole Concentrations in Digesta

The determination of skatole and indole in digesta was conducted according to a slightly modified protocol of Wesoly et al. (2015). In briefly, 1.5 g of intestinal contents were extracted with methanol and purified by solid phase extraction using Amberlite XAD (Merk KGaA, Darmstadt, Germany). A Dionex 3000 Ultra system with FLD detector (excitation and emission wavelength: 275 and 351 nm) was used for quantification. Analytical separations were performed with a Mulospher RP 18 HP- 3 μ column (CS-Chromatographie, Langerwehe, Germany), flow rate 0.4 ml/min, injection volume 10 μ l and column temperature 35°C. Precision was determined by measuring samples spiked with skatole and indole (5 μ g and 10 μ g/g digesta, respectively). The mean recovery was between 86% and 88% for skatole, and between 90% and 94% for indole. The inter- and intra-assay variability was determined for skatole and indole using biological samples, and was less than 15%.

For the determination of the dry matter content of the digesta samples, 1.5 g of sample was dried in an oven (Heraeus RVT 360, Hanef, Germany) at 90 °C for 18 h; the resulting weight is expressed as a percentage of the fresh weight.

pH analyses of digesta

The pH of the digesta samples was determined post-thawing by diluting 1 g of intestinal content with 4 ml of distilled water. After mixing for 30 s and subsequent centrifugation ($1920 \times g$, 15 min, 20 °C), the pH of the supernatant was measured using a WTW (340i) pH meter. Inter- and intra-assay variabilities were assessed using biological samples, with both demonstrating values below 12%.

Analysis of skatole and indole in adipose tissue and plasma

In addition to the back fat samples collected at the end of the experimental phase (day 29), back fat samples were also obtained at weekly intervals throughout the entire experiment (days 0, 7, 14, 21) through punch biopsies. Moreover, blood samples were collected daily. Results of skatole and indole concentrations in blood and adipose tissue are presented in Marro, Wesoly & Stefanski (submitted). However, since the relevant data will be used in this report for the correlation analysis of skatole in fat, plasma and digesta, the method will be briefly described here.

The measurements of skatole and indole in adipose tissue and plasma were performed according to the method described by Wesoly et al. (2015). In short, 100 μ l of melted adipose tissue was dissolved in 1 ml of hexane, followed by solvent partition with acetonitrile:water (4:1). After mixing and centrifugation, the hexane phase was removed, and the sample was measured by ultra-high performance liquid chromatography (Dionex Ultimate 3000 RS, and Dionex Ultimate 3000 RS pump and fluorescence detector) at an emission wavelength of 275 nm and an extinction wavelength of 352 nm. Samples spiked with skatole and indole were used to determine the precision of the measurements. The recovery for skatole and indole was between 93% and 99%. The intra- and inter-assay variability was determined by using biological samples for skatole and indole, and was less than 10%.

Diethyl ether was used to extract skatole and indole from blood plasma. The aqueous phase was frozen before the resulting liquid supernatant was transferred to a vial containing 500 μ l of eluent, and subsequently mixed and centrifuged. The concentrations in the remaining eluent were determined by ultra-high performance liquid chromatography after evaporation of the diethyl ether at 50 °C in a heating block. Blood samples spiked with skatole and indole were used to determine the precision of the measurements. The mean recovery for skatole and indole ranged from 92% to 102% for samples spiked with 125 ng/mL and 250 ng/mL, respectively. Intra-assay and inter-assay variability were determined for skatole and indole using biological samples. Both were less than 10%.

Statistical Analysis

Statistical evaluation of the data was performed using IBM SPSS Statistics Version 23 software (IBM, Armonc, NY, USA). Normal distribution of residuals was checked using the Shapiro-Wilk test. If variables were not normally distributed, an H-test was performed. In order to evaluate whether the treatment had an effect on the concentrations of skatole and indole in the respective sectors of the large intestine, one-way ANOVAs (fixed effect: treatment, pH) were performed. Spearman's rank correlations as well as linear regressions were performed in order to obtain information on the relationship between the parameters.

RESULTS*Influence of the plant extracts on the concentrations of skatole and indole along the large intestine and on the intestinal pH*

Skatole and indole concentrations in each sector of the large intestine are shown in Table 2. No differences ($P > 0.05$, ANOVA) in skatole and indole concentrations per gram of dry matter were observed among the OEO, SC and GEO treatments when compared to the control group. With regard to pH value, a treatment effect was observed for OEO in CDES2 (10 cm proximal to the colon) ($P < 0.05$, ANOVA), with pH value in OEO being higher compared to the control.

Correlations of skatole and indole concentrations in blood, adipose tissue and intestinal samples

The correlations between skatole and indole concentrations in blood, fat, and digesta in the seven sectors of the large intestine are presented in Table 3A and 3B, respectively. Only a few significant correlations between skatole in blood or adipose tissue and large intestine sectors were found (Table 3A). Within the SC treatment group, a positive correlation could be observed between blood and large intestine sector CDES1. In animals fed with GEO, a positive correlation was noted between blood (and adipose tissue) and CAE.

For indole (Table 3B), too, few correlations could be found in the OEO and SC groups. In animals treated with OEO, a positive correlation was found between REC and blood. In the SC group, positive correlations were evident between CDES1, CDES2, REC and adipose tissue, and between COLTR and blood.

Table 2: Skatole and indole concentrations ($\mu\text{g/g}$ dry matter) and pH value in digesta in various sectors of the large intestine following a 14-day treatment with oregano essential oil (OEO), *Schisandra chinensis* extract (SC), garlic essential oil (GEO), or without additive (CON). Data are shown as mean \pm SEM of the raw data without transformation; different superscripts indicate significant differences (* $P < 0.05$) between treatment groups.

Plant additive	OEO (n)	SC (n)	GEO (n)	CON (n)	P-Value
Skatole ($\mu\text{g/g DM}$)					
CAE	26.0 \pm 7.5 (10)	21.5 \pm 6.4 (8)	47.6 \pm 19.5 (6)	33.6 \pm 9.9 (12)	0.44
CASC1	62.1 \pm 26.8 (10)	24.0 \pm 6.4 (8)	64.5 \pm 17.0 (6)	41.0 \pm 7.8 (12)	0.12
CASC2	66.9 \pm 11.8 (10)	52.6 \pm 14.8 (8)	89.7 \pm 24.7 (6)	67.6 \pm 6.8 (12)	0.61
COLTR	95.9 \pm 24.5 (10)	65.5 \pm 11.4 (8)	55.5 \pm 10.6 (6)	72.5 \pm 9.1 (12)	0.53
CDES1	84.3 \pm 10.9 (10)	99.3 \pm 18.3 (8)	63.5 \pm 8.3 (6)	73.2 \pm 7.8 (11)	0.12
CDES2	96.2 \pm 13.6 (9)	77.7 \pm 7.2 (7)	75.1 \pm 7.5 (6)	82.8 \pm 10 (12)	0.54
REC	102.2 \pm 14.3 (10)	72.7 \pm 6.1 (7)	92.8 \pm 9.8 (6)	85.4 \pm 4.9 (10)	0.57
Indole ($\mu\text{g/g DM}$)					
CAE	49.8 \pm 7.5 (10)	58.3 \pm 12.5 (8)	53.4 \pm 10.1 (6)	49.7 \pm 11.6 (12)	0.67
CASC1	50.3 \pm 7.0 (10)	68.1 \pm 9.9 (8)	48.2 \pm 6.6 (6)	42.1 \pm 5.6 (12)	0.19
CASC2	47.5 \pm 7.8 (10)	69.8 \pm 10.5 (8)	56.2 \pm 9.8 (6)	50.4 \pm 5.3 (12)	0.29
COLTR	46.7 \pm 6.7 (10)	63.9 \pm 10.5 (8)	41.3 \pm 5.5 (6)	49.3 \pm 7.1 (12)	0.29
CDES1	31.1 \pm 5.6 (10)	42.0 \pm 9.0 (7)	38.9 \pm 5.2 (6)	31.6 \pm 4.3 (11)	0.45
CDES2	31.6 \pm 5.6 (9)	33.2 \pm 5.7 (7)	33.9 \pm 4.3 (6)	31.9 \pm 4.3 (12)	0.91
REC	30.5 \pm 4.6 (10)	24.5 \pm 3.3 (7)	27.5 \pm 3.8 (6)	30.6 \pm 4.5 (10)	0.63
pH value					
CAE	6.73 (6)	6.58 (8)	6.34 (10)	6.43 (12)	0.38
CASC1	7.26 (6)	7.26 (8)	7.14 (9)	7.20 (12)	0.98
CASC2	6.91 (6)	6.70 (8)	6.61 (9)	6.57 (12)	0.67
COLTR	7.80 (6)	7.45 (8)	7.62 (9)	7.73 (12)	0.67
CDES1	8.13 (6)	7.94 (8)	7.83 (7)	7.91 (12)	0.19
CDES2	8.15 ^a (6)	7.83 ^b (8)	7.73 ^b (10)	7.73 ^b (12)	0.002
REC	7.99 (6)	7.94 (8)	7.81 (10)	7.74 (12)	0.67

* = $P < .05$; **= $p < .01$

§ Due to technical problems, skatole concentrations could not be analysed

CAE (caecum), CASC1 (20 cm distal to the ileocaecal valve), CASC2 (40 cm distal to the ileocecal valve), COLTR (colon transversum), CDES1 (50 cm proximal to the rectum), CDES2 (10 cm proximal to the colon) and REC (rectum)

Table 3A: Spearman rank correlations of skatole concentrations in the digesta with back fat and plasma at various sampling points along the large intestine. Correlation coefficients (rho) are given separately for treatment with oregano essential oil (OEO), *Schisandra chinensis* extract (SC), garlic essential oil (GEO), or without additive (CON)

CON	CAE	CASC1	CASC2	COLTR	CDES1	CDES2	REC
CAE	.86** (11)	.69* (12)	.61* (12)	.71** (12)	.59* (12)	.58 (10)	
CASC1		.57 (11)	.77** (11)	.67* (11)	.47 (9)	.28 (9)	
CASC2			.66* (12)	.69* (12)	.59* (12)	.57 (10)	
COLTR				.81** (12)	.62* (12)	.48 (10)	
CDES1					.94** (12)	.88** (10)	
CDES2						.94** (10)	
Backfat	-.06 (12)	.41 (11)	-.20 (12)	.39 (12)	.13 (12)	.00 (12)	-.12 (10)
Blood plasma [§]							

OEO	CAE	CASC1	CASC2	COLTR	CDES1	CDES2	REC
CAE	.92** (10)	.82** (10)	.43 (10)	.62 (10)	.75* (9)	-.33 (10)	
CASC1		.86** (10)	.44 (10)	.69* (10)	.78* (9)	-.24 (10)	
CASC2			.65 (10)	.69* (10)	.68* (9)	-.18 (10)	
COLTR				.70* (10)	.05 (9)	.30 (10)	
CDES1					.83** (9)	.29 (10)	
CDES2						-.17 (9)	
Backfat	.20 (10)	.35 (10)	.01 (10)	.01 (10)	.21 (10)	.13 (9)	.19 (10)
Blood plasma	-.14 (10)	.00 (10)	.14 (10)	-.21 (10)	.50 (10)	.49 (9)	.39 (10)

GEO	CAE	CASC1	CASC2	COLTR	CDES1	CDES2	REC
CAE	.71 (6)	.54 (6)	.60 (6)	.31 (6)	.03 (6)	.09 (6)	
CASC1		.43 (6)	.94** (6)	.83* (6)	.54 (6)	.66 (6)	
CASC2			.60 (6)	.54 (6)	.49 (6)	.43 (6)	
COLTR				.94** (6)	.77 (6)	.83* (6)	
CDES1					.89* (6)	.94** (6)	
CDES2						.94** (6)	
Backfat	.83* (6)	.26 (6)	.37 (6)	.09 (6)	-.20 (6)	-.49 (6)	-.43 (6)
Blood plasma	.83* (6)	.26 (6)	.37 (6)	.09 (6)	-.20 (6)	-.49 (6)	-.43 (6)

SC	CAE	CASC1	CASC2	COLTR	CDES1	CDES2	REC
CAE	.79* (7)	.43 (7)	.25 (7)	.71 (6)	-.36 (7)	-.26 (6)	
CASC1		.86** (8)	.64 (8)	.75 (7)	-.68 (7)	-.46 (7)	
CASC2			.79* (8)	.57 (7)	-.43 (7)	-.050 (7)	
COLTR				.75 (7)	-.04 (7)	-.29 (7)	
CDES1					.31 (6)	0.00 (7)	
CDES2						.60 (6)	
Backfat	.64 (7)	.60 (8)	.31 (8)	.14 (8)	.71 (7)	-.21 (7)	.15 (7)
Blood plasma	.70 (5)	.66 (6)	.66 (6)	.66 (6)	.89* (6)	.50 (5)	.14 (6)

* = P < .05; ** = p < .01

§ Due to technical problems, skatole concentrations could not be analysed

CAE (caecum), CASC1 (20 cm distal to the ileocaecal valve), CASC2 (40 cm distal to the ileocecal valve), COLTR (colon transversum), CDES1 (50 cm proximal to the rectum), CDES2 (10 cm proximal to the colon) and REC (rectum)

Table 3B: Spearman rank correlations of indole concentrations in the digesta with back fat and plasma at various sampling points along the large intestine. Correlation coefficients (rho) are given separately for treatment with oregano essential oil (OEO), *Schisandra chinensis* extract (SC), garlic essential oil (GEO), or without additive (CON)

CON							OEO								
	CAE	CASC1	CASC2	COLTR	CDES1	CDES2	REC		CAE	CASC1	CASC2	COLTR	CDES1	CDES2	REC
CAE	.48 (11)	.59* (12)	.49 (12)	.45 (12)	.55 (12)	.35 (10)		CAE	.71* (10)	.65* (10)	-.46 (10)	-.06 (10)	-.30 (9)	.25 (10)	
CASC1		.39 (11)	.36 (11)	.36 (11)	.18 (11)	.30 (9)		CASC1		.64* (10)	-.46 (10)	-.14 (10)	-.20 (9)	.09 (10)	
CASC2			.44 (12)	.53 (12)	.51 (12)	.52 (10)		CASC2			-.14 (10)	-.07 (10)	-.43 (9)	.33 (10)	
COLTR				.51 (12)	.73** (12)	.44 (10)		COLTR				.47 (10)	.09 (9)	.31 (10)	
CDES1					.88** (12)	.76* (10)		CDES1					.85** (9)	.71* (10)	
CDES2						.66* (10)		CDES2						.55 (9)	
Backfat	.31 (12)	-.09 (11)	.31 (12)	.25 (12)	.31 (12)	.38 (12)	.10 (10)	Backfat	.25 (10)	.32 (10)	-.18 (10)	-.35 (10)	-.52 (10)	-.35 (9)	-.59 (10)
Blood plasma [§]								Blood plasma	.32 (7)	.29 (7)	.68 (7)	.00 (7)	.50 (7)	.37 (6)	.82* (7)
GEO							SC								
	CAE	CASC1	CASC2	COLTR	CDES1	CDES2	REC		CAE	CASC1	CASC2	COLTR	CDES1	CDES2	REC
CAE	1.0** (6)	.71 (6)	-.03 (6)	-.31 (6)	-.37 (6)	-.03 (6)		CAE	.14 (7)	.14 (7)	-.04 (7)	.26 (6)	.14 (6)	-.09 (6)	
CASC1		.71 (6)	-.03 (6)	-.31 (6)	-.37 (6)	-.03 (6)		CASC1		.64 (8)	.52 (8)	.21 (8)	.00 (7)	-.11 (7)	
CASC2			-.14 (6)	.20 (6)	-.03 (6)	.49 (6)		CASC2			.52 (8)	.04 (7)	-.43 (7)	-.46 (7)	
COLTR				.49 (6)	.66 (6)	.54 (6)		COLTR				-.08 (7)	-.11 (7)	-.32 (7)	
CDES1					.77 (6)	.89* (6)		CDES1					.83* (6)	.71 (7)	
CDES2						.77* (6)		CDES2						.89* (6)	
Backfat	-.20 (6)	-.20 (6)	-.26 (6)	-.09 (6)	.26 (6)	-.31 (6)	-.09 (6)	Backfat	-.04 (7)	.14 (8)	.17 (8)	-.02 (8)	.89** (7)	.82* (7)	.86* (7)
Blood plasma	.26 (6)	.26 (6)	.31 (6)	-.14 (6)	.37 (6)	-.20 (6)	.14 (6)	Blood plasma	.60 (5)	.60 (6)	.49 (6)	.83* (6)	.09 (6)	.40 (5)	.03 (6)

* = $P < .05$; **= $p < .01$

§ Due to technical problems, skatole concentrations could not be analysed

CAE (caecum), CASC1 (20 cm distal to the ileocaecal valve), CASC2 (40 cm distal to the ileocecal valve), COLTR (colon transversum), CDES1 (50 cm proximal to the rectum), CDES2 (10 cm proximal to the colon) and REC (rectum)

DISCUSSION

Essential oils (EOs) and their various bioactive components have attracted considerable attention in the fields of animal nutrition and food preservation, owing to the chemical nature and mode of action of their constituents. Some EOs have been identified to possess antimicrobial properties, leading to suggestions that they act as nutritional antibiotics (Dorman & Deans, 2000b) (Benkeblia 2004; Cui et al. 2019; Hakala et al. 2015; Han 2016; O’Gara, et al. 2000). *In vitro* studies, in particular, demonstrated the antimicrobial efficacy of plant additives against both gram-positive and gram-negative pathogens (Benkeblia 2004; Cui et al. 2019; Hakala et al. 2015; Han 2016; O’Gara, et al. 2000). Given that most skatole-producing bacteria are gram-positive (Li et al. 2019), and considering that gram-positive bacteria are generally more susceptible to EOs due to their cell structure (Omonijo et al., 2018), a substantial impact on skatole was anticipated.

However, our hypothesis that extracts of oregano, *Schisandra chinensis*, and garlic interfere with bacterial growth in the porcine hindgut and thereby affect skatole and indole production could not be confirmed.

Influence on concentrations of skatole and indole in the large intestine

With regard to oregano components, Michiels et al. (2009) have demonstrated the existence of antimicrobial properties *in vitro* by inoculating extracted pig digesta with suspensions of carvacrol (255 mg/l) and thymol (258mg/l), indicating their potential to modulate the microbiota composition and fermentation pattern in the gut. However, in a dietary study, Stelter et al. (2013) could find no effect of a diet containing 93.9 mg carvacrol/kg DM and 7.5 mg thymol/kg DM on the microbiota composition in the jejunum, caecum and colon of piglets. This corresponds to our findings with even higher concentrations of carvacrol and thymol (900 mg/kg and 150 mg/kg, respectively) utilized in the diet.

Likewise, *in vitro* studies of garlic compounds (propyl propane thiosulfonate and propyl propane thiosulfinate) (Ruiz et al, 2010) and garlic powder (Filocamo et al., 2012) have demonstrated that both exhibit bactericidal effects on various bacterial groups, including a reduction of clostridia and enterobacteria, both known to produce skatole. A similar mode of action is reported for oils of SC seeds, which have shown antimicrobial activity against *Escherichia coli* (Teng & Lee, 2014) *in vitro*. While a supplementation with GEO, OEO and SC was expected to similarly reduce skatole concentrations by displacing skatole producing bacteria in our present study, no such effect could be observed here.

Antimicrobial properties of plant extracts could potentially also affect pH values, as the metabolic products of bacteria can influence the acid-base balance in the intestine (Lazar et al., 2022). However, supplementation with plant extracts had very limited effect on pH in the present study, except of OEO in one intestinal sector only. This outcome is in accordance with finding by Zhou et al. (2019) in ruminants, and further supports the notion that the supplemented plant additives did not influence skatole-producing bacteria to an extent which would have affected skatole formation.

The factors contributing to the observed ineffectiveness of the plant additives tested remain unclear. One plausible hypothesis is that the components of the plants may undergo degradation or inactivation before reaching the large intestine. A potential strategy to ensure the delivery of active plant compounds to the large intestine involves employing resistant capsules, which might be tested in the future. Additionally, prior adsorption in the small intestine may have occurred, thereby limiting the quantity of bioactive compounds reaching the hindgut. It must also be

considered that the plant extracts might exhibit diminished or distinct effects on intestinal bacteria in pigs compared to other animal species, potentially accounting for species-specific variations. Furthermore, it is essential to recognize the variability in the quantity of bioactive compounds present in the utilized plant additives, especially given the absence of detailed content information regarding the active constituents on the packaging and in many other studies.

Correlation of skatole and indole concentrations in the digesta along the large intestine

Skatole and indole are absorbed along the colon and subsequently transported to the liver through the bloodstream. As skatole is lipophilic, it is accumulated in adipose tissue (Wesoly and Weiler 2012). We did not find a correlation of skatole concentration between digesta and blood or back fat. However, other studies have demonstrated strong correlations of skatole with blood and feces (Jensen and Jorgensen 1994; Knarreborg et al. 2002; Vhile et al. 2012). Our findings, however, agree with the study conducted by Hawe et al. (1992), indicating that the digesta of the terminal part of the colon does not exhibit any correlation with the skatole in the adipose tissue. The lack of correlation in skatole concentration between digesta and back fat could be attributed to variable absorption, as the liver may degrade significant amounts of skatole in order to maintain low levels in adipose tissue. The treatment with SC might be attributed to correlations between the digesta and accumulations of indole, but an influence on its concentrations remained absent.

Conclusion

This study did not identify a positive influence of OEO, SC and GEO on the skatole concentration of digesta at any part of the large intestine. We thus conclude that the bioactive compounds applied in the tested route of supplementation are not effective in influencing skatole production.

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CHAPTER 5

EFFECT OF PROCESSING ON THE CONCENTRATIONS OF BOAR TAINT COMPOUNDS SKATOLE AND ANDROSTENONE IN DIFFERENT TYPES OF SAUSAGE

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Effect of processing on the concentrations of boar taint compounds skatole and androstenone in different types of sausage

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Abstract

In order to valorise tainted meat from entire male pigs, two options exist in the production of different meat products: either to dilute tainted meat, or to mask off-odours in the final product. Processing steps may also reduce the concentrations of boar taint compounds. This study investigated the impact of different processing methods on the concentrations of boar taint compounds skatole and androstenone in meat products. Three different types of German sausages, raw fermented (salami), boiled (wiener) and cooked meat sausages (liver sausage) were produced from boar meat and fat with either “high” or “low” taint level. Heating during processing, especially the production of wiener and liver sausage, reduced androstenone concentrations between 44% and 87%. In salami, androstenone concentrations were not reduced during production process. In contrast, skatole reductions of up to 26% were observed for salami and up to 44% for wiener, whereas liver sausage was not affected.

Practical applications

The risk of offensive boar taint is one of the main disadvantages of pork production with entire males. If the detection of tainted carcasses at the slaughter line can be improved, a valid strategy to valorise such carcasses is crucial. The study revealed that open heating during processing has the potential to reduce androstenone, whereas smoking of the products seems to reduce skatole concentrations in the final product.

Keywords: boar taint, sausage, processing, skatole, androstenone

1 INTRODUCTION

Surgical castration of boars without pain relief is now considered unacceptable in many countries. Stakeholders of the pork production chain in the EU committed themselves to voluntarily end surgical castration of male pigs by January 2019. The production of entire males (EM) or immunocastrates (IC) requires innovations at all levels of the pork chain to achieve high sustainability and product quality. Some of these aspects have been studied previously, but there is still a range of important unresolved issues. It is generally accepted that carcasses of EM reveal a higher amount of lean meat at the expense of fat (Gispert et al., 2010). Consumer acceptance of pork from entire male pigs, however, is limited due to the occurrence of boar taint, the sex-specific off-odour which is ascribed to two main compounds, skatole and androstenone (Claus, Weiler, & Herzog, 1994; Oskam et al., 2010). These two compounds are the main factors leading to the sex-specific off-odour of pork from EM (Font-i-Furnols, 2012).

Skatole is formed by microbial activity from L-tryptophan in the large intestine of pigs and is partly resorbed. Androstenone is a steroid produced in the Leydig cells of the testis. Both are lipophilic and thus may accumulate in adipose tissue (Wesoly & Weiler, 2012). The levels of formation and further accumulation in adipose tissue are influenced by various endogenous and exogenous factors. Feeding strategies and housing conditions may thus influence skatole accumulation in boars, whereas the formation of androstenone is closely linked to sexual maturity (Wesoly & Weiler, 2012). Even though management strategies are available to reduce skatole and androstenone concentrations in pork production with EM to some extent, a certain amount of tainted carcasses is expected to remain. As a consequence, there is still a need for solutions to valorise carcasses from EM with tainted meat. In this respect, a variety of studies on palatability and possible rejection due to off-odours by sensory expert panels or consumer tests were conducted to evaluate the effects of curing, marinating, smoking, diluting and mixing with untainted pork (Bañón, Costa, Gil, & Garrido, 2003; Bonneau & Chevillon, 2012; Chevillon et al., 2009; Lunde et al., 2008; Meier-Dinkel et al., 2013; Meier-Dinkel, Trautmann et al., 2013; Stolzenbach, Lindahl, Lundström, Chen, & Byrne, 2009; Zepeda et al., 1993). However, the boar taint levels in the final products used for consumer studies remain largely unknown, especially because sausage types, formulas and processing steps differ regionally to a considerable degree. As reviewed by Engesser (2015), up to now only a few studies have analytically evaluated the effect of processing on concentrations of androstenone and skatole in meat products (Bonneau & Chevillon, 2012; Dehnhard, Claus, Herbert, & Hillenbrand, 1995; Müller, Stiebing, & Dederer, 2012; Wauters, Vercruyse, Aluwé, Verplanken, & Vanhaecke, 2016). Most of them, however, indicate potential ways to influence boar taint concentrations in the final product.

The present study assessed whether processing meat and adipose tissue impacts on the off-odours skatole and androstenone of entire male pigs. We tested whether off-odour concentrations were altered by the production process of three model sausages in relation to the starting concentrations of skatole and androstenone. The following three meat products, manufactured with typical recipes, were studied: raw fermented sausages (salami), pasteurised sausages (wiener) and cooked meat sausages (liver sausage), classified in two fortifications (batches): “low” and “high” boar taint.

2 MATERIALS AND METHODS

2.1 Carcass selection for the production of sausages

158 carcasses of entire male pigs were screened for androstenone and skatole from back fat samples to identify the respective taint levels. The analytical determination of skatole was carried out by UHPLC, based on the method described in Wesoly, Jungbluth, Stefanski, & Weiler (2015). The determination of androstenone concentrations in adipose tissue were measured after extraction with an in-house enzyme immunoassay as described by Weiler, Götz, Schmidt, Otto, & Müller (2013). After carcass dissection, meat and fat were frozen and stored at -20°C until the manufacturing process. Analysis of the boar taint compounds in back fat was determined directly after slaughter. The basis of our study was the manufacturing of two different fortifications - batches, ”high” and ”low” for every type of sausage. Out of every batch several “model sausages” were produced in triplicate, duplicate or unique specimen and the concentration of skatole and androstenone determined after processing. The cut-off values for the production of sausages in regard to androstenone concentrations were set for the batch low: 0.40 µg/g to 0.80 µg/g melted back fat and for the batch high: 0.85 µg/g to 2.40 µg/g melted back fat. In case of skatole, the cut-off limits for the batch low were set on 50 ng/g to 80 ng/g melted back fat, whereas for the model sausages of the batch high with 100 ng/g up to 180 ng/g. Out of the screened 158 carcasses thirty-three were selected with regard to the mentioned cut-off levels to manufacture meat products with two different taint levels. The selection of the carcasses was mainly focused on androstenone concentrations. In this background the examination of processing effects regarding skatole needed to be made separately. Therefore, the assignment whether a model sausage was categorized low or high tainted in both compounds differed in some cases and resulted in varying sampling sizes. A detailed description regarding the used carcasses and their respective boar taint concentrations is given in Table 1 for androstenone and in Table 2 for skatole.

TABLE 1: Overview on the androstenone concentration [$\mu\text{g/g}$] in the back fat of the utilized carcasses (Car) for the different androstenone batches and model sausages.

Sausage type	batch	Model sausage	Replicates (n)	mean [$\mu\text{g/g}$]	Carcass				
					I	II	III	IV	V
<i>Salami</i>	low	1	3	0.42	0.27	0.38	0.6		
		2	3	0.61	0.69	0.72			
		3	3	0.58	0.61	0.27	0.89	0.53	
		4	3	0.80	0.94	0.57	0.89		
		5	1	0.60	0.14	1.04	0.60		
	high	1	3	1.15	1.12	0.71	2.31	0.45	
		2	3	0.85	0.96	1.09	0.50		
		3	3	1.01	1.42	0.69	0.92		
		4	3	2.12	3.98	1.40	2.22	0.94	
		5	3	2.14	2.46	0.47	3.48		
		6	1	1.41	0.06	2.31	1.87		
	<i>Wiener</i>	low	1	3	0.37	0.14	0.60		
2			2	0.47	0.14	0.35	0.23	1.04	0.6
3			1	0.58	0.61	0.27	0.89	0.53	
high		1	3	1.15	1.12	0.71	2.31	0.45	
		2	3	1.40	0.92	2.31	1.87	0.50	
		3	2	2.09	2.31	1.87			
		4	1	2.12	3.93	1.40	2.22	0.94	
<i>Liver sausage</i>		low	1	3	0.58	0.61	0.27	0.89	0.53
	2		1	0.47	0.14	0.35	0.23	1.04	0.6
	high	1	3	1.15	1.12	0.71	2.31	0.45	
		2	1	1.40	0.92	2.31	1.87	0.50	
		3	1	2.12	3.98	1.40	2.22	0.94	

2.2 Sausage production

Standard meat products were produced according to Koch, Fuchs, & Gemmer (1992) with slight modifications. Three different types of sausages were produced from carcasses of entire male pigs with known skatole and androstenone concentrations. The sausage types were selected regarding the meat products consumed most frequently in Germany: raw fermented sausages (salami), pasteurised sausages (wiener) and cooked sausages (liver sausage). The production of the meat products started 6 months after dissection of the carcasses and the occurred thawing loss was collected and incorporated in the manufacturing process of the sausages without any further chemical determination. In addition, to confirm the measured concentrations during the first analyses, measurements were repeated with reserve samples. To evaluate processing influences, the final concentrations of boar taint concentrations in the products were compared to the

concentrations of melted back fat prior processing. Regarding the manufacturing of the sausages used in this study, the GEHA- processing material standards (2001) were the frame of reference. This standard is used in Germany to produce pork products. The provided percentage refers to the total amount of the batter and was used for every model sausage.

TABLE 2: Overview on the skatole concentration [ng/g] in the back fat of the utilized carcasses (Car) for the different skatole batches and model sausages.

Sausage type	batch	Model sausage	Replicates (n)	mean [ng/g]	Carcass				
					I	II	III	IV	V
<i>Salami</i>	low	1	3	51	71	53	30	50	
		2	3	64	68	53	89	44	
		3	3	57	73	47	71	35	
		4	3	66	60	70	69		
		5	3	73	35	77	106		
		6	3	69	121	45	41		
	high	1	3	104	37	63	211		
		2	3	127	106	176	99		
		3	3	180	119	43	378		
		4	1	124	47	191	134		
5		1	115	49	158	137			
<i>Wiener</i>	low	1	3	51	71	53	30	50	
		2	3	64	68	53	89	44	
		3	3	57	73	47	71	35	
	high	1	2	91	47	134			
		2	2	147	158	136			
		3	1	111	46	96	89	191	134
		4	1	179	41	158	137	378	
	<i>Liver sausage</i>	low	1	3	51	53	30	50	
2			3	64	68	53	89	44	
3			3	57	73	47	71	35	
high		1	1	111	46	96	89	191	134
		1	1	179	41	158	137	378	

2.2.1 Production of raw fermented sausages: salami

Raw fermented sausages were made of: 60% lean, tendon-free meat trimmings and 40% of lean belly trimmings, breast trip, shoulder and chump trimmings. As spices were added: nitrite and NaCl mixture (1.8%), nitrite salting (2.6%), starter cultures (Starterkulturen Raps opti plus®, Raps GmbH&Co.KG, Kulmbach, Germany, 0.05%), pepper (0.3%), sugar (0.3%) and ascorbic acid (0.05%). The meat was mixed with salt and spices. Afterwards, it was minced with a 5-mm mincer and again mixed properly. The minced meat was filled into smoke-permeable sausage casings

(protein casing, 28 mm) of 90 g each. The maturing process took place over 7 days under the following conditions: day 1 and 2: 20°C and 90% relative air humidity; day 3: 20°C and 90% relative air humidity; after complete colour development, the smoking process occurred 2 hours by smouldering timber wood; drying from day 4 until day 7: 18°C and 88% relative air humidity. The drying was continued until the aspired weight loss of 30-40% was achieved.

2.2.2 Production of pasteurised sausages: wiener

Pasteurised sausages were made of: 55% lean cutter meat with a certain amount of tendons and a maximum of 10 % visible fat, of 25% neck fat and thin, non-lardy back fat and 25% firm back fat. As spices were added: ice (20%), nitrite and common salt mixture (1.8%), ascorbic acid (0.05%), phosphate (0.2%), pepper (0.2%) mace, coriander paprika (0.05%, respectively), ginger (0.03%) and a spice mixture (Matador®, Raps GmbH&Co.KG, Kulmbach, Germany, 0.4%). The frozen raw materials were thawed overnight at 4°C. Meat and fat were then minced with a 2-mm mincer and stored overnight at 1°C. Subsequently, minced meat and fat were mixed slowly in the cutting machine with salt, phosphate and spices (max. temperature 8-10°C). The ice was then added gradually and intermixed at a higher velocity until a temperature of 12°C. After cutting, the filling was portioned into natural sausage casings (sheep, 20/22 mm) of approximately 70 g each. Colour development in curing occurred at 50°C for 45 minutes. Thereafter followed two smoking (smouldering) steps subdivided in 5 min at 50°C and 15 min at 60°C. The last processing step was blanching for 30 min at 70°C with relative air humidity of 90%.

2.2.3 Production of sterilized sausages: liver sausage

The sterilized sausages were made of: 30% of belly without skin and a maximum of 60 % visible fat, 25% Cheek without rind and glands, 20% lean cutter meat with certain amount of tendons and a maximum of 10 % visible fat and 20% pork liver. The liver did not come from entire male boars. Before processing, the bile ducts were removed and the liver was homogenised thoroughly. The following spices were added: nitrite and common NaCl mixture (1.8%), as well as a spice mixture (Matador®, Raps GmbH&Co.KG, Kulmbach, Germany, 0.4%). The meat was cooked in a boil-in-bag package (conventional oven bag made of PET, EDEKA C+C Großhadelsmarkt GmbH) at 95°C for 90 minutes. Together with the cooking loss, the cooked meat was mixed with salt and spices in the cutting machine up to a temperature of 40°C. The prepared liver was then added step by step to the filling. At 35°C, the filling was portioned into sausage casings (Nalo Top Gold, 45/20 mm, Kalle GmbH, Wiesbaden, Germany) of 200 g. The sausages were cooked at 80°C for 75 minutes in a cooking cabinet and then chilled in a water bath.

2.3 Determination of boar taint compounds in sausages

2.3.1 Analytical strategy and sample preparation

To ensure that measurements contained average concentrations of boar taint compounds within the meat product, each sample used for extraction was based on different subsamples which had been minced and thoroughly mixed. Within the experimental strategy a measurement of the collected batter at different stages of production was performed, but unfortunately, due to methodical limits and inadequate quality criterions did not enter the study. In case of raw fermented and liver sausages, we used three subsample sections from each sausage, four in case of wiener. Fat was extracted according to Dehnhard *et al* (1995) and Hillenbrand (2000) with the following slight modifications:

In brief, 1g of sample (sausage) was minced using an Ultra Turrax homogeniser (IKA T25 S5, Staufen, Germany) for 30 seconds in 3 ml Acetonitrile-Water (75:25). In a subsequent step, the sample was further mixed with 4 ml diethyl ether and centrifuged thereafter at 1900 g for 20 minutes. After centrifugation, the samples were frozen for 1 hour to achieve a phase separation with a solvent and solid frozen phase. The liquid phase was transferred into a glass vial and the solvent was evaporated. The remaining fat was used for analyses of skatole and androstenone.

2.3.2 Reagents and chemicals

The reference standards 3-methylindole (skatole), androstenone (5 α -androst-16-ene-3-one) and the internal standard 2-methylindole were obtained from Sigma Aldrich (St. Louis, MO). Stock solutions were prepared in methanol with a concentration of 1 mg mL⁻¹. Working solutions were made for each compound and analysis in methanol. All other reagents described in 2.4 were of analytical grade and obtained from Th. Geyer GmbH & Co. KG (Renningen, Germany) and AppliChem GmbH (Darmstadt, Germany) respectively.

2.3.3 Skatole measurements: instrumentation and analytical conditions

A Dionex Ultimate 3000 RS system was used for UHPLC analysis of skatole and 2-methylindole, coupled with a Florescence Detector (Thermo Fisher, San José, Ca, USA). Separation of the compounds was achieved by using Multohyp C8 as guard column and a Multospher 120 RP 18 HP-3 μ column (CS Chromatographie, Langerwehe, Germany). The protocol used to detect skatole and 2-methylindole in the processed meat products was based on the protocols of Dehnhard *et al.* (1995) and Wesoly *et al.* (2015), with slight modifications. The column temperature was maintained at 20°C, and the injection volume was 2.5 μ l. For the raw fermented and boiled sausages, the mobile phase was composed with water containing 29% acetonitrile, 14.5% isopropanol and 2.5% acetic acid [A] and acetonitrile with 0.7% acetic acid [B]; the flow rate was

0.4 ml/min. The elution condition applied was: -10 to -6 min [B], -6 to -3 min 100-0% [B], -3 to 28 min [A]. In case of liver sausage [A] consisted of water containing 30% acetonitrile, 14.6% isopropanol and 0.4% acetic acid. The condition applied was: -8 to -5 min [B], -5 to -1 min 100-0% [B], -1 to 25 min [A]. The effluent skatole was monitored using the excitation and emission wavelengths 275 and 352 nm.

2.3.4 Androstenone measurements: analytical procedure

The determination of androstenone concentrations was carried out similar to a published method for androstenone measurements in back fat (Weiler et al., 2013), with slight modifications regarding the pipetting volume. In brief, 100 µl of melted fat was added to 900 µl warm (55°C) methanol. The dilutions were mixed and allowed to cool down at room temperature, and centrifuged thereafter (1900 g 10 min, 4°C). 50 µl of the supernatant was transferred into 950 µl assay buffer, 100 µl of which were used to perform enzyme immunological tests. Precision was determined by measuring spiked fat samples (melted back fat) in a range from 50 ng/g to 100 ng/g for skatole, and from 0.2 µg/g to 2.8 µg/g for androstenone. The mean recovery rate for skatole was between 89% and 92%, and for androstenone between 87% and 95%. Inter-assay and intra-assay variabilities were determined with fat from adipose tissue samples (EM) and from sausage samples. Both were below 10%.

2.4 Statistical analysis

The Wilcoxon Signed Rank Test (level of significance $p < 0.05$) was used to detect differences between starting concentrations of skatole and androstenone in back fat before processing and the respective concentrations after processing. Therefore, each type of sausage and its' respective batch was evaluated separately. One batch consisted of different model sausages and varying replicates (Table 1, Table 2).

3 RESULTS AND DISCUSSION

3.1 Influence of processing on skatole concentrations

3.1.1 Raw fermented sausages and pasteurised sausages (salami and wiener)

Our study revealed substantial differences in skatole concentrations of salami and wiener sausages made with low and high tainted starting material (Table 3). In sausages made from the batch high, skatole was reduced in the final product, while this was not the case in the batches low of both types of sausages. Instead, a skatole increase was observed in pasteurised sausages made out of the batch low (+24%). This outcome differs from previous studies investigating raw (fermented) sausages (Dehnhard *et al* 1995; Müller *et al* 2012). The detected increase (14 ng/g in the average)

might be attributed to the utilisation of the drip and meat, involving additional skatole in the sausage and might be considered as an artefact. The reduction of skatole in salami (-26%) and in pasteurised sausages (-44%) in the batches high, might be attributed to the process of smoking, which is known to release formaldehyde. As for the raw fermented sausages the temperature was not high (20-18°C) and the production process short (7 days), the achieved reductions within the batch high probably are the consequence of the exposition to formaldehyde, which can be attributed to the process of smoking. Dehnhard *et al* (1995) clearly demonstrated that formaldehyde is able to interact with 3-methylindole and therefore capable of converting this compound into more polar substances. The exposure of tainted fat samples to a formaldehyde atmosphere provoked a decline of 71% in skatole concentrations within 72 hours (Dehnhard *et al* 1995). Thus, a reduction might have been achieved due to the permeable casing by direct exposure of skatole to formaldehyde. We can therefore suggest that the observed reductions depend on the penetration depth of this compound into the investigated product, providing a possible explanation for the detected variances in salami. Studies of Dehnhard *et al* (1995) and Bonneau, Desmoulin, Frouin, & Bidanel (1980), also investigating pasteurised sausages did use similar concentrations to the batch high utilised in our study and detected skatole reductions in their sausages as well, which can be attributed to the high temperature and the smoking of the production process.

3.1.2 Sterilized meat sausages (liver sausage)

For liver sausage, our data revealed that the process did not manage to reduce skatole concentrations. Instead, the analysis revealed an increase of skatole content (+ 29%) in the final product compared to the concentrations measured in the back fat of the carcasses. This differences may be explained by the specifics of sausage manufacturing, particularly the utilisation of pork belly (30%), which was shown to contain 25% higher skatole concentrations than back fat (Wesoly, Stefanski, & Weiler, 2016). In general, the boiling stage within sealed conditions during processing may prevent a loss of boar taint compounds, whereas the addition of liver (20%) may have contributed to a further increase in skatole concentrations in this type of sausage (Yost, Kuntz, & McGill, 1990).

3.2 Influence of processing on androstenone concentrations

3.2.1 Raw fermented sausage (salami)

As expected the androstenone concentration was not affected by the manufacturing process due to the missing exposure to open heat. The measurements in the final product after processing revealed a significant increase for both batches (Table 4). The batch low seemed to be more affected, showing an increase in androstenone concentration of 60% compared to the batch high with 49%.

Such elevations in androstenone levels were not expected for this type of sausage. The loss of water (up to 30%) leading to a change in a_w values, during the production process, may have contributed to this increase (Corral, Belloch, López-Díez, Salvador, & Flores, 2017) due to more compound left in the sample. The measured increase might also be provoked by the manufacturing, especially in the utilization of different fat depots that are able to vary among anatomical positions. In addition, this might explain the high variability within the measured range. This result, as well as the detection of significantly higher concentrations within the final product, was found in raw fermented sausages only. Similar elevations were found in the study of Wauters et al. (2016), which also investigated the influence of processing on boar taint compounds in meat products, suggesting a redistribution of androstenone from intermuscular towards subcutaneous fat tissue.

TABLE 3. Mean (\pm SD) and range of the boar taint compound skatole in back fat of the used carcasses and in extracted fat of the produced sausages made in two different tainted batches (“low” and “high”); the proportional changes related to starting concentrations are given in %.

Sausage type	Skatole [ng/g]			
	n	Back fat	Sausage fat	%
<i>Salami</i>				
batch “low“	18	63 ^a (8)	70 ^a (50)	+ 11%
range		51-73	21-167	
batch “high”	11	130 ^a (30)	95 ^b (19)	- 26%
range		104-180	64-122	
<i>Wiener</i>				
batch “low“	9	57 ^b (6)	71 ^a (10)	+24%
range		51-64	56-88	
batch “high”	6	132 ^a (39)	73 ^b (13)	-44%
range		91-179	51-84	
<i>Liver sausage</i>				
batch “low“	9	57 ^b (6)	74 ^a (15)	+29%
range		51-64	49-91	
batch “high”	2	145 (48)	159 (26)	
range		111-179	139-179	

Different superscripts indicate significant differences ($p < 0.05$) by Wilcoxon signed-rank test.

3.2.2 Pasteurized sausage (wiener) and sterilized sausage (liver sausage)

In both types of sausages, androstenone concentrations were considerably reduced, thus processing seems to be effective (Table 4). In case of boiled sausages, a decrease of 87% for the batch low and of 66% for the batch high was detected. Levels of androstenone in liver sausages were reduced, although only the batch high reached a level of statistical significance. Androstenone was diminished by 59% (low) and 44% (high), respectively.

These results differ from the data obtained by Dehnhard *et al* (1995), who found no reduction in androstenone concentrations in wiener-type sausage or liver sausage. If we compare the processing methods used for these three types of sausage, the most striking difference in the production process was the exposure to long-time open heat and the use of permeable casings. This production step was already reported by Claus, Fischer, & Vogelbacher (1985) to be most effective in reducing

TABLE 4. Mean (\pm SD) and range of the boar taint compound androstenone in back fat of the used carcasses and in extracted fat of the produced sausages made in two different tainted batches (“low” and “high”); the proportional changes related to starting concentrations are given in %.

Sausage type	Androstenone [$\mu\text{g/g}$]			
	n	Back fat	Sausage fat	%
<i>Salami</i>				
batch “low“	13	0.60 ^b (0.13)	0.96 ^a (0.48)	+60%
range		0.42-0.80	0.5-2.3	
batch “high”	16	1.40 ^b (0.57)	1.99 ^a (0.85)	+49%
range		0.85-2.14	0.7-3.4	
<i>Wiener</i>				
batch “low“	6	0.47 ^a (0.11)	0.06 ^b (0.05)	-87%
range		0.37-0.58	0-0.13	
batch “high”	9	1.69 ^a (0.49)	0.56 ^b (0.24)	-66%
range		1.15-2.12	0.21-0.83	
<i>Liver sausage</i>				
batch “low“	4	0.53 ^a (0.08)	0.22 ^a (0.11)	-59%
range		0.47-0.58	0.12-0.36	
batch “high”	5	1.56 ^a (0.49)	0.54 ^b (0.24)	-44%
range		1.15-2.12	0.28-1.72	

Different superscripts indicate significant differences ($p < 0.05$) by Wilcoxon signed-rank test.

androstenone in meat products, and this is borne out in our study. Thus, canned products, e.g. liver and boiled sausages produced at higher temperature, showed no significant reductions (Dehnhard *et al* 1995).

Processing of tainted meat in regard to meliorate consumers' acceptance is challenging. The present study did not include an organoleptic examination. However, due to the existing body of literature an interpretation in regard to consumer perception can be made. Studies on mini salami and dry sausages (partly) made of boars (Desmoulin *et al* 1982; Meier-Dinkel *et al*, 2013a, 2013b, 2016), utilizing similar or higher boar taint concentrations, were evaluated as acceptable. The investigation of Rudolph & Geßl (2012) showed, that steamed sausages (frankfurter) with androstenone and skatole concentrations exceeding 1 $\mu\text{g/g}$ and 220 ng/g , respectively, led to no significant difference concerning the consumer perception between high tainted and control-sausages. While the recent study of Meier-Dinkel *et al* (2016), using solely high-tainted fat (>4 $\mu\text{g/g}$ androstenone) and a propionate amount of 50% of EM in boiled sausages, reveals a dislike among the consumer panel and an advice against utilization. Taken in concern the achieved reductions due to the boar taint components left in the meat products, a possible evaluation of our model sausages by a testing panel is presumable.

4 CONCLUSION

Comparing the rates of boar taint reduction for the different meat products, two processing methods seem to be important to valorise tainted meat. One is a period of open heating, which reduces androstenone up to 87%. The second is the smoking of the final product, which not only masks off-odours, but also seems to lower high skatole concentrations. This study therefore recommends utilising strongly tainted meat of entire male pigs not for salami, but in particular to produce pasteurised sausages, e.g. wiener.

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CHAPTER 6

GENERAL DISCUSSION

6 GENERAL DISCUSSION

The challenge of boar taint in pork production is primarily attributed to two main components, skatole and androstenone (Doran et al., 2002). While castrating male piglets can significantly reduce the presence of androstenone, its impact on skatole concentration is limited. The only physiological point of contact between the two compounds is their metabolism in the liver (Wesoly & Weiler, 2012a). Despite microbial skatole production in small amounts in the colon, the accumulation of skatole in adipose tissue is a significant concern for the meat industry. Unlike for androstenone, almost all consumers are sensible for skatole (Weiler et al., 2000), posing a threat to product commercialization. In order to ensure complete palatability to the consumer, two interventions are possible: (1) during pig fattening through modified diets and (2) during the processing of the final product.

In recent years, microbiome research has provided important insights into the composition of the gut microbiome and identified possible dietary associations. Essential oils / plant extracts are known to have several properties useful for animal nutrition (Adaszyńska-Skwirzyńska & Szczerbińska, 2017; Windisch et al., 2008). Among anti-inflammatory, antioxidant effects some bioactive components have been discussed as antibiotic alternative (Omonijo et al., 2018). Therefore, they can be considered as a promising agent to interfere with bacterial composition in the hindgut and subsequently suspected as a potential mechanism to prevent skatole formation. Further, investigations were able to demonstrate that essential oils and plant extracts are capable to interfere with enzymes of the P(450)CYP2E1. These group of hemoproteins play a pivotal role in the conversation of endogenous and exogenous substances including the compounds skatole and indole. The present study investigated the effects of supplementing oregano essential oil (OEO), Schisandra chinensis extract (SC) and garlic essential oil (GEO) on their ability to interfere with the skatole pathway.

The plant family Labiatae, to which oregano belongs, has received considerable attention and is regarded as a promising agent in animal nutrition. Various studies have been conducted with oregano, particularly in poultry and pig production, focusing on aspects such as performance, feed efficiency, as well as the modulation of the immune system and microbiota composition (Adaszyńska-Skwirzyńska & Szczerbińska, 2017; Franz et al., 2010; Hall et al., 2021; Westendarp, 2005). The antimicrobial properties of OEO are associated with the incorporated bioactive substances cavacrol and thymol. Both terpene are suspected to interact with the bacterial cell wall by enhancing the permeability of the cytoplasmic membrane, leading to the depletion of ATP in

gram-negative bacteria (Can Baser, n.d.; Lambert et al., 2001). Attributed to various *in vitro* studies demonstrating effectivity against bacterial suspensions (Cui et al., 2019; Lofa & Velasco, 2019; Michiels et al., 2009a) the potential of oregano to unfavourably influence skatole-producing bacteria of the gastrointestinal tract was expected.

Oregano, as dietary ingredient, has been primarily studied for its antimicrobial properties, but less as mediator in the metabolism of xenobiotic substances. However, it is only known to a limited extent how it interferes with P450(CYP). Studies by Nguyen et al., (2014) and Foster et al., (2003) reported that oregano has an inhibitory effect on CYP3A5 and CYP3A7, but none of the studies investigated CYP2E1 or CYP2A.

The results of this study did not confirm this assumption that OEO has potential to reduce skatole concentrations in pigs. A lack of treatment effects was detected on the formation of skatole in the hindgut, which was not affected in any of the investigated sectors in the hindgut. Supplementation with OEO had no effect on CYP enzymatic activity and no discernible effect on plasma skatole levels or accumulation in adipose tissue.

The factors contributing to the observed absence of an effect of the plant additives tested remain unclear. It can be assumed that the components of the plants may undergo physiochemical degradation or inactivation before reaching the large intestine (Omonijo et al., 2018). In recent studies on the use of essential oils, a capsule formulation was employed, ensuring the investigated essential oils to reach their target site (Tian et al., 2021). This assumption could be supported by studies where lower doses of encapsulated oregano essential oils effected on the microbial composition of pigs (Hall et al., 2021; Mo et al., 2022). In addition, essential oils are volatile compounds and are subject to constant degradation when exposed to the environment (Turek & Stintzing, 2013). As our experimental diet composition was prepared before the start of the feeding trial, degradation of the bioactive components of the oils used could not be excluded.

Schisandra chinensis, a plant mostly native to Asia, is widely used in traditional Chinese medicine for a variety of applications, including anticancer, antioxidant, neuroprotective, hepatoprotective and anti-inflammatory activities (Szopa et al., 2017; S. Yang & Yuan, 2021). Although it has been extensively studied, there are few *in vitro* studies on the antimicrobial efficacy of the incorporated dibenzocyclooctadiene (Hakala et al., 2015b; Han, 2016b; Song et al., 2018; Teng & Lee, 2014). Recently, Schisandra chinensis and its constituents have attracted attention for their potential role in drug-herb interaction. Several studies have reported effects of SC on CYPs in rodents (Chiu et al., 2006; Kwan et al., 2017; L. Li et al., 2014; Tesso et al., 2019), attributed to accelerated

enzymatic activity. In addition, research by Zhou et al. (2014) showed that SC is involved in the activation of the Nrf2 pathway, which plays an important role in phase 2 metabolism and has been suggested to influence the hepatic clearance of skatole (Schütz, 2014). An effect on CYP2E1 with SC extract appeared to be dose-dependent in studies by (Su et al., 2013). A similar biphasic effect was demonstrated by where multiple doses inhibited CYP3A (Lai et al., 2009). In contrast to the reported inhibitory effect of SC on rat CYP2E1, the porcine homologue did not appear to be affected. Supplementation did not result in a decrease in CYP2E1 or CYP2A-activity after multiple doses. The reported increase in plasma skatole concentrations may therefore be partly due to the high purine composition of the supplemented diet. An inhibitory effect on skatole formation along the colon was not observed. Correlation analysis revealed positive correlations for the compound indole for digesta with adipose tissue. This abnormality did not occur in any of the treatments except in the SC group. Therefore, a possible effect on hepatic clearance cannot be excluded, as indole metabolism may also occur via CYP2E1 (Banoglu et al., 2001).

However, a major limitation of most studies on *Schisandra chinensis* lack of specific information on the plant parts or constituents used. Similarly, the commercially available plant extract used in this study does not provide detailed information on the proportional composition of plant parts and constituents. Considering that SC extracts are derived from “raw” plant material, various factors such as cultivation area, harvesting method, genetics or prior fermentation of plant compartments play a crucial role in reproducing the intended bioactive activity (Brenes & Roura, 2010; Mocan et al., 2014; Su et al., 2013; Wang et al., 2014). Furthermore, there is still an incomplete understanding of which dibenzo[a,c]cyclooctadiene ligand is the most bioactively efficient. To the knowledge of the authors of the present study, no investigation of SC in pigs or porcine liver cells has been performed to date. Therefore, it must also be considered that the plant extracts might exhibit diminished or distinct effects on CYP in pigs compared to other animal species.

Garlic derivatives and essential oils have been the subject of numerous studies in rodents and humans, demonstrating their inhibitory effect on liver enzymes, particularly CYP2E1 (Davenport & Wargovich, 2005; Morris et al., 2004; Tsai et al., 2012). The inhibitory effect can be attributed to the presence of diallyl sulphide (DAS), a compound present in garlic. The effects of garlic consumption on CYP2E1 have been the subject of extensive study in rodents and humans (Jin & Baillie, 1997; Teyssier et al., 1999). Garlic has been shown to induce irreversible diallyl sulfide (DAS)-mediated inhibition of CYP2E1 in these species. Studies on CYP2E1 in pigs have focused on increasing the expression of CYP mRNA to be translated into active enzymes or to induce

increased CYP-activity and subsequently hepatic clearance of skatole (Brunius et al., 2012; Rasmussen et al., 2011c, 2014). The supplementation of GEO in the present study was aimed in contrary at decreasing CYP2E1-activity, hypothesising that CYP2E1 is similarly impaired in pigs as in other species (rats and mice), and to determine its actual influence on skatole degradation.

Based on the results of Leong et al. (2011), who reported increased skatole accumulation in fat after supplementation with GEO, the authors attributed this effect to reduced CYP2E1 activity. The present study utilized the same concentrations of GEO and obtained similar enhanced skatole concentrations accumulated in adipose tissue. Contrary to expectations, supplementation of GEO did not result in a significant decline of CYP2E1-activity, but for CYP2A. This unexpected outcome might be attributed an adaption of the CYP to a long-term exposure according to Zeng et al. (2009) who conducted a similar study on mice.

This decline in enzyme activity resulted in a significant increase in skatole concentrations in plasma within 8 h and in fat after 360 h. In addition, an inhibitory interaction between DAS and porcine CYP was found to occur after less than 8 hours, and skatole concentrations in plasma and fat increased after 360 hours. An additional factor suggesting a provoked manipulation of hepatic clearance is the concentration of skatole in the digesta. No significant increased production along the large intestine was observed compared to the other groups being tested.

The results obtained support the suggestion of Zeng et al. (2009) that CYP2E1 activity adapts to long-term exposure. The long-term course of skatole and indole concentrations over the entire test period (Chapter 4) shows periodic oscillations, indicating possible enzymatic fluctuations. Another explanation could be that CYP2E1 was not inhibited and that CYP2A plays a more important role than previously thought (Brunius et al., 2012; Rasmussen & Zamaratskaia, 2014; Terner et al., 2006).

One of the most promising results of GEO supplementation is the detection of a non-responder animal. Despite treatment with GEO, there was no effect on CYP-activity. This suggests that the abnormality was caused by genetic polymorphisms in the genes encoding the CYP enzyme Mörlein et al. (2012). The data obtained may show the genetic difference required by Terner et al., (2006) for molecular markers for breeding programmes.

After slaughter, the next stage in the product chain that can influence skatole and androstenone levels is the processing and manufacturing of meat products. This specific stage is crucial for ensuring consumer acceptance of the final product. Unlike many studies focusing on the sensory evaluation of the final product and consumer sensory thresholds with the aim of increasing consumers acceptance (Bonneau et al., 2000; and Županjac et al., 2022), (Font-i-Furnols, 2012b;

Meier-Dinkel et al., 2016; J. Mörlein et al., 2019b; Škrlep et al., 2020), the present study aimed to evaluate the impact of meat processing on the concentration of boar taint components in different sausages. The aim was to determine the effectiveness of the different processes in reducing the levels of skatole and androstenone. In some cases, the results showed an increase in the concentrations of both substances in sausages and salami compared to before processing. This may indicate the lack of studies on residual concentrations and underlines the importance of sensory testing. As no sensory panel was used to evaluate the manufactured sausages, only customer perception could be assumed. Analytically, in this study, two processing methods appeared to be important in the valorisation of contaminated meat, open heating and smoking. Both were effective in reducing boar taint compounds. Thus, the strategy of using smoking procedures seems to not only achieve androstenone masking, but also actively reduce these compounds, leading to higher consumer acceptance (Aaslyng & Koch, 2018; Martínez et al., 2016b; Stolzenbach et al., 2009)

Surgical castration and immunocastration of male piglets, along with dietary interventions, are considered the most effective methods to prevent the accumulation of boar taint in fat tissue (Wesoly & Weiler, 2012; Zamaratskaia & Squires, 2009). As reviewed by Bee et al., (2020), dietary carbohydrate supplementation can reduce skatole and indole levels in pigs in different tissues depending on dose and feeding time. Indeed, the present study has shown that an effective interference with the hepatic clearance of skatole is possible with only 6 g/day (0.2%), with effects within hours depending on the tissue. This makes such an approach with plant extracts economically more feasible. Two possible conclusions for further research can be drawn from this result: screening for additives that strongly increase CYP-activity, or using GEO as a low-cost challenge test for genotypes of non-responders.

Moreover, meat processing represents a promising approach to mitigate the effects of skatole and, consequently, the challenge of meat products with unwanted odor.

Further research should focus on refining dosage forms, understanding degradation and interactions in the different parts of the gastrointestinal tract, and identifying optimal compositions of plant extracts. Additionally, investigations into the long-term effects and potential adaptations of the microbial community and hepatic enzymes are crucial for establishing more reliable strategies to mitigate skatole concentrations. Integrating these findings into practical meat processing steps is also essential for ensuring the successful reduction of boar taint in commercial pork products

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CHAPTER 7

SUMMARY

7 SUMMARY

Until recently, male piglets have commonly been surgically castrated without anesthesia in many European countries, to prevent the development of boar taint in meat. However, since the amendment of animal welfare laws (Tierschutzgesetz), piglet castration without anesthesia and pain relief has been prohibited since the beginning of 2021. As an alternative to surgical castration of male piglets, boar fattening is being discussed. However, this poses a challenge for the entire pork production chain, as there has been insufficient protection for consumers against meat with undesirable odors. The primary substances contributing to the odor of boar meat are androstenone and skatole. Nevertheless, there are ways to reduce the formation of skatole in animals through adjusted diet compositions, as well as in the meat processing stage.

The aim of this study was to examine the impact of adding various plant extracts and/or essential oils to the feed on skatole formation and degradation. Concurrently, the effectiveness of meat processing in diminishing skatole and androstenone was assessed to formulate strategies for mitigating the presence of malodorous carcasses.

In the first part of the study (Chapter 3), the metabolism of skatole in castrated male pigs was explored. The goal was to intervene in the enzymatic activity of the Cytochrome P450 (CYP) complex (specifically CYP2E1 and CYP2A) in the liver by supplementing essential oils and/or plant extracts from oregano, *Schisandra chinensis*, and garlic. The skatole and indole concentrations in plasma and adipose tissue were then determined. The results showed lower activity of CYP2A and higher concentrations of skatole in plasma and adipose tissue after 14 days of garlic oil supplementation. The addition of oregano and *Schisandra chinensis* had no effect on hepatic enzyme activity or the concentration of skatole and indole.

The second part of the study (Chapter 4) investigated the effects of adding essential oils and/or plant extracts from oregano, *Schisandra chinensis*, and garlic on skatole concentrations in the digesta along the large intestine. The results indicated no influence of the added supplements on the skatole concentration of digesta at any part of the large intestine. The correlation analysis between skatole concentrations in plasma and fat from Chapter 3, as well as along the large intestine from Chapter 4, suggested a positive correlation with the addition of garlic and *schisandra chinensis*. The overall results from Chapters 3 and 4 indicate that under the applied conditions, including concentration, form of administration, and duration of use, the addition of essential oils and/or plant extracts from oregano, *Schisandra chinensis*, and garlic cannot reduce skatole concentration in castrated male pigs. On the contrary, the addition of garlic even led to a

higher skatole concentration in plasma and fat tissue. Additionally, there seems to be no difference in skatole production between the caecum, colon, and rectum.

In the third part of the study (Chapter 5), the influence of processing odor-affected meat was examined using three types of sausages (Salami, Wiener Wurst, Leberwurst) on skatole and androstenone concentrations in fat. The results suggest that androstenone concentration can be reduced by heat exposure (Wiener Wurst, Leberwurst), while skatole concentration could be reduced by a smoking process (Salami, Wiener Wurst). A combination of both mechanisms could be considered for processing highly contaminated meat.

This study underscores that the use of essential oils and/or plant extracts from oregano, *schisandra chinensis*, and garlic, under the given conditions, does not contribute to reducing skatole concentration in plasma, adipose tissue or digesta along the small intestine. On the contrary, the use of garlic essential oil even led to an increase in skatole concentration, likely due to a reduction in the activity of CYP2A. Furthermore, this study demonstrates that specific processing methods for odor-affected meat are promising options to mitigate odor and produce a product acceptable to consumers.

CHAPTER 8

ZUSAMMENFASSUNG

8 ZUSAMMENFASSUNG

Die betäubungslose Kastration männlicher Ferkel war bislang in vielen europäischen Ländern gängige Praxis, um Schlachtkörper mit Ebergeruch zu verhindern. Seit der Novellierung des Tierschutzgesetzes ist die betäubungslose Ferkelkastration seit Anfang 2021 jedoch verboten. Als Alternative zur chirurgischen Kastration männlicher Ferkel wird unter anderem die Ebermast diskutiert. Dies stellt jedoch eine Herausforderung für die gesamte Schweinefleischerzeugungskette dar, da bisher kein ausreichender Schutz der Verbraucher vor geruchsbelastetem Fleisch gewährleistet werden kann. Die beiden Hauptsubstanzen, die zu der Geruchsbelastung des Eberfleisches beitragen sind Androstenon und Skatol. Es existieren jedoch Möglichkeiten, die Bildung insbesondere von Skatol sowohl bei lebenden Tieren durch angepasste Fütterungsmaßnahmen, als auch nachfolgend in der Fleischverarbeitung zu reduzieren.

Ziel dieser Arbeit war es daher, den Einfluss verschiedener Pflanzenextrakte und/oder ätherischer Öle als Zusatz zum Futter auf die Bildung und den Abbau von Skatol zu untersuchen. Gleichzeitig sollte die Effizienz der Fleischverarbeitung im Hinblick auf eine Reduktion von Skatol und Androstenon bewertet werden, um daraus Maßnahmen zur Verminderung geruchsbelastender Schlachtkörper ableiten zu können.

Der erste Teil der Arbeit (Kapitel 3) beschäftigte sich mit dem Skatolstoffwechsel in männlichen, kastrierten Schweinen. Ziel war es, mittels Supplementierung essenzieller Öle und/oder Pflanzenextrakte von Oregano, *Schisandra chinensis* und Knoblauch in die enzymatische Aktivität des Cytochrome P450 (CYP) Komplexes (CYP2E1 und CYP2A) der Leber einzugreifen und die Skatol- und Indolkonzentrationen in Plasma und Fettgewebe zu bestimmen. Die Ergebnisse der Studie zeigten eine geringere Aktivität von CYP2A und höhere Konzentrationen an Skatol im Plasma und Fettgewebe nach 14 Tagen Knoblauchölgabe. Der Zusatz von Oregano und *Schisandra chinensis* hatte keinen Einfluss auf die hepatische Enzymaktivität oder die Konzentration von Skatol und Indol.

Der zweite Teil der Arbeit (Kapitel 4) untersuchte die Auswirkungen einer Zugabe von essenziellen Ölen und/oder Pflanzenextrakten von Oregano, *Schisandra chinensis* und Knoblauch auf die Skatolkonzentration im Darminhalt entlang des Dickdarms, um festzustellen, in welchem Abschnitt des Dickdarms diese Zusätze ihre Wirksamkeit entfalten. Die Ergebnisse zeigen keinen Einfluss der eingesetzten Zugaben auf die Skatolkonzentration des Darminhalts entlang des Dickdarms. Die Korrelationsanalyse zwischen den Skatolkonzentrationen im Plasma und Fett aus Kapitel 3, sowie entlang des Dickdarms aus Kapitel 4, weisen bei Zugabe von Knoblauch und *Schisandra chinensis* auf eine positive Korrelation hin. Die Gesamtergebnisse aus Kapitel 3 und

4 deuten darauf hin, dass unter den angewandten Bedingungen, einschließlich der Konzentration, Darreichungsform und Anwendungsdauer, die Zugabe von ätherischen Ölen und/oder Pflanzenextrakten von Oregano, *Schisandra chinensis* und Knoblauch, die Skatolkonzentration bei kastrierten männlichen Schweinen nicht reduzieren kann. Im Gegenteil, der Zugabe von Knoblauch führte sogar zu einer höheren Skatolkonzentration im Plasma und Fett. Darüber hinaus zeigt sich, dass es keinen Unterschied in der Skatolproduktion zwischen Caecum, Colon und Rektum gibt.

Im dritten Teil dieser Arbeit (Kapitel 5) wurde der Einfluss der Verarbeitung von Fleisch mit auffälligem Geruch anhand dreier Wurstsorten (Salami, Wiener Wurst, Leberwurst) auf die Skatol- und Androstenonkonzentration im Fett untersucht. Die Ergebnisse legen nahe, dass die Androstenonkonzentration durch Hitzeexposition reduziert werden kann (Wiener Würstchen, Leberwurst), während die Skatolkonzentration durch einen Räucherungsprozess verringert werden konnte (Salami, Wiener Würstchen). Eine Kombination beider Mechanismen könnte daher für die Verarbeitung von stark belastetem Fleisch in Betracht gezogen werden.

Die vorliegende Arbeit verdeutlicht, dass der Einsatz von essenziellen Ölen und/oder Pflanzenextrakten von Oregano, *Schisandra chinensis* und Knoblauch unter den gegebenen Bedingungen nicht dazu beiträgt, die Skatolkonzentration im Plasma, im Fettgewebe, oder der Digesta entlang des Dickdarms zu reduzieren. Im Gegenteil, der Einsatz von Knoblauch führte sogar zu einer Erhöhung der Skatolkonzentration, wahrscheinlich aufgrund einer Herabsetzung der Aktivität von CYP2A. Darüber hinaus zeigt diese Arbeit, dass spezifische Verarbeitungsprozesse für geruchsbelastetes Fleisch ein vielversprechendes Mittel sind, um die Geruchsbelastung zu mindern und somit ein für den Verbraucher akzeptables Produkt herzustellen.

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Curriculum Vitae

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Place, Date

Signature

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Stuttgart, den 31.01.2024

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Unterschrift

PEER REVIEWED ARTICLES

Published:

- Marro, P., A. Bauer, V. Stefanski, and U. Weiler. 2018. "Effect of Processing on the Concentrations of Boar Taint Compounds Skatole and Androstenone in Different Types of Sausage." *Journal of Food Processing and Preservation* 42(4). doi: 10.1111/jfpp.13580.

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- Marro.P., R. Wesoly, V. Stefanski. 2024. „Influence of different plant extracts on CYPmediated skatole and indole degradation in pigs“ *Animals*
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