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Intestinal integrity characteristics of growing pigs in response to oregano essential oil supplementation

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Dedicated to my family and friends

Der Familie und den Freunden

Abstract

The production of protein of animal origin is of great importance worldwide. In Germany, pig production is of considerable importance to meet this need. To ensure successful animal production, an intact intestinal tract with a balanced microflora is necessary. It is assumed that natural herbal substances, such as the oregano essential oil can positively influence the intestinal integrity. In addition to the general animal production improving effects, which have already been well described in the literature, the molecular genetics and microbial justification of such effects has not been conclusively clarified. To improve this knowledge, three studies were carried out as part of this thesis.

For the first experiment, 16 piglets (German Landrace x Piétrain) have been raised and divided into two groups after weaning. One group was additionally given a powdery flavour additive (DOSTO® powder, DOSTO® FARM, Westerstede, Germany) in a concentration of 1500 mg/kg standard diet for 20 days. The powder contains 7.5% pure oregano essential oil, steam distilled from the aromatic plant: *Origanum vulgare* subsp. *hirtum* var. Vulkan. At the end of the feeding period, the pigs were sacrificed and RNA was isolated from their small intestines. A comparing qPCR analysis of the two groups revealed a significant reduction in the *TNF-α* and *IL-1ß* gene expression in the jejunum. Additionally, a significant increase in the marker gene for *CD8⁺* and a reduction in the gene for *CD4⁺* T-cells in the ileum were found. Furthermore, a significant reduction in the mRNA level for *E. coli* in the small intestines of the oregano group was found. There were no effects in small intestines´ morphology depending on the supplementation.

In the second experiment, pigs were reared under identical test conditions as described in the first experiment. 86 pigs were divided into two groups and raised until the end of fattening with an average weight of 111.1±10.9 kg. The pigs were slaughtered in a conventional abattoir and animals´ tissues of the two groups were examined individually. A chip-based transcriptomic analysis revealed, 70 genes were up- and 23 genes downregulated in the jejunum of pigs which have been supplemented with oregano essential oil. In the ileal tissue we found 48 genes to be up- and only 12 genes to be downregulated. The genes, which have been detected in this analysis are generally related to the intestinal immunological response (e.g. cytokines, chemokines) and the intestinal integrity. However, the administration of oregano essential oil exerted just minor effects on pigs´ carcass traits.

For the third experiment, microbial DNA was isolated from the intestines of 48 pigs, which have been selected from the second experiment. To investigate the phylogenetic microbial composition, the 16S rRNA gene was sequenced. The α-diversity between the individual intestinal segments (jejunum, ileum, caecum, colon and rectal swabs) was highly different, but no differences between the oregano essential oil supplemented and the control group were found. The β-diversity showed significantly differences between the examined intestinal sections and additionally showed significant differences between the test and the control group in the caecum and in the colon. Among others, the analysis revealed that representatives of the *Lactobacillus* genus in the colon and the rectal swab samples were significantly increased and representatives of the *Prevotella* genus in the caecum were reduced by the oregano essential oil supplementation.

These three experiments provide an overview of the effect of oregano essential oil on important genes for intestinal integrity and the microflora, which is essential for intestinal health in pigs.

Kurzfassung

Die Produktion von Protein tierischen Ursprungs nimmt weltweit einen hohen Stellenwert ein. In Deutschland ist zur Deckung dieses Bedürfnisses die Schweineproduktion von erheblicher Bedeutung. Um eine erfolgreiche Tierproduktion zu gewährleisten ist ein intakter Intestinaltrakt mit einer ausgewogenen Mikroflora von Nöten. Es wird angenommen, dass natürliche pflanzliche Substanzen, wie z. B. das aromatische Öl des Oregano die intestinale Integrität positiv beeinflussen können. Neben den in der Literatur schon gut beschriebenen Effekten auf die Verbesserung der Tierproduktion im Allgemeinen ist die molekulargenetische und mikrobielle Begründung solcher Effekte nicht abschließend geklärt. Zur Ergänzung dieses Wissens wurden drei Studien im Rahmen dieser Arbeit angefertigt.

Für das erste Experiment wurden 16 Ferkel (Deutsche Landrasse x Piétrain) aufgezogen und in zwei Gruppen nach dem Absetzen aufgeteilt. Eine Gruppe wurde zusätzlich mit einem pulverförmigen Aromazusatz (DOSTO® Pulver, DOSTO® FARM, Westerstede, Deutschland) in einer Konzentration von 1500 mg/kg Standardfutter über 20 Tage versorgt. Das Pulver enthält dabei 7,5% des reinen ätherischen Oreganoöls, welches aus der Pflanze: *Origanum vulgare* subsp. *hirtum* var. Vulkan dampfdestilliert wurde. Zum Ende der Fütterungsperiode wurden die Schweine beider Gruppen getötet und RNA aus ihren Dünndärmen isoliert. Durch eine die Gruppen vergleichende qPCR konnte eine signifikante Reduktion der Genexpression von *TNF-α* und *IL-1β* im Jejunum und eine signifikante Erhöhung der Expression des Markergens für $CD8^+$ und eine Reduktion der Genexpression für $CD4^+$ T-Lymphozyten im Ileum festgestellt werden. Weiterhin wurde eine signifikante Reduktion des mRNA-Levels für *E. coli* in den Dünndärmen der Oregano-Gruppe verzeichnet. Eine Veränderung der Dünndarmmorphologie wurde nicht festgestellt.

Im zweiten Experiment wurden unter den gleichen Versuchsbedingungen wie im ersten Experiment 86 Schweine in zwei Gruppen aufgeteilt und bis zum Mastende von 111±10,9 kg gehalten. Die Schweine wurden in einem konventionellen Schlachtunternehmen geschlachtet und die Gewebe der Tiere beider Gruppen individuell untersucht. Eine Chip-basierte Transkriptionsanalyse zeigte hierbei, dass in Abhängigkeit von einer Versorgung mit Oreganoöl im jejunalen Gewebe 70 Gene herauf- und 23 Gene herunterreguliert wurden. Im ilealen Gewebe wurden 48 Gene herauf- und nur 12 herunterreguliert. Die hier beobachteten Gene stehen im Allgemeinen im Zusammenhang mit der intestinalen immunologischen Reaktion (so z. B. Zytokine, Chemokine) und der intestinalen Integrität. Es zeigte sich aber weiterhin, dass die Verabreichung des Oreganoöls nur einen geringen Effekt auf die Schlachtkörpermerkmale hatte.

Für das dritte Experiment wurde von 48 Tieren, die aus dem zweiten Experiment ausgewählt wurden, mikrobielle DNA aus deren Därmen isoliert. Die Proben wurden zur Untersuchung der phylogenetischen Zusammensetzung auf das 16S rRNA Gen sequenziert. Es zeigte sich, dass die α-Diversität zwischen den einzelnen Darmabschnitten (Jejunum, Ileum, Caecum, Colon und Rektaltupferproben) hochgradig unterschiedlich war, jedoch kein Unterschied zwischen der mit Oreganoöl gefütterten Gruppe und der Kontrollgruppe festgestellt werden konnte. Die β-Diversität zeigte sich unterschiedlich zwischen den untersuchten Darmabschnitten und wies signifikante Unterschiede zwischen der Test- und der Kontrollgruppe im Caecum und im Colon auf. Weiterhin offenbarte diese Analyse unter anderem, dass Vertreter des Genus *Lactobacillus* im Colon und den Rektaltupferproben signifikant erhöht und Vertreter des Genus *Prevotella* im Caecum durch das Oreganoöl reduziert wurden.

Diese drei Experimente geben einen Überblick über die Wirkung von Oreganoöl auf bedeutende Gene für die intestinale Integrität und die für die Darmgesundheit essenzielle Mikroflora im Schwein.

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List of abbreviations

16S rRNA	16Svedberg ribosomal ribonucleic acid
AKT3	AKT serine/threonine kinase 3
ATP	Adenosine triphosphate
ANOVA	Analysis of variance
Band np.	Band neutrophils
BH	Benjamini and Hochberg correction
Bp	Biological process
Cc	Cellular compartment
CCL21	CC-chemokine ligand 21
CD	Cluster of differentiation
cDNA	Complementary DNA
con	Conductivity
DNA	Deoxyribonucleic acid
DC	Dendritic cell
E. coli	*Escherichia coli*
E. faecalis	*Enterococcus faecalis*
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
F	Forward
F. prausnitzii	*Faecalibacterium prausnitzii*
FDR	False discovery rate
GALT	Gut-associated lymphoid tissue
GRIN3B	Glutamate ionotropic receptor NMDA type subunit 3B
GWAS	Genome-wide association study
H&E	Haematoxylin and eosin stain
HPRT1	Hypoxanthine phosphoribosyl transferase 1
ICAM-1	Intercellular adhesion molecule 1
IFN	Interferon
IL	Interleukin
IT	Intestinal tract
JAK/STAT	Janus kinases/signal transducer and activator of transcription proteins
Limma	Linear models for microarray data
LPS	Lipopolysaccharides
M cells	Microfold cells
MAPK	Mitogen-activated protein kinase

MCH	Mean corpuscular haemoglobin concentration
MCHC	Mean erythrocyte haemoglobin concentration
MCP-1	CC-chemokine ligand 1
MCV	Mean corpuscular volume
Mf	Molecular function
MIG	Monokine induced by gamma interferon
mRNA	Messenger-ribonucleic acid
NaCl	Sodium chloride
NF-κB	Nuclear factor 'kappa-light-chain-enhancer' of activated B-cells
NIRS	Near-infrared spectroscopy
OEO	Oregano essential oil
OTU	Operative taxonomic unit
P.m.	Post mortem
PAS	Periodic acid–Schiff staining
PMCs	Peripheral mononuclear cells
PBLC	Plant bioactive lipid compound
PBS	Phosphate-buffered saline
PCoA	Principal coordinates analysis
Pd	Phylogenetic diversity
PERMANOVA	Permutational multivariate analysis of variance
PI3K/AKT	Phosphoinositide-3-kinase/Akt
PRRSV	Porcine reproductive and respiratory virus syndrome
Qiime	Quantitative insights into microbial ecology
qPCR	Quantitative polymerase chain reaction
R	Reverse
RIN	Ribonucleic acid integrity number
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RPL4	Ribosomal protein l4
ss-cDNA	Single-stranded complementary DNA
T	Thickness
TJP1/ZO-1	Tight junction protein 1/zonula occludens-1
TNF	Tumor necrosis factor
VCAM-1	Vascular cell adhesion protein 1
ZO-1	Zonula occludens-1

Chapter 1: General introduction

1.1 Introduction

Far-reaching changes can be observed in animal production, especially from a German point of view. The increasing demands of the legislature towards animal production and the increasing global need for animal protein, require a rethinking on the part of all participants in the production chain. Due to stress, intensive farming systems negatively affect animal performance (Cappelli et al., 2021). Stressful stimuli activate the immunologic response and thereby the release of immune cells, such as leukocytes, cytokines and immunoglobulins (Wrona et al. 2001). Stressors and also rapid changes in the diet composition can induce local and systemic inflammation and affect the intestinal microbiome (Cappelli et al., 2021). In pig production, especially meat quality and quantity are the most important factors. However, the importance of animal health is growing steadily and is given priority in decisions about breeding (Merks et al., 2012). Besides animal breeding, well adapted feeding is crucial to fulfill these needs. Feed additives have long been used to improve animal productivity, but they were up-surged by the ban on feeding antibiotics by the European Union in 2006 (EU-regulation 1831/2003). Since this decision, interest in alternatives has grown and natural substances, such as plant essential oils have been advertised with several impressive and sometimes not easy to explain effects. Additionally, those oils meet the need to improve animal productivity without obviously damaging the environment. Natural substances are used in the pig production to improve pigs´ performance and to increase the pork quality (Cappelli et al., 2021; Cheng et al., 2017). In this work the effects of oregano essential oil (OEO) from the aromatic plant *Origanum vulgare* subsp. *hirtum* on pigs´ intestinal integrity have been investigated.

The pig (S*us scrofa domesticus*) is a monogastric animal with an outstanding importance for Germanys' meat production. For this, the genetic background, husbandry, feeding, and resulting from this, the animal health in general are crucial factors. In non-ruminants, the small intestines´ task is not only the digestion and the resorption of nutrients, it is also an immunological organ which protects the animal organism from pathogens and houses its´ own bacterial community with far-reaching partially unknown benefits. For the entire life, animals´ intestinal/mucosal immune system protects the body from environmental stressors such as pathogens and toxins. In former studies on

several animal species, OEO was shown to promote anti-inflammatory (Han and Parker, 2017; Gertsch et al., 2008; Cho et al., 2007), antioxidative (Ghormade et al., 2011; Mastelic et al., 2008), antibacterial (Rodriguez-Garcia et al., 2016; Aligiannis et al., 2001), antifungal (Adam et al., 1998), anticancerogenic (Marrelli et al., 2016; Begnini et al., 2014), and cardiovascular health protective (Leyva-López et al., 2017; Alves-Silva et al., 2016) effects. Additionally, OEO has been used as anti-bacterial substance to protect meat and vegetables after their harvest. Animal growth is promoted as well (Losa, 2001), but does not affect further carcass and meat quality attributes (Can Baser, 2008; Janz et al., 2007). Such effects could be useful to improve the intestinal integrity and thereby, the entire animal health.

Even though, supplementing animals with OEO is not a new idea, in many cases the so-called ‘mode of action’ is not adequately explained. Therefore, in this experiment we used current laboratory methods to give new insights about how this plant bioactive lipid compound unfolds its´ effects. Hereinafter, relevant literature depending on the dissertation topic is briefly presented.

1.2 Oregano essential oil; more than the sum of its parts (?)

Prevention is better than cure; it is much cheaper to keep animals healthy through an adapted and well-planned diet instead of having to use expensive pharmaceuticals. Additionally, the use of pharmaceuticals, such as antibiotics, is controversially discussed. In the long-term, the type of animal housing must be adapted in case of suboptimal production. As a short-term solution and cheaper method of influencing animal health, feeding strategies have proven to be more suitable. Therefore, the market for feed additives, such as vitamins, enzymes and aromatic premixes steadily grows. Many of those substances, such as essential oils are clustered to so-called 'plant bioactive lipid compounds' (PBLCs) (Patra et al., 2019; Hausmann et al., 2018). In Germany, aromatic substances, such as OEO, are classified as zootechnical additives. OEO is a complex mixture of different chemical compounds with multiple effects and it is just one fragment in the highly heterogenous group of described phytogenic substances. Phytogenics, which belong to the group of ergotropic substances (Kirchgessner and Roth, 1988), is therefore an umbrella term for plant derived materials with e.g. anti-microbial and anti-inflammatory effects. Well known phytogenic representatives such as carotenoids, capsaicin, flavonoids etc. are advertised as feed additives with growth and health promoting effects. They need to be applied in a specified age or growth period, because some of them are targeted to improve muscular growth in the very first days *postpartum* and some rise the fat weight at the end of the fattening period (Wenk, 2003). Secondary metabolites are not essential for plants´ viability, however they are important for the protection of the plant against microorganism and some herbivores (Pagare et al., 2015; Bakkali et al., 2008). Essential oils can be made available to other organisms through steam-distillation and other extraction techniques (Bakkali et al., 2008). Due to their antioxidant properties, monoterpenes from PBLCs are used in feed preservation (Mastelic et al., 2008). Their antioxidative effects are explained by the ability to split of hydrogen atoms and thus free radicals can be bound (Wojdyło et al., 2007). The effects of phytogenic additives should not be attributed to a potential nutrient, building material or vitamin character. Their impact is rather attributed to their aroma and other functional properties (Karásková et al., 2015), such as effects on the digestibility of nutrients in general (Wenk, 2003), digestion-associated enzymes, e.g. α-

amylase and α-glucosidase (Sarikurkcu et al., 2015), and of course on the transcriptome and/or the microbiome (Wei et al., 2017).

The oregano genus consist of around 61 species and several natural hybrids (Leyva-López et al., 2017; Baser et al., 2008). The origin of oregano is the Mediterranean area and regions of Asia. There are large differences between the oils´ compositions depending on the (regarded) species, the plant heritage, local climate and several other environmental factors (Falco et al., 2013; Azizi et al., 2009). Additionally, the oil extraction technique affects the composition of the oil (Novák et al., 2011) and also within the same species, metabolites´ mixture varies significantly. Therefore, the different species are divided into three chemotypes (Lukas et al., 2015). However, the classification has not yet been finalized (Leyva-López et al., 2017; Fuentes et al., 2011). In this work, we will focus on *Origanum vulgare* subsp. *hirtum*, which is dominated by the monoterpenes carvacrol and thymol with an combined average of 40 – 80% (Asensio et al., 2015; Bonfanti et al., 2012; Adam et al., 1998; Baser et al., 1994; Sezik et al., 1993). An overview about the different compositions of the oil from *Origanum* can be found in the review of Leyva-López et al. (2017) (Figure 1).

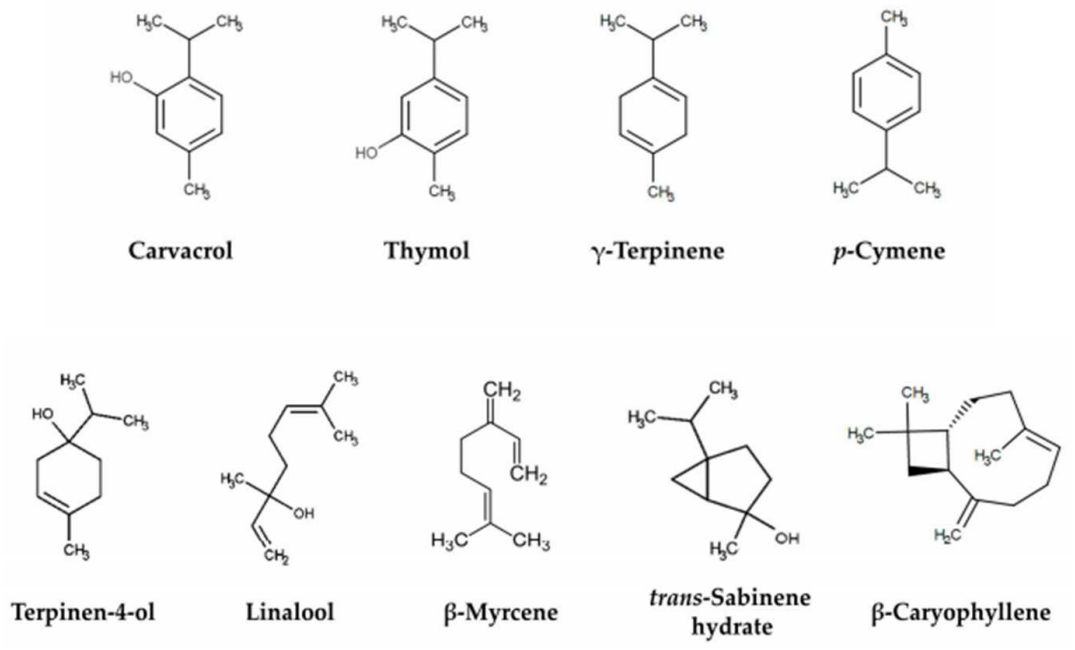

Figure 1: Oregano essential oils´ core chemical compounds and their respective structural formulas (Leyva-López et al., 2017)

It was summarized by Baser et al. (2008), the main biological activities of OEO are achieved by the phenol-ring based monoterpenes. In contrast, more recent studies

suggest that synergistic interactions and the minor substances also need to be regarded to fully reveal OEOs´ potential (Pu et al., 2020; Pezzani et al., 2017; Zeng et al., 2015). The main constituents of OEO are: carvacrol, thymol, γ-terpinene, ρ-cymene, terpinen-4-ol, linalool, β-myrcene, trans-sabinene hydrate, and β-caryophyllene (Sarikurkcu et al., 2015). Because carvacrol and thymol are biosynthesized from γ-terpinene through ρ-cymene, these substances are also fundamental in the oil of oregano (Gertsch et al., 2008; Baser et al., 2008). However, the complexity in the composition makes it difficult to assign the biological properties of action based on individual ingredients. OEOs´ properties depend on the combination of ingredients and their relative proportions and their possible synergistic and antagonistic effects (Zeng et al., 2015).

1.3 Oregano essential oils´ effects on genetics

It is common knowledge that the phenotype of an organism depends on genetic information and environmental factors. Additionally, epigenetic effects must be considered (Remely et al., 2015). First of all, all development processes that take place in the body contain a complex and coordinated signal pattern on the intracellular and the cell-environmental level. Countless genes are involved in this network (Dauncey et al., 2001), which are regulated by metabolic signals that cells receive from internal and environmental factors (Asmelash et al., 2018). Muller and Kersten (2003) showed in their review different opportunities to modulate the gene expression, protein production, the metabolome modification etc. by dietary signals. Therefore, nutrition is one of the most important factors in the aforementioned cascade. The kind of research in which nutritional effects on animals´ genetics are investigated, is called: ‘Nutrigenomics’. However, this is just an umbrella term for studies, that focus to reveal diet-related modulations to molecular-genetic levels and therefore combine different fields of research. A basis for such work is the knowledge of the genetic code of the organism investigated. The first time the pig genome was sequenced was in 2009 (Archibald et al., 2010). Ghormade et al. postulated in 2011, it could be possible to improve the immune system and by that the entire animal health by the aforementioned processes. Nutrients and other food components play key roles in controlling gene expression and transcription (Sales et al., 2014). It is assumed that ingredients in plant essential oils exerts ligand-like effects and therefore are able to

affect gene expression (Gonçalves et al., 2020). Other substances exert hormone like effects and by that bind to cellular receptors and stimulate metabolic processes (Pakalapati et al., 2009). Chemogenetic activities, which include amongst others the genetic reaction to chemical substances (Tobin et al., 2020; Strobel, 1998), can be displayed for more or less every compound within OEO. A major task in such studies is the fact, that the feed is usually a complex mixture of several ingredients with superimposing and negating effects. It was shown by Mariman (2006) and Raskin et al. (2002), diet-related disorders do not originate from the interaction of a single nutrient with a single gene, but rather from complex interactions of several nutrients with several genes. A general overview about inter- and intra-cellular interactions in response to dietary chemogenetic signals can be found in figure 2. One general target of this field of research is the improvement of the animal health and by this the productivity. Therefore, it is necessary to understand non-energetically nutritional impacts (Ghormade et al., 2011; Zduńczyk and Pareek, 2009).

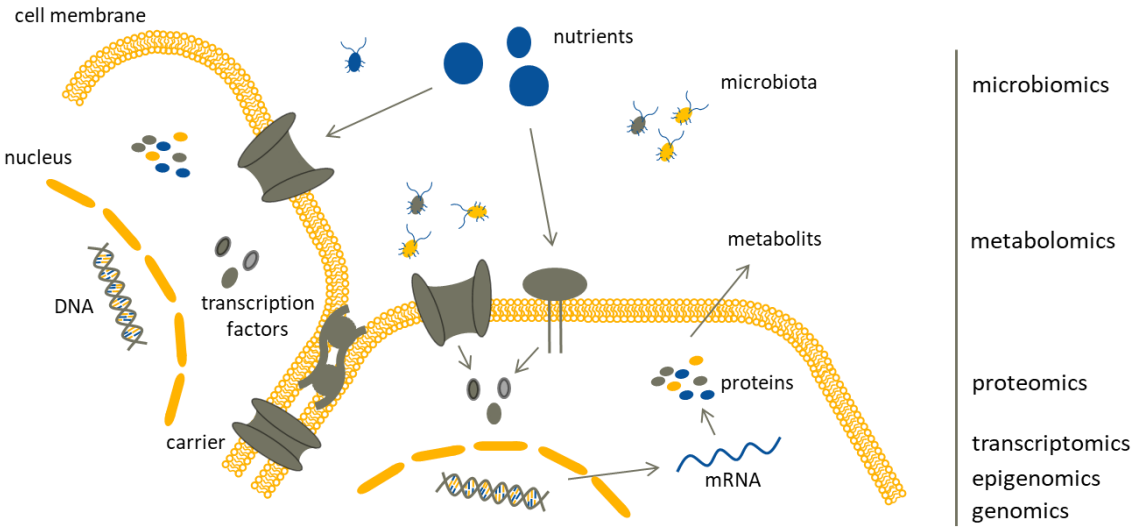

Figure 2: Dietary and microbial signals affect whole metabolite production cascade due to their functions as transcription factors and ligands. Modulations can be observed at several molecular genetic levels, such as the transcriptomics. Ramifications are not just limited to directly affected cells. Due to intercellular communication systemic effects must be expected. Modified (Muller and Kersten, 2003)

PBLCs´ showed effects on several pathways, such as '*Janus kinase / signal transduction and activator of transcription*' (*JAK/STAT*) and '*Phosphatidylinositol 3-kinase / serine / threonine kinase Akt*' (*PI3K/AKT*)-pathways, which are involved in cell proliferative and immunologic processes (Urasaki et al., 2020; Zou et al., 2016; Pianetti et al., 2002). It

was shown by da Silva Lima et al. (2013), OEOs´ core component, carvacrol, exerts anti-inflammatory effects by the reduction of the expression of genes such as *cyclooxygenase-2*, *interleukin (IL)-1β* etc. (Cho et al., 2012). In addition, the expression of the proinflammatory mediator *tumor necrosis factor (TNF)-α* can be significantly reduced by a carvacrol-thymol supplementation in piglets (Wei et al., 2017). Carvacrols´ and thymols´ postulated anti-inflammatory effects are not simply limited to the modulation of gene expression. Thus it was shown by Ocana-Fuentes et al. (2010), both substances decrease proinflammatory *TNF-α*, *IL-1β*, and *IL-6* mediator synthesis and increase the production of anti-inflammatory cytokine *IL-10* in a dose dependent manner. The aforementioned mediators also affect proteins, which are responsible for the epithelial integrity (*Occludin* and *Zonula Occludens* (*ZO-1*)) (Zou et al., 2016; Suzuki, 2013). Therefore, modulating the expression of those genes could lead to manipulation of the intestinal integrity and consequently enhance or decrease protection against microorganisms and the absorbency of nutrients. For the sesquiterpene β-caryophyllene it was shown, that it selectively binds to cellular receptors such as the cannabinoid (CB)2-receptor in the central nervous system and inhibits proinflammatory cytokine expression (e.g. *TNF-α* and *IL-1β*) and decreases lipopolysaccharide (LPS)-stimulated *mitogen-activated protein* (*MAP*)-*kinase* pathway activation (Gertsch et al., 2008). Although CB2-receptor is predominantly found in the central nervous system (Mackie, 2006), it was shown to prevent colitis in the intestinal tract (Kimball et al., 2006). β-caryophyllene can be found in cinnamon, black pepper and various other spices (Mockute et al., 2001). In OEO it can be found in an average of 5% but in some species with up to 15% (Mockute et al., 2001). Anti-inflammatory properties are also associated with the preliminary stage of carvacrol and thymol, the ρ-cymene (Quintans et al., 2013). Especially considering the different OEO species, the knowledge of OEOs´ impact on mechanisms of genetic pathways and the transcriptome modulating functions is limited. This chapter only provides an overview of the effects of OEO on gene expression. Further examples can be found in the following chapters two and three.

1.4 Small intestinal integrity

In addition to the absorption of nutrients, electrolytes, and water and the secretion of mucus and immunoglobulins, the intestine fulfills additional tasks. In terms of animal

health, the small intestine is a major immunologic organ and takes on a key role due its´ segregation between the body and the environment (Lallès et al., 2004). The particular challenge for the intestinal immune system is to ensure a balance between important nutrient intake and protection against pathogens (Mayer, 2003; Börsch, 1984). The development of tolerances and the limitation to a local immune response in case of an immune defence are essential and characteristic for the intestinal immune system (Börsch, 1984). This is possible by a selective composition of antigens and a highly specific population of T-cells (Mayer, 2003). The structure of pigs´ intestinal tract can be found in figure 3. The intestinal immunity is characterized of a 'trialog' between hosts´ microflora, the gastrointestinal-associated lymphoid tissue (GALT), and the epithelial barrier (Falk et al., 1998). The GALT is the major player for the intestinal health / integrity. It is functionally independent of the systemic immune defence and occurs in higher vertebrates (Asmuth et al., 2012). GALTs´ mucosa consists of a single layer of highly prismatic epithelial cells (dominantly enterocytes, goblet -, Paneth -, M cells etc.). The highly proliferative enterocytes are rejected after a short lifetime and pass into the intestinal mucus. These rapid restorative processes are necessary to guarantee the protective function, which is mainly based on the intestinal mucus, the epithelial barrier, and the epithelial connective proteins (Sobotta and Welsch, 2006). Especially tight junction proteins, *Occludin* and *ZO-1* have been shown to react on PBLCs (Zou et al., 2016; Suzuki, 2013). They serve as a selectively permeable barrier in the intestine to prevent the invasion of the body by pathogenic microorganisms and antigens, while at the same time promoting the flow of required nutrients (Smith et al., 2010; Shin et al., 2006). The fewer tight junctions there are between the epithelial cells, the worse the integrity and the higher the permeability of the epithelial layer will be (Oswald, 2006). They are a subclass of T-lymphocytes which maintain the epithelial barrier by cytolytic, purifying and regenerative effects (Burkey et al., 2009). Due to the antigen-presenting properties of epithelial cells, communication between them and T-lymphocytes is important. Underneath the epithelial layer, the *Lamina propria* can be found, which is a connective tissue layer pervaded with lymph follicles. It contains a large number of lymphocytes that aggregate into lymph follicles and thus form Peyers´ patches, which can be found predominantly in the terminal ileum (Neutra et al., 2001). These are lymphatic nodes whose main task is to recognize antigens followed by the activation of

lymphocytes. The mucosa is completed by a final and continuous layer of smooth muscle cells, the *Muscularis mucosae* (Kleessen et al., 2003). The goblet cells, which belong to the group of epithelial cells, permanently secrete a mucus layer on the apical side of the epithelium (Mayer, 2003). The glycoprotein rich intestinal mucus itself prevents pathogens from attaching to the intestinal barrier and for some commensal bacteria, it serves as an energy source. Hence, there is a fragile balance between hosts´ mucosa and its´ intestinal microflora (Fabrellas et al., 2014; Dohms, 2004). In addition to the integrity of the epithelium, intestine-associated health also includes various mediators such as chemokines, cytokines and immunological cells e.g. lymphocytes etc. In healthy animals´ intestinal tract, the epithelial cells form a protective layer in combination with mucins. If this layer is damaged due to stress, pathogenic bacteria or bacterial imbalance in general, chronic inflammation, harmful substances etc., it can be observed that those beforementioned stressors can easily invade the animal organism and by that reduce animal productivity (Dohms, 2004; Farhadi et al., 2003).

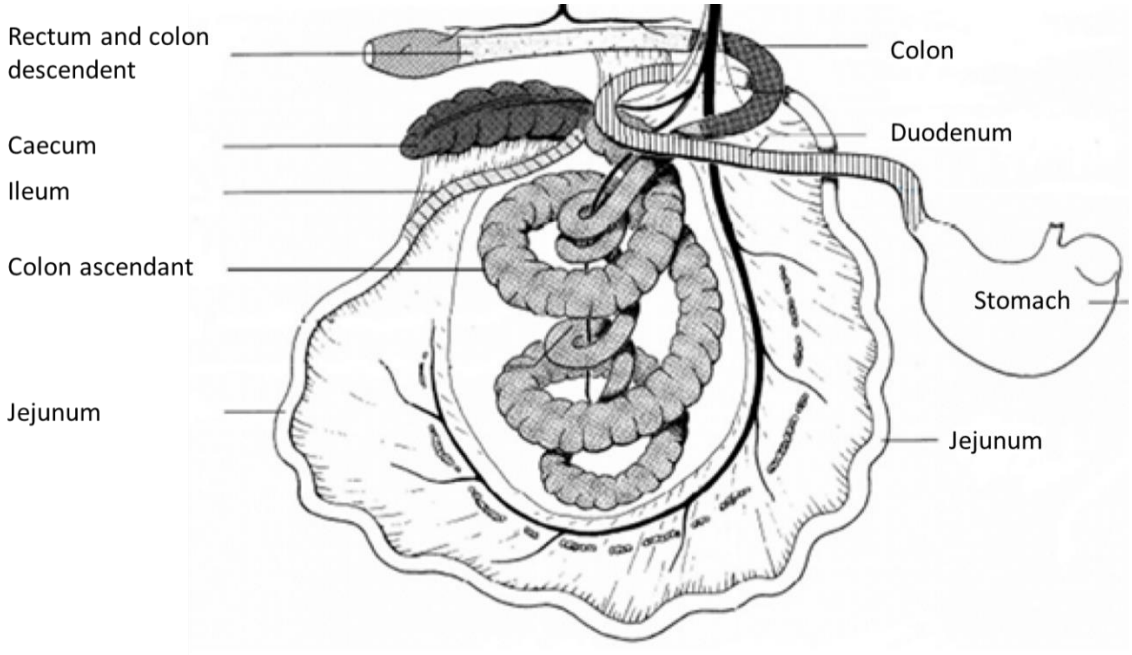

Figure 3: Structure of pigs´ gastrointestinal tract (König and Liebich, 2018)

PBLCs´ regulate the activity of lymphocytes and immunoglobulins and by this the entire immune response. Additionally, anti-inflammatory action of OEO has been shown by several authors as aforementioned (Han and Parker, 2017; Gertsch et al., 2008; Cho et al., 2007). However, in response to cellular damage, inflammation is a physiological

response and it needs to be discussed, if the inhibition of such natural processes is beneficial to the animal organism. In cases of chronic inflammatory processes or the uncontrolled overproduction of proinflammatory mediators, anti-inflammatory substances could help to negate those pathogenic cascades (Leyva-López et al., 2017). In this thesis, two small intestinal segments, jejunum and ileum, were examined predominantly, but in order to investigate the intestinal microflora large, intestine-associated samples have been additionally examined.

1.5 Our body is not ours alone; the intestinal microbiome

The intestinal microbiome is made up of many different microorganisms. It is considered to be our second genome (Bonder et al., 2016) and interacts with the dietary composition and with hosts´ intestinal cells (Hall et al., 2021; Fabrellas et al., 2014). It can influence the absorption of nutritional salts such as zinc, selenium, iodine or, through metabolism, improve the bioavailability of bioactive food components (Tomás-Barberán et al., 2017). A guaranteed thermostable living space and a continuous supply of nutrients is the symbiotic benefit for the intestinal microflora. The importance of the intestinal microbiome is steadily increasing from the point of view of animal production (Rauth, 2016; Seifert, 2016). The intestinal microbiome contains the entirety of all microorganisms living in the intestinal tract of an organism (Fabrellas et al., 2014; Turnbaugh et al., 2008). The highest proportion of intestinal tracts´ microbial mass resides in the large intestine (Rauth, 2016). *Postpartum*, the intestine is largely sterile and its´ flora develops immediately after birth (Seifert, 2016). With the beginning of suckling, the intestine gets colonized by *Lactobacillus* (*Firmicutes*) and other Gram-negative bacteria (e.g. *Proteobacteria* and *Bacteroidetes*). As the number of solid foods increases in piglets´ diet, the microflora develops to an increasingly mature stage (Looft et al., 2014; Snel et al., 2002). Especially at an early stage of life, the microflora is particularly prone to stress (Leclercq et al., 2016). In a mature stage, the predominant bacterial phyla are *Firmicutes* and *Bacteroidetes* in pigs´ intestine (Hall et al., 2021; Hill et al., 2005). However, the intestinal microbial population is a highly dynamic community of hundreds of different species that is in constant change throughout the life of the host (Gaskins, 2001; Mackie et al., 1999). Currently, up to 1000 different bacterial species have been identified so far in the intestine by investigating the 16S

rRNA gene sequence (Quast et al., 2012; Ley et al., 2006). The composition of this community depends on various factors. Stress, health, age and housing conditions can lead to significant differences in the microflora composition within animals of the same breed. Additionally, diet composition is another major regulator of hosts´ microbial community (Richards et al., 2005). The microbiome is a highly specific and fragile ecosystem. Controlling the composition of the intestinal flora is an important approach in animal production because it is ultimately crucial for optimizing the animal productivity and health (Hall et al., 2021; Fabrellas et al., 2014; Schachtschneider et al., 2013).

With the discovery of microorganisms in the 17th century, scientific research was focused for a long time on their pathogenic activity. Nowadays however, it is known, that animals live in symbiosis with their intestinal microflora, which is crucial for the host. Microorganisms support their host in digesting carbohydrates and supplying them with vitamins. Additionally, they are also involved in detoxifying the intestinal tract (Rauth, 2016). The hosts´ metabolism and immune system are particularly influenced in the area of the interfaces of the microbiota and hosts´ cells, such as the intestinal mucosa (Ribet and Cossart, 2015). In addition to its function of digesting indigestible food components for the host, the microbiome has the task to protect the host from pathogens by occupying the adhesion sites on the intestinal epithelium. Therefore, pathogenic microorganisms cannot colonize this region (Looft et al., 2014; Börsch, 1984). This resistance to colonization leads to an increased integrity of the intestinal immune system (Seifert, 2016). *Lactobacillus* and other bacteria acidify the intestinal environment and thus kill acid-prone pathogens that have survived passage through the stomach. Other commensal intestinal bacteria are able to kill pathogens through the production of antimicrobial substances such as bacteriocins and microcins. Studies at the microbiome level showed that the microflora produces bioactive molecules e.g. butyrate, biotin and acetate, which play a role in transcriptomic and epigenetic processes (Conlon and Bird, 2015; Frese et al., 2015; Gibson and Roberfroid, 1995). Short-chain fatty acids, produced by the microflora (e.g. *Faecalibacterium prausnitzii*), serve on the one hand as a source of nutrients for intestinal epithelial cells, on the other hand they are also involved in the surface enlargement of the intestinal epithelium through a growth-promoting effect on the intestinal villi and promote intestinal

peristalsis. Additionally, those short-chain fatty acids have an anti-inflammatory effect by decreasing proinflammatory cytokines (Fukumoto et al., 2003).

A highly heterogenous microflora is supposed to be beneficial for hosts´ intestinal health. If the flora shifts, the immune systems balance in the host can be disturbed (Hill et al., 2005). Illnesses or various external influences, such as medication and animal husbandry decisions, e.g. rapid weaning, can lead to a dysregulation of the microbiome. Overpopulation with acidic intestinal bacteria can result in chronic inflammation. Needless to say, antibiotics can affect the microbiome as well (Looft et al., 2014). Dense colonization with certain representatives of *Enterococcus*, *Escherichia coli (E. coli)* and *Salmonella* is to be assessed as negative for the pig. They lead to a reduction in digestion and absorption of nutrients, but morphological damage such as lesions can also arise in the intestine (Williams et al., 2001). The regulation of the intestinal flora is therefore of crucial importance in order to avoid inflammatory processes in the intestinal tract (Schachtschneider et al., 2013). A statement about the quality of the intestinal flora can be made based on the density of the population of different types of bacteria such as *Lactobacillus spp.* or also different *E. coli* species. Changing the composition of the diet also regulates the intestinal flora. Within three to four weeks after a feed change, the microbiome has adapted to the new feed (Metzler-Zebeli et al., 2015).

In addition to the aforementioned effects of OEO, several PBLCs, in former studies generally termed just as essential oils, exert antibacterial actions (Chowdhury et al., 2018; Rodriguez-Garcia et al., 2016). Especially carvacrol antibacterial activities are manifold. By depleting intracellular adenosine triphosphate (ATP) from the bacterial membrane via the reduction of ATP synthesis and increase of ATP hydrolysis, it eliminates bacteria (Helander et al., 1998). Additionally, it decreases internal bacterial pH from 7.1 to 5.8 (Ultee et al., 1999) and increases the permeability of the cytoplasmic membrane in Gram-negative bacteria (Gill and Holley, 2006; Helander et al., 1998). ρ-cymene showed comparable antimicrobial effects. However, the effects are 3-fold lower compared to carvacrol (Veldhuizen et al., 2006). Most of the studies with the aim to reveal OEOs´ antimicrobial activity were carried out under *in-vitro* conditions on selected culturable bacteria. Currently, investigations on the entire intestinal microbiome to OEO supplementation in pigs´ intestine are limited.

1.6 Scope of the thesis

Diet has an impact on the activity of certain genes and it has been shown several times that carvacrol and thymol can regulate immune parameters (Wei et al., 2017). However, if we stick to a one-sided, biased view of the actions of PBLCs, or in this case of OEO, we could also risk to worsen animal productivity. Certain secondary ingredients, such as carvacrol, with usually positive effects can become toxic above a certain concentration (Kara et al., 2015; Wald, 2004). Thus, it was reported by Sivropoulou et al. (1996), that eukaryotic cells can be critically stressed by high concentrations of the oil from *O.* v*ulgare* subsp. *hirtum*. In carcinoma cells, OEO showed antiproliferative activity in several studies (Marrelli et al., 2016; Begnini et al., 2014) and cell viability was also negatively affected by OEO (Spagnoletti et al., 2016). It cannot be excluded that the antiproliferative effects are also present in other cells and therefore it is important to develop precise feeding recommendations for such PBLCs.

Therefore, we performed three experiments to reveal OEOs´ effects on several factors, which are associated with the intestinal integrity.

The experiments presented in this thesis are part of the project 'Darmgesundheit' (no. 17-06.02.01 – 05/2018), which was financially supported by the 'Ministerium für Umwelt, Landwirtschaft, Natur- und Verbraucherschutz des Landes Nordrhein-Westfalen (MULNV)' and comprises three manuscripts addressing the fields of research and problems formulated in the general introduction (chapter one). The following chapters two – four have been submitted to or prepared for scientific journals, but have not been published yet.

Hypothesis:

- Oregano essential oil from *O. vulgare* subsp. *hirtum* affects pigs´ intestinal integrity by the regulation of immunologic gene expression in piglets, as well as in growing pigs
- By positively affecting the intestinal health, pigs´ carcass quality metrics will also be improved
- OEO shifts the microflora composition and promotes several beneficial species, such as short-chain fatty acid producing bacteria

Chapter 2: Oregano Essential Oil affects Piglets´ intestinal Integrity

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Key words: Oregano essential oil; piglets; gene expression; microbiota

2.1 Abstract

Oregano essential oil affects expression of genes associated with piglets´ immune system, regulates the activity of mucus producing cells and has an antimicrobial impact on some bacteria, e.g. *E. coli*, in the intestine. Especially weaning and the very first days after this harsh intervention in piglets´ life are critical for the intestinal integrity. We hypothesized that oregano essential oil has a positive influence on early intestinal integrity development. Therefore, we investigated the activity of immune related genes, classification of microbiota and intestinal morphology. To conduct our experiment, we produced 16 crossbreed piglets (German Landrace x Piétrain). The treated group (n=8) was fed oregano essential oil; all other parameters were identical to the control group (n=8). After 20 days feeding period, pigs were euthanized. PAS staining of jejunal and ileal mucosa revealed a lower production of mucus in the treated group compared to the control. Intestinal morphology was not affected by oregano essential oil. The qPCR of jejunal tissue showed significantly lower expression levels of *TNF-α* ($p<0.01$) as well as *IL-1β* ($p<0.01$) in the treated group. In the ileal tissue, we found a significant higher expression of *CD8* ($p<0.001$) and a reduction of *CD4* ($p<0.01$) and *E. coli* ($p<0.01$) mRNA level within the treated group. Gene expression of the *Fas ligand* was significantly increased ($p<0.05$) in spleen of the treated group. In summary, we were able to demonstrate that oregano essential oil alters the expression of genes associated with immune response in pigs´ small intestine and spleen after weaning. These findings show, that piglets´ intestinal integrity is affected by oregano essential oil within a short feeding trial.

2.2 Introduction

Modern ecological animal production depends on a balance between performance, animal welfare and health, which directly influences the product quality. Feed efficiency and intestinal integrity regulating effects, e.g. gene expression, of some feed ingredients are important key factors for that balance. The improvement of animal welfare and health without accidental losses in performance represents a principal challenge of modern animal production. Basic requirement for this is a healthy intestinal tract (IT) (Bachinger et al., 2019; Fabrellas et al., 2014; Dohms, 2004).

Non-energetical nutritional components from oregano essential oil (OEO) are recognized by cellular sensors and can thereby modulate target gene expression by stimulating multiple pathways. Such targets can be used for an early warning in nutrition-induced disorders of immunological homeostasis (Kaput and Rodriguez, 2004). OEO from the aromatic plant *Origanum vulgare* subsp. *hirtum* contains at least 34 chemical compounds, e.g. carvacrol, thymol and β-caryophyllene. Within OEO, carvacrol (5-isopropyl-2-methylphenol) is the core component with > 60% (Rychen et al., 2017). Because of OEOs´ antioxidative (Chen et al., 2009), anti-inflammatory (Kaput and Rodriguez, 2004), antiapoptotic (Wang et al., 2017) effects, its ability to change the activity of proteases and lysozymes (Mabrok and Wahdan, 2018) as well as its capacity to protect and the repair cells, carvacrol and thymol have been targets of several studies. Other major effects of those additives are the regulation of gene expression (Kaput and Rodriguez, 2004), elevation of feed efficiency, nutrient digestibility and conversion (Zhang et al., 2012). It was shown by Wei et al. (2017) that those chemicals affect expression of several cytokines such as *Interleukins* (e.g. *IL-1β*, *IL-10*, *IL-21*), *Tumor Necrosis Factor-α* (*TNF-α*) and they direct influence epithelial integrity proteins such as *Occludin* and *Zonula Occludes* (Al-Ghadban et al., 2016; Kagnoff, 2014; Suzuki, 2013).

Functions of cytokines are heterogeneous, but all are crucial for normal physiological immune system function. *IL-1β*, a proinflammatory cytokine, is primarily produced in blood monocytes, but was also found to be regulated in intestinal tissue after essential oil treatment (Wei et al., 2017). The importance of *IL-10* in regard to immunomodulation in the small intestinal tract has been described several times. It is involved in T-cell reaction, e.g. $CD4^+$ T helper cells, $CD8^+$ T cytotoxic cells, and it is important for B-cell

proliferation. Nevertheless, if pathogens were able to penetrate the protective epithelial barrier, the acquired immune system, which is activated by the innate immunity, would intervene by activating of $CD4^+$ T helper cells, $CD8^+$ T cytotoxic cells and B lymphocytes. *CD178* (*Fas ligand*), expressed by $CD8^+$ cells, belongs to the same family as *TNF-α* and unfolds anti-inflammatory properties in combination with *IL-10*. This activation can also be addressed by dietary signals, e.g. oregano essential oil (Kaput and Rodriguez, 2004). Additionally, these dietary effects are not limited to their local impact. Thus, it was shown that OEO supplementation affects the composition of body fluids and modulates gene expression of several organs and their specific tissues (Andersen, 2006; Walter and Bilkei, 2004).

The small intestine in monogastric animals plays a key role in terms of health. Not only its function to absorb nutrients but also it is also an immunological organ with multiple lymphoid follicles such as Peyers´ patches in the terminal ileum. IT disorders are responsible for around 90% of the non-infectious diseases in piglets (Dohms, 2004). Particularly the small intestine, especially the mucosal barrier, protects against the entry of pathogens due to its epithelium and its cellular components (e.g. macrophages and neutrophils). The luminal side of the IT belongs to the environment and is thereby subject to permanent stress. Intestinal mucus protects the outer membrane from desquamation due to nutritional particles. Mucins can also bind pathogens and are feed for some commensal bacteria (Gibson and Roberfroid, 1995). Wei et al. (2017) showed that functional ingredients in OEO, such as carvacrol and thymol, have an effect on small intestinal morphology and cellular activity. Besides the importance of the mucosal barrier for the immune system, its function for the directed transport of nutrients and the protection against a passive nutrient loss is crucible for the animal organism.

The intestinal flora has an important impact on conventional intestinal function and animal health (Fabrellas et al., 2014). The microbial composition within several intestinal segments is different and it is shaped by individual genetic effects (Camarinha-Silva et al., 2017). Environmental factors, e.g. weaning, nutritional composition (Conlon and Bird, 2015) and dietary signals (Yang et al., 2018; Trevisi et al., 2015; Zhang et al., 2013) regulate the microbiota profile, too. It was summarized by Round and Mazmanian (2009) that a higher diversity of intestinal microorganisms provides a better defense against pathogens. Several stressors disturb the natural heterogeneity and lead to high

numbers of *E. coli*, which have been described to negatively affect the intestinal integrity. However, some bacteria, such as higher amounts of lactic acid producing *Lactobacillus*, promote intestinal functionality (Xu et al., 2014). Due to their ability to decompose the outer membrane of bacteria, carvacrol and thymol, derived from OEO, have antimicrobial properties (Helander et al., 1998). In pigs´ intestine, they have an antibacterial effect against *E. coli* and *E. faecalis* (Wei et al., 2017; Mastelic et al., 2008). Abreu (2010), Goto and Kiyono (2012) found direct genetic cross interactions between intestinal cells and the intestinal microbiota. Thus, it was shown by Yan et al. (2010), that development and maturation of the host immune system also depends on the microbial community. Wolowczuk et al. (2008) showed the activity of major transcription factors, e.g. *Nuclear Factor-κB* depend on the microflora.

Weaning is a critical stage in piglets´ live, negatively influencing intestinal health. The first days (body weight: 8-12 kg) following the weaning are crucial for healthy IT development and thereby for the development of the whole animal. We hypothesized that OEO, derived from *O. vulgare* subsp. *hirtum* with its non-energetical chemical compounds affects intestinal integrity shortly after weaning. Therefore, we investigated small intestinal morphology and activity of mucosal cells. Additionally, we proofed described antibacterial actions on selected microbiota in the chime of piglets and immune associated transcriptomic modulations in small intestinal and splenic tissue.

2.3 Material and methods

For this study eight female and eight male piglets (25 days old; half-siblings; German Landrace x Piétrain) from two sows were selected after weaning by their initial weight (7.85±0.9 kg). The animals were randomly allocated to two groups with eight piglets per pen with self-feeders and nipple-drinkers for *ad libitum* access for a 20-day feeding trial. Groups were kept under identical environmental conditions (basal diets can be found in supplementary Tables 1 and 2), but one group (treated) was fed additionally with an oregano flavour additive (DOSTO® powder, DOSTO® FARM, Westerstede, Germany), steam distilled from *O. vulgare* subsp. *hirtum* var. Vulkan to the basal diets (Table 1). The concentration was 1500 mg flavour additive (7.5% oregano essential oil) per kg basal diet, corresponding to the recommended concentration for piglets and not expected to pose a risk for the environment (Rychen et al., 2017). Both groups were fed with ground

feed to prevent segregation of the flavour additive within the experimental phase. OEO is registered as a feed additive according to the entry in the European Union Register of Feed Additives under Regulation (EC) No 1831/2003 (2b natural products – botanically defined) (Rychen et al., 2017).

Blood samples were taken by jugular venipuncture 20 days after weaning and mixed with Ethylenediaminetetraacetate (EDTA) to prevent coagulation. Additional blood samples were taken at the end of the experiment and all pigs were euthanized for tissue sample collection. Blood, tissue segments and small intestinal chymus, stabilized in NaCl+0.1% Tween 80 (Carl Roth, Karlsruhe, Germany), were immediately stored at -80 °C after collection. For histological dissection, jejunal and ileal segments were transported in cold (4 °C) phosphate-buffered saline (PBS) to a sterile environment. For mucosa microscopy the samples were fixed in Tissue-Tek® O.C.T.™ Compound (Sakura Finetek, Torrance, USA) and stored at -80 °C. After sectioning samples at 6 µm thickness for each sample 3 technical replicates were stained in Hematoxylin & Eosin (H&E) and Periodic Acid-Schiff (PAS)-reagent (Carl Roth, Karlsruhe, Germany). Histological investigation was performed with a Leica DMLB 100S microscope (Leica Microsystems GmbH, Wetzlar, Germany). Staining intensity and morphological measurements were performed with the DISKUS (MIL 7.5, 4.80 (Büro Hilgers, Königswinter, Germany) microscopic discussion software.

For the expression analysis, total RNA was isolated from jejunum, ileum and spleen by using the GF-1 Nucleic Acid Extraction Kit (Vivantis, Subang Jaya, Malaysia) and genomic bacterial DNA with the QIAamp DNA Stool Mini Kit (Qiagen, Hilden, Germany) following the manufactures protocols. Blood RNA was isolated using QIAzol Lysis Reagent (Qiagen, Hilden, Germany) and cleaned up with RQ1 RNase-Free-DNase (Promega, Mannheim, Germany). After measuring total RNA concentration and quality with a NanoDrop 8000 spectrophotometer (Thermo Fisher Scientific, Schwerte, Germany), equally diluted RNA was in accordance with the manufactures protocol reverse-transcribed with the First Strand cDNA Synthesis kit (Thermo Fisher Scientific, Schwerte, Germany).

Using their respective primer pairs, several immune related genes and internal controls were investigated in a qPCR. The full list of primers can be found in table 2. Primers targeting the 16S rRNA gene have been utilized to discriminate against some specific

bacteria in small intestinal chime. qPCR was performed in the StepOnePlus™ Real-Time PCR system (Thermo Fisher Scientific, Schwerte, Germany) using an iTaqTM Universal SYBR® Green super mix (Bio-Rad, München, Deutschland) in accordance to the manufactures instructions and $2^{-\Delta\Delta Ct}$ ratios were generated by usage of two different housekeeping genes [*Ribosomal Protein L4* (*RPL4*), *Hypoxanthin-Guanin-Phosphoribosyltransferase 1* (*HPRT1*)].

Statistical analyses were carried out using R (www.r-project.org, version: 3.3.1). Comparisons among treated and control groups were performed using a one-way ANOVA followed by linear contrasts. Differences were considered significant when $p<0.05$.

Table 1: Composition of the flavour additive. Concentration of oregano essential oil is 7.5%

Composition of the product based on manufacturer´s specification	
Carrier substances	
Wheat flour, silicic acid, precipitated, dried	
Natural oregano oil (%)	
Carvacrol	60.0 – 65.0
Thymol	1.0 – 3.0
γ-terpinene	4.0 – 9.0
p-cymene	5.0 – 10.0
Linalool	max. 5.0
β-caryophyllene	2.0 – 5.0
α-terpinene	max. 1.5
Terpinene-4-ol	max. 2.0
Trans-sabinene hydrate	0.3 – 1.0

Oregano essential oil: 75000 mg/kg, DOSTO® powder, DOSTO® FARM, Westerstede, Germany

Table 2: List of primers used for qPCR

Target	Sequence (5´ - 3´)	Annealing Temperature (°C)	Product Size (bp)
HPRT1	F: AACCTTGCTTTCCTTGGTCA	60	150
	R: TCAAGGGCATAGCCTACCAC		
RPL4	F: AGGTGACACTATAGAATATC	60	185
	R GTACGACTCACTATAGGGAT		
TNF-α	F: TCCTCACTCACACCATCAGC	60	235
	R: CCAAAATAGACCTGCCCAGA		
IL-1β	F: GTACATGGTTGCTGCCTGAA	59	210
	R: CTAGTGTGCCATGGTTTCCA		
IL-10	F: CTCTACCATGCCCAGCTCAG	60	157
	R: GAAACTCTTCACTGGGCCGA		
IL-21	F: TCATCTTCTCAGGCACAGTGG	59	151
	R: ACATCTTCTGGAGCTGGCAG		
CD4	F: CTGGGTCTGCATCGTCTGT	54	237
	R: ATTCTCTGGTCTGCAGGCC		
CD8	F: GGCTCATCCTCCACAGCATT	57	154
	R: ACTTACTGCATTGCCTCCCC		
CD23	F: GAGTCAAGGATCGGGCCAAA	60	220
	R: GAGGCTGGAGTGTGAGTTCC		
CD154	F: AGGATCCTCAAATTGCGGCA	60	228
	R: AGAGGCTGGCTATGAAGGGA		
CD178	F: GGAAAAGCAGCTACCAGGGT	59	238
	R: AGAAGATGCTGCCAACACGA		
Total bacteria	F: CGGCAACGAGCGCAACCC	60	130
	R: CCATTGTAGCACGTGTGTAGCC		
Enterococcus spec.	F: CCCTTATTGTTAGTTGCCATCATT	60	144
	R: ACTCGTTGTACTTCCCATTGT		
Escherichia coli	F: CATGCCGCGTGTATGAAGAA	60	96
	R: CGGGTAACGTCAATGAGCAAA		
Lactobacillus spec.	F: AGCAGTAGGGAATCTTCCA	55	341
	R: CACCGCTACACATGGAG		
F. prausnitzii	F: AATTCCGCCTACCTCTGCACT	61	248
	R: GGAGGAAGAAGGTCTTCGG		

HPRT1 = Hypoxanthin-Guanin-Phosphoribosyltransferase 1; *RPL4 = Ribosomal Protein L4*; *TNF-α = Tumor Necrosis Factor-α*; *IL = Interleukin*; *CD = Cluster of Differentiation*; *total bacteria* (Denman and McSweeney, 2006); *Enterococcus spec.*, *Escherichia coli*, *Lactobacillus spec.* (Wei et al., 2017); *F. prausnitzii = Faecalibacterium prausnitzii* (Ramirez-Farias et al., 2008); F = forward; R = reverse

2.4 Results

Growth performance is not negatively affected by pungent Smell

In this study, we fed piglets with OEO in addition to their basal diet to investigate OEOs´ effect on intestinal morphology, relative expression of immune related genes and the abundance of selected microbiota. Within the experimental period, none of the groups suffered on any illnesses, symptoms such as diarrhea or animal losses. In this short feeding trial there were no differences between the two groups in case of body weight and average daily gain (Table 3). The aromatic smell of the DOSTO® powder is perceptible, also within our mixed diet. However, piglets of the OEO treated group showed no caution about the feed which is documented by our results.

Table 3: Average daily gain and weights of piglets fed with basal diet (control) or basal diet + oregano essential oil (treated)

	control	treated	p-value
Birth weight (kg)	1.45±0.17	1.40±0.22	0.69
Initial experimental weight (kg)	8.02±0.79	7.57±0.75	0.38
Weight after 20 d (kg)	12.50±0.79	12.00±0.91	0.38
Average daily gain (g/day) *	224.17±13.04	221.67±17.24	0.91

All results are presented as mean ± standard deviation (n=6)

* Average daily gain within 20 days feeding trial

OEO reduces small intestinal mucus

Intestinal mucosa is important for nutritional resorption and protects the animal organism against bacteria. To investigate ileal and jejunal morphology, two staining procedures were carried out. No damage to the tissues was found in any group. Investigation of H&E stained small intestinal cells showed no differences in villus : crypt ratio nor villus height between groups. However, a comparative consideration of PAS, docking on mucopolysaccharides and glycogen, stained ileal and jejunal sections revealed a less intensive activity of mucus producing goblet cells in the oregano treated group in both small intestinal segments (Figure 1). The amount of mucin seems to be reduced in the treated group.

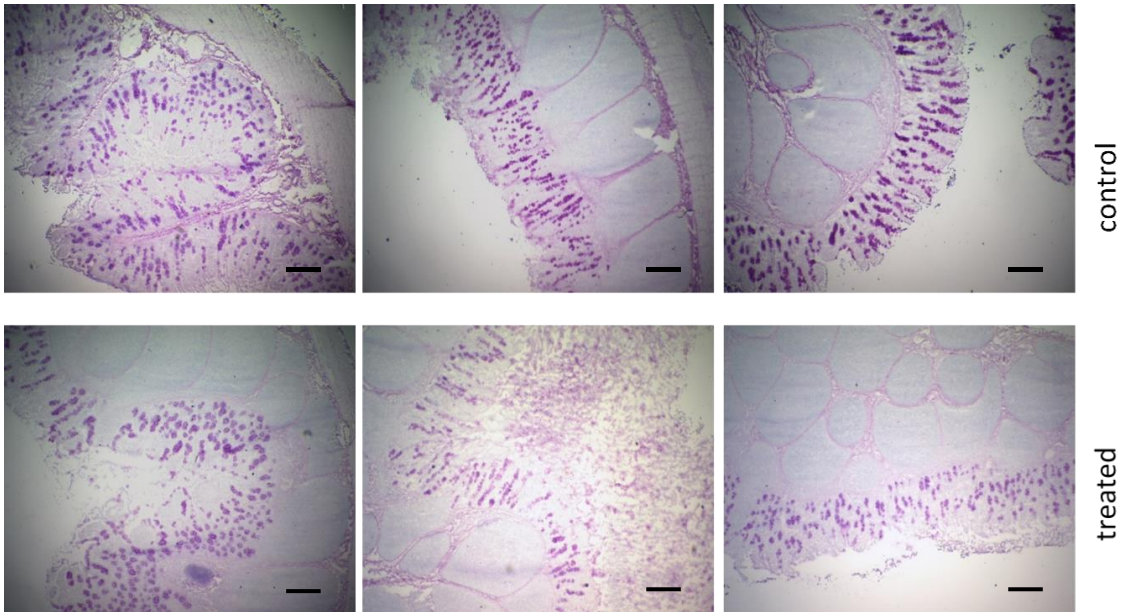

Figure 1: Periodic acid-Schiff (PAS) staining of jejunal tissue of 6 animals from two different groups. Upper row=control (basal diet), bottom row=treated (basal diet + oregano essential oil). Bars=200 µm

Immune related genes in jejunum and ileum are affected by OEO treatment

In order to investigate intestinal function of OEO treated and non-treated piglets in detail, the expression of immune related genes was quantified by qPCR. Two different intestinal sections (jejunum and ileum) have been considered in this experiment. We could show that they react differently in terms of relative gene expression patterns after an oregano supplementation. As shown in figure 2, there are significant lower expressions of the inflammatory cytokines *TNF-α* and *IL-1β* in the OEO treated groups´ jejunum compared with the control group ($p<0.01$). The mRNA level of *CD4⁺ T helper cells* ($p<0.01$) and *CD8⁺ cytotoxic T-cells* ($p<0.05$) are significant lower in the treated group. In the ileum we found no differences between groups considering *TNF-α* and *IL-1β* but we recognized a significant reduction ($p<0.05$) of *CD4⁺ T helper cells* in the treated groups´ ileum, which is in accordance to the jejunum. We found a 3-fold higher expression of *CD8* in the OEO groups´ ileum ($p<0.001$). In both small intestinal segments *CD154* was significant higher expressed in the treated group ($p<0.05$).

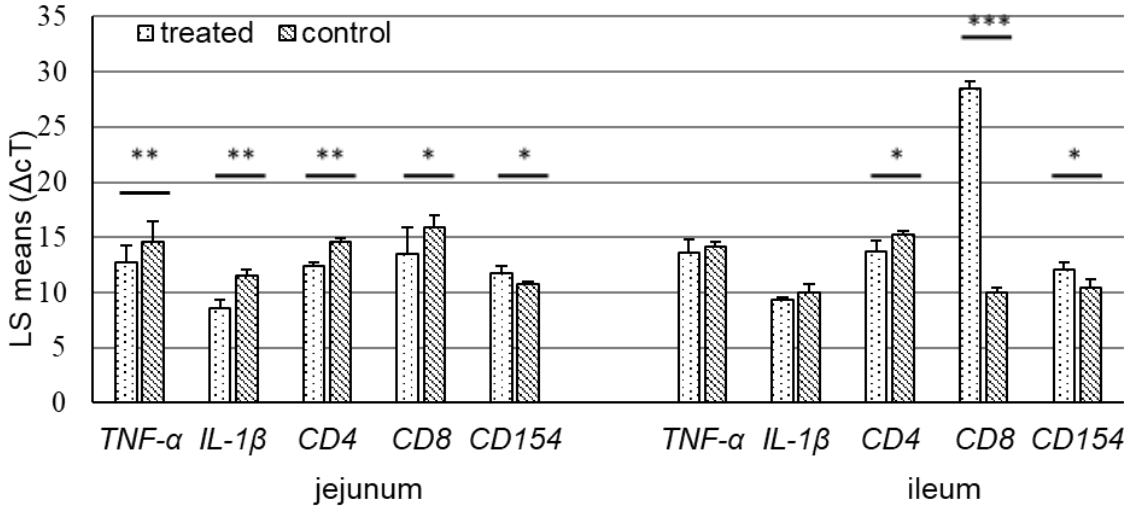

Figure 2: Relative mRNA levels of immunologic genes in pigs´ small intestine depending on OEO supplementation after 20 day feeding trial. *Tumor Necrosis Factor-α* (*TNF-α*), *Interleukin-1β* (*IL-1β*) and *Clusters of Differentiation 4* (*T helper cells*), *8* (*cytotoxic T-cells*) & *154* (*CD40-ligand*). Treated = basal diet + oregano essential oil; control = basal diet. Samples have been collected at an age of 45-days. All results are presented as means + SEM. * $p<0.05$, ** $p<0.01$, *** $p<0.001$

Reduction of Inflammation associated genes in splenic tissue

To proof postulated systemic effects of OEO, we also investigated relative gene expression in spleen tissue since we found local effects in both small intestinal segments. Figure 3 shows the expression of selected candidate genes in spleen. Thus, we were able to measure a significant higher expression of the *Fas-ligand* (*CD178*) in the OEO groups´ spleen ($p<0.01$; Figure 3). In addition, the relative expression levels of *IL-10* ($p<0.001$) and *Il-21* ($p<0.01$) were significantly higher after inclusion of OEO in the basal diet of the treated group. There was no effect on B-cell specific surface / activation markers, e.g. *CD23* and *CD154*. Identical genes have also been investigated in piglets´ blood, but no significant effect has been detected.

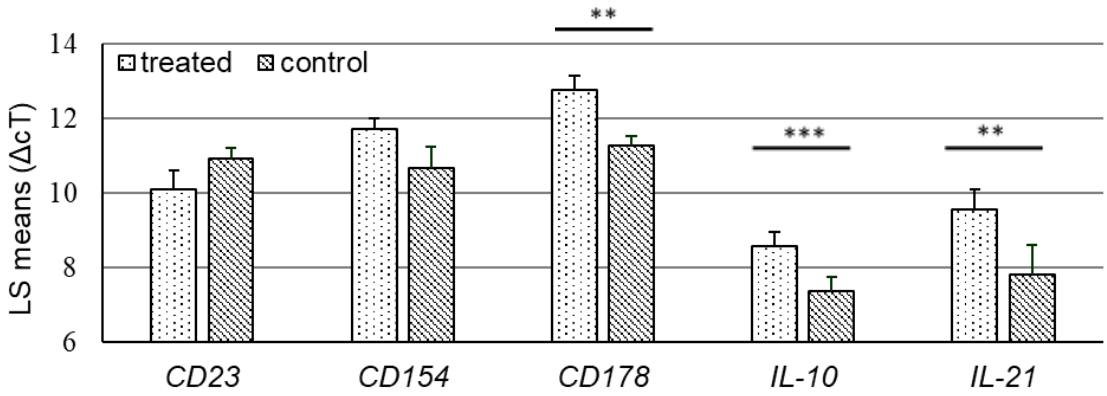

Figure 3: Relative mRNA levels of immunologic genes in pigs´ spleen depending on OEO supplementation after 20 day feeding trial. Oregano essential oil treated group shows a changed gene expressions patterns in the spleen compared to the control group. *Clusters of Differentiation 23* (*Fcε-receptor II*), *154* (*CD40-ligand*), *178* (*Fas-ligand*), *Interleukin-10* (*IL-10*) and *21* (*IL-21*). Spleen tissue of both groups was collected at an age of 45-days. All results are presented as means + SEM. ** $p<0.01$, *** $p<0.001$

OEO reduces *E. coli* counts in piglets chyme

A heterogeneous intestinal microflora is more robust against several stressors. Hosts´ intestinal integrity is influenced by microbial composition, which is shaped by natural substances, such as carvacrol from OEO. Therefore, we examined 4 key microbiota and found the microflora to be highly heterogeneous between different individuals. Supplementation of OEO led to a significant reduction of Gram-negative *E. coli* in the treated groups´ small intestinal chyme ($p<0.01$). The colonization with *Lactobacillus spec.* tended to be reduced in the OEO group, but we found elevated counts of Gram-positive *Faecalibacterium prausnitzii* ($p<0.05$). The counts of *Enterococcus spec.* did not differ between groups (Figure 4).

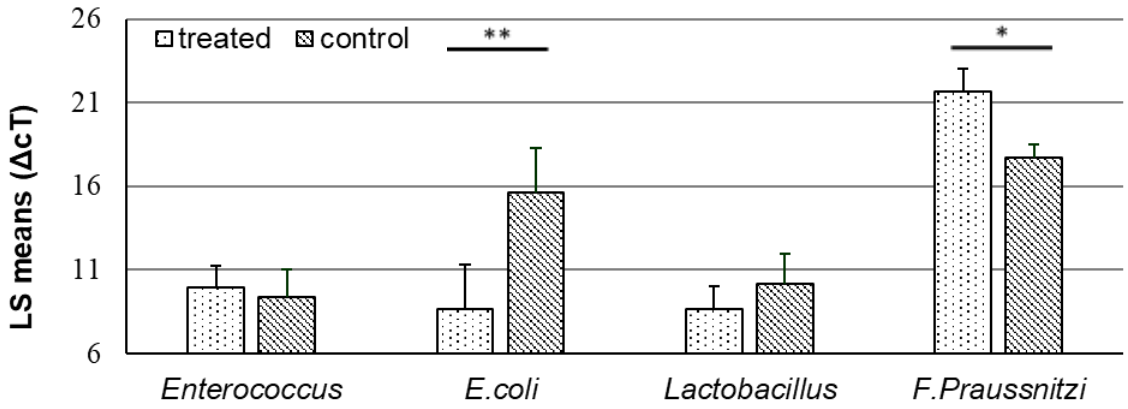

Figure 4: Relative population of selected small intestinal microbiota after 20 days feeding trial. Small intestinal chyme of both groups (treated = basal diet + oregano essential oil; control = basal diet) was collected at an age of 45-days. All results are presented as means + SEM. * $p<0.05$, ** $p<0.01$

2.5 Discussion

The main component of OEO is carvacrol with > 60%. Therefore, many studies focus on this chemical compound. Bimczok et al. (2008) concluded, that previous observed effects of OEO are not only dependent on carvacrol; the other chemical substances have to be noticed too and synergistic effects have to be expected. In our study, we used a medium dosage of OEO (112.5 mg/kg) in the piglet diet. Rychen et al. (2017) showed, that a concentration of 150 mg OEO per kg feed had no negative effect on weaned piglets. In the present study, we investigated several OEO effects on intestinal health parameters, confirming the results.

Namkung et al. (2004) found a significant reduction of the average daily gain depending on an oregano oil containing herb mixture within three weeks after weaning. The authors partially attribute this effect to the strong smell of the herbs. However, it was indicated by Zhang et al. (2012) that natural oil compounds will reduce the feed intake but not affect the body weight in general which might be explained by an improved feed conversion. In contrast to the indications by Namkung et al. (2004) and Zhang et al. (2012) we observed no differences in feed consumption during the feeding period and thereby in feed conversion between groups which could be partial explained by the short feeding period. Stelter et al. (2013) fed dried oregano plants and they did not measure an effect on weaned piglets growth performance, too. The aromatic smell of the DOSTO® powder was perceptible, also within our moderate mixed diet. However, we did not recognize any anticipatory behavior of our piglets against the oregano treated feed. In Broilers it was shown, that supplementation of 15 or 20 mg/kg of carvacrol / thymol can improve feed expenses and also the growth performance (Abdel-Wareth et al., 2012). Van der Aar et al. (2017) explained this performance promoting effect through higher feed intake, better absorption of nutrients and in general improved intestinal integrity. In addition, anti-inflammatory, intestinal integrity improving and positive effects on the commensal microbiota composition within the intestine have been described (Wei et al., 2017; van der Aar et al., 2017).

Small intestinal morphology

In the current study we did not find any effects on small intestines´ morphology, e.g. villus height or crypt depth. Our results are mostly in line with findings from Wei et al. (2017), who used a 100 mg/kg (1:1; carvacrol and thymol) dosage in their two weeks feeding trial in piglets. However, they reported a reduced villus height and villus : crypt ratio according to their supplementation. By contrast, four weeks feeding trial with 25 mg/kg OEO showed increased villus height, which could protect the small intestine against epithelial necrosis in finishing pigs (Zou et al., 2016). According to those results, the question is whether application age, duration time or concentration of natural oils is important. Zhang et al. (2012) showed that chemicals from OEO enhance the digestibility of feeds´ dry matter, which is likely due to the positively affected villous height. Beside some positive effects on small intestinal morphology in *in-vitro* studies, negative effects have also been reported. A dosage of > 5 mM carvacrol damaged the epithelial barrier in a cell line (IPEC-1; ACC 705; DSMZ) (Roselli et al., 2007) and it induced apoptosis in porcine lymphocytes (Bimczok et al., 2008). Although there was no morphological effect in the current study, we noticed a lower activity of mucus-producing goblet cells in jejunum via glycogen staining PAS. We expect that the intestinal wall protecting mucus would be produced in a higher amount in the control group, which could also positively influence the absorbency of the tissue. A stronger barrier could be beneficial against pathogen invasion but could also reduce intestinal absorption (Ribeiro et al., 2016; Smirnov et al., 2005).

OEO affects several genes associated with piglet intestinal integrity

Substances, such as carvacrol and thymol, which are isopropyl cresols, can penetrate the intestinal mucin layer and will cross the epithelial wall into deeper intestinal layers and thereby affect various cellular processes such as gene expression in epithelial and myeloid cells (Bimczok et al., 2008; Andersen, 2006; Walter and Bilkei, 2004). This is likely due to the reversible chemogenetic action of dietary signals as e.g. ligands. In both investigated small intestinal segments, we found significant differences of gene expressions associated with piglet intestinal integrity. In the jejunum of the treated group, we measured a reduction of *TNF-α*, an important cytokine that is involved in systemic inflammatory processes and is part of the *NF-κB* signaling pathway. After essential oil treatment, Li et al. (2012) found in an ELISA-assay elevated values of *TNF-α* and reduced levels of *IL-6*. A mouse model also revealed inhibitory and thereby anti-inflammatory effects of carvacrol on *TNF-α* (Guimarães et al., 2012). Another factor within this pathway is *IL-1β*, which is also expressed significantly lower in the OEO group. These findings are in accordance with the observations by Wei et al. (2017). In summary, these findings show that carvacrol and thymol have a significant influence on the regulation of genes associated with immune cell activity. The highest amount of these substances is absorbed in the mid-jejunum. This might explain why results in the ileum are not similar. Peyers´ patches are lymphoid follicles. They can be found in the whole small intestine but are concentrated in the terminal ileum. The high number of lymphocytes may explain the high reactivity of *CD8* in the ileum. T-cells are involved in the maintenance of the intestine and can be found along mucosal surface (Kabelitz et al., 2005). *CD154* is expressed on active $CD4^+$ cells and binds on *CD40*, which can be found on B-cells surface. Interaction between *CD40-CD154* is crucial for activation and differentiation of B-cells and thereby for humoral immune response (Pinelli and Ford, 2015; Song and Buchwald, 2015). Walter and Bilkei (2004) found a higher percentage of $CD4^+$ and $CD8^+$ cells in blood of finishing pigs after supplementation with OEO but in gestation sows treatment with 250 mg/kg OEO did not affect T-cells (Ariza-Nieto et al., 2011). There was also no effect on $CD4^+$ and $CD8^+$ cells in blood after feeding dried oregano plants in weaned piglets (Stelter et al., 2013). Nevertheless, we were able to measure OEO effects on intestinal *CD4* and *CD8* expression but it seems that T-cells in blood are not affected by this. In the spleen we found a higher expression of *IL-21*,

produced by $CD4^+$ cells and natural killer (NK) cells, regulating the activity of NK cells and of cytotoxic $CD8^+$ cells (Mehta et al., 2004). Increased *IL-21* expression could therefore be interpreted as a sign of increased reactivity of the humoral immune response in the event of an antigen contact. *CD178* (*Fas-ligand*) was expressed significantly higher in OEO supplemented group. In a complex with $CD8^+$ cells it induces apoptosis in infected cells and in complex with $CD4^+$ cells it leads to the deactivation of auto reactive B-cells (Gajate and Mollinedo, 2015). Saraiva and O'garra (2010) showed in a mouse model, that low concentrations of *IL-10* could induce chronic inflammatory disease. In the current study, we found higher levels of *IL-10* in OEO groups´ spleen. As indicated by previous studies, OEO can affect gene expression of several genes. Not only are these local events in the small intestine, but also effects can be displayed in other organs, too. This is a potential indicator for OEOs´ systemic effects.

Reduction of *E. coli* by OEO

The intestinal microflora is easily influenced by feed (Abdel-Wareth et al., 2012; Maslowski and Mackay, 2011) and weaning stress (Guevarra et al., 2018). Stress will lead to decreased counts of *Lactobacillus* and higher abundances of *E. coli* (Xu et al., 2014). The microflora is shaped by different treatments and dietary signals. Furthermore, it has a crucial impact on intestinal morphology and immune response which was shown in a germ-free piglet model with 16 animals by Shirkey et al. (2006). An *in-vitro* study showed; the minimum inhibitory concentration of carvacrol against *E. coli* is 250 mg/kg (Guarda et al., 2011). We measured a significant reduction of *E. coli* depending on our 20 days OEO feeding trial with a concentration of ≈ 70.3 mg/kg carvacrol. Our findings are in accordance with previous studies that also reported an *E. coli* reducing effect with a quite similar dosage (Wei et al., 2017; Zou et al., 2016). Furthermore, very low concentrations of thymol (18 mg/kg) have also shown a significant effect on the reduction of *E. coli* (Li et al., 2012). The ambivalence between *in-vivo* and *in-vitro* findings considering *E. coli* may be partially explained by synergistic effects between substances in essential oils. Best synergistic effects were achieved by a carvacrol, thymol (1:1) mixture (Guarda et al., 2011). Another explanation may be that pure carvacrol loses its activity after 6 h storage in aqueous solutions such as culture medias (Si et al., 2006). In our study we did not measure significant differences between groups regarding *Enterococcus* and *Lactobacillus*, which is in contrast to several other studies and may be

explained by variable dosages of OEO, or its functional components, in different studies. Because of their competitive exclusion against *E. coli*, *Lactobacillus* have several beneficial effects on hosts´ intestinal health. However, the effect of natural oils, in a wide range of concentrations, on *E. coli* is clear, reaction of *Lactobacillus* on dietary treatments seems to be more complicated. A 100 mg/kg essential oil mixture (thymol: 18 mg/kg) showed no effect on *Lactobacillus* (Li et al., 2012). Usage of OEO (1000 mg/kg; ≈ 600 mg/kg carvacrol + others) significantly improved *Lactobacillus* counts in weaning piglets (Zhang et al., 2012). Wei et al. (2017) also found elevated *Lactobacillus* counts in weaned piglets after treatment with 100 mg/kg (1:1; carvacrol and thymol) blend. However, in broiler chickens *Lactobacillus* counts were reduced after treatment with OEO (Abdel-Wareth et al., 2012). Those different results imply, that the function of OEO on the bacterial community has not fully been explained yet. Another beneficial bacterium is *Faecalibacterium prausnitzii*, a butyrate-producing bacterium (butyrogenic effect) (Ramirez-Farias et al., 2008), which reacts sensitively on substances such as thymol (Thapa et al., 2012). Butyrate can be absorbed by epithelial cells and thereby improves intestinal health (Ramirez-Farias et al., 2008). In this study *F. prausnitzii* tended to be positively affected by our treated groups´ feeding regime, which might be beneficial for the host.

2.6 Conclusion

Oregano essential oil affects immune system related genes and several bacteria. In the present *in-vivo* study, we were able to illustrate that a flavour additive comprising OEO can alter expression of immunologic genes in piglets´ small intestine after weaning. Thus, we found a significant higher *CD8* expression in ileal tissue and a reduction of inflammation related genes in the spleen. Experiments´ data show potential anti-inflammatory effects according to OEO treatment at a genetic level. Whereas there was no effect on the small intestinal morphology, we observed the mucin layer in the jejunum to be reduced. To exploit the performance potential of farm animals, an optimization of the diet is indispensable. It is possible to affect gene expression of immunological factors, bacterial composition and to reduce the negative effects of weaning stress by prior administration of certain additives. Feed manufacturers can use this knowledge to develop nutrition lines for specific stages of animal production.

Therefore, it is possible to improve the performance and to reduce diet-related drugs. However, the efficiency of stimulating effects of natural oils is dependent on their concentration, duration of supply, composition of the standard diet, age and race of the consuming animal. Translation of nutrigenomic data into precise predictions for animal breeding is a further goal for which additional research is needed.

Statements

Animal care followed the general guidelines outlined in the European Animal Welfare Regulations and the Directive 2010/63/EU. It was conducted according to the institutional guidelines and animal husbandry regulations of Germany. The State Agency of Nature, Environment and Consumer Protection, North Rhine-Westphalia, Germany (permission no. 84-02.05.04.14.027), approved the blood sampling protocol.

2.7 Appendix

Supplementary table 1: Composition of the basal diet 1 (0-7 d)

Item	Amount
Ingredients (%)	
Sour whey powder	21.4
Wheat	17.8
Maize	15.0
Soybean meal, peeled	10.0
Soybean meal, steamed	6.4
Soybeans, steamed	5.5
Oatmeal	5.0
Soyoil	5.0
Sugar	3.7
Potato protein	2.0
Wheat gluten	1.6
Wheat, extruded	1.6
Coconut oil	1.0
Dicalcium phosphate	0.7
Wheat bran	0.3
Sodium chloride	0.3
Monocalcium phosphate	0.3
Calcium carbonate	0.1
Yeast	0.04
Plant oil	0.02
Yeast cell walls, inactivated	0.002
Nutrition levels	
Digestible Energy (MJ ME/kg)	15.2
Crude protein (%)	21.0
Crude fat (%)	9.0
Crude fibre (%)	2.0
Crude ash (%)	6.0
Calcium (%)	0.6
Phosphorus (%)	0.6
Sodium (%)	0.2
Magnesium (%)	0.1
Lysine (%)	1.4
Methionine (%)	0.5
Threonine (%)	0.9
Tryptophan (%)	0.3

Per kg diet: vitamin A 20000 IU, vitamin D3 2000 IU, vitamin E 100 IU, potassium iodide 2.5 mg/kg, selenium 0.4 mg/kg, copper-(II)-sulfate pentahydrate 150 mg/kg, manganese (II)-oxide 50 mg/kg, zinc oxide 105 mg/kg, iron (II)-sulfate monohydrate 125 mg/kg; Blattivit® Ferkel-Pre-Classic; Blattin, Dormagen, Germany

Supplementary table 2: Composition of the basal diet 2 (7-20 d)

Item	Amount
Ingredients (%)	
Barley	35.0
Wheat	26.3
Soybean meal, steamed	17.4
Wheat bran	6.0
Maize	5.0
Bakery products	5.0
Calcium carbonate	0.66
Monocalcium phosphate	0.56
Sodium chloride	0.5
Rape oil	0.5
Sodium bicarbonate	0.1
Nutrition levels	
Digestible Energy (MJ ME/kg)	13,6
Crude protein (%)	17.5
Crude fat (%)	3.5
Crude fibre (%)	4.0
Crude ash (%)	5.0
Lysine (%)	1.25
Methionine (%)	0.42
Calcium (%)	0.6
Phosphorus (%)	0.5
Sodium (%)	0.25

Per kg diet: vitamin A 16000 IU, vitamin D3 2000 IU, vitamin E 100 mg, copper-(II)-sulfate pentahydrate 80 mg, iron (II)-sulfate monohydrate 90 mg, zinc sulfate monohydrate 105 mg, manganese (II)-oxide 80 mg, calcium iodine 0.8 mg, selenium 0.3 mg; EuroStart®, Agravis, Münster, Germany

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Chapter 3: OEO modulates several biological processes in growing pigs´ small intestine

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Under revision

Running title: Oregano essential oil showed limited effects on pigs´ carcass quality and haematology whereas a transcriptome analysis revealed significant modulations in the jejunum and the ileum

Key words: Oregano essential oil; growing pigs; transcriptome analysis; carcass quality

3.1 Abstract

Pig production depends on a health and performance balance. An approach to improve intestinal health is the oregano essential oil (OEO) supplementation within a conventional diet. Intestinal integrity regulating effects, e.g. gene expression, of some feed ingredients are important key factors for that balance. We hypothesized that OEO affects the expression of genes associated with pigs´ intestinal integrity. In four trials, a total of 86 pigs have been used. From weaning, the 'treated' group (n=42) was additionally fed an oregano flavour additive [1500 mg/kg (7.5% pure OEO)] within the basal diet. The 'control' group (n=44) was kept under identical environmental conditions, except the OEO. At age of 6 months, pigs were slaughtered with an average weight of 111.1±10.9 kg. In addition to automatically generated 'Fat-o-Meter' (AutoFOM) data, carcass quality factors have been measured manually. Valuable cuts of meat, such as ham and belly were significantly reduced in the OEO group. Effects of OEO on pigs´ haematologic parameters were very limited. For transcriptome analysis, the most interesting microarray expression results have been listed in a table (topTable). Selected genes were technically validated by qPCR. As a result, few significant differences in animal development and meat quality have been found between the OEO treated and the control group. Depending on OEO supplementation, we found 93 differently regulated genes in the jejunal tissue (70 up, 23 down) and 60 in the ileal tissue (48 up, 12 down). Just three genes [*GRIN3B* (*glutamate ionotropic receptor NMDA type subunit 3B*), *TJP1/ZO-1* (*tight junction protein ZO-1*) and one uncharacterized gene] were affected by OEO both in jejunum and ileum. qPCR validation revealed *AKT serine/threonine kinase 3* (*AKT3*), *Interferon* (*IFN*) *-ε*, *-ω*, *TJP1/ZO-1* to be up-regulated in the jejunum and *C-C motif chemokine ligand 21* (*CCL21*) was up-regulated in the ileum of pigs that were supplemented with OEO. OEO supplementation had limited effects on pigs´ performance traits. However, we were able to demonstrate that OEO alters the expression of genes associated with adaptive immune response in pigs´ small intestine. These findings help to explain OEOs´ beneficial impact on pigs´ intestinal integrity.

3.2 Introduction

The increasing need for high-quality animal protein in human nutrition can only be achieved through modern animal production. Additionally, an increasing interest in sustainable food can be observed globally (Grunert et al., 2018). Substitutes for synthetic antioxidants and other additives to improve meat quality have been investigated. Herbal medicines, or phytogenics and their bioactive compounds can be used to improve animal productivity by promoting intestinal health. However, secondary plant metabolites composition depends on plant species, genus and local environmental conditions (Dhama et al., 2018; Pushpa et al., 2013). Oregano essential oil (OEO) has already been used to improve meat quality and quantity in several species (Simitzis et al., 2010; Janz et al., 2007; Namkung et al., 2004), but often with highly heterogeneous results. OEO from the aromatic plant *Origanum vulgare* subsp. *hirtum* contains at least 34 chemical compounds, e.g. carvacrol, thymol and β-caryophyllene. With a share of > 60% carvacrol (5-isopropyl-2-methylphenol) is the core component within OEO (Rychen et al., 2017). Because of OEOs´ antioxidative (Chen et al., 2009) and anti-inflammatory (Landa et al., 2009) effects, its ability to change the activity of proteases and lysozymes (Mabrok and Wahdan, 2018) as well as its capacity to protect and repair cells, carvacrol and thymol have been targets of several studies (Wei et al., 2017, Chen et al., 2009). However, due to synergistic effects, natural essential oils seem to be more effective compared to the single components (Pu et al., 2020; Pezzani et al., 2017) and the 'mode of action' has not been fully described.

Intestinal integrity depends on several epithelial cells, e.g. enterocytes, their linkage through tight junction proteins and mucus (DeMeo et al., 2002). Therefore, the small intestine is an important immunological organ in addition to its purpose of digesting and absorbing nutrients, metabolic and barrier function. Dendritic cells (DCs) can be commonly found in lymphoid follicles, tissue and Peyers´ patches, which are important for the ileal immunity. They are highly important for immune systems functionality due to their antigen-presenting function (Jang et al., 2006). Humoral factors, such as interferons and other cytokines are directly involved in the small intestinal immunity and protect the organism against viruses and other pathogens (Huang et al., 2014). It was shown by several authors that gene expression of the aforementioned parameters was affected by plant essential oils (Wang et al., 2017; Jung et al., 2015; Jang et al.,

2006). Non-energetical nutritional components from OEO are recognized by intestinal cellular sensors and can thereby modulate gene expression. Intestinal gene expression modulations, identified by high throughput technologies (e.g. microarray-based transcriptome analysis), might explain effects on characteristics such as intestinal integrity. Oral administration often showed an influence on the composition of the blood and cells, which are important for the immune system, such as T-cells (Gómez-Gómez et al., 2017; Stelter et al., 2013; Bilkei, 2004). However, the assessment of effects on the immune system is often not easy. Therefore, auxiliary characteristics, such as carcass quality can help to reveal biological activities, cellular responses and effects on signal pathways depending on essential oils (Urasaki et al., 2020).

Intestinal integrity is a key regulator for animal performance and health. We hypothesized that OEO, derived from *O. vulgare* subsp. *hirtum* with its non-energetical chemical compounds improve intestinal integrity of jejunal and ileal tissue. Therefore, the objective of this study was to investigate OEOs´ effects on production parameters, growth, carcass quality, haematologic profile and transcriptomic modulations for the whole fattening period in pigs.

3.3 Materials and methods

OEO is registered as a feed additive according to the entry in the European Union Register of Feed Additives under Regulation (EC) No 1831/2003 (2b natural products – botanically defined) (Rychen et al., 2017). Animal care followed the general guidelines outlined in the European Animal Welfare Regulations and the Directive 2010/63/EU. The experiment was conducted according to the institutional guidelines and animal husbandry regulations of Germany. The State Agency of Nature, Environment and Consumer Protection, North Rhine-Westphalia, Germany (permission no. 81-02.04.2019.A307), approved the blood sampling protocol. During the entire experiment, the animals showed no symptoms of illness or other abnormalities.

To evaluate the influence of oregano essential oil on pigs´ transcriptome, carcass quality and blood profile, we used closely related pigs. For this, nine gilts (German Landrace, siblings) have been paired with one Piétrain boar. In four methodically identical trials (four quarters of the year, average time = 140±3 d), a total of 86 offspring pigs (43 female, 43 uncastrated males) were used. Pigs have been selected according to their birth weight (1.4±0.2 kg) and their weight at age 28 d (8.4±1.6 kg). Henceforth, in each trial, pigs´ have been equally divided into two feeding groups. Groups were kept under identical conditions, such as lighting, pen size, ventilation with maximal four pigs per pen (sexes divided), nipple-drinkers and self-feeders for *ad libitum* access in a climate controlled stable. The feeding regime was also identical for both groups with one exception, the 'treated' group was additionally fed an oregano flavour additive, steam distilled from *O. vulgare* subsp. *hirtum* var. Vulkan (DOSTO® powder, DOSTO® FARM, Westerstede, Germany, chapter two: Table 1), directly after weaning (28 d) until end of fattening. The concentration of the flavour additive, which was given as a topping, within the basal diet was 1500 mg/kg (7.5% pure oregano essential oil). Pigs were fed a grower (28 d – 130 d) and a finisher (130 d – 170 d) diet following the generally applied feeding schedule. The grower diet consisted of (% as fed) 54 wheat, 21 barley, 22 soybean meal, 1 soybean oil and 2 mineral-vitamin mix. The finisher diet consisted of (% as fed) 42 wheat, 40 barley, 15 soybean meal, 1 soybean oil and 2 mineral-vitamin mix. The diets were fed dry as coarsely ground meal (particle size ≈ 300 – 2000 µm).

Full blood and carcass quality analysis

During the fattening period, the pigs were randomly weighed every week by the animal husbandry staff. Exactly weights have been measured at the blood sampling time points (average ages: 28 d = weaning, 70 d = fattenings' start, 130 d = mid-fattening, 170 d = fattenings´ end). Blood samples have been taken from the jugular vein. To prevent coagulation during the blood sampling process, S-Monovettes containing ethylenediaminetetraacetic acid (EDTA) KE (Sarstedt, Nümbrecht, Germany) have been used. Blood samples were taken by jugular venipuncture. We collected 3 ml full blood of each pig and stored the samples immediately at 4 °C. After 3 h chilling, samples have been sent to an analytical lab (SYNLAB Vet, Leverkusen, Germany) and complete blood counts (CBC), using flow cytometry technique, were generated on the same day of sampling.

At an average age of six months, pigs were transported from the experimental farm 'Frankenforst' (Vinxel, Germany) to a conventional abattoir within a transportation time of 2 ½ h and were slaughtered with an average weight of 111.1±10.9 kg. After 45 min of resting time, the slaughter began by using carbon dioxide anaesthesia. Carcasses were measured by AutoFOM technique, which forms a three-dimensional ultrasound image to measure carcasses fatty tissue fully automatically (Brøndum et al., 1998). 45 min and 24 h post mortem meat quality characteristics were measured in *Longissimus thoracis et lumborum* between the 13th and the 14th rib according to the protocol of Kayan et al. (2013) using a portable pH-, con- and chroma-meter (PH-Star, LF-Star and OPTO-Star, Matthäus, Eckelsheim, Germany). From the 13/14th rib longissimus dorsi, 600 g muscle pieces were taken from each animal, packed in standardized plastic bags and hanging transported in cooled boxes to the institutional meat quality lab. Drip loss was evaluated by bag-method described by Kayan et al. (2013). The percentage of collagen, fat, protein and water was determined with a Foss FoodScan^{TM} Lab (Near Infrared Spectroscopy (NIRS), Foss, Hamburg, Germany) device. Shear force was recorded with an Instron universal testing machine, equipped with a Warner Bratzler shear blade (Instron, Darmstadt, Germany). Drip, cooking and thawing loss have been calculated as the percentage of weight loss based on the initial sample weights (Kayan et al., 2013). Additionally, valuable cuts of meat were measured by AutoFOM. They are correlated to the carcass weight and have therefore been corrected for this.

Transcriptomic profiling (RNA isolation and chip preparation)

For transcriptomic analysis, jejunum (an average 15 cm long piece, 30 cm before the ileum) and ileum (an average 8 cm long piece, 5 cm before samples the ileo-caecal junction) of each pig have been collected. Samples have been washed with phosphate-buffered saline (PBS, Capricorn Scientific, Ebsdorfergrund, Germany) and were immediately stored at -196 °C for transportation. At the Institute of Animal Science (Bonn, Germany) samples have been stored at -80 °C until transcriptome profiling. However, for transcriptome analysis the intestinal samples of eight homogenous pigs (104.3±2.0 kg, from one sow, four pigs as control, four pigs for treatment) have been selected from one trial. For nucleic acid extraction, 5 g of each small intestinal sample have been ground to a fine powder with sterile and diethyldicarbonat (DEPC) treated mortars and pistils. Sample temperature was <-40 °C during the whole grinding procedure. Total RNA was extracted from 30 mg powder per sample using the NucleoSpin RNA Plus mini kit (Macherey-Nagel, Düren, Germany) according to the manufacturers´ protocol. RNA concentration and quality were checked with a NanoDrop 8000 spectrophotometer (Thermo Fisher Scientific, Schwerte, Germany) and afterwards RNA integrity number (RIN) was evaluated by microcapillary electrophoresis on an Agilent 2100 Bioanalyzer with the RNA 6000 Nanochip kit (Agilent Technologies, Waldbronn, Germany). The average RIN was 8.3±0.3. RNA isolation of samples < RIN 8 was repeated. Biotin-labelled single-strand cDNA (ss-cDNA) was synthesized using the GeneChip WT plus reagent kit (Thermo Fisher Scientific, Schwerte, Germany) according to the manufacturers´ protocol from 100 ng total RNA per sample. 130 µL ss-cDNA probes were injected into the GeneChip Porcine Gene 1.0 ST array (81/4 format, 23,256 transcripts from 20,201 *sus scrofa* genes, Thermo Fisher Scientific, Schwerte, Germany) and incubated for 16 h in a GeneChip hybridization oven 640 (Thermo Fisher Scientific, Schwerte, Germany) at 45 °C with 60 rpm for hybridization. The hybridized chips were washed and stained in a GeneChip fluidics station 450 (Thermo Fisher Scientific, Schwerte, Germany) and scanned by GeneChip scanner 3000 7G (Thermo Fisher Scientific, Schwerte, Germany). The image data were evaluated, regarding the internal quality controls, using Affymetrix GeneChip command console software. The intensity data were exported into .CEL file format.

Transcriptome data processing and statistical analysis

For microarray data normalization and background correction the 'oligo' Bioconducter package (version 1.48.0, Carvalho and Irizarry, 2010) was used in R project software (v. 3.6.1, Gentleman et al., 2004). The 'arrayQualityMetrics' package (v. 3.44.0, Kauffmann et al., 2009) has been used to prove array quality. Robust Multi-array Average (RMA) based quantile normalization (log2) of microarray data was performed at the transcript level. Probe to gene transcript annotation was accomplished with a common Affymetrix annotation file (PorGene-1_0-st-v1, Liu et al., 2003). Transcriptional modifications investigation in response to feeding regime and the differential gene expression analysis was performed by using the 'Linear Models for Microarray Data (limma)' technique (v. 3.44.3, Ritchie et al., 2015; Smyth, 2005) with empirical Bayes adjustment to the variance, followed by Benjamini and Hochberg (BH) correction for multiple testing (Benjamini and Hochberg, 1995). Pairwise contrast for treated vs. control groups was considered for differential gene expression false discovery rate (FDR) of <0.05 and log2 fold-change either >1.5 or <-1.5 were considered as the threshold for differential expression of genes. The adjusted p-value has been set to <0.1 for topTable generation.

For the functional analysis of differentially expressed genes and the biological interpretation of the transcriptome data, the dataset has been linked with the 'Ensembl' databank (Yates et al., 2020). The 'biomaRt' package (v. 2.44.1, Durinck et al., 2009) from Bioconducter was used for analysis. Significantly represented gene ontology terms and biological pathways were explored with 'DAVID Bioinformatics Resources 6.8' (Huang et al., 2009; Sherman and Lempicki, 2009) and the 'reactome' pathway website (Jassal et al., 2020; Fabregat et al., 2018). For this analysis, the normalized and filtered expression dataset containing human orthologous 'Ensembl' gene identifiers and gene symbols were uploaded into the mentioned online tools. The main parameters for the functional annotation in the DAVID online application were Gene_Ontology and Pathways. 'Gene_Ontology for biological processes', 'Gene Ontology for cellular components' and 'Gene_Ontology for molecular functions' were the secondary parameters in Gene_Ontology analysis and for the Pathway analysis, the parameter 'KEGG_Pathway' has been used.

qPCR validation of selected genes

The microarray expression results were technically validated by qPCR within a higher number of samples (n=36, equally divided to both groups). 50 ng total RNA has been used for cDNA reverse transcription with the First Strand cDNA synthesis kit (Thermo Fisher Scientific, Schwerte, Germany) according to the manufacturers´ protocol. Primer design is based on Primer3web (version 4.1.0, http://primer3.ut.ee/) open-source software. The full list of primers can be found in table 1. qPCR was performed in the StepOnePlus™ Real-Time PCR System (Thermo Fisher Scientific, Schwerte, Germany) using 12.5 µl of the primaQUANT CYBR qPCR-Mastermix (high ROX, Steinbrenner, Wiesenbach, Germany) and 10 µM of each primer for every reaction. Thermal cycling conditions were 95 °C for 10 min, followed by 40 cycles with 10 s at 92 °C, 20 s at 60 °C and 1 min at 72 °C. The melt curve was generated for quality control. Relative expression was calculated with the $2^{-\Delta\Delta CT}$ method for qPCR (Livak and Schmittgen, 2001) with two housekeeping genes [*Hypoxanthin-Guanin-Phosphoribosyltransferase 1* (*HPRT1*) and *beta-Actin* (*ß-Actin*)].

Table 1: Primer sequences used for array qPCR validation

Target	Sequence (5´ - 3´)	Annealing Temperature (°C)	Product Size (bp)
HPRT1	F: AACCTTGCTTTCCTTGGTCA	60	150
	R: TCAAGGGCATAGCCTACCAC		
β-Actin	F: AAGGACCTCTACGCCAACAC	60	207
	R: CTTGCTGATCCACATCTGCT		
AKT 3	F: TGAGAACACACACGTACGCA	60	156
	R: CAGTGGTGGGCTCATGACTT		
CCL 21	F: CACGAGGGTCCACTTCACTC	60	186
	R: CTTCCGGTAGCTGCGTACAA		
GRIN3B	F: CGGAGCCAAGATGTCCTGAA	60	164
	R: CTCATCCACCTCCGCATCTG		
IFN-β1	F: TTGGCATGTCAGAAGCTCCT	60	250
	R: GGTCATCCATCTGCCCATCA		
IFN-ε	F: CACTCATGAGACTGCAGGCA	60	174
	R: ACACAAAGAATAGGCACCGGT		
IFN-ω	F: AGAAGACCCAGGCCATCTCT	57	175
	R: TGCTCTTCCATCTCCTGCAC		
ZO-1	F: CCAAAGGTCCTGCACAGAGT	60	224
	R: CTCTGAGAGATGGCTGGCAG		

HPRT1 = Hypoxanthin-Guanin-Phosphoribosyltransferase-1; *AKT3 = AKT serine/threonine kinase 3*; *CCL21 = CC-chemokine ligand 21*; *GRIN3B = glutamate ionotropic receptor NMDA type subunit 3B*; *IFN-β1 = Interferon-*β1; *IFN-ε = Interferon-*ε; *IFN-ω = Interferon-*ω; *tight junction protein (TJP1)/ZO-1 (ZO-1)*; F = forward; R = reverse

Statistical Analysis

Statistical analysis of complete blood count, meat quality and qPCR data were realized using an animal mixed model as follows:

$$\boldsymbol{y} = \boldsymbol{X\beta} + \boldsymbol{Zm} + \boldsymbol{e},$$

where $\boldsymbol{y}$ contained the observations for the traits for each individual. The vector $\boldsymbol{\beta}$ included the fixed effects of sex (1-2), group (1-2) and herd-year-season-sow (1-4). The covariate was modeled as age at the time of sample collection. Maternal genetic effects were represented by $\boldsymbol{m}$ whilst vector $\boldsymbol{e}$ described the environmental residual effects. $\boldsymbol{X}$ and $\boldsymbol{Z}$ were the incidence matrices that linked fixed and random effects to the observations in $\boldsymbol{y}$.

Analysis of variance was performed using ANOVA function via R [cran.r-project.org/bin/windows/base/; version 3.6.1 (2019-07-05)]. Group comparisons have been drawn by Tukeys´ Test. Differences were considered significant when $p<0.05$. For the microarray-transcriptome analysis, $p<0.1$ was set as a trend.

3.4 Results

To describe nutritional effects on animal health in general, performance data can be used as auxiliary characteristics. Improvement of animal performance will lead to a countervailing benefit for farmers. Therefore, it was examined whether dietary immunologic manipulation might be beneficial for meat quality and intestinal integrity. During the whole experiment none of the pigs suffered any illnesses and therefore it was not necessary to treat them with antibiotics and other drugs.

Oregano supplementation had limited effects on the haematological profile

Based on blood profiles evaluated in this study, animals of both groups can be assessed as healthy. At the beginning of the feeding trial (weaning, age 28 days) we found significantly reduced ($p<0.05$) haemoglobin, haematocrit, MCV (mean corpuscular volume) and MCH (mean corpuscular haemoglobin concentration) values in the treated group. However, band neutrophil value was significantly increased ($p<0.05$) in the treated group at this time point. The number of samples at 28 days was lower, because of coagulation (Table 2). Blood samples were also taken at an age of 70 days, 130 days and 180 days. We found increased platelets values in the oregano supplemented group at the mid-fattening (130 days, Table 4) time point. There were no other significant effects on haematological profiles comparing the treated to the control group.

Table 2: Descriptive statistic of complete blood counts at an age of 28 days (weaning) depending on the feeding regime

Trait	Unit	Min-Max treated (n=36)	Mean±SD treated (n=36)	Min-Max control (n=39)	Mean±SD control (n=39)	p-value
Leukocytes	G/l	6.90-34.67	16.72±6.58	7.20-30.90	15.13±5.22	0.852
Erythrocytes	T/l	5.14-7.42	6.29±0.62	4.63-7.45	6.36±0.63	0.078
Haemoglobin	g/l	72.00-135.00	103.79±15.47	69.00-130.00	107.60±15.64	**0.009**
Haematocrit	l/l	0.25-0.44	0.34±0.05	0.22-0.42	0.35±0.05	**0.023**
MCV	fl	43.40-63.50	54.25±4.37	45.90-62.90	54.96±4.54	**0.050**
MCH	pg	11.80-19.90	16.47±1.63	13.60-19.20	16.90±1.62	**0.026**
MCHC	g/dl	27.20-33.10	30.34±1.23	28.30-32.70	30.75±1.07	0.157
Platelets	G/l	163.00-1314.00	601.40±270.04	134.00-1148.00	543.90±232.00	**0.045**
Neutrophils	%	23.00-67.00	38.78±11.13	24.00-74.00	41.18±12.79	0.481
Band np.	%	0.00-0.00	0.00±0.00	0.00-2.00	0.08±0.35	0.346
Lymphocytes	%	25.00-72.00	54.08±10.86	19.00-70.00	51.59±12.51	0.330
Monocytes	%	0.00-6.00	3.61±1.25	1.00-7.00	3.90±1.31	0.285
Eosinophils	%	0.00-8.00	1.83±1.44	0.00-6.00	1.74±1.27	0.282
Basophils	%	0.00-2.00	1.00±0.53	0.00-3.00	0.92±0.70	0.655

MCV = mean corpuscular volume, MCH = mean corpuscular haemoglobin, MCHC = mean erythrocyte haemoglobin concentration, Band np. = Band neutrophils

Table 3: Descriptive statistic of complete blood counts at an age of 70 days (fattenings´ start) depending on the feeding regime

Trait	Unit	Min-Max treated (n=40)	Mean±SD treated (n=40)	Min-Max control (n=44)	Mean±SD control (n=44)	p-value
Leukocytes	G/l	13.70-38.30	22.16±7.26	12.70-55.90	22.16±55.90	0.624
Erythrocytes	T/l	4.92-7.36	6.66±0.54	4.52-7.50	6.66±7.50	0.550
Haemoglobin	g/l	69.00-120.00	112.10±8.26	79.00-124.00	112.10±124.00	0.983
Haematocrit	l/l	0.24-0.40	0.37±0.03	0.25-0.40	0.37±0.40	0.446
MCV	fl	48.50-60.90	55.51±2.46	50.80-60.40	55.51±60.40	0.793
MCH	pg	14.00-18.70	16.85±0.84	15.30-18.70	16.85±18.70	0.431
MCHC	g/dl	28.90-31.80	30.34±0.65	29.20-31.60	30.34±31.60	0.227
Platelets	G/l	155.00-925.00	463.10±141.93	193.00-761.00	463.10±761.00	0.947
Neutrophils	%	29.00-56.00	43.23±5.83	29.00-55.00	43.23±55.00	0.520
Band np.	%	0.00-3.00	0.11±0.44	0.00-2.00	0.11±2.00	0.999
Lymphocytes	%	4.00-60.00	47.75±5.86	35.00-64.00	47.75±64.00	0.687
Monocytes	%	3.00-9.00	6.09±1.94	2.00-10.00	6.09±10.00	0.575
Eosinophils	%	0.00-5.00	1.66±0.91	0.00-4.00	1.66±4.00	0.341
Basophils	%	0.00-2.00	0.91±0.52	0.00-2.00	0.91±2.00	0.128

MCV = mean corpuscular volume, MCH = mean corpuscular haemoglobin, MCHC = mean erythrocyte haemoglobin concentration, Band np. = Band neutrophils

Table 4: Descriptive statistic of complete blood counts at an age of 130 days (mid-fattening) depending on the feeding regime

Trait	Unit	Min-Max treated (n=40)	Mean±SD treated (n=40)	Min-Max control (n=44)	Mean±SD control (n=44)	p-value
Leukocytes	G/l	14.50-30.70	21.35±4.07	13.40-75.40	23.39±10.37	0.107
Erythrocytes	T/l	6.45-8.04	7.31±0.37	6.44-8.15	7.32±0.38	0.893
Haemoglobin	g/l	116.00-144.00	129.90±6.94	115.00-145.00	130.60±7.24	0.641
Haematocrit	l/l	0.38-0.47	0.41±0.02	0.36-0.48	0.41±0.03	0.804
MCV	fl	52.50-62.30	56.49±2.43	52.70-61.50	56.58±2.09	0.795
MCH	pg	16.80-19.40	17.77±0.69	16.40-19.70	17.84±0.63	0.610
MCHC	g/dl	28.50-33.70	31.51±1.44	29.10-32.90	31.56±1.13	0.519
Platelets	G/l	72.00-518.00	341.80±117.65	63.00-527.00	318.60±95.48	**0.002**
Neutrophils	%	20.00-49.00	30.33±6.24	14.00-46.00	29.35±5.98	0.361
Band np.	%	0.00-1.00	0.03±0.16	0.00-6.00	0.25±0.98	0.599
Lymphocytes	%	42.00-72.00	58.95±6.76	44.00-80.00	60.55±6.88	0.430
Monocytes	%	2.00-8.00	5.21±1.24	0.00-8.00	5.08±1.80	0.734
Eosinophils	%	1.00-18.00	3.95±2.67	0.00-5.00	3.35±1.21	0.768
Basophils	%	0.00-2.00	1.15±0.54	0.00-2.00	1.03±0.58	0.269

MCV = mean corpuscular volume, MCH = mean corpuscular haemoglobin, MCHC = mean erythrocyte haemoglobin concentration, Band np. = Band neutrophils

Table 5: Descriptive statistic of complete blood counts at an age of 180 days (fattenings´ end) depending on the feeding regime

Trait	Unit	Min-Max treated (n=40)	Mean±SD treated (n=40)	Min-Max control (n=44)	Mean±SD control (n=44)	p-value
Leukocytes	G/l	7.80-26.90	19.25±3.32	11.10-27.70	18.60±3.18	0.573
Erythrocytes	T/l	6.24-8.23	7.36±0.44	6.28-8.40	7.37±0.42	0.678
Haemoglobin	g/l	113.00-146.00	131.50±8.19	119.00-151.00	132.70±7.62	0.851
Haematocrit	l/l	0.36-0.45	0.41±0.02	0.37-0.48	0.41±0.02	0.826
MCV	fl	52.90-59.30	55.78±1.76	51.70-60.00	56.23±1.74	0.220
MCH	pg	16.60-19.70	17.90±0.78	16.50-19.60	18.02±0.62	0.342
MCHC	g/dl	30.90-33.60	32.07±0.67	31.00-33.30	32.05±0.53	0.874
Platelets	G/l	172.00-531.00	360.30±79.57	87.00-544.00	334.40±89.20	0.077
Neutrophils	%	19.00-39.00	26.15±4.09	17.00-36.00	27.45±3.86	0.122
Band np.	%	0.00-1.00	0.03±0.16	0.00-1.00	0.02±0.15	0.948
Lymphocytes	%	51.00-71.00	63.05±4.22	53.00-77.00	62.57±4.60	0.487
Monocytes	%	3.00-9.00	5.15±1.10	3.00-7.00	5.07±0.93	0.706
Eosinophils	%	2.00-9.00	4.25±1.45	0.00-7.00	3.80±1.39	0.205
Basophils	%	0.00-2.00	1.00±0.23	0.00-1.00	0.95±0.21	0.458

MCV = mean corpuscular volume, MCH = mean corpuscular haemoglobin, MCHC = mean erythrocyte haemoglobin concentration, Band np. = Band neutrophils

Meat quality tended to be more valuable in control group

The phenotype values of interest, including muscle pH, conductivity, water holding capacity parameters and carcass composition were collected for the oregano treated and the control group. The initial weight after birth and at an age of 70 days, fattenings´ start, tended to be higher in the control group (Table 6). We started with oregano essential oil supplementation immediately after weaning. Therefore, trends in birth weight cannot be explained by dietary composition, but probably of random maternal and environmental effects. The average weight was always higher in the control group but was never significant. As a result of this investigation, few significant differences in animal development and meat quality have been found in the control group compared to the group with oregano essential oil supplementation. Attributes such as pH-value, the electrical conductivity and the meat colour measured at the cross-sectional area between the 13th/14th rib 24 h after slaughter are important to describe meat quality. In this experiment, we did not find any significant effects on pH and con values or meat colour (Table 6). In addition to the aforementioned values, the degree of fatness at the loin, back and neck as well as the number of valuable meat pieces (ham, shoulder, loin, belly, chop) are important for the value of the carcass. Neckfat (mm) tended to be thicker in control animals ($p<0.08$, Table 6) and the weight of ham and belly cuts was significantly lower ($p<0.05$, Figure 1) in the treated group.

NIRS-measurements (Near Infrared Spectroscopy) provide important information for the industry to gauge meat quality. In this experiment percentage of collagen, fat, protein and water were determined in the carcasses of the pigs investigated. There were no significant differences between the oregano treated pigs compared to the control group in the aforementioned traits. Thawing loss tended ($p<0.08$) to be higher in the oregano treated group, whereas there was no difference between both groups in drip $loss_{96h}$ and cooking loss. We found also no effect on meat tenderness measured by shear force between groups.

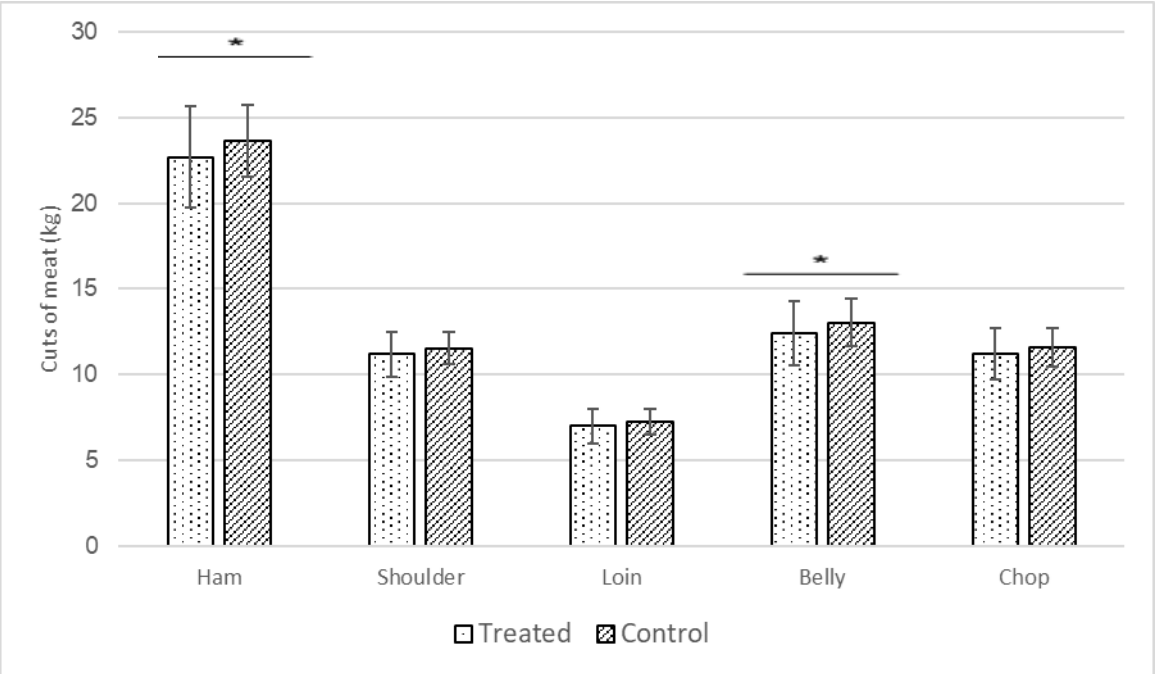

Figure 1: Weight of cuts of meat depending on oregano essential oil supplementation. AutoFOM measurement. * = $p<0.05$

Table 6: Descriptive statistic of animal weight development and meat quality parameters depending on the feeding regime, n=86

Trait	Min-Max treated	Mean±SD treated	Min-Max control	Mean±SD control	p-value
Birth (kg)	0.90-1.70	1.35±0.23	0.80-1.90	1.44±0.25	0.069
Weaning (kg)	5.30-10.70	8.29±1.57	5.40-12.00	8.61±1.69	0.472
Fattenings´ start (kg)	10.70-30.50	19.93±4.67	12.20-30.10	21.23±4.05	0.055
Mid-fattening (kg)	43.00-83.00	69.06±9.29	51.00-90.00	71.72±8.19	0.082
Fattenings´ end (kg)	74.00-129.00	109.60±12.53	95.00-133.00	112.60±9.18	0.108
$pH_{1\ h\ p.m.}$	5.42-6.37	5.88±0.27	5.28-6.51	5.91±0.29	0.765
$pH_{24\ h\ p.m.}$	4.91-5.63	5.46±0.12	5.01-5.61	5.43±0.16	0.151
$Con_{1\ h\ p.m.}$ (mS/cm)	1.70-7.60	3.02±1.11	1.40-4.70	3.07±0.90	0.924
$Con_{24\ h\ p.m.}$ (mS/cm)	2.10-5.90	3.88±1.04	2.00-6.40	3.99±1.25	0.301
Meat $colour_{24\ h}$	51.90-72.60	60.10±5.28	50.70-72.60	59.89±4.70	0.842
Loinfat t. (mm)	5.45-20.06	11.56±3.71	4.10-19.61	11.87±3.40	0.649
Backfat t. (mm)	6.41-27.22	16.03±5.01	5.01-29.33	16.44±5.26	0.684
Neckfat t. (mm)	14.72-33.16	23.95±4.42	18.69-42.74	25.94±4.91	0.057
Ham (kg)	13.62-26.26	22.69±2.96	18.92-28.44	23.64±2.10	**0.042**
Shoulder (kg)	7.50-12.88	11.19±1.29	9.42-13.78	11.55±0.96	0.081
Loin (kg)	4.02-8.52	7.00±1.00	5.92-8.90	7.26±0.73	0.098
Belly (kg)	7.40-15.18	12.40±1.85	9.90-16.32	13.02±1.39	**0.026**
Chop (kg)	6.84-13.50	11.20±1.51	9.48-14.06	11.59±1.10	0.119
NIRS collagen (%)	0.41-0.89	0.68±0.11	0.47-0.84	0.69±0.10	0.779
NIRS fat (%)	1.19-2.63	1.86±0.31	1.13-2.40	1.77±0.27	0.237
NIRS protein (%)	23.11-27.70	24.62±0.80	23.75-25.40	24.82±0.45	0.211
NIRS H_2O (%)	72.54-75.09	73.48±0.64	72.52-74.42	73.34±0.47	0.369
Drip $loss_{96\ h}$ (%)	1.01-11.12	3.35±1.73	1.19-5.73	2.95±1.57	0.389
Thawing loss (%)	0.64-36.22	12.99±7.48	1.83-34.56	10.40±6.24	0.076
Cooking loss (%)	9.43-52.89	33.45±7.66	10.86-51.93	31.76±6.35	0.371
Shear force (kg)	21.36-40.70	28.32±4.68	23.02-43.96	30.54±5.47	0.389

Con = Conductivity, t = thickness, NIRS = Near Infrared Spectroscopy, p.m. = *post mortem*

Transcriptomic profiling of jejunal and ileal tissue following OEO supplementation

To reveal the transcriptional modification profile of pigs´ small intestine responding to OEO treatment, we conducted a global transcriptome profiling of OEO treated and untreated pigs after slaughter using the Affymetrix GeneChip Porcine Gene 1.0 ST array (81/4 format). These porcine specific arrays are encoded with up to 26 probes per gene (median: 22 probes/gene, total: 394,580 probes) representing a total of 19,212 genes. After technical normalization 27,558 probes had higher signal intensity than the background. Probes were filtered and arrays divided into small intestinal tissues, jejunum and ileum. After annotation, we found 140 genes in the jejunum and 585 in the

ileum. For the comparative transcriptomic investigation associated with the dietary treatment (oregano vs. control), the probes have been filtered by thresholds of FDR<0.05 and log2 fold-change >1.5 or <-1.5. 108 transcripts remained in the jejunum and 390 in the ileum. 93 genes were differentially expressed in the jejunum and 60 in the ileum. The number of up-regulated genes in the jejunum was 70 and 23 were down-regulated. In the ileum 48 were up- and 12 were down-regulated. The intersecting Venn diagram (Figure 2) shows, only three genes [*GRIN3B* (*glutamate ionotropic receptor NMDA type subunit 3B*), *TJP1/ZO-1* (*tight junction protein ZO-1*) and one uncharacterized gene] have been modulated by oregano treatment in both small intestinal segments. Hierarchical clustering of differentially expressed genes in jejunal and ileal tissue depending on OEO supplementation has provided a clear image of genes that were directed in response to the dietary regime (Figures 3 + 4).

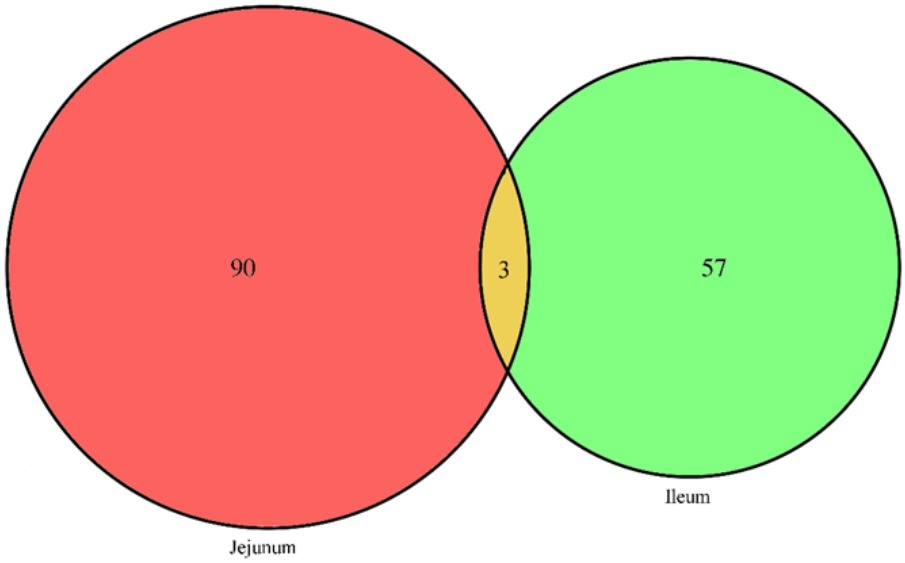

Figure 2: Intersecting Venn diagram of the differentially expressed genes in the jejunum and the ileum. The overlap shows three genes affected by the feeding regime in both small intestinal segments

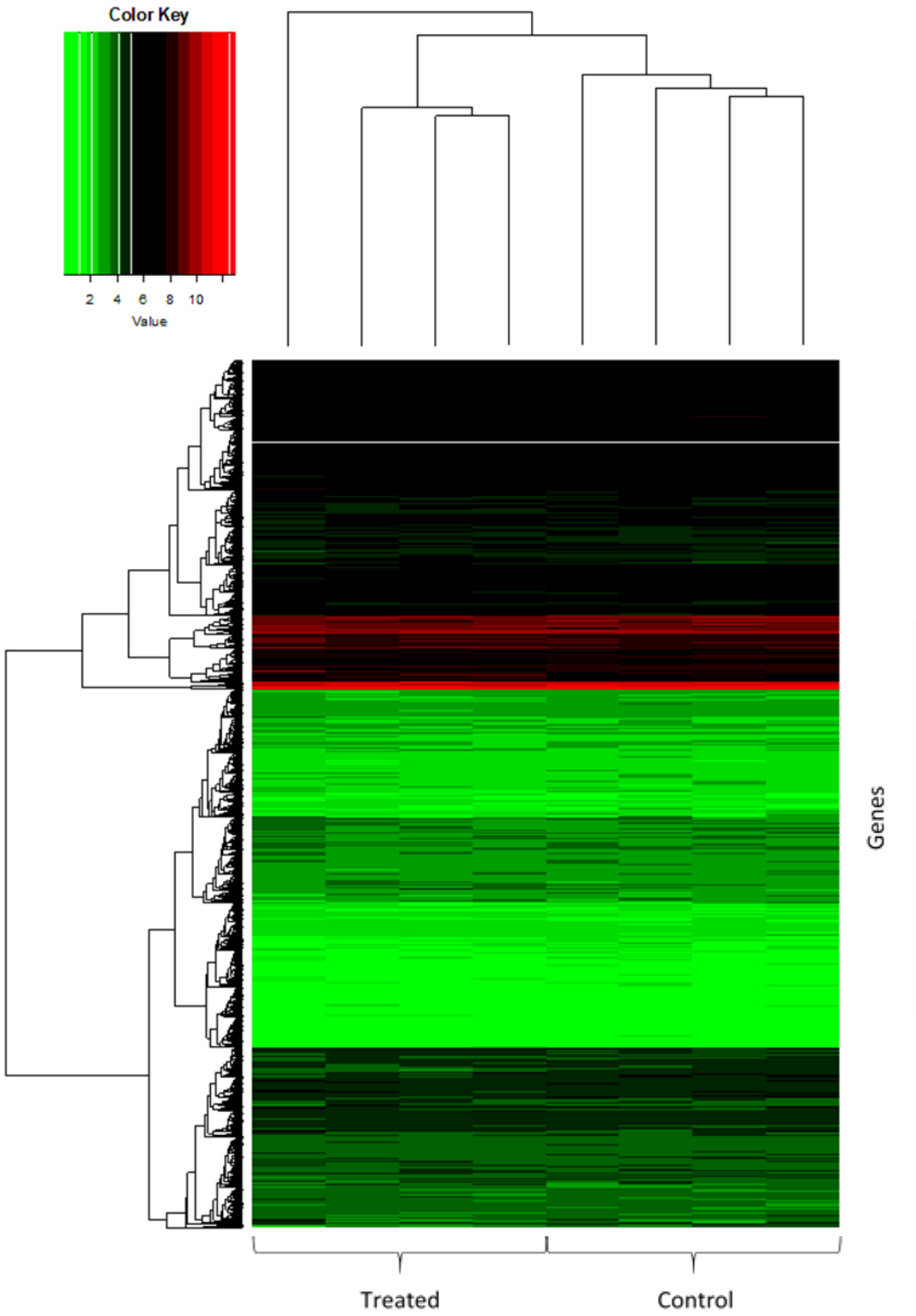

Figure 3: Jejunum: Hierarchical heat map showing differential gene expression depending on dietary treatment (oregano vs. control). Normalized log2 transformed values of jejunal tissue. The cutoff values of log2 fold change as either >1.5 or <-1.5 and FDR<0.05 were considered for statistical significance. Each column represents one array. The first 4 arrays are the oregano treated animals, remaining arrays are control

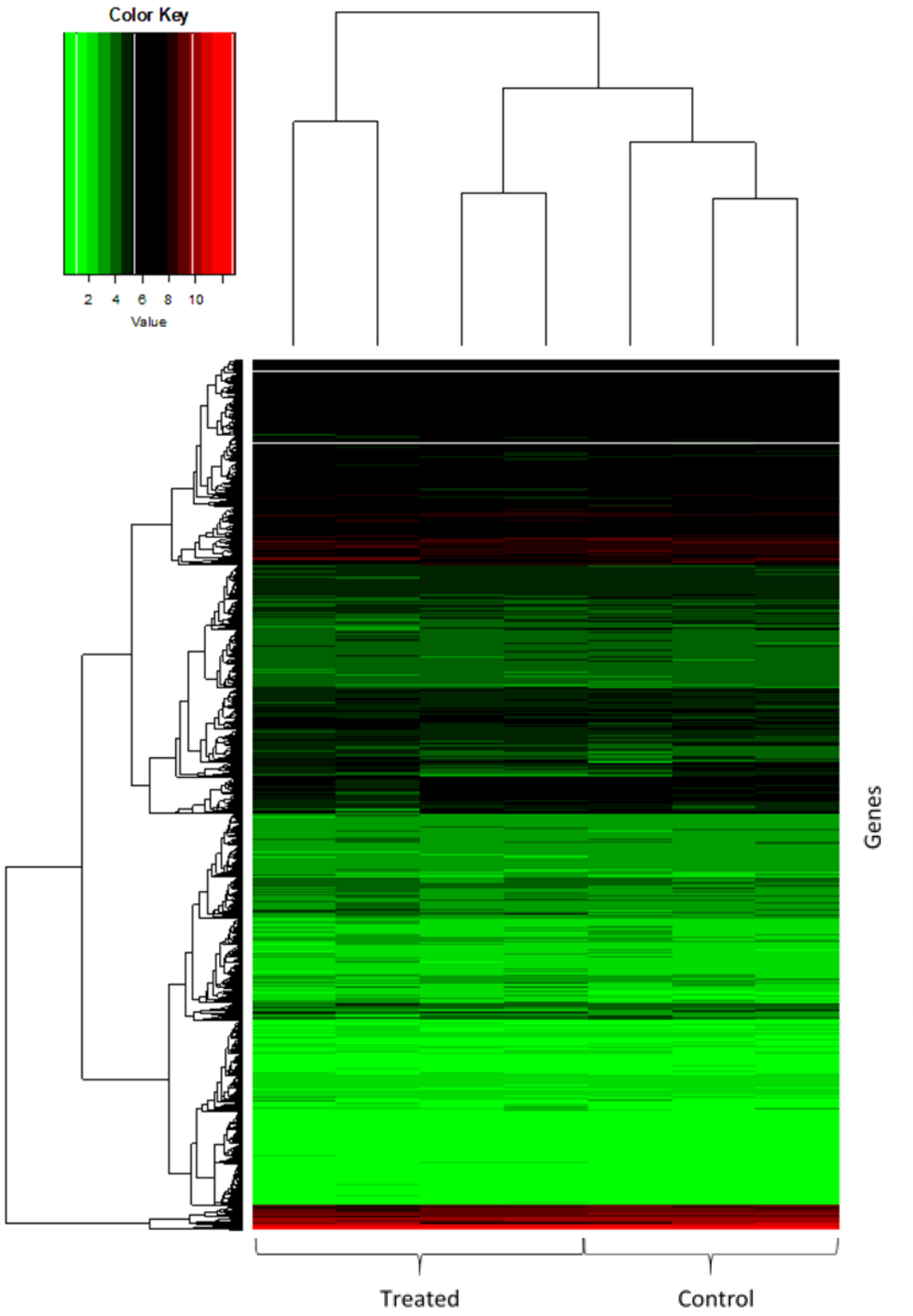

Figure 4: Ileum: Hierarchical heat map showing differential gene expression depending on dietary treatment (oregano vs. control). Normalized log2 transformed values of ileal tissue. The cutoff values of log2 fold change as either >1.5 or <-1.5 and FDR<0.05 were considered for statistical significance. Each column represents one array. The first 4 arrays are the oregano treated animals, remaining arrays are control

Biological processes and pathways revealed by Gene Ontology analysis

Modulations in biological processes (BP) and pathways depending on the OEO supplementation have been investigated in the jejunum and the ileum. In the jejunum, we found 30 biological processes, 7 cellular components, 4 molecular functions, 3 KEGG pathways and in the ileum 9 BPs, 4 cellular components and 3 molecular functions to be significantly affected (Tables 7 + 8). According to their biological importance we highlighted 5 biological processes per intestinal segment. Depending on the dietary addition of OEO in the jejunum we found BPs of 'humoral immune response' (3 genes up-, 1 gene down-regulated; $p<0.05$), 'leukocyte differentiation' (5 genes up-, 1 gene down-regulated; $p=0.01$), 'intrinsic apoptotic signalling pathway' (4 genes up, 1 gene down-regulated; $p<0.01$) and 'response to organic substance' (11 genes up-, 2 genes down-regulated; $p<0.05$) significantly affected. In the ileum 'negative regulation of vascular permeability' (2 genes up-regulated; $p<0.05$), 'response to glucose' (3 genes up-regulated, $p<0.05$), 'positive regulation of cell differentiation' (5 genes up-regulated; $p<0.1$), 'anatomical structure formation involved in morphogenesis' (5 genes up-, 1 gene down-regulated; $p<0.1$) and 'cell development' (7 genes up-, 1 gene down-regulated; $p<0.1$) top 5 processes have been found. Exact NCBI gene IDs and fold changes for selected BPs can be found in tables 10 + 11. Additionally, DAVID pathway analysis revealed 3 KEGG pathways, 'RIG-I-like receptor signalling pathway' ($p<0.05$), 'Cytokine-cytokine receptor interaction' ($p<0.05$) and 'JAK-STAT signalling pathway' ($p<0.1$), to be affected by OEO treatment. Depending on the set conditions in this experiment, no such pathway was found in the ileum.

For closer investigation and technical validation, four genes [*CCL 21* (*C-C motif chemokine ligand 21*), *IFN-β1* (*Interferon*), *IFN-ε* and *IFN-ω*], which were found in the jejunum and one gene [*AKT 3* (*AKT serine/threonine kinase 3*)], which was exclusively found in the array data of the ileum, have been selected. Due to their importance for pigs´ health, these five genes and *TJP1/ZO-1* have been analysed by qPCR technique in both intestinal tissues. Our qPCR investigation revealed highly significant ($p<0.001$) expression differences in all genes investigated between jejunal and ileal tissues independent of dietary supplementation. In general, we recognized more differentially expressed genes in the jejunum. Especially, we found the *AKT3* gene to be significant ($p<0.005$), *IFN-ε*, *IFN-ω*, *TJP1/ZO-1* significant ($p<0.05$) up-regulated and *CCL21* tended ($p<0.08$) to be higher expressed in oregano treated groups´ jejunum (Figure 5). Except for *IFN-ε* expression, which was shown to be negatively affected by OEO in the array data, the remaining observations in qPCR in the jejunum confirm the array results. In the ileum, we found a significantly higher ($p<0.05$) mRNA level of *CCL21* in comparison to the control group (Figure 6). The other genes were not significantly affected by OEO supplementation in the ileum.

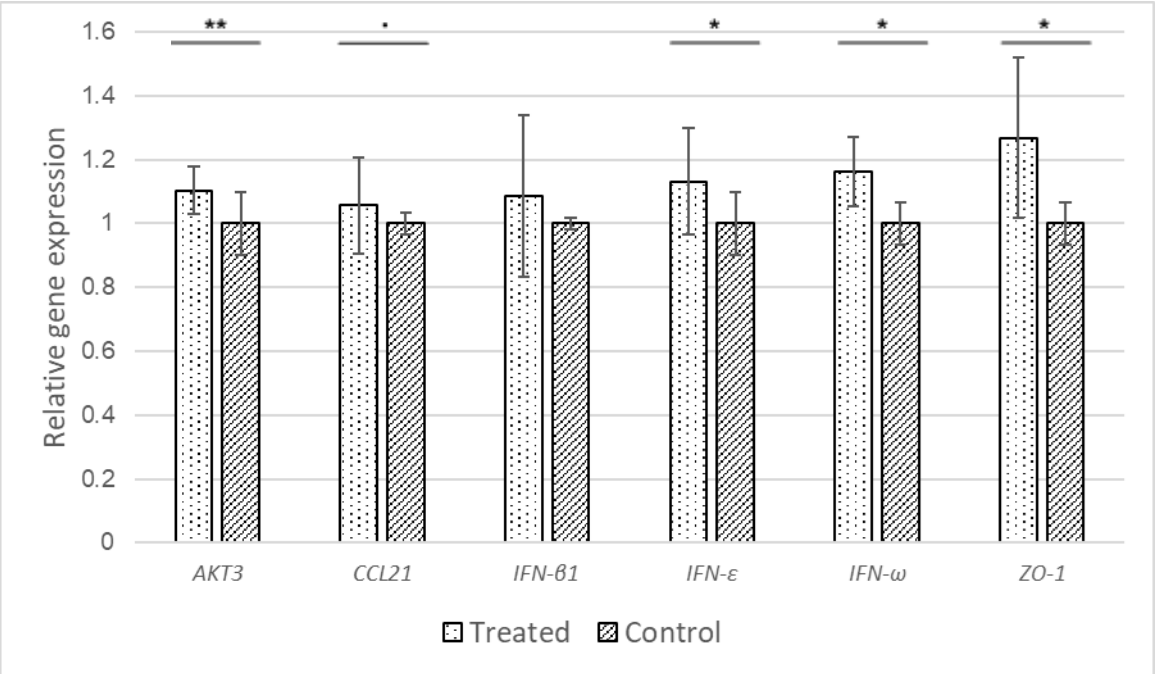

Figure 5: LS-Means (based on $2^{-\Delta\Delta CT}$ values) of differentially expressed genes in jejunal tissue (n=36) depending on oregano essential oil supplementation. *AKT serine/threonine kinase 3* (*AKT3*), *Interferon-* (*IFN*)*-β1*, *-ε*, *-ω*, *tight junction protein* (*TJP1*)*/ZO-1* (*ZO-1*), *C-C motif chemokine ligand 21* (*CCL21*). ** = p<0.01, * = p<0.05, · = p<0.08

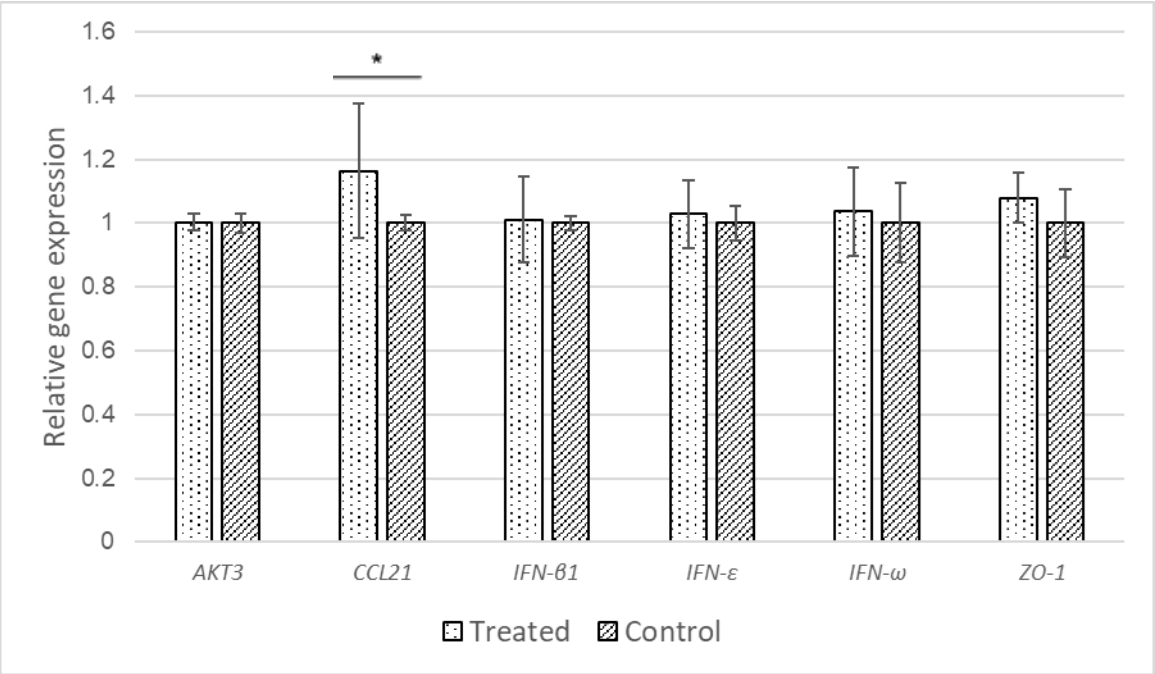

Figure 6: LS-Means (based on $2^{-\Delta\Delta CT}$ values) of differentially expressed genes in ileal tissue (n=36) depending on oregano essential oil supplementation. *AKT serine/threonine kinase 3* (*AKT3*), *Interferon-* (*IFN*)*-β1*, *-ε*, *-ω*, *tight junction protein (TJP1)/ZO-1* (*ZO-1*), *C-C motif chemokine ligand 21* (*CCL21*). * = p<0.05

Table 7: TopTable of biological processes, cellular compartments and molecular functions depending on OEO supplementation in the jejunum

Intestinal segment	Category	GO ID	GO term	Nr. of genes	p-value
Jejunum	Bp	GO:0048583	Regulation of response to stimulus	17	**0.034**
	Bp	GO:0010033	Response to organic substance	15	**0.019**
	Bp	GO:0002521	Leukocyte differentiation	6	**0.010**
	Bp	GO:0097193	Intrinsic apoptotic signaling pathway	5	**0.008**
	Bp	GO:0042100	B-cell proliferation	4	**<0.001**
	Bp	GO:0006959	Humoral immune response	4	**<0.001**
	Bp	GO:0033141	Positive regulation of peptidyl-serine phosphorylation of STAT protein	3	**0.001**
	Bp	GO:0002323	Natural killer cell activation involved in immune response	3	**0.001**
	Bp	GO:0002286	T-cell activation involved in immune response	3	**0.002**
	Bp	GO:0043330	Response to exogenous dsRNA	3	**0.004**
	Bp	GO:0051607	Defense response to virus	4	**0.011**
	Bp	GO:0030183	B-cell differentiation	3	**0.015**
	Bp	GO:0007409	Axonogenesis	3	**0.031**
	Bp	GO:0006346	DNA methylation-dependent heterochromatin assembly	2	**0.033**
	Bp	GO:0042711	Maternal behavior	2	**0.036**
	Bp	GO:0009615	Response to virus	3	**0.038**
	Bp	GO:0044030	Regulation of DNA methylation	2	**0.038**
	Bp	GO:0048568	Embryonic organ development	2	**0.047**
	Bp	GO:0030318	Melanocyte differentiation	2	0.055
	Bp	GO:0043044	ATP-dependent chromatin remodeling	2	0.062
	Bp	GO:0002250	Adaptive immune response	3	0.064
	Bp	GO:0051924	Regulation of calcium ion transport	3	0.065
	Bp	GO:2001235	Positive regulation of apoptotic signaling pathway	3	0.068
	Bp	GO:0007568	Aging	3	0.078
	Bp	GO:0001662	Behavioral fear response	2	0.078
	Bp	GO:0042771	Intrinsic apoptotic signaling pathway in response to DNA damage by p53 class mediator	2	0.083
	Bp	GO:0031667	Response to nutrient levels	2	0.083
	Bp	GO:2001244	Positive regulation of intrinsic apoptotic signaling pathway	2	0.088
	Bp	GO:0097192	Extrinsic apoptotic signaling pathway in absence of ligand	2	0.091
	Bp	GO:0007420	Brain development	17	**0.034**
	Cc	GO:0043025	Neuronal cell body	6	**0.002**
	Cc	GO:0005615	Extracellular space	9	**0.040**
	Cc	GO:0005882	Intermediate filament	3	**0.043**
	Cc	GO:0035371	Microtubule plus-end	2	**0.048**
	Cc	GO:0000792	Heterochromatin	2	0.059
	Cc	GO:0005783	Endoplasmic reticulum	6	0.092
	Cc	GO:0005737	Cytoplasm	21	0.097
	Mf	GO:0005132	Type I interferon receptor binding	3	**0.001**
	Mf	GO:0008327	Methyl-CpG binding	2	0.059
	Mf	GO:0005515	Protein binding	32	0.075
	Mf	GO:0005125	Cytokine activity	3	0.093
	KEGG		RIG-I-like receptor signaling pathway	3	**0.023**
	KEGG		Cytokine-cytokine receptor interaction	4	**0.046**
	KEGG		Jak-STAT signaling pathway	3	0.084

Bp = biological process, Cc = cellular compartment, Mf = molecular function

Table 8: TopTable of biological processes, cellular compartments and molecular functions depending on OEO supplementation in the ileum

Intestinal segment	Category	GO ID	GO term	Nr. of genes	p-value
Ileum	Bp	GO:0048468	Cell development	8	0.096
	Bp	GO:0048646	Anatomical structure formation involved in morphogenesis	6	0.080
	Bp	GO:0045597	Positive regulation of cell differentiation	5	0.088
	Bp	GO:0007411	Axon guidance	3	**0.041**
	Bp	GO:0009749	Response to glucose	3	**0.043**
	Bp	GO:0043116	Negative regulation of vascular permeability	2	**0.024**
	Bp	GO:0031290	Retinal ganglion cell axon guidance	2	**0.038**
	Bp	GO:0071480	Cellular response to gamma radiation	2	**0.042**
	Bp	GO:0007389	Pattern specification process	2	0.055
	Cc	GO:0005654	Nucleoplasm	11	**0.046**
	Cc	GO:0005667	Transcription regulator complex	3	0.058
	Cc	GO:0030054	Cell junction	4	0.066
	Cc	GO:0005794	Golgi apparatus	5	0.096
	Mf	GO:0005515	Protein binding	24	**0.018**
	Mf	GO:0042162	Telomeric DNA binding	2	**0.045**
	Mf	GO:0051539	4 iron, 4 sulfur cluster binding	2	0.077

Bp = biological process, Cc = cellular compartment, Mf = molecular function

Table 9: TopTable of genes involved in selected biological processes in the jejunum. Positive fold changes represent up-regulated and negative fold changes represent down-regulated genes in the OEO treated group

GO ID	GO term	p-value	Gene symbol	NCBI gene ID	Gene name	Fold change[2]
GO:0006959	Humoral immune response	**0.032**	*BCL2*	596	*BCL2, apoptosis regulator*	1.07
			IFNB1	3456	*Interferon beta 1*	1.07
			IFNE	3467	*Interferon epsilon*	-1.63
			IFNW1	338376	*Interferon omega 1*	1.19
GO:0002521	Leukocyte differentiation	**0.010**	*BCL2*	596	*BCL2, apoptosis regulator*	1.07
			CD109	135228	*CD109 molecule*	1.23
			IFNB1	3456	*Interferon beta 1*	1.07
			IFNE	3467	*Interferon epsilon*	-1.63
			IFNW1	338376	*Interferon omega 1*	1.19
			PDE1B	5153	*Phosphodiesterase 1B*	1.03
GO:0097193	Intrinsic apoptotic signalling pathway	**0.008**	*BCL2*	596	*BCL2, apoptosis regulator*	1.07
			CASP2	835	*Caspase 2*	1.47
			PRKRA	8575	*Protein activator of interferon induced protein kinase EIF2AK2*	1.03
			RPS27L	51065	*Ribosomal protein S27 like*	1.07
			SHISA5	51246	*Shisa family member 5*	-1.11
GO:0010033	Response to organic substance	**0.019**	*ATP6V0A2*	23545	*ATPase H+ transporting V0 subunit a2*	-1.57
			BCL2	596	*BCL2, apoptosis regulator*	1.07
			BRINP1	1620	*BMP/retinoic acid inducible neural specific 1*	1.02
			CCL21	6366	*C-C motif chemokine ligand 21*	1.32
			CD109	135228	*CD109 molecule*	1.23
			IFNB1	3456	*Interferon beta 1*	1.07
			IFNE	3467	*Interferon epsilon*	-1.63
			IFNW1	338376	*Interferon omega 1*	1.19
			MBD2	8932	*Methyl-CpG binding domain protein 2*	1.08
			MYO5A	4644	*Myosin VA*	1.26
			PDE1B	5153	*Phosphodiesterase 1B*	1.03
			PRKRA	8575	*Protein activator of interferon induced protein kinase EIF2AK2*	1.03
			SFRP4	6424	*Secreted frizzled related protein 4*	1.06
			TJP1/ZO-1	7082	*Tight junction protein 1/ZO-1*	1.34
GO:0048583	Regulation of response to stimulus	**0.034**	*BNIP2*	663	*BCL2 interacting protein 2*	1.56
			BCL2	596	*BCL2, apoptosis regulator*	1.07
			CCL21	6366	*C-C motif chemokine ligand 21*	1.32
			CD109	135228	*CD109 molecule*	1.23
			CASP2	835	*Caspase 2*	1.47
			DLX2	1746	*Distal-less homeobox 2*	1.29
			IFNB1	3456	*Interferon beta 1*	1.07
			IFNE	3467	*Interferon epsilon*	-1.63
			IFNW1	338376	*Interferon omega 1*	1.19
			MBD2	8932	*Methyl-CpG binding domain protein 2*	1.08
			MYO5A	4644	*Myosin VA*	1.26
			NRG4	145957	*Neuregulin 4*	1.21
			PRKRA	8575	*Protein activator of interferon induced protein kinase EIF2AK2*	1.03
			SFRP4	6424	*Secreted frizzled related protein 4*	1.06
			SHISA5	51246	*Shisa family member 5*	-1.11

Table 10: TopTable of genes involved in selected biological processes in the ileum. Positive fold changes represent up-regulated and negative fold changes represent down-regulated genes in the OEO treated group

GO ID	GO term	p-value	Gene symbol	NCBI gene ID	Gene name	Fold change[2]
GO:0045597	Positive regulation of cell differentiation	0.088	*CD83*	9308	*CD83 molecule*	2.21
			EPHB2	2048	*EPH receptor B2*	1.67
			KDM1A	23028	*Lysine demethylase 1A*	1.29
			OTP	23440	*Orthopedia homeobox*	1.07
			SLIT2	9353	*Slit guidance ligand 2*	1.17
GO:0048646	Anatomical structure formation involved in morphogenesis	0.080	*EPHB2*	2048	*EPH receptor B2*	1.67
			PERP	64065	*PERP, TP53 apoptosis effector*	1.09
			SOX4	6659	*SRY-box 4*	1.41
			SLIT2	9353	*Slit guidance ligand 2*	1.17
			TXNDC8	255220	*Thioredoxin domain containing 8*	-1.24
			TJP1/ZO-1	7082	*Tight junction protein 1/ZO-1*	1.05
GO:0043116	Negative regulation of vascular permeability	**0.024**	*SLIT2*	9353	*Slit guidance ligand 2*	1.17
			TJP1/ZO-1	7082	*Tight junction protein 1/ZO-1*	1.05
GO:0048468	Cell development	0.096	*EPHB2*	2048	*EPH receptor B2*	1.67
			SOX4	6659	*SRY-box 4*	1.41
			KIF5B	3799	*Kinesin family member 5B*	1.05
			KDM1A	23028	*Lysine demethylase 1A*	1.29
			OTP	23440	*Orthopedia homeobox*	1.07
			SLIT2	9353	*Slit guidance ligand 2*	1.17
			TXNDC8	255220	*Thioredoxin domain containing 8*	-1.24
			TJP1/ZO-1	7082	*Tight junction protein 1/ZO-1*	1.05
GO:0009749	Response to glucose	**0.043**	*SOX4*	6659	*SRY-box 4*	1.41
			KIF5B	3799	*Kinesin family member 5B*	1.05
		0.088	*TJP1/ZO-1*	7082	*Tight junction protein 1/ZO-1*	1.05

3.5 Discussion

We aimed to answer the question if oregano essential oil supplementation has effects on carcass quality, haematology and gene expression in the small intestine. For this purpose, we investigated meat quality parameters, blood counts and the intestinal transcriptomic profile. The usage of high-throughput genomic technologies helped to reveal some very interesting transcriptomic processes. Taken together, we chose several parameters as indicators for OEOs´ effects on pigs´ health. Gāliņa and Valdovska (2017) postulated, OEO exerts its´ effects only during pathological conditions. However, we did not challenge our animals with any stimulants to give insights on pigs´ reaction under conventional circumstances. In this study, we used a dosage of pure OEO (112.5 mg/kg) in fattening pigs´ diet and we found very limited effects on carcass quality and haematology. Nevertheless, transcriptomic investigation revealed some highly interesting modulations in jejunal and ileal tissue after slaughter. The main component of OEO in our study is carvacrol with > 60%. The composition of OEO depends on plant variety and origin (Aligiannis et al., 2001; Mockute et al., 2001; Adam et al., 1998). Therefore, comparability between some studies is limited. Bimczok et al. (2008) concluded that previously observed effects of OEO are not only dependent on carvacrol; the other chemical substances within natural oils have to be noticed too and synergistic effects have to be expected (Pu et al., 2020; Burt, 2004).

Haematologic profile is barely affected by OEO treatment

Functional ingredients from OEO, e.g. carvacrol, thymol and β-caryophyllene, can cross the intestinal mucosal barrier and affect cells in layers beneath the epithelium (Bimczok et al., 2008). Interaction with immunologic cells can lead to mutual impacts with cells from the blood. Savoini et al. (2002) used a thyme mixture, which is similar to the explained health-promoting effects of oregano, in pigs´ diet. Contrary to their hypothesis, they did not find growth performance-boosting effects and blood biochemistry was poorly affected, too. As it can be seen by our results, OEO supplementation had, with exception of the significantly higher platelets value at age 130 d, no effect on pigs´ haematological profile. Significant differences at weaning age cannot be explained by the dietary regime. Stelter et al. (2013) conducted an experiment with 80 male castrated pigs and fed half of the group dried whole oregano instead of

OEO, which was used in our experiment, for three weeks. Within each feeding group, 20 animals have been additionally treated with immune-activating lipopolysaccharide (LPS). 2 h after LPS treatment, blood samples have been taken by the researchers. There were no effects on animal performance during the whole experimental period. However, they found lymphocyte values, as well as $CD4^+$ and $CD8^+$ T-cells to be affected by OEO supplementation and by LPS treatment. Double positive T-cells were exclusively enhanced by LPS, but not by oregano treatment. However, Walter and Bilkei (2004) found double positive $CD4^+$ $CD8^+$ T-cells positively affected by a mixture of oregano flour and cold-pressed oil in finishing pigs. In accordance with our results, they also found no effects on erythrocytes and leucocytes. By contrast, *Origanum* extract administration (7.5 and 15 mg/l) in the drinking water of 15 weaned piglets showed increased red and white cell counts (Gómez-Gómez et al., 2017). The researchers concluded beneficial effects on performance and health. Blood parameters were also investigated by Seirafy and Sobhanirad (2017) in Holstein suckling calves. Some of the calves were supplemented with 5 ml/d/calf oregano (thymol: 29.06%, carvacrol: 37.30%, γ-terpinene: 9.6%, p-cymene: 4.5%) in milk. While there was no difference in red blood cell counts, the oregano treated group showed increased total blood cell, haemoglobin and MCV values compared to the control group. Méndez Zamora et al. (2017) fed broiler chickens additionally to the basal diet with 0.4 g/kg Mexican oregano oil (carvacrol: 60.62%, 1,8-cineole: 24.63%, p-cymene: 10.57%, thymol: 4.06%, γ-terpinene: 0.11%). The researchers reported, that the oregano treated group showed slightly increased white blood cell, erythrocyte and haemoglobin values. A higher number of red blood cells results in an increased percentage of transported oxygen through the body, which leads to improved animal efficiency. Broiler chickens reared under stress conditions fed with 250 mg/kg OEO (*O. vulgare* subsp. *hirtum*) equally showed beneficial effects in some blood parameters. Especially in combination with vitamin C, OEO had an antioxidative impact in broilers´ serum (Ghazi et al., 2015). In contrast, broiler chickens, which were fed with an herbal mixture containing oregano, showed no effects in haematological parameters (Khattak et al., 2014). Some researchers interpreted increased white blood cell counts as an indicator for stimulation of the immune system. These results cannot be reinforced by our experiment. Taken together, OEOs´ impact on pigs´ haematologic profile is still not really clear and as it can be seen by our results,

blood parameters do not help to explain the potential health benefits of OEO on the small intestinal integrity.

Slight differences in carcass & meat quality parameters depending on OEO treatment

In Germany pigs serve as a valued source of food. The balance of the cost of production, meat quality, animal health and thereby animal welfare is crucial for modern meat production (Zhang et al., 2015). In this experiment we found no effect on animal weight at the end of the fattening period. As mentioned before, experimental oregano supplementation was started immediately after weaning and after the first blood sampling. Therefore, differences between groups cannot be explained by the feeding regime. The aromatic odour of the OEO product was perceptible, even within the diets. However, we did not recognize any reluctant behavior of the pigs against the oregano treated feed but the animal keepers noted a higher water consumption (no data collected). Only at the beginning of fattening animals of the OEO treated group tended ($p<0.08$) to be lighter compared to the control. As mentioned before, experimental oregano supplementation was started immediately after weaning and after the first blood sampling. Therefore, differences between groups cannot be explained by the feeding regime. Phytogenics, such as OEO, improve the apparent ileal digestibility of nutrients (Cheng et al., 2018; Amad et al., 2011). Simitzis et al. (2010) produced 64 pigs for their experiment, equally divided into four groups and fed for 35 days with different concentrations (0.25, 0.5, 1.0 ml/kg) of OEO supplementation (carvacrol > 80%, thymol > 2%, β-caryophyllene < 1%). They did not recognize any effect on body weight, drip loss and general sensory attributes. By contrast, Cheng et al. (2018) reported that OEO supplementation improved body weight in pigs in an experiment with protein-reduced basal diets and had similar growth-promoting effects as the antibiotics-treated group. Simitzis et al. (2010) postulated, well-nourished and healthy animals do not respond to growth-promoting supplements when they are held under good environmental conditions. This could explain, why there were no effects on carcass quantity. In the experiment of Zou et al. (2016b), finishing pigs with an initial average body weight of 74 kg received OEO in a dosage of 25 mg/kg for four weeks in addition to their basal diet. They reported, that OEOs´ group carcass weight was significantly higher after 5 h transportation stress in comparison to the control group. Besides, pH value 45 min after

slaughter, as well as meat colour were increased and drip loss (24 h) was reduced after OEO treatment. However, Alarcon-Rojo et al. (2013) and Janz et al. (2007) showed, OEO supplementation does not affect pork colour. In our study we used 112.5 mg/kg pure OEO but we were not able to confirm those previously described observations. Our results are mostly in accordance with the findings of Alarcon-Rojo et al. (2013). In their study 48 Landrace x Yorkshire were fed in different concentrations with OEO and they did not find effects on any meat quality factor. Khattak et al. (2014) found in broiler chickens, that carcasses of oregano supplemented (45 – 60 mg/kg) animals were significantly heavier. They stated, that this effect depends on the high OEO concentration, which was used in their experiment and promoted the secretion of digestive enzymes, leading to better nutrient digestion and passage through the intestinal barrier (Lee et al., 2004). Meat quality improving effects have been partially described by the reduction of lipid peroxidation, indicated by higher activity of antioxidant enzymes and thereby reduction of reactive oxygen species (ROS) (Yan et al., 2010). Botsoglou et al. (2002) explained in 2002 contrary results in OEO studies by the differences in the source of the used plants, supplementation dosages in combination with basal diet composition and the individual intestinal microflora.

The value of the carcass depends on quality parameters like percentage of valuable cuts of meat, meat composition, tenderness etc. (van Laack et al., 2001). Good predictors for these factors are pH and conductivity measurements (Fernandez and Tornberg, 1994). Popp et al. (2015) showed, that higher pH_1 values are an indicator for better meat quality. Meat colour and water holding capacity are also affected by the pH value. Drip loss and thereby reduced water holding capacity are indicators for reduced pork quality. Sensory and technological properties are negatively affected by this (Schellander et al., 2010). Drip loss *post mortem* itself is affected by time and amount of pH degradation in association with cooling technique and protein degradation (Fischer, 2007). Drip loss and pH are negatively correlated (Alarcon-Rojo et al., 2013). There was no difference between groups regarding pH and drip loss by OEO supplementation in our experiment. The pH-value in tissue is a good indicator for fresh and well-processed meat. Aforementioned processes accelerate a decrease of pH-value, which negatively affects the quality. The pH-value is one of the most influential factors for muscle protein functionality (Alarcon-Rojo et al., 2013). The physiological pH optimum for enzyme

activity and transportation processes in muscles is at 7.4 and 45 min post mortem 6.3 is usual. Transportation stress also negatively affects meat quality (Schellander et al., 2010). In our study, we measured an average pH of 5.9 one-hour *post mortem* and there was no significant difference between groups. This is in accordance with the results of Alarcon-Rojo et al. (2013).

In addition to conventional methods, NIRS technology can be used to measure fat distribution in the carcass and in the tissue itself. Distributions are influenced by factors such as feeding regime, especially before late fattening, gender, age, weight at slaughter and other environmental factors (Zamora-Rojas et al., 2012). In this study, we used a NIRS-device to investigate the effect of OEO supplementation on the percentage of collagen, fat, protein and water in pork. None of the parameters was significantly affected by OEO treatment. It was shown, that 0.1% carvacrol, a major ingredient within OEO, treatment in mices´ diet reduced fat-pad weights by inhibition of lipogenesis signalling cascades (Cho et al., 2012). It was described by Simitzis et al. (2010) and Janz et al. (2007), that OEO treatment positively affects the tenderness of meat by reducing the shear force. In our experiment a higher dosage of OEO was used compared to the aforementioned studies but we were not able to confirm those results. As previously described by Stelter et al. (2013), dose- and age-related effects have to be expected.

Gene expression modulations by OEO supplementation in small intestinal tissue

Some authors reported, phytogenics, such as OEO, have several positive effects on pigs' traits and health (Liu et al., 2014; Wei et al., 2013; Pilau et al., 2011). Such components, e.g. carvacrol, thymol and β-caryophyllene, can penetrate the intestinal mucosa, cross the epithelial wall into deeper layers and will thereby affect various cellular and intestinal physiological functions. Due to their chemogenetic action, processes such as gene expression can be affected (Bimczok et al., 2008; Andersen, 2006; Walter and Bilkei, 2004). OEO differently modulates gene expression of several targets in the small intestinal tissue by modifications of intracellular signal transduction. The benefit of phytochemicals is not only the potential improvement of animal health but also direct protective effects against viruses via their inhibition of the expression of viral coat proteins and by blocking RNA and protein synthesis (Dhama et al., 2018; Lai et al., 2012; Pilau et al., 2011). It was shown by Gao et al. (2013), that phytochemicals, e.g. from

fungus, can suppress specific RNA polymerase activity and thereby inhibit Porcine Reproductive and Respiratory Syndrome Virus (PRRSV) infection. However, the 'mode of action' from a molecular genetic point of view, especially in OEO, is still unclear. Additionally, Swaggerty et al. (2020) showed, that the two small intestinal segments react differently to dietary signals. These results are in accordance with our findings and might be due to the fact, that the highest amount of these OEOs´ ingredients are absorbed in the mid-jejunum and do not reach the terminal ileum. Additionally, the partially different cellular composition between jejunum and ileum might explain why results in the two segments are not similar. Therefore, we performed an array-based transcriptomic analysis. The results in the two small intestinal segments indicated that OEO had major effects on transcripts related to several genes of biological processes and pathways. As it can be seen in table 10 we found two BPs in the jejunum (GO:0048583 and GO:0010033) describing the cellular response to organic substances or external stimulus. This could be likely due to our OEO supplementation. Additionally, immune-related BPs, such as 'humoral immune response' (GO:0006959) etc., were also affected by OEO. Doyle et al. (2011) showed in their experiment, that OEO activates diverse intracellular signal pathways, e.g. *NF-κB* and *MAPK pathways* (Pu et al., 2018). In the present experiment '*janus kinase / signal transduction and activator of transcription* (*JAK* / *STAT*)' pathway, which is responsible for a cellular immune response (Urasaki and Le, 2019), was significantly affected by OEO supplementation. Especially *AKT3*, which is crucial for the '*phosphatidylinositol 3- kinase* / *serine* / *threonine kinase* (*PI3K* / *AKT*)' pathway, is a key regulator in the *JAK* / *STAT* pathway, in addition to *IFN-ε* und *IFN-ω* (Zou et al., 2016a). Several plant extracts can affect the transcriptomic profile, which was shown by Liu et al. (2014). In their array-based transcriptomic investigation, three different natural plant extracts (capsicum oleoresin, garlic botanical and turmeric oleoresin) were fed just for 9 d in weaned pigs' diet and they found diverse genes, associated with immune-related BPs, to be significantly regulated.

In the jejunum, we found the expression of a serine and threonine kinase, *AKT3*, to be significantly upregulated in the oregano treated group. This gene directs hosts´ metabolism, up-regulates protein synthesis and is involved in the *NF-κB* pathway, a key regulator for various cellular processes e.g. immune cell maturation and cytokine expression (Zhang et al., 2017; Albers et al., 2014). There was no effect on *AKT3* in the

ileum. Swaggerty et al. (2020) showed contrary results for *AKT3* gene expression. In their experiment, they fed thymol containing phytogenic substance and showed *AKT3* to be up-regulated in the ileum of broilers, but not in the jejunum. The main component of copaiba oil is β-caryophyllene (> 50%), which is also an important functional ingredient within OEO exerting anti-inflammatory activities (Jung et al., 2015). In an *in-vitro* assay, copaiba oil did not affect the expression of *AKT3* in intestinal cells (Urasaki et al., 2020). This could indicate, that other substances within OEO with exception of β-caryophyllene regulate the *AKT3* expression. The direct inhibition of *AKT3* leads to a cell cycle arrest and apoptosis, which is due to its kinase activity (Wang et al., 2017). By contrast, higher abundances of this gene promote myoblast proliferation and muscle differentiation (Wei et al., 2013).

In pigs, detection of illnesses, welfare and the interpretation of immune systems activity status is often not clear. For this, beta-chemokine (e.g. *CCL21*, or *CCR7* ligand) gene expression is a valuable marker to interpret the response to natural infections etc. and can be found in lymphoid and mucosal tissue. *CCL21* is necessary for lymphocyte and dendritic cell (DCs) trafficking (Noor and Wilson, 2012; Fleming-Canepa et al., 2011). In the small intestine, DCs can be found abundant, where they are organized in lymphoid tissues (Peyers´ patches) and follicles. Sufficient levels of *CCL21* are necessary for normal lymphoid tissue architecture and DC migration (Mikulski et al., 2015; Song et al., 2009; Jang et al., 2006). Natural compounds, such as long-chain fatty acids (Tsuzuki et al., 2006) and turmeric oleoresin (Liu et al., 2014) up-regulate also *CCL21* activity and thereby DC chemotaxis. In our experiment, we found *CCL21* expression to be up-regulated in both small intestinal tissues in the qPCR but limited just to the jejunum in our array assay. The higher amount could therefore be an indicator for an enhanced immunological activity depending on OEO supplementation. However, we suppose, OEO has a balancing function on *CCL21* expression. It was shown by Jung et al. (2015) that overexpression of *CCL21* was inhibited by the sesquiterpene β-caryophyllene, which is assumed to have major effects within OEO, supplementation in mice cells.

Type I interferons (*IFNs*), e.g. *IFN-β*, *-ε* and *-ω*, are cytokines that are crucial for a humoral and adaptive immune response (Sang et al., 2010). *INF-ω* is produced by leukocytes after viral infection (Mege et al., 1991). Some IFNs are anti-inflammatory, proinflammatory, or both, e.g. *IFN-β1* (Bolívar et al., 2018; Billiau, 2006). IFN production depends on the

NF-κB signalling pathway. This pathway has an important impact on humoral and adaptive immunity and it is a key regulator of several cellular processes, such as apoptosis (Huang et al., 2014). However, in our study the previously mentioned *NF-κB* pathway was not affected by OEO supplementation in the small intestine of pigs. High expression levels of *IFNs*, such as *IFN-β1*, are an indicator of immune systems´ reaction against viral infections (Hafidh et al., 2015). Sang et al. (2010) found that the porcine *IFN* family consists of 39 functional genes with seven subclasses and they have antiviral effects against PRRSV in pigs. The superior meaning of *IFN*-complexes in pigs, depending on their immunomodulatory and molecular diversity, was also proofed by Jennings and Sang (2019). In our experiment we found *IFN-β*, *IFN-ω* to be significantly up-regulated and *IFN-ε* to be down-regulated in the jejunum depending on OEO supplementation in our array-based transcriptomic investigation. They were not affected in the ileum. Validation of these genes with qPCR technique showed, *IFN-ε* and *IFN-ω* to be significantly up-regulated in jejunal tissue but not in the ileum. There was no significant effect on *IFN-β* in both small intestinal segments. However, the higher expression of the aforementioned cytokines could be an important indicator for OEOs´ immunomodulatory activity in pigs. In broilers it was shown, OEO (60% carvacrol, 4% thymol) supplementation in two different dosages (0.01% / 0.005% in the diet) had a positive effect on humoral immune response through positive *IFN* activity manipulation. *IFNs* are also expected to have antiviral activity in chicken, too. Therefore, OEO could protect the chicken from viral infections, or at least clinical signs due to its immunomodulatory effects in some major cytokine pathways, such as the *IFN* pathway (Galal et al., 2016).

The intestinal barrier consists of a single layer of cells, the epithelium. It is composed of enterocytes (ca. 80%), enteroendocrine and goblet cells. Inter-epithelial tight junctions connect these cells and they are responsible for the selective passage of macromolecules and solutes (Blikslager et al., 2007). Dietary components and several other stimuli affect the epithelial permeability. However, cellular damages in case of inflammation or pathogen invasion impair the ability of the epithelium to absorb nutrients and to protect the cells lying beneath (Grilli et al., 2015; Boudry et al., 2002). Disturbances in the intestinal barrier can lead to long-term damages to the entire animal health by translocation of antigens, luminal bacteria and toxins into the subepithelial

tissue (Pu et al., 2020; Blikslager et al., 2007; Moeser et al., 2007). Intestinal tight junction proteins are indicators for a functional intestinal barrier (Mao et al., 2011). In our experiment, the transcriptomic analysis revealed significant up-regulation of *TJP1*/*ZO-1* in the jejunum and the ileum of pigs after oregano treatment. The increased expression of *TJP1*/*ZO-1*, a tight junction protein, indicates a potential benefit of feeding OEO on gut barrier function. However, pro-inflammatory mediators, such as some IFNs, can disrupt the epithelial barrier and thereby facilitate the invasion of the organism by pathogens (Suzuki, 2013; Al-Sadi et al., 2009; Pié et al., 2004). In the experiment of Pu et al. (2020), 10 piglets were assigned to two OEO-containing diets for 26 d. They found the intestinal barrier disruption enhancing cytokines, such as *TNF-α* and *IL-1β* to be reduced by OEO. In the intestine mRNA level of occludin was enhanced, but there was no effect on *TJP1*/*ZO-1*. In an earlier experiment where piglets were challenged with *E. coli*, they also found, that OEO prevented the downregulation of *claudin-1* and *occludin*. But there was also no effect on *TJP1*/*ZO-1* (Pu et al., 2018). In a rat experiment, OEO (> 80% carvacrol) was fed for several days and effects on several intestinal barrier indicators have been investigated. The mRNA expression of *TNF-α*, *IL-6* was reduced and the level of *occludin* was higher in OEO treated group. However, there was no effect on *TJP1*/*ZO-1* in the jejunal mucosa (Wei et al., 2015). Two years later, the research group published another experiment in which piglets were fed with a 1:1 carvacrol-thymol blend (100 mg/kg). In this study, they did not find significant effects on *occludin* nor *TJP1*/*ZO-1* (Wei et al., 2017). These results indicate again, that OEOs´ effects cannot be limited to one or two functional ingredients and synergistic effects must be expected. Taken together, OEOs´ effects on the gene expression of genes investigated can be judged as indicators for OEOs´ small intestinal health-promoting activity. However, more research is needed to explain OEOs´ effects on pigs´ intestinal health. In particular, the large differences in gene expression between the jejunum and the ileum can only be partially explained. Additionally, other auxiliary features have to be found in further studies that facilitate the determination of effects on intestinal health.

3.6 Conclusion

In conclusion, carcass and meat quality attributes were mostly not affected by OEO supplementation, indicating that dietary administration for the whole fattening period did not exert direct valuable effects on pig meat parameters in the present experiment. Only few studies have been conducted aiming to show OEOs´ impact on animals' haematological profile, particularly in pigs. However, our findings provide new insights into transcriptome modulatory properties. Feeding OEO significantly affected diverse biological processes and regulated the mRNA level of genes related to the activation of the immune response, especially intestinal immunity and barrier function. However, effects on the transcriptomic level may not correlate with actual protein production, which depends on several other regulative processes. As it can be noticed, haematological, meat quality and transcriptional results are inconsistent across different studies. Difficulty for the comparisons between studies, using natural products such as OEO is the high heterogeneity within those substances, duration of supplementation, the composition of the basal diet (synergistic effects) and animal genetics.

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Chapter 4: Does Oregano essential oil affect the microbial community in pigs´ intestinal tract?

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Running title: Does Oregano essential oil affect the microbial community in pigs´ intestinal tract?

Key words: Oregano essential oil; growing pigs; microbiome, 16S rRNA sequencing

4.1 Abstract

This study has been focused to explore the effects of oregano essential oil (OEO) on the microbial composition in pigs´ jejunum, ileum, caecum, colon, and swab samples at the same timepoint in an 16S rRNA sequencing approach. Previous studies failed to show OEOs´ microbiota regulating effects, because classic microbiology is limited to cultivable bacteria. In this study, we provide insights into the phylogenetic composition of pigs´ microbiome at an average age of six months and dependent on oregano essential oil supplementation. Therefore, the microbiota composition was studied from 48 fattening pigs in five different intestinal compartments by 16S rRNA gene V3/V4 regions sequencing with the Illumina Miseq platform. We investigated the alpha- and beta-diversity of the intestinal microbial communities. These metrics have been used as indicators for the impact of oregano essential oil supplementation on microbial composition. However, alpha-diversity was highly significant different between the investigated intestinal segments but there was no difference between the OEO treated and the control group. Additionally, the beta-diversity between the different investigated intestinal body sites was significantly different and we found significant differences between the treated and the control group in caecum and colon samples. The taxonomic analysis showed the most interesting observations in the large intestine associated samples. We found members of the *Lactobacillus* genus to be significantly enhanced by OEO supplementation in colon and swab samples. *Faecalibacterium* and *Prevotella* genera were significant reduced in the treated groups´ caecum. This study demonstrates OEOs´ impact on pigs' intestinal microbiota profiles.

4.2 Introduction

Phytogenics, such as Oregano essential oil (OEO) from the aromatic plant *Origanum vulgare* subsp. *hirtum* and its´ bioactive compounds are used to improve animal productivity by promoting intestinal health (Simitzis et al., 2008; Janz et al., 2007; Namkung et al., 2004). OEO was shown to have anti-inflammatory (Landa et al., 2009), as well as anti-bacterial effects (Bakkali et al., 2008), and is therefore beneficial for pigs´ small intestinal health. Nowadays, it is well accepted that the integrity of the small intestine is crucial for entire animal health. Besides the absorption of nutrients, the intestine exerts a barrier function against pathogens and protects the animal organism. The whole animal body is inhabited by microorganisms, especially the intestinal tract is a place of residence for countless bacteria, archaea, funguses, and viruses, which have various effects on animal health (Kim and Isaacson, 2015; Gill et al., 2006). In humans and other animals, it is suggested that the body contains ten times as many microbial as endogenous cells (Guevarra et al., 2018). However, not all microorganisms are harmful to the animal, the commensals digest complex macronutrients which otherwise would be indigestible for the animal organism and exerts various other beneficial aspects for the host, e.g. the regulation of the host immune system (Nicholson et al., 2012). Short-chain fatty acids are partially released by microbial digestion, some of which stabilize epithelial integrity (Puertollano et al., 2014; Daly and Shirazi-Beechey, 2006). Additionally, colonization resistance is represented by the commensal microflora, which leads to an inhibition of the colonization of pathogenic microorganisms (van der Waaij et al., 1971). The hosts´ immune system is trained not to attack its´ commensal flora, but sometimes auto-immune failures lead to the disintegration of this natural symbiosis (Guevarra et al., 2018). Therefore, preservation of microflora homeostasis or the improvement of beneficial bacteria must be a principal target in animal production.

In the last century, antibiotics have been used as growth promoters and to modulate the phylogenetic composition of intestinal microorganisms. Since the European Union banned preventively given antibiotics in animal production in 2006 (EU-regulation 1831/2003), researchers tried to replace industrially produced antibiotics with natural substances with microbiota regulating effects. Understanding the microbiome of monogastric animals and its´ reaction to feeding regimes allows control over the intestinal microbial composition. In the long run, this could help to improve digestion,

physiology and several immunologic processes in livestock animals and thereby productivity (Rothschild et al., 2018; Clemente et al., 2012). The microbial community in pigs´ intestines is shaped by age, diet (Hall et al., 2021; Frese et al., 2015) and several environmental effects (Filippo et al., 2010). Therefore, natural substances, such as OEO, with health-promoting and antimicrobial effects can be used to positively stimulate the microbiota composition (Wei et al., 2017; Aligiannis et al., 2001).

This study brings new insights into pigs´ microbiota profile in different intestinal segments at an average age of six months and the modulating effect of OEO supplementation. For this, microbial DNA of chyme and faecal samples was sequenced at the 16S rRNA gene.

4.3 Materials and methods

In this experiment, 16S rRNA-sequencing technology was used to investigate pigs´ intestinal microflora in a case-control study after OEO supplementation (DOSTO® powder, DOSTO® FARM, Westerstede, Germany, chapter two: Table 1). The flavour additive, steam distilled from *O. vulgare* subsp. *hirtum* var. Vulkan was supplemented with 1500 mg/kg (7.5% pure oregano essential oil) in a medium dosage directly after weaning (28 d). We produced 48 closely related crossbreed (German Landrace x Piétrain) piglets (22 female, and 26 uncastrated male pigs) from 5 sibling-sows. Pigs´ have been equally divided into two groups. The OEO supplemented group was kept under identical conditions compared to the control group.

Sample collection and DNA isolation

The pigs have been slaughtered with an average weight of 111.9±11.7 kg. Three days before the transportation to a conventional slaughterhouse, sterile swabs have been used to collect individual stool samples of each pig for DNA isolation. Six hours before slaughter, access to feed was stopped. Transportation time to the slaughterhouse was less than 2 ½ h. The pigs have been narcotized by carbon dioxide after 45 min of resting time. Immediately after slaughter, we removed the complete gastro-intestinal tract for sample preparation. Samples have been prepared at 4 °C room temperature to delay environmental bacterial contamination and spoiling of the tissue. For 16S rRNA microflora investigation we collected 15 ml chyme and a 5 cm^2 tissue sample of the jejunum (40 cm ahead of the ileum), the ileum (5 cm ahead of the caecum), the caecum and the colon (15 cm ahead of the rectum) of each pig. The chyme and the associated tissue have been stored in 50 ml tubes. The tubes were filled up with 30 ml of an isotonic NaCl (9 g/l) + 0.1% Tween80 (Merck, Darmstadt, Germany) sterile-filtered buffer (Wei et al., 2017). The samples were transported on ice to the institutional lab and have been processed according to the protocol by Wei et al. (2017). In brief, samples were homogenised by rapid shaking for 5 min and then centrifuged at 800 g for 5 min at a temperature of 4 °C. After centrifugation we collected 15 ml of the supernatant, added 15 ml of the NaCL+0.1% Tween80 buffer and repeated the whole process two more times.

After these washing steps, we collected 15 ml supernatant of each sample. The pooled supernatants have been centrifuged at 12000 g for 15 min at a temperature of 4 °C. Afterwards, the supernatant was discarded and the pellet was resuspended in 1.5 ml sterile PBS (Capricorn Scientific, Ebsdorfergrund, Germany). Immediately after preparation, the samples have been stored at -80 °C for two months.

Microbial DNA was isolated with the QIAamp PowerFecal Pro DNA kit (Qiagen, Hilden, Germany) according to the manufactures protocol and the mechanical disruption was performed in a Precellys24 tissue homogenizer (Bertin instruments, Frankfurt am Main, Germany). DNA quality and concentration were measured with a NanoDrop 8000 spectrophotometer (Thermo Fisher Scientific, Schwerte, Germany). DNA was stored at 4 °C for less than two weeks until sequencing.

Barcode labelling and 16S rRNA sequencing

DNA samples have been sent to the Next Generation Sequencing (NGS) Core Facility of the Medical Faculty of the University of Bonn and were checked for purity and quantity with a Qubit dsDNA HS Assay kit (Thermo Fisher Scientific, Schwerte, Germany). For 16S library preparation samples were labelled with the NexteraXTIndex kit (Illumina, San Diego, USA). The V3/V4 hypervariable region of the bacterial 16S rRNA was amplified using 341F (5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG-3') and 806R (5'- GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC-3') as forward and reverse primers (Klindworth et al., 2013), respectively. Finally, amplicons were sequenced on the Illumina Miseq (2x 300 bp paired-end reads) device (Illumina, San Diego, USA) with the V3/V4 chemistry kit.

Data processing and statistical analysis

The free software R (version: 4.0.2 (2020-06-22)) has been used for sequence assembly and raw sequence exploration. Reads < 50 bp were removed and paired end reads were merged with an overlap of 15 bp. Farther, the maximal allowed mismatch ratio was set to 0.2. For core statistical analysis the open-source bioinformatics pipeline 'Quantitative Insights into Microbial Ecology2' (QIIME2, version: 2020.8, Bolyen et al., 2019) was used. In brief, the DADA2 plugin (Callahan et al., 2016) was used for quality and chimeric sequences filtering and for further analysis, a rooted phylogenetic tree was built with

the QIIME 'q2-phylogeny' plugin. According to our dataset-quality, sampling depth for α- (Shannon´s diversity index, Faith´s phylogenetic diversity, and Pielou´s evenness) and β-diversity analysis was set to 21100. The analysis was performed with the 'q2-diversity' plugin in QIIME. The statistic test for β-diversity was the unweighted (qualitative) UniFrac (Unique fraction) method (Lozupone and Knight, 2005) with a pairwise Permutational Multivariate Analysis of Variance (PERMANOVA, Anderson, 2001) and 999 permutations. For the taxonomic investigation, the operative taxonomic units (OTUs) have been mapped against the SILVA 138 SSU (https://www.arb-silva.de/documentation/release-138/) classifier with a 99% confidence threshold. Venn diagrams were created with Venny (version: 2.1, https://bioinfogp.cnb.csic.es/tools/venny/index.html). R has been used for the analysis of variance using the ANOVA function. Group comparisons have been drawn by Tukeys´ Test. Differences were considered significant when $p<0.05$.

Statements

OEO is registered as a feed additive according to the entry in the European Union Register of Feed Additives under Regulation (EC) No 1831/2003 (2b natural products – botanically defined) (Rychen et al., 2017). Animal care followed the general guidelines outlined in the European Animal Welfare Regulations and the Directive 2010/63/EU. The experiment was conducted according to the institutional guidelines. Animal husbandry regulations of Germany and the State Agency of Nature, Environment and Consumer Protection, North Rhine-Westphalia, Germany (permission no. 81-02.04.2019.A307), approved the sampling protocol.

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4.4 Results

Core metrics and quality filtering of the dataset

After demultiplexing, we worked with 28,905,351 sequence counts in total and there was an average of 100,366 sequences per sample. 52,165 reads were the minimum in one sample and the maximum was 173,406 reads. In 288 samples (six different samples have been taken from each of the 48 animals) we detected 21,633 OTUs, in one ileum-sample 87,968 OTUs have been found and the mean within our dataset was 42,309 OTUs per sample (Figure 1). One OTU was only once detected in the whole dataset, whereas another OTU has been found in 439,852 sequences in 288 different samples. The mean frequency per OTU was 563. Because of low quality (quality score < 20), < 80,000 reads per sample and low feature numbers, three samples have been excluded from our analysis. Finally, 285 samples have been used for further investigations.

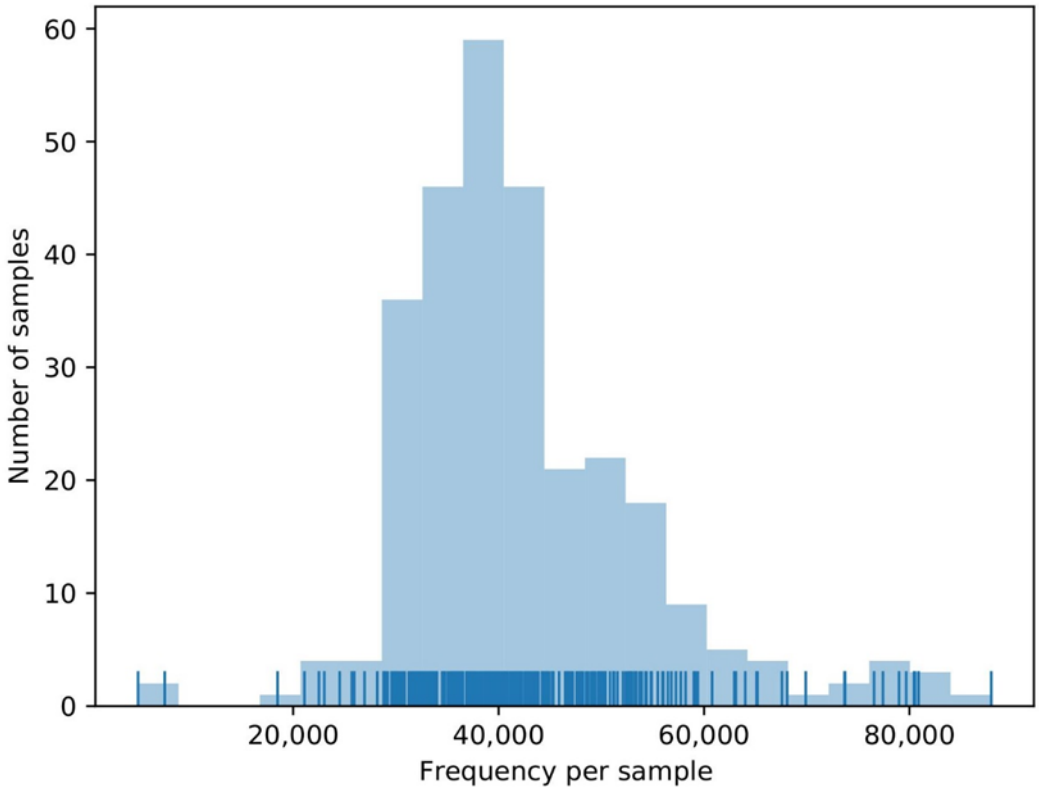

Figure 1: Distribution of OTUs (frequency) per sample. The mean of OTUs detected was 42,309 per sample, n=285

Alpha diversity, exploring the complexity of communities

For alpha diversity investigation three different metrics have been used. Shannon´s diversity index is purely quantitative measurement of community complexity regardless of the phylogeny. However, Faith´s phylogenetic diversity is a qualitative measurement that includes phylogenetic information and community richness. Additionally, Pielou´s evenness of community was tested. We found, there was no difference between the caecum, colon and swab samples regarding alpha-diversity. Also, jejunum and ileum communities were not statistically different. However, we are able to show, small intestinal samples from the jejunum and the ileum are highly statistically different (Table 1) to large intestine-associated samples (caecum, colon, and swab samples, which have been taken at the rectum). This result was confirmed by Pielou´s evenness analysis. However, taking phylogeny into account, we recognized that all investigated sample types are significantly different from each other (supplementary Figure 2 and Table 2). Using these three metrics, we found no significant differences between the sows, which were the mothers of the animals used in this experiment. Because the α-diversity in the different body sites was heterogeneous (supplementary Figure 1), for group comparison (treated vs. control) diversity metrics have been investigated individually in every community. There was no difference between the OEO-treated and the control group in any sample type (Table 3).

Table 1: Pairwise (Kruskal-Wallis) estimation of Shannon´s diversity index for alpha-diversity

Group 1	Group 2	H	p-value	q-value
Caecum (n=47)	Colon (n=48)	0.73	0.392	0.436
	Ileum (n=48)	45.38	**1.62E-11**	3.25e-11
	Jejunum (n=47)	53.20	**3.02E-13**	7.55e-13
	Swab (n=95)	0.44	0.506	0.506
Colon (n=48)	Ileum (n=48)	43.30	**4.70E-11**	7.83e-11
	Jejunum (n=47)	53.43	**2.68E-13**	7.55e-13
	Swab (n=95)	2.53	0.112	0.140
Ileum (n=48)	Jejunum (n=47)	2.96	0.086	0.122
	Swab (n=95)	65.70	**5.27E-16**	2.63e-15
Jejunum (n=48)	Swab (n=95)	73.42	**1.05E-17**	1.05e-16

Table 2: Pairwise (Kruskal-Wallis) estimation of Faith´s phylogenetic diversity for alpha-diversity

Group 1	Group 2	H	p-value	q-value
Caecum (n=47)	Colon (n=48)	18.00	**2.21E-05**	3.68e-05
	Ileum (n=48)	54.09	**1.92E-13**	1.92e-12
	Jejunum (n=47)	27.58	**1.51E-07**	3.01e-07
	Swab (n=95)	49.97	**1.57E-12**	5.22e-12
Colon (n=48)	Ileum (n=48)	42.34	**7.70E-11**	1.92e-10
	Jejunum (n=47)	10.87	**9.75E-04**	1.39e-03
	Swab (n=95)	4.66	**0.038**	0.038
Ileum (n=48)	Jejunum (n=47)	5.39	**0.020**	0.025
	Swab (n=95)	50.72	**1.06E-12**	5.22e-12
Jejunum (n=48)	Swab (n=95)	8.249	**0.004**	0.005

Table 3: Pairwise comparison between OEO-treated (n=24) and control (n=23) samples of core-metrics for alpha-diversity analysis (Kruskal-Wallis test)

Body site	Metric	H	p-value	q-value
Ileum	Shannon´s diversity index	0.00	0.949	0.949
	Faith´s phylogenetic diversity	2.22	0.136	0.136
	Pielou´s evenness	0.13	0.718	0.718
Jejunum	Shannon´s diversity index	0.00	1.000	1.000
	Faith´s phylogenetic diversity	2.35	0.126	0.126
	Pielou´s evenness	0.12	0.734	0.734
Caecum	Shannon´s diversity index	0.08	0.782	0.782
	Faith´s phylogenetic diversity	0.28	0.594	0.594
	Pielou´s evenness	1.43	0.233	0.233
Colon	Shannon´s diversity index	2.63	0.105	0.105
	Faith´s phylogenetic diversity	0.88	0.348	0.348
	Pielou´s evenness	1.66	0.197	0.197
Swab	Shannon´s diversity index	1.22	0.270	0.270
	Faith´s phylogenetic diversity	0.19	0.666	0.666
	Pielou´s evenness	0.56	0.457	0.457

Beta diversity, revealing differences between microbial communities from different 'environments'

The beta-diversity between the different body sights was highly significant in this study (Table 4). As it was also shown in the alpha-diversity analysis, the body sites were dissimilar. Therefore, for group comparison, the different body sites have been investigated individually. We found significant differences between the OEO supplemented and the control group in the colon ($p<0.05$) and beta-diversity tended to be different in the caecum ($p<0.08$). In this experiment, we did not find an effect of OEO supplementation on the jejunum, the ileum, and the swab samples regarding the beta-diversity (Table 5). Additionally, we did not recognize any differences between the sexes (data not shown). As can be seen in figure 2, the small intestinal samples (jejunum and ileum) are more similar compared to the large intestinal associated samples (caecum, colon, and rectal swabs).

Table 4: Beta-diversity as an indicator for different bacterial communities in intestinal compartments, pairwise PERMANNOVA, 999 permutations

Group 1	Group 2	Pseudo-F	p-value	q-value
Caecum	Colon (n=48)	8.656	**0.001**	0.001
	Ileum (n=48)	43.958	**0.001**	0.001
	Jejunum (n=47)	38.967	**0.001**	0.001
	Swab (n=95)	18.208	**0.001**	0.001
Colon	Ileum (n=48)	43.608	**0.001**	0.001
	Jejunum (n=47)	39.453	**0.001**	0.001
	Swab (n=95)	8.370	**0.001**	0.001
Ileum	Jejunum (n=47)	2.535	**0.007**	0.007
	Swab (n=95)	67.575	**0.001**	0.001
Jejunum	Swab (n=95)	61.822	**0.001**	0.001

Table 5: Pairwise comparison between the Oregano essential oil treated and the control group in the different body sites (PERMANOVA, 999 permutations)

Organ	Pseudo-F	p-value	q-value
Jejunum (n=47)	1.192	0.215	0.215
Ileum (n=48)	1.140	0.279	0.279
Caecum (n=47)	1.490	0.054	0.054
Colon (n=48)	1.332	**0.034**	0.034
Swab (n=95)	1.140	0.211	0.211

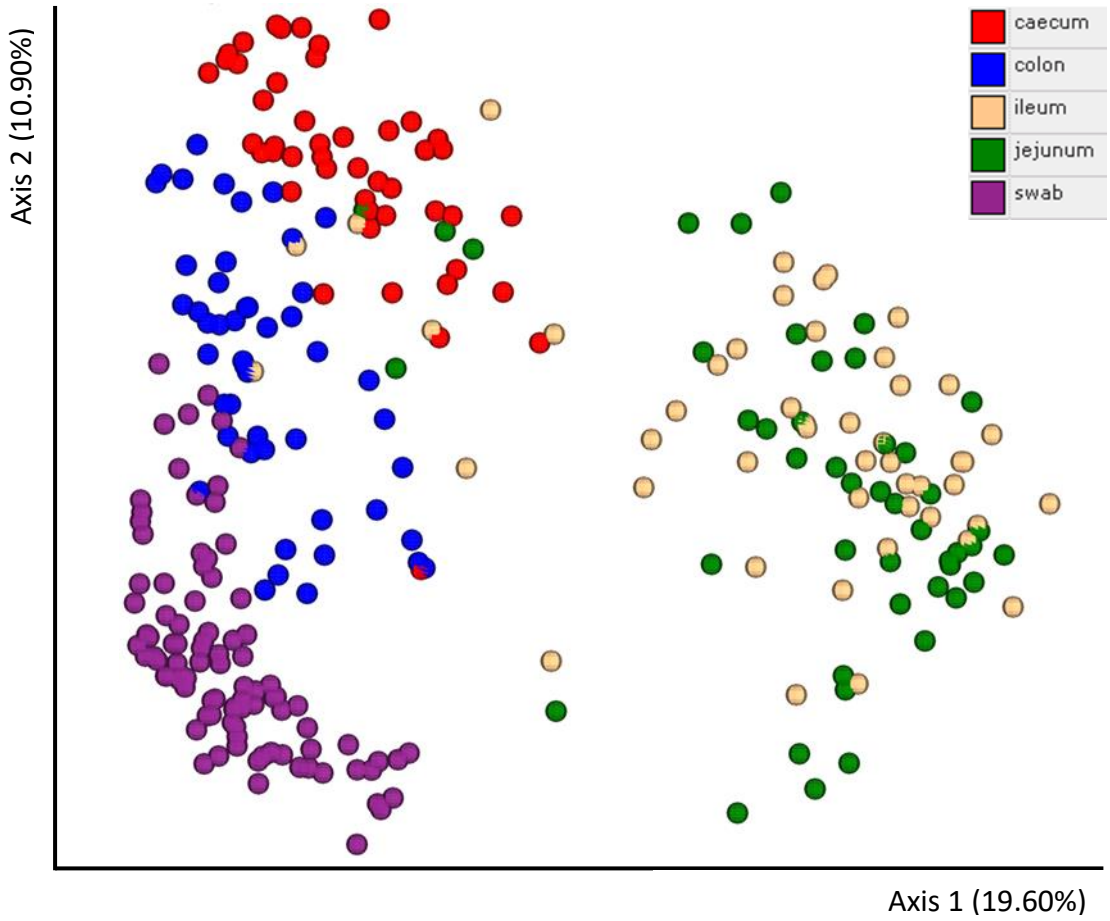

Figure 2: Principal coordinates (PCoA) plot shows the relative distribution of microbial samples. Additionally, it shows the similarity within small intestinal samples (jejunum and ileum) and the relative dissimilarity within large intestinal associated samples (caecum, colon, and rectal swabs). The two compartment groups (small vs. large intestine) can be divided from another. In total, the two shown axes explain 30.5% of the variance (Bray-Curtis dissimilarity), n=285

As shown before, the microbial communities between small intestinal and large intestine-associated samples are highly different. Therefore, we compared these two groups separately. Additionally, we used a conventional classifier (SILVA 138 SSU) to align the explored OTUs. As it can be seen in figure 3, jejunal and ileal shared 81.3% of total explored OTUs and 59.8% were shared between the large intestine-associated samples (Figure 4) at the order level. As a result of this investigation, 80.2% of the OTUs were shared by jejunal and ileal samples, and 56.4% were shared between the large intestine-associated samples at the family level. Relative bacterial compositions in the different investigated intestinal segments can be found in supplementary figures 3 to 7. In this experiment, we found *Firmicutes* to be the most abundant phylum with an average of 65.21% across all investigated body sites. In the jejunum and the ileum, the

Proteobacteria phylum was the second highest present (19,39%) and in the large intestine-associated samples it was the *Bacteroidota* phylum with an average of 24.02% (supplementary Table 1). To investigate nutritional effects on bacteria, we selected the most abundant top 10 at the phylum, class, order and family level. The relative abundance of *Spirochaetota* tended ($p<0.08$) to be significantly higher in caecum samples of the treated group and the abundance of *Actinobacteriota* was significantly higher in the swab samples of the treated group in comparison to the control (supplementary Table 2). However, the abundance of the phylum '*Spirochaetota*' was significantly lower in treated groups´ swab samples. At the class level, we found the counts of *Clostridia* and *Spirochaetia* to be significantly downregulated in the swab samples. On the contrary, the abundance of *Bacilli*, *Negativicutes* and *Actinobacteria* was significantly higher in the treated group. There were no further differences at this level in the other investigated body sites (supplementary Table 2). At the order level, *Pasteurellales* tended ($p<0.08$) to be more abundant in the treated group in comparison to the control group in both small intestinal segments (jejunum and ileum). In the colon, *Erysipelotrichales* was significantly more abundant in the control group (supplementary Table 9). The swab samples were most affected by the feeding regime. We found significant differences between the OEO treated animals and the control group in *Lactobacillales*, *Clostridiales*, *Veillonellales-Selenomonadales*, *Erysipelotrichales*, and *Spirochaetales* (supplementary Table 3). The bacterial family: '*Lactobacillaceae*' was significantly more abundant in treated groups´ caecum, colon and swab samples. There were no differences between the dietary groups between the top 10 bacterial families in the ileum and again, swab samples showed the most differences (supplementary Table 4). At the genus level (supplementary Table 5), we found significantly higher counts of *Actinobacillus* in the OEO treated groups´ ileum. Again, the effects on the small intestine were very limited. However, in the colon and swab samples, we found significantly higher abundances of *Lactobacillus* in the treated group and *Turibacter*, as well as *Terrisporobacter* were significantly reduced.

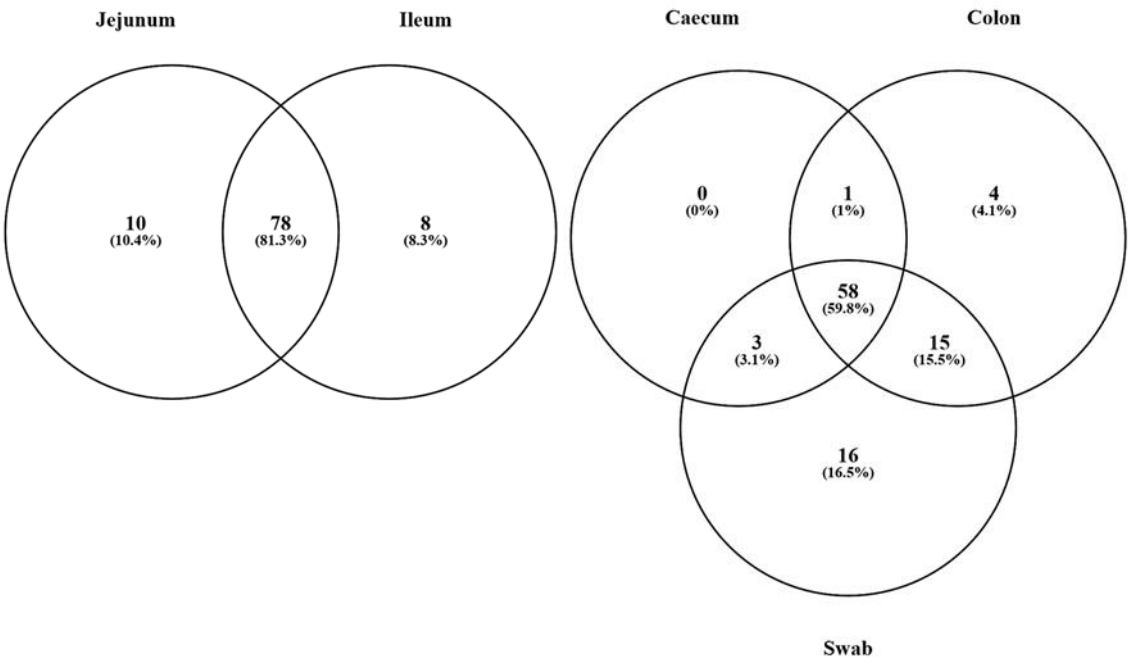

Figure 3: Venn diagrams of small intestinal (jejunum and ileum) and large intestine associated (caecum, colon, and swab) samples showing the percentage of shared OTUs at the order level

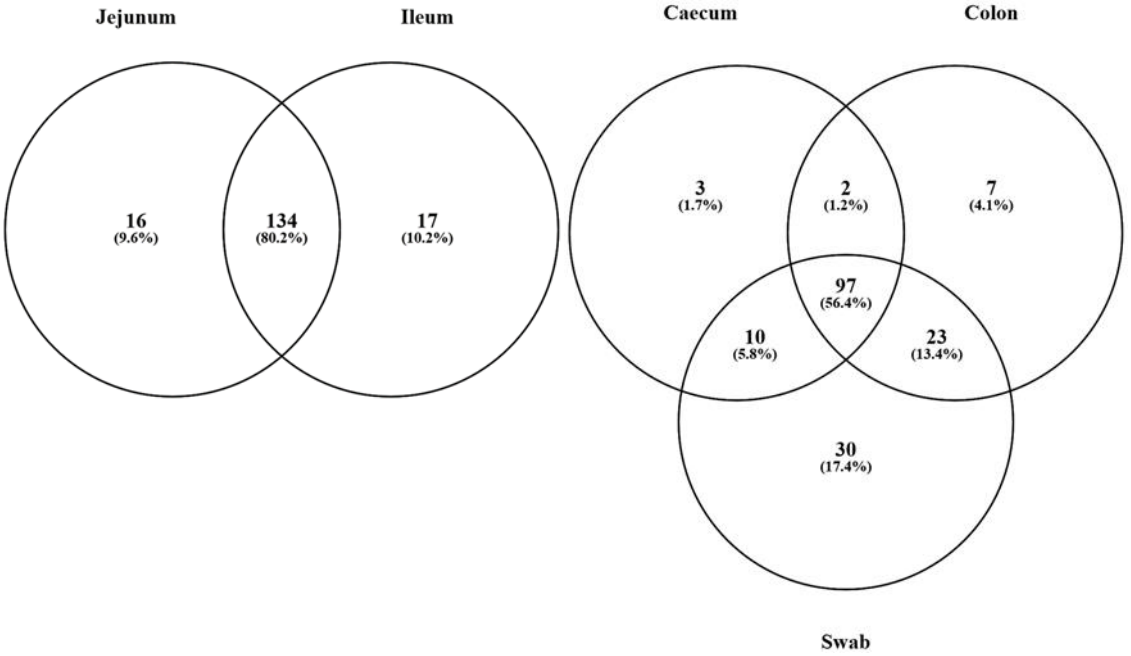

Figure 4: Venn diagrams of small intestinal (jejunum and ileum) and large intestine associated (caecum, colon, and swab) samples showing the percentage of shared OTUs at the family level

4.5 Discussion

Oregano essential oils´ effects on pigs´ intestinal microbiota profile is yet not fully understood. Antibacterial effects on selected microorganisms have been shown by several classic microbiological experiments. Since these former studies were only able to show OEOs´ impacts on cultivable bacteria, the effects were limited. Currently, investigation of the microbiome is only assessable by 16S rRNA relationship analysis.

It was summarized by Round and Mazmanian (2009), that the gastrointestinal tract is the most important location for interactions between the host and its´ microflora. Such interactions can be beneficial or negative for the host. Therefore, this 16S rRNA sequencing experiment was performed to reveal and to compare the microbial composition in pigs´ intestinal tract. The targeted manipulation of the microbiome could be a highly efficient tool to improve animal production and health. It was summarized by Round and Mazmanian (2009), that the mammalian immune system is controlled by microorganisms. This may be regarded as somewhat one-sided but underlines the importance of the microbial community. Frese et al. (2015) showed that environmental factors, such as stress, rearing conditions, and diet structure affect the intestinal microbial flora. The phylogenetic profile changes throughout aging and reaches its´ highest heterogeneity at an early stage of life (Yatsunenko et al., 2012). Pigs´ intestinal microflora was suggested to be mature with an average age of 80 days (Ke et al., 2019). However, each of the aforementioned impacts on phylogenetic profile depends on the age of the organism investigated (Yatsunenko et al., 2012). In addition, there are genetic effects on the microbial composition, but these are difficult to determine (Rothschild et al., 2018; Khachatryan et al., 2008). Natural substances, such as plant oils with partially chemogenetic actions could help to shift the microbial composition (Wei et al., 2017; Rodriguez-Garcia et al., 2016; Aligiannis et al., 2001). In this study, OEO was supplemented as an aromatic powder (DOSTO® powder) in a dosage of 1500 mg/kg conventional feed.

We found very limited effects on 16S rRNA sequencing core metrics. The alpha-diversity was significantly different between the investigated body sites in this study. Jejunal and ileal samples are closely related, as well as caecum, colon, and swab samples. We found clear differences between those small intestinal and large intestine-associated samples not just only in the alpha-diversity, but also in beta-diversity and the taxonomic

investigation. However, alpha diversity between the OEO treated and the control group was not different in any of the body sites examined. This result is in accordance with the study of Hall et al. (2021), who also did not find any differences between treatment groups and time points in these metrics. In their study, sows were fed with 1% OEO (*O. vulgare* subsp. *hirtum*) in addition to their commercial feeding regime for the whole lactation period and piglets were supplemented with the same concentration of OEO after 14 days of age. The authors investigated fresh faecal samples from the sows and rectal swabs of the piglets in a conventional 16S rRNA sequencing experiment. Their Bray-Curtis and unweighted UniFrac distance analysis for beta-diversity showed sample clustering depending on the sampling time point. They explained this by changes in dietary composition and the housing of the sows. In our experiment, we found significant differences between the body sites investigated. As shown before, the most significant differences can be found between small intestinal and large intestine-associated samples. Additionally, PERMANOVA analysis revealed significant differences between the OEO treated group and the control in the colon samples, and beta-diversity tended to be different in the caecum samples. There were no differences in the jejunum, ileum, and swab samples. This is also in accordance with the results by Hall et al. (2021). They did not find differences in beta-diversity metrics in swab samples, too. In general, high heterogeneity in the phylogenetic composition is associated with hosts´ health and fitness (Ke et al., 2019; Le Chatelier et al., 2013).

As a result of this investigation, we can confirm *Firmicutes* and *Bacteroidetes* to be the most dominate phyla across all stages of life in pigs´ intestinal tract (Ke et al., 2019). Accordingly, Hall et al. (2021) showed that the *Proteobacteria* phylum was dominant in piglets' intestinal tract. They refer to the many pathogen representatives in this phylum and summarize several disorders (Hall et al., 2021; Rizzatti et al., 2017). In the study of Ke et al. (2019), *Proteobacteria* counts were considerably high in an adult age of eight months. Our results confirm the *Firmicutes* and the volatile fatty acid-producing *Bacteroidetes* (Rajilić-Stojanović and Vos, 2014) to be dominant. The *Proteobacteria* phylum was additionally dominating in pigs' intestinal tract with an average abundance of > 20% in the small intestine and < 5% in large intestine associated samples. There was no difference between the OEO supplemented group and the control regarding *Proteobacteria* phylum. Ke et al. (2019) showed, the *Lactobacillus*, *Prevotella*,

Ruminococcus and *Treponema* genera to be the core bacteria until an age of eight months. We can partially confirm those results. However, in our dataset, the *Treponema* genus from the *Spirochaetales* order was not present in the most abundant top 10 of bacteria investigated.

Cheng et al. (2018) showed long-term supplementation with 250 mg OEO powder (25 mg pure OEO from Greek *O. vulgare* subsp. *hirtum*) per kg of a protein-reduced feed did not affect total bacteria but improved intestinal bacteria composition, such as relatively elevated *Lactobacillus* counts. The authors consider that OEO can be used as an alternative to antibiotics in a protein-reduced diet to improve intestinal integrity and regulation of microbiota. In contrast, our data rather hints toward a modulating effect. However, increased relative abundances of members of the *Lactobacillaceae*, *Spirochaetaceae*, *Peptostreptococcaceae*, and *Lachnospiraceae* families depending on OEO supplementation were also shown by Hall et al. (2021). In addition to that, OEO fed sows showed increased relative abundances of *Fibrobacteriaceae* and *Akkermansiaceae*, which are important for fibre digestion, during the lactation. Those bacterial families are not listed in our investigation because their relative abundance was lower than 1%. Suckling piglets of these OEO fed sows had decreased *Enterobacteriaceae* counts and decreased *Enterococcus* counts. However, the counts of butyrate-producing *Lachnospiraceae* increased (Hall et al., 2021). *Lactobacillus* from the *Lactobacillaceae* family can digest plant-derived mono- and disaccharides (Guevarra et al., 2018; Schwab and Gänzle, 2011). Our study confirms, that members of the *Lactobacillaceae* family were significantly increased by OEO supplementation in caecum, colon and swab samples. There was no effect in small intestinal samples. However, we cannot confirm, increase of *Lachnospiraceae* and a decrease of *Enterobacteriaceae* which are associated with intestinal inflammation after OEO supplementation. In both small intestinal segments, we found high amounts of *Enterobacteriaceae*. Therefore, this could be an indicator for intestinal inflammation (Hall et al., 2021). In other monogastric animals, such as broilers, the total counts of *Lactobacillus* were elevated after carvacrol supplementation, which is the main ingredient of OEO (Jamroz et al., 2005). Members of the *Erysipelotrichaceae* family are negatively associated with the digestion of fat and protein (Bermingham et al., 2017) but positively correlated with carbohydrate digestion. However, McCormack et al.

(2017) showed, that the *Erysipelotrichaceae* family is correlated with lower feed efficiency in general and in humans it is associated with several diseases and inflammatory processes (Kaakoush, 2015). In our study, we found *Erysipelotrichaceae* to be significant reduced by OEO in the colon and swab samples. Therefore, OEO supplementation could help to reduce the aforementioned physiological negative effects of the *Erysipelotrichaceae* family for hosts´ health. Further research is needed to understand the effects of this family on the host. The Gram-negative *Pasteurellaceae* bacterial family belongs to the order of *Proteobacteria* (Christensen et al., 2015) and was the only group of microorganisms that was significantly increased in the treated groups´ ileum. This family is shaped by commensal representatives, some of which have a positive influence on the host. However, there are also important pathogens within this family such as *Actinobacillus pleuropneumoniae* (Kielstein et al., 2001). There were no further effects on small intestinal samples at this level. The Gram-negative and anaerobic *Prevotellaceae* family of the *Bacteroidales* order are common in monogastric animals´ intestinal tract (Morotomi et al., 2009). In OEO supplemented pigs´ caecum, we found *Prevotellaceae* to be significantly reduced. It was shown by Scher et al. (2013), that high abundances of *Prevotella copri*, a member of this family, is associated with rheumatoid arthritis in humans. The *Prevotella* genus is associated with the production of short-chain fatty acids from non-starch polysaccharides from plants via fermentation (Guevarra et al., 2018; Ivarsson et al., 2014; Liu et al., 2012) and β-glucanase, mannase, and xylanase enzymes (Flint and Bayer, 2008). In our experiment we found *Prevotella* to be significant reduced in the caecum samples of the treated group. There was no effect on this genus in the other investigated body-sites. In the swab samples, we found *Clostridiaceae* of the *Clostridiales* order to be significantly reduced in OEO treated pigs. Members of the *Clostridiaceae* family are associated with macronutrient digestibility (Bermingham et al., 2017), spore-forming, obligate anaerobic and mostly Gram-negative bacteria (Wiegel et al., 2006). However, the highly diverse *Clostridiaceae* family includes some pathogenic members (Rajilić-Stojanović and Vos, 2014). Therefore, the reduction of those bacteria by OEO supplementation in the swab samples could be beneficial for hosts´ health but negative for nutrient digestibility. Additional research is needed to interpret the local bacterial effects in the different intestinal segments. *Lachnospiraceae* and *Ruminococcaceae* are involved in the digestion of polysaccharides and the

production of short-chain fatty acids which are a valuable source of energy for the animal organism. We found these families to be highly abundant in the large intestine-associated samples, but there was no difference between the investigated groups. By contrast, the butyrate metabolizing *Faecalibacterium* genus (Bermingham et al., 2017; Vital et al., 2015) was significantly reduced in the caecum of OEO supplemented pigs. It was summarized by (Bermingham et al., 2017), that reduced counts of *Faecalibacterium* are typically associated with acute diarrhoea. We did not measure the faecal score during this experiment but we also did not observe acute diarrhoea in the animals investigated.

Our study shows the high heterogeneity between the investigated body-sites. OEO supplementation affects phylogenetic composition but dominantly targeted the large intestine-associated samples. It is necessary to understand why OEO has no impact on the bacterial composition of the small intestine but on the large intestine. Additionally, the question needs to be answered, if the microbiome gets used to a long term OEO supplementation and shifts back to its´ initial structure. Therefore, a time-course study is needed to determine this postulated reaction. Previous studies aimed to show this, yet only investigated the microbial composition in faecal samples. As shown by our experiment, the intestinal segment profiles are fundamentally different. Therefore, focusing on faecal samples only provides limited results. Another issue in 16S rRNA-experiments is the continuous development and improvement of taxonomic classifiers, such as the SILVA or Greengenes databases. Therefore, comparability to older studies is conditionally limited. Another limitation is, that the knowledge about the beneficial and opportunistic effects of the microorganisms and the different families is sometimes very limited (Hall et al., 2021). Our experiment is one of the first in which the microbial profile is examined in five different types of intestinal samples at the same time in pigs.

4.6 Conclusion

Oregano essential oil affected the microbial composition in jejunal, ileal, caecal, colon and swab samples. Thereby, previous studies where OEOs´ microbiota regulating effect were shown in classic microbiological approaches are partially confirmed by our results. However, the modulative activity of oregano was strongest in the large intestine-associated samples and further studies are needed to explain the result of this investigation. Furthermore, it needs to be noted, most of natural substances, such as OEO, are digested at the beginning of the small intestine in monogastric animals. Comparability between 16S rRNA sequencing approaches right now is only partially given, since it depends on different hypervariable regions of the 16S rRNA gene, the amount of different taxonomic classifiers, the heterogeneous genetic backgrounds of the hosts investigated, and of course the differences (duration of supplementation, composition of the oil etc.) of the oregano essential oils. Nevertheless, our experiment provides new insights into pigs´ microbiota profile in several intestinal segments with an average age of six months and its´ reaction to a long-term OEO supplementation.

Acknowledgments

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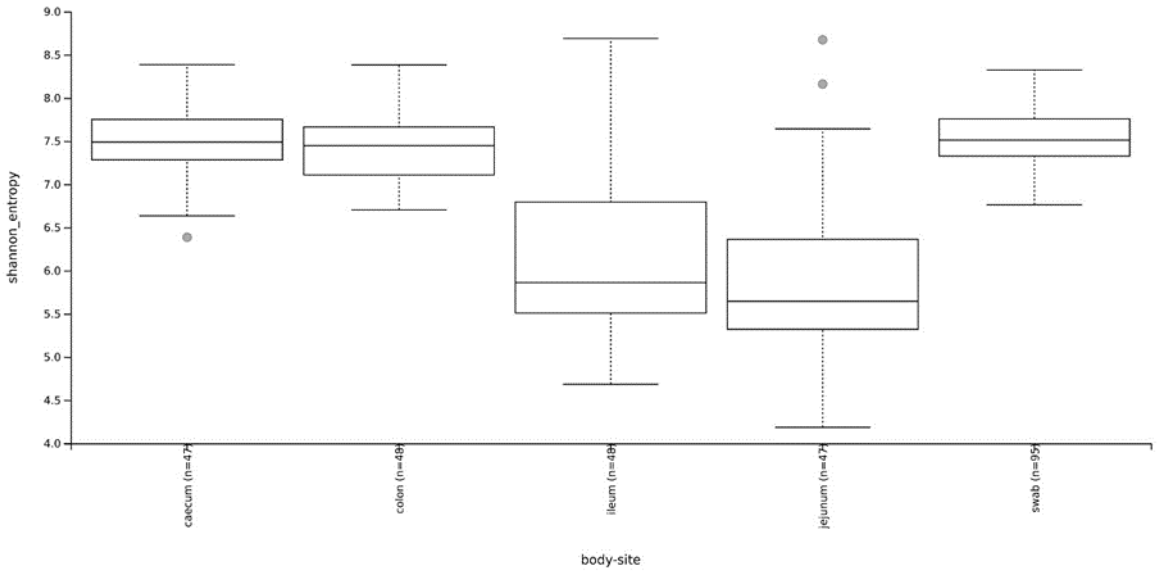
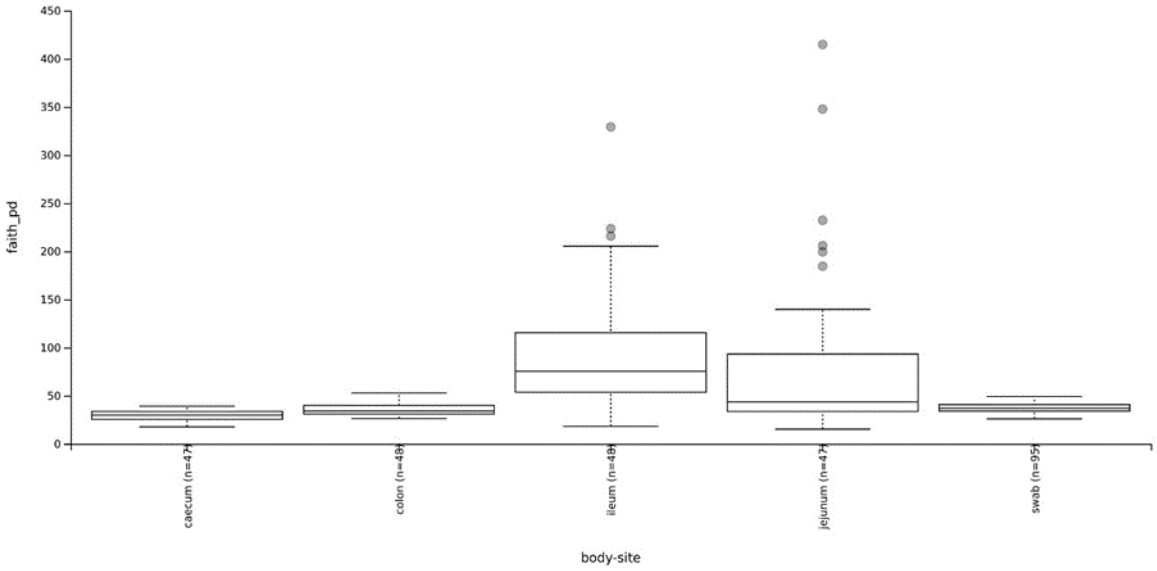
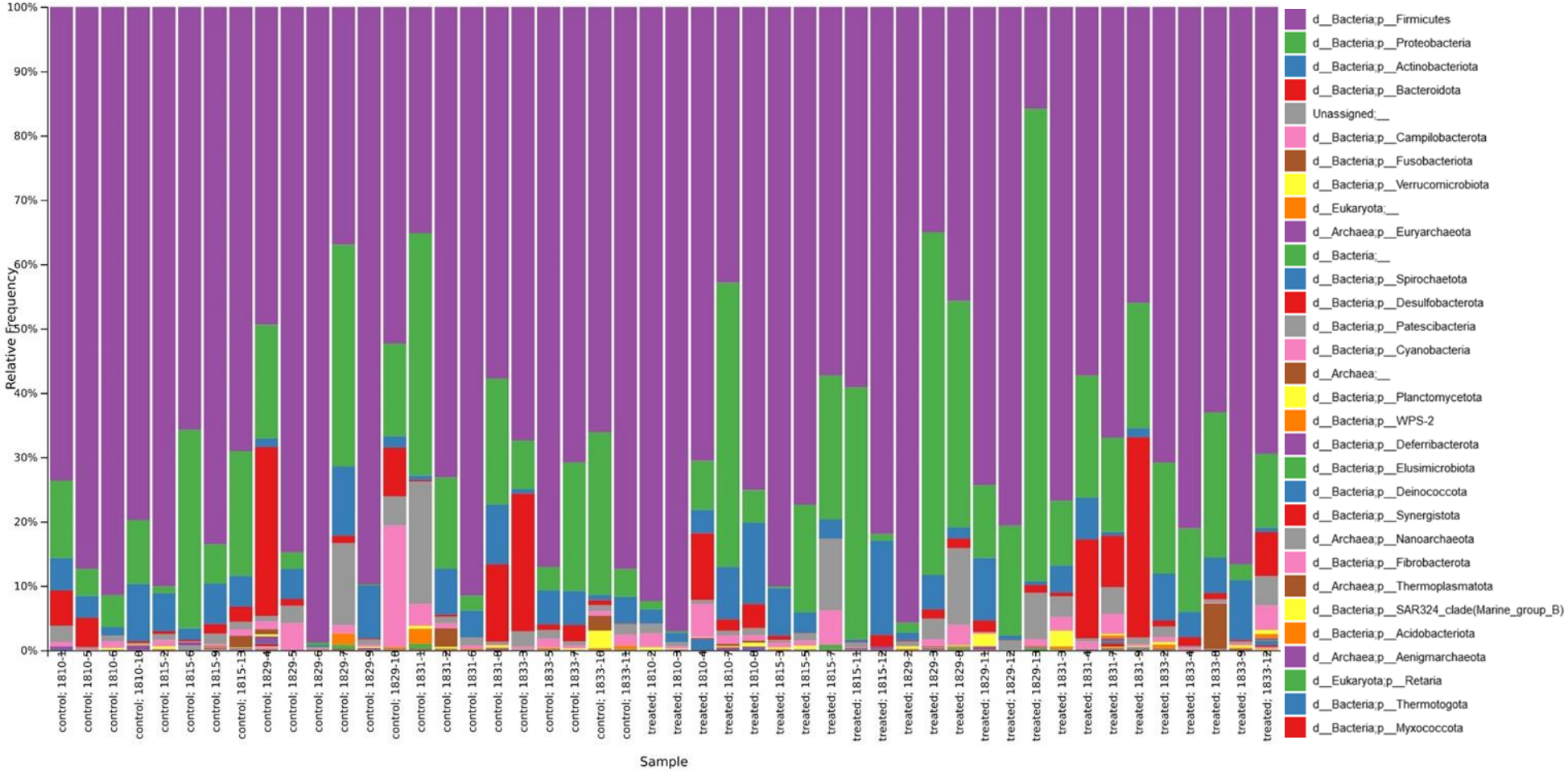
4.7 Appendix



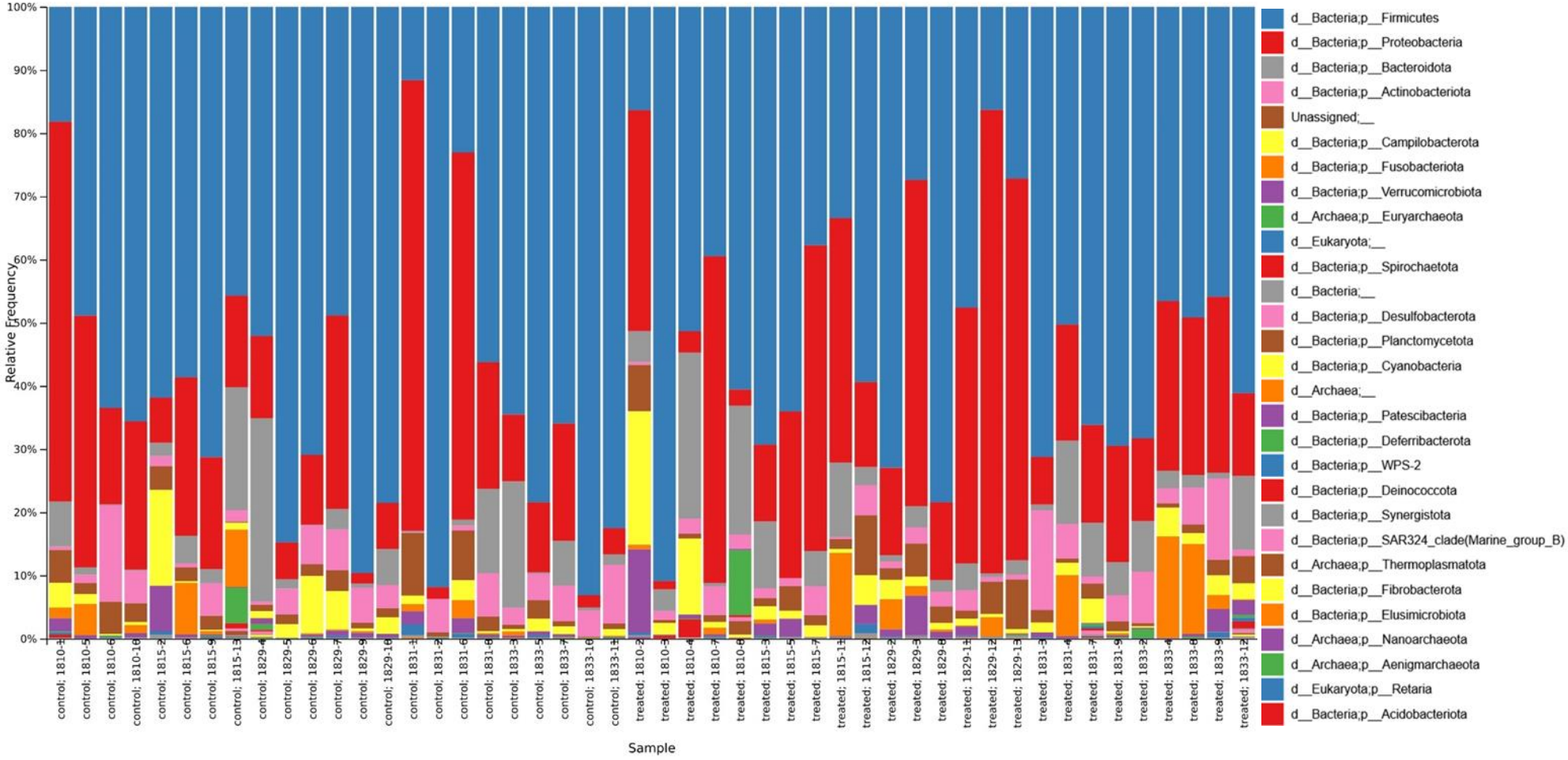
Supplementary figure 1: Shannon´s diversity index (entropy) across the four investigated intestinal segments (caecum, colon, ileum, jejunum) and the swab samples showing heterogeneity in alpha-diversity of the communities, n=285



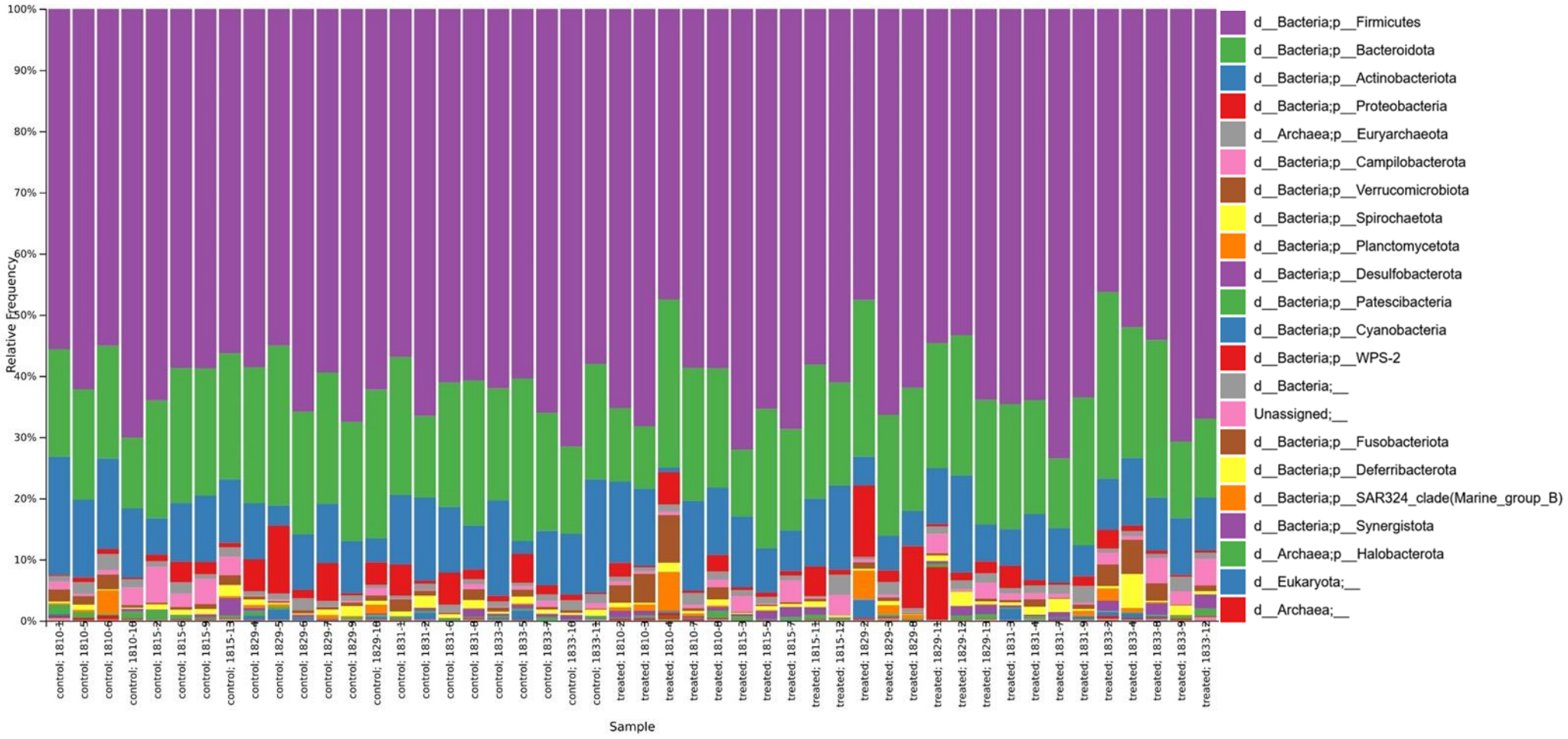
Supplementary figure 2: Faith´s phylogenetic diversity (pd) across the body sites showing heterogeneity in alpha-diversity of the communities, especially between small intestinal and large intestine-associated samples, n=285



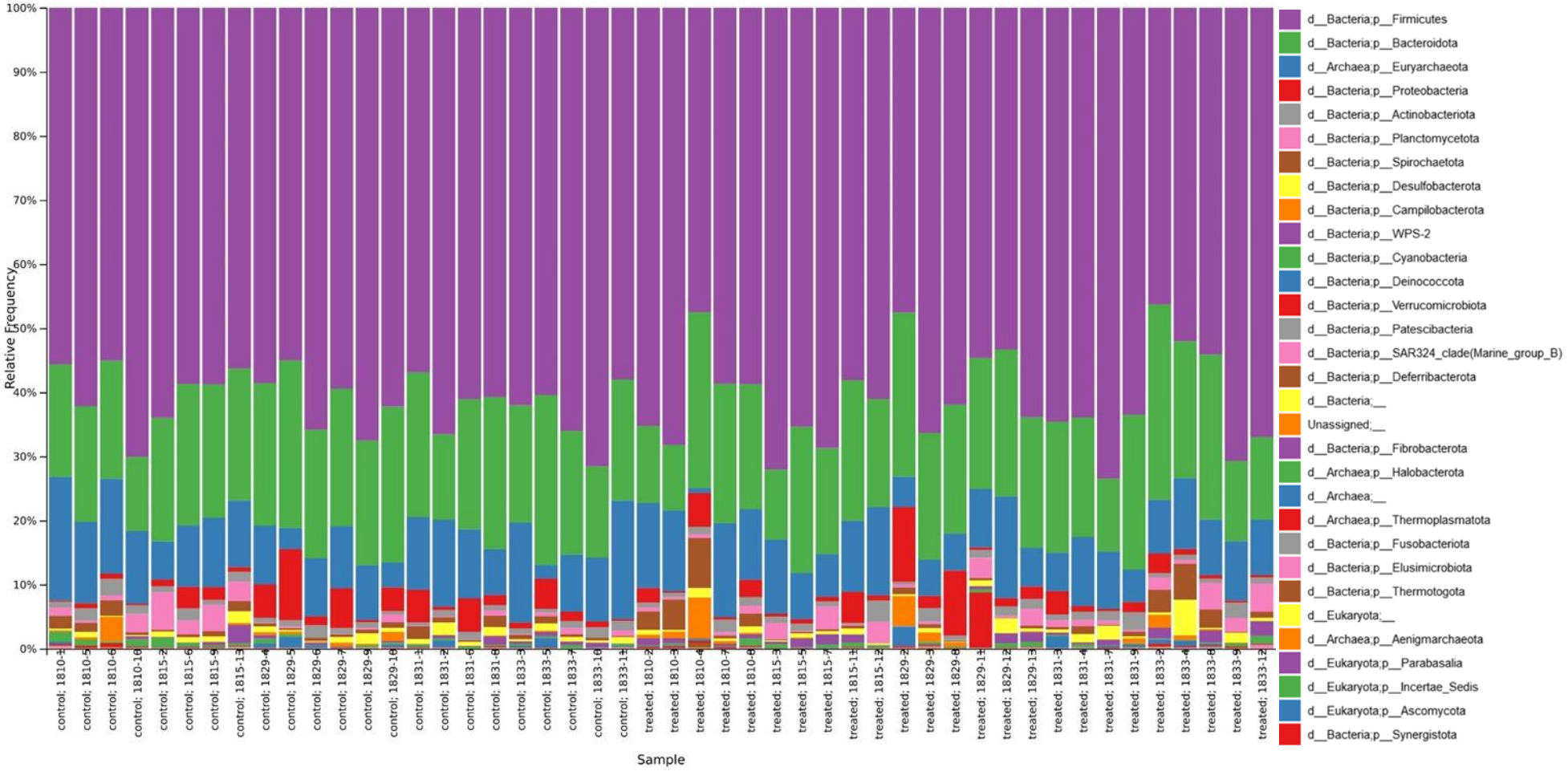
Supplementary figure 3: Relative taxonomic composition in the jejunum. d = domain, p = phylum, x-axis shows the experimental group and the ID of the animals investigated, n=48



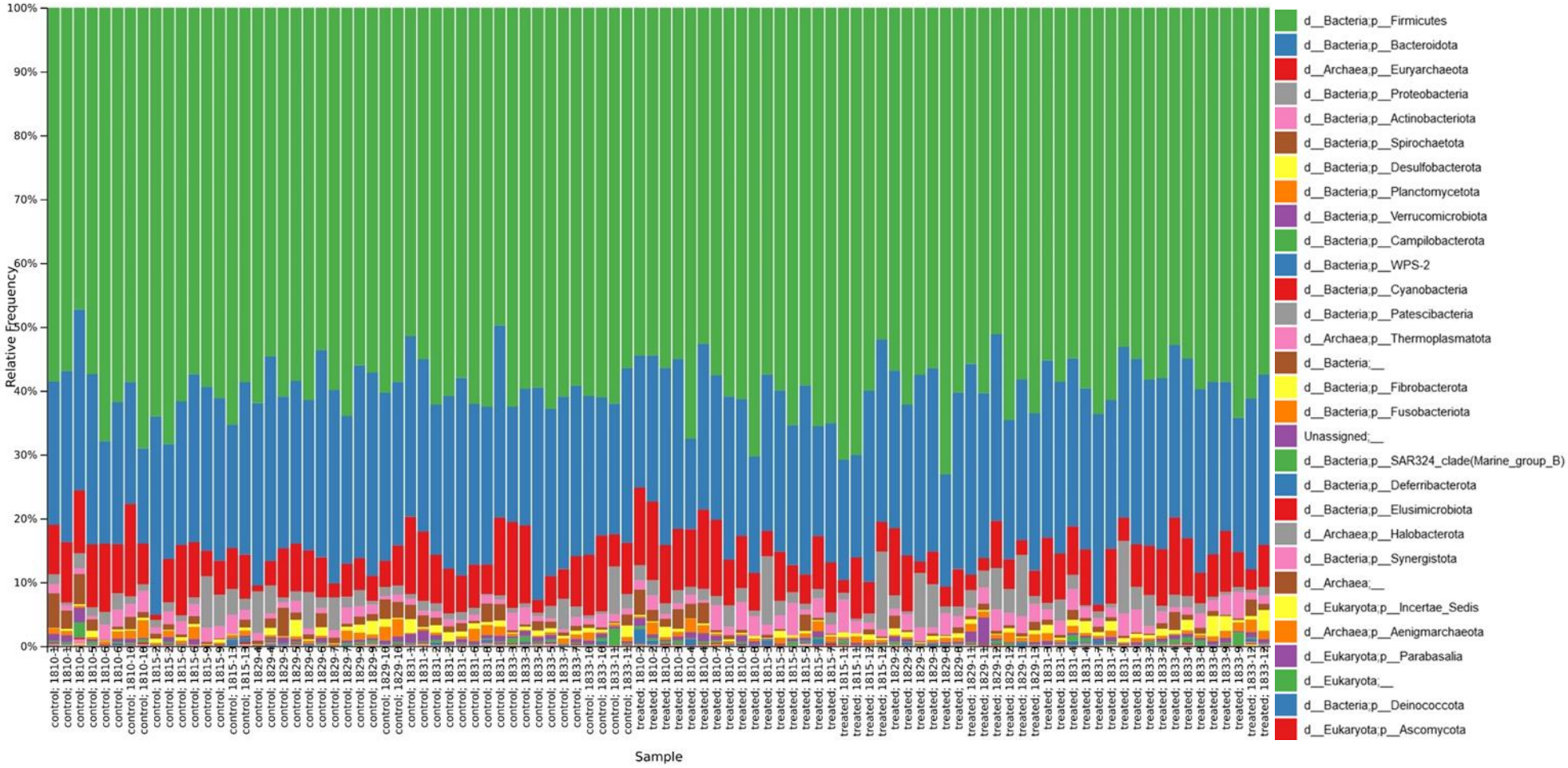
Supplementary figure 4: Relative taxonomic composition in the ileum. d = domain, p = phylum, x-axis shows the experimental group and the ID of the animals investigated, n=48



Supplementary figure 5: Relative taxonomic composition in the caecum. d = domain, p = phylum, x-axis shows the experimental group and the ID of the animals investigated, n=48



Supplementary figure 6: Relative taxonomic composition in the colon. d = domain, p = phylum, x-axis shows the experimental group and the ID of the animals investigated, n=48



Supplementary figure 7: Relative taxonomic composition in the swab samples. d = domain, p = phylum, x-axis shows the experimental group and the ID of the animals investigated, n=96

Supplementary table 1: Top 10 bacterial phyla in the investigated body-sites. Ranked according to their total abundance in the respective intestinal segment

Body site	Species	Relative abundance (%)				p-value
		Treated	± SEM	Control	± SEM	
Jejunum	*Firmicutes*	68.36	19.58	73.43	17.35	0.357
	Proteobacteria	18.39	17.85	12.73	10.99	0.199
	Actinobacteriota	4.63	3.90	4.44	2.99	0.854
	Bacteroidota	3.59	6.73	3.87	6.82	0.890
	Campilobacterota	1.38	1.54	1.73	3.78	0.687
	Fusobacteriota	0.32	1.39	0.35	0.80	0.939
	Verrucomicrobiota	0.37	0.58	0.25	0.56	0.478
	Unassigned bacteria	0.08	0.15	0.08	0.19	0.875
	Spirochaetota	0.12	0.33	0.02	0.06	0.164
	Desulfobacterota	0.04	0.11	0.02	0.08	0.513
Ileum	*Firmicutes*	52.83	18.97	62.00	21.90	0.138
	Proteobacteria	26.02	18.93	20.43	19.07	0.324
	Bacteroidota	6.11	6.40	5.26	7.64	0.686
	Actinobacteriota	3.64	3.71	4.22	3.38	0.585
	Campilobacterota	2.97	4.39	2.34	3.45	0.592
	Fusobacteriota	2.73	4.82	1.37	2.51	0.231
	Verrucomicrobiota	1.62	2.77	0.90	1.48	0.273
	Spirochaetota	0.21	0.59	0.08	0.20	0.305
	Unassigned bacteria	0.12	0.14	0.09	0.12	0.537
	Desulfobacterota	0.09	0.20	0.04	0.12	0.330
Caecum	*Firmicutes*	86.06	82.38	67.61	22.42	0.300
	Bacteroidota	29.29	27.15	24.98	9.06	0.468
	Actinobacteriota	2.69	2.57	1.85	1.19	0.155
	Proteobacteria	1.94	2.12	2.06	1.87	0.844
	Campilobacterota	1.40	4.15	1.21	3.15	0.860
	Verrucomicrobiota	1.31	4.24	0.02	0.03	0.150
	Spirochaetota	1.03	1.65	0.30	0.63	**0.053**
	Planctomycetota	0.42	1.18	0.03	0.07	0.119
	Desulfobacterota	0.21	0.38	0.12	0.16	0.294
	Patescibacteria	0.16	0.23	0.07	0.09	0.104
Colon	*Firmicutes*	61.07	7.61	61.41	4.62	0.857
	Bacteroidota	19.48	5.34	19.97	3.60	0.715
	Proteobacteria	2.28	2.90	2.50	2.64	0.789
	Actinobacteriota	1.27	0.73	1.18	0.52	0.656
	Planctomycetota	1.47	1.35	1.15	1.49	0.440
	Spirochaetota	1.48	1.98	0.86	0.64	0.160
	Desulfobacterota	0.95	1.11	0.80	0.51	0.558
	Campilobacterota	0.79	1.51	0.38	0.80	0.249
	Bacteria_WPS-2	0.75	0.70	0.41	0.61	0.085
	Cyanobacteria	0.39	0.31	0.49	0.39	0.337
Swab	*Firmicutes*	59.62	5.06	59.71	4.25	0.928
	Bacteroidota	24.96	3.95	25.41	4.32	0.600
	Proteobacteria	2.77	2.93	2.12	1.92	0.206
	Actinobacteriota	2.13	1.15	1.42	0.75	**5.0E-04**
	Spirochaetota	0.92	0.94	1.39	1.22	**0.042**
	Desulfobacterota	0.73	0.71	0.68	0.60	0.669
	Planctomycetota	0.72	0.50	0.74	0.68	0.862
	Verrucomicrobiota	0.55	0.61	0.56	0.44	0.928
	Campilobacterota	0.30	0.34	0.32	0.50	0.760
	Bacteria_WPS-2	0.21	0.37	0.11	0.17	0.105

Supplementary table 2: Top 10 bacterial classes in the investigated body-sites. Ranked according to their total abundance in the respective intestinal segment

Body site	Species	Relative abundance (%)				p-value
		Treated	± SEM	Control	± SEM	
Jejunum	*Bacilli*	34.68	20.00	41.82	26.53	0.312
	Clostridia	35.94	35.88	32.35	24.03	0.680
	Gammaproteobacteria	21.60	23.96	13.03	12.34	0.131
	Bacteroidia	4.35	8.58	4.86	10.35	0.857
	Actinobacteria	4.07	3.10	3.95	2.65	0.887
	Negativicutes	3.31	4.04	3.32	4.62	0.994
	Unassigned	2.90	4.15	2.74	4.92	0.903
	Campylobacteria	1.69	2.69	2.32	5.84	0.647
	Fusobacteriia	0.55	2.50	0.42	1.06	0.814
	Chlamydiae	0.34	0.68	0.34	1.03	0.978
Ileum	*Bacilli*	25.81	16.18	35.08	22.47	0.119
	Gammaproteobacteria	25.60	18.91	20.24	18.96	0.343
	Clostridia	23.02	12.92	22.41	14.70	0.882
	Bacteroidia	6.11	6.40	5.26	7.64	0.686
	Negativicutes	4.00	3.88	4.51	6.16	0.742
	Actinobacteria	3.34	3.59	3.97	3.25	0.537
	Campylobacteria	2.97	4.39	2.34	4.45	0.592
	Unassigned	2.69	2.47	2.60	2.38	0.898
	Fusobacteriia	2.73	4.82	1.37	2.51	0.231
	Chlamydiae	1.55	2.80	0.89	1.48	0.323
Caecum	*Clostridia*	36.72	4.20	36.27	6.79	0.790
	Bacilli	26.13	7.59	26.31	5.36	0.926
	Bacteroidia	23.04	4.59	24.66	5.21	0.271
	Negativicutes	4.83	2.21	5.66	3.44	0.342
	Gammaproteobacteria	1.56	1.50	1.98	1.55	0.361
	Campylobacteria	1.26	3.59	1.39	3.95	0.908
	Actinobacteria	1.37	0.74	1.01	0.68	0.093
	Coriobacteriia	0.79	0.31	0.82	0.47	0.802
	Verrucomicrobiae	0.97	3.35	0.00	0.00	0.171
	Spirochaetia	0.75	1.27	0.29	0.58	0.115
Colon	*Clostridia*	33.59	5.60	35.54	3.29	0.152
	Bacilli	23.14	6.09	21.71	3.97	0.346
	Bacteroidia	19.48	5.34	19.97	3.60	0.715
	Negativicutes	4.31	2.26	4.10	2.09	0.746
	Gammaproteobacteria	2.23	2.89	2.45	2.63	0.783
	Planctomycetes	1.47	1.35	1.15	1.48	0.439
	Spirochaetia	1.48	1.98	0.86	0.64	0.160
	Actinobacteria	0.70	0.44	0.64	0.54	0.707
	Desulfuromonadia	0.72	1.05	0.51	0.49	0.396
	Coriobacteriia	0,57	0,50	0,54	0,21	0.799
Swab	*Clostridia*	35.87	5.50	39.63	4.20	**3.14E-04**
	Bacteroidia	24.96	3.95	25.41	4.42	0.600
	Bacilli	19.81	3.31	17.11	3.90	**5.26E-04**
	Negativicutes	3.88	2.16	2.92	1.76	**0.020**
	Gammaproteobacteria	2.71	2.93	2.06	1.94	0.205
	Actinobacteria	1.74	1.14	1.07	0.75	**0.001**
	Spirochaetia	0.92	0.94	1.34	1.22	**0.042**
	Planctomycetes	0.72	0.50	0.74	0.68	0.868
	Desulfuromonadia	0.59	0.72	0.55	0.61	0.800
	Kiritimatiellae	0.43	0.34	0.50	0.41	0.381

Supplementary table 3: Top 10 bacterial orders in the investigated body-sites. Ranked according to their total abundance in the respective intestinal segment

Body site	Species	Relative abundance (%)				p-value
		Treated	± SEM	Control	± SEM	
Jejunum	*Lactobacillales*	30.13	22.03	30.51	22.06	0.953
	P.T.	20.17	19.92	16.44	13.87	0.462
	Clostridiales	10.49	10.56	9.27	9.95	0.690
	Erysipelotrichales	6.85	4.99	10.06	10.49	0.201
	Pasteurellales	10.62	18.06	3.14	4.31	**0.059**
	Bacteroidales	4.96	11.52	4.68	9.49	0.929
	Burkholderiales	4.94	8.78	4.36	8.70	0.824
	Enterobacterales	4.36	8.38	4.72	7.40	0.877
	Bifidobacteriales	4.42	3.86	3.90	2.97	0.605
	V.S.	3.58	5.31	3.18	4.10	0.776
Ileum	*Lactobacillales*	19.59	15.94	26.85	20.07	0.184
	Enterobacterales	10.69	11.92	12.60	19.36	0.692
	P.T.	12.45	15.34	11.25	13.32	0.777
	Clostridiales	7.97	7.93	7.41	6.73	0.797
	Pasteurellales	10.24	17.18	3.31	4.60	**0.068**
	Erysipelotrichales	6.41	5.78	8.01	7.13	0.410
	Bacteroidales	5.59	5.44	5.82	10.90	0.931
	Burkholderiales	5.12	5.55	4.30	5.64	0.626
	V.S.	3.36	3.43	5.34	10.85	0.419
	Bifidobacteriales	3.09	3.80	4.09	3.91	0.382
Caecum	*Bacteroidales*	23.04	4.60	24.66	5.21	0.270
	Lactobacillales	19.10	8.64	18.41	6.46	0.757
	Lachnospirales	13.10	2.28	13.29	3.50	0.830
	Oscillospirales	13.35	2.20	13.39	2.05	0.951
	Erysipelotrichales	6.65	2.86	7.61	3.10	0.284
	P.T.	5.75	3.42	4.51	4.69	0.314
	V.S.	3.49	2.39	4.37	3.20	0.300
	Clostridiales	1.84	1.31	2.56	3.62	0.380
	Acidaminococcales	1.34	0.83	1.29	1.04	0.853
	Campylobacterales	1.26	3.59	1.39	3.95	0.908
Colon	*Bacteroidales*	19.48	5.33	19.97	3.60	0.712
	Lactobacillales	18.67	6.64	15.91	4.41	0.102
	Oscillospirales	9.76	2.28	10.02	1.96	0.676
	Lachnospirales	8.57	2.35	9.54	2.57	0.190
	P.T.	7.92	3.43	8.63	3.05	0.460
	Clostridiales	5.89	3.85	5.53	3.67	0.749
	Erysipelotrichales	4.04	1.60	5.44	1.30	**0.002**
	V.S.	3.30	2.26	3.03	2.11	0.677
	Aeromonadales	1.67	2.78	2.00	2.64	0.681
	Pirellulales	1.47	1.35	1.15	1.48	0.439
Swab	*Bacteroidales*	24.95	3.97	25.41	4.32	0.593
	Lactobacillales	16.99	3.55	13.87	4.21	**2.06E-04**
	Clostridiales	13.26	4.12	15.73	4.02	**0.004**
	P.T.	8.99	4.79	10.13	3.71	0.200
	Oscillospirales	6.32	1.90	6.54	1.57	0.540
	Lachnospirales	6.15	2.24	6.00	2.15	0.748
	V.S.	3.31	2.09	2.29	1.77	**0.012**
	Erysipelotrichales	2.32	0.75	2.77	0.67	**0.003**
	Aeromonadales	2.39	3.00	1.71	1.97	0.191
	Spirochaetales	0.92	0.94	1.39	1.21	**0.042**

Supplementary table 4: Top 10 bacterial families in the investigated body-sites. Ranked according to their total abundance in the respective intestinal segment

Body site	Species	Relative abundance (%)				p-value
		Treated	± SEM	Control	± SEM	
Jejunum	*Lactobacillaceae*	18.90	16.21	19.38	15.30	0.919
	Peptostreptococcaceae	18.90	15.80	19.01	16.99	0.982
	Clostridiaceae	9.10	6.64	8.78	7.82	0.884
	Streptococcaceae	8.33	10.96	9.66	10.60	0.678
	Pasteurellaceae	8.44	13.81	3.84	5.74	0.144
	Erysipelotrichaceae	4.37	3.86	5.62	4.08	0.293
	Enterobacteriaceae	4.15	9.29	3.90	6.55	0.916
	Bifidobacteriaceae	3.96	3.48	3.86	2.81	0.916
	Prevotellaceae	2.93	5.78	3.58	6.30	0.716
	Alcaligenaceae	2.01	3.05	2.09	5.05	0.969
Ileum	*Lactobacillaceae*	11.47	12.05	17.59	18.71	0.199
	Enterobacteriaceae	10.15	13.42	11.33	15.95	0.788
	Peptostreptococcaceae	10.77	8.10	11.39	14.16	0.859
	Streptococcaceae	7.97	10.04	9.29	9.12	0.641
	Clostridiaceae	7.28	5.94	7.02	5.99	0.883
	Pasteurellaceae	9.14	14.19	3.42	4.88	**0.073**
	Erysipelotrichaceae	4.74	3.82	4.40	4.06	0.777
	Prevotellaceae	4.01	4.43	4.21	6.46	0.902
	Bifidobacteriaceae	3.09	3.57	3.72	2.97	0.514
	Lachnospiraceae	2.61	3.12	2.45	3.76	0.873
Caecum	*Prevotellaceae*	16.99	4.60	19.86	5.05	**0.051**
	Lachnospiraceae	13.10	2.29	13.29	3.50	0.830
	Streptococcaceae	9.84	5.68	11.54	6.51	0.353
	Ruminococcaceae	9.87	3.05	9.93	2.57	0.937
	Lactobacillaceae	9.22	4.81	6.87	4.64	**0.008**
	Erysipelotrichaceae	6.35	2.78	7.14	3.11	0.372
	Peptostreptococcaceae	5.58	3.35	4.37	4.52	0.312
	Muribaculaceae	4.10	2.12	2.46	0.97	**0.002**
	Selenomonadaceae	1.74	1.00	2.56	2.27	0.131
	Oscillospiraceae	2.15	1.50	1.74	1.33	0.323
Colon	*Streptococcaceae*	12.07	5.97	11.28	4.05	0.599
	Prevotellaceae	10.14	4.21	11.31	4.38	0.360
	Lachnospiraceae	8.57	2.34	9.54	2.57	0.189
	Peptostreptococcaceae	7.61	3.43	8.33	3.00	0.447
	Ruminococcaceae	5.99	2.54	6.10	2.09	0.867
	Clostridiaceae	5.89	3.85	5.53	3.67	0.752
	Lactobacillaceae	6.59	3.47	4.63	2.64	**0.036**
	Muribaculaceae	5.72	2.25	5.09	1.34	0.244
	Erysipelotrichaceae	3.70	1.45	5.04	1.38	**0.003**
	Oscillospiraceae	2.37	0.94	2.38	0.77	0.997
Swab	*Prevotellaceae*	16.96	4.13	16.61	4.20	0.689
	Clostridiaceae	13.26	4.11	15.72	4.03	**0.004**
	Lactobacillaceae	9.58	3.91	7.32	4.06	**0.007**
	Peptostreptococcaceae	6.72	2.06	8.02	1.75	**0.001**
	Streptococcaceae	7,15	3.06	6.23	2.62	0.124
	Lachnospiraceae	6.15	2.24	6.00	2.15	0.748
	Muribaculaceae	4.20	1.56	4.33	1.58	0.699
	Ruminococcaceae	3.30	1.58	3.30	1.43	0.991
	Rikenellaceae	1.98	0.87	2.43	1.30	**0.051**
	Erysipelotrichaceae	1.83	0.59	2.31	0.56	**1.00E-04**

Supplementary table 5: Top 10 bacterial genera in the investigated body-sites. Ranked according to their total abundance in the respective intestinal segment

Body-site	Species	Relative abundance (%)				p-value
		Treated	± SEM	Control	± SEM	
Jejunum	*Lactobacillus*	18.90	16.20	19.38	15.30	0.919
	Romboutsia	10.08	9.02	11.39	11.40	0.670
	Clostridium_sensu_stricto	9.09	6.64	8.76	7.82	0.876
	Streptococcus	8.33	10.96	9.66	10.61	0.678
	Terrisporobacter	8.39	9.26	7.21	7.34	0.633
	Actinobacillus	7.44	13.35	3.33	5.15	0.172
	Turicibacter	4.02	3.91	5.39	4.19	0.260
	Escherichia-Shigella	4.15	9.27	3.90	6.55	0.917
	Bifidobacterium	3.28	2.94	3.00	2.22	0.719
	Achromobacter	2.01	3.05	2.06	5.05	0.969
Ileum	*Lactobacillus*	11.47	12.05	17.59	18.71	0.199
	Escherichia-Shigella	10.15	13.41	11.33	15.95	0.787
	Streptococcus	7.97	10.04	9.29	9.12	0.641
	Clostridium_sensu_stricto	6.93	5.46	6.49	5.56	0.786
	Romboutsia	6.18	5.52	6.82	9.33	0.783
	Actinobacillus	8.46	13.45	2.98	4.51	**0.070**
	Turicibacter	4.15	3.89	3.99	4.17	0.892
	Terrisporobacter	4.08	3.70	4.30	6.90	0.897
	Bifidobacterium	2.61	3.13	3.03	2.59	0.623
	Prevotella	2.31	2.50	2.79	4.26	0.645
Caecum	*Streptococcus*	9.84	5.68	11.54	6.51	0.353
	Lactobacillus	9.22	4.81	6.87	4.64	0.098
	Prevotella	5.39	2.98	9.14	4.14	**0.001**
	Subdoligranulum	6.98	2.83	6.61	3.04	0.673
	Alloprevotella	6.47	3.52	6.46	2.99	0.992
	Muribaculaceae	4.10	2.12	2.46	0.97	**0.002**
	Romboutsia	3.75	2.27	2.68	2.50	0.137
	Turicibacter	3.30	1.98	3.38	2.57	0.909
	Blautia	2.55	1.16	2.75	0.90	0.524
	Faecalibacterium	1.54	1.48	2.35	1.56	**0.079**
Colon	*Streptococcus*	12.07	5.97	11.28	4.05	0.599
	Clostridium_sensu_stricto	5.88	3.83	5.51	3.59	0.738
	Lactobacillus	6.59	3.47	4.63	2.64	**0.036**
	Terrisporobacter	5.41	2.51	5.86	2.42	0.539
	Muribaculaceae	5.72	2.24	5.06	1.34	0.232
	Prevotella	4.40	2.88	5.82	2.93	0.105
	Subdoligranulum	3.56	1.97	3.55	1.61	0.973
	Turicibacter	1.69	1.07	2.45	1.27	**0.035**
	Romboutsia	1.92	1.01	2.17	1.14	0.444
	Alloprevotella	1.51	1.48	1.88	0.91	0.308
Swab	*Clostridium_sensu_stricto*	13.24	4.11	15.70	4.03	**0.004**
	Prevotella	10.47	3.42	10.07	3.51	0.581
	Lactobacillus	9.58	3.91	7.32	4.06	**0.007**
	Streptococcus	7.15	3.06	6.23	2.62	0.124
	Terrisporobacter	5.27	1.66	6.17	1.43	**0.006**
	Muribaculaceae	4.19	1.55	4.29	1.59	0.743
	Prevotellaceae_NK3B31	2.41	1.11	2.71	1.35	0.246
	Rikenellaceae_RC9	1.78	0.81	2.18	1.18	**0.064**
	Megasphaera	1.84	1.12	1.10	0.94	**7.40E-04**
	Succinivibrionaceae_UCG-001	1.70	2.61	1.14	1.71	0.224

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Chapter 5: General discussion and conclusion

For many years, the focus on the nutrition has been on optimizing the composition of energetic substances, vitamins, and other nutritive elements in order to improve quantitative traits and to avoid diseases and deficiency symptoms in animal production. In animal breeding, breeding goals are defined using theoretical models, but environmental influences such as diet are often given to less an argumentative value. In general, in conventional animal production the quantitative traits have a predominate status. As a result of current research, improving animal health is given greater importance (Merks et al., 2012). Improving animal health and welfare will also enhance customers' acceptance for animal products. Another opportunity to improve animal health is, as it is shown in this experiment and several formers studies, the usage of nutritional effects on pigs genetics. Those non-energetical ligand-like side effects can positively stimulate intestinal integrity and the role of the microbiome should not be underestimated (Wei et al., 2017; Ghormade et al., 2011; Muller and Kersten, 2003). In the following, discussion key points from chapters two to four are discussed in a general context considering OEOs´ effects on pigs´ small intestinal integrity traits. For this, candidate genes have been investigated with the qPCR technique in piglets with an average weight of 12.3±0.9 kg and a 'GeneChip Porcine Gene 1.0 ST' array-based transcriptome analysis was performed in finishing pigs with an average weight of 111.1±10.9 kg. Additionally, 16S rRNA sequencing technique has been used to reveal OEOs´ effects on finishing pigs´ intestinal microflora. The complete blood counts and carcass characteristics were used as auxiliary traits to grasp animal health and productivity in general. The results of these studies provide new insights of OEOs´ multifaceted effects on pigs´ genetics with an average age of four weeks or six months and on the microbial composition in five different intestinal segments. The studies presented here are *in-vivo* approaches. However, the general discussion will also cover *in-vitro* studies to discuss studies´ results in a broader context.

5.1 Oregano essential oil, principal issues

The feed supplement which has been used in this study, DOSTO® powder (DOSTO® FARM, Westerstede, Germany), is obtained from the essential oil of *Origanum vulgare* subsp. *hirtum* var. Vulkan. When comparing studies on the subject of OEO and other PBLCs, it must be noted that oils´ composition depends on several environmental-, and species related factors (Leyva-López et al., 2017; Falco et al., 2013; Azizi et al., 2009). Especially in plants of the *Lamiaceae* family, the 'mode of action' varies depending on the composition of the essential oil (Yen et al., 2015). Additionally, the extraction and especially the drying process affect the ingredients in OEO (Novák et al., 2011). Some studies, showing OEOs´ effects to modulate pigs´ genetics, are lacking basic information about plant origin, extraction method or oils´ composition, which are needed to provide comparability. The studies´ OEOs´ functional components consist largely of the monoterpenes, carvacrol and thymol, and β-caryophyllene. Pure substances, such as carvacrol and thymol, are often used in very specialized *in-vitro* experiments or *in-vivo* models to modulate gene expression (Wei et al., 2017; Zduńczyk and Pareek, 2009). The transferability of such results from pure substances to PBLCs is not always guaranteed due to the aforementioned synergistic effects (Pu et al., 2020; Pezzani et al., 2017; Burt, 2004). In this study, we were not able to allocate the singular effects to OEOs´ ingredients. Thus, we are only able to detect the combined effects in OEO on the aforementioned traits. However, because of higher costs, usage of pure substances in conventional animal production are only conditionally suitable. Additionally, they need to be formulated in such a way that they actually reach the desired target organs (Stevanović et al., 2018). The authors summarized, PBLCs need to be microencapsulated or at least dissolved in some oil carriers to improve the physio-chemic stability (e.g. oxidative stability, thermo stability, photo stability, shelf-life, and biological activity). Without this specific protection of the PBLCs, they will not/or just limited reach the desired target organ (Stevanović et al., 2018). Additionally, it was shown by Cairo et al. (2018), encapsulation is beneficial for natural substances with a strong odour, which usually negatively affects animals´ acceptance for the feed and by this reduces the feed intake. Especially young animals, such as weaning piglets tend to show an anticipatory behaviour for very aromatic substances (Cairo et al., 2018; Stevanović et al., 2018). However, as explained in chapter two and three, in this experiment we did not

recognized a reduced acceptance for the OEO supplemented feed. For upcoming experiments, however, ethological investigations should also be carried out and the water uptake must necessarily be recorded. No data was collected for this, but the animal keepers reported a more frequent visit to the nipple drinkers of the OEO supplemented pigs compared to the control. Nevertheless, *in-vitro* studies and the investigation of pure substances in general are crucial to reveal the manifold cellular mechanisms in response to such natural stimuli.

5.2 Limitations in OEOs´ usage and inflammatory processes

Due to the low bioavailability, the worse solubility in water and the high concentrations of carvacrol and thymol, which are required to achieve therapeutic effects, the effectiveness of these two monoterpenes is said to be limited in animal nutrition (Suntres et al., 2015; Mastelic et al., 2008). In contrast to these previous results, despite the use of a moderate dosage of OEO of 112.5 mg/kg, significant effects on intestinal integrity and the microbial composition were found in the present study. When administering PBLCs attention must be paid to their limitation as feed additives due to side effects and potential cytotoxicity (Zou et al., 2016). However, it was shown by Mastelic et al. (2008), OEOs´ core component, carvacrol, exerts limited cytotoxic effects and Suntres et al. (2015) summarized, that consumption of carvacrol is generally save. Just high dosages, such as 690 mg/kg in the feed of mice exerted a lethality enhancing effect. However, the knowledge about mutagenicity and genotoxicity is still limited (Suntres et al., 2015; Andersen, 2006). In previous studies, OEOs´ anti-inflammatory activities are predominantly positive described (Han and Parker, 2017; Cho et al., 2012; Ocana-Fuentes et al., 2010). The essential oil from *O. vulgare* significantly reduced inflammatory biomarkers, such as *monocyte chemoattractan protein-1* (*MCP-1*), the *vascular cell adhesion molecule-1* (*VCAM-1*) and the *intracellular cell adhesion molecule-1* (*ICAM-1*) in human neonatal fibroblasts, additionally showing OEOs´ anti-inflammatory activities (Han and Parker, 2017). It was concluded by Han and Parker (2017), that OEO modulated the global gene expression and thereby several inflammatory-, tissue remodelling- and cancer signalling pathways. Especially, the *monokine induced by gamma interferon* (*MIG*) ligand was significantly inhibited at both the protein and the gene expression level. The researchers however used a significant

lower dosage of OEO (0.0037%), which was the highest tested concentration without cytotoxic effects in their *in-vitro* experiment. For comparison, 7.5% pure OEO has been used in this experiment in pigs. In the experiment, described in chapter two, we found significant reductions of the *TNF-α* and *IL-1β* expressions after OEO supplementation in the small intestine of piglets with an average weight of 12.3±0.9 kg. As part of the *NF-κB* pathway, both mediators are involved in several inflammatory processes. Inflammatory mediators, such as cytokines are recruited by inflammatory processes, which are conventional biological responses to pathogenic invasion, physical and chemical stress, and tissue damage (Leyva-López et al., 2017; Medzhitov, 2008). OEO showed the ability to inhibit *NF-κB* expression after bacterial stimulation of the associated inflammatory pathway (Paur et al., 2008). Of course, in case of chronic inflammation or in general by overproduction of proinflammatory mediators, a reduction of these by OEOs´ ingredients could be beneficial for the animal organism (Li et al., 2015; Liang et al., 2014; Hart et al., 2000). Additionally, the question remains open at which stage of the pigs´ life OEO should be given. As it was indicated by the aforementioned results and reports, piglets and finishing pigs reaction to OEO supplementation is not always identical.

5.3 The intestinal integrity in dependence of OEO

The sections of the intestine examined in this study differ in their lining with villi, and crypts. Intestinal morphology is affected by digestion enzymes (Jolma et al., 1980) and PBLCs, such as OEO (Zou et al., 2016). The jejunum is supplied with larger arteries, but the ileum contains a significantly higher number and the immunologic very important lymphoid clusters, the Peyers´ patches, which can be found predominantly here (Conley et al., 2010; Reynolds and Morris, 1983). In a study with five to six-week-old lambs, it was shown that the mucosal immunity and thereby the intestinal integrity of the small intestinal segments, jejunum and ileum, react differently depending on antigen stimulation, such as nutritional effects, and revealed a higher immunologic competence of the jejunal tissue in ruminants (Mutwiri et al., 1999). Kroismayr et al. (2005) showed, PBLCs negatively affected the surface of those clusters in the ileum and reduced the expression of the multifunctional transcription factor *NF-κB*. The authors explained the decreased immunologic activity by the antimicrobial actions of essential oils in the small

intestinal segments. However, in this study (chapter four), OEO supplementation had limited effects on the bacterial genera in the jejunum and the ileum. As it was shown in chapter two and three and by Swaggerty et al. (2020) (experiment in broilers), the different intestinal segments react differently to OEO supplementation which is partially explained by the major digestion of those substances in the mid-jejunum. In the jejunum we found 93 genes and in the ileum 60 genes to be differentially expressed. The gene expression between both small intestinal segments was significantly different ($p<0.001$, chapter three). The reduced effect on the ileum could be explained by the fact, that in this study OEO was supplemented in a fine powder like structure. As it was summarized by Stevanović et al. (2018), the supplementation could have lost a part of its´ activity by physio-chemic processes, which can be attributed by the improper protection of the OEO. Another problem with powder like supplements is the segregation within the feed (Witkowska et al., 2013). Therefore, to prevent segregation pigs were fed with ground feed in this experiment. Because of the very fine structure of the supplemented powder, the standard diet was ground to a particle size ranging from 300 µm up to 2000 µm. As is was summarized by Vukmirović et al. (2017), feed particle size < 400 µm negatively affects intestinal integrity by increasing ulcerations and thereby negatively affecting the daily feed intake. Additionally, feed particle size affects the intestinal microbial composition. It was shown, larger particles enhance the abundance of lactic acid producing bacteria in the small intestine and reduce the counts of *Enterobacteriaceae* in the caecum of pigs (Canibe et al., 2005). Another effect on the gene expression could have been the effects of OEO on small intestines´ morphology (Zou et al., 2016; Wei et al., 2015). As it was shown in chapter two, we did not find any effects on the small intestinal morphology nor of inflammatory processes in piglets' intestinal tissue in both groups. Additionally, there were just minor effects on pigs´ carcass quality and quantity (chapter three). This shows, the OEO supplementation and the small feed particle sizes had no negative effects on the most important commercial trait in pig fattening (Simitzis et al., 2010). For further studies, encapsulated OEO should be used to reach the caudal segments of the intestine and to prevent the intestinal integrity of pigs from being damaged by food particle sizes that are too small. Average particle sizes between 500 and 1600 µm are recommended (Cappai et al., 2013).

The separation of the bodies´ interieur from the environment in the intestinal tract is of great importance for animals. Although the selective permeability of the intestinal tissue enables the absorption of nutrients, it can also enable pathogens to infiltrate the organism. However, another approach to improve pigs intestinal integrity could be to strengthen the intestinal epithelial barrier by improving tight junction proteins. By this, pathogens and other stressors cannot easily invade the organism and inflammatory processes will be reduced. An enhanced intestinal permeability is an indicator for malfunction of the barrier (Shin et al., 2006). The intestinal integrity depends on a complex interaction of several proteins, which are responsible for the cell-cell connection (Suzuki, 2013). In this study, we found the expression of *ZO-1*, a protein which connects tight junctions with the cytoskeleton (Harhaj and Antonetti, 2004), to be significantly up-regulated in the OEO treated group in both small intestinal segments investigated. In this study, no effects on further tight junction proteins, such as *occludin* and *claudin* have been found. However, Zou et al. (2016) reported a positive effect of OEO on the expression of *ZO-1* as well as *occludin*. Pu et al. (2018) found OEO to prevent *occludin* and *claudin* to be dysregulated by *E. coli* challenge but they did not find any effect on *ZO-1*. After the supplementation of carvacrol and thymol, *ZO-1* and *occludin* expression was significantly reduced in piglets (Wei et al., 2017). Of course, age dependent differences in pigs´ reaction to such a supplementation must be considered. It was shown by Amrik and Bilkei (2004), OEO improves the renewal rate of intestinal cells, such as enterocytes and thereby improve the intestinal barrier. A low expression of inflammatory genes, e.g. *IFNs*, was shown to improve the intestinal barrier (Al-Sadi et al., 2009; Pié et al., 2004) and Pu et al. (2020) found the cytokines *Tκ-α* and *IL-1β*, which disrupt the intestinal integrity, to be reduced by OEO. In this study, type-I-interferons *IFN-β1, -ε, -ω*, which are essential for the innate and the adaptive immune response in case of viral infection, have been found to be affected by OEO supplementation in pigs´ small intestine. The transcriptome analysis of OEO supplemented pigs and its´ respective control group showed higher expression of *IFN-β1* and *IFN-ω* in the OEO supplemented group whereas the expression of *IFN-ε* was reduced. We did not find any significant differences in the aforementioned mediator *TNF-α* in the fattenings pigs´ transcriptome analysis described in chapter three. However, as it was shown in chapter two, *TNF-α* and *IL-1β* expression was significantly reduced in piglets jejunum after OEO

supplementation. An age depend effect must be assumed here. Additionally, the duration of the supplementation and the timepoint of sampling, especially for microbial investigation are also important (Canibe et al., 2005). The migration of immune cells is partially regulated by chemokines such as *CCL21* (Palomino and Marti, 2015). As it was shown in chapter three, the expression of the aforementioned chemokine was significantly enhanced after OEO supplementation in the jejunum. However, the expression of surface markers of immunologic cells, such as $CD4^+$ and $CD8^+$ cells, were significantly reduced after OEO supplementation in piglets (chapter two). Only in the ileum of OEO fed piglets, the relative abundance of *CD8*$^+$ marker gene was enhanced. In the blood of finishing pigs, those cells were up-regulated after OEO supplementation (Walter and Bilkei, 2004). However, Ariza-Nieto et al. (2011) found no effect on the T-cells in OEO fed pigs. Nevertheless, in this study, OEOs´ effects on intestinal T-cell surface marker expression were significant. In the study of Cappelli et al. (2021) OEOs´ effects on peripheral mononuclear cells (PBMCs) have been investigated. They revealed diverse modulations in the gene expression of mediators, such as *TNF-α*, *IL-1β*, *IL8* etc. by qPCR technique. This suggests that dietary OEO can have an effect on the blood in general. In contrast, in the present study, the composition of immunologic cells in the blood was not affected by OEO supplementation (compare chapter two and three). With those experiments, we showed new effects on pigs´ intestinal gene expression after a long-term OEO supplementation. However, the influence of OEO on the activity of genes associated with the intestinal barrier and the mucosal immunity has not yet been conclusively clarified and further research is needed.

5.4 OEOs´ modulation of the intestinal microflora

Previous studies have successfully shown for several times, that OEO exerts an antimicrobial effect on specific/selected microorganisms (Chowdhury et al., 2018; Wei et al., 2017; Zou et al., 2016; Rodriguez-Garcia et al., 2016; Helander et al., 1998). In living beings, however, it is not only important that specific microorganisms are repressed/killed, but the determination of which organisms are taking their place is much more essential. Therefore, in this work we tend to talk of a microbiome-modulating effect due to OEO instead of pure antimicrobial effects. As it is shown in chapter three, OEOs´ effects on the microbial composition can be displayed at several

phylogenetic levels. For an initial overview of OEOs´ effects on the microbial composition, we have only shown the top 10 clusters at each level. Of course, the lower the level that is considered, the more modulations can be found.

The intestinal microbiome directly affects the intestinal immune system and by this the entire animal health (Schachtschneider et al., 2013). Due to the fact that the auxiliary characteristics (carcass traits and blood characteristics, compare chapter three), which have been examined, showed only minor changes in dependence of OEO supplementation, it is not possible for us to determine whether the influence of OEO on the microbial composition can be assessed as positive in this study. For this, the investigated microbial composition has been discussed with findings from the literature (compare chapter four). Members of the *Lactobacillaceae* family are beneficial for the animal health, due their ability to digest plant-derived mono- and disaccharides, which otherwise would not be digestible for monogastric animals (Guevarra et al., 2018; Schwab and Gänzle, 2011). We found higher relative abundances of those bacteria in the large intestine associated samples. However, in our first experiment, where key-microbiota were investigated in the chyme of piglets, we found no differences in the *Lactobacillus* species in dependence of OEO supplementation. In chapter two, short-chain fatty acids producing *Faecalibacterium prausnitzii* was significantly enhanced in piglets small intestinal chyme after OEO supplementation. In the fattening pigs small intestinal samples, the *Faecalibacterium* genera was not in the top 10 genera investigated. However, in caecum samples of the oregano fed group, lower relative abundances of this genus have been found. The intestinal microbial composition is subject to constant change (Yatsunenko et al., 2012). Therefore, it is not surprising that the two stages of life investigated here show inconsistent results. In this study, we found the Gram-negative and pathogenic *Actinobacillus* from the *Pasteurellaceae* family to be enhanced by OEO supplementation. A low level of members of *Enterobacteriaceae* family, which are associated with intestinal inflammation, is important for a healthy intestinal tract in pigs (Hall et al., 2021). In this study, there was no effect on this family in pigs´ small intestine and we did not find this bacteria family in the large intestine associated samples. One of the remaining questions is, how the microbial colonization influences the gene expression of an organism and whether, for example, the transcription profile of the intestinal mucosa directly influences the microbial

community of the intestine. Accordingly, it would also be interesting to see whether the intestinal flora has a direct effect on the expression of tight junction proteins, such as *ZO-1*. To understand host-microbiota interactions, some research has already been performed (Bonder et al., 2016; Dąbrowska and Witkiewicz, 2016). However, those interactions in pigs´ intestine are still not clear, especially bringing additional dietary effects, such as OEO into account.

Microbiota sequencing studies by itself provide new insights in the microbial community without the limitation of culturing techniques. The chip-based transcriptomic profiling revealed several modulations in pigs´ intestinal cells depending on OEO supplementation. However, diverse environmental factors affect the expression of genes on the transcriptional and posttranscriptional level. Only in connection with other members of the so called 'omics' (proteomics, metabolomics etc.) it is possible to substantiate postulated effects, which have been advertised by the transcriptional modulations. Therefore, we propose a combined approach in which genomic and metagenomic data are examined under the influence of PBLCs, such as OEO in a genome wide association study (GWAS). The combination of the animals´ own genetic data and the knowledge about the microbiome enables an approximate holistic approach to explain nutritional effects on the intestinal integrity.

5.5 Conclusion

We performed a comparative feeding experiment to reveal the effect of oregano essential oil on pigs´ intestinal integrity and its´ microbial composition. For this, piglets have been sacrificed to investigate OEOs´ impact on the intestinal gene expression after a short supplementation time. For further analysis of long-term activities of OEO on pigs´ intestine, pigs have been supplemented for the whole fattening period and the respective slaughterhouse material has been used for transcriptomic and 16S rRNA sequencing analysis. Additionally, carcass traits were evaluated to investigate if the supplementation affects the carcass quality. Complete blood counts have been used to monitor pigs´ health across the experimental period. In this study, we found several modulations in the transcriptomic profiles of piglets and finishing pigs. Especially, biological processes and genes associated with the intestinal immunity and its´ barrier function were affected by OEO supplementation. Additionally, in response to oral OEO supplementation we found the expression of genes involved in inflammatory processes to be affected in the spleen. These actions indicate an effect that promotes intestinal integrity. There were only minor impacts on the carcasses and the complete blood counts of the pigs investigated. We found the intestinal microflora to be highly heterogenous. Interestingly, the major modulation depending on OEO supplementation was found in the large intestine associated samples and only minor effects have been found in the jejunum and the ileum. Right now, we have no explanation for OEOs´ 'skipping' of the small intestine. However, especially the increasement of members of the *Lactobacillaceae* family in the caecum, colon and in rectal swab samples is an indicator for the positive regulation of pigs´ intestinal microbiota composition.

In general, PBLCs such as OEO continue to be of interest due to their diverse benefits in animal production. These advantages are not necessarily reflected in production traits, but in part in health characteristics which are difficult to determine. In continuation of the current research as well as to resolve some questions yet to be answered, several experiments could be followed:

- The determination of the host-microbiome-diet interactions by a genome-wide association study (GWAS)
- Investigation of the reaction of different pig breeds on essential oil supplementation by whole genome sequencing
- Identification of transgenerational effects of OEO across several generations
- Direct comparative investigation of whole OEO and its´ functional ingredients in the same experiment to reveal the postulated synergism
- Feeding OEO in different forms (e.g. powder vs. encapsulated) to see if it charges influence on the small intestine

Chapter 6: References

For chapters one and five

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