The porcine intestinal microbiota: studies on diversity and dietary impact

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Universität Hohenheim

Institut für Nutztierwissenschaften, Prof. Dr. Jana Seifert

Institut für Mikrobiologie, Prof. Dr. Julia Fritz-Steuber

vorgelegt von

Katharina Burbach

aus *Esslingen*

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Dekan:	Prof. Dr. Uwe Beifuß
1. berichtende Person:	Prof. Dr. Jana Seifert
2. berichtende Person:	Prof. Dr. Julia Fritz-Steuber
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Zusammenfassung

Die Gesamtheit der mikrobiellen Gemeinschaften im Gastrointestinaltrakt wird als intestinale Mikrobiota bezeichnet und setzt sich vorwiegend aus Bakterien zusammen. Wechselwirkungen zwischen der Mikrobiota, dem Wirt und der Ernährung sind unerlässlich für ein gesundes und funktionelles Ökosystem im Darm. Das übergeordnete Ziel dieser Thesis war die Charakterisierung der intestinalen Mikrobiota des Schweines und Kenntnisse über die Auswirkungen unterschiedlicher Diäten zu erweitern.

Hochdurchsatz-Sequenzierung des 16S rRNA Gens erleichtert die Erforschung der taxonomischen Zusammensetzung der Mikrobiota. Allerdings können die jeweiligen Ergebnisse durch methodische Abweichungen beeinträchtigt sein. Aufgrund dessen werden im Rahmen dieser Thesis kommerzielle DNA Extraktions-Kits für ihre Eignung in der Analyse der Schweine-Mikrobiota bewertet. Die getesteten Extraktionen bringen Unterschiede in der DNA Quantität und Qualität hervor. Im Weiteren wird die DNA zur Aufklärung der Mikrobiota-Struktur, durch eine schnelle Fingerprinting-Methode (Terminales Restriktionsfragmentlängen-polymorphismus) und einem hochauflösenden Sequenzierungsansatz (Illumina Amplikon Sequenzierung), eingesetzt. Zwar variieren unterschiedliche variable Regionen des 16S rRNA Gens in der taxonomischen Auflösung, doch belegen Sequenzierungsanalysen eine gute Vergleichbarkeit von V1-V2 und V5-V6. Des Weiteren offenbaren die Mikrobiota-Profile eine hohe Übereinstimmung zwischen Fingerprinting und Sequenzierung, unterscheiden sich aber zwischen den unterschiedlichen DNA Extraktions-Kits. Basierend auf Kriterien der DNA Extraktion und der aufgeklärten Mikrobiota-Zusammensetzung, wird das FastDNA SPIN Kit for Soil für weitere Analysen der intestinalen Mikrobiota des Schweines empfohlen.

Diese methodischen Erkenntnisse werden anschließend angewendet, um die Auswirkungen durch verschiedene Diäten zu untersuchen. So zeigt Illumina Amplikon Sequenzierung der V1-V2 Region des 16S rRNA Gens unterschiedliche Mikrobiotastrukturen auf, wenn Diäten sich nur aus Roggen oder Triticale zusammensetzen. Neben der taxonomischen Analyse von Chymusproben aus dem Ileum und dem Kot, werden auch Konzentrationen von bakteriellen Metaboliten bestimmt. Zusammenfassend fördert Roggen eine erhöhte Abundanz von saccharolytischen Bakterien wie *Lactobacillus*, *Bifidobacterium* und *Prevotella* und führt zu höheren Konzentrationen von bakteriellen Metaboliten im Kot. Im Gegensatz dazu ist eine Triticale-Diät mit einer erhöhten Abundanz von *Clostridium* sensu stricto assoziiert, was ein erhöhtes cellulolytisches Potential der Mikrobiota andeutet.

Bei einem erhöhtem Rohproteingehalt (18%) in der Diät, im Vergleich zu einem geringeren Gehalt (14%), wird eine erhöhte *Lactobacillus*-Abundanz im Chymus des Ileums aufgezeigt. Der Gehalt des Rohproteins beeinflusst allerdings die Gesamtheit der Mikrobiota nicht signifikant. Auch zeigt die diätische Ergänzung von probiotischen *Bacillus* spp. keinen Effekt. Abschließend werden diese diätischen Effekte auf die Mikrobiota gemeinsam mit den Ergebnissen der Proteinverdaulichkeitsanalyse betrachtet.

Im Weiteren wird, anhand eines Fingerprinting-Ansatzes, ein Einfluss von Calcium und Phosphat in Kombination mit verschiedenen Proteinquellen in Chymusproben untersucht. Hier zeigt der Calcium-Phosphat Gehalt einen signifikanten Effekt auf die Mikrobiota im Chymus des Blinddarms. Die dafür verantwortlichen Bakterien werden durch einen Klonierung-Sequenzierung-Ansatz identifiziert. Ähnlich hierzu, weist die 16S rRNA Gen Sequenzierung von Kotproben einen signifikanten Einfluss des diätischen Calcium-Phosphors auf, ohne dabei auf bestimmte beeinflusste Bakterien hin zu deuten. Gemeinsam mit Ergebnissen eines Meta-Proteomik Ansatzes wird auf eine graduelle Anpassung an diätische Veränderungen hingewiesen und daher wird eine verlängerte Adaptationszeit von drei bis vier Wochen für Diät-Mikrobiota Studien empfohlen.

Diese Thesis enthält auch eine umfassende Analyse der Mikrobiota quer durch und entlang des Gastrointestinaltraktes von Ferkeln und untersucht hier vier verschiedene Stufen einer Rationszulage von Insektenlarvenmehl. Die Verfütterung von Insekten stellt eine alternative Quelle von Protein dar, wobei, im Vergleich zu einer Kontrolldiät, der erhöhte Chitingehalt auf eine potentielle Veränderung der Mikrobiota hinweist. Hier zeigt allerdings nur ein paarweiser Vergleich zwischen den Diäten einen signifikanten Einfluss auf die Mikrobiota in Chymus Proben aus dem Dünndarm. Diätische Zulage von 5% Insektenmehl erhöht die Abundanz von *Lactobacillus*, wohingegen die Kontrollgruppe *Bifidobacterium* fördert.

Abschließend heben die Ergebnisse dieser Thesis die Bedeutung einer Standardisierung innerhalb 16S rRNA Gen basierten Studien der intestinalen Mikrobiota des Schweines hervor. Des Weiteren wird die Notwendigkeit der Untersuchung verschiedener Probenentnahmestellen zusammen mit dem Einsatz von interdisziplinären Ansätzen aufgezeigt.

Abstract

The entirety of microbial communities within the gastrointestinal tract is referred to as intestinal microbiota and is predominantly composed of bacteria. Interactions between the microbiota, the host and the diet are essential for maintaining a healthy and functional intestinal ecosystem. The overarching aim of this thesis was the characterization of the porcine intestinal microbiota and further to enhance knowledge about the effects of varying diets.

High-throughput sequencing of the 16S rRNA gene facilitates exploration of the taxonomic composition of the microbiota. However, the respective findings may be impaired by methodological variations. Thus, within this thesis, commercial DNA extraction kits are evaluated for their suitability in porcine microbiota analysis. The tested extractions yield into variations of quantity and quality of DNA. The DNA extracts are further used to elucidate the structure of the microbiota by a rapid fingerprinting (Terminal Restriction Fragment Length Polymorphism) and high-resolution sequencing (Illumina amplicon sequencing). While different variable regions of the 16S rRNA gene vary in the taxonomical resolution, sequencing analyses exhibit a good comparability of the two regions V1-V2 and the V5-V6. Furthermore, the microbiota profiles reveal a high consistency by the fingerprinting and sequencing approach but are distinguished by the different DNA extraction kits. Based on criteria of DNA extraction and the depicted microbiota composition, it is recommended to use the FastDNA SPIN Kit for Soil for further analysis of porcine intestinal microbiota.

Subsequently, these methodological findings are applied to investigate the impact of varying diets. Illumina amplicon sequencing of the V1-V2 region of the 16S rRNA gene reveals different microbiota structures when diets are solely composed of rye or triticale. Besides the taxonomic analyses of ileal digesta and fecal samples, the concentrations of bacterial metabolites in feces are determined. In summary, rye promotes an increased abundance of saccharolytic bacteria like *Lactobacillus*, *Bifidobacterium,* and *Prevotella* and results in higher concentrations of bacterial metabolites in fecal samples. In contrast, a diet based on triticale is associated with an increased abundance of *Clostridium* sensu stricto, which may indicate an enhanced cellulolytic potential of the microbiota.

When the crude protein content is increased (18%), compared to a lower content (14%), an increased abundance of *Lactobacillus* is demonstrated in microbiota of ileal digesta samples. However, the content of crude protein did not affect the overall microbiota significantly. In addition, dietary supplementation with probiotic *Bacillus* spp. shows no effect. In conclusion,

these dietary effects on microbiota are considered together with results of a protein digestibility analysis.

Moreover, an impact of dietary calcium and phosphorus in combination with different sources of dietary protein is analyzed by fingerprinting approach of digesta samples. Here, the content of calcium-phosphorus shows significant effects on the microbiota of caecal digesta and the putative identities of discriminative variables are determined by a cloning-sequencing approach. Similar, 16S rRNA gene sequencing reveals a significant impact of dietary calcium-phosphorus on the overall fecal microbiota without indicating specific discriminating variables. In combination with the results of a meta-proteomic approach, a gradual adaptation on dietary changes is indicated and consequently, a prolonged adaptation time of three to four weeks is recommended for diet-microbiota studies.

This thesis includes a comprehensive analysis of the microbiota across and along the gastrointestinal tract of piglets and explores the dietary inclusion of four levels of insect larvae meal. Feeding insects represent an alternative source of dietary protein, whereby the increased content of chitin indicates a potential shift in microbiota composition compared to a control diet. However, in this case, the structural analysis demonstrates no effects on the overall microbiota's structure. However, a pairwise comparison between diets reveals significant effects on the microbiota of digesta samples of the small intestine. Dietary inclusion of 5% insect meal increases the abundance of *Lactobacillus*, whereas the control treatment promotes *Bifidobacterium*.

In conclusion, the results of the present thesis emphasize the importance of standardization within 16S rRNA gene based studies of the porcine intestinal microbiota. Furthermore, the necessity of studying various sampling sites combined with multidisciplinary approaches is demonstrated.

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Chapter 1 Introduction

1.1 The ecological environment of the porcine gastrointestinal tract

Microorganisms are capable to cope with extreme abiotic conditions and inhabit various ecosystems, ranging from aquatic habitats through the soil to the gastrointestinal tract (GIT) of animals. The community of microorganisms in a defined environment, like the GIT, is referred to as ‘microbiota’ (Marchesi and Ravel 2015). This collective term includes bacteria, archaea, fungi and viruses. Bacteria are the most abundant and as well most studied microbiota’s members (Cox et al. 2013). Recently, the number of bacterial cells in the human GIT has been estimated to $4x10^{13}$ (forty trillion), which indicates a bacteria-host cell ratio of around 1:1, emphasizing the significance in studying host-microbiota interactions (Sender et al. 2016). The monogastric structure of the human GIT is highly similar to the porcine GIT and pig models are suitable for translational research with a focus on nutrition and health (Heinritz et al. 2016; Zhang et al. 2013).

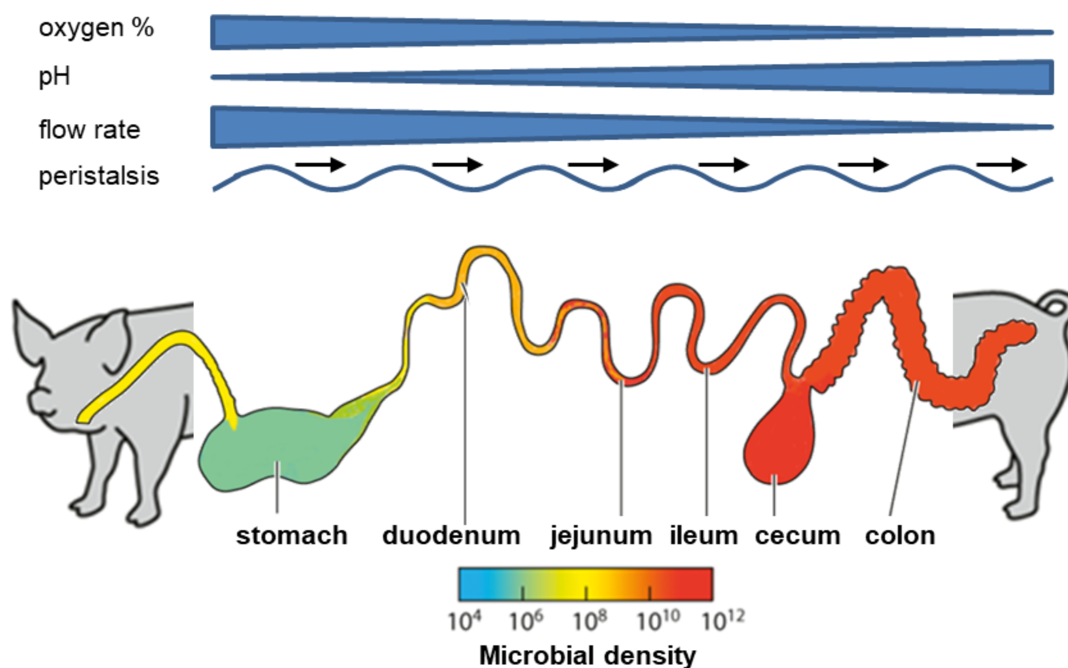

Figure 1-1 Longitudinal profile of the porcine GIT ecosystem. The graphical representation was modified from (Yeoman and White 2014). Gradients of abiotic factors like dissolved oxygen, pH and the flow rate of digesta affect the microbial density in sections along the GIT. In addition, peristaltic movements transport digesta and associated bacteria along the GIT through changing environmental conditions.

The ecosystem of the GIT is characterized by a complex and dynamic interplay between microbial communities, host cells and dietary nutrients. The anatomical structure is accompanied by a biogeographical distribution of abiotic factors like oxygen content, pH,

nutrient supply and digesta flow rate and this influences the microbial density along (Figure 1-1) and across the GIT (Figure 1-2). A decline in the concentration of dissolved oxygen, both in longitudinal and radial axes, supports varying microhabitats for aerobic, facultative aerobic and anaerobic bacteria (Hillman et al. 1993). Through the GIT's lumen, digesta, including nutrients and associated bacteria, is transported by peristaltic wave-movements and with a decreasing flow rate in rectal direction (Loeffler and Gäbel 2009). The association with the mucus layer enables bacteria to prolong their transition time within the GIT.

Along the GIT, epithelial cells are coated with a mucus layer, hereinafter referred to as mucosa, and some bacteria occupy this layer as ecological niche and show close interaction with host cells (Walter 2008; Mann et al. 2014).

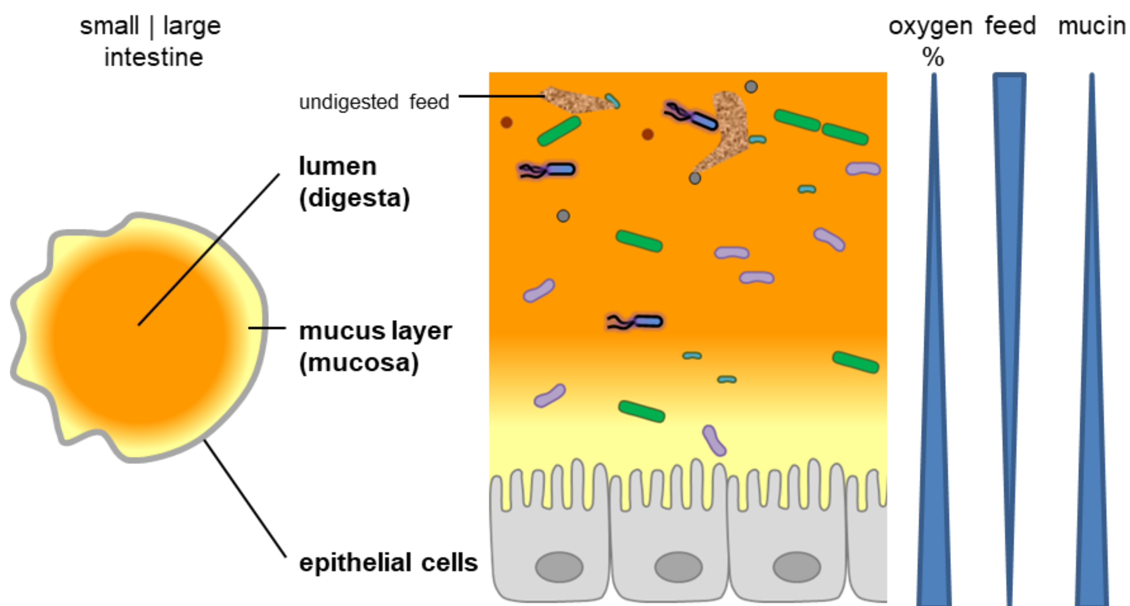

Figure 1-2 Micro-environments across the porcine GIT ecosystem. Gradients of oxygen and nutrient availability along the radial axis of the GIT reflect varying environmental conditions for the microbiota associated with digesta and mucosa, both in the small and large intestine.

Although the stomach has a high nutrient content, bacterial density is here the lowest due to strongly acidic conditions. Gastric cells secrete hydrochloric acid, which acidifies the gastric lumen and activates gastric proteases like pepsin, an important step for protein digestion (Yen 2001). The mucosa protects host epithelial cells against acidic content.

From the stomach, digesta is passed in controlled portions into the small intestine, which is subdivided into duodenum, jejunum and ileum. Within the small intestine the main proportions of feed derived proteins, fats and carbohydrates will be digested and absorbed by the host. An increased nutrient absorption is enabled by the surface expansion of the mucosa forming villi structures. Fat digestion is facilitated by bile salts, which act as emulsifiers. As

not only feed derived fat but as well phospholipids of bacterial cell walls may be digested, bile salts have an antimicrobial activity. However, some bacteria produce hydrolases to deconjugate bile salts, conferring an advantage on colonizing the small intestine (Begley et al. 2005).

In contrast to the small intestine, the mucosa of the large intestine forms no villi and the main function here is the absorption of water and therefore the fluidity is reduced and the retention time of the digesta is extended. The large intestine is subdivided into caecum, colon and rectum. So far none-digested carbohydrates and proteins serve as substrates for bacterial fermentation activity. By the absorption of bacterial fermentation products, the host may obtain about 30% of its energy requirement for maintenance (Rérat et al. 1987). Still, undigested feed substances, metabolic end products and as well host and bacteria cells, are voided as feces.

Next, to digestion and absorption of nutrients, the GIT takes the function as an organ of the immunological system of the host. The mucosal immune system responds to intestinal contents, both feed components and microbes, resulting either in a tolerance or defense reaction (Niewold 2015). Important effector sites are for example the Peyer’s patches (PP) in the small intestine, which contain multiple types of immune cells. Some bacterial fermentation products affect both host and other intestinal bacteria and so may support the intestinal immune system in the defense of pathogens (Niewold 2015).

1.2 Studying microbiota using 16S rRNA based techniques

Mimicking the environmental conditions of the intestinal microbiota in a culture flask is very challenging and the acquired knowledge by culture-dependent studies is strongly restricted. More comprehensive insights into the structure of microbiota are gained by culture-independent, molecular approaches. Since the 1970s, the taxonomical composition of microbiota is commonly identified by analysis of the 16S rRNA gene. This gene sequence is about 1550 bp long, encodes the rRNA of the small subunit of the ribosome and is commonly present in prokaryotes. A comparative analysis revealed distinct differences between bacteria and archaea in their 16S rRNA sequences (Woese and Fox 1977). The structure of the 16S rRNA gene is composed of highly conserved regions and nine hypervariable regions (V1-V9), with approximately 7000 times increased substitution rate in nucleotides of the variable regions, which enables taxonomic resolution down to species level (Van de Peer et al. 1996). In PCR-based microbiota studies, primers are designed to target the conserved sequences flanking the hypervariable regions, which enable the universal amplification of bacterial

sequences and a subsequent taxonomical identification of bacteria. However, the taxonomical accuracy varies between the different variable regions and this has to be considered when comparing studies targeting different variable regions (Hamady and Knight 2009).

Fingerprinting

Molecular fingerprinting techniques use features of the 16S rRNA gene to reveal microbiota's structure. So in a denaturing gradient gel electrophoresis (DGGE) gel, nucleotide variations in 16S rRNA gene amplicons cause varying melting temperatures and in result in specific separation patterns in a gel electrophoresis (Muyzer et al. 1993). The DGGE method was capable of monitoring differences in the gut microbiota of pigs of different ages and diets and as well among different sampling sites of the porcine GIT (Simpson et al. 1999).

Another gel-based fingerprinting method is the amplified rDNA restriction analysis (ARDRA). Here, amplicons are digested by a restriction enzyme and, based on sequence variations, restriction fragments of different length are generated and subsequently, microbiota structure is visualized as specific band pattern on a gel (Vaneechoutte et al. 1992). This technique is as well applicable to evaluate a large number of 16S rDNA clones for further, more cost intensive, analyses (Weidner et al. 1996).

A more frequently used fingerprinting technique is the terminal-restriction fragment length polymorphism (T-RFLP) analysis. The applied workflow of T-RFLP analysis in the here presented studies are shown in Figure 1-3. The 16S rRNA gene is amplified using a fluorescently labeled primer, followed by digestion with restriction enzymes, resulting in one labeled terminal-restriction fragment (TRF) and several unlabeled fragments per amplified sequence (Liu et al. 1997). Restriction fragments are separated by capillary electrophoresis and by laser-detection a profile of TRFs is obtained, the so-called electropherogram. The area of the fluorescence peaks reflects the relative abundance of each TRF, which is a feature lacked by other fingerprinting techniques. Previous studies demonstrated the applicability of T-RFLP analysis to assess the dietary impact on porcine intestinal microbiota (Metzler-Zebeli et al. 2010; Ivarsson et al. 2012; Liu et al. 2012).

Sequencing

Forty years ago, 1977, already before the first thermocycler instrument was manufactured, Frederick Sanger and colleagues published the development of a seminal technique determining the nucleotide sequence of DNA molecules (Sanger et al. 1977). This sequencing technique is based on an amplification reaction which is terminated by incorporation of

labeled dideoxynucleotides (ddNTPs) resulting in different amplicon lengths. The reaction products are separated by gel-electrophoresis, each type of ddNTP reaction in a separate lane and due to labeling, the terminal nucleotide may be determined. An improvement of the original Sanger sequencing was the replacement of a radioactive-labeling by fluorescence (Ansorge et al. 1986) and the development of large-scale DNA sequencer running capillary electrophoresis (Hunkapiller et al. 1991).

Further enhancements in high-throughput sequencing were achieved by several developments, which are collectively referred to the “next-generation sequencing” (NGS). The history of DNA sequencing was previously summarized (Heather and Chain 2016). Commonly, NGS techniques are based on sequence-by-synthesis either by pyrosequencing (454/Roche), fluorescently labeled reversible terminators (Solexa/Illumina), ligation (“SOLiD”, Life Technologies) or ion semiconductor (“Ion Torrent”, Life Technologies). The pioneer of NGS was the pyrosequencing method by the company 454 Life Science (later acquired by Roche).

For the 454-pyrosequencing, DNA-fragments are bound to beads and are clonally amplified by an emulsion-PCR (Margulies et al. 2005). Nucleotides flow in a specific order through the system, the incorporation of one dNTP releases a molecule of pyrophosphate, this is converted by a Sulfurylase-Luciferase reaction in a photon, which is then detected by a camera (Margulies et al. 2005). This sequencing method was used in a large-scale project investigating the human microbiota of different body sites (HumanMicrobiomeProjectConsortium 2012). Other high-throughput sequencing systems followed to the 454-pyrosequencing and actually overtop, as Roche shut down the pyrosequencing-platform (GenomeWeb 2013). Among these, the Illumina-sequencing (originally developed by the company Solexa) is widely used and was the technique of choice of ongoing, large-scale research projects (Gilbert et al. 2010; Qin et al. 2010).

The Illumina sequencing approach uses a solid phase, a flow cell, where DNA molecules bind via adaptor molecules. Here clusters of clonal DNA are bound to the flow cell and are generated by a bridge-amplification (Fedurco et al. 2006). Illumina sequencing uses fluorescently tagged dNTPs, so all four types are present during each sequencing cycle. This feature is advantageous over pyrosequencing which exhibits undesirable high base-call error-rate in homopolymers. Furthermore, Illumina sequencing provides paired-end reads which increases identification accuracy when assigning reads to reference sequences. Comparisons of 454 and the Illumina sequencing platforms verified a high agreement in assessing sequence

diversity, however, each method has certain advantages and limitations (Luo et al. 2012; Claesson et al. 2010).

In the here presented studies, Illumina amplicon sequencing was the method of choice to characterize microbial communities based on the 16S rRNA gene. Prior sequencing itself, libraries were prepared in a two-step PCR approach (Camarinha-Silva et al. 2014), as represented in Figure 1-3. In a first PCR, the primer sequences targeted the V1 to V2 region of the 16S rRNA gene and further included a two nucleotide linker, followed by a sample-specific barcode, sequences complementary to flow cell adaptor oligonucleotides and a further Illumina specific sequence. The PCR product of the first PCR was used as the template for a second PCR, integrating multiplexing sequence, index sequence and again an Illumina specific sequence. Using barcode and index sequences allows the simultaneous sequencing of large numbers of samples, which is cost-effective and supports accurate maintenance of high-quality data. Bioinformatic procession of the sequencing data was performed using the Mothur pipeline (Kozich et al. 2013; Camarinha-Silva et al. 2014). Sequences were clustered into an operational taxonomic unit (OTU) at 97% similar identity. The taxonomical assignment of OTUs was based on the naïve Bayesian classifier (Wang et al. 2007) of the ribosomal database project (RDP) and using previously suggested thresholds (Yarza et al. 2014).

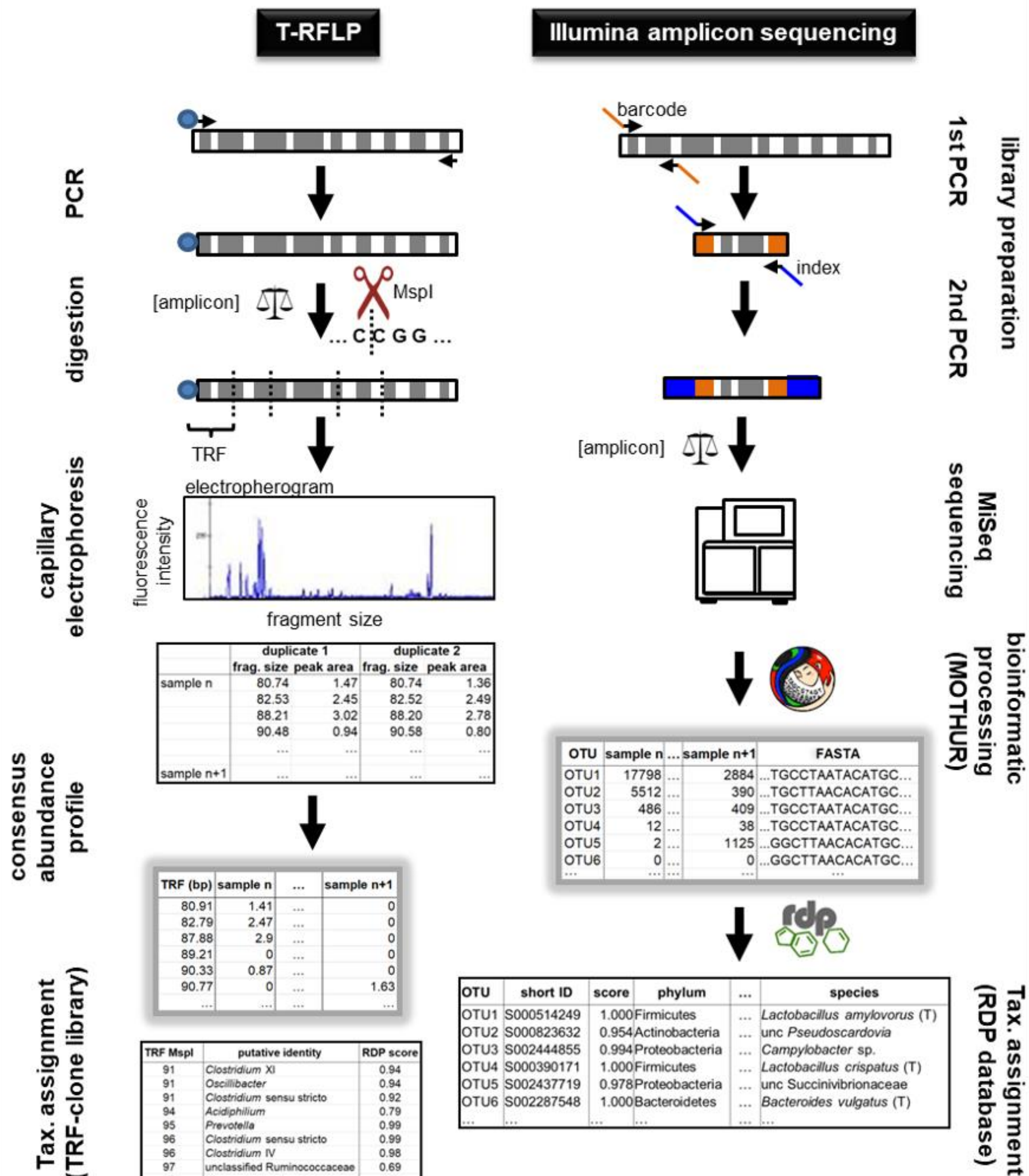

Figure 1-3 16S rRNA gene based methods to study microbiota. Workflows, as applied in the here presented studies are depicted with the final output tables being highlighted in grey. Left: terminal-restriction length polymorphism (T-RFLP), 16S rRNA gene is amplified using fluorescently labeled forward primer (27f-FAM) and unlabeled reverse primer (1492r). An equal amount of purified amplicons are digested with restriction enzyme MspI. The peak area of a detected terminal restriction fragment (TRF) represents its abundance. Consensus profiles are generated to compare multiple profiles. Taxonomical assignment of TRFs relies on a clone-TRF library. Right: Illumina amplicon sequencing, sequence libraries of the V1 to V2 region of the 16S rRNA gene are prepared by a two-step PCR approach. Inserting a sample-specific barcode-sequence and index-sequence, allows simultaneous sequencing of several samples with individual evaluation, subsequently. Equimolar amplicons are sent to run on a MiSeq platform and resulting sequence reads are processed by the bioinformatic software package MOTHUR. At 97% similarity sequences are clustered to OTU and RDP database is used for taxonomical assignment.

The latest developments of sequencing techniques are based on direct detection of nucleotide sequence. The single molecule, real-time (SMRT) sequencing by PacBio is the most successful platform using amplification-independent sequencing. This method uses so-called

zero-mode waveguides (ZMWs), which represent holes in a metallic film, where a DNA polymerase is located at the bottom. The narrow diameter of the hole allows a real-time base-calling when a fluorescently-labeled nucleotide is incorporated in a DNA strand. The SMRT sequencing runs within a few hours and produces long reads of 20 kb, however, the high costs are highly unattractive for users (van Dijk et al. 2014).

1.3 Multivariate data analysis on microbiota studies

Addressing whole microbial communities of multiple samples by high-throughput technologies provides a large amount of multivariate data. Statistical approaches attempt to identify patterns and causal factors, explaining such patterns and various useful routines are collected in software packages like PRIMER-E, R (vegan) and Past (Clarke and Warwick 2001; Dixon 2003; Hammer et al. 2001). In the following, an overview of some common multivariate analysis methods is given.

Prior to statistical analysis, samples are further characterized by a priori defined groups (e.g. sampling time point, site, experimental treatment) and the detected variables (here TRF or OTU) are standardized per object. Visualizing the relative abundance data by a color scale supports the decision of data pre-treatment, which might be relevant to balance common and rarer variables (Clarke et al. 2014).

One starting point in multivariate analysis is to compare how similar the community structure between pairs of samples is. Similarities are calculated using coefficients like the Bray-Curtis algorithm, which considers both presence/absence and the abundance of variables (Bray and Curtis 1957). Values of similarity might be computational converted into dissimilarities. Based on (dis-)similarity matrices, patterns within multivariate data may be revealed by varying exploratory analyses as previously reviewed (Ramette 2007). Common ordination methods are multidimensional scaling (MDS) and principal coordinate analysis (PCoA or PCO). In a MDS plot, the rank order of dissimilarities is used to map objects in a non-metric and low-dimensional space. Whereas in PCoA the calculation of eigenvalues is involved, projecting objects onto axes in a metric-way.

In addition to ordination methods, dissimilarity structures can be assessed by hypothesis-driven tests (Ramette 2007; Anderson and Walsh 2013) providing statistical evidence on patterns. The analysis of similarities (ANOSIM; (Clarke 1993)) compares the ranks of dissimilarities within and between a priori defined groups. In contrast to ANOSIM, the permutational analysis of variance (PERMANOVA; (Anderson 2001)) is based on the actual

dissimilarity values within and between a priori groups, this allows to test more specific null-hypotheses and a greater robustness to heterogeneity. Two dissimilarity matrices, on the same objects but based on different sets of variables (e.g. OTU abundances and metabolite concentrations of the same GIT sample), are compared by the Mantel test (Mantel 1967), which corresponds to RELATE routine by PRIMER-E (Clarke 2006).

To identify discriminating variables, the similarity percentage routine (SIMPER; (Clarke 1993)) can be used as a post-hoc test of between-group differences. The relative contributions of variables to dissimilarities between groups of samples are determined, however, the interpretation of SIMPER results is limited due to a lack of hypothesis-driven test and its reduced power in detecting variables of low mean-variances (Warton et al. 2012). An alternative approach is the linear discriminant analysis effect size (LEfSe) method, which uses the Kruskal-Wallis rank-sum test to identify variables with different abundances between groups (Segata et al. 2011).

Analysing the dissimilarities between samples is also referred to as β-diversity of communities. The diversity within samples is called α-diversity and a common measurement is the Shannon index, considering both the numbers of community members and the proportion of each.

1.4 The microbiota along the porcine GIT

Before molecular techniques have been broadly applicable, culture-dependent and labor-intensive methods were used to explore microbiota of the porcine GIT. By comparing colony counts on selective media of varying energy substrates, differences were indicated in bacteria populations of porcine cecum and colon and as well in populations associated with digesta or mucosa (Allison et al. 1979). A cloning and Sanger sequencing approach revealed 375 phylotypes in the porcine GIT with a reduced power in the classification, as only 17% of these phylotypes could be assigned to a reference database (Leser et al. 2002). By pyrosequencing and Illumina amplicon sequencing deeper insights in microbiota taxonomy and similarity structure have been gained, revealing distinct differences accompanied by GIT organization (Looft et al. 2014; Zhao et al. 2015).

Development of the microbiota

Like other mammals, pigs are born sterile and the development of intestinal microbiota in piglets occurs in a successional process. A recent pyrosequencing study on fecal samples of piglets revealed a quite simple microbiota composition in the first three days after birth (Bian

et al. 2016). In this early time span, the relative proportion of Firmicutes is predominated, followed by the abundance of Proteobacteria and as well Fusobacteria, who drastically decrease in the following days. From day three of birth on, Bacteroidetes increases in abundance and become the second dominant phyla after Firmicutes. Facultative anaerobic bacteria, like microaerophilic Proteobacteria, are assumed to act as pioneers in intestinal colonization, generating a reduced, more anaerobic environment, which enables successional colonization by anaerobic bacteria like Bacteroidetes (Houghteling and Walker 2015). It has been shown, that as well in adult pigs the relative proportion of the microbiota's members changes with the aging of the host (Zhao et al. 2015; Kim and Isaacson 2015). Apart from variations in composition, Firmicutes, Bacteroidetes and Proteobacteria represent the major phyla in the porcine GIT.

The microbiota of the upper GIT

The harsh environmental conditions in the stomach and small intestine effect low bacterial density, as depicted in Figure 1-1, however, some bacteria overcome well these challenges. So the abundance of acidic-tolerant *Lactobacillus*, belonging to Firmicutes, show high affinity to gastric mucosa (Holman et al. 2017). It has been suggested, that *Lactobacillus* form biofilms, which allow them to persist and occupy the ecological niche of the porcine stomach (Walter 2008). In addition, bile resistance enables *Lactobacillus* an enriched abundance in digesta of the small intestine (Ruiz et al. 2013). Here, *Streptococcus* is as well highly abundant (Metzler-Zebeli et al. 2010; Holman et al. 2017). Both genera form lactic acid by metabolizing dietary carbohydrates. Furthermore, the small intestine is enriched by facultative anaerobs, like *Escherichia* and other *Enterobacteriaceae*, plus aerobic bacteria, like *Acinetobacter* and *Psychrobacter* (Zhao et al. 2015).

The microbiota of the lower GIT

Caecum and colon harbor a high density of anaerobic and strictly anaerobic bacteria, with dominating proportion of Clostridiales, members of Firmicutes phyla (Leser et al. 2002; Zhao et al. 2015). Even if some severe intestinal pathogens belong to Clostridiales, the majority of this bacteria group is known for its contribution to the gut homeostasis (Velasquez-Manoff 2015). For Bacteroidetes, predominant members in the large intestine are Prevotella and Bacteroides (Looft et al. 2014; Zhao et al. 2015). Within the three major intestinal phyla, *Prevotella* (Bacteroidetes), *Oscillibacter* (Firmicutes) and *Succinivibrio* (Proteobacteria) showed to drive differences between colon and other GIT sections by enriched colonic proportions (Looft et al. 2014).

The lower GIT is characterized by an increased amount of undigested feed components, which serve as substrates for carbohydrate and protein fermentation by bacteria and thus is associated with an increased metabolic activity (Figure 1-4). Further, compared to upper GIT, the anaerobic environment of the lower GIT has been suggested to drive metabolic cross-feeding between bacteria and this, in turn, leads to a more complex ecosystem (Heinken and Thiele 2015). For example a co-culture study demonstrated that lactate, which is produced by *Bifidobaterium* degrading starch from culture medium, is converted by *Eubacterium* into butyrate (Belenguer et al. 2006). So based on this relation, in microbiota studies the abundance of *Bifidobaterium* might be linked to the abundance of butyrate-producing bacteria and butyrate concentration. Further cross-feeding interactions have recently been reviewed and are here illustrated in Figure 1-4 (Ríos-Covián et al. 2016). Furthermore, nutritional strategies have been suggested to influence the production of bacterial metabolites in the porcine lower GIT (Jha and Berrocoso 2016) (see Chapter 1.5).

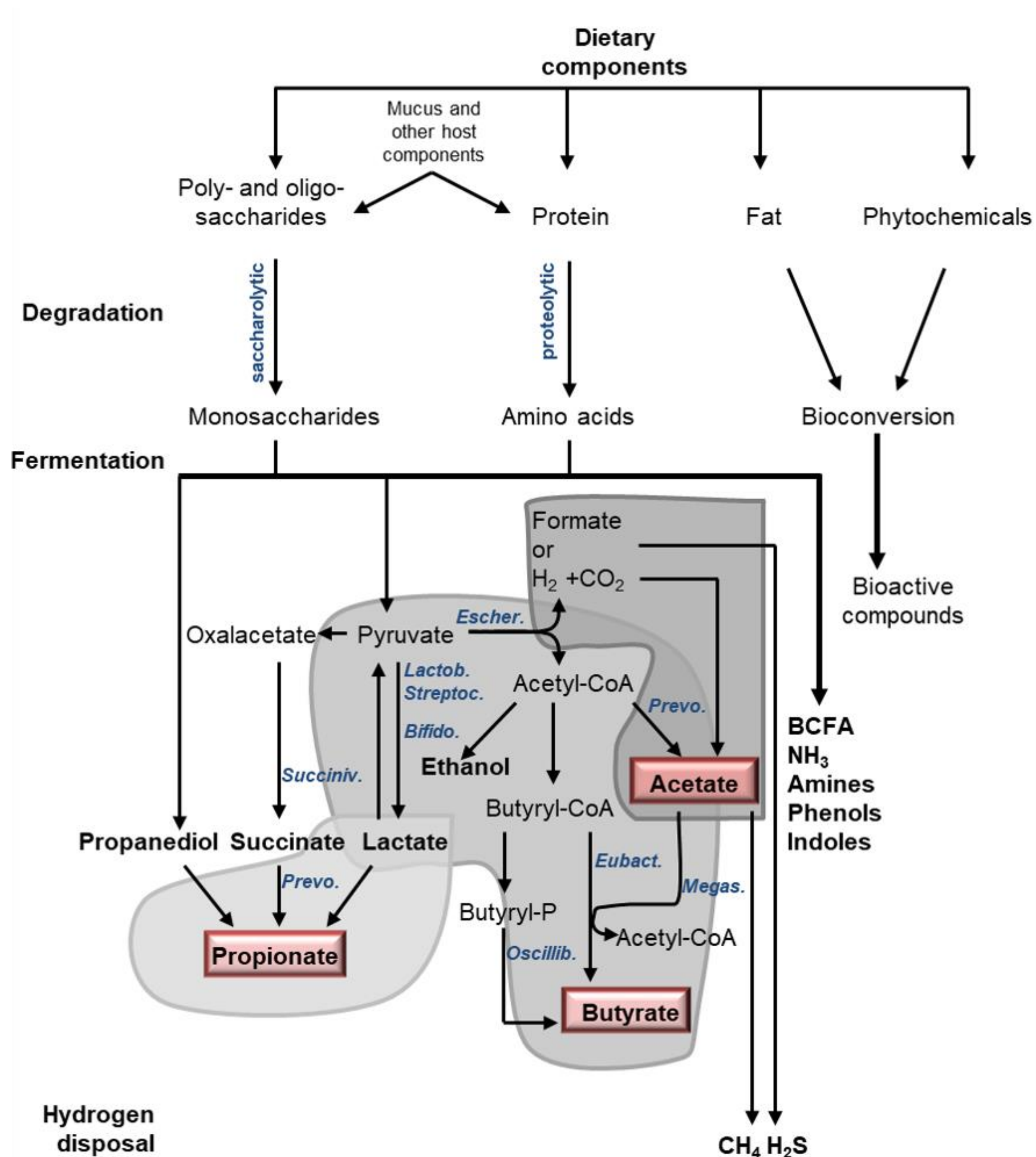

Figure 1-4 Overview of bacterial metabolic pathways contributing to diet degradation. The graphic was modified from (Salonen and de Vos 2014; Ríos-Covián et al. 2016). Grey shaded spaces represent metabolic cross-feeding of the main short-chain fatty acids. A bacteria representative, forming a certain metabolite, is indicated in blue italic on the right site of a reaction arrow: *Bifidobacterium*, *Escherichia*, *Eubacterium*, *Lactobacillus*, *Megasphaera*, *Oscillibacter*, *Prevotella*, *Succinivibrio*.

The mucosa-associated microbiota

In addition to the longitudinal GIT, varying environmental conditions in the radial axis also affect distinct communities of mucosa and lumen (Albenberg et al. 2014; Looft et al. 2014). A pyrosequencing study revealed Firmicutes being predominant in gastric mucosa, Proteobacteria in ileal mucosa and Bacteroidetes in colonic mucosa (Mann et al. 2014). A cross-section through ileum showed higher abundances of *Prevotella*, *Campylobacter* and *Coprococcus* in ileal mucosa than in ileal digesta, where the proportion of *Anaerobacter* is enriched (Looft et al. 2014). Whereas in caecum, mucosa-associated sites are more enriched by *Helicobacter* plus *Campylobacter* and caecal digesta by *Prevotella* (Kelly et al. 2017).

At species level, *Campylobacter coli* has been reported to dominate the mucosa-associated microbiota along the entire GIT (Kelly et al. 2017). *Campylobacter* is commonly isolated from the pig's GIT and a transmission may cause infections with diarrhea in humans (Alter et al. 2005). Similar, *Escherichia coli* is a mucosa-associated commensal of the porcine GIT and known for its pathogenic potential (Conway and Cohen 2015).

1.5 Microbiota structure and host's diet

Research in the field of microbiota flourished in the last decade. NCBI PubMed database reports 3390 "gut microbiota" publications in 2016 in contrast to 84 publications in 2006. Similar, the interest in "pig gut microbiota" increased, with 84 publications in 2016 whereas only two are reported for 2006 (https://www.ncbi.nlm.nih.gov/pubmed; 14.11.2017). In general, the coevolution between mammals and their gut microbiota with a direct link between diet and microbiota composition is recognized (Ley et al. 2008).

Within the life of a pig, the transition from suckling to weaning is accompanied by multiple, challenging stress factors, including the dietary change from highly digestible milk to less digestible complex feed (Lalles et al. 2007). It has been shown, that abrupt weaning at day 21 shapes both taxonomical structure and enzymatic functional capacities of their fecal microbiota, with *Enterobacteriaceae* decreasing and *Prevotellaceae* increasing in abundance (Frese et al. 2015). Furthermore, weaning is associated with disorders of the porcine GIT resulting in severe diarrhea and increased effort has been focused on nutritional strategies stimulating a gut health-promoting microbiota, instead of applying in-feed antibiotics (Lalles et al. 2007; de Lange et al. 2010).

In this context, diets of reduced crude protein content are considered to enhance the health of porcine GIT. This feeding approach yields a reduction of substrates for bacterial protein

fermentation resulting in a reduced production of branched chain fatty acids (BCFA) and potential detrimental metabolites, like ammonia, amines, phenols and indols (Figure 1-4). When compared to increased dietary protein content, low protein diets enhanced the proportion of *Prevotella*, in piglets' microbiota in ileal digesta and in growing pigs in caecal digesta (Rist et al. 2013; Zhou et al. 2016).

Next to the level of dietary protein, as well the protein source affects the GIT microbiota structure. For example, increased bacteria gene copy numbers in feces of piglets fed with a soybean meal-based diet compared to a higher digestible casein-based diet, indicated an increased nutrient-availability for the microbiota (Rist et al. 2014). In general, soybean meal is one of the most important protein source in diet formulations of pigs. Furthermore, grain legumes and cereal grains are used as dietary protein sources. The protein digestibility of these plants is lower than of soybean meal, but the associated dietary inclusion of fermentable substrates for bacteria is appreciated to promote a healthy gut (Aumiller et al. 2015).

Similar to varying contents of dietary protein, diets of varying fat content showed to affect microbiota composition and metabolic activity, with high-fat content being linked to a reduced health status of the porcine GIT (Heinritz et al. 2016).

In swine nutrition, the main energy sources are carbohydrates derived from cereal grain. By host non-digestible plant carbohydrates, like non-starch polysaccharides (NSP), resistant starch and oligosaccharides, are summed up as dietary fibers and serve as substrates for bacterial fermentation resulting in the production of short-chain fatty acids (SCFA), see Figure 1-4. Variations in dietary fibers composition affect profiles of porcine intestinal microbiota and metabolites (Castillo et al. 2007; Metzler-Zebeli et al. 2010).

Besides these macronutrients, as well minerals and trace elements may impact the microbiota of the porcine GIT. Dietary calcium and phosphorus are known to form complexes and feeding varying levels of calcium-phosphorus have been linked both to alteration in bacteria population abundances and metabolites in the upper GIT of pigs (Metzler-Zebeli et al. 2013). Dietary supplementation of increased zinc oxide levels promotes animal growth, similarly to in-feed antibiotics, and affects alteration in the overall microbiota profile and metabolite distribution along the GIT (Hojberg et al. 2005; Yu et al. 2017).

Furthermore, in microbiota-focused feeding strategies, additives like prebiotics and probiotics are used for their beneficial health effects. By definition, a prebiotic is a substrate that is selectively utilized by host microorganisms conferring a health benefit (Gibson et al. 2017). In swine nutrition, inulin from chicory is recognized as prebiotic, enhancing the abundances of

lactic acid producing bacteria (Liu et al. 2012; Ivarsson et al. 2012). This group of bacteria contains several probiotic strains (Casey et al. 2007). Probiotics are defined as live microorganisms, which when administered in adequate amounts, confer a health benefit on the host (FAO/WHO Group 2002). Besides lactic acid producers, probiotic activity has been attributed to bacteria strains of different taxonomical origin when added to porcine diet (Schroeder et al. 2006; Pajarillo et al. 2015).

1.6 Aim of this thesis

The overall aim of studies presented in this thesis was to improve the knowledge on the diversity in the microbiota of the porcine GIT and elucidate influences of varying dietary compositions. To address the microbiota structure, molecular genetic methods targeting the 16S rRNA gene were used.

Methodological variations are known to impair study findings. Therefore standardization is considered to encourage the comparability between different studies. So with regard to studies on porcine GIT microbiota, the first objective of this thesis was to compare multiple DNA extraction protocols and evaluate the recovered microbiota structure by a cost-effective, rapid fingerprinting (T-RFLP) and a more cost-intensive sequencing approach (Illumina amplicon sequencing), which provides greater resolution (Chapter 2.1).

Subsequently, the methodological findings were used to elaborate the impact of the diet on the intestinal microbiota of pigs and identify both significant factors and responding variables, when the diet is composed of:

- solely one cereal grain type, either rye or triticale (Chapter 2.2)
- varying contents of crude protein and supplemented probiotic Bacillus species (Chapter 2.3)
- varying contents of calcium-phosphorous and fermentable substrates (Chapter 2.4) and
- in addition, investigate the dietary impact over the experimental time of four weeks (Chapter 2.5)
- innovative porcine feed component, insect-meal of Hermetia illucens and within this study, comprehensively characterize the microbiota along and across the porcine GIT (Chapter 2.6).

1.7 References (Introduction)

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Chapter 2 Publications and manuscripts

Results of this thesis have been incorporated into publications and manuscripts. In the following, the own contribution is briefly described.

2.1 Evaluation of DNA extraction kits and phylogenetic diversity of the porcine gastrointestinal tract based on Illumina sequencing of two hypervariable regions

Katharina Burbach, Jana Seifert, Dietmar H. Pieper, Amélia Camarinha-Silva

MicrobiologyOpen 2016; 5(1): 70–82

DOI: 10.1002/mbo3.312

In this work methodological protocols have been evaluated for microbiota analysis of sample materials from the porcine GIT. Jointly with Amélia Camarinha-Silva I performed the DNA extraction procedures. Further contribution included T-RFLP analysis and preparation of the manuscript, including the Table 2.1-1 and -2 and Figure 2.1-1, -2 and proportionally the Figure 2.1-3. Findings of this work have been considered in subsequent studies of this thesis.

2.2 Porcine intestinal microbiota is shaped by diet composition based on rye or triticale

Katharina Burbach, Elisa J.P. Strang, Rainer Mosenthin, Amélia Camarinha-Silva, Jana Seifert

Journal of Applied Microbiology 2017; 123(6): 1571–1583

DOI: 10.1111/jam.13595

This work described significant dietary effect on the porcine microbiota of ileal digesta and feces when pigs are fed with a diet composed of a sole energy source, either rye or triticale. My own contribution included DNA extraction, preparation of Illumina sequencing libraries, analysis of the OTU data set, correlation between bacteria relative abundance data and bacteria metabolite concentration and preparation of the manuscript.

2.3 Effect of *Bacillus subtilis* and *Bacillus licheniformis* supplementation in diets with low- and high-protein content on ileal crude protein and amino acid digestibility and intestinal microbiota composition of growing pigs

Chanwit Kaewtapee, Katharina Burbach, Georgina Tomforde, Thomas Hartinger, Amélia Camarinha-Silva, Sonja Heinritz, Jana Seifert, Markus Wiltafsky, Rainer Mosenthin, and Pia Rosenfelder-Kuon

Journal of Animal Science and Biotechnology 2017; 8(37)

DOI: 10.1186/s40104-017-0168-2

This work focused on dietary supplementation of probiotic species in combination with varying contents of dietary crude protein. My own contributions included DNA extraction, preparation of Illumina sequencing libraries, analysis of the OTU data set and, in addition, providing Figure 2.3-1, -2 and -3 and Table 2.3-6.

2.4 Phytate degradation, intestinal microbiota, microbial metabolites, and immune values are changed in growing pigs fed diets with varying calcium-phosphorus concentration and fermentable substrates

Charlotte Maria Elisabeth Heyer, Sonja Schmucker, Katharina Burbach, Eva Weiss, Meike Eklund, Tobias Aumiller, Filippo Capezzone, Julia Steuber, Markus Rodehutscord, Ludwig Eduard Hoelzle, Jana Seifert, Rainer Mosenthin, Volker Stefanski

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DOI: 10.1111/jpn.13088

This work studied a dietary impact both on the porcine immune system and intestinal microbiota. Here my own contribution included T-RFLP analysis and providing the Figure 2.4-1 and -2 to the manuscript.

2.5 Dietary changes in nutritional studies shape the structural and functional composition of the pig's fecal microbiome - from days to weeks

Bruno Tilocca, Katharina Burbach, Charlotte M. E. Heyer, Ludwig E. Hoelzle, Rainer Mosenthin, Volker Stefanski, Amélia Camarinha-Silva, Jana Seifert

Microbiome 2017; 5:144

DOI: 10.1186/s40168-017-0362-7

This work used both a meta-proteomic and taxonomic approach to determine a temporal adaptation of bacterial communities. My contribution included the taxonomical approach by 16S rRNA gene sequencing: DNA extraction from fecal samples, preparation of 16S rRNA gene libraries for Illumina amplicon sequencing and analysis of the OTU data set. Further, I provided information for Figure 2.5-1 and -2.

2.6 Exploring effects of dietary inclusion of insect meal on the microbiota of piglets' gastrointestinal tract

Katharina Burbach, Amélia Camarinha-Silva, Andreas Berk., Sven Dänicke, Jana Seifert, Jeannette Kluess

Manuscript in preparation

This work described piglets' microbiota along and across the GIT and further studied effects by dietary inclusion of varying levels of insect larvae meal. My own contributions included DNA extraction, preparation of Illumina sequencing libraries, analysis of the OTU data set and preparation of the manuscript, so far.

2.1 Burbach et al. 2016

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ORIGINAL RESEARCH

Evaluation of DNA extraction kits and phylogenetic diversity of the porcine gastrointestinal tract based on Illumina sequencing of two hypervariable regions

Katharina Burbach[1], Jana Seifert[1], Dietmar H. Pieper[2] & Amélia Camarinha-Silva[1]

[1]Institute of Animal Science, University of Hohenheim, Stuttgart, Germany
[2]Microbial Interactions and Processes Research Group, Helmholtz Centre for Infection Research, Braunschweig, Germany

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Evaluation of DNA extraction kits and phylogenetic diversity of the porcine gastrointestinal tract based on Illumina sequencing of two hypervariable regions

2.1.1 Abstract

A robust DNA extraction method is important to identify the majority of microorganisms present in environmental microbial communities and to enable a consistent comparison between different studies. Here, fifteen manual and four automated commercial DNA extraction kits were evaluated for their efficiency to extract DNA from porcine feces and ileal digesta samples. DNA yield, integrity and purity varied among the different methods. Terminal restriction fragment length polymorphism (T-RFLP) and Illumina amplicon sequencing were used to characterise the diversity and composition of the microbial communities. We also compared phylogenetic profiles of two regions of the 16S rRNA gene, one of the most used regions (V1-2) and the V5-6 region. A high correlation between community structures obtained by analysing both regions was observed at genus and family level for ileum digesta and feces. Based on our findings we want to recommend the FastDNA™ SPIN Kit for Soil (MP Biomedical) as a suitable kit for the analyses of porcine gastrointestinal tract samples.

2.1.2 Introduction

The gastrointestinal tract is a complex ecosystem harbouring a huge variety of microorganisms that influence the metabolism of nutrients and health of the host (Henderson et al. 2013). For instance the degradation of complex dietary carbohydrates by gut microbes is an important source of energy and fermentation products that may stimulate immunity of the host. Factors like ageing, host genetics, diet, environmental conditions and medical treatments define the composition of the intestinal microbial community (Zhao et al. 2015; Looft et al. 2014; Ley et al. 2008). Previous studies of the porcine gut microbiota have focussed on the influence of feeding strategies and antibiotic treatments on the community (Rist et al. 2013; Holman and Chenier 2014). However, recently, the pig has been recognized as a suitable animal model for human related research and is therefore of increasing interest (Heinritz et al. 2013).

The analysis of microbial community structures is typically performed through culture-independent methods. Insights into phylogenetic composition of the porcine intestinal microbiota were obtained by using different approaches such as clone libraries (Leser et al.

2002), terminal restriction fragment length polymorphism (T-RFLP) and quantitative PCR (qPCR) (Metzler-Zebeli et al. 2010; Pedersen et al. 2013a), microarrays (Arnal et al. 2014) and high-throughput sequencing technologies (Illumina and 454-pyrosequencing) (Lamendella et al. 2011; Pajarillo et al. 2014; Looft et al. 2014; Holman and Chenier 2014). DNA extraction from intestinal samples has a crucial effect on the determination of community composition of the intestinal microbiota. Combined with the phylogenetic diversity, the diverse morphological and physicochemical properties of bacterial cells might affect the extraction. The efficient DNA extraction is challenging and requires some effort (Ariefdjohan et al. 2010; Maukonen et al. 2012).

A classical and inexpensive method of DNA extraction uses a phenol-chloroform-mixture to purify DNA (Kirby 1956). This protocol is rather time consuming and demands hazardous chemicals that even inhibit downstream amplification reactions (Katcher and Schwartz 1994). Today, a wide range of commercial DNA extraction kits are available and are easy to use. Apart from differences in the protocols, the commercial kits have some general steps in common: cell lysis, washing and DNA capture. Cell lysis occurs either through chemical lysis and/or by mechanical cell disruption with bead beating. In most cases, the released DNA is captured on a silica matrix in the presence of a solution with high concentration of salt. In automated systems, silica-cladded magnetic beads are commonly used. Since PCR inhibitors like humic substances, polysaccharides, bile salts and haemoglobin may be co-extracted with DNA and affect downstream PCR application (Tsai and Olson 1992; Monteiro et al. 1997; Lantz et al. 1997; Akane et al. 1994) some DNA extraction kits include a PCR inhibitor removal step to avoid these complications.

Standardisation of methods is an important step to gain reliable knowledge on bacterial community profiles. In the last years some research consortia were formed as initiatives to investigate the earth microbiome (EMP) (Gilbert et al. 2011), human microbiome (HMP) (HumanMicrobiomeProjectConsortium 2012) and human gut metagenome (MetaHit) (Qin et al. 2010). Such large-scaled projects have to deal with multiple samples, whose analysis and interpretation need efficient techniques and standardised methods. A general protocol was suggested for studies of the HMP, using the MoBio PowerSoil™ kit for the DNA extraction from intestinal samples (McInnes and Cutting 2010). Other studies compared different protocols of DNA extraction from intestinal samples source and the effect on downstream applications (Guo and Zhang 2013; Sergeant et al. 2012; Maukonen et al. 2012; Claassen et al. 2013; Ariefdjohan et al. 2010; Desneux and Pourcher 2014; Ferrand et al. 2014; Rubin et al. 2014). So far, there are no standardized molecular genetic methods for extracting DNA of

the porcine gastrointestinal tract. In this study we compared 15 manual and four automated commercial DNA extraction kits to extract DNA from porcine feces and ileal digesta. The DNA extraction protocols were evaluated based on yield and purity of extracted DNA. Bacterial community profiles were analysed with terminal restriction fragment length polymorphism (T-RFLP) and bacterial community composition by Illumina amplicon sequencing spanning the V1-2 and V5-6 regions of the 16S rRNA gene.

2.1.3 Materials and Methods

Sample collection

The animal experiment was approved by the Animal Welfare commission of the University of Hohenheim in accordance to the German Animal Welfare legislation (Lorz and Metzger 1999). One feces and one ileal digesta sample were obtained from a pig, fed with temperature pre-treated soybeans to study ileal digestibility of this animal feed. The pig was surgically fitted with a simple T-cannula at the distal ileum (Li et al. 1993). For collection of the ileal digesta a plastic bag was attached to the barrel of the cannula. Freshly voided feces were taken without being in contact with the floor. The samples were immediately put on ice and stored at -80°C.

DNA extraction

Samples were thawed on ice, homogenized and 250 mg each were used to extract DNA with commercial kits (Table 2-1) according to the respective manufacturer's instruction with slight modification as follows: as suggested in the protocol of the FastDNA™ SPIN Kit for Feces and FastDNA™ SPIN Kit for Soil (MP Biomedical) an extended centrifugation for 15 minutes at 14,000 x g was performed after homogenization in the FastPrep®-24 instrument (MP Biomedical) at 6 m/s for 40 seconds. DES (DNase/Pyrogen-Free Water) was warmed up at 55°C prior to DNA elution from the SPIN™ Filter. No Proteinase K was added to the NukExPure RNA/DNA (Gerbion) protocol. When using the Precellys Soil DNA Kit (PEQLAB) lysis was performed using the FastPrep®-24 instrument at 4 m/s for 20 seconds, followed by centrifugation at 17,000 x g for 5 minutes. Lysis with the protocols of the PowerLyzer™ PowerSoil®, PowerFecal™ and PowerSoil® DNA Isolation Kit (Mo Bio Laboratories) was in all three cases performed with FastPrep®-24 at 6.5 m/s for 45 seconds. In case of DNA extraction from fecal material using the PowerLyzerTM PowerSoil® and PowerSoil® DNA Isolation Kit an additional centrifugation at 10,000 x g for 2 minutes was performed to achieve a pellet. The NucleoSpin® Soil (Macherey-Nagel) protocol was

evaluated in four variations of two alternative lysis buffers, SL 1 and SL 2, and in combination with or without the Enhancer SX. Lysis was always achieved using a FastPrep®-24 at 5 m/s for 30 seconds. To obtain clear supernatant after the lysis of fecal material centrifugation was performed twice for 2 minutes at 11,000 x g. The InnuPREP Stool DNA Kit (Analytik Jena) was used according to the manufacturer's instructions, whereas the innuSPEED Stool DNA Kit and innuSPEED Soil DNA Kit (Analytik Jena) required an additional centrifugation for 2 minutes at 13,000 x g after lysis using the FastPrep®-24 instrument at 6 m/s for 40 seconds. The QIAamp® DNA Stool mini kit (Qiagen) was used according to the manufacturer instructions.

In addition, DNA was extracted using four variations of the Maxwell® 16 FFPE Plus LEV DNA Purification Kit (Promega) with the automatic DNA extraction by the Maxwell® 16 Instrument (Promega). For the extraction named Promega Maxwell® 16 A (K16) 80 mg feces or ileal digesta were added to 400 µl lysis buffer and mixed thoroughly by vortexing. Twenty five µl of these solutions were loaded into the machine and eluted with 100 µl nuclease free water. Promega Maxwell® 16 B (K17) followed the same procedure but with 80 mg of source material in 800 µl lysis buffer. For Promega Maxwell® 16 C (K18) and Promega Maxwell® 16 D (K19), 80 mg source material were thoroughly resuspended in 400 µl lysis buffer, followed by a bead beating step using FastPrep™ Lysing Matrix D and 50 µg beads of 0.5 mm diameter, respectively, in the FastPrep®-24 instrument at 6 m/s for 40 seconds. One hundred µl of these solutions were used for DNA extraction followed by an elution in 100 µl nuclease free water.

Table 2.1- 1 Evaluated DNA extraction kits.

Kit no.	Name	Company	Procedure	Lysis	DNA capture	Elution (µl)
K1	FastDNA™ SPIN Kit for Feces	MP Biomedical	manual	BB	SM	50
K2	FastDNA™ SPIN Kit for Soil	MP Biomedical	manual	BB	SM	50
K3	NukExPure RNA/DNA	Gerbion	manual	CL	SM	50
K4	Precellys Soil DNA Kit	PEQLAB	manual	BB	SM	80
K5	PowerLyzer™ PowerSoil® DNA Isolation Kit	Mo Bio	manual	BB	SM	70
K6	PowerFecal® DNA Isolation Kit	Mo Bio	manual	BB	SM	70
K7	PowerSoil® DNA Isolation Kit	Mo Bio	manual	BB	SM	70
K8	Nucleo Spin® Soil SL1	Macherey Nagel	manual	BB	SM	50
K9	Nucleo Spin® Soil SL1+Enhancer SX	Macherey Nagel	manual	BB	SM	50
K10	Nucleo Spin® Soil SL2	Macherey Nagel	manual	BB	SM	50
K11	Nucleo Spin® Soil SL2+Enhancer SX	Macherey Nagel	manual	BB	SM	50
K12	innuPREP Stool DNA Kit	Analytik Jena	manual	CL	SM	80
K13	innuSPEED Stool DNA Kit	Analytik Jena	manual	BB	SM	80
K14	innuSPEED Soil DNA Kit	Analytik Jena	manual	BB	SM	50
K15	QIAamp® DNA Stool mini kit	Qiagen	manual	CL	SM	100
K16	Promega Maxwell® 16 A	Promega	automatic	CL	MB	100
K17	Promega Maxwell® 16 B	Promega	automatic	CL	MB	100
K18	Promega Maxwell® 16 C	Promega	automatic	BB + CL	MB	100
K19	Promega Maxwell® 16 D	Promega	automatic	BB + CL	MB	100

BB: bead beating, CL: chemical lysis, MB: magnetic beads, SM: silica membrane

DNA quality and quantity

The concentration of extracted DNA (absorbance at 260 nm) and its purity (absorbance ratio 260/230 and 260/280) were measured using NanoDrop (Thermo Fisher Scientific). Integrity of extracted DNA was analysed by gel electrophoresis.

T-RFLP analysis

16S rRNA gene fragments were amplified from DNA extracts using the primer set 27F, labelled at the 5’ end with 6-carboxyfluorescein (FAM), and 1492R (Lane 1991). Polymerase chain reaction (PCR) was performed in a 50 µl mixture containing 1 µl of a 1:10 dilution of DNA template, 10 µM of each primer and 10 µl of Taq 5X Master Mix (New England

BioLabs). The PCR was run under the following conditions: initial denaturation at 95°C for 4 min, followed by 30 cycles of denaturation at 95°C for 30 s, annealing at 52°C for 30 s, extension at 68°C for 30 s and a final extension step at 68°C for 10 min. The size of amplified PCR products was verified by 1% agarose gel electrophoresis and four replicates of each PCR were pooled for purification with the QIAquick PCR Purification Kit (Qiagen). Seventy ng of purified PCR products were digested with 5 U MspI (New England BioLabs) for 1 h at 37°C. Triplicates of 2 µl digested PCR product were mixed with 17 µl HiDi Formamid (Applied Biosystems), 0.5 µl Map Marker 1500-ROX (BioVentures) and 0.5 µl Tracking Dye (BioVentures), followed by denaturation at 95°C for 5 min and subsequent cooling on ice for 5 min before further analysis on an ABI 3130xI Genetic Analyzer (Applied Biosystems). The peaks of separated terminal restriction fragments (TRFs) were exported with a threshold of 50 and analysed with GeneMapper® v.4.1. For further analysis TRFs beyond the range of 45 bp to 1400 bp were excluded. The abundance of each fragment was standardised by the total fragment area within each sample. Two replicate profiles were used to generate a consensus profile using the T-align program (Smith et al. 2005).

Illumina amplicon sequencing

Library preparation with PCR amplification, purification and equimolar pooling of samples for amplicon sequencing of the V1-2 region of the 16S rRNA gene was performed as previously described (Camarinha-Silva et al. 2014). For Illumina amplicon sequencing of the V5-6 region, the 807F and 1050R primers were used (Bohorquez et al. 2012). The reaction mixture (20 µl) contained PrimeSTAR buffer (Clontech Laboratories), each deoxynucleoside triphosphate at a concentration of 2.5 mM, each primer at a concentration of 0.2 µM, 5% dimethyl sulfoxide (DMSO), 1 µl of template DNA and PrimeSTAR HS DNA polymerase (2.5 U, Clontech Laboratories). An initial denaturation at 95°C for 3 minutes was followed by 20 cycles of denaturation at 98°C for 10 seconds, annealing at 51°C for 10 sec, extension at 72°C for 45 sec and a final extension for 2 minutes at 72°C. One µl of the PCR product was used on a second PCR (15 cycles), following the same PCR conditions, where the forward primer contains a 6 nucleotide (nt) barcode (Hamady et al. 2008) and a 2 nt GT linker (Meyer and Kircher 2010). Both primers contained a sequence complementary to the Illumina specific adapters at the 5'-ends ((Camarinha-Silva et al. 2014), Table 2.1-S1). A third PCR (10 cycles) was performed in a 50 µl reaction under the same conditions as previously described using primers that integrate the sequence of Illumina multiplexing sequencing and Illumina index primers (Camarinha-Silva et al. 2014). Amplicons were purified with QIAquick PCR Purification Kit, quantified with QuantiFluor dsDNA System (Promega) and equimolar ratios

(30 ng) of amplicons were pooled. Libraries were sequenced using 250 bp paired-end sequencing chemistry on an Illumina MiSeq platform. A total of 4,3 million sequence reads were quality filtered and assembled using the RDP pipeline (Wang et al. 2007). Sequences were excluded if they have any primer or barcode mismatch and an N character. Sequences were assigned to a specific sample via the barcode, aligned and checked for chimeras using uchime and clustered into operational taxonomic units (OTU) at ≥ 97% similarity (Wang et al. 2007). Low abundance OTUs, if present in less than 5 samples in relative abundances lower than 0.01%, were removed. Sample K3 ileal digesta was discarded from the analysis due to low number of reads (<350).

A total of 474 and 995 phylotypes (V1-2 region, ileal digesta and feces samples respectively), and 1,366 and 5,281 phylotypes (V5-6 region, ileal digesta and feces samples respectively) were taxonomically assigned using the naïve Bayesian RDP classifier (Wang et al. 2007). Sequences were submitted to the European Nucleotide Archive (ENA) under accession number PRJEB9411 (http://www.ebi.ac.uk/ena/data/view/PRJEB9411). All samples analysed with V1-2 region comprised more than 11,000 sequence reads, where the mean number of reads per sample was 35,026±2,042. In regards to V5-6 region, all samples have more than 25,000 reads being the mean number of reads per sample 67,325±3,550.

Statistical analysis

T-RFLP and Illumina amplicon sequencing datasets were statistical analysed using PRIMER (v.6.1.16, PRIMER-E; Plymouth Marine Laboratory, UK) (Clarke and Warwick 2001). Abundance data, obtained from T-RFLP and Illumina amplicon analysis, were standardised by total and sample resemblance matrixes were generated using Bray-Curtis similarity coefficient. Community structures were explored by non-metric multidimensional scaling (MDS) and hierarchical clustering. Phylotype richness (Pielou's evenness) and diversity (Shannon diversity) were analysed using univariate measures through PRIMER. Significant differences in diversity were evaluated by analysis of similarity (ANOSIM) (999 permutations) and considered significantly different if P-value < 0.05.

A Mantel-type test (Relate routine in Primer) was used to measure how closely related were the results of the two evaluated regions of the 16S rRNA gene at the genus level (Clarke and Warwick 2001). Rho values were significant if less than 5% of 999 permutations were greater than the real rho. Multivariate dispersion analysis was used to calculate the intra-variation among the same DNA extraction analysed with each 16S rRNA region where a low

dispersion index point to a higher similarity within sample. Species responsible for observed differences were identified by similarity percentages (SIMPER) (Clarke and Warwick 2001).

Illumina amplicon sequencing heat maps, were generated with heatmap.2 provided by gplots package (Venables et al. 2012) implemented in R (version 3.1.2) and depict all the families present in relative abundance higher than 1% in at least one of the 16S rRNA regions analysed.

2.1.4 Results and Discussion

DNA yield and purity

Fifteen manual and four automated DNA extraction procedures were selected to extract total DNA from one pig fecal and one ileal digesta sample (Table 2.1-1, Figure 2.1-1). We applied approximately 250 mg of sample for all commercial methods (K1-K15) and 80 mg for the automated procedure (K16-K19). Spectrophotometric measurements of the extracted DNA showed differences in quantity and quality according to the extraction kit and the sample source (Table 2.1-2). The DNA yield (ng DNA per mg source material) extracted from the fecal sample was higher than from the ileal digesta sample. For ileal digesta, DNA extraction kits K2, K3, K12 and K13 obtained the highest DNA yields with a mean yield of 16.6 ng/mg sample. The other kits showed lower mean DNA yields of 1.5 ng/mg sample. For feces the highest yields were obtained with K1, K2, followed by K3 and K15. With these extraction kits there was a mean DNA yield of 47.9 ng/mg sample, where other kits yielded an average of 6.3 ng/mg sample. Previous studies have also analysed DNA extraction using some of the same kits reported here in mice feces (Ferrand et al. 2014). The yields obtained for K1, K2 and K15 were similar to the ones reported in Table 2.1-2. Another important parameter that can affect the PCR-based community profiling is the quality of extracted DNA. Some of the analysed kits showed values lower than 1 for the A260/280 ratio (Table 2.1-2) that can be associated with protein contamination or some reagent used during the extraction (Hansen et al. 2007; Neary et al. 2014). These kits were also the ones giving low DNA yield from the ileal digesta samples. In this study we used the A260/230 values to have a measure of non-nucleic acid contaminations in each DNA extraction (Table 2.1-2). The results indicated non-nucleic acid contaminations in each DNA extract (A260/230: ileal digesta=0.4±0.1; feces=0.44±0.1). These low values may be due to impurities, buffer salts and/or residual guanidine that are commonly used in column based kits (Hansen et al. 2007; Neary et al. 2014).

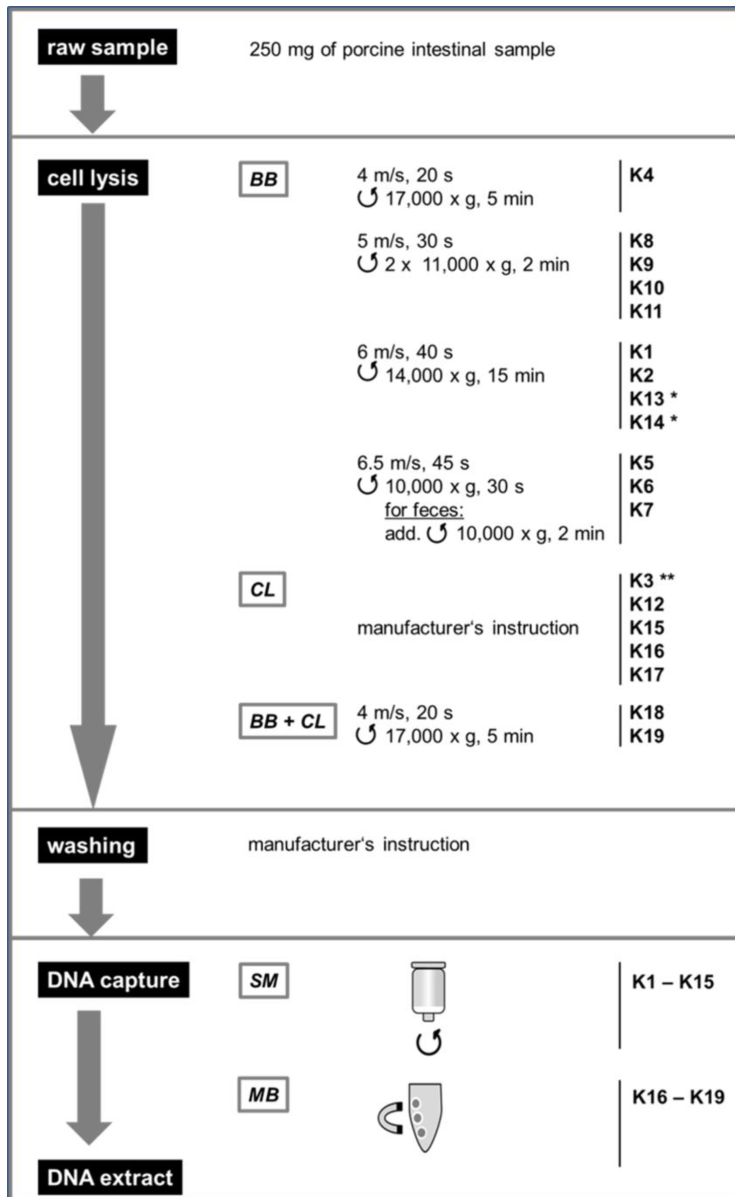

Figure 2.1-1 Flow chart comparing the main steps in DNA extraction protocols of the evaluated kits. BB: bead beating, BB+CL: chemical lysis with an additional bead beating treatment, CL: chemical lysis buffer, MB: magnetic beads, SM: silica membrane, ↺ centrifugation, * for fecal sample an additional centrifugation step for 2 min with 13,000 x g was required to obtain a pellet, ** no Proteinase K was added.

However this was not affecting further DNA usage, as a 1:10 dilution was suitable for successful amplification of the 16S rRNA gene for all DNA extracts using either T-RFLP or Illumina amplicon sequencing. Overall, our results indicate that some kits show better yields, purity and quality of the DNA extracted being more suitable for downstream use. These results were in line with a study comparing DNA extraction kits for denaturing gradient gel electrophoresis (DGGE) that also concluded that DNA extraction efficiency is crucial for comprehensive reflection of microbial communities (Ariefdjohan et al. 2010).

Table 2.1- 2 Yield and purity of extracted DNA.

	ileal digesta				feces			
	yield[a]	concentration (ng/µl)	$A_{260/280}$	$A_{260/230}$	yield[a]	concentration (ng/µl)	$A_{260/280}$	$A_{260/230}$
K1	2.1	12.1	6.1	0.0	54.8	301.0	1.9	0.6
K2	16.6	83.6	1.9	0.6	71.6	411.7	1.9	0.6
K3	20.5	108.8	2.2	1.2	34.7	178.2	2.2	1.2
K4	1.3	4.6	2.1	0.1	1.6	5.9	2.1	0.1
K5	0.5	2.1	0.7	-0.2	3.1	11.1	0.7	-0.2
K6	0.7	2.7	0.9	0.6	3.7	14.1	0.9	0.6
K7	0.2	0.8	0.5	-0.1	2.5	9.3	0.5	-0.1
K8	2.4	12.1	1.9	0.7	2.3	11.9	1.9	0.7
K9	1.6	8.1	2.2	0.6	6.4	34.6	2.2	0.6
K10	2.1	11.5	1.8	0.5	1.7	8.3	1.8	0.5
K11	2.4	13.5	1.8	0.3	11.7	60.6	1.8	0.3
K12	16.6	58.6	1.9	0.7	12.9	42.1	1.9	0.7
K13	12.8	42.8	2.1	0.9	10.2	31.0	2.1	0.9
K14	1.3	7.0	1.8	0.0	1.9	9.3	1.8	0.0
K15	3.2	8.1	2.0	1.5	30.5	71.0	2.0	1.5
K16	0.4	0.4	-4.5	0.1	7.3	5.8	-4.5	0.1
K17	0.9	0.7	4.1	0.0	7.0	5.6	4.1	0.0
K18	2.5	2.0	1.8	0.1	8.1	6.5	1.8	0.1
K19	1.3	1.0	1.9	0.1	14.8	11.8	1.9	0.1

[a] yield: DNA (ng) per source material (mg)

Comparing bacterial communities based on diversity

To compare bacterial communities recovered by the tested DNA extraction kits, T-RFLP analysis and Illumina 16S rRNA amplicon sequencing were performed. There was a heterogeneous distribution in the mean numbers of TRFs and OTUs among the tested DNA extraction kits and type of sample (Figure 2.1-2A).

This indicates a large variation in the capability of recovering a certain given bacterial community. The mean numbers of TRF and OTU units showed great standard deviation for DNA extractions from ileal digesta and feces samples. The mean TRF number of DNA extracted was 11±1 and 26±3 from ileal digesta and feces respectively. Illumina amplicon sequencing of the V1-2 region of the 16S rRNA gene generates an average of 339±11 OTUs (V5-6 region: 777±66 OTUs) from ileal digesta and 682±30 OTUs from feces (V5-6 region:

2964±173 OTUs). The ileum digesta sample, extracted with the automated procedures showed the lowest number of OTUs, regardless of the 16S region analysed, however, this was not observed for the feces sample where these procedures gave similar numbers of OTUs as the majority of the tested kits. In general sequencing of the 16S rRNA gene region V5-6 resulted in higher numbers of OTUs than sequencing the V1-2 region.

Regardless the kit used, higher numbers of TRFs and OTUs were obtained from fecal samples than from ileal digesta (Figure 2.1-2A). This finding of reduced richness in ileal digesta is in line with the study of Looft and colleagues who compared bacterial communities in different sections of porcine intestine (Looft et al. 2014).

The mean Shannon diversity across all DNA extraction kits and analysing methods for ileal digesta was 3.1±0.70. The top three DNA extraction kits based on the highest Shannon index, for T-RFLP, are K9, K11 and K14. Although K11, K12 and K13 gave amplicons that indicated a higher diversity in case of the V1-2 region, amplicons obtained by K2, K8 and K11 extractions showed the highest diversity when analysing the V5-6 region (Figure 2.1-2B). The lowest diversity was observed in the DNA extractions using the automated kit. For feces the mean Shannon diversity across all DNA extraction kits and both T-RFLP and Illumina amplicon sequencing was 3.9±0.81. The highest diversity was observed with K2, K5 and K7 with T-RFLP and V1-2 amplicon sequencing analysis, while V5-6 region showed more diversity when K1, K2 and K11 extractions had been used (Figure 2.1-2B). The differences observed on the Shannon diversity index result suggest that the fecal sample analysed with the different kits do not demonstrate similar diversity profile.

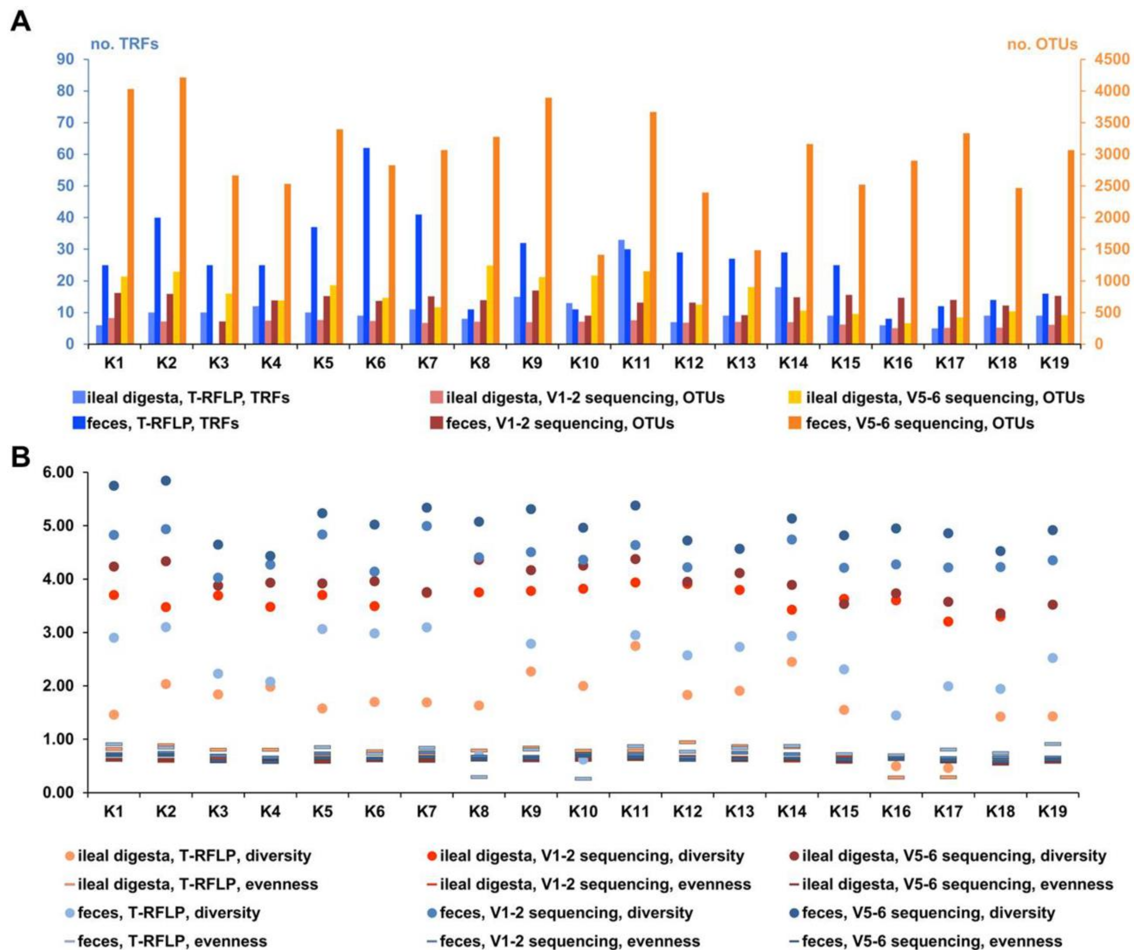

Figure 2.1-2 Numerical comparison of T-RFLP analysis and Illumina 16S rRNA gene amplicon sequencing. (A) Numbers of TRFs and OTUs obtained. TRFs are plotted in blue on the left y-axis and OTUs are plotted in red-orange on the right. (B) Diversity indices of bacterial communities recovered by evaluated DNA extraction kits. Circle symbols show Shannon diversity and bars show Pielou's evenness. Diversity indices of fecal samples are plotted in shades of blue and ileal digesta samples in shades of red.

The differences in global composition of bacterial communities of ileal digesta and feces samples evaluated after DNA extraction using different kits are depicted in Figure 2.1-3 and -S1. A main group, that show similar microbial communities, contains 12 DNA extraction kits that combine cell lysis with bead beating and DNA capture with a silica membrane spin filter. This group clusters together with some outliers in T-RFLP analysis and V1-2 amplicon sequencing of feces material. The clustering shown in Figure 2.1-3, suggests an effect of lysis and DNA recovering on the recovered microbial community composition. Significant differences in bacterial communities with respect to DNA extraction kits using different lysis approaches were observed in the ileum digesta extractions (T-RFLP: R=0.67, p=0.001; V1-2: R=0.8, p=0.001; V5-6: R=0.66, p=0.002). There were no significant differences in the communities recovered from feces extractions, where the R-statistics were close to 0 and p-values > 0.1, however at 60% similarity the dendrograms of feces samples extracted with

different methods showed 8 (T-RFLP), 3 (V1-2) and 2 (V5-6) clusters of samples (Figure 2.1-S1). Kits that have a bead beating step were clustering together suggesting that different species abundance patterns were found consistently in those groups (Figure 2.1-S1). Independently of the analysis method the bacterial community of the ileal digesta of K9 and K11 clustered together (Figure 2.1-3 and -S1) showing that the addition of enhancer SX in the NucleoSpin® Soil (Macherey-Nagel) protocol leads to the extraction of similar bacterial communities even if different lysis buffers are used. Also, this addition resulted in higher DNA yields and Shannon diversity. These results suggest that it may be worth to add the enhancer when this kit is chosen for DNA extraction from porcine intestine samples. Such a variation of the DNA extraction protocol was previously recommended by Desneux and Pourcher (2014).

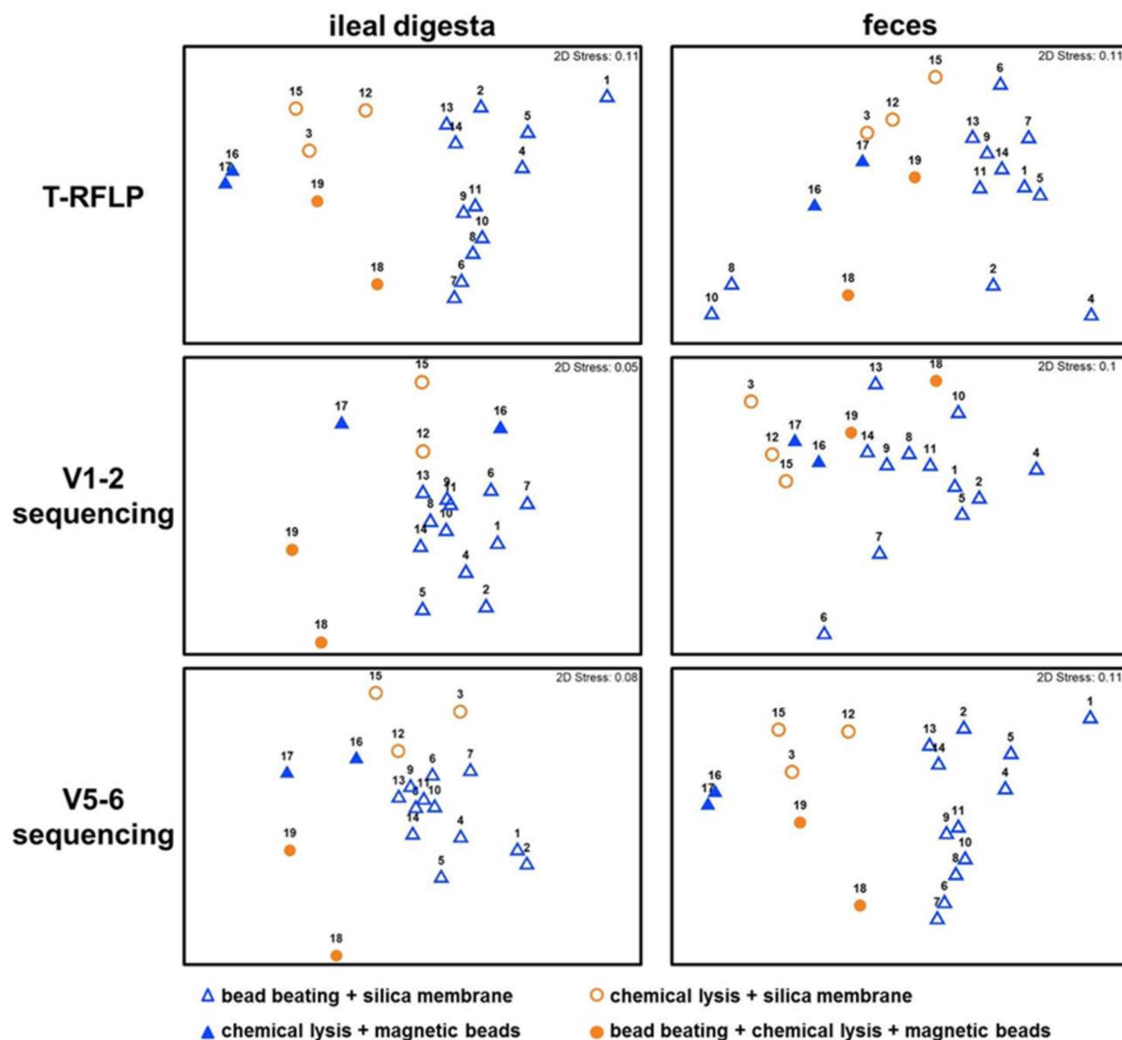

Figure 2.1-3 Non-metric multidimensional scaling (MDS) plot comparing the global bacterial community of all samples analysed with T-RFLP and Illumina amplicon sequencing. Abundance data were standardised with Bray-Curtis similarity algorithm. Symbols refer to cell lysis and DNA capture technique of the DNA extraction protocol.

Comparing bacterial communities based on phylogenetic structure

A recent review article lists several studies surveying porcine intestine samples using different 16S rRNA gene regions (Kim and Isaacson 2015). One of the most used region to study bacterial communities of porcine gut and feces samples is the V1-3 region (Kim and Isaacson 2015). The majority of these studies used 454-pyrosequencing to analyse porcine gut contents and feces samples at different life stages of the animal. Although the V5 region has low resolution, one study analysed colon and cecum samples using Illumina sequencing spanning this region (Pedersen et al. 2013b). Other studies characterise these niches using different regions (V4-5, V4-6 and V5-6) (Riboulet-Bisson et al. 2012; Upadrasta et al. 2013; Cousin et al. 2012). Overall different variable regions are used across the world by different research groups, their choice mainly depends on their experience and knowledge. A comparison of the different variable regions has shown that none of them gives equal results (Claesson et al. 2010). Each 16S rRNA gene region gives different phylogenetic accuracy and the direct comparison of studies is hindered by the absence of using a consensus region (Hamady and Knight 2009).

In the present study, DNA extraction protocols were compared in regards to the bacterial community profiles obtained with Illumina amplicon sequencing spanning the V1-2 and V5-6 region of the 16S rRNA gene. A new Illumina amplicon approach based on a previous study (Camarinha-Silva et al. 2014) was developed using the V5-6 region. A high correlation between community structures obtained by analysing both regions was observed at genus (Rho=0.875, p=0.001) and family level (Rho=0.862, p=0.001) for ileal digesta and also for feces samples (genus: Rho=0.935, p=0.001; family: Rho=0.921, p=0.001) suggesting that both regions are appropriate to analyse the porcine gastrointestinal tract.

Ileal digesta samples showed microbial communities where most of the members belonged to the phyla Firmicutes, Proteobacteria, Bacteroidetes and Fusobacteria. Due to its similarity to the major phyla occurring in the human gut (Firmicutes and Bacteroidetes) (Karlsson et al. 2013), the pig has already been used as an animal model for humans (Heinritz et al. 2013). Bacterial families belonging to these phyla and present in abundances higher than 1%, in at least one of the 16S rRNA community analyses are depicted in Figure 2.1-4. Regardless of the different extraction methods tested, ileal sample analysed within V1-2 and V5-6 region showed 67-68% similarity at genus and 71-72% at family level. Some variances were observed in the community composition detected with each variable region and within DNA extraction procedures. Only one family was identified in the ileal digesta sample using the V5-6 region that was not detected with V1-2 region. *Rhodospirillaceae*, a family of the α-

Proteobacteria, was not detected in the porcine intestine of other studies, but it is known to be a member of communities in soybean-planted soils (Ge et al. 2014). Since pigs were fed with soybeans the recovered *Rhodospirillaceae* might be of feed-origin.

The relative abundances of the most abundant families are shown in Figure 2.1-4. Despite the fact that bead beating improves the detection of Gram-positive bacteria, this effect was not uniformly observed at genus level of ileal digesta sample, across the extractions methods that use mechanical lysis, leading to the differences in the detection of the families that account for 63-73% of total community abundance. Similarity percentage analyses indicated that OTUs belonging to uncultured *Veillonellaceae*, *Streptococcus*, *Clostridium* cluster XI and *Actinobacillus* were responsible for the observed differences between the extractions. The genus *Clostridium* XI of the *Peptostreptococcaceae* family was not equally identified by all extraction kits, with K2 giving the highest abundance (24-26%) and K18 the lowest (4-5%) (Figure 2.1-S2). Streptococcus was detected in higher abundances using the K5 extraction method (29-34%) but almost not presented in the samples extracted with K15 (<0.7%) (Figure 2.1-S2). Both genera were previously found in the lumen of the ileum in abundances close to 5% (Looft et al. 2014). As previously recognized by Sergeant and colleagues it is still uncertain which protocol will give us the most correct description of community structure (Sergeant et al. 2012). At genus level, ileal digesta extracted with K2 showed the highest average community similarity (73%) and the lowest dispersion index (0.1), indicating little difference between the microbial communities analysed by both regions.

DNA extraction from feces revealed 69-71% and 75-77% similarity in community structure within each 16S region at genus and family level. All evaluated DNA extracts from porcine feces showed the presence of five bacterial phyla (Firmicutes, Bacteroidetes, Proteobacteria, Spirochaetes and Actinobacteria) that occurred in a relative abundance higher than 1%, for at least one of the 16S rRNA regions analysed (Figure 2.1-4). This result is in agreement with previous microbiome studies of porcine feces (Holman and Chenier 2014; Looft et al. 2014; Lamendella et al. 2011). A higher representation of *Peptostreptococcaceae* (K4) was observed in feces, once compared to the other extractions kits. K4, K5 and K2 have shown the highest similarity percentage (67%; 65% and 62%, respectively) in the bacterial community structure analysed with both regions, showing the closeness of the samples within the same extraction and analysed with different 16S rRNA regions. Clostridiales Incertae Sedis XIII and

Peptococcaceae 1 were only observed in average abundances lower than 0.09% using the V1-2 region. Microorganisms belonging to the families *Bacteroidaceae*, *Defluviitaleaceae*,

Enterobacteriaceae and *Marinilabiliaceae* were only detected in average abundances lower than 0.9% with V5-6 region.

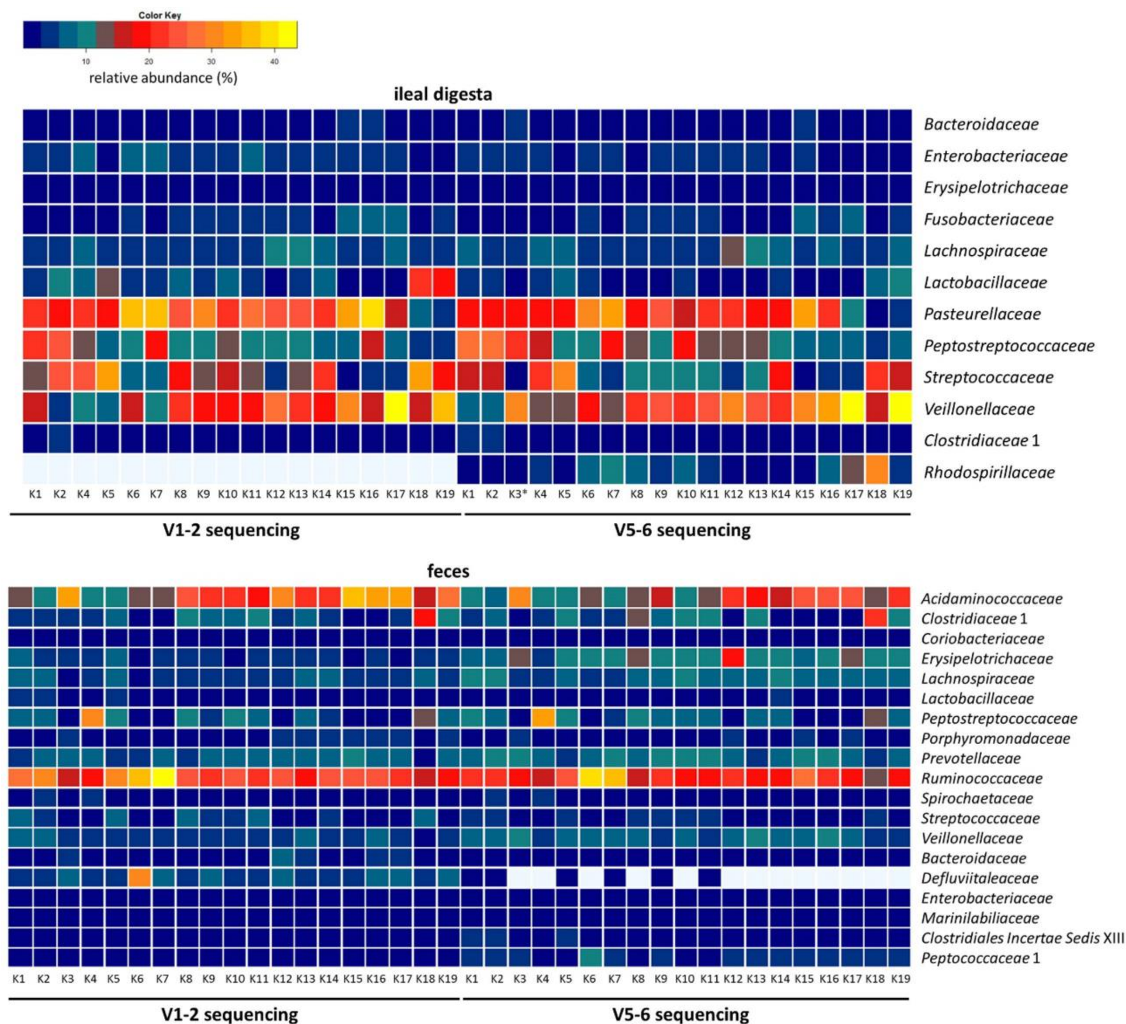

Figure 2.1- 4 Heat map plots depicting the bacterial communities of the ileal digesta and fecal sample at family level. OTUs detected by Illumina sequencing in a relative abundance higher than 1%, in at least one of the 16S rRNA regions, are assigned to bacteria families and listed on the right of the heat maps. The relative abundance of these bacteria families in each DNA extract is coded according to the colour key on the top of the figure. K3* indicates missing data for sequencing 16S rRNA gene region V1-2 of DNA extracted with K3 from ileal digesta, data was excluded because of low read numbers.

Regardless the region, bacterial community profiles, of ileal digesta sample, obtained with DNA extraction protocols using bead beating and compared with the ones of chemical lysis extraction revealed that the group of OTUs contributing for 24-26 average dissimilarity belong to Firmicutes. This is consistent with previous comparative studies of DNA extraction protocols that showed that bead beating improves the detection of Firmicutes (Santiago et al. 2014; Wu et al. 2010; Henderson et al. 2013). Firmicutes members, like *Blautia* were detected in higher relative abundance when using bead beating to disrupt cells compared to chemical lysis. Thus, both type of lysis detect differently some groups of microorganisms.

A previous study on the effect of different DNA extraction conditions on the community structure showed that mechanical cell lysis by bead beating increases relative abundance of Gram-positive bacteria even if this effect did not occur to be uniform within a genus (Sergeant et al. 2012). Such underestimation was found by all 16S rRNA gene sequencing analysis at the phyla level with K5 (PowerLyzer™ PowerSoil®DNA Isolation Kit). Underestimation of one bacterial cell wall type might be crucial for obesity studies that often use the comparison of Firmicutes and Bacteroidetes ratio (Pedersen et al. 2013a). In a previous work, evaluating DNA extractions from porcine intestinal samples, the usage of a modified QIAamp® DNA Stool Mini Kit (Qiagen) was suggested (Li et al. 2003), despite all conclusions lead to similar DGGE results and bacterial detection (12 clones) irrespective of the lysis approach. Also, this kit was compared to a bead beating protocol where severe bead conditions were used (bead beating was performed twice for 2 min) and these conditions are normally not applied in commercial kits.

To study bacterial communities of complex intestinal environments the DNA extraction protocol of choice should be able to extract DNA from different types of source material with a similar efficiency. One evaluated DNA extraction kit that revealed unequal efficiency of ileal digesta and feces sample source was K7, PowerSoil® DNA Isolation Kit (Mo Bio Laboratories), which is used in two large scale microbiome studies, EMP and HMP (McInnes and Cutting 2010). Surprisingly, in the present study, the lowest DNA yield from ileal digesta was obtained using this kit, resulting in low numbers of TRFs and OTUs. However, the diversity indices of the K7 fecal DNA extract showed a good efficiency compared to the results obtained from other kits.

Thus, according to our findings optimisations of DNA extraction are recommendable to ensure comprehensive representation of bacterial communities. Caution should be taken when data are compared across different studies as differences in community structure were observed depending on the extraction method chosen. Finally, based on the results obtained with both samples types and among all tested DNA extraction kits, the FastDNA™ SPIN Kit for Soil (K2) from MP Biomedical emerged as the most suitable one for analysing microbial diversity of the porcine gastrointestinal tract. It gives a high species richness, good DNA yield and quality and it shows, at genus level, high similarity percentage of bacterial community within both regions for the porcine ileal digesta and feces samples and a low dispersion index.

2.1.5 Conclusion

This study compared nineteen different DNA extraction protocols to identify the most suitable one for a comprehensive investigation of the porcine gastrointestinal microbiota. The criteria chosen were based on the DNA yield and purity as well as on the diversity indices and phylogenetic compositions identified by T-RFLP and Illumina sequencing. A high correlation between the community structures obtained V1-2 and V5-6 16S rRNA regions at family and genus level, for ileal digesta and feces samples, showed that both are suitable for future studies. The evaluation of the results showed that the FastDNA™ SPIN Kit for Soil (K2) was the most efficient for bacterial community characterisation and should be the kit of choice in future DNA-based analyses of porcine gastrointestinal samples.

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Conflict of interest

None declared.

2.1.6 References (Burbach *et al.* 2016)

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2.1.7 Supporting information

Table 2.1-S 1 Primers used in this study for amplicon sequencing of the V5-6 region.

Primer name	Primer Sequence (5'-3')	Reference
IlluBC1	ACACTCTTTCCCTACACGACGCTCTTCCGATCTTTGATTGTGGATTAGATACCCBRGTAGTC	
IlluBC2	ACACTCTTTCCCTACACGACGCTCTTCCGATCTTTAATGGTGGATTAGATACCCBRGTAGTC	
IlluBC3	ACACTCTTTCCCTACACGACGCTCTTCCGATCTAACCAGGTGGATTAGATACCCBRGTAGTC	
IlluBC4	ACACTCTTTCCCTACACGACGCTCTTCCGATCTAAGACCGTGGATTAGATACCCBRGTAGTC	
IlluBC5	ACACTCTTTCCCTACACGACGCTCTTCCGATCTAATGGAGTGGATTAGATACCCBRGTAGTC	
IlluBC6	ACACTCTTTCCCTACACGACGCTCTTCCGATCTACGTTGGTGGATTAGATACCCBRGTAGTC	
IlluBC7	ACACTCTTTCCCTACACGACGCTCTTCCGATCTAGTCTCGTGGATTAGATACCCBRGTAGTC	
IlluBC8	ACACTCTTTCCCTACACGACGCTCTTCCGATCTCAACTTGTGGATTAGATACCCBRGTAGTC	
IlluBC9	ACACTCTTTCCCTACACGACGCTCTTCCGATCTCAGTAAGTGGATTAGATACCCBRGTAGTC	
IlluBC10	ACACTCTTTCCCTACACGACGCTCTTCCGATCTCCTAACGTGGATTAGATACCCBRGTAGTC	
IlluBC11	ACACTCTTTCCCTACACGACGCTCTTCCGATCTCGAGAGGTGGATTAGATACCCBRGTAGTC	
IlluBC12	ACACTCTTTCCCTACACGACGCTCTTCCGATCTCGCATAGTGGATTAGATACCCBRGTAGTC	This study
IlluBC13	ACACTCTTTCCCTACACGACGCTCTTCCGATCTGCTCCAGTGGATTAGATACCCBRGTAGTC	
IlluBC14	ACACTCTTTCCCTACACGACGCTCTTCCGATCTGGTTATGTGGATTAGATACCCBRGTAGTC	
IlluBC15	ACACTCTTTCCCTACACGACGCTCTTCCGATCTAACGCAGTGGATTAGATACCCBRGTAGTC	
IlluBC16	ACACTCTTTCCCTACACGACGCTCTTCCGATCTAACTGCGTGGATTAGATACCCBRGTAGTC	
IlluBC17	ACACTCTTTCCCTACACGACGCTCTTCCGATCTAGTTGGGTGGATTAGATACCCBRGTAGTC	
IlluBC18	ACACTCTTTCCCTACACGACGCTCTTCCGATCTATAACTGTGGATTAGATACCCBRGTAGTC	
IlluBC19	ACACTCTTTCCCTACACGACGCTCTTCCGATCTATAGACGTGGATTAGATACCCBRGTAGTC	
IlluBC20	ACACTCTTTCCCTACACGACGCTCTTCCGATCTATATTGGTGGATTAGATACCCBRGTAGTC	
IlluBC21	ACACTCTTTCCCTACACGACGCTCTTCCGATCTCAAGAGGTGGATTAGATACCCBRGTAGTC	
IlluBC22	ACACTCTTTCCCTACACGACGCTCTTCCGATCTCAATTCGTGGATTAGATACCCBRGTAGTC	
IlluBC23	ACACTCTTTCCCTACACGACGCTCTTCCGATCTCAGGCCGTGGATTAGATACCCBRGTAGTC	
IlluBC24	ACACTCTTTCCCTACACGACGCTCTTCCGATCTCCAACGGTGGATTAGATACCCBRGTAGTC	This study
IlluBC25	ACACTCTTTCCCTACACGACGCTCTTCCGATCTGTTCCGGTGGATTAGATACCCBRGTAGTC	

Primer name	Primer Sequence (5'-3')	Reference
Illu Rev	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTAGYTGDCGACRRCCR TGCA	

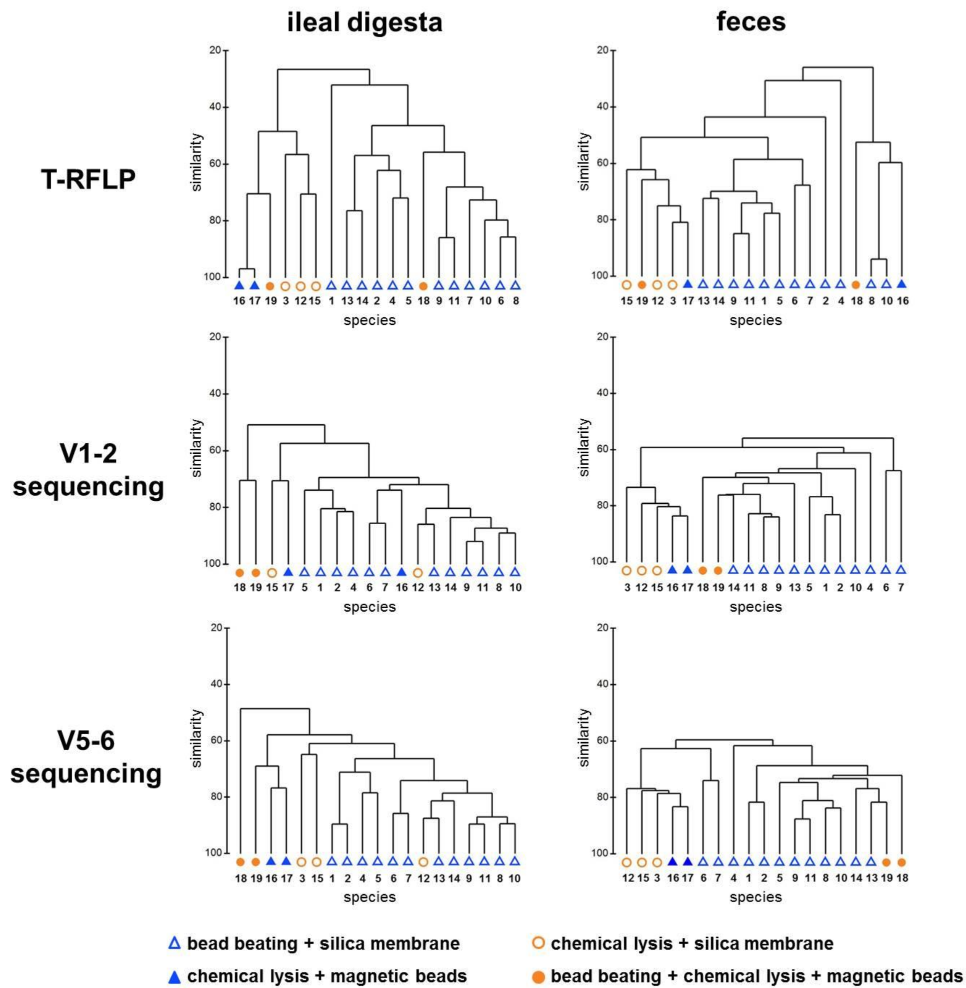

Figure 2.1-S 1 Dendogram for hierarchical clustering of analysed samples based on Bray –Curtis similarity.

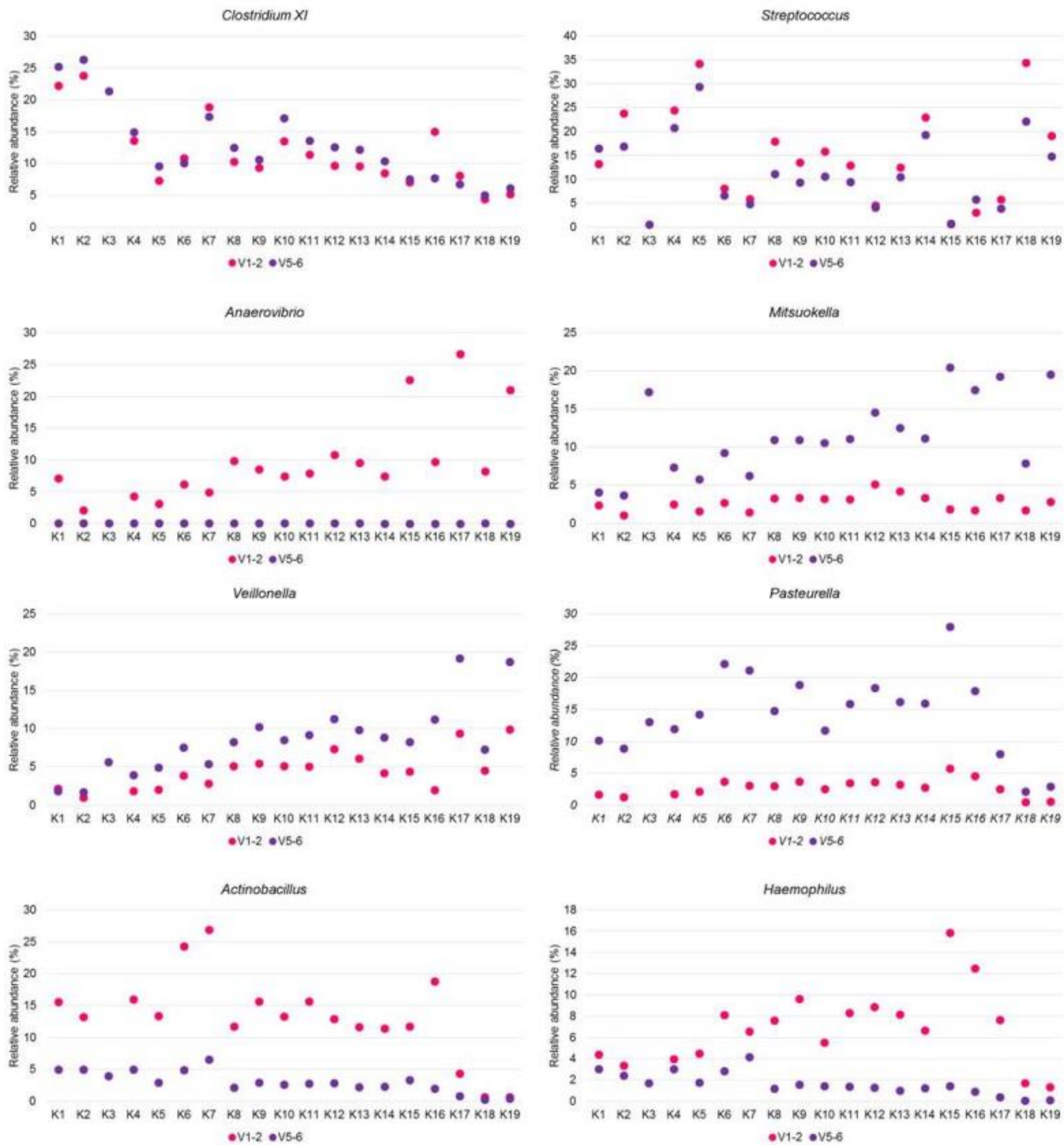

Figure 2.1-S 2 Relative abundance of genus of interest across the ileum digesta sample analysed with different extraction kits.

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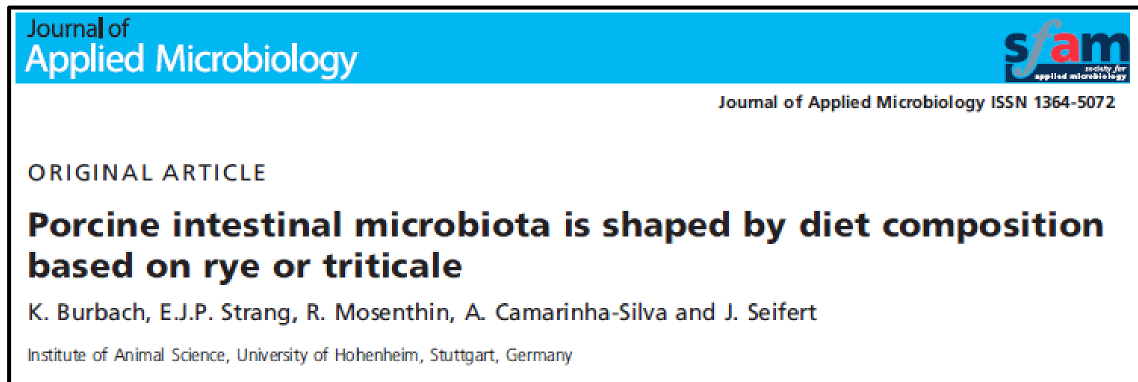

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ORIGINAL ARTICLE

Porcine intestinal microbiota is shaped by diet composition based on rye or triticale

K. Burbach, E.J.P. Strang, R. Mosenthin, A. Camarinha-Silva and J. Seifert

Institute of Animal Science, University of Hohenheim, Stuttgart, Germany

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Porcine intestinal microbiota is shaped by diet composition based on rye or triticale

2.2.1 Abstract

Aims: The present study aimed to compare the microbiota composition from pigs fed different cereal grain types, either rye or triticale, as sole energy source.

Methods and Results: Ileal digesta and faeces were sampled from eight pigs of each experiment. Illumina amplicon sequencing of the 16S rRNA gene was used to analyse the microbiota. Concentrations of short chain fatty acids and ammonia were determined from faecal samples. The grain type revealed significant alterations in the overall microbiota structure. The rye-based diet was associated with an increased abundance of *Lactobacillus* in ileal digesta and *Streptococcus* in faeces and significantly higher concentrations of faecal short-chain fatty acids and ammonia compared to triticale. Whereas triticale significantly promoted the abundance of *Streptococcus* in ileal digesta and *Clostridium* sensu stricto in faeces.

Conclusions: Diets based on rye or triticale affect varying intestinal microbiota, both of taxonomical and metabolic structure, with rye indicating an enhanced saccharolytic potential and triticale a more cellulolytic potential.

Significance and Impact of the Study: Nutrient composition of rye and triticale are attractive for porcine nutrition. Both cereal grains show varying stimuli on the microbiota composition and microbial products of the ileum and feces.

2.2.2 Introduction

The gastrointestinal tract (GIT) is a highly efficient ecosystem, in which host cells and microbial cells are interacting to extract nutrients and energy from dietary ingredients. With alterations in the dietary input, the environmental conditions of this ecosystem may change and consequently, the taxonomic composition and functional activities of microbial communities will adapt to the new conditions (Ley et al. 2008), which, in turn, may affect gut homoeostasis, health status and performance of the host (de Lange et al. 2010).

Diets based on cereal grains are rich in carbohydrates and serve as an important source of energy both in human and livestock nutrition. Starch is the main cereal carbohydrate and is hydrolysed by alpha amylase secreted by animals (Lunn and Buttriss 2007). In addition to starch, cereal grains contain high levels of dietary fibres like the non-starch polysaccharides

(NSP) (Rodehutscord et al. 2016). As nutrient utilisation and energy supply are negatively associated with an increased proportion of dietary NSP, dietary fibres are considered as anti-nutritional substances for animals (Noblet and Le Goff 2001). However, the intestinal microbiome can use NSP as a substrate and releases its metabolites and energy for the host (Noblet and Le Goff 2001). The great majority of starch is already hydrolysed by host-secreted amylases in the small intestine, while most of the NSP proportion cannot be degraded in the small intestine, but will be fermented by the microbiota in the large intestine (Bach Knudsen and Hansen 1991; Lunn and Buttriss 2007). Taxonomical composition and metabolic features of faecal microbiota have shown to be affected by dietary fibres like purified NSP fractions causing alterations of the digesta flow into distal intestine sections. This involves the availability of fermentable substrates and supports microbiota members individually (Metzler-Zebeli et al. 2010; Yang et al. 2013).

For nutritional purposes, wheat is a favoured cereal grain due to its high starch content. However, the nutritional quality with regard to amino acid composition is more favourable in rye. Crossing wheat and rye resulted in the hybrid cereal grain triticale. Triticale combines positive attributes of both crops, like high yield potential from wheat and low demands of growth conditions from rye (McGoverin et al. 2011). A recent comprehensive evaluation of nutritive values of different genotypes of cereal grains showed higher NSP fraction in rye than triticale (Rodehutscord et al. 2016). In particular, the proportions of NSP fractions of arabinoxylan (AX) and β-glucans are higher in rye compared to triticale, whereas triticale is enriched by starch and cellulose compared to rye (Rodehutscord et al. 2016). Furthermore, rye is characterised by higher concentrations of non-cellulosic polysaccharides and sugars which can serve as easily available substrates for microbial fermentation processes. The content of crude protein in triticale is higher than in rye, but the protein quality is advantageous in rye due to an elevated content of lysine. In comparison to protein-rich feeds like soybeans, cereal grains deliver a relatively low proportion of crude protein, however, due to high inclusion levels in the diets, cereal grains contribute significantly to pigs' amino acid requirement (Myrie et al. 2008). The standardised ileal digestibility of amino acids in eight genotypes of rye and triticale, respectively, was determined in growing pigs in previous studies (Strang et al. 2016; Strang et al. 2017). Within the grain type, the ileal digestibility of the genotypes did not differ for the majority of amino acids. Lysine, which is the first limiting amino acid in pig nutrition, was not among those amino acids that showed differences.

With regards to health-conscious nutrition, cereal grains and the microbiota of the intestine play a beneficial role. The knowledge of the effect of rye and triticale on the intestinal

microbiota of humans and animals is limited (Sullivan et al. 2007; Le Gall et al. 2009; Sandberg et al. 2016). The present study aimed to characterise the microbiota composition in ileal digesta and faeces of pigs fed rye- or triticale-based diets and to assess the effect of these diets on fermentation products in faeces.

2.2.3 Materials and Methods

Animals, feeding and sample collection

The experiments were carried out at the University of Hohenheim, Germany, and are described in detail in previous publications for the rye experiment (Strang et al. 2016) and for the triticale experiment (Strang et al. 2017).

Briefly, in total 16 barrows (German Landrace x Piétrain) were fitted with a simple T-cannula at the distal ileum. The pigs were housed individually in stainless steel metabolism crates and randomly allocated to an 8 x 8 Latin square design with 8 periods and 8 experimental diets each based on different genotypes of rye or triticale. The experimental diets contained about 95% of rye or triticale. Growth, harvest and grinding conditions were the same for all genotypes of each grain. In the rye experiment, each period lasted 6 days and samples were collected on day 5 and 6. In the triticale experiment, each period lasted 7 days with sample collection on day 6 and 7. Ileal digesta samples were collected in plastic bags attached to the cannula, and freshly voided faeces were collected individually for each pig. Samples were frozen at -80°C until further analyses.

Analysis of faecal metabolites

For chemical analysis of the faecal components, dry matter of faeces was prepared. Concentrations of short-chain fatty acids (SCFA) were determined by gas chromatography (GC grade; Fluka, Taufkirchen, Germany) following previously described sample preparation (Zijlstra et al. 1977) and measurements using methylvaleric acid as internal standard (Wischer et al. 2013). NH3-N was considered as ammonia and analysed according to Kjeldahl method using steam distillation (Vapodest; Gerhardt GMbH and Co. KG, Königswinter, Germany). Faecal sample material was digested by sulphuric acid, distilled ammonia was trapped in 3% boric acid and titrated with 0.05 N HCl. Concentrations were calculated based on dry matter (DM) content (mmol kg-1 of DM).

DNA extraction and Illumina amplicon sequencing

Genomic DNA from ileal digesta and faeces, each 250 mg, was extracted using the FastDNATM SPIN Kit for soil (MP Biomedicals, Solon, OH, USA) following the manufacturer's instructions. DNA was quantified using NanoDrop 2000 spectrophotometer (Thermo Scientific, Waltham, MA, USA).

Amplicon libraries of the V1-2 region of the 16S rRNA gene were performed according to previously described procedures (Camarinha-Silva et al. 2014) with a slightly modified sequence of primer 27F (AGRGTTHGATYMTGGCTCAG). Amplicons were verified by agarose gel electrophoresis and normalised using SequalPrepTM Normalization Plate Kit (Invitrogen, Thermo Fisher Scientific, Waltham, USA). Amplicons were pooled by index, purified using MinElute PCR Purification Kit (Qiagen, Hilden, Germany) and quantified using Qubit® 2.0 Fluorometer (Invitrogen, Carlsbad, CA, USA). Amplicon libraries were sent for sequencing on an Illumina MiSeq platform using 250 bp paired-end sequencing chemistry.

Bioinformatic and statistical analysis

In total, 11.6 million sequence reads were obtained and bioinformatically processed using Mothur pipeline (Kozich et al. 2013). Sequences with 97% similarity were clustered into operational taxonomic units (OTUs) and those with low occurrence, up to ten reads among ten samples, were excluded from further analysis. The closest phylogenetic representative was manually identified using Seqmatch tool from Ribosomal Database Project. The taxonomical classification was based on the threshold suggested by Yarza et al (Yarza et al. 2014) and applied on both cultured and uncultured bacteria. OTUs classified as Chloroplast/Cyanobacteria were assumed to represent undigested grain material and were therefore removed from the dataset. Four samples were discarded due to insufficient reads. Lastly, bacterial community analysis was based on 2,493 OTUs with an average of 46,396 sequence reads per sample. Sequences were submitted to the European Nucleotide Archive (ENA) under the accession number PRJEB19511 (http://www.ebi.ac.uk/ena/data/view/PRJEB19511). In the text, relative abundance is reported as mean ± standard error of the mean (sem).

Multivariate analysis of data derived from Illumina amplicon sequencing was performed by using PRIMER (v.6.1.16, PRIMER-E, Plymouth Marine Laboratory, Plymouth, UK) (Clarke and Warwick 2001) with the PERMANOVA+ add-on (Anderson et al. 2008). OTU abundance data were standardised by the total. Shannon index was used to determine bacterial diversity based on OTUs. Similarities between the structure of bacterial communities were

examined based on Bray-Curtis similarity matrix and displayed by using multidimensional scaling plot. Individual variabilities along the experimental time were assessed by calculating relative dispersion using multivariate dispersion routine (Warwick and Clarke 1993). Increased dispersion values indicate greater variability between samples from the same animal, meaning a higher temporal heterogeneity. PERMANOVA (permutational multivariate analysis of variance) routine was used to analyse the differences among multivariate community structure in response to experimental factors. Differences were considered to be significant at $P < 0.05$. Differences between univariate data sets (bacterial diversity, abundance of certain taxa, faecal metabolite concentration, relative dispersion) derived from different dietary treatment groups were evaluated using Welch's t-test (Welch 1947) and considered to be significant at $P < 0.05$. Correlations between faecal microbiota and metabolites were computed on normalised variables, by the mean and standard deviation across all pigs. Pearson correlation analysis was performed in the R studio v1.0.136 (https://www.rstudio.com) and visualised by using the corrplot package (Wei 2012). Functional profiles of microbiota were predicted based on 16S rRNA gene sequencing results using Tax4Fun (Asshauer et al. 2015). This metagenomic prediction tool transforms OTU abundance data based on the reference database SILVA (release 123) to a taxonomic profile of organisms collected in Kyoto Encyclopedia of Genes and Genomes. Venn diagram was generated using an online tool (http://bioinformatics.psb.ugent.be/cgi-bin/liste/Venn/calculate_venn.htpl).

2.2.4 Results

Overall bacterial composition is influenced by sampling site

Eight different genotypes of the two grain species rye and triticale were used in the experiments. These different genotypes showed no significant effect on microbiota structure, both at ileal and faecal level ($P > 0.05$). Therefore, the grain genotypes were considered as replicate treatments for each pig in the subsequent data analyses. After data filtering, microbiota analysis was based on times.

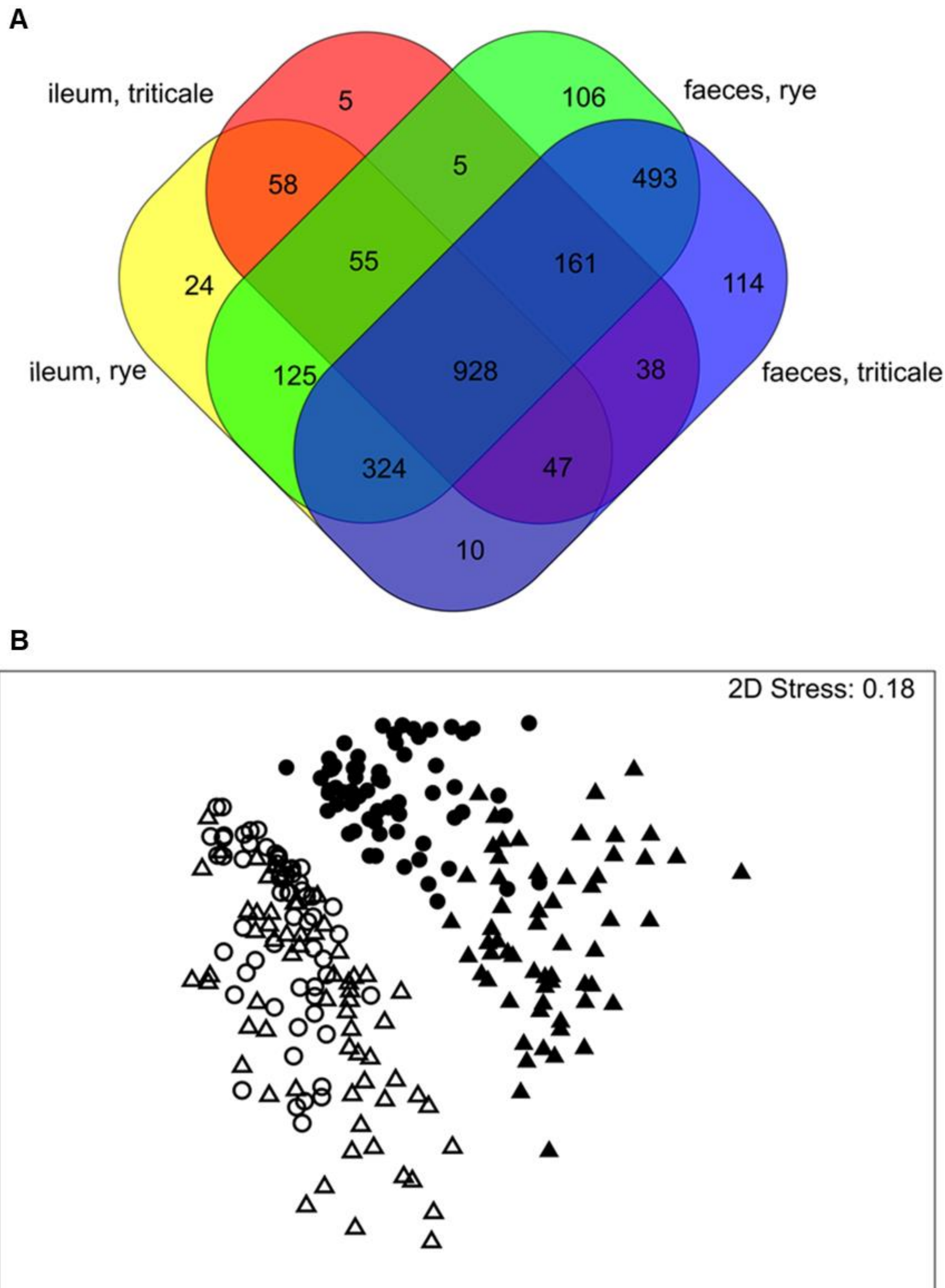

Figure 2.2-1 Microbiota structure across intestine site and feeding treatment. Microbiota is represented on OTU level. (A) Venn-diagram depicting numbers of common and specific OTUs of ileum digesta and faeces from the two feeding treatments based on rye or triticale. (B) Multidimensional scaling plot based on Bray-Curtis similarity algorithm calculated on OTU abundance data from two feeding treatments, each containing eight pigs and eight sample collections at two different sites. Microbiota is represented by ileal digesta: shaped icons, faeces: filled icons and experimental treatment based on rye (triangles) or triticale (circles).

A bacterial core community of 928 OTUs was detected in all samples from the experiments based on rye and triticale (Figure 2.2-1A). In general, the total number of OTUs and the Shannon diversity index were higher in faecal samples than in ileal digesta ($P < 0.001$) (Figure 2.2-S1). Multidimensional scaling revealed a distinct clustering of ileal and faecal samples ($P = 0.001$) (Figure 2.2-1B). Within ileal and faecal samples, the community structures were significantly different based on the grain type fed to pigs ($P = 0.001$) (Figure 2.2-1B).

The overall taxonomic structure of intestinal microbiota was predominated by Firmicutes (mean ± sem; 76% ± 0.8%) (Figure 2.2-S2). In addition, ileal digesta was enriched by the relative abundance of members of Proteobacteria (9% ± 0.8%) and faeces by Bacteroidetes (14% ± 0.7%). Members of Fusobacteria (3% ± 0.3%) were exclusively detected in ileal digesta, whereas the phyla Spirochaetes (5% ± 0.4%) was solely found in faecal samples.

Bacterial composition in the ileal digesta

More than 70% of the total bacterial community in the ileum digesta sample was represented by ten bacterial genera. The average abundance of these bacteria was significantly different between rye- and triticale-based treatment ($P < 0.05$), so the increased abundance indicates an enhanced association with one grain species (Figure 2.2-2, Table 2.2-S1).

Ileal digesta from pigs fed rye were significantly enriched in *Lactobacillus*, *Escherichia/Shigella*, *Bifidobacterium*, *Prevotella*, and *Veillonella* in comparison to samples of triticale, with higher abundances of *Streptococcus*, *Clostridium* sensu stricto, *Clostridium* XI, *Sarcina* and *Bacteroides*. The *Escherichia/Shigella* group was dominated by the species *E. coli* (rye: 10% ± 1.2%, triticale: 5% ± 0.6%).

When comparing the two treatments, samples from the rye-based diets showed a stronger association with lactic acid producing bacteria, like *Lactobacillus*, *Streptococcus* and *Bifidobacterium* than samples from triticale-based diets (cumulative abundance 43% ± 3.1 % versus 35% ± 2.8%).

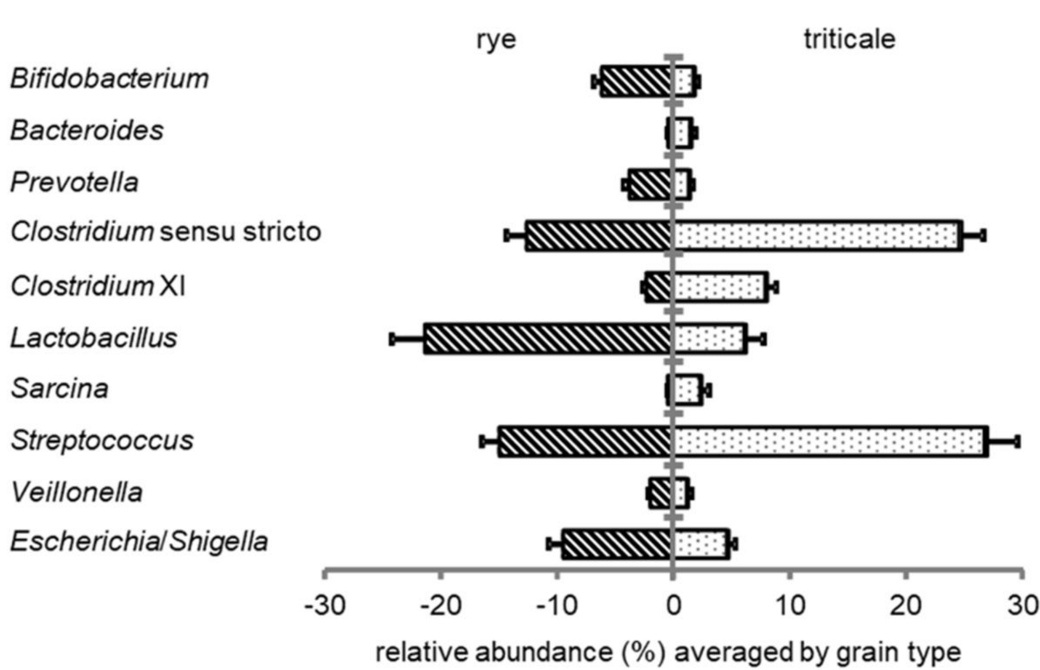

Figure 2.2-2 Genera-grain associated abundance in ileal digesta. The bar chart represents the relative abundance of bacteria genera with an average abundance ≥ 1% derived from rye-based (hatched) and triticale-based feeding treatment (dotted) and proportions being significantly different ($P < 0.05$).

Bacterial composition in faeces

In faecal microbiota, the abundance of 15 genera, with an average abundance >1%, showed a significant association with either rye- or triticale-based treatment (Figure 2.2-3, Table 2.2-S2), representing cumulatively more than 60% of the total faecal composition. *Streptococcus* was the predominant genus in faeces of pigs fed rye-based diet, whereas *Clostridium* sensu stricto was predominant in faeces of pigs fed triticale-based diet. Furthermore, rye promoted the relative abundance of *Prevotella*, *Lactobacillus*, *Bifidobacterium* and *Olsenella*, while the relative abundance of *Turicibacter*, *Clostridium* XI and *Sarcina* was closely associated with feeding triticale.

The faecal abundance of *Bifidobacterium* and *Prevotella* showed to be increased 5-fold and 2.5-fold, in faeces of pigs fed diets with rye and triticale, respectively. In faecal samples from triticale-based treatment, *Clostridium* sensu stricto was the predominant genera and showed a fourfold increase in abundance compared to rye-based treatment.

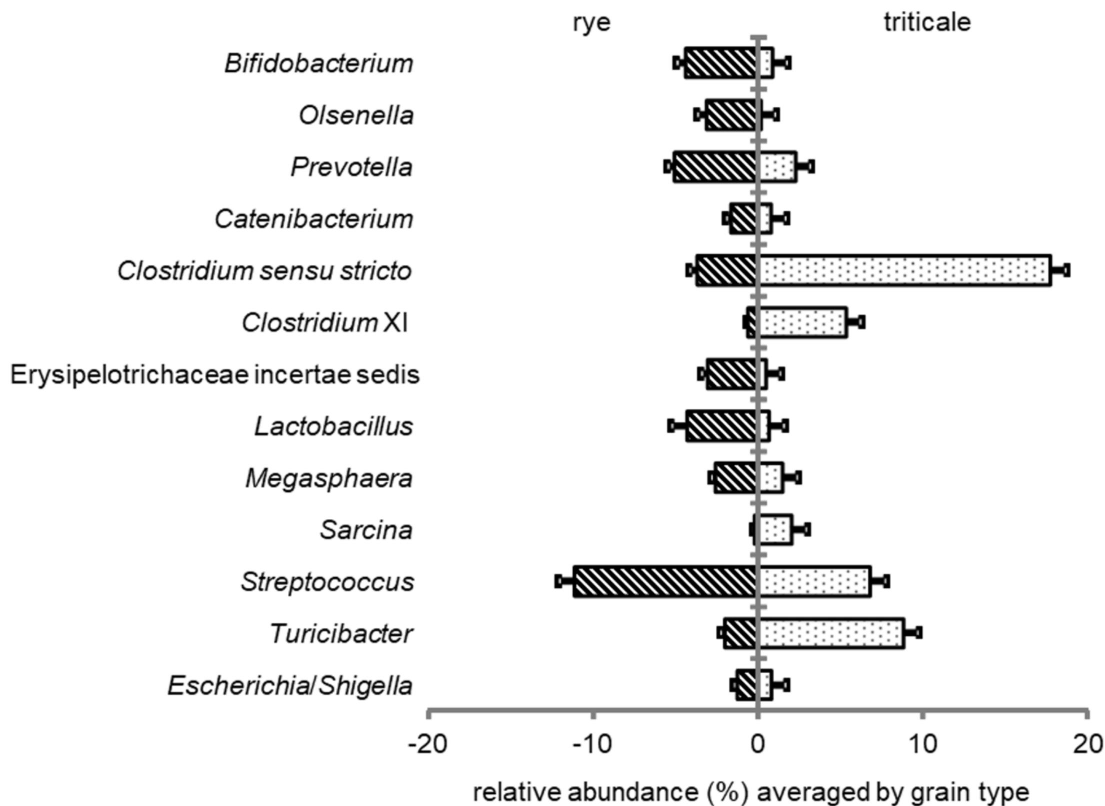

Figure 2.2-3 Genera-grain associated abundance in faeces. The bar chart represents the relative abundance of bacteria genera being significantly different ($P < 0.05$) in faeces derived from rye-based (hatched) and triticale-based feeding treatment (dotted) and with an average abundance ≥ 1%.

Additionally, to the taxonomical structure of the faecal microbiota, profiles of metabolites showed variations in response to the grain species. The significantly higher concentration of SCFAs (acetate, propionate, butyrate, isobutyrate, isovalerate and valerate) and ammonia were determined in faeces of pigs fed rye-based diets compared to those fed triticale-based diets ($P < 0.05$), Table 2.2-1. Acetate was the predominant SCFA in both treatments (rye versus triticale: 180.3 versus 141.7 mmol kg-1 of DM). The concentration of ammonia was in a similar range to faecal acetate (rye: 178.9 mmol kg-1 of DM, triticale: 127.2 mmol kg-1 of DM).

Table 2.2- 1 Concentrations of metabolites in faecal samples.

mmol kg^{-1} of DM	rye	triticale
acetate	180.3	141.7
propionate	58.5	43.7
isobutyrate	16.0	13.1
butyrate	10.6	8.3
isovalerate	13.9	8.1
valerate	34.0	21.0
ammonia	177.9	127.2

All $P < 0.002$

Pearson correlation coefficients were computed to analyse the relationships among bacterial community members and, in addition, between bacterial abundance and concentration values of faecal metabolites (Table 2.2-S3, Table 2.2-S4). The results demonstrated variable correlation patterns between rye and triticale treatments (Figure 2.2-4).

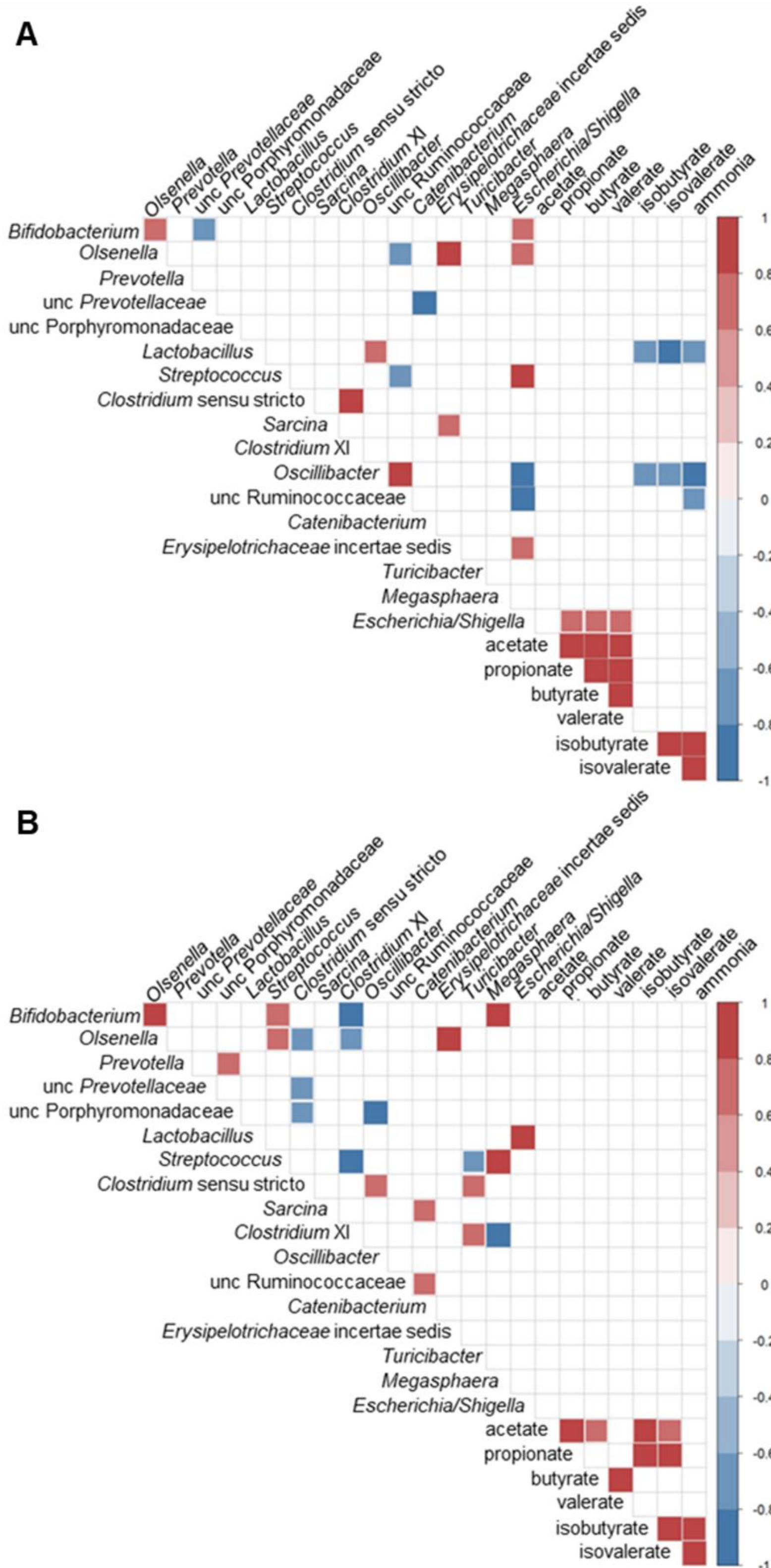

Figure 2.2-4 Correlations between faecal bacteria abundance and metabolites concentration. A subset of microbiota members was chosen, with each being present with an average abundance of ≥ 1% across all faecal samples and showing significant association with one grain type ($P < 0.05$). (A) Correlogram on data derived from rye-based treatment and (B) from triticale-based treatment. The colour code on the right represents the strength of correlation, only significant correlations ($P< 0.05$) are displayed. The abbreviation 'unc' represents uncultured bacteria.

For the rye treatment, the faecal abundance of *Escherichia*/*Shigella* was significantly correlated with various bacteria (Figure 2.2-4A). *Escherichia*/*Shigella* was positively linked

to the abundance of *Bifidobacterium*, *Olsenella*, *Streptococcus* and *Erysipelotrichaceae* incertae sedis, but negatively correlated with uncultured *Ruminococcaceae* and *Oscillibacter*. In addition, faecal abundance of *Oscillibacter* was positively correlated with *Lactobacillus* and uncultured *Ruminococcaceae*.

Faecal samples of pigs fed triticale-based diets showed a higher abundance of *Clostridium* sensu stricto that positively correlated with the abundance of *Oscillibacter* and *Turicibacter* (Figure 2.2-4B). *Clostridium* sensu stricto was negatively linked to the abundance of *Olsenella*, uncultured *Prevotellaceae* and uncultured *Porphyromonadaceae*, which were only detected in an average abundance $< 0.1\%$.

Regardless grain type, *Olsenella* and *Erysipelotrichaceae* incertae sedis were positively correlated, with a significantly increased average abundance by rye-based diets compared to triticale (rye versus triticale, *Olsenella*: 3.1% versus 0.1%, *Erysipelotrichaceae* incertae sedis: 3.1% versus 0.4%).

The correlation analyses of faecal microbiota of the rye treatment (Figure 2.2-4A) revealed positive relationships between the abundance of *Prevotella*, *Streptococcus* and *Escherichia*/*Shigella* to all determined metabolites, though only *Escherichia*/*Shigella* showed statistical significance with propionate, butyrate and valerate ($P < 0.05$). Significant negative correlation was found for the abundance of *Lactobacillus* and *Oscillibacter* with isobutyrate, isovalerate and ammonia. Although not statistically significant, the abundance of Clostridia (*Clostridium* sensu stricto, *Sarcina*, *Clostridium* XI, *Oscillibacter* and uncultured *Ruminococcaceae*) and concentrations of SCFAs were negatively related (Table 2.2-S3).

The results of the correlation analyses of the triticale treatment showed no significant links between abundance of bacterial genera and faecal metabolites (Figure 2.2-4B). However, significant positively and negatively correlations were observed among genera abundances and as well among metabolites. When grouping OTUs at family level, then significant correlations were determined between bacteria and metabolites in the triticale-based treatment (Fig. S3). Significant positive correlation was found for the abundance of uncultured Bacteroidales with acetate, propionate, isobutyrate and isovalerate. And the correlation between *Lachnospiraceae* and acetate and butyrate was significantly negative.

A functional prediction analysis based on 16S rRNA gene amplicon sequencing using Tax4Fun proposed a total of 266 KEGG ortholog (ko) pathways. The overall predicted function of faecal microbiota was statistically affected by the cereal grain type (PERMANOVA, $P = 0.001$) but limited to minor variations of certain predicted pathways

(Table 2.2-S5). The predominant group was the two-component system (ko02020), being 1.20-fold promoted in faeces derived from triticale-treatment compared to rye. Phosphotransferase system (ko02060), a regulatory system of carbohydrate uptake into bacterial cells, was 1.29-fold more abundant in samples of pigs fed rye-based diets. Degradation pathway of valine, leucine and isoleucine (ko00280) was predicted to be 1.28-fold increased for the triticale treatment. The butanoate metabolism (ko00650) was predicted to be slightly promoted by triticale (1.11-fold).

Temporal variations among microbiota

In the present study, the effect of diet composition on gut microbiota was studied in pigs, with 12 weeks of age, over a period of 8 weeks. PERMANOVA test showed a significant period effect on microbiota structure at OTU level ($P < 0.05$). A multivariate dispersion analysis was performed on faecal microbiota structure, both on the level of OTUs and metabolites, to assess individual variation in faecal communities along the present experiment. When comparing the average dispersion of individuals fed rye- and triticale-based diets, the faecal microbiota of the rye treatment showed increased dispersion values (Table 2.2-S6, rye versus triticale, OTU: 1.19 versus 0.86, metabolites: 1.24 versus 0.87, both $P < 0.05$). High variations in the structure of faecal microbiota of the rye treatment have as well been demonstrated by multidimensional scaling plot (Figure 2.2-1B), as samples of the rye treatment show greater distance to each other compared to samples of the triticale treatment, which cluster more closely together.

2.2.5 Discussion

The microbiota' response to a sole source nourishment was studied with 16 cannulated pigs for eight weeks, with eight pigs solely fed with rye and other eight pigs solely with triticale. The comparison between microbiota from both feeding groups revealed distinct different bacterial community structures by the cereal grain type. Rye and triticale are closely related cereal grains, however, the relative variations in chemical composition showed to promote microbiota members differently. The chemical variations among genotypes of both rye and triticale did not affect the microbiota composition and the ileal amino acid digestibility, as was investigated in previous studies (Strang et al. 2016; Strang et al. 2017). When pigs are fed with cereal grain based diets, the lower intestinal segments will be enriched in proportions of low digestible dietary fibres, which serve as substrates for bacterial growth, contributing to a higher bacterial diversity in faecal samples compared to ileal digesta, which was here observed in both feeding experiments. This finding of the diversity of porcine microbiota is in

accordance with previous studies (Looft et al. 2014; Burbach et al. 2016). Next to locations and dietary conditions, variations among the intestinal microbiota may be influenced by different factors like the host's age, genetic background, physiological status and circadian rhythms (Lozupone et al. 2012; Voigt et al. 2014; Zhao et al. 2015; Camarinha-Silva et al. 2017; Kubasova et al. 2017). The majority of factors can be excluded from our study as the pigs were bred and housed under the same conditions, sampling was done at the same time of the day and the same animal caretakers were in contact with the animals. But the physical development of the host during the 8 week of experiment showed a significant effect. In previous longitudinal investigations on faecal microbiota, an age-dependent composition was also identified (Kim et al. 2011). In the present study, larger values of multivariate dispersion correspond to a greater within-group dispersion, indicating higher temporal variations especially in faecal samples from the rye based treatment (Clarke and Warwick 2001). Before the start of the experiment, pigs were fed with a commercial starter feed (Porcigold SMA 134; RKW Süd GmbH, Würzburg, Germany), containing easily digestible components. Subsequently, rye as a source of nutrients might represent rather challenging conditions for the intestinal ecosystem. Such challenges may be represented by a high viscosity of rye cereals, which is positively correlated with its NSP fractions (Rodehutscord et al. 2016). Pigs fed with rye-bread showed a high viscosity of the ileal digesta resulting in lower digestibility of proteins and fat (Bach Knudsen et al. 2005). An increased viscosity impairs the capacity of digestive enzymes to degrade substrates and this reduction in available nutrients might initiate changes in composition of the intestinal microbiota.

Bacterial polysaccharide and protein degradation differs based on cereal composition

As triticale is a hybrid of rye and wheat, its chemical composition is influenced by the composition of both. Triticale contains 7.4 g kg-1 DM more cellulose than rye (Rodehutscord et al. 2016) and this increased proportion might reflect a dietary support of cellulolytic species of the taxonomic group of *Clostridium* sensu stricto (Varel and Pond 1992), dominating ileal digesta and faeces. Furthermore, an increased level of dietary crude protein in diets based on triticale (rye versus triticale: 124 versus 117 g kg-1 DM, (Rodehutscord et al. 2016)), may promote the faecal abundance of *Clostridium* XI. The taxonomic group of *Clostridium* XI is known to include proteolytic species that may ferment amino acids by Stickland-reaction (Bouillaut et al. 2013; Poehlein et al. 2014). This proteolytic fermentation is considered to be important for spore germination (Lawley et al. 2009).

The general predominant prediction of ABC transporters reflects an active substrate transport between bacterial cells and their intestinal environment. In the rye-based treatment, the prediction of phosphotransferase system was increased, indicating a promoted sugar metabolism by microbiota when rye was fed, which is in accordance with higher concentrations of faecal SCFA compared to triticale-based treatment. The faecal microbiota from triticale-based treatment predicted an increased degradation of valine, leucine and isoleucine compared to rye. This amino acid degradation yields in isobutyrate, isovalerate and valerate (Macfarlane et al. 1992), which were present in higher concentration in faeces of pigs fed diets with rye rather than triticale. This is in contrast to the higher crude protein content of triticale described above, but a decreased detection of metabolites concentrations in faeces and an increased prediction of the pathway in faecal microbiota, might be due to an increased absorption of metabolites into host cells from the GIT (Topping and Clifton 2001; Vogt and Wolever 2003).

Formation of short chain fatty acids and gut health

In both feeding experiments, the ileal microbiota was dominated by lactic acid bacteria like *Lactobacillus* and *Streptococcus*, with the abundance of *Lactobacillus* being increased in rye-based treatments and *Streptococcus* promoted by triticale. Next to the two genera listed above, *Bifidobacterium* produce lactic acid by fermentation of carbohydrates. Lactic acid may reduce the pH in the GIT, which inhibits the growth of pathogens. Consequently *Lactobacillus*, *Streptococcus* and *Bifidobacterium* are considered to be beneficial for the host's health by a complex cross-talk with the host (Pessione 2012; Yang et al. 2015). Here rye promoted the abundance of *Bifidobacterium* and *Prevotella* both in samples from ileal digesta and faeces. This can be explained by genetic capacity of both genera to break down AX (Dodd et al. 2010; Rivière et al. 2014), which proportion is higher in rye than in triticale (85% in rye versus 55% in triticale, (Rodehutscord et al. 2016)). Arabinoxylan-oligosaccharides have been proven to support a bifidogenic and as well butyrogenic response in microbiota due to mutual cross feeding of bifidobacteria and butyrate-producing bacteria (Rivière et al. 2015). In addition, an in vivo study showed that rye bread, enriched in AX, stimulated butyrate production and butyric plasma concentration when compared to consuming wheat bread, which contains higher contents of cellulose (Bach Knudsen et al. 2005). Similar to these findings, the rye-based treatment of this study here yielded in higher faecal concentrations of butyrate when compared to faeces from pigs fed triticale. Interestingly, the prediction analysis of functional faecal profiles suggested slight increased butanoate metabolism of the microbiota by triticale, including the biomarker genes but and buk for butyrate-producing

communities (Vital et al. 2013). Previously, a missing detection of faecal butyrate concentration was reviewed to occur due to colonic uptake during an extended transit time (Topping and Clifton 2001). In a human study on the relationship between faecal SCFAs concentrations and colonic SCFAs absorption, faecal acetate showed an inverse relation to acetate absorption. Therefore it was suggested to consider the concentration in faeces as an indicator of the absorption rate in the colon, rather than the rate of production (Vogt and Wolever 2003).

Correlation analysis between faecal bacterial abundances and metabolite concentrations aimed to identify bacteria of high metabolic activity by one of the cereal grains. Positively correlation between the faecal abundance of *Escherichia*/*Shigella*, *Prevotella* and *Streptococcus* with faecal metabolites concentrations, indicated an active role of these bacteria in metabolising the rye-based diets. However, only the positive correlations of *Escherichia*/*Shigella* were statistically significant. Characterization of the porcine microbiota across the whole GIT indicated a prevalent role of *Escherichia*/*Shigella* in digestion processes in the small intestine (Zhao et al. 2015). The main representative of the *Escherichia*/*Shigella* was the facultative anaerobe species *E. coli*, which pathogenic potential often relegates its importance as intestinal commensal (Conway and Cohen 2015). Under anaerobic conditions *E. coli* uses a mixed acid fermentation with the main end products acetate, ethanol, lactate, formate, succinate, hydrogen and carbon dioxide. Here the correlation analysis between the abundance of *Escherichia*/*Shigella* and the concentration of acetate showed no significance ($P>0.05$) but a positive correlation was found with propionate, butyrate and valerate. *E. coli* is known to carry genes of cryptic pathways, which convert succinate to propionate (Haller et al. 2000) such metabolic activity might be indicated by the here presented data. Furthermore, *Escherichia*/*Shigella* reflected symbiotic relationships with some anaerobic bacteria by positive correlations of abundances. Above mentioned cross feeding between degraders of complex carbohydrates and fermenters of simple sugars might support multiple correlations with the abundance of *Escherichia*/*Shigella* in faeces of pigs fed with rye.

Prevotella was detected among the most abundant bacteria in porcine faeces and is known for its high abundance in the faecal metagenome of pigs exhibiting proteolytic and saccharolytic activities (Purushe et al. 2010; Lamendella et al. 2011; Pajarillo et al. 2015). By a quantitative PCR approach in ileal digesta from pigs fed a diet with chicory pectin, a positive correlation between the abundance of *Bacteroides–Prevotella–Porphyromonas* and butyrate concentration was found and a stimulating effect on butyrate-producing bacteria by cross-feeding was hypothesised (Ivarsson et al. 2014). *Streptococcus* revealed a variable promotion

by the cereal grain treatments here. In ileal digesta, the abundance of *Streptococcus* was more promoted by triticale than rye, but in faeces *Streptococcus* was more enriched by rye. This suggests a wide substrate utilisation of *Streptococcus*, using both the highly digestible nutrients of rye in ileal digesta and the low digestible nutrients of triticale in lower intestinal segments. The correlation analysis of the triticale-based treatment missed revealing any significant correlations between bacterial abundance and metabolite concentration in faeces. However, an enhanced faecal abundance of *Clostridium* sensu stricto might have indicated a potential metabolic importance as this bacterial group includes SCFA producers (Bruce et al. 2014). In general, the rye-based diets resulted in higher faecal concentrations of SCFAs and ammonia compared to feeding triticale. The proportions of the SCFAs were similar to those in previous studies of porcine microbiota (Metzler-Zebeli et al. 2010; Heinritz et al. 2016).

In the rye-based treatment, the negative correlation between *Oscillibacter* and *Escherichia/Shigella* reflected the relationship between bacteria with putative beneficial and detrimental effects on the intestinal health. Previously, *Oscillibacter* was detected as a phylogenetic marker of the intestinal health status, as the qualitative and quantitative detection was significantly higher in healthy humans compared to ileal Crohn's disease patients, suffering from a dysbiosis in faecal microbiota (Mondot et al. 2011). Additionally to human microbiota, a high abundance of *Oscillibacter* in faeces of pigs has also been suggested to be important for porcine health.

Both feeding treatments showed a positive correlation between the faecal abundance of *Olsenella* and *Erysipelotrichaceae* incertae sedis, which might be explained by an interaction between the families *Coriobacteriaceae* (that includes Olsenella genus) and *Erysipelotrichaceae* as described in a study on diet-microbiota-host interactions in hamsters (Martínez et al. 2013). Increased concentrations of faecal cholesterol, caused by diet enriched in plant sterol esters, decreased the faecal abundance of *Coriobacteriaceae* and *Erysipelotrichaceae*. Similar to these findings in faecal microbiota of hamsters, the lower abundance of both, *Olsenella* and *Erysipelotrichaceae* incertae sedis, when triticale-based diets have been fed compared to rye, might be explained by different cholesterol levels of the cereal grains. Rye bread in comparison to wheat bread has been demonstrated to reduce plasma cholesterol levels in pigs (Lærke et al. 2008). Studies on the effect of triticale on lipid metabolism are still needed.

Outcomes with relevance for human nutrition

Dietary response of porcine intestinal ecosystem may in some extent be transferred on issues considering human nutrition, due to similar intestinal anatomy and intestinal microbial ecology (Heinritz et al. 2013). The pig was used as a model to study the intestinal digestion of wheat and rye bread for humans and showed a lower digestibility for rye bread, which is in agreement with reduced digestibility of crude protein from rye compared to triticale in growing pigs (Le Gall et al. 2009; Strang et al. 2016; Strang et al. 2017). Furthermore, with regards to metabolic response, pigs and humans showed very similar metabolic profiles in plasma after the consumption of bread made from wheat- or rye-based flour (Nielsen et al. 2014). In a study on healthy males, which consumed a rye-based evening meal, an increased colonic overnight fermentation and increased numbers of *Bifidobacterium* were observed and combined with a subsequently reduced energy intake, an appetite lowering potential by rye was indicated (Ibrügger et al. 2014). Furthermore, whole grain based diets, like rye and triticale in this study, may alter human gut microbiome beneficially influencing immunological functions, by anti-inflammatory effects and improved glycemic response (Martinez et al. 2013).

However, the authors would like to emphasize that the here reported study was designed as an observational study. It is the first study comparing the effects of rye and triticale on the intestinal microbiota from pigs. This approach provides no casual links to putative health benefits by feeding rye or triticale. In summary, by analysing bacterial abundances and metabolite concentrations insights in the potential of these cereal grains are given. Diets based on rye promoted an increased abundance of saccharolytic bacteria like *Lactobacillus*, *Bifidobacterium* and *Prevotella* and reveal higher faecal concentrations of SCFAs when compared to triticale. Whereas triticale-based diets yielded in microbiota with a dominant proportion of *Clostridium* sensu stricto, indicating an enhanced cellulolytic potential of the microbiota.

Author statements

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Conflicts of interest

The authors declare that they have no competing interests.

Ethical statement

The protocol for the animal experiments was approved by the German Ethical Commission for Animal Welfare. The experiments were carried out at the University of Hohenheim, Germany, and are described in detail by previous publication for the rye experiment (Strang et al. 2016) and for the triticale experiment (Strang et al. 2017).

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2.2.7 Supporting Information

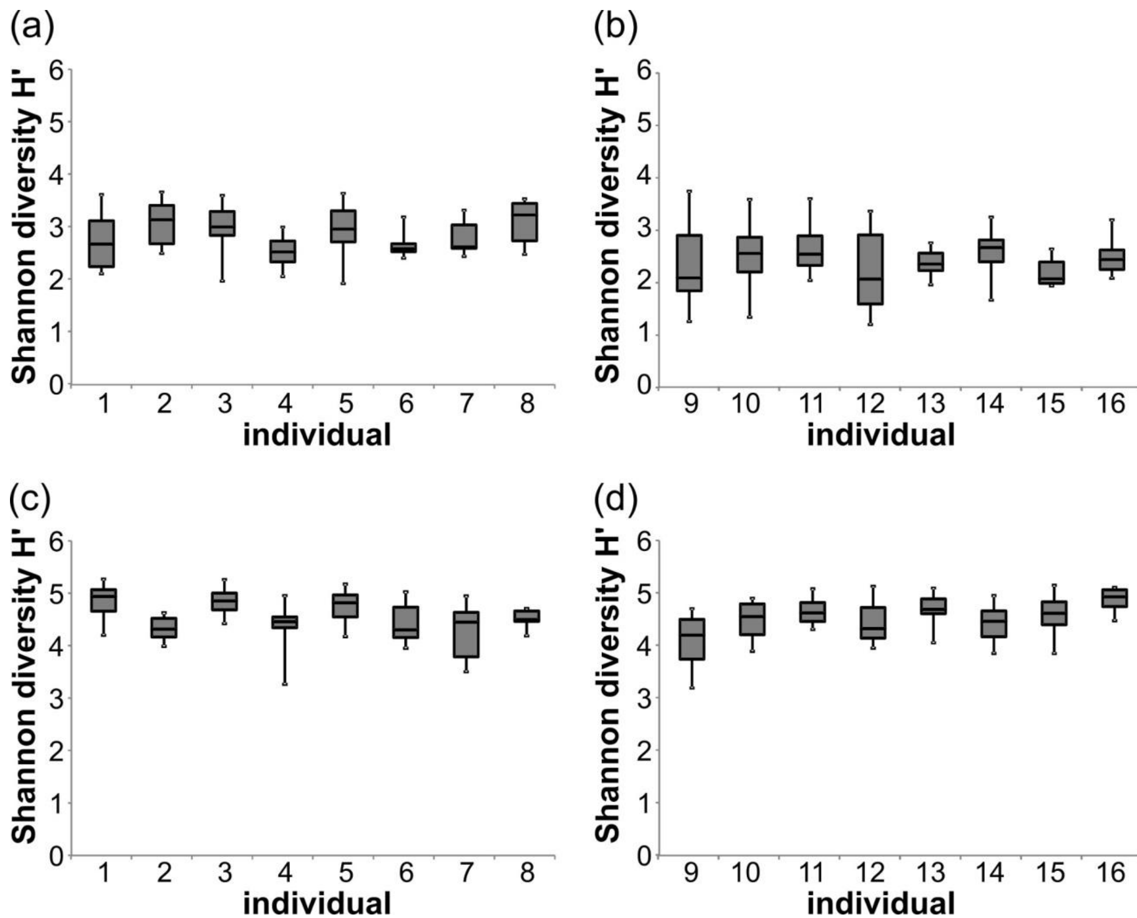

Figure 2.2-S1 Bacterial diversity across intestine site and feeding trial. Boxplots of Shannon diversity indices are based on OTUs from ileal digesta of rye- (a) and triticale-based trial (b) and from faeces of rye- (c) and triticale-based treatment (d). The boxes depict the range from the first quartile (0.25) to the third quartile (0.75), the horizontal line marks the median and the whiskers represent minimum and maximum values.

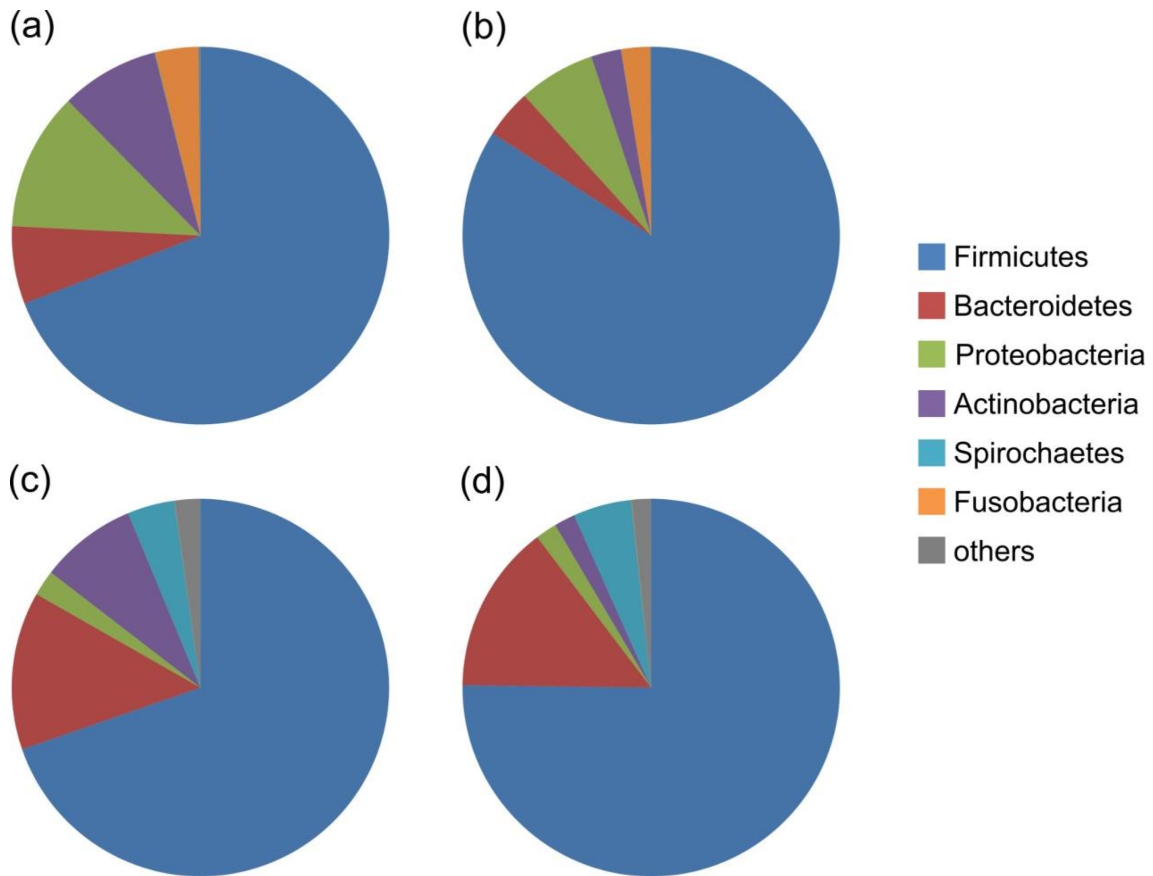

Figure 2.2-S2 Phyla composition. Pie charts depicting the relative proportion of bacteria phyla with an average abundance >1% from ileal digesta of rye- (a) and triticale-based trial (a) and from faeces of rye- (c) and triticale-based trial (d).

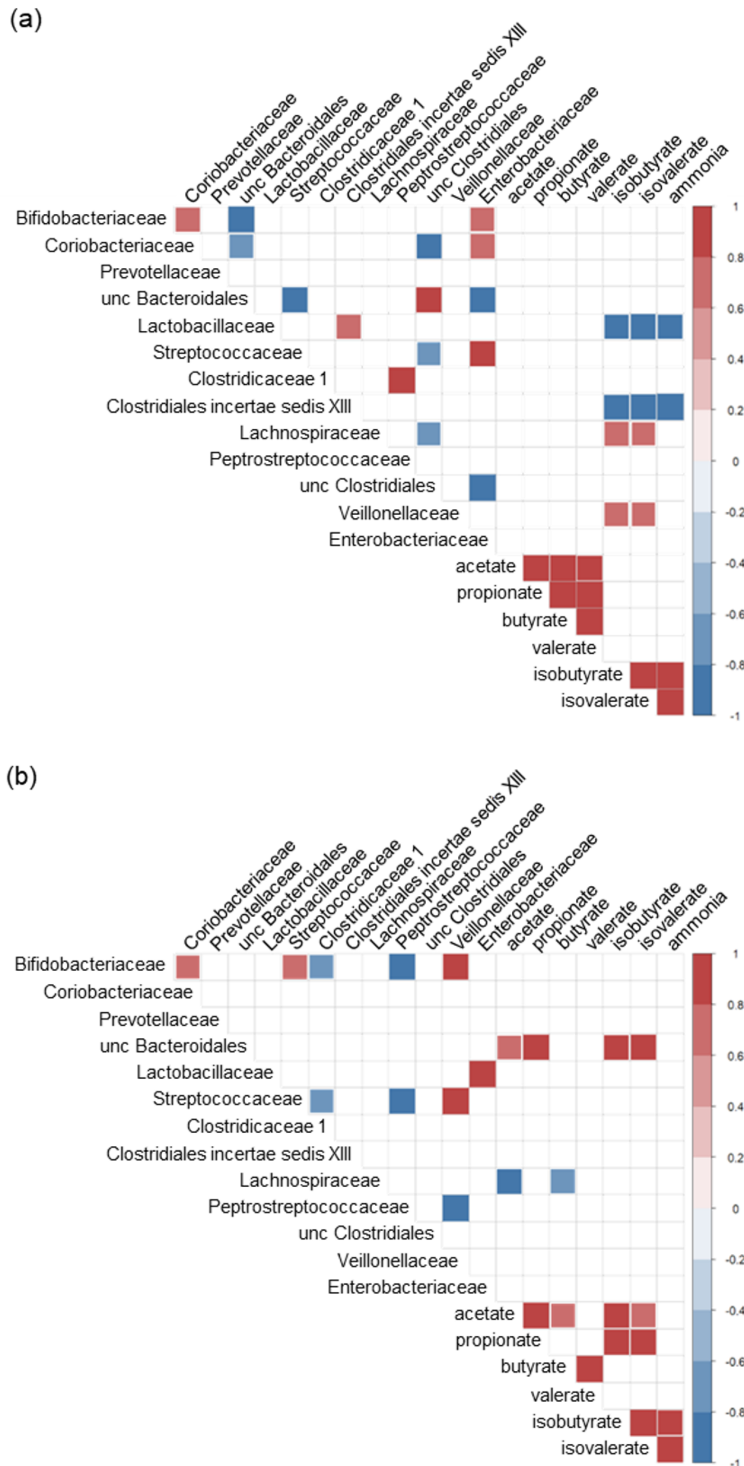

Figure 2.2-S3 Correlations between faecal bacteria family abundance and metabolites concentration. A subset of bacteria families was chosen, with each family being present with an average abundance of ≥ 1% across all faecal samples and showing significant association with one grain type ($P < 0.05$). (a) Correlogram on data derived from rye-based treatment and (b) from triticale-based treatment. The colour code on the right represents the strength of correlation, only significant correlations are displayed ($P< 0.05$). The abbreviation 'unc' represents uncultured bacteria.

Table 2.2-S1 Grain associated abundance of bacteria genera in ileal digesta. Bacteria genera with an average abundance > 1% in ileal digesta and significant different abundance between rye-based and triticale-based treatment are listed (P < 0.05). The values represent the mean ± sem of the relative abundance.

genus	phylum	rye (%)	triticale (%)
Bifidobacterium	Actinobacteria	6.2 ± 0.7	2.0 ± 0.3
Bacteroides	Bacteroidetes	0.5 ± 0.1	1.4 ± 0.3
Prevotella	Bacteroidetes	3.8 ± 0.4	1.2 ± 0.2
Clostridium sensu stricto	Firmicutes	12.7 ± 1.7	25.0 ± 1.9
Clostridium XI	Firmicutes	2.3 ± 0.3	8.3 ± 0.7
Lactobacillus	Firmicutes	21.4 ± 2.8	6.0 ± 1.5
Sarcina	Firmicutes	0.5 ± 0.1	2.4 ± 0.6
Streptococcus	Firmicutes	15.0 ± 1.4	27.0 ± 2.7
Veillonella	Firmicutes	2.0 ± 0.2	1.2 ± 0.2
Escherichia/Shigella	Proteobacteria	9.5 ± 1.2	4.9 ± 0.6

Table 2.2-S2 Grain associated abundance of bacteria genera in faeces. Bacteria genera with an average abundance ≥ 1% in faeces and significant different abundance between rye-based and triticale-based treatment are listed (P < 0.05). The values represent the mean ± sem of the relative abundance.

genus	phylum	rye (%)	triticale (%)
Bifidobacterium	Actinobacteria	4.4 ± 0.5	0.8 ± 0.1
Olsenella	Actinobacteria	3.1 ± 0.6	0.1 ± 0.03
Prevotella	Bacteroidetes	5.1 ± 0.4	2.2 ± 0.4
Catenibacterium	Firmicutes	1.7 ± 0.3	0.7 ± 0.2
Clostridium sensu stricto	Firmicutes	3.7 ± 0.5	17.7 ± 1.0
Clostridium XI	Firmicutes	0.7 ± 0.1	5.3 ± 0.4
Erysipelotrichaceae incertae sedis	Firmicutes	3.1 ± 0.3	0.4 ± 0.1
Lactobacillus	Firmicutes	4.3 ± 0.9	0.6 ± 0.1
Megasphaera	Firmicutes	2.6 ± 0.3	1.5 ± 0.3
Sarcina	Firmicutes	0.3 ± 0.1	2.0 ± 0.2
Streptococcus	Firmicutes	11.1 ± 1.0	6.8 ± 0.8
Turicibacter	Firmicutes	2.0 ± 0.2	8.8 ± 0.5
Escherichia/Shigella	Proteobacteria	1.3 ± 0.2	0.8 ± 0.1

Table 2.2-S3 Pearson correlations between faecal abundance of bacteria and concentration of metabolites from faeces of rye-based treatment. * indicates a significant correlation value ($P < 0.05$), the abbreviation "unc" represents uncultured bacteria classified on family level.

	Olsenella	*Prevotella*	unc Prevotellaceae	unc Porphyromonadaceae	*Lactobacillus*	*Streptococcus*	*Clostridium* sensu stricto	*Sarcina*	*Clostridium* XI	*Oscillibacter*	unc Ruminococcaceae	*Catenibacterium*	Erysipelotrichaceae incertae sedis	*Turicibacter*	*Megasphaera*	*Escherichia/Shigella*	acetate	propionate	butyrate	valerate	isobutyrate	isovalerate	ammonia
Bifidobacterium	0.77*	-0.57	-0.77*	0.14	-0.11	0.67	-0.58	0.60	-0.45	-0.36	-0.68	0.52	0.66	-0.06	0.24	0.72*	0.15	0.39	0.34	0.47	0.22	0.13	0.06
Olsenella		-0.25	-0.30	0.30	-0.38	0.47	-0.28	0.66	-0.08	-0.64	-0.77*	0.11	0.96*	0.36	-0.18	0.78*	0.10	0.32	0.32	0.39	0.37	0.33	0.31
Prevotella			0.64	-0.26	-0.27	-0.03	0.25	-0.24	0.03	-0.42	-0.02	-0.65	-0.09	-0.06	-0.30	0.11	0.56	0.29	0.39	0.26	0.48	0.54	0.62
unc Prevotellaceae				0.20	-0.33	-0.36	0.57	-0.06	0.60	-0.12	0.31	-0.88*	-0.18	0.49	-0.49	-0.35	-0.23	-0.47	-0.38	-0.48	0.05	0.15	0.25
unc Porphyromonadaceae					-0.35	-0.02	0.05	0.34	0.32	-0.11	-0.26	-0.44	0.19	0.62	0.29	-0.04	-0.39	-0.50	-0.56	-0.50	0.08	0.10	0.17
Lactobacillus						-0.36	-0.34	-0.66	-0.51	0.74*	0.58	0.61	-0.49	-0.66	0.14	-0.46	-0.10	0.03	-0.02	0.15	-0.79*	-0.82*	-0.80*
Streptococcus							-0.13	0.45	-0.23	-0.69	-0.78*	0.03	0.38	-0.24	-0.09	0.83*	0.50	0.54	0.51	0.54	0.42	0.35	0.44
Clostridium sensu stricto								-0.26	0.88	-0.12	0.08	-0.52	-0.28	0.25	-0.54	-0.25	-0.11	-0.20	-0.23	-0.43	-0.02	-0.01	0.19
Sarcina									0.09	-0.50	-0.49	-0.13	0.72*	0.62	-0.04	0.53	-0.23	-0.17	-0.10	-0.07	0.43	0.44	0.30
Clostridium XI										-0.10	0.08	-0.56	-0.05	0.64	-0.47	-0.28	-0.45	-0.50	-0.51	-0.69	-0.01	0.04	0.14
Oscillibacter											0.87*	0.43	-0.68	-0.23	0.34	-0.87*	-0.56	-0.52	-0.56	-0.47	-0.79*	-0.77*	-0.86*
unc Ruminococcaceae												0.08	-0.71	-0.09	0.01	-0.92*	-0.55	-0.60	-0.56	-0.53	-0.70	-0.63	-0.71*
Catenibacterium													0.03	-0.53	0.29	0.05	0.01	0.32	0.26	0.38	-0.38	-0.46	-0.59
Erysipelotrichaceae incertae sedis														0.45	-0.23	0.76*	0.12	0.31	0.36	0.38	0.49	0.47	0.40
Turicibacter															-0.16	-0.05	-0.57	-0.61	-0.55	-0.61	0.24	0.32	0.24
Megasphaera																-0.15	0.05	-0.07	-0.12	-0.07	0.13	0.11	-0.03
Escherichia/Shigella																	0.62	0.72*	0.72*	0.73*	0.65	0.59	0.62
acetate																		0.91*	0.91*	0.83*	0.61	0.54	0.63
propionate																			0.98*	0.94*	0.45	0.36	0.42
butyrate																				0.95*	0.50	0.43	0.46
valerate																					0.34	0.26	0.29
isobutyrate																						0.99*	0.94*
isovalerate																							0.94*

Table 2.2-S4 Pearson correlations between the faecal abundance of bacteria and concentration of metabolites from faeces of triticale-based treatment. * indicates a significant correlation value (P < 0.05), the abbreviation "unc" represents uncultured bacteria classified on family level.

	Olsenella	*Prevotella*	unc Prevotellaceae	unc Porphyromonadaceae	*Lactobacillus*	*Streptococcus*	*Clostridium* sensu stricto	*Sarcina*	*Clostridium* XI	*Oscillibacter*	unc Ruminococcaceae	*Catenibacterium*	Erysipelotrichaceae incertae sedis	*Turicibacter*	*Megasphaera*	*Escherichia/Shigella*	acetate	propionate	butyrate	valerate	isobutyrate	isovalerate	ammonia
Bifidobacterium	0.81*	0.28	0.32	0.30	-0.26	0.79*	-0.69	-0.57	-0.90*	-0.42	-0.51	-0.11	0.63	-0.56	0.85*	-0.12	-0.07	-0.27	0.25	0.31	-0.35	-0.43	-0.16
Olsenella		0.37	0.21	0.57	-0.10	0.72*	-0.75*	-0.31	-0.73*	-0.52	-0.07	0.14	0.94*	-0.70	0.70	0.08	-0.22	-0.29	0.15	0.25	-0.34	-0.37	0.01
Prevotella			0.42	0.77*	-0.44	-0.11	-0.51	0.09	-0.08	-0.56	0.39	0.58	0.50	-0.29	-0.13	-0.38	0.21	0.50	-0.13	-0.20	0.33	0.30	0.33
unc Prevotellaceae				0.48	0.05	0.36	-0.77*	-0.58	-0.31	-0.60	-0.06	-0.17	0.10	-0.36	0.23	-0.10	0.02	0.10	0.03	0.04	-0.04	-0.02	0.15
unc Porphyromonadaceae					0.05	0.05	-0.72*	-0.10	-0.10	-0.88*	0.60	0.51	0.70	-0.31	-0.06	0.00	-0.19	0.06	-0.26	-0.20	0.12	0.12	0.32
Lactobacillus						-0.06	0.09	-0.27	0.34	-0.02	0.15	-0.47	-0.02	0.49	-0.19	0.92*	-0.10	-0.26	0.30	0.32	0.13	0.22	0.44
Streptococcus							-0.65	-0.66	-0.86*	-0.24	-0.60	-0.43	0.46	-0.71*	0.94*	0.03	-0.29	-0.52	0.23	0.34	-0.67	-0.69	-0.33
Clostridium sensu stricto								0.46	0.67	0.77*	-0.02	-0.13	-0.64	0.73*	-0.57	0.09	0.15	0.10	-0.01	-0.13	0.22	0.24	-0.06
Sarcina									0.40	0.42	0.57	0.71*	-0.09	0.10	-0.49	-0.14	0.38	0.58	-0.07	-0.05	0.55	0.54	0.25
Clostridium XI										0.22	0.55	0.09	-0.49	0.76*	-0.96*	0.18	-0.01	0.22	-0.29	-0.43	0.39	0.46	0.24
Oscillibacter											-0.38	-0.29	-0.51	0.34	-0.09	0.16	0.49	0.28	0.47	0.39	0.23	0.23	0.07
unc Ruminococcaceae												0.73*	0.19	0.10	-0.67	0.05	-0.13	0.25	-0.46	-0.40	0.38	0.43	0.33
Catenibacterium													0.32	-0.24	-0.30	-0.43	0.09	0.41	-0.41	-0.32	0.37	0.32	0.12
Erysipelotrichaceae incertae sedis														-0.54	0.43	0.19	-0.11	-0.10	0.16	0.23	-0.09	-0.11	0.25
Turicibacter															-0.69	0.39	0.19	0.13	0.08	-0.07	0.46	0.49	0.29
Megasphaera																-0.03	-0.07	-0.37	0.36	0.49	-0.50	-0.56	-0.28
Escherichia/Shigella																	0.12	-0.12	0.58	0.59	0.23	0.30	0.60
acetate																		0.88*	0.73*	0.66	0.82*	0.79*	0.70
propionate																			0.38	0.29	0.87*	0.85*	0.68
butyrate																				0.96*	0.43	0.42	0.64
valerate																					0.36	0.35	0.58
isobutyrate																						0.99*	0.83*
isovalerate																							0.87*

Table 2.2-S5 Tax4Fun prediction of functional profiles of microbiota from faeces. The triangle symbol indicates increased pathway abundance averaged among samples from rye- or triticale-based treatment.

predicted pathway	rye	triticale	P-value	fold change
ko02020; Two-component system		▲	0.000	1.20
ko00910; Nitrogen metabolism		▲	0.002	1.11
ko03420; Nucleotide excision repair	▲		0.000	1.11
ko02030; Bacterial chemotaxis		▲	0.002	1.16
ko00720; Carbon fixation pathways in prokaryotes		▲	0.000	1.10
ko00052; Galactose metabolism	▲		0.002	1.18
ko02040; Flagellar assembly		▲	0.000	1.18
ko00760; Nicotinate and nicotinamide metabolism	▲		0.000	1.10
ko02060; Phosphotransferase system (PTS)	▲		0.029	1.29
ko00650; Butanoate metabolism		▲	0.000	1.11
ko00511; Other glycan degradation	▲		0.001	1.16
ko00350; Tyrosine metabolism		▲	0.000	1.12
ko00360; Phenylalanine metabolism		▲	0.000	1.19
ko00362; Benzoate degradation		▲	0.000	1.20
ko00280; Valine, leucine and isoleucine degradation		▲	0.000	1.28
ko00540; Lipopolysaccharide biosynthesis	▲		0.007	1.29

Table 2.2-S6 Relative dispersion in faecal microbiota from rye- and triticale-based treatment.

individuals	OTU	metabolites
rye		
pig 1	1.5	1.0
pig 2	0.6	1.5
pig 3	1.4	1.2
pig 4	1.4	1.0
pig 5	1.2	1.1
pig 6	0.8	1.4
pig 7	1.8	1.1
pig 8	0.8	1.5
mean ± sem	1.2 ± 0.13	1.2 ± 0.07
triticale		
pig 9	0.9	0.6
pig 10	0.9	0.9
pig 11	1.0	1.2
pig 12	0.9	1.2
pig 13	0.9	0.9
pig 14	0.5	0.6
pig 15	0.8	0.5
pig 16	0.9	0.6
mean ± sem	0.9 ± 0.05	0.9 ± 0.10
P-value (rye/triticale)	0.05	0.01

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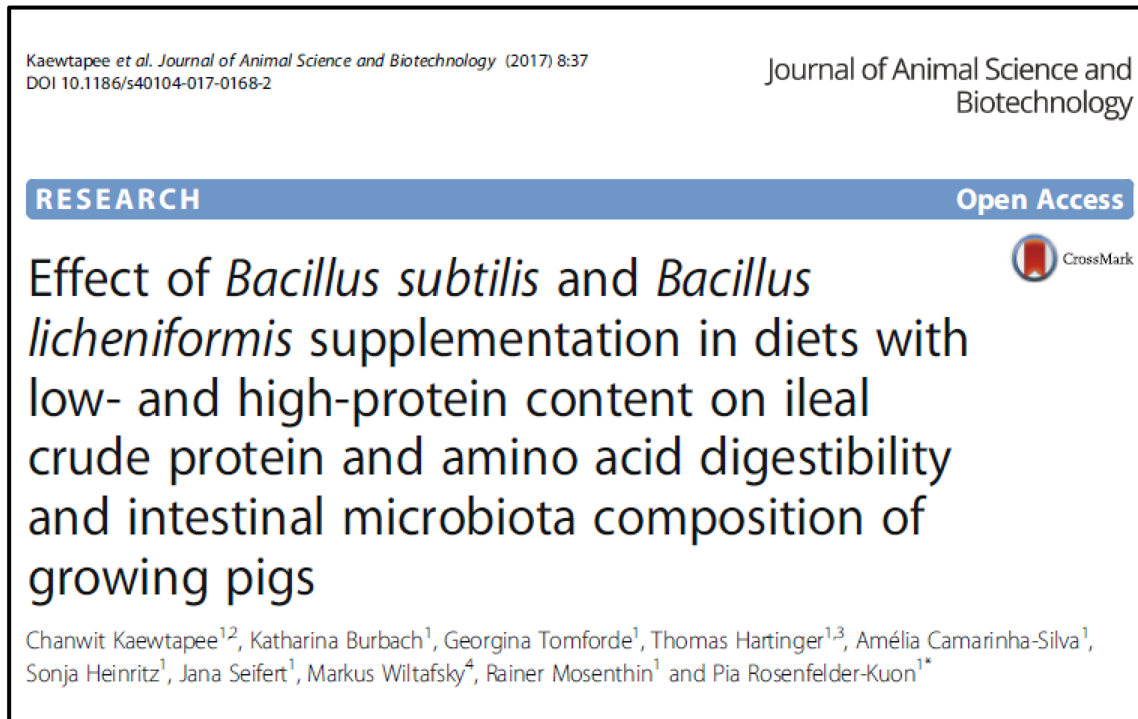

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RESEARCH Open Access

Effect of *Bacillus subtilis* and *Bacillus licheniformis* supplementation in diets with low- and high-protein content on ileal crude protein and amino acid digestibility and intestinal microbiota composition of growing pigs

Chanwit Kaewtapee[1,2], Katharina Burbach[1], Georgina Tomforde[1], Thomas Hartinger[1,3], Amélia Camarinha-Silva[1], Sonja Heinritz[1], Jana Seifert[1], Markus Wiltafsky[4], Rainer Mosenthin[1] and Pia Rosenfelder-Kuon[1*]

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Effect of *Bacillus subtilis* and *Bacillus licheniformis* supplementation in diets with low- and high-protein content on ileal crude protein and amino acid digestibility and intestinal microbiota composition of growing pigs

2.3.1 Abstract

Background: *Bacillus* spp. seem to be an alternative to antimicrobial growth promoters for improving animals health and performance. However, there is little information on the effect of *Bacillus* spp. in combination with different dietary crude protein (CP) levels on the ileal digestibility and microbiota composition. Therefore, the objective of this study was to determine the effect of *Bacillus* spp. supplementation to low- (LP) and high-protein diets (HP) on ileal CP and amino acid (AA) digestibility and intestinal microbiota composition. Methods: Eight ileally cannulated pigs with an initial body weight of 28.5 kg were randomly allocated to a row-column design with 8 pigs and 3 periods of 16 d each. The assay diets were based on wheat-barley-soybean meal with two protein levels: LP (14% CP, as-fed) and HP diet (18% CP, as-fed). The LP and HP diets were supplemented with or without Bacillus spp. at a level of 0.04% (as-fed). The apparent ileal digestibility (AID) and standardized ileal digestibility (SID) of CP and AA was determined. Bacterial community composition from ileal digesta was analyzed by Illumina amplicon sequencing and quantitative real-time PCR. Data were analyzed as a 2 × 2 factorial design using the GLIMMIX procedures of SAS. Results: The supplementation with *Bacillus* spp. did not affect both AID and SID of CP and AA in growing pigs. Moreover, there was no difference in AID of CP and AA between HP and LP diets, but SID of cystine, glutamic acid, glycine, and proline was lower ($P < 0.05$) in pigs fed the HP diets. The HP diets increased abundance of *Bifidobacterium* spp. and *Lactobacillus* spp., ($P < 0.05$) and by amplicon sequencing the latter was identified as predominant genus in microbiota from HP with *Bacillus* spp., whereas dietary supplementation of *Bacillus* spp. increased ($P < 0.05$) abundance of *Roseburia* spp.. Conclusions: The HP diet increased abundance of *Lactobacillus* spp. and *Bifidobacterium* spp.. The supplementation of *Bacillus* spp. resulted in a higher abundance of healthy gut associated bacteria without affecting ileal CP and AA digestibility, whereas LP diet may reduce the flow of undigested protein to the large intestine of pigs. Keywords: *Bacillus* spp., Growing pigs, Ileal digestibility, Microbiota, Protein levels Background Due to the ban of antimicrobial growth promoters in livestock feeding by the European Union in 2006 [1], probiotics are considered as an alternative for improving animals' health and performance [2, 3]. Within this regard, *Bacillus* spp. have the ability to sporulate, thereby making them stable

during thermal treatment of feed, and resistant to enzymatic digestion along the gastrointestinal tract (GIT) [4]. Thus, *Bacillus* spp. such as *Bacillus subtilis* (*B. subtilis*) and *Bacillus licheniformis* (*B. licheniformis*) are frequently supplemented to pig diets [4–6] as these two species have been listed to be added as non-toxigenic, biological supplements to livestock diets [7], and additionally, they are widely used for the large-scale industrial production of proteins including extracellular enzymes [8]. Positive effects of dietary supplementation of *B. subtilis* and *B. licheniformis* on pigs' growth performance have been reported before [9, 10]. Activity of probiotics is influenced by diet composition [11] and variations in dietary protein supply, thereby possibly affecting microbial composition in the gut [12, 13]. Accordingly, reducing the dietary crude protein (CP) level has been reported to markedly reduce the production of potentially harmful microbial metabolites such as ammonia and amines due to the lower availability of undigested protein for microbial fermentation [14]. Thus, excessive nitrogen (N) excretion by pigs is mitigated, resulting in a decrease of environmental pollutants [15, 16]. In contrast, increasing the dietary CP intake may stimulate the proliferation of almost all bacteria groups over the entire GIT including beneficial bacteria, such as *Bifidobacterium* spp., and potentially pathogenic bacteria, such as *Bacteroides* groups [17]. Furthermore, there is increasing evidence that interactions of supplemental probiotics with dietary CP level affect the intestinal microbiome at the ileal level [18]. According to the results of previous studies [19, 20] *Bacillus* spp. enhanced the development and activities of digestive enzymes in the GIT, which was associated with a numerical increase in apparent ileal digestibility (AID) and standardized ileal digestibility (SID) of some amino acids (AA) in weaning pigs [21]. However, studies with growing pigs in which *Bacillus* spp. were supplemented to diets varying in CP content are still lacking. Therefore, the objective of the present study was to test the hypothesis, if *B. subtilis* and *B. licheniformis* supplementation to low- and high-protein diets will affect ileal CP and AA digestibility and intestinal microbiota composition in growing pigs.

2.3.2 Methods

The research protocol was reviewed and approved by the German Ethical Commission for Animal Welfare, and care of the animals throughout this experiment was in accordance with guidelines issued by the Council Directive [22].

Animals, housing, and surgical procedures

Eight pigs were obtained from the University of Hohenheim Research Station. The average initial and final body weight (BW) of the experimental animals were 28.5 ± 0.8 and 64.3 ± 1.5

kg, respectively. The pigs were housed individually in stainless steel metabolic crates (0.8 m× 1.5 m). Each metabolic crate was equipped with an infrared heating lamp and a low pressure drinking nipple which allowed free access to water. The research unit was equipped with an automated temperature control system kept at 20 °C. Until the beginning of the experiment, the pigs were fed a commercial starter diet at a daily level of 4% (as-fed)/kg of average BW (Porcigold® SMA 134, Raiffeisen Kraftfutterwerke Süd GmbH, Würzburg, Germany; 17.5% CP and 13.4 MJ metabolizable energy (ME)/kg, as-fed). After arrival at the research unit, the pigs were surgically fitted with a simple T-cannula at the distal ileum as described by Li et al. [23]. The pigs were allowed a recovery period of at least 7 d. During this period, the feed allowance was gradually increased, starting from 50 g/d the day after surgery until 1000 g/d (as-fed) were consumed.

Experimental design, diets, and procedures

The experiment was arranged as a row-column design with 8 pigs and 3 experimental periods of 16 d each. Pigs were fed assay diets twice daily at 0700 and 1900 h at a level of 4% (as-fed)/kg of their average BW corresponding to 3 times their energy requirement for maintenance (i.e. 0.44 MJ ME/kg BW0.75) [24]. Pigs' BW was determined at the beginning of each experimental period. The assay diets were based on wheat, barley, and soybean meal with 2 protein levels resulting in a lowprotein (14% CP, as-fed; LP) and a high-protein diet (18% CP, as-fed; HP). The LP diet was accomplished by blending the HP diet with 25% of native cornstarch. The contents of oil, minerals, vitamins, and titanium dioxide were the same for all diets. The *Bacillus* spp. product is comprised of a mixture of spray-dried spores of *B. licheniformis* and *B. subtilis*. The LP and HP diets were supplemented with (+) or without (-) *Bacillus* spp. at a level of 0.04% (as-fed). All assay diets were formulated (Table 2.3-1) to meet or exceed the dietary threshold levels for CP and AA according to Fan et al. [25] and NRC [26] nutrient recommendations for pigs from 25 to 50 kg BW. Vitamins and minerals were supplemented to all diets to meet or exceed NRC [26] standard, and all diets contained titanium dioxide at a level of 0.4% (as-fed basis) as an indigestible marker. The assay diets were in a mash form mixed with water (1/1, w/v). During each of the 3 experimental periods, the pigs were allowed to adapt to their assay diets for 14 d before ileal digesta was collected for a total of 24 h from 0700 to 1900 h on d 15 and from 1900 on d 16 to 0700 h on d 17. Digesta collection procedure was adapted from Li et al. [23] using soft plastic bags attached to the barrel of the cannula by elastic bands. The bags were changed at least every 20 min. To minimize further bacterial fermentation 4 mL of 2.5 mol/L formic acid were added to the sampling bags and then immediately frozen at -18 °C. The individual digesta samples of each

pig were pooled for each sampling period, freeze-dried, and ground to 0.5 mm prior to analyses. For analyses of intestinal microbiota composition, ileal digesta and feces samples were taken prior to the first experimental period (starter period) and on d 15 once for each experimental period. Ileal digesta and feces samples for microbial community analysis were immediately put on ice before being stored in a freezer at -80 °C for subsequent treatment and analyses.

Table 2.3- 1 Ingredient composition of assay diets, % as-fed basis

Item	High-Protein	Low-Protein
Barley	20.00	15.00
Wheat	51.00	38.24
Soybean meal	21.51	16.13
Oil[1]	1.50	1.50
Cornstarch[2]	2.08	25.55
Vitamins and minerals premix[3]	0.76	0.76
Sodium chloride	0.07	0.07
Monocalcium phosphate	0.66	0.66
Calcium carbonate	0.65	0.65
Vitamin E[4]	0.03	0.03
L-Lysine-HCl[5]	0.61	0.46
DL-Methionine[5]	0.22	0.16
L-Isoleucine[5]	0.03	0.02
L-Leucine[5]	0.13	0.10
L-Threonine[5]	0.22	0.17
L-Tryptophan[5]	0.01	0.01
L-Valine[5]	0.12	0.09
Titanium dioxide	0.40	0.40
Bacillus spp.[6]	-	-
Calculated chemical composition[7]		
Metabolizable energy, MJ/kg	13.43	14.25
Crude protein, %	18.00	14.00
Calcium, %	0.66	0.63
Available Phosphorus, %	0.27	0.25
SID[7] Lysine, %	1.20	0.92
SID[7] Methionine, %	0.43	0.33
SID[7] Threonine, %	0.73	0.56

1Blend of rapeseed oil (75%) and soybean oil (25%)

2Roquette, Lestrem, France

3Vilomin® 18950, Deutsche VilomixTierernährung GmbH, Neuenkirchen-Vörden, Germany; provided the following quantities of minerals and vitamins per kg of diet: Ca, 1.86 g; P, 0.38 g; Na, 0.42 g; Mg, 76.00 mg; Fe, 30.40 mg (FeSO4.H2O); Cu, 3.80 mg (CuSO4.5H2O); Mn, 20.29 mg (MnO); Zn, 25.38 mg (ZnO); I, 0.51 mg (Ca(IO3)2); Se, 0.10 mg (Na2SeO3); Co, 0.06 mg (2CoCO3.3Co(OH)2.H2O); vitamin A, 3,040 IU; vitamin D3, 456 IU; vitamin E, 19.00 mg; vitamin B1, 0.38 mg; vitamin B2, 1.18 mg; vitamin B6, 0.95 mg; vitamin B12, 7.60 µg; vitamin K3, 0.76 mg; niacin, 4.75 mg; calcium pantothenate, 2.85 mg; folic acid, 0.19 mg; choline chloride, 57 mg.

4LutavitE 50, BASF, Ludwigshafen, Germany

5All crystalline amino acids (AA) were supplied by Evonik Industries AG (Hanau-Wolfgang, Germany). The purity of all crystalline AA was 99%, with the exception of L-Lysine-HCl (78%).

6High- and low-protein diets were supplemented with or without 0.04% (as-fed) of Bacillus spp. product at the expense of cornstarch.

7SID standardized ileal digestibility

Chemical analyses

Official standard methods [27] were used to determine contents of proximate nutrients, neutral detergent fiber (NDF), acid detergent fiber (ADF), acid detergent lignin (ADL), and

microbial numbers of B. subtilis and B. licheniformis in assay diets. The assay diets and digesta samples were analyzed for DM (method 3.1). In addition, assay diets were analyzed for ash (method 8.1); ether extract (EE; method 5.1.1 using petroleum ether), NDF assayed with a heat-stable amylase and expressed inclusive of residual ash (method 6.5.1), ADF expressed inclusive of residual ash (method 6.5.2), and ADL determined by solubilization of cellulose with sulphuric acid (method 6.5.3). Moreover, microbial numbers of B. subtilis and B. licheniformis in assay diets were determined by method 28.2.2 [27]. Nitrogen contents in assay diets and ileal digesta samples were analyzed using a gas combustion method according to official method 990.03 of the AOAC International [28] (FP-2000, Leco Corp., St Joseph, MI, US). Ethylenediaminetetraacetic acid was used as a reference standard before and after all N analyses. Crude protein contents were calculated by multiplying the content of N with 6.25. Amino acid contents in assay diets and ileal digesta samples were determined by using ion-exchange chromatography with postcolumn derivatization with ninhydrin [29]. Tryptophan was determined by HPLC with fluorescence detection (extinction 280 nm, emission 356 nm), after alkaline hydrolysis with barium hydroxide octahydrate for 20 h at 110 °C according to the procedure as outlined by Commission Directive [30]. The titanium dioxide content in the assay diets and ileal digesta samples was performed according to the procedure described by Brandt and Allam [31].

DNA extraction of ileal digesta and feces samples

Genomic DNA was extracted from 250 mg ileal digesta and feces using Fast DNA Spin Kit for Soil (MP Biomedicals GmbH, Heidelberg, Germany). Extraction procedure was performed with slight modifications to manufacturer's instructions as described by Burbach et al. [32].

Amplicon sequencing analysis

Illumina amplicon sequencing libraries of the V1-2 region of the 16S rRNA gene was performed similar to procedures described previously [33]. Library preparation, however, was modified as follows: the V1-2 region was amplified with a 27 F-modified forward primer (AGRGTTHGATYMTGGCTCAG) in a 20 µL reaction. 1 µL of this first PCR was used as template in a second PCR using multiplexing and indexing primers as described previously [33]. Amplicons were verified by agarose gel electrophoresis and normalized using SequalPrepTM Normalization Plate Kit (Invitrogen, Thermo Fisher Scientific, Waltham, USA). Libraries were pooled by index, purified with MinElute PCR Purification Kit (Qiagen, Hilden, Germany), quantified with Qubit® 2.0 Fluorometer (Invitrogen) and sequenced on

Illumina MiSeq platform using 250 bp paired end sequencing chemistry. All analyzed samples comprised around 2.8 million reads, with an average of 43,646 reads per sample. Reads were quality filtered, assembled and aligned using Mothur pipeline [34]. UCHIME was used to find possible chimeras and reads were clustered at 97% identity into 2601 operative taxonomic units (OTU). The closest representative was manually identified with seqmatch from RDP [35]. Sequences classified as Chloroplast/ Cyanobacteria were removed from OTU dataset as it was assumed that they represent undigested plant material. Sequences were submitted to European Nucleotide Archive under the accession number PRJEB14413 (http://www.ebi.ac.uk/ena/data/view/PRJEB14413).

Quantitative real time PCR

Quantitative real-time PCR (qPCR) was used to analyze the following bacteria groups in the ileal digesta samples: Total bacteria, Lactobacillus spp., Bifidobacterium spp., Roseburia spp., Enterobacteriaceae, Bacteroides-Prevotella-Porphyromonas group, Clostridium Cluster IV, and Bacillus spp.. All used primers were selected from literature and are listed in Table 2.3-2. Optimization of primer conditions was done in order to determine optimal annealing temperatures and primer concentrations by running a standard PCR with diverse primer concentrations (200 nmol/L, 400 nmol/L, 600 nmol/L) and a temperature gradient from 55.0 °C to 65.0 °C. According to melt curves on standard PCR and the agarose gel electrophoresis results, optimal primer concentration and annealing temperature was set for each primer. Standard curves for each primer were designed using serial dilutions of the purified and quantified PCR products generated by standard PCR and genomic DNA from pig feces. The PCR products were checked by agarose gel electrophoresis (2% agarose) to ensure correct primer specific products. Quantity of purified PCR amplification products was determined using Qubit® 2.0 Fluorometer (Invitrogen). Quantification was carried out using the CFX Connect ™ Real-Time System (Bio-Rad Laboratories GmbH, Munich, Germany), associated with the Bio Rad CFX Manager™ Software 3.1 (Bio-Rad Laboratories GmbH, Munich, Germany). All samples were determined in duplicate and all standards were pipetted in triplicate on each plate. The order of samples and standards on the plates was randomized. The reaction mixture for each bacterial group consisted of 10 µL of KAPA SYBR FAST (PEQLAB Biotechnologie GmbH, Erlangen, Germany), 1 µL template DNA (ileal digesta samples and standards), the optimized primer concentrations of forward and reverse primers (Table 2.3-2), and was filled up to a total volume of 20 µL with PCR grade water (Carl Roth GmbH, Karlsruhe, Germany). Amplification conditions were: activation of polymerase at 95.0 °C for 3 min, followed by 40 cycles consisting of denaturation at 95.0 °C for 5 s, primer

annealing for 20 s (at optimized temperatures, Table 2.3-2), and extension at 72.0 °C for 1 s. Subsequently, a final elongation step at 72.0 °C for 1 min followed. The melt curve was obtained by stepwise (0.5 °C) increase of temperature from 55 °C to 95 °C. Results were reported as log10 16S rRNA gene copies/g digesta.

Calculations

The AID of CP and AA in the assay diets was calculated according to the following equation:

$$AID_D = [1 - (I_D \times A_I) / (A_D \times I_I)] \times 100\%$$

where AID_D = AID of CP or AA in the assay diet (%), I_D = marker content in the assay diet (g/kg DM), A_I=CP or AA content in ileal digesta (g/kg DM), AD=CP or AA content in the assay diet (g/kg DM), and I_I=marker content in ileal digesta (g/kg DM). According to Stein et al. [36] and Jansman et al. [37], the basal ileal endogenous loss of CP and AA (IAA_{end}) is considered to be constant among groups of pigs, and therefore, mean values for IAA_{end} [37] can be used for transformation of AID into their SID values. The SID of CP and AA in assay diets was estimated according to the following equation:

$$SID_D = AID_D + (IAA_{end} / A_D) \times 100\ \%$$

where SIDD = SID of CP or AA in the assay diet (%).

Table 2.3-2 Oligonucleotide primers used for real-time PCR

Target group	Item	Oligonucleotide sequence (5′–3′)	Primer conc., nmol/L	Annealing temp., °C	Product size, bp	Reference
Total bacteria	Forward	GTGSTGCAYGGYYGTCGTCA	600	52	147	Fuller et al. [80]
	Reverse	ACGTCRTCCMCNCCTTCCTC				
Lactobacillus spp.	Forward	AGAGGTAGTAACTGGCCTTTA	400	59	391	Malinen et al. [81]
	Reverse	GCGGAAACCTCCCAACA				
Bifidobacterium spp.	Forward	TCGCGTCYGGTGTGAAAG	400	59	243	Rinttilä et al. [82]
	Reverse	CCACATCCAGCRTCCAC				
Roseburia spp.	Forward	AGGCGGTACGGCAAGTCT	400	59	353	Veiga et al. [83]
	Reverse	AGTTTYATTCTTGCGAACG				Rinttilä et al. [82]
Enterobacteriaceae	Forward	CATTGACGTTACCCGCAGAAGAAGC	200	59	195	Bartosch et al. [84]
	Reverse	CTCTACGAGACTCAAGCTTGC				
Clostridium Cluster IV	Rflbr730F	GGCGGCYTRCTGGGCTTT	400	65	147	Ramirez-Farias et al. [85]
	Clep866mR§	CCAGGTGGATWACTTATTGTGTTAA				Lay et al. [86]
Bacteroides-Prevotella-Porphyromonas	Forward	GGTGTCGGCTTAAGTGCCAT	600	58	140	
	Reverse	CGGAYGTAAGGGCCGTGC				Rinttilä et al. [82]
Bacillus spp.	Forward	CCTACGGGAGGCAGCAGTAG	600	59	78	
	Reverse	GCGTTGCTCCGTCAGACTTT				Fernández-No et al. [87]

Statistical analyses

Homogeneity of variances and normal distribution of the data were confirmed using the UNIVARIATE procedure of SAS (SAS Inst., Inc., Cary, NC). Data were analyzed as a 2×2 factorial using the GLIMMIX procedures of SAS. The model included the protein level, probiotic supplementation, and the interactive effects of protein level and probiotic supplementation as the fixed effects, and pig and period as the random effects. In case of interaction, the significant differences between treatments based on a t-test were set at $\alpha = 0.05$ using the algorithm for letter-based representation of all pair-wise comparisons according to Piepho [38]. For microbiota analyses, bacterial 16S rRNA gene copy numbers in pre-treatment period was considered as covariate. Least squares means and standard error of the means are presented, and a probability level of $P < 0.05$ was considered to be statistically significant, whereas a $P < 0.10$ was considered to constitute a tendency. Illumina amplicon sequencing data were analyzed using statistic software PRIMER (v.6.1.16, PRIMER-E; Plymouth Marine Laboratory, Plymouth, UK) [39]. Samples were standardized by total and resemblance matrix was calculated using Bray-Curtis coefficient. Overall community structures were explored by nonmetric multidimensional scaling (MDS). One way analysis of similarity (ANOSIM) and permutational multivariate analysis of variance (PERMANOVA) were used to evaluate similarity between different dietary groups, different protein levels and probiotic treatments, and a probability level of $P \leq 0.05$ was considered to be significant different. The ANOSIM R values range from -1 to 1; the farer from zero the more distinct and the closer to zero the more similar are the compared groups. Variables contributing to observed differences were identified by similarity percentages routine. The bacterial families contributing to overall 70% of dissimilarities among treatment groups were considered to be the most important and their abundance data were graphically plotted according to a color key from zero to maximal abundance. Shannon index was used to measure diversity in bacterial communities from different sample groups, taking into account the number of OTUs and the proportion of each OTU. A Mantel-type test (RELATE) on Bray-Curtis matrices was used to quantify the correlation between results from bacterial community analysis. To enable comparison between amplicon sequencing and qPCR approaches, RELATE routine was run on untransformed datasets, restricted to bacteria groups targeted by qPCR primers and the generated Spearman Rho was considered to be significant if $P \leq 0.05$.

2.3.3 Results

All pigs remained healthy throughout the experiment and readily consumed their daily feed allowances. The analyzed CP and AA contents of the assay diets and microbial numbers of *B. subtilis* and *B. licheniformis* in assay diets are presented in Table 2.3-3. As expected, CP and AA contents in LP were approximately 76.5 and 76.6% that of HP, respectively. The contents of ash, EE, NDF, ADF, and ADL in the HP diets were also greater than in the LP diets. The *Bacillus* spores determined in the experimental diets amounted to 1.54×10^9 CFU/kg feed for HP + and LP + diets, whereas HP - and LP - diets contained 0.02×10^9 and 0.04×10^9 CFU/kg feed, respectively.

The AID and SID of CP and AA in the assay diets are shown in Tables 2.3-4 and -5, respectively. The supplementation with Bacillus spp. did not affect both AID and SID of CP and AA. Furthermore, there was no difference in AID of CP and AA between HP and LP diets, but SID of cystine, glutamic acid, glycine, and proline was lower ($P < 0.05$) in the HP diets than in the LP diets. Moreover, SID of CP, alanine, aspartic acid, and serine also tended to be lower ($P < 0.10$) in the HP diets. However, no interactions between CP level and Bacillus spp. supplementation could be observed for AID and SID of CP and AA in the present study.

Table 2.3-3 Analyzed chemical composition and *Bacillus* cell numbers in assay diets

	High-Protein		Low-Protein	
***Bacillus* spp.**	**-**	+	**-**	+
Dry matter, %	88.6	88.7	88.3	88.6
Crude protein, % DM	20.6	20.3	15.2	16.1
Ash, % DM	6.1	6.0	5.2	5.3
Ether extract, % DM	3.7	3.6	3.2	3.3
Neutral detergent fiber, % DM	12.7	13.1	10.1	10.5
Acid detergent fiber, % DM	7.0	6.6	5.4	5.1
Acid detergent lignin, % DM	1.1	0.9	0.8	0.8
Indispensable amino acids, % DM				
Arginine	1.26	1.25	0.93	0.99
Histidine	0.46	0.46	0.35	0.36
Isoleucine	0.80	0.80	0.61	0.63
Leucine	1.53	1.53	1.15	1.19
Lysine	1.48	1.50	1.12	1.12
Methionine	0.50	0.51	0.38	0.37
Phenylalanine	0.95	0.95	0.69	0.74
Threonine	0.91	0.91	0.68	0.70
Tryptophan	0.27	0.27	0.20	0.21
Valine	1.02	1.01	0.76	0.79
Dispensable amino acids, % DM				
Alanine	0.80	0.79	0.60	0.63
Aspartic acid	1.73	1.71	1.29	1.37
Cystine	0.33	0.33	0.25	0.26
Glutamic acid	4.08	4.03	3.05	3.20
Glycine	0.81	0.80	0.61	0.64
Proline	1.32	1.31	0.99	1.04
Serine	0.93	0.91	0.69	0.73
Bacillus cell numbers, CFU/kg feed				
Bacillus subtilis	0.022×10^9	0.860×10^9	0.038×10^9	0.970×10^9
Bacillus licheniformis	$< 0.002\times10^9$	0.680×10^9	0.006×10^9	0.570×10^9

Table 2.3-4 Apparent ileal digestibility of crude protein and amino acids of the assay diets[1]

	High-Protein		Low-Protein		SEM	*P*-Value		
***Bacillus* spp.**	**-**	+	**-**	+		**P[2]**	**B[3]**	**P × B[4]**
Crude protein	76.4	75.4	80.0	76.6	2.09	0.273	0.310	0.573
Indispensable amino acids								
Arginine	85.4	84.8	87.1	84.7	1.35	0.563	0.286	0.534
Histidine	80.4	79.2	82.9	79.7	1.70	0.398	0.211	0.551
Isoleucine	79.3	78.6	82.5	79.1	1.96	0.356	0.313	0.513
Leucine	81.1	80.3	83.8	80.6	1.73	0.389	0.269	0.501
Lysine	84.8	84.3	87.5	84.2	1.38	0.351	0.194	0.327
Methionine	89.7	89.3	91.4	89.0	1.03	0.492	0.200	0.349
Phenylalanine	78.6	78.1	82.0	79.0	1.99	0.295	0.386	0.522
Threonine	76.6	75.7	79.5	75.3	2.09	0.546	0.235	0.446
Tryptophan	75.1	73.2	78.1	73.8	2.47	0.470	0.226	0.628
Valine	78.8	78.9	81.9	78.1	1.93	0.403	0.232	0.471
Dispensable amino acids								
Alanine	70.6	68.9	75.5	71.0	2.66	0.197	0.257	0.605
Aspartic acid	74.6	73.4	78.6	74.7	2.32	0.263	0.284	0.566
Cystine	74.3	72.6	78.9	74.5	2.33	0.176	0.200	0.568
Glutamic acid	86.4	85.7	88.9	86.9	1.22	0.148	0.267	0.601
Glycine	65.5	63.4	70.1	64.3	2.91	0.352	0.188	0.521
Proline	82.7	81.4	85.3	82.2	1.64	0.312	0.197	0.611
Serine	76.7	75.2	79.7	76.3	2.10	0.341	0.244	0.665

[1]LS means and standard error of the means, %
[2]*P*-value of protein level
[3]*P*-value of probiotic supplementation with *Bacillus* spp.
[4]*P*-value of interaction between protein level and probiotic supplementation with *Bacillus* spp.

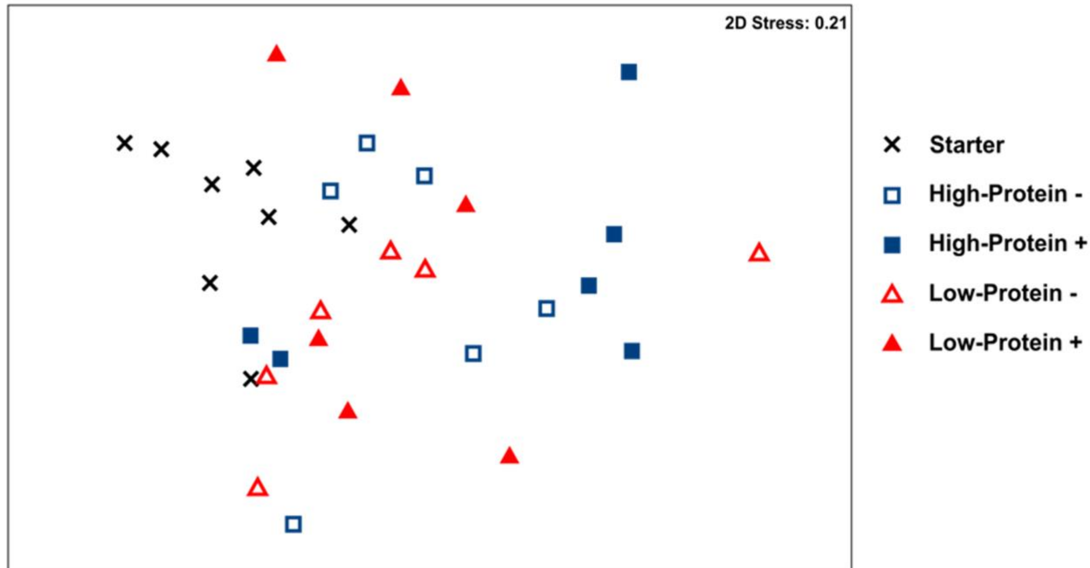
Table 2.3-5 Standardized ileal digestibility of crude protein and amino acids of the assay diets[1]

	High-Protein		Low-Protein		SEM	*P*-Value		
***Bacillus* spp.**	**-**	+	**-**	+		**P[2]**	**B[3]**	**P × B[4]**
Crude protein	82.1	81.3	87.8	83.9	2.09	0.063	0.274	0.488
Indispensable amino acids								
Arginine	88.5	87.9	91.3	88.7	1.35	0.210	0.254	0.474
Histidine	84.5	83.4	88.4	84.9	1.70	0.128	0.187	0.492
Isoleucine	84.0	83.3	88.8	85.2	1.96	0.110	0.289	0.474
Leucine	84.3	83.5	88.0	84.7	1.73	0.164	0.255	0.472
Lysine	87.5	87.0	91.1	87.8	1.38	0.126	0.192	0.333
Methionine	91.9	91.5	94.3	92.0	1.03	0.166	0.201	0.363
Phenylalanine	82.2	81.7	86.9	83.6	1.99	0.112	0.348	0.474
Threonine	83.3	82.4	88.5	84.1	2.09	0.114	0.212	0.410
Tryptophan	80.3	78.4	85.0	80.3	2.47	0.195	0.202	0.584
Valine	84.2	83.2	89.0	85.0	1.93	0.106	0.218	0.436
Dispensable amino acids								
Alanine	76.9	75.2	83.9	79.0	2.66	0.056	0.232	0.549
Aspartic acid	79.3	78.2	85.0	80.7	2.32	0.094	0.259	0.509
Cystine	80.7	78.9	87.5	82.5	2.33	0.037	0.166	0.499
Glutamic acid	89.5	88.8	93.1	90.8	1.22	0.034	0.242	0.539
Glycine	76.8	74.8	85.3	78.7	2.91	0.045	0.159	0.436
Proline	91.4	90.1	96.8	93.2	1.64	0.018	0.158	0.486
Serine	84.1	82.6	89.6	85.6	2.10	0.055	0.209	0.547

[1]LS means and standard error of the means, %
[2]*P*-value of protein level
[3]*P*-value of probiotic supplementation with *Bacillus* spp.
[4]*P*-value of interaction between protein level and probiotic supplementation with *Bacillus* spp.

The overall structure in bacterial communities from ileal digesta was evaluated by 16S rRNA gene amplicon sequencing. Analysis of similarity revealed significant differences in

microbiota composition due to different dietary treatments (P = 0.05), but a statistic R value close to zero (R = 0.176) suggests a weak separation of the different treatment groups (Figure 2.3-1A).



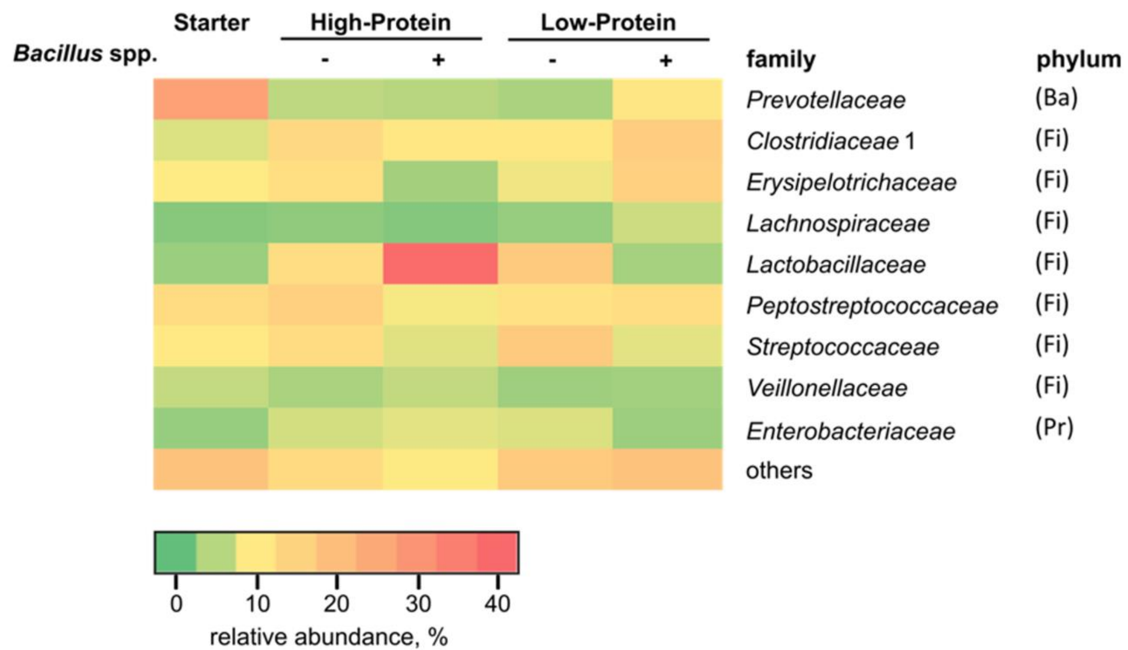


Figure 2.3-1 Microbiota composition in ileal digesta samples from pigs fed starter diet and assay diets. (A) Multidimensional scaling plot based on Bray Curtis similarity matrix of 16S rDNA sequence data from ileal digesta. (B) Abundance plot of most important bacterial families in overall microbiota structure of ileal digesta. Phyla: *Firmicutes* (Fi), *Bacteroidetes* (Ba), *Proteobacteria* (Pr).

When compared with the starter period, bacterial communities were different (P < 0.01) between dietary treatments. Within assay diets, however, there were no effects (Table 2.3-6).

Table 2.3-6 Results from PERMANOVA test for dietary effect on 16S rRNA sequencing data from ileal digesta

Source	Degrees of Freedom	Sum of Squares	Mean Square	Pseudo-F	*P*(perm)	Unique perms
P[1]	1	2022.4	2022.4	0.770	0.638	998
B[2]	1	1340.1	1340.1	0.511	0.901	998
P × B[3]	1	2691.9	2691.9	10.255	0.424	999
Res	20	52497	2624.8			
Total	23	58551				

[1]*P*(perm)-value of protein level,
[2]*P*(perm)-value of probiotic supplementation with *Bacillus* spp.
[3]*P*(perm)-value of interaction between protein level and probiotic supplementation with *Bacillus* spp.

Taxonomical composition of ileal digesta samples demonstrated some variation among dietary treatments. At phylum level, the bacterial communities were dominated by Firmicutes and Bacteroidetes. Within the assay diets from periods 1 to 3, the relative abundance of Firmicutes was higher than Bacteroidetes when compared to the starter period. The reduction of Bacteroidetes was mainly due to lower abundance of Prevotellaceae, with an average abundance of 27% in the starter diet compared to 5% in the HP diets, 4% in LP - and 11% in LP +. Nine bacterial families contributed to the overall dissimilarities among microbiota structure in ileal digesta samples of different dietary treatments (Figure 2.3-1B). Ileal microbiota from dietary treatments without probiotic supplementation consisted mainly of *Peptostreptococcaceae*, *Clostridiaceae* 1, *Streptococcaceae*, *Lactobacillaceae* and *Erysipelotrichaceae* with even proportions, except for *Peptostreptococcaceae* and *Streptococcaceae* being the predominant family in the HP and LP treatment, respectively. *Streptococcus alactolyticus* accounted for 15% of total microbiota in samples of LP - treatment. Compared to this, ileal digesta samples from LP + were enhanced in *Clostridiaceae* 1, *Erysipelotrichaceae* and *Prevotellaceae*. In HP +, the bacterial composition was dominated by *Lactobacillaceae*, with an average abundance of 40%. Here, an uncultured *Lactobacillus* from porcine intestine (relative abundance of 21.5%) and *Lactobacillus amylovorus* (14.2%) were the predominant species.

Lactobacillus spp. and other bacteria groups of interest were quantified in ileal digesta by qPCR (Table 2.3-7). Mantel test showed a significant correlation between the two approaches, sequencing and qPCR (Rho = 0.852, $P < 0.01$), thus confirming that both methodological approaches resulted in comparable results. The HP diets increased abundance of *Lactobacillus* spp. and *Bifidobacterium* spp. ($P < 0.05$). No effects of CP content on ileal gene copy numbers of total bacteria, *Roseburia* spp., *Enterobacteriaceae*, *Bacteroides-Prevotella-Porphyromonas*, *Clostridium* cluster IV and *Bacillus* spp. were found. Likewise, no

significant effect of supplementation of *Bacillus* spp. was observed for ileal gene copy numbers of total bacteria, *Lactobacillus* spp., *Bifidobacterium* spp., *Enterobacteriaceae*, *Clostridium* cluster IV and *Bacillus* spp.. However, dietary supplementation of *Bacillus* spp. increased ($P < 0.05$) abundance of *Roseburia* spp., while it tended ($P < 0.10$) to promote *Bacillus* spp. and total bacteria. Furthermore, there was an interaction ($P < 0.05$) of protein level and *Bacillus* spp. supplementation for ileal gene copy numbers of *Bacteroides-Prevotella-Porphyromonas*. The LP+ resulted in higher ($P < 0.05$) abundance of *Bacteroides-Prevotella-Porphyromonas* than the LP-, but did not differ from the HP diets.

Table 2.3-7 Ileal gene copy numbers[1] in ileal digesta of growing pigs

	High-Protein		**Low-Protein**		**SEM**	***P*-value**		
***Bacillus* spp.**	**-**	**+**	**-**	**+**		**P[2]**	**B[3]**	**P × B[4]**
Total bacteria	8.9	9.1	8.4	9.1	0.30	0.286	0.070	0.226
Lactobacillus spp.	7.9	8.8	6.9	7.1	0.44	0.002	0.109	0.279
Bifidobacterium spp.	6.2	6.4	5.3	6.0	0.32	0.024	0.179	0.354
Roseburia spp.	7.1	7.3	6.5	7.7	0.33	0.834	0.033	0.111
Enterobacteriaceae	7.7	7.9	7.4	8.3	0.43	0.836	0.139	0.274
Bacteroides-Prevotella-Porphyromonas	8.1[ab]	8.2[ab]	7.6[b]	8.6[a]	0.26	0.968	0.013	0.042
Clostridium cluster IV	5.6	5.7	5.2	5.9	0.30	0.735	0.185	0.324
Bacillus spp.	8.0	8.3	7.5	8.1	0.23	0.100	0.054	0.498

[1]$\log_{10}$ 16S rRNA gene copies/g digesta (LS means and standard error of the means)
[2]*P*-value of protein level,
[3]*P*-value of probiotic supplementation with *Bacillus* spp.
[4]*P*-value of interaction between protein level and probiotic supplementation with *Bacillus* spp.
[a, b]Within a row, LS means with a common superscript are not different at $\alpha = 0.05$

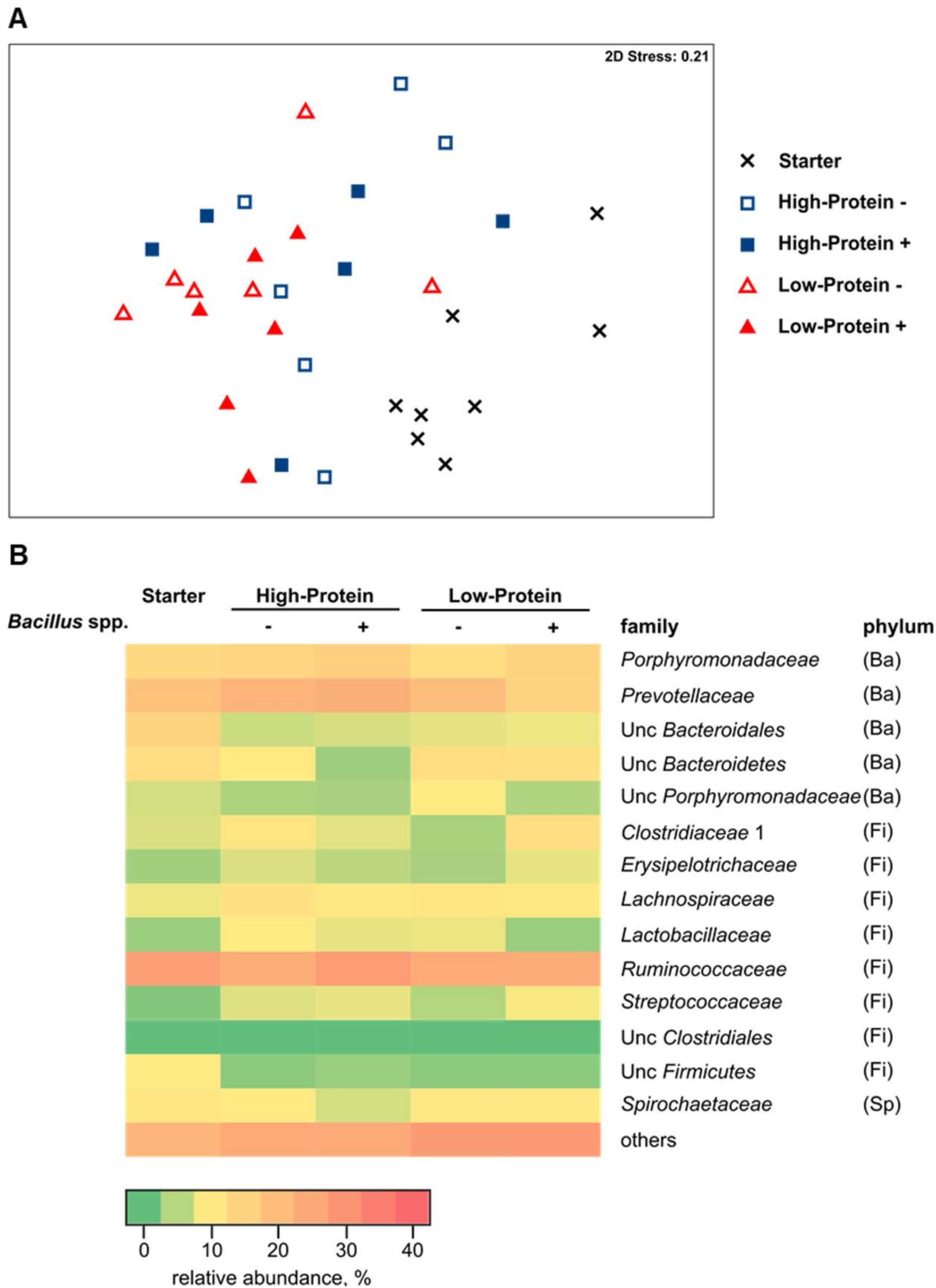


Figure 2.3-2 Microbiota composition in fecal samples from pigs fed starter diet and assay diets. (A) Multidimensional scaling plot based on Bray Curtis similarity matrix of 16S rDNA sequence data from fecal samples. (B) Abundance plot of most important bacterial families in overall microbiota structure of feces. Phyla: *Firmicutes* (Fi), *Bacteroidetes* (Ba), *Spirochaetes* (Sp).

The analysis of fecal microbiota by 16S rRNA gene amplicon sequencing showed no statistical effect on overall community structure. However, feces microbiota from each assay diet revealed to be significant different to that from the starter period ($P < 0.01$; Figure 2.3-

2A). At family level, *Prevotellaceae* exhibited the strongest impact on these dissimilarities (Figure 2.3-2B), with *Prevotella* being the predominant genus. The average abundances of *Prevotella* showed slight variations for treatment groups with different protein levels; starter (15%), LP diets (13%), and HP diets (19%).

Comparing sequencing results from porcine ileal digesta and feces revealed distinct differences in bacterial communities structure ($R = 0.924$, $P < 0.01$) (Figure 2.3-3A). Samples from ileal digesta showed a lower diversity compared to feces (Shannon index in average 2.9 vs. 4.7) (Figure 2.3-3B and C). Mainly *Streptococcus alactolyticus* contributed to the dissimilarity with an average abundance of 9.7% in ileal digesta compared to 2.0% in feces. At family level differences were mainly due to *Lactobacillaceae* and *Ruminococcaceae*. The abundance of *Lactobacillaceae* was higher in ileal digesta (16%) than in feces (2%), and contrary the abundance of *Ruminococcaceae* was higher in feces (23%) than in ileal digesta (0.5%).

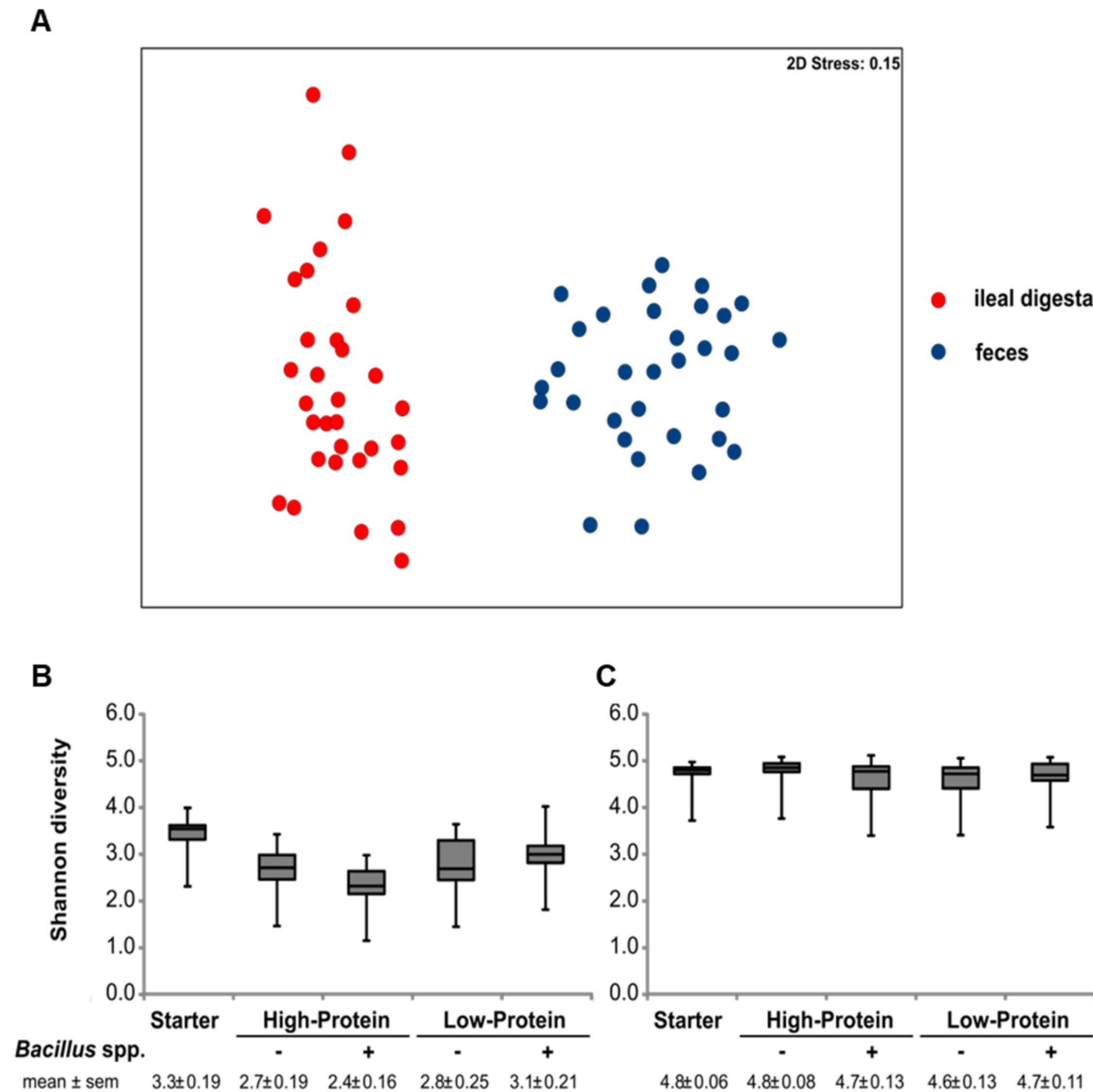


Figure 2.3-3 Comparison of microbiota from ileal digesta and feces. (A) MDS plot based on Bray-Curtis similarity matrix of all samples from ileal digesta and feces. (B) Shannon diversity calculated on operative taxonomic units data from ileal digesta samples (C) and from fecal samples.

2.3.4 Discussion

According to previous studies, *B. subtilis* and *B. licheniformis* produce extracellular enzymes including proteases and α-amylase [19, 20], which may enhance nutrient digestibility resulting in improved feed conversion in finisher pigs [40]. In addition, *B. subtilis* exceeds *B. licheniformis* in production of glycosyl hydrolases [4], which assist in the hydrolysis of glycosidic bonds in complex sugars. However concerning antibiotic resistance, which is considered to be an important key requirement for probiotics, a higher concentration of antibiotics is tolerated by *B. licheniformis* than by *B. subtilis* [4]. Recently, probiotic characteristics were described for spores of *B. subtilis*, although interactions with porcine epithelial cells are not understood so far [4]. For example, the supplementation of *B. subtilis*

to a soybean meal diet showed slight improvements in AID and SID of some AA in weaning pigs as described by Kim et al. [21]. However, in the present study, there was no improvement in AID and SID of CP and AA in growing pigs fed diets supplemented with *B. subtilis* and *B. licheniformis*. Similarly, previous studies [6, 41] failed to demonstrate that the inclusion of *B. subtilis* and *B. licheniformis* in diets would affect apparent total tract digestibility of CP in growing-finishing pigs. The lack of probiotic treatment effects may be due to low quantity of the supplemented bacterial species in porcine intestine, as in treatments with probiotic supplementation the *Bacillus* spp. numbers were not significantly higher compared to numbers in treatments without probiotic supplementation. The gene copy numbers of *Bacillus* spp. in the treatments without probiotic supplementation correspond to results of a study by Dowd et al. [42] on *Bacillus* spp. in the ileum of piglets using 16S rRNA gene sequencing. In addition to the qPCR results, further *Bacillus* species (*B. pumilus* and *B. cereus*) were identified by amplicon sequencing. Operative taxonomic units corresponding to *Bacillus* genus appeared in very low abundance (<1%), and were present in samples with and without *Bacillus* spp. supplementation. These results are in accordance with previous studies demonstrating the ability of germinated *Bacillus* spores to proliferate in mammal GIT, even if only at a low rate [5], and therefore might not be persistent [43]. Positive effects of diets supplemented with *B. subtilis* and *B. licheniformis* on feed conversion in pigs have been reported before [40, 44], however, the underlying mechanisms of *Bacillus'* probiotic activity are little understood, and may be attributed to competitive adhesion and immunomodulation by *Bacillus* spores or to enzymes and other substances produced by the germinated, vegetative cells of *Bacillus* [5]. Notably, probiotic supplements may be more effective under stress such as practical field conditions [45, 46]. This might be one reason for the missing effect of *Bacillus* spp. supplementation on digestibility values in the present study, as pigs were individually housed and kept in a clean environment under optimal temperature and minimal stress conditions. Furthermore, the age of pigs may be associated with probiotic efficacy [47]. The use of probiotics tended to be more effective in early age of pigs rather than the growing period [48, 49]. In the present study, grower pigs (13- and 20-week old at the initial and final BW, respectively) fed diets supplemented with *Bacillus* spp. did not show any differences in ileal digestibility of CP and AA. It has been suggested that increasing age may be a contributing factor in building up the complexity of the microbial community [50] with growing pigs being more resistant to intestinal disorders than young pigs [51]. Dietary content of CP has been reported to be associated with AID due to the variation in endogenous CP and AA losses in ileal digesta [52]. Previous research [53] suggests that AID shows segmented

quadratic with plateau relationships as the level of CP and AA in the diet increased from 4 to 24% (as-fed). Alternatively, SID has been widely accepted to overcome this problem by correcting AID values for basal endogenous losses of CP and AA [54]. In general, SID values are higher in comparison to their corresponding AID values as the basal endogenous losses of CP and AA are subtracted from ileal CP and AA outflow [36]. In the present study, SID of some AA was lower in HP diets than in LP diets. Apparently, higher fiber contents in HP diets, associated with enhanced secretion of endogenous AA [55, 56], may have contributed to higher rate of digesta passage in the digestive tract of pigs [57], thereby, decreasing SID values. This is confirmed by the results of a recent study [58], where SID of CP and most AA decreased linearly with increasing dietary CP from 6.8 to 21.4% (as-fed) due to the greater NDF and ADF contents. The higher numbers of *Lactobacillus* spp. and *Bifidobacterium* spp. in ileal digesta of HP treatments are in agreement with a recent study by Rist et al. [17], where piglets fed high dietary CP levels showed an increased growth and proliferation of lactic-acid bacteria in ileal digesta. As content of soybean meal in the present study was greater in HP than in LP diets, enhanced availability of fermentable carbohydrates in the small intestine can be suggested, thereby stimulating ileal growth of lactobacilli and bifidobacteria [17]. Furthermore, HP diets could increase the availability of free AA in the small intestine [17], contributing much more preformed AA of dietary and endogenous origin to bacterial growth in the upper part of the digestive tract than microbial *de novo* synthesis of AA [13]. Furthermore, analysis of overall microbiota composition in ileal digesta by amplicon sequencing supported an increasing effect on *Lactobacillus* proportion upon feeding of HP + diets. The presence of *Lactobacillus* spp. and *Bifidobacterium* spp. in the GIT has been reported to be beneficial for the host animal [17] due to their ability for bacteriocin production [59]. Moreover, proliferation of pathogenic bacteria may be inhibited through the production of short-chain fatty acids (SCFA) and lactic acid, being associated with a lower pH, causing a hostile environment for some acid-sensitive bacteria strains [60]. The presented sequencing results for *Lactobacillus* spp. are supported by qPCR results, which revealed a higher number of *Lactobacillus* gene copies in HP diets. The identified *Lactobacillus* spp. were dominated by an uncultured bacterium, previously isolated from porcine intestine [61], and the species *L. amylovorus*. *L. amylovorus* is a synonym expression for *Lactobacillus sobrius*, which is characterized by amylolytic activity, and being previously identified with high prevalence in porcine intestine [62–65]. Application of an oral probiotic mixture including a *L. amylovorus* strain has been shown to promote growth performance of pigs [66]. In general, the enhancement of potential beneficial *Lactobacillus* spp. is considered to promote gut health.

However, the above described supporting effect of HP + diet on abundance of *Lactobacillus* caused a reduced community diversity compared to microbiota from ileal digesta of other dietary treatments. A high diversity in intestinal microbiota might be preferable to cope effectively with potential challenging conditions [67]. Regardless of dietary protein level, the supplementation of *B. subtilis* and *B. licheniformis* had a stimulating effect on targeted quantity of *Roseburia* spp., known as an important butyrate producer [68]. Butyrate represents the most preferential energy source of colonocytes [69], resulting in the stimulation of epithelial cell proliferation and mucus secretion [70]. Therefore, the supplementation of *B. subtilis* and *B. licheniformis* may contribute to an improved gut health of pigs. Assay diets did not significantly impact overall microbiota, but influence was demonstrated for bacterial copy numbers of *Bacteroides-Prevotella-Porphyromonas*. In the present study, the dietary CP level and the supplementation of *B. subtilis* and *B. licheniformis* showed an interaction, as supplementation of *B. subtilis* and *B. licheniformis* increased *Bacteroides-Prevotella-Porphyromonas* in the LP diets when compared to HP diets. The *Bacteroides-Prevotella-Porphyromonas* group includes phylogenetic related species from Bacteroidetes phylum that commonly inhabit GIT. Sequencing results confirmed an increased abundance of *Prevotella* in ileal digesta from LP + treatment when compared to the other assay diets. This finding is in agreement with other studies, which showed an enhancing effect of low protein diets on gene copy numbers of *Bacteroides-Prevotella- Porphyromonas* group in ileal digesta [17], and a significant increase in the abundance of *Prevotella* genus in cecum [71] when compared to samples of treatments with a higher protein level [17, 71]. *Prevotella* dominate the porcine fecal metagenome [72], play an important role in intestinal carbohydrate fermentation [73] and also show proteolytic activity [74]. Sequencing results also revealed members of *Prevotella* as main discriminators of community structure from ileal microbiota of starter and experimental periods. The observed decrease over experimental time is in agreement with a longitudinal study of Kim et al. [75]. Thus, the observations on relative proportion of *Prevotella* represent the general impact of diet and age on porcine intestinal microbiota. Contrary to ileal digesta, where abundance of *Prevotella* was highest in LP +, the fecal proportion of *Prevotella* was higher in HP than LP treatment and slightly increased over experimental time. This variation along sampling sites is in agreement with a previous study, investigating as well ileal digesta and fecal samples from pigs [17], where abundance patterns of *Prevotella* species in the GIT of pigs were different between ileal digesta and fecal samples. The results of this study demonstrate an overall lower bacterial diversity for ileal digesta compared with fecal samples. Metagenome studies on porcine microbiota collected

from different intestine sites revealed different contributions of bacterial species and activities along the GIT [76, 77]. The fecal collection is an easy accessible sampling site with samples showing high similarity to microbiota composition from proximal intestine. However, microbiota composition from fecal samples is not identical representatives to those from ileal digesta. Therefore, collecting samples of different sites of the GIT, where close interactions between the microbiome and the digestive processes occur, will improve understanding of probable functional changes and the effects of dietary treatments such as the addition of probiotics. Undigested dietary components passing into the large intestine are subjected to fermentation by the intestinal microbiota [17]. As a result, fermentation products such as SCFA are rapidly absorbed across the gut wall, contributing up to 30% of growing pigs' maintenance requirement for energy [78]. On the other hand, increasing protein fermentation may result in the formation of detrimental fermentation products such as ammonia and amines in the colon [79]. A lower dietary protein level may reduce ammonia production, as observed by Htoo et al. [14] in cecal samples of pigs, while supplementation of diets with *B. subtilis* and *B. licheniformis* showed similar results in slurry samples from pigs due to a lowering effect on the pH [6]. Therefore, LP diets supplemented with *B. subtilis* and *B. licheniformis* might be used to reduce the production of harmful microbial metabolites in the large intestine of pigs.

2.3.5 Conclusions

Supplementation with *Bacillus* spp. did not affect both AID and SID of CP and AA in growing pigs. The higher SID of some AA in the LP diets when compared to HP diets hints towards the possibility of reducing N excretion through diet manipulation. Regarding microbiota, the assay diets had no significant effect on overall community structure, neither in ileal digesta nor feces. Nevertheless, dietary protein content and *Bacillus* spp. supplementation may enhance various community members in ileal digesta. Within this regard, feeding of the HP diet resulted in a higher abundance of *Lactobacillus* spp. and *Bifidobacterium* spp., whereas LP diet may support bacteria important for carbohydrate degradation such as *Prevotella*. Furthermore, relative proportion of *Prevotella* was altered during pig's age. The supplementation of *Bacillus* spp. promoted gene copy numbers of *Roseburia* spp., which may be beneficial due to ascribed health promoting properties of this butyrate producer, and this phenomenon may be more effective under stress condition. The LP diet supplemented with *B. subtilis* and *B. licheniformis* may be used as an alternative feeding strategy to support gut health in pigs.

Abbreviations

AA: Amino acid; ADF: Acid detergent fiber; ADL: Acid detergent lignin; AID: Apparent ileal digestibility; ANOSIM: Analysis of similarity; BW: Body weight; CFU: Colony forming units; CP: Crude protein; DM: Dry matter; GIT: Gastrointestinal tract; HP: High-protein diet; LP: Low-protein diet; ME: Metabolizable energy; N: Nitrogen; NDF: Neutral detergent fiber; OTU: Operativ taxonomic unit; PERMANOVA: Permutational multivariate analysis of variance; qPCR: Quantitative real-time PCR; RELATE: Mantel type test; SCFA: Short chain fatty acids; SID: Standardized ileal digestibility

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Availability of data and materials

All data generated or analyzed during this study are available from the corresponding author on reasonable request. Authors' contributions CK, KB, PRK, SH, JS and RM conceived the study, performed the statistics and drafted the manuscript. KB, GT and TH participated in the animal care and digesta collection. KB, ACS, TH and SH conducted the DNA extraction and quantitative real-time PCR. MW analyzed contents of CP and AA in diets and ileal digesta. All authors read and approved the final manuscript.

Competing interests The authors declare that they have no competing interests. Consent for publication Not applicable. Ethics approval and consent to participate The research protocol was approved by the German Ethical Commission for Animal Welfare. Author details 1University of Hohenheim, Institute of Animal Science, Emil-Wolff-Strasse 10, 70599 Stuttgart, Germany. 2Present address: Department of Animal Science, Faculty of Agriculture, Kasetsart University, 50 Ngam Wong Wan Rd, Chatuchak, Bangkok 10900, Thailand. 3Present address: University of Bonn, Institute of Animal Science, Endenicher Allee 15,

53115 Bonn, Germany. 4Evonik Nutrition & Care GmbH, Rodenbacher Chaussee 4, 63457 Hanau-Wolfgang, Germany. Received: 14 September 2016 Accepted: 8 April 2017

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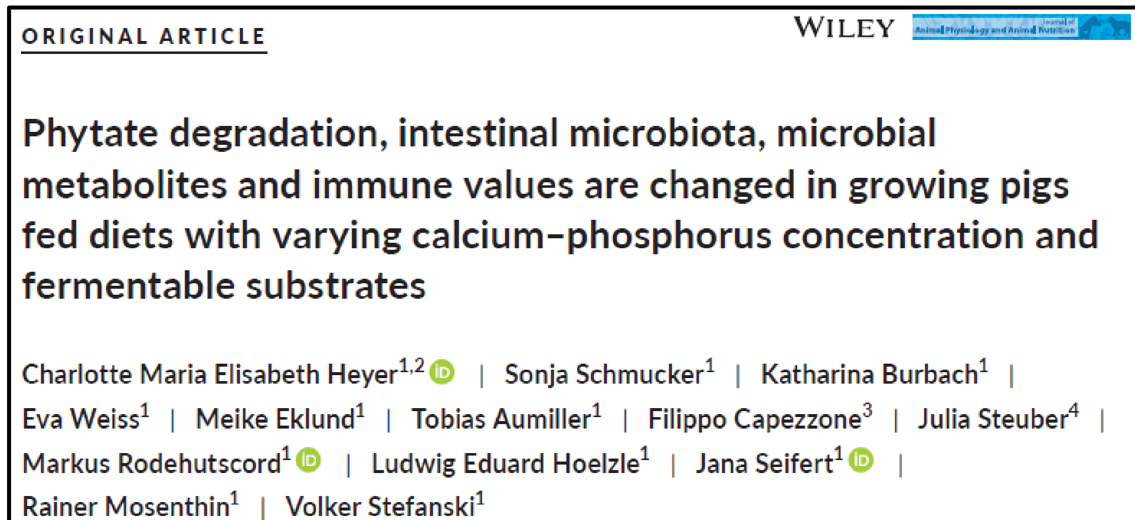
ORIGINAL ARTICLE

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Phytate degradation, intestinal microbiota, microbial metabolites and immune values are changed in growing pigs fed diets with varying calcium–phosphorus concentration and fermentable substrates

Charlotte Maria Elisabeth Heyer[1,2] | Sonja Schmucker[1] | Katharina Burbach[1] | Eva Weiss[1] | Meike Eklund[1] | Tobias Aumiller[1] | Filippo Capezzone[3] | Julia Steuber[4] | Markus Rodehutscord[1] | Ludwig Eduard Hoelzle[1] | Jana Seifert[1] | Rainer Mosenthin[1] | Volker Stefanski[1]

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Phytate degradation, intestinal microbiota, microbial metabolites, and immune values are changed in growing pigs fed diets with varying calcium-phosphorus concentration and fermentable substrates

2.4.1 Abstract

The present study assessed effects of diets containing varying calcium-phosphorus (CaP) concentration and fermentable substrates on digestibility of diets, intestinal microbiota, and immune system using 32 crossbred pigs (initial BW 54.7 kg). In a 2×2 factorial arrangement, pigs were fed either a corn-soybean meal (CSB) or corn-field pea (CFP) diet with either low [-] (4.4 g Ca/kg; 4.2 g total P/kg) or high [+] (8.3 g Ca/kg; 7.5 g total P/kg; supplemented with monocalcium phosphate) CaP content for a period of 9 weeks. In week 8, blood samples were taken and at the end of the trial all pigs were euthanized to collect digesta and mesenteric lymphoid tissue. Apparent total tract digestibility (ATTD) of P was greater ($p < 0.05$) for pigs fed the CaP+ and CFP diets than CaP- and CSB diets. The *myo*-inositol 1,2,3,4,5,6-hexakis (dihydrogen phosphate) ($InsP_6$) concentration in jejunal digesta was higher ($p < 0.05$) for CaP+ than in CaP- fed pigs. In addition, caecal and faecal $InsP_5$ isomer concentration were greater ($p < 0.05$) for CSB than CFP diets. In the caecum, gene copy numbers of saccharolytic bacteria, such as *Eubacterium rectale* and *Roseburia* spp., as well as SCFA concentration were higher ($p < 0.05$) for CaP+ than CaP- diets. In particular, innate immune cell numbers such as natural killer cells, dendritic cells, monocytes and neutrophils, were greater ($p < 0.05$) for CaP+ than CaP- fed pigs. Diets high in CaP resulted in higher abundance of potential beneficial bacteria and might promote the first line of defence enhancing the activation of the cellular adaptive immune response, thereby possibly decreasing the risk for intestinal disturbances. These results strongly suggest that both, CaP supply and dietary ingredients (protein, carbohydrate) differing in fermentability may beneficially affect gut health through increase in SCFA producing bacteria and/or bacteria with anti-inflammatory properties.

Keywords: fermentable substrates, immune system, inositol phosphate, intestinal microbiota, phosphorus

2.4.2 Introduction

There is increasing interest in dietary ingredients that are appropriate to support digestive and immune functions, but also maintain a stable microbial ecosystem in the gastrointestinal tract

(GIT). In general, a high diversity in gut microbial composition is considered to be beneficial for the host health (Konstantinov et al., 2004). Among other methods, the use of terminal restriction fragment length polymorphism (T-RFLP) has been proven to characterize the structure of the porcine gut microbiota (Ivarsson, Liu, Dicksved, Roos, & Lindberg, 2012; Burbach, Seifert, Pieper, & Camarinha-Silva, 2016). The impact of variations in dietary carbohydrate concentration and protein sources on the intestinal microbiota in pigs has been reported in several *in vivo* studies (Rist, Weiss, Sauer, Mosenthin, & Eklund, 2014; Weiss et al., 2016). In piglets, graded levels of soybean meal at the expense of corn-starch seem to have a positive linear effect on the growth of saccharolytic and potentially beneficial genera such as *Bifidobacteria* in ileal digesta and faecal samples (Rist et al., 2014). Evidence that dietary P modulates the porcine intestinal microbiota and immune defence is emerging, although results are not always consistent, as recently reviewed (Heyer et al., 2015). Physiologic effects of dietary CaP in combination with different fermentable substrates on immune cell abundance and functions, and the intestinal microbiota have not been investigated in pigs. Thus, our objective was to assess the effects of diets differing in their CaP concentration as well as protein and carbohydrate sources on apparent total tract digestibility (ATTD) of dietary nutrients, degradation of myo-inositol 1,2,3,4,5,6-hexakis (dihydrogen phosphate) ($InsP_6$), peripheral and gut-associated immune system, jejunal, caecal, and colonic bacterial communities, and intestinal short-chain fatty acid (SCFA) concentrations in pigs. We hypothesized that a minimum concentration of dietary CaP is needed to ensure normal immune functioning as well as a stable microbial ecosystem, and as a consequence, a diet low in CaP may negatively affect host health.

2.4.3 Materials and Methods

The study was conducted at the experimental unit of the department of Behavioural Physiology of Livestock of the University of Hohenheim. All experimental procedures involving animals were approved by the local authorities (Regierungspräsidium Stuttgart, Germany; permit number: V308/13 TH) in accordance with the German Welfare Legislation.

Experimental design, diets and sampling

Animals and diets

In total, 32 German Landrace × Piétrain pigs (initial BW: 54.7 ± 4.1 kg) were used in a 2 × 2 factorial arrangement of dietary treatments in two periods with 16 animals per period. Pigs were fed one of four diets based on corn and soybean meal (CSB) or corn and field peas

(CFP), and formulated to meet the nutrient requirement of National Research Council for growing pigs (NRC, 2012), except for Ca and P (Table 2.4- 1). Diets were supplemented with two different Ca and P concentrations, referred to as low (-) and high (+), with CaP concentrations amounting to 66% and 120% of pigs' actual Ca and P requirement, respectively, based on animals' BW in the range from 50 to 75 kg (NRC, 2012). The composition of the experimental diets has been published in Heyer et al. (2016). Pigs were housed in individual pens (each 3.25 m^2) under controlled environmental conditions (approximately 20°C and a 12 h dark, 12 h light cycle). Each pen was equipped with a drinking nipple and a stainless steel feeder. The health status of the animals was monitored daily. One pig was removed before the trial started due to increased body temperature. Feed was offered twice daily at 0800 h and 1500 h, and pigs had free access to water. Pigs were weighed each week to adjust their daily feed allowances to an amount of 4% of the average BW of all pigs within each week.

Sample collection

Each period was composed of 9 weeks, including an adaptation of 19 days to the diets. In week 4, 6 and 8, faecal samples (spot sampling) of 3 randomly selected pigs per treatment in each period were sampled. Each faecal sampling period consisted of 4 days per week from 0800-1600 h. In week 6 and 8, blood samples were collected between 0900-1030 h from all animals using a nose snare to fix the pig. Approximately 18 ml blood was collected by jugular vein puncture in heparinized tubes for immunological measurements. Two ml of the heparinized blood were centrifuged (1,000 × g for 10 min at 10°C) and plasma samples were stored at -20°C until further analysis. In week 4 and 6, all pigs were immunized with a 2 ml intramuscularly injection of 2.5 mg keyhole limpet hemocyanin (KLH) (Sigma-Aldrich Corporation, St. Louis, MO, USA) in 1 ml incomplete Freund's adjuvant (Sigma-Aldrich Corporation, St. Louis, MO, USA).

Table 2.4- 1 Chemical composition of the experimental diets (g/kg DM)†

	CSB		CFP	
Item	CaP-	CaP+	CaP-	CaP+
Metabolizable energy (kcal/kg)	3,667	3,581	3,801	3,686
Dry matter	905	901	901	897
Starch	535	514	561	536
Crude protein	168	167	153	148
Ether extract	71	79	80	89
Neutral detergent fibre	138	138	102	107
Acid detergent fibre	65	58	59	65
Calcium	4.3	8.3	4.5	8.3
Total phosphorus	4.1	7.5	4.2	7.4
$InsP_6$-P	2.1	2.0	2.0	2.0
Ins(1,2,3,4,5)P_5 (µmol/g DM)	<LOQ	<LOQ	ND	ND
Ins(1,2,4,5,6)P_5 (µmol/g DM)	0.7	0.7	0.6	0.7
$InsP_6$-P (µmol/g DM)	11.4	10.8	10.9	10.6
Indispensable amino acids				
Arginine	103.0	101.0	108.0	105.0
Histidine	49.0	48.5	41.5	41.0
Isoleucine	67.0	65.0	57.5	54.0
Leucine	163.0	160.0	130.0	125.5
Lysine	93.5	92.5	95.0	94.0
Methionine	27.5	28.0	34.0	33.5
Phenylalanine	86.5	84.5	74.0	72.0
Threonine	67.0	67.5	57.5	56.5
Valine	75.5	73.5	67.0	63.0
Dispensable amino acids				
Alanine	97.0	95.5	79.5	76.5
Aspartic acid	165.0	162.0	160.0	156.5
Cysteine	28.0	27.0	22.5	21.0
Glutamic acid	313.0	307.0	264.5	257.0
Glycine	68.5	67.0	63.5	61.5
Proline	113.5	110.5	81.0	77.0
Serine	88.0	87.0	77.0	75.5
Tyrosine	60.5	59.5	51.0	49.5

CSB, corn-soybean meal; CFP, corn-field pea; $InsP_6$, *myo*-inositol 1,2,3,4,5,6-hexakis (dihydrogen phosphate); $InsP_5$, *myo*-inositol pentakisphosphate; ND, not detected; LOQ, limit of quantification.
†Calculated total dietary fibre content (g/kg DM) in CSB CaP-, CSB CaP+, CFP CaP- and CFP CaP+ was 125, 121, 128, 124, respectively. Values were calculated based on table values for corn, soybean meal and field peas (NRC, 2012; Wang et al., 2008).

At the end of trial, pigs received the last meal 3 to 4 h before being euthanized to ensure that equal amounts of digesta had reached each section of the GIT. General anaesthesia was induced in pigs with an injection of ketamin (20 mg/kg BW, Serumwerk Bernburg AG, Bernburg, Germany) and azaperon (2 mg/kg BW, Lilly Deutschland GmbH, Bad Homburg, Germany). Pigs were euthanized by intravenous injection via the ear vein with pentobarbital (about 70 mg/kg BW, CP-Pharma Handelsgesellschaft mbH, Burgdorf, Germany). Immediately thereafter, the abdominal cavity was opened and the entire GIT was removed. The intestinal sections (jejunum, ileum, caecum, colon) were separated using clamps to

prevent mixing of digesta. First, all jejunal mesenteric lymph nodes (MLN) were taken and transferred into ice cold phosphate-buffered saline without Ca^{2+}/Mg^{2+} and with 50 µg/ml gentamycin (Biochrom GmbH, Berlin, Germany). Then, digesta from the jejunum (80 cm from the *Plica ileocaecalis*), ileum, caecum and ascending colon (10 cm in both directions from cone tip) were aseptically collected. Digesta of each intestinal section were immediately stored at -20°C and transferred to -80°C after sampling procedure. In addition, other subsamples of digesta for measurements of SCFA were stored at -20°C.

Analysis methods

Leukocyte distribution and functionality

Approximately 4 g of randomized jejunal MLN were separated into small pieces and processed by gentleMACS Dissociator (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) in phosphate-buffered saline without Ca^{2+}/Mg^{2+} supplemented by 50 µg/ml gentamycin (Biochrom GmbH, Berlin, Germany). Cell suspensions were extracted of cell clumps by 100 µm sterile cell strainer and stored in RPMI supplemented by 10% fetal calf serum and 50 µg/ml gentamycin (Biochrom GmbH, Berlin, Germany) until processing. Cell numbers were determined by a Z2 Coulter Counter (Beckman Coulter GmbH, *Krefeld*, Germany).

For discrimination of various leukocyte types, whole blood samples and single-cell suspension of MLN were labelled with antibodies and analysed by flow cytometry as described previously (Grün et al., 2013). The following fluorochrome-labelled antibodies were used: CD3 (clone PPT3), CD4 (clone 74-12-4), CD8α (clone 76-2-11), and CD 172a (clone 74-22-15) (Biozol Diagnostica Vertrieb GmbH, Echingen, Germany). In detail, granulocytes were identified by their forward and side scatter characteristics and further differentiated into neutrophils and eosinophils by autofluorescence characteristics within the unstained control sample. The PBMC and MLN subpopulations were classified by light scatter properties and the combination of surface marker expression as follows: T cells (SSC- CD3+), naive T helper cells (SSC- CD3+ CD4+ CD8α-), antigen-experienced T helper cells (SSC- CD3+ CD4+ CD8α+), cytotoxic T cells (SSC- CD3+ CD4- $CD8\alpha^{high}$), γδ T cells (SSC- CD3+ CD4- $CD8\alpha^{dim}$), natural killer cells (SSC- CD3- CD8α+ CD172a-), monocytes (SSC- CD3 CD8α- $CD172a^{high}$), and dendritic cells (SSC- CD3 CD8α- $CD172a^{dim}$) and B cells (SSC- CD3 CD8α CD172a-). In total, at least 10,000 cells, adjusted by exclusion of debris, were analysed per sample.

The PBMC were isolated from heparinized whole blood by density centrifugation using LeucosepTM (Greiner Bio-One; Frickenhausen, Germany) and mitogen-induced lymphocyte proliferation assays were used to assess *in vitro* activity of these blood PBMC as well as MLN cells according to Grün et al. (2013) with few modifications. Briefly, 1.5×10^5 immune cells were seeded per well of a U-bottom 96-well cell culture plate in triplicates per treatment and stimulated subsequently with either 5 µg/ml of the mitogen concanavalin A (ConA; Biochrom GmbH, Berlin, Germany), 5 µg/ml of the pokeweed mitogen (PWM; Sigma-Aldrich Corporation, St. Louis, MO, USA) or left without stimulation. After 48 h of incubation (37°C, 5% CO_2), 0.25 µCi titrated thymidine ([6-3H], PerkinElmer Inc., Waltham, MA, USA) was added to each well. One day later, cells were harvested on glass fibre filters and radioactivity was measured by liquid scintillation analyser (PerkinElmer Inc., Waltham, MA, USA). The Δ cpm for ConA and PWM were determined for each individual (Δ cpm = stimulated (cpm) - unstimulated cells (cpm)).

Concentrations of plasma anti-KLH immunoglobulin (Ig) G and anti-KLH IgM were measured by ELISA as described in Grün, Schmucker, Schalk, Flauger, & Stefanski (2014) with few modifications for anti-KLH IgM analysis. ELISA plates were coated with 100 ng/well KLH (Sigma-Aldrich Corporation, St. Louis, MO, USA) in coating buffer (15 mM $NaHCO_3$ and 35 mM Na_2CO_3, pH 9.6) and incubated for 1 hour at 4°C. Diluted plasma samples were added to the plates after blocking with BSA (Albumin Fraction V, Carl Roth GmbH + Co. KG, Karlsruhe, Germany). Bound plasma anti-KLH antibodies were detected with HRP-labelled goat anti-pig IgM (GeneTex Inc., Irvine, CA, USA). Plasma samples were quantified by reference to standard curves constructed with a pooled plasma control and calculated as arbitrary units.

Intestinal microbiota

Genomic DNA was isolated from jejunal, ileal, caecal and colonic digesta samples as previously described (Weiss et al., 2016). Profiles of bacterial communities in digesta samples were obtained by analysis of T-RFLP as described by Burbach et al. (2016) with few modifications. Bacterial 16S rRNA gene fragments were amplified from the genomic DNA using primer pair 27F, 5'labeled with 6-carboxyfluorescein, and 1492R (Lane, 1991). The PCR mixture included 3% DMSO and the PCR was performed with 33 cycles. 200 ng amplicon DNA were digested with 5 U MspI and analysed on an ABI 3130 Genetic Analyzer (Applied Biosystems; Foster City, CA, USA).

To assign single terminal restriction fragments (TRF) clone libraries from ileal digesta and faecal samples of one pig fed with CSB$^-$ were constructed. 16S rRNA gene fragments were amplified with primer 27F and 1492R. Amplicons were gel-purified using Double Pure Kit (Bio&Sell GmbH, Feucht bei Nürnberg, Germany) and cloned into pGEM®-T Easy Vector System (Promega, Madison, WI, USA) according to manufactures instructions. Randomly selected clones were screened by amplified rDNA restriction analysis for variable phylogenetic clusters using RsaI (New England BioLabs, Ipswich, MA, USA). Representatives of the different restriction patterns were sent to Sanger sequencing (Eurofins Genomics, Ebersberg, Germany). Sequence assembly and manual editing was done in Codon-Code Aligner (http://www.codoncode.com/aligner/) and BioEdit (http://www.mbio.ncsu.edu/bioedit/bioedit.html). Sequences were taxonomically assigned using RDP Sequence Match and submitted to NCBI GenBank under the accession number KU705874-KU705900.

Furthermore, genomic DNA from jejunal, caecal, and colonic digesta samples was used to quantify certain bacteria groups according to Weiss et al. (2016) using previously described primer sets (Supplemental Table 2.4- 1). Results were reported as $\log_{10}$ 16S rRNA gene copies/g fresh matter.

Concentration of SCFA in jejunal and caecal samples was analysed by gas chromatography according to Wischer et al. (2013). A mean for each detected bacteria and each SCFA was calculated only if the individual bacteria or SCFA was detected in at least three out of the six samples of one treatment group. If the detected value was below the limit of quantification (LOQ) in more than 1/3 or more samples, this was noted as less than the LOQ in the tables, and means were not calculated.

Chemical analysis

Concentration of proximate nutrients were determined according to the official methods in Germany (VDLUFA, 2007). Diets were analysed for dry matter, crude ash, crude protein, and crude fat. Content of GE in the diets was measured using a bomb calorimeter (IKA calorimeter, C200, IKA®-Werke GmbH & Co. KG, Staufen, Germany). The concentration of P, Ca, Titanium dioxide (Ti), and InsP isomers in diets, faeces and digesta were determined as described by Zeller, Schollenberger, Kühn, & Rodehutscord (2015). A mean for an InsP isomer was calculated only if the isomer was detected in at least 3 out of the 6 samples of one treatment group. If the detected value was below the limit of quantification (LOQ) in more

than 1/3 or more samples, this was noted as less than the LOQ in the tables, and means were not calculated.

Calculations and statistical analyses

Calculations

The apparent total tract digestibility (ATTD) of dietary nutrients was calculated using the indicator method (Adeola, 2001): ATTD, % = 100 − [100 × (concentration of Ti in diet × concentration of nutrient in faeces or digesta/concentration of Ti in faeces or digesta × concentration of nutrient in diet)]. Diet metabolizable energy (ME) values were calculated with the analysed content of ash, crude protein, ether extract and neutral detergent fibre (Noblet & Perez, 1993), as adopted by NRC (2012).

Statistics

The following linear mixed effects model was fitted to the data:

$$y_{ijklm} = \mu + d_i + s_j + \pi_k + \rho_l + (\pi\rho)_{lk} + e_{ijklm}, \quad (1)$$

Where y_{ijklm} is an observation on the *m*-th animal from the j-th sow in the i-th period treated with the lk-th combination of CaP concentration and corn-protein diet,

μ is the intercept,

d_i is the effect of the i-th experimental period,

s_j is the effect of the j-th sow,

π_k is the effect of the *k*-th corn-protein diet,

ρ_l is the effect of the *l*-th CaP concentration,

$(\pi\rho)_{lk}$ is the interaction of corn-protein diet and CaP concentration, and

e_{ijklm} are the residual errors associated with y_{ijklm}.

d_i, s_j and e_{ijklm} were estimated as random effects with means zero and variances σ_d^2, σ_s^2 and σ_e^2. For the data collected after euthanasia, day of euthanasia was added as random effect.

The model was fitted in the statistical package R version 3.1.0 (Development Core Team R, 2014) using the function lmer() from the R-package 'lme4' (Bates, Maechler, Bolker, & Walker, 2014), where variance components are estimated by restricted maximum likelihood. Fixed effects were tested for significance in Wald-Type-III F-tests. Denominator Degrees of freedom were adjusted using the method of Kenward and Roger, leading to more precise F-

tests (Littell, Milliken, Stroup, Wolfinger, & Schabenberger, 2007). The levels of factors found significant ($p < 0.05$) in the F-test were compared by pairwise Tukey-tests using the function lsmeans() in combination with the function cld() for generating letter displays. Levels of main effects (corn-protein diet and CaP concentration) were only compared, if interaction was not significant ($p > 0.05$) in the F-test. A significance level of $\alpha = 5\%$ was used throughout the whole analysis. Model assumptions of homogeneity of variance and normal distribution of residuals were assessed by an inspection of plots of Pearson-residuals versus predicted values and QQ-plots, respectively. If not fulfilled, the model was re-fitted to the transformed response variable to stabilize variance and meet distribution assumption (Piepho, 2009). Adjusted mean estimates from models with transformed response variable are reported on the original scale after a back transformation. Means estimated on the transformed scale constitute estimates of medians on the original scale (*ibid.*). Standard errors on the original scale are approximated using the Delta-method (Johnson, Kemp, & Kotz, 2005).

Statistical analysis of multivariate T-RFLP data sets were carried out using PRIMER v6 (Clarke & Warwick, 2001). Abundance data of TRF in the range of 70 bp to 1 400 bp were standardized by total and Bray-Curtis similarity matrix. Bacterial community structures based on T-RFLP data were explored by principal coordinate analysis. Group-average cluster were superimposed onto principal coordinate analysis plot to show similarity within groups of samples. TRF contributing to dissimilarity between sample groups were determined by similarity percentage analysis and bubbles representing relative abundance of mostly contributing TRF were superimposed onto principal coordinate analysis plots. Significant differences ($p < 0.05$) in bacterial communities were evaluated by analysis of similarity (ANOSIM). The R statistic value expresses the separation between groups in range from -1 to 1, with the higher R value the more distinct are the groups.

2.4.4 Results

Effect of varying calcium-phosphorus concentration and fermentable substrates on nutrient digestibility and phytate degradation

The ATTD of P was higher for CaP+ ($p < 0.001$; Table 2.4- 2) compared to CaP-, as well as higher for the CFP compared to CSB diets ($p < 0.05$). However, the ATTD of Ca was greater ($p < 0.001$) for CaP- compared to CaP+, independent of corn-protein diet. The ATTD of neutral detergent fibre was higher ($p < 0.001$) for CSB compared to CFP. In jejunal digesta,

the concentration of $InsP_6$ was greater ($p < 0.05$; Table 2.4- 3) in CaP+ compared to CaP-. Furthermore, the interaction of corn-protein diet and CaP concentration resulted in a higher $InsP_6$ concentration ($p < 0.05$) in CSB- and CFP+ in caecal digesta. In addition, caecal and faecal Ins(1,2,3,4,6)P_5 and Ins(1,2,4,5,6)P_5 concentration were greater ($p < 0.05$) in CSB compared to CFP.

Table 2.4- 2 Apparent total tract digestibility (ATTD) of diets.

	CSB		CFP			p-value		
Item	CaP-	CaP+	CaP-	CaP+	Pooled SEM	Protein	CaP	CaP × Protein
ATTD %								
Dry matter	81.1	82.0	82.3	82.7	0.8	0.096	0.209	0.625
Crude protein	74.4	75.0	68.9	74.4	1.8	0.107	0.109	0.192
Neutral detergent fibre	52.0^{A}	57.7^{A}	42.5^{B}	42.2^{B}	1.6	<0.001	0.108	0.081
Acid detergent fibre	14.0	15.0	13.2	19.5	6.8	0.610	0.319	0.466
Calcium	45.1^{a}	35.2^{b}	42.7^{a}	36.1^{b}	2.6	0.684	<0.001	0.333
Total phosphorus	2.0^{Bb}	20.8^{Ba}	4.8^{Ab}	23.8^{Aa}	1.3	0.033	<0.001	0.958
$InsP_6$-P	9.3	14.3	12.6	10.3	4.8	0.845	0.461	0.056

CSB, corn-soybean meal; CFP, corn-field pea; $InsP_6$, *myo*-inositol 1,2,3,4,5,6-hexakisphosphate; SEM, Standard error of the mean.
Least-square means based on 6 observations per diet.
Within a row, means of protein that do not share a common capital superscript letter differ ($p < 0.05$); within a row, means of CaP concentration that do not share a common lower case superscript letter differ ($p < 0.05$). Mean estimates of protein within each level of the factor CaP that do not share a common capital superscript letter differ significantly ($p < 0.05$); mean estimates of CaP concentration within each source of the factor protein that do not share a common lower case superscript letter differ significantly ($p < 0.05$).

Table 2.4- 3 Concentration of different inositol phosphate (InsP) isomers (µmol/g DM) in digesta and faecal samples

	CSB		CFP			p-value		
Item	CaP-	CaP+	CaP-	CaP+	Pooled SEM	Protein	CaP	CaP × Protein
Jejunum								
Ins(1,2,4,5,6)P_5	1.40	2.01	ND	1.32	0.56	0.069	0.095	-
InsP_6	28.45^{b}	33.22^{a}	16.79^{b}	30.53^{a}	6.12	0.068	0.023	0.215
Caecum								
Ins(1,2,3,4,6)P_5	0.98^{A}	1.09^{A}	0.51^{B}	0.64^{B}	0.21	0.003	0.372	0.958
Ins(1,2,4,5,6)P_5	2.22^{A}	2.15^{A}	1.51^{B}	1.95^{B}	0.26	0.018	0.329	0.176
InsP_6	36.90^{Ab}	38.31^{Bb}	29.21^{Bb}	39.24^{Ba}	2.47	0.088	0.007	0.045
Faeces								
Ins(1,2,3,4,6)P_5	1.57^{A}	1.65^{A}	1.12^{B}	1.01^{B}	0.13	<0.001	0.859	0.182
Ins(1,2,4,5,6)P_5	3.25^{A}	3.26^{A}	2.83^{B}	2.85^{B}	0.17	0.005	0.893	0.995
InsP_6	54.59	55.42	54.87	54.92	2.58	0.940	0.753	0.769

CSB, corn-soybean meal; CFP, corn-field pea; InsP_6, *myo*-inositol 1,2,3,4,5,6-hexakisphosphate; ND, not detected; SEM, Standard error of the mean.
Least-square means based on 3-6 observations per diet.
Within a row, means of protein that do not share a common capital superscript letter differ ($p < 0.05$); within a row, means of CaP concentration that do not share a common lower case superscript letter differ ($p < 0.05$). Mean estimates of protein within each level of the factor CaP that do not share a common capital superscript letter differ significantly ($p < 0.05$); mean estimates of CaP concentration within each source of the factor protein that do not share a common lower case superscript letter differ significantly ($p < 0.05$).
Ins(1,2,3,4,5)P_5 in jejunal digesta (mean 0.91 µmol/g DM) and Ins(1,2,3,4,6)P_5 in caecal digesta (mean 0.49 µmol/g DM) were detectable only for CSB, high CaP; no statistical analysis performed.

Intestinal bacterial communities

The overall structure of intestinal microbiota was evaluated by T-RFLP profiles from jejunal, ileal, caecal and colonic digesta samples. A total number of 156 TRF was detected, with a core community of 11 TRF being present in all intestinal sections. ANOSIM test revealed significant differences between all four analysed intestinal sections (R = 0.598, p = 0.001). In a principal coordinate scaling plot (Figure 2.4- 1), the bacterial communities from digesta samples are grouped by small or large intestine origin showing in average 79% dissimilarity. This dissimilarity was mainly caused by TRF 521, which predominated in all intestinal sections, with a fourfold higher average abundance in the small intestine than the large intestine.

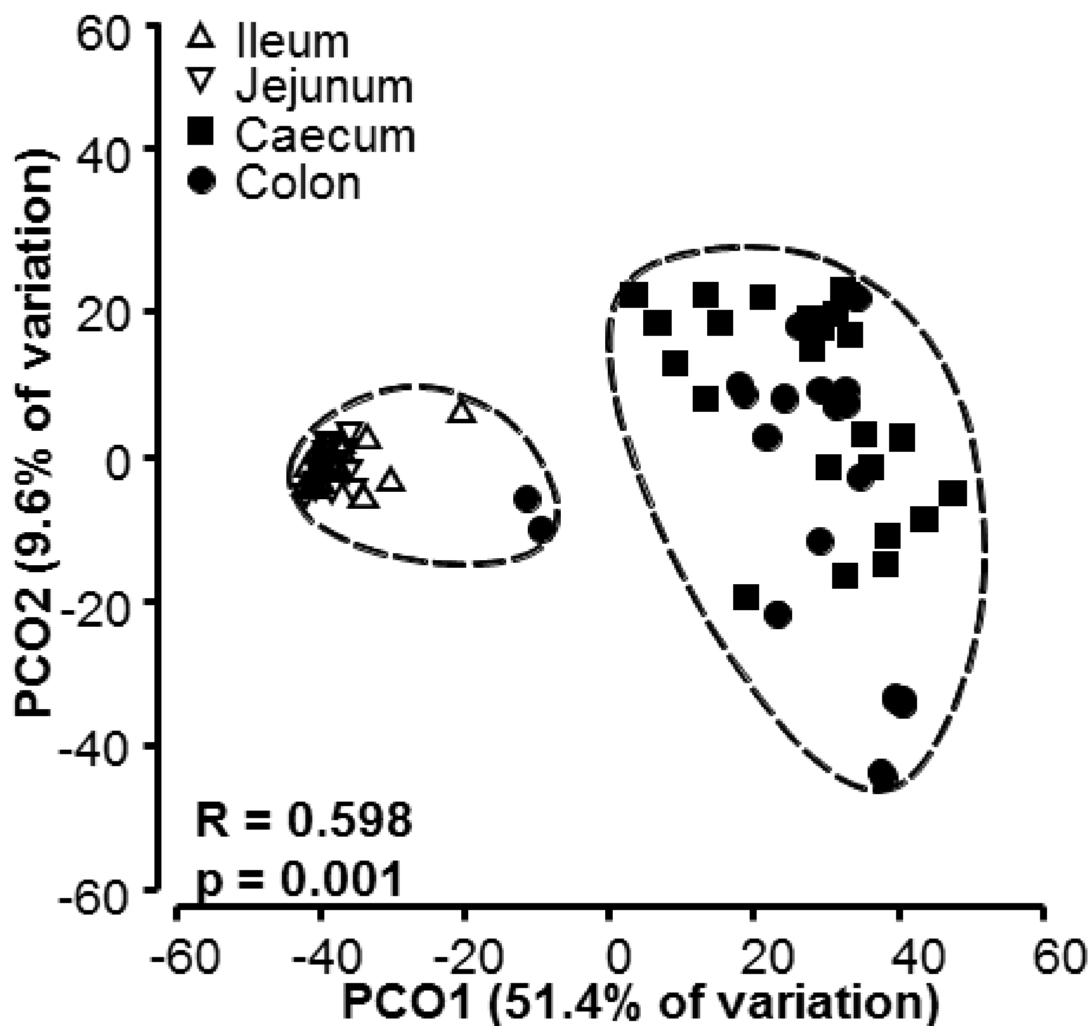

Figure 2.4- 1 Principal coordinate analysis plot of bacterial community structure by T-RFLP profiles from different intestinal sections. Jejunal profiles are displayed as downturned triangles, ileal as upturned triangles, caecal as squares and colonic as circles. Clusters of 30% similarity are overlaid as determined by group-average clustering.

The bacterial community structures in each intestinal section were further investigated in respect to diet-associated effects and the caecal structure was significantly impaired. The T-RFLP patterns of the caecal digesta samples showed separations of communities based on the dietary treatment ($R = 0.387$, $p = 0.001$) (Figure 2.4- 2). These differences were not associated with the protein source ($p > 0.05$) but associated with CaP concentration ($p = 0.001$). We identified discriminating TRFs that contributed to more than 5% to these dissimilarities: TRF 92, TRF 219 and TRF 521. By comparing T-RFLP profiles with clone sequences, TRF 521 was assigned to an uncultured bacterium related to unclassified Porphyromonadaceae (Supplemental Table 2.4- 2). TRF 92 bp can probably be assigned to a member of the Clostridiales as several clones sequences share relevant sequence similarity and showed an experimental TRF of 91 bp, which can be considered to be equivalent, by accepting technical bias of +/-1 bp. The discriminating TRF 219 was not assigned to any known sequence. TRF 521 was less abundant in samples low in dietary CaP concentration and more abundant in samples of treatments with CaP-rich diets (13.0% *vs.* 23.5%). For the abundance of TRF 92 the opposite ratio was observed with a higher abundance in samples with low dietary concentration of CaP compared to those with high concentration of CaP (12.6% *vs.* 5.5%).

Quantification of 16S rRNA gene copy numbers and short-chain fatty acids

In caecal digesta, 16S rRNA gene copy numbers of *Eubacterium rectale* and *Roseburia* spp. were higher ($p < 0.05$; Table 2.4- 4) in CaP+ compared to CaP- fed pigs. The number of *Lactobacillus* spp. was greater ($p < 0.01$) in CaP+ in caecal and colonic digesta compared to CaP-. Furthermore, colonic *Clostridium* cluster IV was greater ($p < 0.05$) in CaP+ compared to CaP-. Colonic *Clostridium* cluster XIVa was higher ($p < 0.01$) in CaP- compared to CaP+. Gene copy number of caecal *Bifidobacterium* spp. and colonic *Roseburia* spp. were greater ($p < 0.05$) in CSB than in CFP fed pigs

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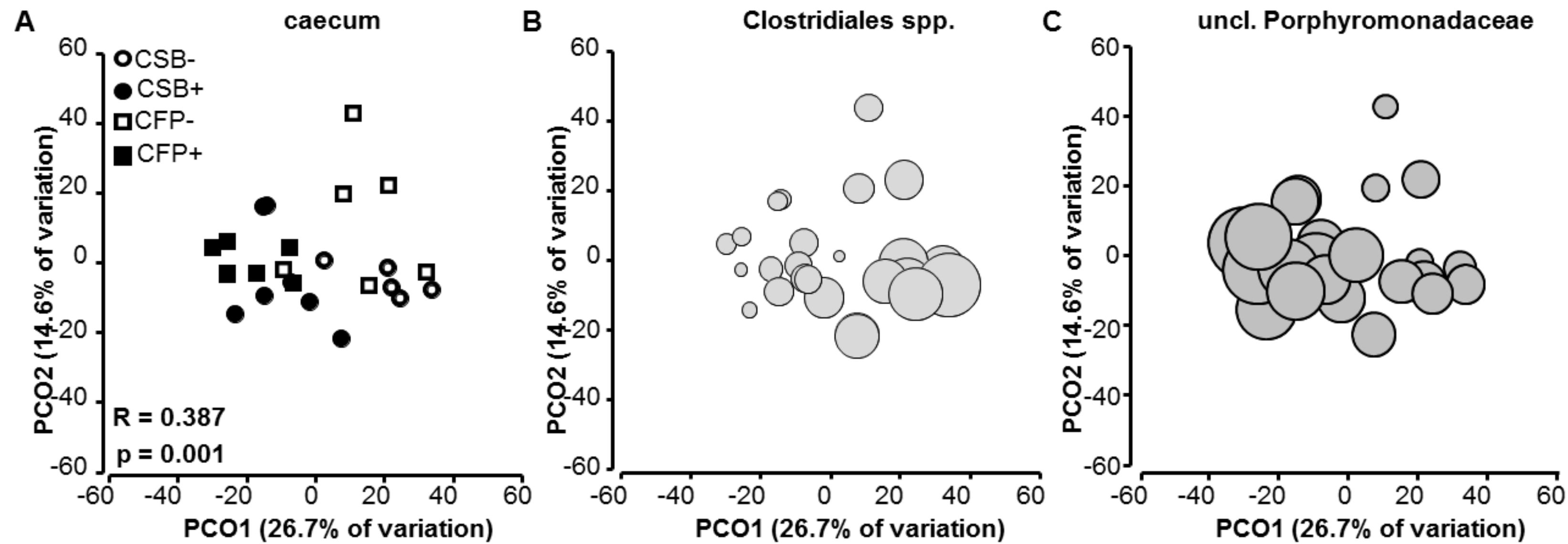

Figure 2.4- 2 Dietary effect on microbiota profile of caecal digesta. (A) In a principal coordinate analysis plot, based on T-RFLP profiles, samples from dietary treatment with CSB- are shown as lined circles, CSB+ as filled circles, CFP- as lined squares and CFP+ as filled squares.(B) Bubble area representing the relative abundance of TRF 92 assigned to Clostridiales spp. (C) Bubble area representing the relative abundance of TRF 521 assigned to unclassified Porphyromonadaceae.

Table 2.4- 4 Bacterial numbers (log_{10} 16S ribosomal RNA gene copies/g fresh matter) in jejunal, caecal, and colonic digesta

	CSB		CFP			p-value		
Item	CaP-	CaP+	CaP-	CaP+	Pooled SEM	Protein	CaP	CaP × Protein
Jejunum								
Lactobacillus spp.	7.2	6.3	7.2	6.8	0.6	0.477	0.129	0.512
Clostridium cluster IV	<LOQ	4.4	4.9	4.7	0.5	0.697	0.872	-
Clostridium cluster XIVa	6.2	5.9	6.6	5.2	0.5	0.724	0.079	0.291
Eubacterium rectale	<LOQ	<LOQ	<LOQ	<LOQ	-	-	-	-
Roseburia spp.	5.2	4.9	5.1	<LOQ	0.4	0.879	0.640	-
Bifidobacterium spp.	7.0	6.6	6.9	5.5	0.5	0.219	0.106	0.424
Caecum								
Lactobacillus spp.	7.7^{b}	8.2^{a}	7.6^{b}	7.9^{a}	0.2	0.176	0.004	0.295
Clostridium cluster IV	7.9	8.1	8.0	8.2	0.1	0.339	0.114	0.930
Clostridium cluster XIVa	9.9	9.8	9.8	9.9	0.1	0.953	0.826	0.377
Eubacterium rectale	5.9^{b}	6.5^{a}	5.2^{b}	6.2^{a}	0.4	0.074	0.011	0.510
Roseburia spp.	8.5^{b}	8.7^{a}	8.1^{b}	8.6^{a}	0.2	0.137	0.022	0.404
Bifidobacterium spp.	7.4^{A}	6.9^{A}	6.6^{B}	6.4^{B}	0.3	0.038	0.199	0.806
Colon								
Lactobacillus spp.	7.1^{b}	8.4^{a}	7.4^{b}	8.1^{a}	0.3	0.995	0.002	0.251
Clostridium cluster IV	8.3^{b}	8.6^{a}	8.5^{b}	8.6^{a}	0.1	0.055	0.013	0.077
Clostridium cluster XIVa	10.1^{a}	10.0^{b}	10.3^{a}	9.9^{b}	0.1	0.416	0.009	0.065
Eubacterium rectale	6.1	6.5	6.1	6.1	0.4	0.497	0.494	0.428
Roseburia spp.	8.7^{A}	8.8^{A}	8.4^{B}	8.6^{B}	0.1	0.043	0.352	0.621
Bifidobacterium spp.	7.2	6.7	6.9	6.6	0.4	0.488	0.129	0.839

CSB, corn-soybean meal; CFP, corn-field pea; LOQ, limit of quantification; SEM, Standard error of the mean.
Least-square means based on 3-6 observations per diet.
Within a row, means of protein that do not share a common capital superscript letter differ ($p < 0.05$); within a row, means of CaP concentration that do not share a common lower case superscript letter differ ($p < 0.05$). Mean estimates of protein within each level of the factor CaP that do not share a common capital superscript letter differ significantly ($p < 0.05$); mean estimates of CaP concentration within each source of the factor protein that do not share a common lower case superscript letter differ significantly ($p < 0.05$).

Table 2.4- 5 Short-chain fatty acid concentrations (mmol/kg DM) in jejunal and caecal digesta

	CSB		CFP			p-value		
Item	CaP-	CaP+	CaP-	CaP+	Pooled SEM	Protein	CaP	CaP × Protein
Jejunum								
Acetic	272.2^{Ab}	258.9^{Bb}	110.7^{Bb}	229.4^{Ba}	32.1	0.007	0.087	0.041
Propionic	<LOQ	1.7†	<LOQ	<LOQ				
Butyric	34.5	26.5	11.9	29.8	7.3	0.168	0.430	0.067
Isobutyric	<LOQ	<LOQ	<LOQ	<LOQ				
Valeric	<LOQ	<LOQ	<LOQ	<LOQ				
Isovaleric	1.8	2.5	<LOQ	1.9	0.8	0.504	0.467	-
Total	304.4^{A}	285.6^{A}	127.6^{B}	258.4^{B}	37.3	0.015	0.134	0.057
Caecum								
Acetic	671.8^{b}	792.9^{a}	671.6^{b}	742.1^{a}	49.3	0.052	0.002	0.323
Propionic	175.6^{b}	230.0^{a}	180.1^{b}	214.7^{a}	15.4	0.716	0.007	0.523
Butyric	141.3	117.9	99.6	107.2	13.7	0.056	0.533	0.256
Isobutyric	4.7	5.1	4.4	4.7	0.7	0.617	0.607	0.869
Valeric	6.3	9.8	5.9	7.3	1.4	0.287	0.079	0.419
Isovaleric	5.3	4.7	3.9	3.5	0.8	0.112	0.534	0.934
Total	1,004.7^{b}	1,160.4^{a}	812.0^{b}	1,079.3^{a}	75.7	0.085	0.010	0.481

CSB, corn-soybean meal; CFP, corn-field pea; LOQ, limit of quantification; SEM, Standard error of the mean.

Least-square means based on 4-6 observations per diet.

Within a row, means of protein that do not share a common capital superscript letter differ ($p < 0.05$); within a row, means of CaP concentration that do not share a common lower case superscript letter differ ($p < 0.05$). Mean estimates of protein within each level of the factor CaP that do not share a common capital superscript letter differ significantly ($p < 0.05$); mean estimates of CaP concentration within each source of the factor protein that do not share a common lower case superscript letter differ significantly ($p < 0.05$).

†Calculated mean.

The interaction of corn-protein diet and CaP concentration resulted in a greater jejunal acetate concentration ($p < 0.05$; Table 2.4- 5) in CSB- and CFP+. Jejunal total SCFA were greater ($p < 0.05$) in CSB compared to CFP fed pigs. In caecal digesta, acetate, propionate and total SCFA were greater ($p < 0.05$) in CaP+ compared to CaP- fed pigs.

Leukocyte distribution and functionality

Numbers of T cell populations in jejunal mesenteric lymph nodes were not affected by the dietary treatment ($p > 0.05$; Supplemental Table 2.4- 3). However, lymphocyte proliferation using the mitogen ConA was greater ($p < 0.05$) in CSB than in CFP fed pigs.

In the blood, numbers of antigen-experienced T helper cells, natural killer cells, B cells, dendritic cells, monocytes, granulocytes and neutrophils were greater ($p < 0.05$; Table 2.4- 6) in CaP+ compared to CaP-. Furthermore, the naive : antigen-experienced T helper cell ratio was greater ($p < 0.05$) in CaP- than in CaP+ fed pigs. The lymphocyte proliferation using mitogen ConA and PWM as well as anti-KLH IgM and anti-KLH IgG were not affected by the dietary treatment ($p > 0.05$).

Table 2.4- 6 Numbers of leukocytes (number/µl blood) in whole blood and in vitro proliferation of peripheral blood mononuclear cells using the mitogen Concanavalin A and Pokeweed mitogen, and plasma anti-KLH antibody concentration (arbitrary units)

	CSB		CFP			p-value		
Item	CaP-	CaP+	CaP-	CaP+	Pooled SEM	Protein	CaP	CaP × Protein
T cells	8,753	9,328	8,805	8,923	812	0.734	0.482	0.633
naive T helper cells	1,606	1,519	1,550	1,380	156	0.503	0.354	0.755
T helper cells	2,843	3,385	2,891	2,997	232	0.458	0.149	0.317
antigen-experienced T helper cells	1,238[b]	1,874[a]	1,367[b]	1,568[a]	131	0.503	0.004	0.103
naive : antigen-experienced T helper cell ratio	1.3[a]	0.9[b]	1.2[a]	0.9[b]	0.1	0.781	0.010	0.539
Cytotoxic T cells	1,278	1,795	1,794	1,618	237	0.342	0.330	0.056
Regulatory T cells[†]	0.10	0.09	0.06	0.08	0.03	0.177	0.659	0.500
γδ T cells	4,518	4,292	4,021	4,342	708	0.512	0.879	0.377
Natural killer cells	203[b]	414[a]	263[b]	347[a]	74	0.952	0.019	0.291
B cells	1,403[b]	1,518[a]	1,346[b]	1,757[a]	187	0.444	0.029	0.175
Dendritic cells[§]	880[b]	1,238[a]	1,078[b]	1,154[a]	95	0.429	0.017	0.089
Monocytes	788[b]	1,006[a]	837[b]	906[a]	57	0.651	0.018	0.199
Granulocytes[§]	3,899[b]	5,195[a]	3,780[b]	4,367[a]	382	0.284	0.021	0.483
Neutrophils	3,553[b]	5,092[a]	3,526[b]	4,059[a]	423	0.221	0.020	0.239
Eosinophils[§]	478	488	500	551	100	0.565	0.663	0.773
Lymphocyte proliferation[‡]								
Concanavalin A[§]	9,312	9,740	8,947	8,721	3,895	0.612	0.944	0.810
Pokeweed mitogen[§]	16,978	16,998	15,968	14,526	8,561	0.342	0.679	0.672
Antibody concentration								
Anti-KLH IgM (week 6) [§]	2,893	3,000	2,752	3,023	651	0.922	0.762	0.892
Anti-KLH IgG (week 8) [§]	2,078	2,657	3,329	5,140	911	0.280	0.169	0.698

CSB, corn-soybean meal; CFP, corn-field pea; Ig, immunoglobulin; KLH, keyhole limpet hemocyanin; SEM, Standard error of the mean.
Least-square means based on 7-8 observations per diet. Within a row, means of protein that do not share a common capital superscript letter differ ($p < 0.05$); within a row, means of CaP concentration that do not share a common lower case superscript letter differ ($p < 0.05$). Mean estimates of protein within each level of the factor CaP that do not share a common capital superscript letter differ significantly ($p < 0.05$); mean estimates of CaP concentration within each source of the factor protein that do not share a common lower case superscript letter differ significantly ($p < 0.05$). [†]regulatory T cells × 10^3/µl related to all lymphocytes. [‡]The Δ counts per minute (cpm) for Concanavalin A and Pokeweed mitogen were determined for each individual (Δ cpm = stimulated (cpm) - unstimulated cells (cpm)).[§]Adjusted mean estimates from models with transformed response variable are reported on the original scale after back transformationA and Pokeweed mitogen were determined for each individual (Δ cpm = stimulated (cpm) - unstimulated cells (cpm)).[§]Adjusted mean estimates from models with transformed response variable are reported on the original scale after back transformation.

2.4.5 Discussion

The microbial profile and microbial activity are dependent on substrate availability and microbial substrate preference. The modulation of the GIT microbial ecosystem by dietary intervention often aims to increase host commensal microbiota, such as Bifidobacterium and Lactobacilli, to beneficially affect immune function and GIT development and to inhibit pathogenic microbes (De Filippo et al., 2010; Gibson & Wang, 1994). Furthermore, the interaction between the intestinal microbiota including their metabolic products such as butyrate, the integrity of the epithelial barrier function, and the local and systemic immunity describes the close relationship between the intestinal microbiota and the immune system (Grenham, Clarke, Cryan, & Dinan, 2011). Several studies have shown interactions between dietary CaP supply and the microbial ecosystem of the GIT in pigs (Heyer et al., 2015). However, studies with pigs focusing on potential relationships between dietary CaP supply and the animals' immune system are rare. There is increasing evidence that P has to be considered as part of an integrated approach to support digestive and immune function, with special focus on maintaining the intestinal eubiosis of pigs (Heyer et al., 2015). In the present study, the higher dietary concentration of CaP increased the abundance of one TRF that was assigned to an uncultured bacterium related to Porphyromonadaceae. This certain clone sequence was assigned by its similarity to a 16S rRNA sequence isolated from faeces of pigs with high levels of antibiotic resistance (Kalmokoff et al., 2011). Members of Porphyromonadaceae are common members of porcine intestine and found in increased abundance in human intestine of Crohn's disease patients (Manichanh et al., 2006) as well as in malnourished children (Gupta et al., 2011). A mice study revealed negative correlation between BW gain and Porphyromonadaceae abundance (Ryan et al., 2014). This is in contrast to our findings, where the abundance of the related TRF showed an increase along with a greater final BW in dietary groups of CaP+ concentration ($p < 0.001$; CSB-: 92.6; CSB+: 106.8; CFP-: 91.5; CFP+: 102.8 kg). Furthermore, one TRF assigned to bacterial order Clostridiales was identified as discriminator in bacterial communities of CaP- diets. By clone library matches, this TRF is assumed to be affiliated to *Clostridium sensu stricto* and *Clostridium* cluster XI. With the two main representatives *Clostridium perfringens* and *Clostridium difficile*, the potential identity of TRF 92 is conditionally associated with a less healthy microbiota (Songer & Uzal, 2005). By qPCR, *Clostridium* cluster IV and *Clostridium* cluster XIVa were examined, but here no significant CaP effect in caecal digesta was found, whereas colonic gene copy numbers of *Clostridium* cluster IV have been promoted by the

CaP+ diets and colonic gene copy numbers of *Clostridium* cluster XIVa were lower in the CaP+ diets. These groups of Clostridiales contain many butyrate producers and therefore are associated with a healthy microbiota (Louis & Flint, 2009). The measured butyrate concentrations did not differ between dietary treatments. A missing link between butyrate producing bacteria and butyrate concentrations might be due to limitations of the applied analysis. Deeper insights in the effects of dietary CaP on microbial butyrate production might be obtained by a metagenomic-transcriptomic approach. By such an in-depth, integrative approach, both the genetic potential of butyrate production and expressed butyrate metabolism are revealed. In general, *Clostridiales* dominate the porcine microbiota in the large intestine (Zhao et al., 2015) and low numbers of pathogenic species can be found in healthy subjects (Ozaki et al., 2004).

Dietary supplementation with P and Ca has been suggested as a potential strategy to modulate the GIT microbiota in pigs (Mann et al., 2014) based on studies with rats, where a deceased abundance of pathogens and a promotion of lactobacilli in the small intestine in CaP-rich diets have been observed (Bovee-Oudenhoven, Wissink, Wouters, & Van der Meer, 1999). In pig nutrition, an oversupply of dietary CaP above the pigs" actual requirements is considered to be disadvantageous compromising gastric barrier function in piglets (Lawlor, Lynch, Gaffrey, O'Reilly, & O'Connell, 2005). However, evidence exists that the CaP availability and the formation of individual inositol phosphates affects the intestinal microbiota in pigs (Heyer et al., 2015). In the present study, the effect of dietary CaP supply by adding different amounts of monocalcium phosphate has been studied. The increase in ATTD of P by adding a higher amount of dietary inorganic P was expected and is in agreement with other studies (Johnston et al., 2004; Stein, Kadzere, Kim, & Miller, 2008), and thereby increasing P availability for the microbes. However, the P digestibility might be negatively affected due to the formation of insoluble Ca-$InsP_6$ complexes and the entailed limited efficiency of mucosal phytase, in particular in the proximal gut. In our study, greater $InsP_6$ concentration was analysed in jejunal CFP samples resulting in lower $InsP_5$ isomer concentrations in caecal digesta and faeces, indicating a reduced degradation of $InsP_6$.

In contrast, with higfher dietary CaP, both caecal *Eubacterium rectale* and *Roseburia* spp., caecal and colonic *Lactobacillus* spp. were increased, whereas colonic *Clostridium* cluster XIVa decreased. The genus *Lactobacillus* is one of the most abundant beneficial bacterial groups in the porcine GIT (Konstantinov et al., 2006). In agreement with rat (Bovee-Oudenhoven et al., 1999) and piglet (Mann et al., 2014) studies, CaP-rich diets did improve

colonization resistance against intestinal pathogens and promote lactobacilli in different GIT segments. While the present study focuses on the effect of variations in dietary P supply, specific effects of Ca, such as the potential of free Ca^{2+} ions to inhibit the proliferation of specific bacterial groups in the proximal part of the GIT, have to be considered (Larsen, Nissen, & Willats, 2007; Metzler-Zebeli, Vahjen, Baumgärtel, Rodehutscord, & Mosenthin, 2010). This might be of specific importance with regard to competition for adhesion sites, such as the mucosa, and as a consequence, for the colonisation resistance against potential pathogens. Moreover, in the large intestine CaP- diets decreased microbial activity resulting in a lower acetate, propionate and total SCFA concentration. The decrease in P availability for the intestinal microbiota can be associated with a lowered fermentation activity, similar to studies with rumen microbes. Komisarczuk, Merry, & McAllan (1987) examined the effects of variations in P supply on rumen microorganisms in sheep and determined that the available P in the surrounding medium affects the activity of bacterial fibrolytic enzymes. The authors concluded that a P deficiency may cause a reduced SCFA synthesis due to a decreased fermentation of cellulose by the microorganisms. Short-chain fatty acids, especially acetate, propionate and butyrate, are the major end-products of carbohydrate fermentation, lowering the pH in the colon and thereby preventing the growth and activity of pathogenic bacteria. In particular, butyrate plays an important role in cell growth, differentiation, intestinal barrier function, and also immune regulation. These findings suggest that the intestinal microbiota, in particular specific bacteria, affects the host immune system in a different manner, which is in agreement with studies showing the presence of a gut-brain-axis (Grenham et al., 2011). In the present work, the butyrate concentration in digesta samples was not significantly affected by the dietary treatment, whereas numerical butyrate concentration was greater in CSB diets compared to CFP with a potential effect on immune values determined in the present study. Future research should focus on the role of active microbial fractions and their functions, possibly causing activation of the immune system due to for example lipopolysaccharides present on the outer membrane of gram-negative bacteria.

An additional major finding in the present study was that the CaP+ diets had the potential to distinctly increase the number of several blood leukocytes such as dendritic cells, monocytes and neutrophils. Furthermore, results demonstrated that the CaP- diets resulted in a higher ratio of naive:antigen-experienced T helper cells caused by a lower number of antigen-experienced T helper cells in the blood indicating an impaired immunological memory functioning and probably a limited protection against pathogens. Blood lymphocyte

proliferation using the mitogen ConA and PWM were not affected by the dietary treatment, whereas the lymphocyte proliferation induced by ConA in jejunal mesenteric lymph nodes was greater for CFP diet, suggesting a promotion of the adaptive arm of the immune system. Thus, the dietary treatment had local and systemic modulatory impact. In accordance with the present study, Kegley, Spears, & Auman (2001) observed in weaned pigs no effect on PWM-induced proliferation with increasing dietary P supply (monocalcium-dicalcium phosphate). Similarly, in the present study the *in vitro*-proliferative response of lymphocytes to PWM was not influenced by the dietary treatment. Thus, it can be assumed that the greater number of blood antigen-experienced T helper cells in CaP+ fed pigs most probably did not result from a modulated reactivity of lymphocytes per se in these pigs, but probably from an increased number of innate immune cells potentially promoting the adaptive immune response. Correspondingly, CaP+ diets led to an increase in innate immune cells within the present study. Further studies are needed to elucidate, whether function of antigen-presenting cells, such as migration patterns as well as cytokine secretion profiles, are also affected by the dietary treatment and thus causing or mediating the observed modulations in the adaptive immune response.

Beside the effect of dietary CaP supply on the microbial ecosystem, the impact of type and amount of fermentable dietary carbohydrates on the intestinal microbiota has been observed in several studies (Varley, McCarney, Callan, & O'Doherty, 2010; Metzler-Zebeli, Zijlstra, Mosenthin, & Gänzle, 2011; Metzler-Zebeli et al., 2013). Dietary fibre provides the majority of carbohydrates that flow from the small to the large intestine where they are degraded by microbes (Bach Knudsen, Lærke, & Jørgensen, 2013). In the caecum and proximal colon, the microbial activity is high and almost all sugars, nondigestible oligosaccharides, residual starch, and soluble nonstarch polysaccharides are degraded (Bach Knudsen, Jensen, Andersen, & Hansen, 1991; Bach Knudsen, Jensen, & Hansen, 1993). Partly due to the structural organisation and cross linkage of the cell wall polysaccharides to lignin, the degradation of insoluble nonstarch polysaccharides is slower (Glitsø et al., 1999; de Vries et al., 2014). Based on table values (CVB, 2016; NRC, 2012), the concentration in corn, soybean meal, and field peas is 13.7; 16.7; and 15.5% for total dietary fibre (Wang, Hatcher, & Gawalko, 2008), 11.1, 27.3, and 16.6% for nonstarch polysaccharides, and 0.03, 3.81, and 0.59% galacto-oligosaccharides (sum of raffinose, stachyose and verbascose). Overall, the total dietary fibre content among the diets did not vary greatly (coefficient of variation 2.1 %). The main carbohydrates in field peas include starch (41.6 %; CVB, 2016) and non-starch

polysaccharides, whereas field peas are rich in slowly digestible or resistant starch (Jezierny et al., 2011), thereby possibly affecting selectively specific bacterial groups. In an *in vitro* study with colonic bacteria isolated from pigs (Jha, Bindelle, Van Kessel, & Leterme, 2011) using the gas technique to determine fermentation characteristics of different feed ingredients differing in carbohydrate and protein composition, field peas and field pea fibres increased bacterial protein synthesis. It needs to be taken into account that N incorporated into bacterial mass would not be immediately available for metabolite production such as ammonia. This suggests that increased bacterial N assimilation might be beneficial for host health (Mosenthin, Sauer, Henkel, Ahrens, & De Lange, 1992). Moreover, the experimental diets contained different amounts of corn, resulting in different amounts of carbohydrates, mainly resistant starch, available for microbial fermentation. In a study by Rist et al. (2014), an increased supply of corn starch may have supported starch escaping digestion in the small intestine to be subsequently utilized by the microbiota in the large intestine. Therefore, a shift from protein-fermenting to carbohydrate-fermenting bacterial groups occurs. These results are in agreement with our present study with higher caecal gene copy numbers of *Enterobacteriaceae* (data presented in Heyer et al. (2016)) as well as lower caecal gene copy numbers of *Bifidobacterium* spp. and colonic gene copy numbers of *Roseburia* spp. for pigs fed the CFP diets compared to CSB. Although our results showed no effect on SCFA concentration in the proximal gut sections, the bacterial shift might have modulated the bacterial metabolite production, resulting in a greater total SCFA in jejunal digesta for CSB fed pigs compared to CFP, potentially promoted by differences in available fermentable substrates such as nondigestible oligosaccharides and nonstarch polysaccharides. It can be concluded that regardless of Ca and P concentration variations in fermentable substrates might have a modulating impact on the intestinal microbiota, thereby influencing the host immune system (Grenham et al., 2011).

2.4.6 Conclusions

The present study demonstrated that CaP and fermentable substrates have a distinct effect on the peripheral and gut-associated immune system, as well as microbial composition and activity in pigs. In particular, the inorganic CaP concentration seems to affect the outcome of innate and adaptive immune response, alter the composition of the caecal microbiota, especially on saccharolytic bacteria that might be beneficial for animal’s health. In agreement with the hypothesis of the study, it seems to be that a minimum concentration of available P

is needed to ensure a normal immune functioning as well as a stable microbial ecosystem to promote animal's health.

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2.4.8 Supporting information

Supplemental Table 2.4- 1 Oligonucleotide primers used for quantitative real-time PCR

Target bacterial group	Primer sequences (5′–3′)	Amplicon length (bp)	Annealing temperature (°C)	Reference
Total eubacteria	**F:** GTG STG CAY GGY YGT CGT CA	147	52	Fuller et al., 2007
	R: ACG TCR TCC MCN CCT TCC TC			
Lactobacillus spp.	**F:** AGA GGT AGT AAC TGG CCT TTA	391	59	Malinen et al., 2003
	R: GCG GAA ACC TCC CAA CA			
Bifidobacterium spp.	**F:** TCG CGT CYG GTG TGA AAG	243	59	Rinttilä et al., 2004
	R: CCA CAT CCA GCR TCC AC			
Roseburia spp.	**F:** AGG CGG TAC GGC AAG TCT	353	59	Rinttilä et al., 2004; Veiga et al., 2010
	R: AGT TTY ATT CTT GCG AAC G			
Clostridium cluster IV	**F:** GGC GGC YTR CTG GGC TTT	147	65	Ramirez-Farias et al., 2009; Lay et al., 2005
	R: CCA GGT GGA TWA CTT ATT GTG TTA A			
Clostridium cluster XIVa	**F:** CGG TAC CTG ACT AAG AAG C	429	63	Rinttilä et al., 2004
	R: AGT TTY ATT CTT GCG AAC G			
Eubacterium rectale	**F:** AAG GGA AGC AAA GCT GTG AA	200	65	Balamurugan et al., 2008
	R: TCG GTT AGG TCA CTG GCT TC			

Supplemental Table 2.4- 2 16S rRNA gene clone library analyzed with T-RFLP and putative identity aligned to closest relative in RDP database

genebank accession number	TRF	clone name	putative identity	similarity score	order	family
KU705900	91	HomPi_107	*Clostridium* sensu stricto	0.92	Clostridiales	Clostridiaceae 1
KU705883	91	HomPi_127	*Clostridium* cluster XI	0.94	Clostridiales	Peptostreptococcaceae
KU705885	95	HomPi_129	*Prevotella*	0.98	Bacteroidales	Prevotellaceae
KU705878	96	HomPi_92	*Clostridium* sensu stricto	0.99	Clostridiales	Clostridiaceae 1
KU705897	97	HomPi_162	*Clostridium* sensu stricto	1.00	Clostridiales	Clostridiaceae 1
KU705887	131	HomPi_135	unclassified_Ruminococcaceae	0.90	Clostridiales	Ruminococcaceae
KU705875	155	HomPi_84	*Clostridium* cluster XI	0.98	Clostridiales	Peptostreptococcaceae
KU705894	192	HomPi_157	*Clostridium* sensu stricto	0.93	Clostridiales	Clostridiaceae 1
KU705876	193	HomPi_86	*Clostridium* cluster XI	0.99	Clostridiales	Peptostreptococcaceae
KU705881	194	HomPi_105	*Clostridium* sensu stricto	0.98	Clostridiales	Clostridiaceae 1
KU705884	212	HomPi_128	unclassified_Clostridiales	0.98	Clostridiales	unclassified Clostridiales
KU705877	281	HomPi_88	*Clostridium* cluster XI	0.94	Clostridiales	Peptostreptococcaceae
KU705879	283	HomPi_96	*Clostridium* sensu stricto	0.92	Clostridiales	Clostridiaceae 1
KU705896	284	HomPi_160	unclassified_Ruminococcaceae	0.97	Clostridiales	Ruminococcaceae
KU705893	295	HomPi_153	*Clostridium* sensu stricto	0.94	Clostridiales	Clostridiaceae 1
KU705890	297	HomPi_147	unclassified "Porphyromonadaceae"	0.98	Bacteroidales	Porphyromonadaceae
KU705882	308	HomPi_125	unclassified Erysipelotrichaceae	0.96	Erysipelotrichales	Erysipelotrichaceae
KU705874	455	HomPi_74	unclassified Clostridiales	0.95	Clostridiales	unclassified Clostridiales
KU705899	458	HomPi_190	*Clostridium* cluster XI	0.99	Clostridiales	Peptostreptococcaceae
KU705889	466	HomPi_143	*Clostridium* sensu stricto	0.94	Clostridiales	Clostridiaceae 1
KU705880	469	HomPi_97	*Clostridium* sensu stricto	0.94	Clostridiales	Clostridiaceae 1
KU705886	472	HomPi_130	*Clostridium* sensu stricto	0.97	Clostridiales	Clostridiaceae 1
KU705895	519	HomPi_158	*Clostridium* cluster XI	0.91	Clostridiales	Peptostreptococcaceae
KU705892	520	HomPi_152	*Clostridium* sensu stricto	0.99	Clostridiales	Clostridiaceae 1
KU705891	521	HomPi_148	unclassified "Porphyromonadaceae"	0.98	Bacteroidales	Porphyromonadaceae
KU705888	522	HomPi_136	*Oscillibacter*	0.99	Clostridiales	Ruminococcaceae
KU705898	523	HomPi_181	*Clostridium* sensu stricto	1.00	Clostridiales	Clostridiaceae 1

Supplemental Table 2.4- 3 Numbers of T cell populations (number/g tissue × 10^6) in jejunal mesenteric lymph nodes and *in vitro* proliferation of mesenteric lymph node cells using the mitogens Concanavalin A and Pokeweed mitogen

	CSB		CFP			p-value		
Item	CaP-	CaP+	CaP-	CaP+	Pooled SEM	Protein	CaP	CaP × Protein
Leukocytes‡	160	152	147	114	30	0.324	0.401	0.580
T cells‡	91	66	86	70	21	0.976	0.186	0.760
T helper cells‡	58	59	59	44	11	0.181	0.192	0.346
naive T helper cells	28	20	26	21	6	0.968	0.121	0.717
antigen-experienced T helper cells‡	32	35	34	22	7	0.174	0.213	0.081
Cytotoxic T cells‡	32	35	34	22	7	0.535	0.120	0.488
γδ T cell‡	13	12	13	11	3	0.958	0.407	0.982
naive:antigen-experienced T helper cell ratio‡	0.7	0.4	0.7	0.7	0.1	0.156	0.150	0.054
Lymphocyte proliferation†								
Concanavalin A‡	13,249^{A}	15,005^{A}	12,335^{B}	9,444^{B}	3,909	0.035	0.614	0.102
Pokeweed mitogen	14,404	12,784	14,258	11,446	4,797	0.698	0.248	0.756

CSB, corn-soybean meal; CFP, corn-field pea; SEM, Standard error of the mean.
Adjusted mean estimates based on model (1); estimates are based on 7-8 observations per diet.
Within a row, means of protein that do not share a common upper case superscript letter differ ($p < 0.05$); within a row, means of CaP concentration that do not share a common lower case superscript letter differ ($p < 0.05$). Mean estimates of protein within each level of the factor CaP that do not share a common upper case superscript letter differ significantly ($p < 0.05$); mean estimates of CaP concentration within each source of the factor protein that do not share a common lower case superscript letter differ significantly ($p < 0.05$).
†The Δ counts per minute (cpm) for Concanavalin A and Pokeweed mitogen were determined for each individual (Δ cpm = stimulated (cpm) - unstimulated cells (cpm)).
‡Adjusted mean estimates from models with transformed response variable are reported on the original scale after back transformation.

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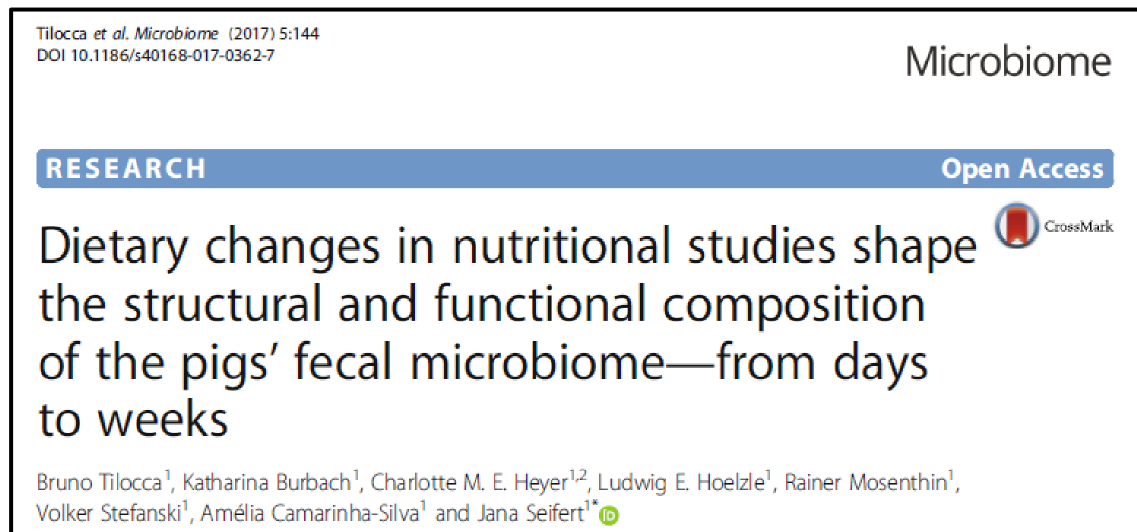
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Dietary changes in nutritional studies shape the structural and functional composition of the pigs' fecal microbiome—from days to weeks

Bruno Tilocca[1], Katharina Burbach[1], Charlotte M. E. Heyer[1,2], Ludwig E. Hoelzle[1], Rainer Mosenthin[1], Volker Stefanski[1], Amélia Camarinha-Silva[1] and Jana Seifert[1*]

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Dietary changes in nutritional studies shape the structural and functional composition of the pigs' fecal microbiome – from days to weeks

2.5.1 Abstract

Background: The possible impact of changes in diet composition on the intestinal microbiome is mostly studied after some days of adaptation to the diet of interest. The question arises if a few days are enough to reflect the microbial response to the diet by changing the community composition and function. The present study investigated the fecal microbiome of pigs during a time span of 4 weeks after a dietary change to obtain insights regarding the time required for adaptation. Four different diets were used differing in either protein source (field peas meal vs. soybean meal) or the concentration of calcium and phosphorus (CaP).

Results: Twelve pigs were sampled at seven time points within 4 weeks after the dietary change. Fecal samples were used to sequence the 16S rRNA gene amplicons to analyse microbial proteins via LC-MS/MS and to determine the SCFA production. The analysis of OTU abundances and quantification values of proteins showed a significant separation of three periods of time ($p = 0.001$). Samples from the first day are used to define the 'Zero period'; samples of weeks 1 and 2 are combined as 'metabolic period' and an 'equilibrium period was defined based on samples from weeks 3 and 4. Only in this last period, a separation according to the supplementation of CaP was significantly detectable ($p = 0.001$). No changes were found based on the corn-soybean meal or corn-field peas administration. The analysis of possible factors causing this significant separation showed only an overall change of bacterial members and functional properties. The metaproteomic approach yielded a total of about 9700 proteins, which were used to deduce possible metabolic functions of the bacterial community.

Conclusions: A gradual taxonomic and functional rearrangement of the bacterial community has been depicted after a change of diet composition. The adaptation lasts several weeks despite the usually assumed time span of several days. The obtained knowledge is of a great importance for the design of future nutritional studies. Moreover, considering the high similarities between the porcine and human gastrointestinal tract anatomy and physiology, the findings of the current study might imply in the design of human-related nutritional studies.

2.5.2 Background

The intestinal microbiota is involved in a variety of physiological processes of primary importance for the host metabolism and growth, such as nutrient absorption, metabolism and utilisation [1, 2]. Other vital processes including host immune-modulation and prevention from metabolic and neoplastic diseases have often been related to the intestinal microbiota composition and activity [3].

Due to these important implications, several investigations of the microbiota are nowadays carried out on humans and other animal models, in the attempt to elucidate the onset mechanisms of impactful pathological conditions such as obesity, inflammatory bowel disease, diarrhea, necrotizing enterocolitis and many others [3, 4]. In the recent years, animal scientists started to perform in-depth microbiota investigations for the optimisation of the animal husbandry strategies as well as the improvement of animal's health status [2, 5].

Diet represents one of the major environmental factors shaping the intestinal microbiota. Here, a varying ratio of carbohydrates and proteins or a change of the source of these basal feed components were important key factors [6]. Besides these main feed components, minerals and trace elements are known to influence the intestinal microbiota [7]. Due to the intrinsic incapability for an autonomous phosphorus (P) uptake, standard pig diet contains a supplemental level of calcium and phosphorus (CaP) [8]. This results in a higher excretion of the respective minerals, contributing to the environmental problem of water eutrophication, and besides, being responsible for a useless raise of husbandry costs and a waste of valuable P resources [9, 10]. These reasons pose the need to reduce the P excretion by reducing the dietary CaP-supplementation. However, changes in the diet formulation may be associated with the alteration of microbiota composition and activity due to the needs to fulfil nutritional requirements or by the alteration of the physicochemical condition of the gut lumen resulting in an awkward milieu for microbial colonisation and growth [9, 11, 12]. Studies performed in rats which were kept on a diet with high CaP levels indicated an increased amount of beneficial lactobacilli and an increased resistance to intestinal pathogen colonisation [13]. These results are in agreement with other studies performed at the luminal [9, 11] and mucosal [14] level of the pig's gastrointestinal tract.

Although various studies investigated the dietary-induced modulation of the pigs intestinal microbiota composition and activity, it is still being discussed how and how long the microbiota adaptation process lasts. This fact is of great importance for nutritional studies

including the translational research for human health. Pigs and rodents are the foremost microbiota models for translational studies into the human field [15]. Pigs resemble humans more than rodents in terms of dietary regimen, gastrointestinal tract anatomy, physiology and nutrient digestibility [16, 17]. Moreover, similar to the human intestinal microbiota, the intestinal microbiota of pigs is primarily composed of the phyla of Firmicutes and Bacteroidetes with a varying composition depending on the concerned section along the gastrointestinal tract [2].

So far, to the best of our knowledge, no studies were performed to investigate the progressive adaptation of the pigs' intestinal microbiota challenged by feeding different experimental diets. Therefore, it was our objective to investigate the expected gradual adaptation of the fecal microbiota over an experimental period of 4 weeks. 16S rRNA gene sequencing along with a metaproteomic approach were employed to provide an exhaustive description of the structural and functional changes of the intestinal microbiota triggered by the experimental treatments. The experimental diets fed varied in the composition of the protein source and the amount of supplemented CaP. The results of this study provide novel insights into the structural and functional changes during the adaptation periods and show that weeks rather than days are required to observe a significant change in the microbial community composition and function.

2.5.3 Methods

Animal experiment and experimental diets

Twelve pigs (German Landrace x Piétrain, initial body weight 54.7 kg ± 4.1 kg) were randomly assigned to four experimental diets. The diets were formulated to meet or exceed the animal's nutrient requirements and differed among each other in the protein source and the CaP levels. Two out of four diets contained low digestible (LD) corn-field pea meal as a protein source whereas the remaining two diets comprised highly digestible (HD) corn-soybean meal as a protein source. Each of these dietary groups was further supplied with high and low CaP levels. Diets with high and low CaP levels were formulated to contain 120 and 66% of the requirements for 50–75 kg pigs (NRC, 2012). In all diets, the Ca:P ratio was kept at 2:1 constantly. Gross energy content of the corn-field peas-based diets was 18.83 MJ/kg whereas the energetic content of the corn-soybean based diets was 19.60 MJ/kg. Further details on the animal experiments and experimental diets are provided in [18].

All animals were initially fed with a conventional diet until week 12 of age. At week 13 of age, the animals were randomly assigned to the four experimental diets (three animals per diet) which were fed until week 20 of age. The pigs were individually allocated in pens, and fecal samples were collected constantly, before and during the experimental treatments. Eighty-four fecal samples across the whole experimental time span were selected for the investigation of the fecal microbiota adaptation to the experimental diets. Samples were collected on ice and immediately stored at − 80 °C until subsequent analysis. Details on the experimental trial and sampling time are reported in Table 2.5-1.

Table 2.5- 1 Fecal samples were collected seven times across an experimental time span of 4 weeks and independently subjected to both 16S rRNA gene sequencing and metaproteomic investigation (X).

Animal number	Diet		Experimental period						
		week	0	1			2	3	4
		day	3	8	10	12	17	25	32
01	R4		X	X	X	X	X	X	X
03	R3		X	X	X	X*	X	X	X
04	R1		X	X	X	X	X	X	X
05	R4		X	X	X	X	X	X	X
06	R2		X	X	X	X	X	X	X
07	R1		X	X	X	X	X*	X	X
08	R4		X	X	X	X	X	X	X
11	R2		X	X	X	X	X	X	X
12	R3		X*	X	X	X	X	X	X
13	R1		X	X	X	X	X	X	X
15	R2		X	X	X	X	X	X	X
16	R3		X	X	X	X	X	X	X

Failed DNA analyses are indicated by X*. R1 corn-soybean, high-digestible (HD), high CaP; R2 corn-soybean, HD, low CaP; R3 corn-field pea, low digestible (LD), high CaP; R4 corn-field pea, LD, low CaP.

DNA extraction and amplicon sequencing

In accordance with a previous study [19], the FastDNA™ SPIN Kit for Soil (MP Biomedicals, Heidelberg, Germany) was used for DNA extraction by following the manufacturer's instruction with slight modifications. Briefly, 250 mg of feces were added to a Lysing Matrix E tube supplied with the provided buffers. Bead beating was performed twice in a Fast Prep®-24 Instrument (6 m/s, 40 s). Cell lysates were separated by centrifugation (14,000×g, 15 min) and proteins were precipitated from the supernatant. DNA was bound to a silica matrix on a spin filter and eluted with 55 °C pre-warmed DES water. DNA extracts were quantified in a NanoDrop instrument (Thermo Fisher Scientific, Waltham, MA).

The V1-V2 region of the 16S rRNA gene was amplified once for each sample and sequenced as previously described [20]. The primer pair 27F-338R was used to amplify the target region, with a slightly modified sequence of the primer 27F (AGRGTTHGATYMTGGCTCAG). Obtained amplicons were verified by agarose gel

electrophoresis, purified and normalised with SequalPrep™ Normalization Plate kit (Invitrogen, Carlsbad, CA, USA). Libraries were pooled by index, purified with MinElute PCR Purification Kit (Qiagen, Hilden, Germany) and quantified by using QuantiFluor® dsDNA System (Promega, Madison, USA). Amplicons were sequenced on an Illumina MiSeq in paired-end mode (2 × 250 base pairs). Sequence reads were quality filtered and assembled using Mothur software package [21]. Sequences were quality filtered by excluding reads that had an average quality score lower than 20, a total length of more than 355 base pairs (bp), any primer or barcode mismatch, more than eight homopolymer stretches or an N character. Reads were checked for chimeras and were clustered into operational taxonomical units (OTUs) at 97% identity [22]. OTUs appearing only once across the samples as well as those with less than 10 reads each were manually deleted. The remaining OTUs were finally assigned to the closest taxonomical representative using seqmatch from RDP [22].

OTU abundances were subjected to statistical investigation using Primer6 v.6 statistical software (PRIMER-E, Plymouth, UK) [23]. Prior to statistical analysis, the amplicon sequencing data was standardised by abundances of all sequences, square root transformed and the principal coordinate analysis (PCoA) was calculated on the basis of the Bray-Curtis dissimilarity matrix. A Good's coverage index greater than 98% indicated sufficient sampling of our data and adequate depth. Statistical differences across time points over the experimental time frame and between diets were calculated by performing ANOVA with permutations (PERMANOVA).

Sample preparation for LC-MS/MS analyses

Procedures for sample preparation, including protein extraction and the in-gel digestion of the proteins were performed as previously described in Tilocca *et al*. [12].

Tryptic peptides were purified and desalted by using self-assembled C18 Stage Tips [24]. Tips containing the C18 membranes with the bounded peptide mixture were stored at − 20 °C and resuspended in 5% acetonitrile (5% ACN/ 0.1% TFA) prior to the LC-MS/MS measurements.

LC-MS/MS analysis

A volume of 1.5 μL of the resuspended peptides mixture was measured by using a Q-Exactive HF mass spectrometer (Thermo Fisher Scientific, Darmstadt, Germany) faced with an EasyLC 1000 nano-UHPLC (Thermo Fisher Scientific, Darmstadt, Germany) as described previously [25]. Separation of peptides was performed on a 20-cm fused silica column of 75-

μm inner diameter (Proxeon Bio-systems). The column has been *in-house* packed with reversed-phase ReproSil-Pur 120 C18-AQ 1.9 μm resin (Dr. Maisch GmbH, Ammerbuch, Germany). Peptides were loaded onto the column in solvent A (0.1% formic acid) at a flow rate of 500 nl/min and subsequently eluted with an 87-min segmented gradient of 10–50% HPLC solvent B (80% ACN in 0.1% formic acid).

The MS/MS instrument was set to positive ion mode. Full scans were acquired in the mass range from m/z 300 to 1650 in the Orbitrap mass analyser at a resolution of 120,000 followed by HCD fragmentation of the 12 most intense precursor ions. High-resolution MS/MS spectra were acquired with a resolution of 30,000. The target values were 3 * 106 charges for the MS scans and 1 * 105 charges for the MS/MS scans with a maximum fill time of 25 and 45 ms, respectively. Fragmented masses were excluded for 30 s after MS/MS. Spectra de-noising was performed prior to peptides identification by considering only the top 12 peaks in a window of 100 Da width.

Bioinformatics analysis of protein data

Out of the total LC-MS/MS raw data inventory, a restricted number of samples were selected for a preliminary investigation of the bacterial protein composition. Selected samples were representative of the potential variability induced by the experimental treatments and the potential variability across the experimental time frame.

Sorted raw data were processed through Thermo Proteome Discoverer software (v.1.4.1.14) and searched against NCBInr bacteria database (release 19 October 2015) in order to evaluate the overall taxonomic composition and to export a consensus fasta database. Methionine oxidation was set as variable modification and carbamidomethylation of cysteine as fixed modification. The Mascot significance threshold was set to 0.05, and a filter considering only entries with at least one peptide per protein was chosen. All other filters and settings of the software were kept as default, including protein grouping with peptide confidence set on “high” and delta Cn of 0.1. The Percolator node supporting a strict maximum parsimony principle was activated with a false discovery rate of 1%.

The consensus protein fasta database obtained from the previous Proteome Discoverer processing of the raw files was employed as an *in-house* database (14,535 entries) for a second search performed on the MaxQuant software. The use of a custom database for processing the whole raw data inventory maximises the protein identification rate and reduces the false discovery rate by including only protein entries that exclusively belong to the bacterial specimen of our interest [26]. Additionally, an independent database-dependent search of all raw files was performed against UniProtKB database (release March 2016) Sus

scrofa (UniProt ID 9823; 61,019 entries). MaxQuant software (v.1.5.3.8), set on LFQ modality, was used for peptide identification and protein inference. Cysteine carbamidomethylation was set as fixed modification and methionine oxidation as variable modification. Two missed cleavage sites were allowed for in silico protease digestion and peptides had to be fully tryptic. All other parameters of the software were set as default, including a peptide and protein FDR < 1%, at least 1 peptide per protein, precursor mass tolerance of 4.5 ppm after mass recalibration and a fragment ion mass tolerance of 20 ppm. Taxonomic information was inferred according to the protein description obtained from the MaxQuant search results. These in turn were gathered from the protein annotation of the chosen database (*i.e.* NCBInr). Identified proteins were functionally classified into COG and KEGG categories via WebMGA [27] with an e-value cut-off of 10−3 considering exclusively the best hits. Qualitative evaluation of the resulting DNA-based and metaproteomic datasets have been performed by sorting the OTUs and protein accession numbers into the respective adaptation period. Comparisons between adaptation periods were performed for each dataset and presented as Venn diagrams using the Venny online tool. Protein abundance indexes of the identified proteins (LFQ values) were subjected to statistical investigation through the use of Primer6 v.6 statistical software (PRIMER-E, Plymouth, UK) [23]. Principal coordinate analysis (PCoA) was calculated on the basis of the Bray-Curtis dissimilarity matrix which in turn was calculated on the square root transform of the protein LFQs [28]. Statistical differences across time points over the experimental time frame and between diets were calculated by performing a PERMANOVA. Similarity percentage analysis (SIMPER) was also performed in order to isolate proteins driving dissimilarities between adaptation periods [29]. Heat maps visualising microbial community composition across the adaptation periods and functional classification of the identified proteins were drawn using heatmap.2 provided by the gplots package [30] implemented in R v.3.1.2 software (http://www.R-project.org).

Analysis of microbial metabolites

Short-chain fatty acids (SCFAs) were analysed by direct measurements of feces. Samples were prepared as previously described [31] followed by gas chromatography (GC) with flame ionization detector (HP 6890 Plus; Agilent, Waldbronn, Germany) measurements using fatty acids (GC grade; Fluka, Taufkirchen, Germany) as internal standards [32]. A capillary column (HP 19091F-112, 25 m × 0.32 mm × 0.5 μm) was used with the following oven program: 80 °C, 1 min; 155 °C in 20 °C/min; 230 °C in 50 °C/min., constant for 3.5 min to separate the metabolites and helium as carrier gas. Concentration of the major SCFA (*i.e.* acetate, propionate and butyrate) was registered as referred to kilogram feces.

Quantitative evaluation of these metabolites was also inferred via investigation of proteins that are commonly recognised as being related to SCFA biosynthesis [33]. Here, abundances of proteins related to major SCFA were cumulatively considered to provide a quantitative estimation for each metabolite.

Results from direct and inferred estimation were standardised and subjected to Spearman correlation analysis by using the corrplot package of R v.3.1.2 software (http://www.R-project.org).

2.5.4 Results

16S rRNA gene sequencing and metaproteome analysis revealed three adaptation periods

Sequencing of the V1-V2 region of the 16S rRNA gene produced 4.8 million reads (57,916 ± 2139 reads per sample). A Good's coverage index greater than 98% showed sufficient sampling of our data and adequate depth. Reads were filtered and trimmed before being clustered into 3497 operative taxonomical units (OTUs) (Additional file 1: Table 2.5-S1).

Adopted protocols for the metaproteomic investigation enabled a total of 9703 and 38,239 bacterial protein and peptide identification, respectively. Insights into the protein and peptide profile of each sample, as well as the respective abundance indexes for each of the identified entries are provided in Additional file 1: Table 2.5-S2.

Both datasets, based on DNA and metaproteomic investigation, were depicted in a PCoA plot on a sample basis (Figure 2.5-1). The samples ordination revealed a highly comparable clustering on

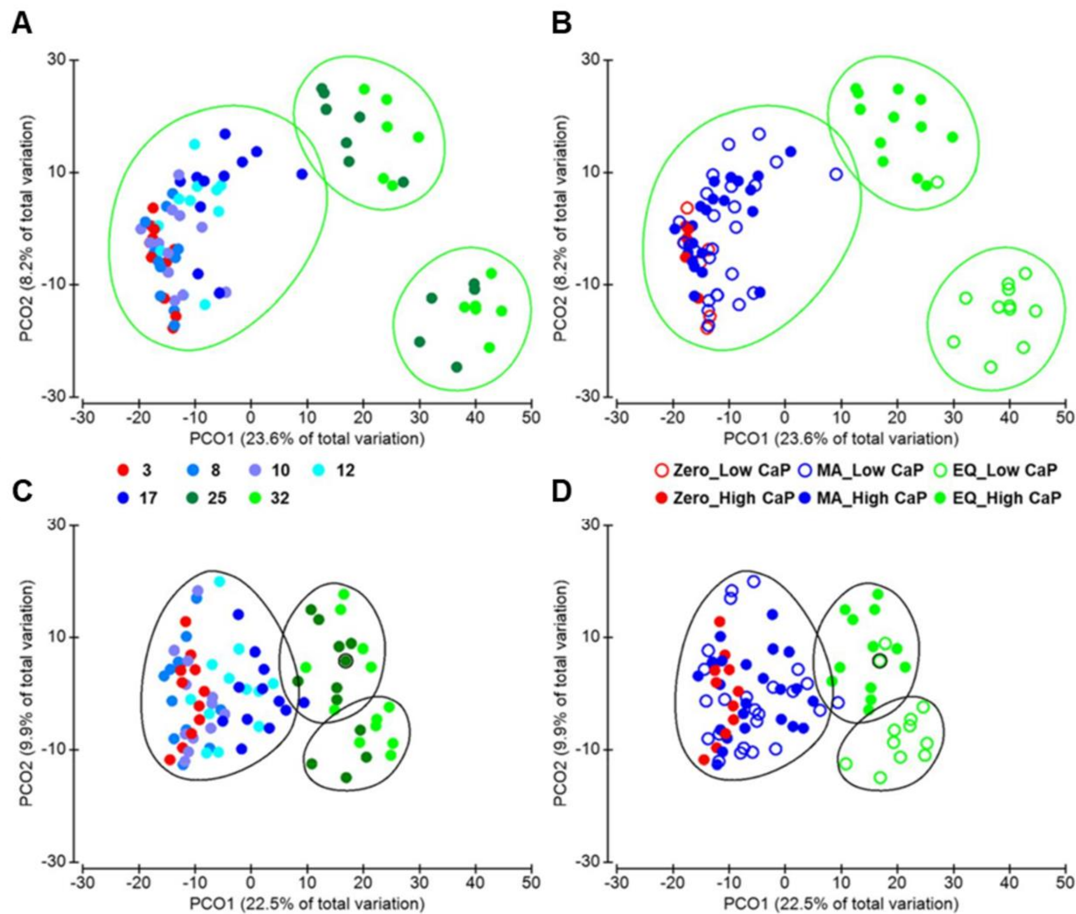

Figure 2.5-1 Samples ordination reveals three adaptation periods. a and b panels illustrate the ordination of the dataset obtained from the 16S rRNA gene sequencing approach. c and d panels show the metaproteomic dataset ordination. Datasets from both approaches are ordered on a sample basis. The time-dependent aggregation into three clusters is shown in panels a and c. Panel b and d include information in respect to the experimental diets. Similarity analysis showed 40% similarity in the sequencing dataset (green clusters) and 60% similarity in the metaproteomics dataset (grey clusters).

a time point dependent manner along the PCO1 axis (Figure 2.5-1A, C). Samples grouped into three clusters over the experimental time span ($p = 0.001$ for both datasets) suggesting that the adaptation process of the intestinal microbiota evolved throughout three main adaptation periods: Zero (*i.e.* the phase prior to the experimental diet administration), metabolic adaptation (MA, adaptation period to the challenging diets) and equilibrium (EQ, last experimental period, where a new suited microbiota is established). The equilibrium achieved in the bacterial community at the EQ period is also supported by the PCO2 ordination where only EQ samples are further clustered according to the CaP supplementation of the experimental diets ($p = 0.001$) (Figure 2.5-1B, D, Additional file 2: Figure 2.5-S1C, D). Identified OTUs were sorted according to the three adaption periods. This prior qualitative evaluation indicates the presence of a period-specific architecture of the micro-biota featured by a gradual adaptation of the microbial communities, as suggested by the lower number of shared OTUs between the Zero-EQ periods when compared to the Zero-MA and MA-EQ

pairs (Additional file 2: Figure 2.5-S1A). Similarly, sorting of the protein dataset revealed that a variable number of proteins was uniquely identified in each of the three adaptation periods (1521, 595, 1927, respectively, for Zero, MA and EQ period) whereas an equal number of proteins is shared between the MA-Zero and MA-EQ periods. Only 13 proteins are shared between the Zero and EQ periods supporting the achievement of a new homeostatic balance (Additional file 2: Figure 2.5-S1B).

Taxonomic distribution based on 16S rRNA gene sequencing and metaproteomics

OTUs with more than 10 associated reads appearing in more than one sample were selected to investigate the structure of the fecal microbiota. Taxonomic distribution based on the whole metaproteomic dataset did not provide noticeable shifts among the adaptation periods (Additional file 2: Figure 2.5-S2), probably because of the presence of highly abundant housekeeping proteins. Thus, we focused on the unique proteins of each adaptation period to highlight the dynamics featuring the taxonomic composition of the fecal microbiota across the adaptation periods.

The DNA-based investigation revealed a dynamic composition of the fecal microbiota over the experimental time frame (Figure 2.5-2) featured by an increased abundance of the *Clostridiaceae* and *Prevotellaceae* families in the EQ period (29.2 ± 2.21% and 8.9 ± 1.22%). The *Peptostreptococcaceae* increased in abundance with the administration of the experimental diets, showing a higher abundance in the MA (11.9 ± 0.74%) and EQ. (12.2 ± 1.42%) periods. Similarly, *Bifidobacteriaceae* showed a time-dependent increase in abundance. At the Zero period, the abundance of *Bifidobacteriaceae* sequences were 0.1% ± 0.06, whereas in the EQ period this family showed an abundance of 4.3% ± 1.07. Contrarily, the gut microbiota re-structuration triggered by the challenging diets showed a gradual decrease of the family Lactobacillaceae from 22.4 ± 1.76% of abundance observed in Zero period to 3 ± 0.52% in the EQ period (Figure 2.5-2).

The metaproteomic investigation confirmed the restructuring of the fecal microbiota architecture on a time point dependent manner. Compared to the DNA-based approach, a higher bacterial heterogeneity is shown in the description of the active bacterial community. The abundance of proteins affiliated to *Clostridiaceae* and *Bifidobacteriaceae* family reflects the observed results at the 16S rRNA gene level. Proteins of *Clostridiaceae* (10.5 ± 1.0% at the Zero period to 18.6 ± 1.2% of the total protein abundance scored in the EQ period) and *Bifidobacteriaceae* (0, 2.4 ± 0.03% and 10.2 ± 0.7% in Zero, MA and EQ periods respectively) increased gradually within time (Figure 2.5-2).

Proteins affiliated to *Erysipelotrichaceae* indicate a reduced abundance in the EQ period (respectively 1.6 ± 0.6% and 0.8 ± 0.01% of protein abundance in the Zero and EQ period). None of the proteins related to *Peptostreptococcaceae* family passed the filters and thresholds applied to the dataset, leading to its exclusion from the taxonomic assessment of the active bacterial community.

Proteins associated with *Bacteroidaceae* strongly increase in abundance with time. The reverse trend is shown for *Lachnospiraceae* and *Veillonellaceae* members, whose protein abundances are firmly reduced in the EQ period. Similarly, the abundance of proteins affiliated to *Burkholderiaceae* is progressively reduced during the MA period until the EQ, where it was not detectable with the investigation method (Figure 2.5-2).

The abundance of proteins related to *Prevotellaceae* members collapse during the MA period and are restored in the EQ period, suggesting Prevotellaceae as a bacterial family involved in important housekeeping functions carried out in both homeostatic balances (Zero and EQ). The opposite effect was observed for Lactobacillaceae, Spirochaetaceae and Acidaminococcaceae members, whose protein repertoire is highly pronounced in the MA period, indicating these families are potentially involved in driving the shift from the Zero to the EQ microbiota (Figure 2.5-2).

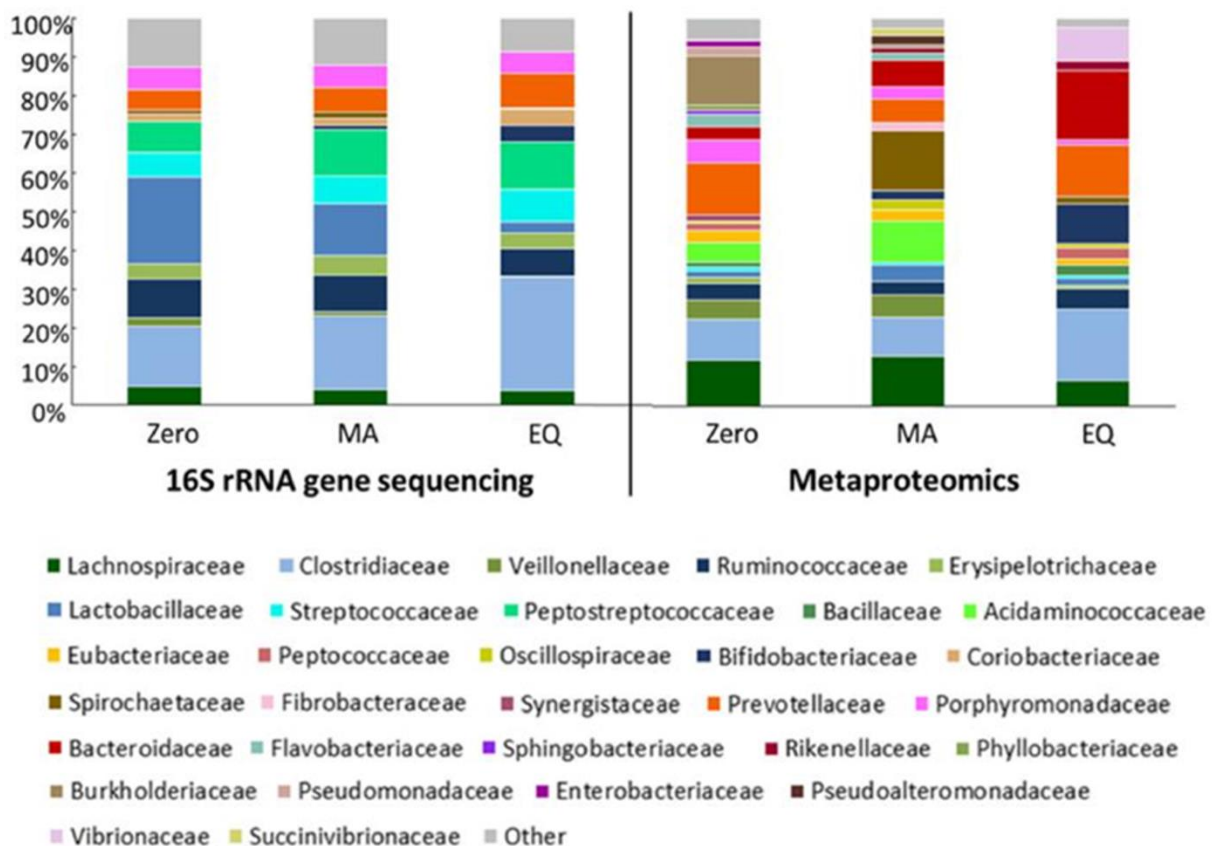

Figure 2.5-2 Gut microbiota composition changes in a time point-dependent manner.

Figure 2.5-2 Gut microbiota composition changes in a time point-dependent manner. Bar chart displays the relative abundance of the bacterial families as assessed by 16S rRNA gene sequencing and label-free quantification metaproteomics. Both methods display a dynamic taxonomic composition among the adaptation periods. A higher taxonomic variability is visualized in the metaproteomic-based assessment when compared to the DNA-based approach.

Functional adaptation of the intestinal microbiota

Out of the total protein repertoire, the “unique proteins” for each of the three adaptation periods (Additional file 2: Figure 2.5-S1B), were considered for a functional categorization in order to investigate the overtime functional shift of the fecal microbiota. Here, shared proteins were excluded since they are most likely involved in the highly conserved house-keeping functions taking place in all the adaptation periods. Moreover, shared proteins account for most of the total protein abundance (Additional file 2: Figure 2.5-S3), therefore, their consideration hinders a clear visualisation of the hypothesised gradual functional shift of the microbiota (Additional file 2: Figure 2.5-S4), as also supported by statistical results (*i.e.* three adaptation periods, $p = 0.001$).

The LFQ values of the sorted proteins in each adaptation period were compared to each other to identify the major proteins responsible for the observed statistical differences. Only proteins scoring at least 5-fold changes between adaptation periods were considered for a further functional classification into KEGG biochemical pathways. Global representation of the screened proteins according to their LFQ ratio, as well as their functional categorization into KEGG biochemical pathways is provided in Additional file 2: Figures 2.5-S5 and 2.5-S6. Functional profiles drawn for the three adaptation periods clearly show a dynamic change of the microbiota, as supported by the fluctuating expression levels of diverse pathways among the adaptation periods as well as the emergence of new, other paths in the MA and EQ periods (Additional file 2: Figure 2.5-S6).

The heat map shown in Figure 2.5-3 summarises the biochemical pathways with the highest variability in abundance between the adaptation periods including along with the bacterial families that contributed to their expression.

The Clostridiaceae family, whose abundance increases in the EQ period, showed a concomitant increase in abundance in some of the selected pathways such as the pentose and glucuronate interconversion pathway (ko00040), and the glyoxylate and dicarboxylate metabolism (ko00630). Proteins of Bifidobacteriaceae members showed only low to medium abundance in the aminoacyl-t-RNA biosynthesis (ko00970) and the pentose phosphate pathway (ko00030) once achieving the new equilibrium (EQ period). This evidence suggests that the gradual increase of the Bifidobacteriaceae registered in the metaproteomic-based phylogenetic taxonomic assessment reflected a bacterial activity concerned in other aspects of

the functional adaptation of the gut bacterial community. The reduced abundance of the family of Lachnospiraceae is functionally reflected by its sudden drop in the galactose metabolism (ko00052) and glycerolipid metabolism (ko00561). Similarly, the reduced abundance of Veillonellaceae results in a decreased abundance in the glycerolipid metabolism and pentose phosphate pathway. The increased abundance of Lactobacillaceae members in the MA period is accordingly related to a boosted number of proteins in the pentose, glucuronate interconversion and pentose phosphate pathway (Figures 2.5-3 and -4).

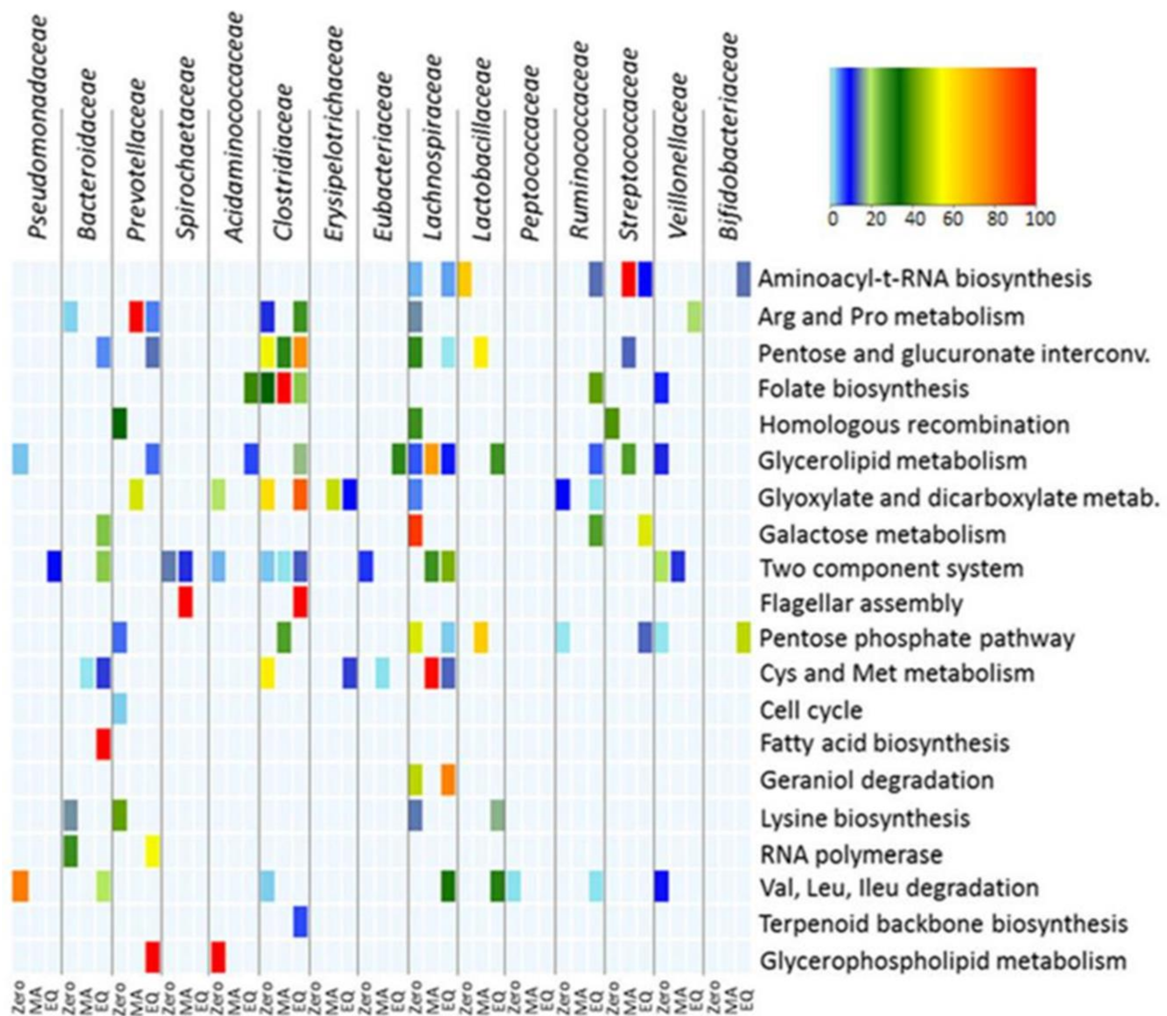

Figure 2.5-3 Microbiota members are involved in a variety of diverse biochemical pathways. The heat map shows the contribution of the top 10 most abundant bacterial families in the selected pathways in each adaptation period. Bacterial families exhibit specific involvement in the biochemical pathways, with a contribution that changes in dependence of the considered bacterial family and adaptation period.

A further focus on the carbohydrate metabolic pathways and the related bacterial families is summarised in Figure 2.5-4 and Additional file 2: Figure 2.5-S6. In accordance with the previous evidence, a diverse fraction of the bacterial community is concerned in the

carbohydrate metabolism in each of the adaptation periods. Furthermore, a detailed investigation reveals quantitative differences in the portion of the major biochemical pathways for each of the adaptation periods, underlining a different impact of every adaptation period on the selected carbohydrate pathways. In the pentose and glucuronate interconversion KEGG pathway (Figure 2.5-4A), proteins of the Zero samples showed high abundances throughout the whole path, highlighting that Zero bacterial community is mainly focused on facing complex substrates and improving the carbon and energy uptake. Proteins affiliated to glycerolipid metabolism indicate Zero and MA samples as being concerned in the biosynthesis of triglycerides and glycerolipids. A similar functional profile was also identified in EQ samples along with their strong implication in using glycerol as a carbon and energetic source (Figure 2.5-4B).

Investigation of proteins of the pentose phosphate pathway highlighted that diverse bacterial proteins are involved in common functions (Figure 2.5-4C). From a quantitative point of view, the identified protein repertoire showed a higher efficiency of the EQ-related bacterial community in the production of intermediates entering the glycolytic route when compared to the other adapta-tion periods. Nevertheless, a similar function is also achieved by the MA-related microbiota using a different route within the same biochemical pathway.

Proteins categorised into the galactose metabolism KEGG pathway showed a major abundance in the Zero and EQ samples (Figure 2.5-4D). The bacterial community of the Zero samples, in line with the previous observation (Figure 2.5-4A), is almost exclusively concerned in widening the array of substrates through the production of more easily digestible metabolites. The EQ-related bacterial community, in contrast, is involved in the production of N-acetylgalactosamine-specific component IIA (EC 2.7.1.-) and tagatose 1,6-diphosphate aldolase (EC 4.2.1.40). The first is a component of the phosphotransferase system, one of the major bacterial mechanisms for the uptake of complex sugars whereas the latter enzyme is a class I aldolase also involved in essential metabolic pathways such as gluconeogenesis and glycolysis [34, 35].

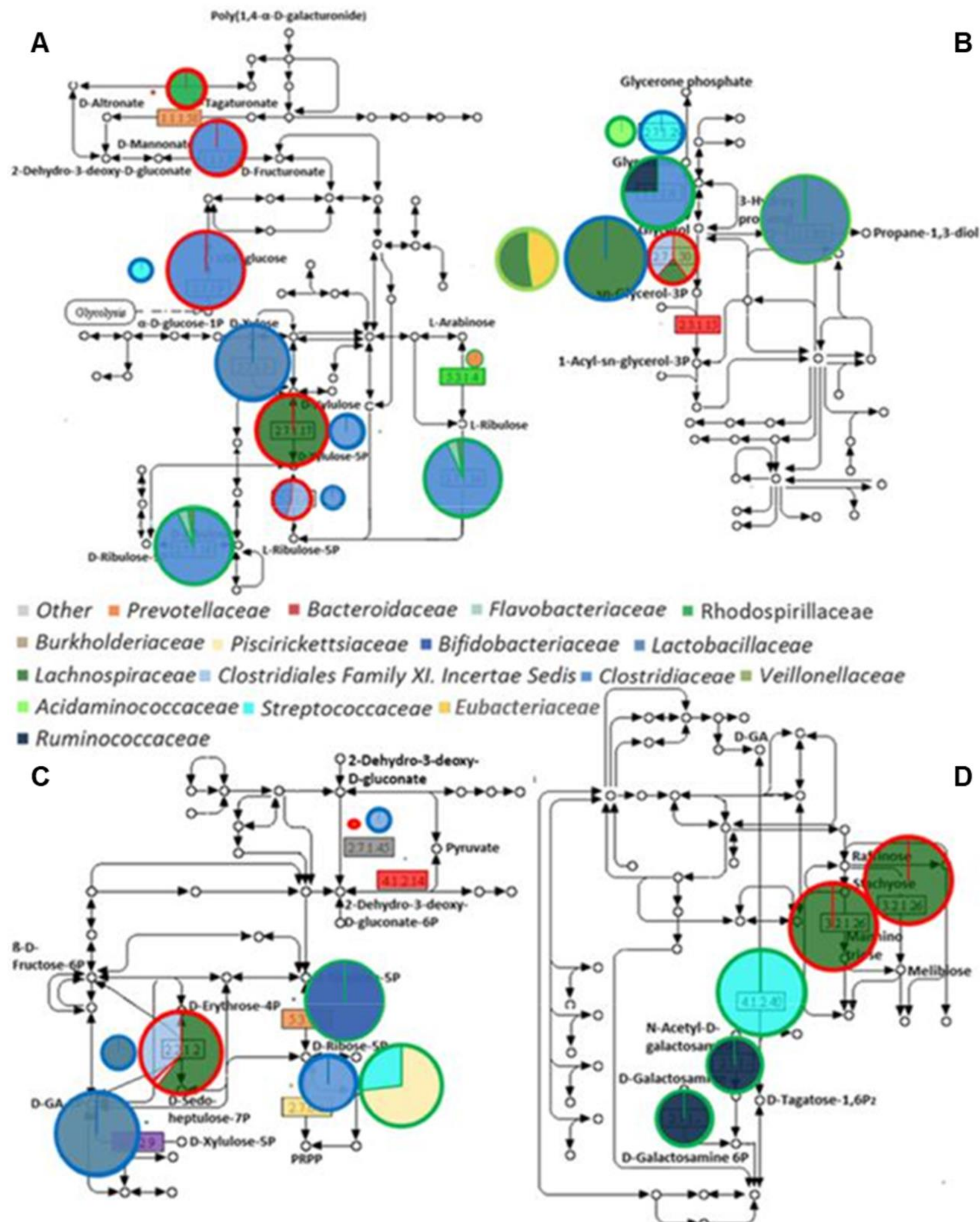

Figure 2.5-4 Microbial community exhibit a varying concern in selected carbohydrates pathways. Panel „a" Pentose and glucuronate interconversions, „b" glycerolipid metabolism, „c" pentose phosphate pathway and „d" galactose metabolism. Differently colored fillings of the pies indicate the bacterial families involved in the production of each of the identified proteins. Pie size is representative of the protein abundance, normalised on a time point basis. This normalisation highlights the portion of path of major concern for each of the three adaptation periods. The color code of the pie framing stands for red: Zero, blue: MA and green: EQ. Colored squares, indicating the identified proteins, are used when pies contour does not allow for a clear distinction between the adaptation periods due to the reduced pie size. Red: Zero; blue: MA; green: EQ; yellow: all periods; grey: Zero/MA shared; orange: Zero/EQ shared; purple: MA/EQ shared.

The protein dataset was also analysed for the presence of glycosyl hydrolases (GH) and glycosyl transferases (GT) via the CAZy database [36] (Additional file 1: Table 2.5-S3). In general, qualitative identification of the GH and GT families is not changing between the adaptation periods. Enzymes of the families GH13 and GH36 are more abundant in the EQ samples, indicating a higher concern of the EQ bacterial community in the hydrolysis of the alpha-bond of glycosylated macromolecules (glyco-lipids, glycoproteins) and large polysaccharides (starch and glycogen) when compared to the Zero counterpart. In contrast, Zero samples exhibited a higher abundance of enzymes affiliated to GH1, GH43, GH3 and GH95 families enabling a broad range of functions [36]. In line with the taxonomic results, Prevotellaceae and Lachnospiraceae were among the major producers of the GHs identified in the Zero samples, whereas Prevotellaceae, Clostridiaceae and Bacteroidaceae were found to be some of the main contributors to the GHs of the EQ-related bacterial community (Additional file 1: Table 2.5-S3). The abundance of proteins related to the GT5 family is higher in Zero samples, indicating a strong concern of the microbiota in the formation of the alpha-1,4-bonds required in the biosynthesis of polysaccharides such as glycogen and starch. Their production is needed in enteric bacteria to ensure a rapid growth in the intestinal environment where there is high competition and occasional lack of nutrients [37]. An opposite trend is observed in the EQ bacterial community, showing a higher abundance of enzymes affiliated to the GT35 family, whose main function is the phosphorylation-mediated degradation of starch and glycogen [36]. Similarly to GHs, the identified bacterial families showed a different contribution to the production of the GTs depending on the adaptation period. Lachnospiraceae members produced a higher abundance of GT5 in Zero samples when compared to the EQ counterpart. Bifidobacteriaceae and Bacteroidaceae, in contrast, are strongly involved in the EQ-related GTs and did not show participation in the production of GTs of the Zero samples. Equal contributions of Prevotellaceae members are observed in the production of the GTs in all the adaptation periods (Additional file 1: Table 2.5-S3).

Short-chain fatty acids biosynthesis

The whole metaproteomic datasets of the Zero, MA and EQ periods were checked for the presence of proteins which are indicators of SCFA production as previously reported [33] and listed in the legend of Additional file 2: Figure 2.5-S7. Investigated enzymes are involved in the biosynthesis of formate, acetate, propionate and butyrate. A minor part of the whole dataset concerned the SCFA indicators of our choice, corresponding to 3.7 ± 0.1%, 2.8 ± 0.1% and 3.0 ± 0.09% of the total LFQ of Zero, MA and the EQ period, respectively. The three adaptation periods accordingly indicated proteins involved in the propionate synthesis

pathway as the most abundant followed by proteins of the butyrate, acetate and formate production pathways. The abundance of the butyrate-producing enzymes was not changing over time, whereas the abundance of the propionate indicators showed a gradual decrease, counterbalanced by the progressive increase in the abundance of acetate and formate-producing enzymes (Additional file 2: Figure 2.5-S7).

Details on the bacterial specimen involved in the SCFA production are provided in Additional file 2: Figure 2.5-S8. Results of the GC measurements of SCFA showed acetate as the most abundant SCFA followed by propionate and butyrate with an average abundance of 59 ± 3.3, 22.6 ± 1.1 and 16.2 ± 1.1 mmol/kg feces regardless the experimental time points (Additional file 1: Table 2.5-S4 and Additional file 2: Figures 2.5-S7 and 2.5-S8). Similar to the metaproteomics outcomes, no notable overtime changes were observed for butyrate concentration, but a dietary effect is observed with an increase of butyrate in all samples from diets with low CaP levels. Propionate was registered with an increased amount in EQ samples (24.4 mmol/kg feces) when compared to the Zero counterpart (20.9 mmol/kg feces) but no dietary effect was observed. No gradual changes could be shown for acetate but diets with low CaP levels exhibited a lower acetate concentration at day 32 (Additional file 1: Table 2.5-S4 and Additional file 2: Figures 2.5-S7 and -S8). Correlation of the metabolite measurements with the protein abundances revealed a scarce correlation between the results, with a Pearson correlation coefficient either positive or negative close to Zero (ranging from − 0.33 to + 0.13) (Additional file 2: Figure 2.5-S9).

Host proteome is affected by changes in the intestinal microbiota

In this study, a total of 513 pig proteins were identified and functionally categorised into proteomaps according to the adaptation periods described above [38]. A quantitative representation of the ongoing host functions (visualized as gene names (GN)) over the experimental time span is shown in Figure 2.5-5.

In general, abundance profiles of the animal proteins confirmed the previously observed gradual adaptation process highlighting two distinct representations for Zero and EQ samples, whereas, MA period samples recorded intermediate abundance values ($p = 0.001$, Figure 2.5-5).

Abundance of proteins identified in the Zero period depicted a strong implication of the host in cell growth, motility and cell cycle, as supported by the high abundance of proteins such as actin alpha1 (GN = ACTA1) and annexin A4 (GN = ANXA4). The functional profile assessed in the MA period showed common functions to the two equilibrium conditions (Zero and EQ). However, the higher abundance of proteins such as phospholipase A2 (GN =

PLA2G1B) in MA samples suggests a host effort in preserving the functional homeostasis of the gastric mucosa by monitoring the structure of its microbial community. Similarly to the Zero period, EQ samples were also involved in cell motility and cell cycle. However, the higher abundance of tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta (GN = YWHAZ), annexin A11 (GN = ANXA11) and tubulin beta 4B class IVb (gene name TUBB4B), involved in mitotic cell cycle, cell division and cytoskeletal organization respectively, leads us to the assumption that there is an important concern of the host in organ enlargement and animal growth at the EQ period.

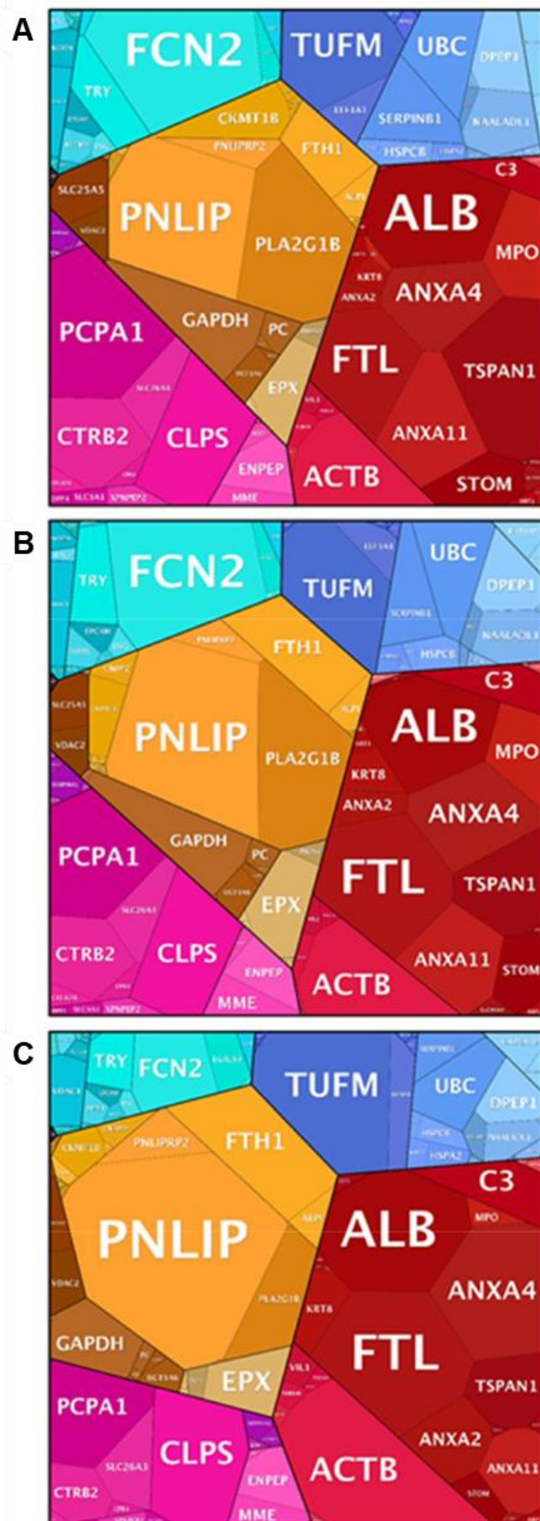

Figure 2.5-5 Host proteome changes along with the remodelling of its gut metaproteome. Voronoi diagrams show the host proteome of Zero „a“, MA „b“ and EQ „c“ samples. Identified proteins are visualised as polygons, whose area reflect their relative abundance. Gene IDs are detailed for each polygon.

2.5.5 Discussion

An improved knowledge about the intestinal microbiota of pigs is of interest for translational research, animal husbandry optimisation and animal health improvement. Kim et al. [39] described the natural, age-dependent shift of the fecal microbiota composition of commercial swine, emphasizing the importance of animal's age as a factor shaping the pigs intestinal microbiota. The same study also determined the trustworthiness of results obtained from pig groups rather than results arising from the same trial conducted at the level of individual animals [39]. The current study investigated the gradual adaptation mechanisms of the pigs' gut bacterial community during a shift of experimental diets differing protein sources and levels of CaP. The statistical analyses of both DNA- and protein-based datasets showed a clustering of the investigated samples overtime, revealing a gradual adaptation of the fecal microbiota to the experimental diets. The microbiota adaptation process was hypothesised throughout three main periods; the first of which (*i.e.* Zero) represents the unaltered gut microbial community prior to the administration of the experimental diets. The second period (*i.e.* MA) describes the structural and functional transition of the

fecal microbiota in the attempt to face the challenging factor. The third period (*i.e.* EQ) depicts the newly established equilibrium of the gut microbial community, as supported by further clustering of the samples according to the diverse levels of CaP administered to the diets. Previous studies on the intestinal microbiota of animals kept at diets with high CaP levels reported an increased amount of *Prevotella* spp. along with other *Enterobacteriaceae* and *Clostridiaceae* members [9, 14]. At the stomach level, high CaP levels were associated with an increased amount of *Lactobacillaceae* and a reduced portion of *Prevotella* and *Streptococcus* [14]. A further study concluded that the overall bacterial community rather than specific groups is affected by feeding diets with varying levels of CaP [40]. This could be observed in the present study where an overall remodelling of the bacterial community was observed without identifying specific factors, like OTUs or proteins, which may cause this effect. We believe that the fecal microbiota is shaped by the changing CaP levels through a multitude of ways, ranging from the modified physicochemical environment to altered relationships between microorganisms and the host. Here, we measured a decreased amount of SCFA, especially acetate, at low CaP levels but an enhanced concentration of butyrate (Additional file 1: Table 2.5-S4). This could indicate a functional shift with beneficial effects for the host as butyrate serves as an energy source for the colonic epithelium [41]. In broilers, it was demonstrated that the stress induced by a reduced CaP supplementation is subsequently mirrored in the gastrointestinal tract-related microbial community [13]. As no increase of stress-related proteins were found during the present study, the change in the microbial community was probably caused by an altered metabolism of the host linked to a modified secretion of host metabolites into the gut lumen. Thus, further investigations are required to define specific factors involved in the CaP-dependent alteration of the intestinal microbiota. In contrast to CaP, no effect imputable to the diverse protein sources over the experimental time frame was identified. This is probably due to a large fraction of corn (33–67%) in both diet formulations, which masks the possible effect of the soybean meal and field peas supplementation, as already highlighted in other studies [42, 43]. In contrast, Rist et al. observed a shift in the intestinal microbiota composition due to increased dietary corn supplementation [44].

The structure of the fecal microbiota was investigated through 16S rRNA gene sequencing and metaproteomic analyses. Changes in the abundance of some bacterial families such as *Clostridiaceae*, *Bifidobacteriaceae* and *Lactobacillaceae* are detected by both investigation strategies indicating a parallel structural and functional remodelling of the gut bacterial

community. Even though, a diverse general microbiota composition is drawn by the two adopted approaches.

The protein-based microbiota assessment described a very dynamic structure of the bacterial community, highlighting the disappearance of some bacterial families and the presence of new ones along the complete experimental time span. The emergence of new bacterial families and the strong changes of functions observed during the three adaptation periods are a clear example of how the process of microbiota re-structuration occurs overtime and how the diverse bacterial entities synergistically co-operate to form a balanced microbial community. This enables a better facing of the challenging diets and adaptation to the new surrounding environment. Compared to the DNA-based investigation, the metaproteomic-based taxonomic assessment identified a higher bacterial heterogeneity at both family and phyla level. The reported diver-gence of results is most likely imputable to the different principles these methods are based on. Both methods target different biological macromolecules and thus, are destined to diverse technical issues [12]. Moreover, we retain that metaproteomics enables the identification of a higher bacterial complexity since the changes in the abundance of expressed proteins are detected earlier than changes in the number of the DNA copies targeted by 16S rRNA gene sequencing. Similar evidence was observed in previous investigations. Tang and colleagues highlighted inconsistencies between the DNA and protein-based assessment of the microbiota composition [45]. Moreover, other studies described a higher bacterial complexity in metaproteomic datasets than in 16S rRNA gene sequencing data [12, 46].

Uniquely identified proteins for each of the three adaptation periods were subjected to functional classification. A functional classification of the whole metaproteome has been attempted, but a clear description of the gradual functional shift of the gut bacterial community was not possible. A plausible reason for this is that the shared proteins are involved in housekeeping functions, thus their consideration masks the statistically predicted gradual shift of the fecal microbiota. Moreover, the abundance of shared proteins counts for the most of the total LFQ indexes of each adaptation period, hampering the masking effect arising from the consideration of the shared proteins.

Functional profiles of the bacterial communities in the diverse adaptation periods reveal a dynamic change of the bacterial activity. In line with the taxonomic assessment, the bacterial families responsible for a phase-specific architecture of the fecal microbiota are also among those families active in the biochemical pathways causing the diverse functional profiles of each adaptation period. We focused our attention on the biochemical pathways showing the

highest abundance variability is the major factor responsible for the phase-specific functional profiles of the fecal microbiota. Interestingly, almost all the changing bacterial families highlighted in the taxonomic assessment of the fecal microbiota are involved in carbohydrate-related pathways such as pentose and glucuronate interconversion, glycerolipid metabolism, pentose phosphate pathway and galactose metabolism. Focusing on these pathways highlighted that for each adaptation period, different reactions of the paths are concerned in a quantitatively different manner. Therefore, even though the diverse bacterial communities appeared to be involved in common pathways, internal investigation of the paths revealed a diverse array of functions performed by the bacterial community depending on the adaptation periods, thus the variety of systems enrolled to achieve convergence points (for example, entering the glycolysis).

Based on the identified protein repertoire and their functional categorization, we speculate that the Zero period represents a thriving bacterial community whose composition and functional equilibrium have not been altered by external factors. This enables a deep specialization of the bacterial community, as supported by the high abundance of phosphoribulose isomerase (EC 5.1.3.4), tagaturonate reductase (EC 1.1.1.58), beta-fructofuranosidase (EC 3.2.1.26) and mannonate oxidoreductase (EC 1.1.1.57) suggesting a strong involvement of the Zero bacterial community in facilitating sugar uptake and digestion [47]. In addition, this enlarges the substrate array to maximise feed conversion, by improving carbon and energy uptake [48, 49].

The EQ period in contrast, describes a bacterial community in a stage of freshly achieved homeostasis, thus still refining its functional profile for a better adaptation to the surrounding environment. Functions related to the widening of the substrates array and facing complex carbohydrates are still expressed, but at a lower level than observed in Zero microbiota. Nevertheless, the high abundance of the glycerol dehydrogenase (EC 1.1.1.6) and 1,3-propanediol dehydrogenase (EC 1.1.1.202) observed in the glycerolipid metabolism KEGG pathway reveals a possible implication of the EQ bacterial community in alternative strategies to improve carbon and energy yield through the use of glycerol as a carbon and energetic source [50, 51].

On the other hand, the increased abundance of phosphoriboisomerase (EC 5.3.1.6) and tagatose 1,6-diphosphate aldolase (EC 4.2.1.40) suggests a higher concern of the EQ-related bacterial community in entering the glycolytic route [52] in order to yield the energy required to complete the specialization process for an optimal settlement in the new host environment.

Investigation of the MA period proteins describes a transitory bacterial community featured by intermediate evidence in terms of both composition and function. Here, the overtime increase of the abundance of the enzyme ribose-phosphate diphosphokinase (EC 2.7.6.1) suggests an increasing ability of the bacterial community of numerous biosynthetic processes, such as the de novo biosynthesis of purines and pyrimidines [53].

Indicators of SCFA production were sorted out of the total metaproteomic dataset, in order to infer the SCFA production in the different adaptation periods. Correlation analysis of the predicted SCFA production with the direct measurements of the metabolites indicated a high correlation coefficient for acetate exclusively. The scarce correlation scored for all other metabolites is probably due to the fact that bacteria can produce SCFAs through a variety of metabolic routes, each of which is featured by a diverse array of enzymes [54]. Based on this finding, we believe that only acetate was produced through the route targeted by the indicators of our choice; whereas the other metabolites were produced through metabolic routes (*i.e.* enzymes) that were not identified by the set of indicators used in our investigation.

Alteration of the intestinal microbiota, as well as its gradual adaptation, is also reflected in the host proteome. Protein profile of the Zero and MA samples showed a strong participation of the host in shaping the intestinal microbiota composition for a better facing of the new diets. EQ samples instead are involved in host cell division and organ enlargement. In this regard, we retain that the freshly assessed bacterial community built an optimal growth environment by providing nutrients and energy to its host. This determines an increased tendency in intestine enlargement in EQ samples rather than Zero ones. However, care should be taken when comparing the growth capability of Zero and EQ samples since these samples do not belong to animals of the same age. Therefore, some of the variability observed in their protein profile could be age-related and not exclusively due to the intestinal microbiota changes. Moreover, the sample preparation protocols applied in the current study preferentially target bacterial proteins, resulting in a lower coverage of the host proteome that does not allow for a deep and complete investigation of the complex interaction network established between the intestinal microbiota and its host.

2.5.6 Conclusion

For the first time, this study presents insights into the gradual adaptation of the porcine intestinal microbiota challenged by experimental diets. Taxonomic and functional dynamics of the bacterial community have been depicted through 16S rRNA gene sequencing and metaproteomics until the achievement of a stable bacterial community. Besides the dynamic

changes of the microbiota, this study defines the duration of the metabolic adaptation process required by the intestinal microbiota. This is of a great importance for the design of future nutritional studies. Moreover, considering the high similarities between the porcine and human gastrointestinal tract anatomy and physiology, the findings of the current study might imply in the design of human-related nutritional studies as well as the characterization of the human intestinal microbiota when challenged by the alteration of external factors such as the diet. Nevertheless, this study focused on the investigation of the major changes of the fecal microbiota, therefore further complementary studies investigating other structural and functional aspects of the challenged microbial community are desirable.

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Availability of data and materials

Sequences are available at the European Nucleotide Archive under the accession number PRJEB19477 (http://www.ebi.ac.uk/ena/data/view/ PRJEB19477). The mass spectrometry data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository [55] with the dataset identifier PXD006224.

Authors' contributions

BT, CMEH, LEH, RM, VS and JS conceived and designed the project. BT, KB and CMEH collected samples and performed the experiments. BT, KB, ACS and JS analysed and interpreted the data. BT, KB, ACS and JS wrote the paper. All authors commented on the manuscript. All authors read and approved the final manuscript.

Ethics approval

Animal husbandry, as well as experimental treatments conducted on the animals used for this study, was approved by the animal welfare commission of the University of Hohenheim.

Competing interests

The authors declare that they have no competing interests

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Author details

[1]Institute of Animal Science, University of Hohenheim, Emil-Wolff-Str. 6-10, 70593 Stuttgart, Germany. [2]Department of Agricultural, Food and Nutritional Science, University of Alberta, Edmonton, Canada.

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2.5.8 Supporting information - Additional Files

See https://doi.org/10.1002/mbo3.312.

Additional file 1: Table 2.5-S1**.** Table report the fast sequence of each OTU. Table 2.5-S2. Metaproteomic dataset. A) Table report a summary of information on the peptides/proteins identified. B) Table include further insights on peptide identification and their implication in protein IDs inference. Table 2.5-S3. Glycosyl hydrolase and glycosyl transferase production.

A) Panel reports the identified GHs and GTs. The relative abundance (%) is detailed for each GH and GT family in each adaptation period. B) Panel shows the relative contribution (%) of the bacterial specimens encoding for the major GH and GT families identified over the three adaptation periods. C) Panel report the list of proteins classified in each of the identified GH and GT, along with the LFQ index and the relative bacterial families. Table S4. SCFA concentration. Table of the total SCFA, acetate, propionate and butyrate concentration of all animals at days 3 and 32. (ZIP 108295 kb)

Additional file 2: Figure 2.5-S1. Venn diagrams display the number of OTUs (A) and proteins (C) attributed to the three adaptation periods. B, D are tables showing the respective p values calculated by a pairwise comparison to show the significant differences between the time points and diets. Figure 2.5-S2. Taxonomic assessment of the samples at each of the selected experimental time points (days). The entire metaproteomic dataset (*i.e.* both unique and shared proteins) is considered for the fecal microbiota taxonomic assessment. Figure S3.

LFQ distribution among the adaptation periods. Pie charts represent the relative distribution of the abundance index of the proteins identified in Zero (A) MA (B) and EQ (C) samples. Figure S4. Functional classification of the identified proteins by their categorization into COG classes (A) and KEGG biochemical pathways (B). Only categories with a cumulative abundance higher that 1% of the total LFQ abundance index are included in the visualisation. A functional classification of the samples at all the selected experimental time points (days) is provided. Figure 2.5-S5. Heat map displays a list of proteins whose abundance ratio is changing between adaptation periods of at least 5-fold. Abundance indexes of each protein in the diverse adaptation periods are shown as log LFQ. Figure 2.5-S6. Protein classification into KEGG biochemical pathways. Abundance of the pathways is expressed as a relative percentage for each of the adaptation periods. The only pathways scoring at least 2.5-fold change between the adaptation periods are visualised. Figure 2.5-S7. SCFA production as assessed through the metaproteomic (A and B) and conventional approach (C and D). A Abundance of the enzymes, selected as indicators of SCFA production, out of the total LFQ abundance indexes. B Distribution of the indicators for the major SCFA production, across the diverse adaptation periods. C Summary of the SCFA measurements in the Zero and EQ period, on an animal basis. D Relative production of the major SCFA as assessed through GC measurement. The proteins involved in the prediction of the SCFAs production are formate production: COG1882. Acetate production: COG0282; COG0280; COG1012. Propionate production: COG0777, COG4799; COG2185, COG1884; COG4577. Butyrate production: COG4770; COG0183; COG1028, COG1064; COG3426; COG1250, COG1024. Figure 2.5-S8. SCFA production by gut microbial commensals. In the metaproteomic approach, the SCFA production has been inferred through investigation of the quantitative expression of enzymes involved in SCFA biosynthesis. A Formate production: COG1882. B Acetate production: COG0282; COG0280; COG1012. C Propionate production: COG0777, COG4799; COG2185, COG1884; COG4577. D Butyrate production: COG4770; COG0183; COG1028, COG1064; COG3426; COG1250, COG1024. Figure 2.5-S9. A Correlogram displays the relationships occurring between the investigation approaches, as well as the relationships between metabolites production as measured according to either metaproteomics or the conventional GC-based approach. B The correlation coefficient for each of the compared pair is also provided. (ZIP 1560 kb).

2.6 Burbach et al.

Exploring effects of dietary inclusion of insect meal on the microbiota of piglets' gastrointestinal tract

Burbach K.[1], Camarinha-Silva A.[1], Berk A.[2], Dänicke S.[2], Seifert J.[1], Kluess J.[2]

[1]*Institute of Animal Science, University of Hohenheim, 70593 Stuttgart, Germany*

[2]*Institute of Animal Nutrition, Friedrich-Loeffler-Institute, 381116 Braunschweig, Germany*

Manuscript in preparation

Exploring effects of dietary inclusion of insect meal on the microbiota of piglets' gastrointestinal tract

2.6.1 Introduction

The world population is expected to reach approximately 9.8 billion in 2050 (UN 2017). This prospection is accompanied by an immense increase in the global demand for meat (Alexandratos and Bruinsma 2012). To meet these nutritional demands, the inclusion of insects to the feed for livestock is a promising approach. An appropriate candidate in terms of nutrient supply and mass production is the larvae of the Black soldier fly, *Hermetia illucens* (van Huis 2016). Its nutrient digestibility has been reported for aquaculture (Lock et al. 2016), poultry (Hale 1973; Cullere et al. 2016) and swine (Newton et al. 1977). Findings of these studies suggest the inclusion of *Hermetia* larvae meal to be suitable for livestock nutrition. However, currently in the European Union, insects are restricted to fish production and not yet allowed for swine and poultry (van Huis 2016).

Next, to the nutrient digestibility, effects on the microbiota inhabiting the gastrointestinal tract (GIT) are acknowledged in the evaluation of feed. The GIT microbiota plays an essential role in efficient digestion of feed components and maintaining the health status of its host. *Hermetia* larvae meal contains chitin, a polysaccharide which forms the exoskeleton of insects, which is indigestible by mammals but may be degraded by some members of the *Clostridium* genus (Simunek et al. 2001). Furthermore, bactericidal activity has been reported for chitin and its derivates on selected bacteria species in an in-vitro study (Benhabiles et al. 2012). The microbiota composition of chicken manure was beneficially influenced by *Hermetia* larvae, as the proportion of pathogenic bacteria was reduced (Erickson et al. 2004). So far, effects by dietary inclusion of *Hermetia* larvae meal on the porcine GIT and its microbiota are unknown.

Studies on the spatial organization along and across the porcine GIT revealed varying taxonomical composition of the microbiota (Looft et al. 2014; Zhao et al. 2015; Kelly et al. 2017). Further, the age of the host, the dietary composition and as the well the host's genetics are known to affect the microbiota's structure of porcine GIT (Zhao et al. 2015; Burbach et al. 2017; Camarinha-Silva et al. 2017). Besides varying factors driving microbiota a common core microbiota of the porcine GIT was recently characterized (Holman et al. 2017).

The aim of this study was to investigate effects of different inclusion levels of *Hermetia illucens* larvae meal in rearing diets on piglets' performance and the overall microbiota structure of the GIT.

2.6.2 Material and Methods

Experimental Design

Weaned barrows with an initial body weight (BW) of 7.0 ± 0.4 kg were housed in floor-pens (4 pigs/pen) and allocated to one of four diets (n=20/diet) varying in the level of *Hermetia illucens* larvae meal (Hermetia Baruth GmbH, Baruth/Mark): control feed (CON), diet with 2.5% (HERM-2.5), 5% (HERM-5.0) or with 10% *Hermetia* meal (HERM-10). Larvae were raised on rye and wheat bran, defatted mechanically and ground, thus containing 12.5% crude fat and 57.9% crude protein (DM-basis) in the final product. *Hermetia* meal was included to the expense of soya protein concentrate and nutrient content was kept constant for all diets. Piglets were fed ad libitum for five weeks. Piglets' performance (feed intake, weight gain, feed efficiency, faecal consistency) were determined weekly for the experimental period of five weeks.

Sampling the gastrointestinal tract

After five weeks pigs (n=10/diet) were slaughtered, dissected and samples collected: intestinal wall from stomach, duodenum, proximal and mid-jejunum, proximal and terminal ileum, caecum and colon, chyme from stomach, small intestine (divided equally in 3 thirds), caecum and colon as well as feces. An overview on the samples included in the microbiota analysis is represented in Table 2.6-1.

DNA extraction

Based on findings of a previous study (Burbach et al. 2016), FastDNA SPIN Kit for Soil (MH Biomedicals, Solon, OH, USA) was used to extract genomic DNA from the GIT samples. DNA extraction from chymus and fecal samples were performed according to manufacturer's instructions with an additional bead beating step in a Fast Prep®-24 Instrument (6 m/s, 40 s). To release mucosa-associated bacteria from tissue, samples were pretreated: 978 µl Sodium Phosphate Buffer, supplied by the kit, was added and sonicated for 30 seconds. Subsequently, samples were chilled on ice and centrifuged at 2500 g for 10 minutes. The supernatant was transferred to a Lysing Matrix E tube and the following procedure was performed according to the kit manufacturer's instructions.

Table 2.6-1 List of samples included in microbiota analysis.

			numbers by sample group				
sampling site	**type**	**abbreviation**	**total**	**CON**	**HERM-2.5**	**HERM-5**	**HERM-10**
gastric	mucosa	GA.m	39	10	10	10	9
duodenum	mucosa	DU.m	34	9	9	8	8
jejunum proximal	mucosa	JEp.m	29	8	7	6	8
jejunum middle	mucosa	JEm.m	24	6	7	5	6
ileum proximal	mucosa	ILp.m	36	8	10	10	8
ileum terminal	mucosa	ILt.m	11	3	3	3	2
caecum	mucosa	CA.m	34	9	8	9	8
colon	mucosa	CO.m	39	10	10	10	9
gastric	chymus	GA.c	31	8	9	7	7
small intestine proximal	chymus	SIp.c	35	10	10	8	7
small intestine middle	chymus	SIm.c	33	9	8	8	8
small intestine distal	chymus	SId.c	29	8	7	7	7
caecum	chymus	CA.c	33	7	9	9	8
colon	chymus	CO.c	38	10	9	9	10
feces	feces	FE	31	8	8	8	7

Illumina amplicon sequencing

The V1-V2 region of the 16S rRNA gene was amplified according to a previously published protocol (Camarinha-Silva et al. 2014), using a 2-step approach to insert sample-specific barcode, index sequences, and Illumina-specific sequences. Amplicons were verified on an agarose-gel, concentrations were normalized using the SequalPrep Normalization Kit (Invitrogen Inc., Carlsbad, CA, USA) and pooled by index. Amplicon libraries were purified by the MinElute PCR Purification Kit (Qiagen, Hilden, Germany) and sequenced with 250 bp paired-end sequencing chemistry on an Illumina MiSeq platform.

The bioinformatics processing of the sequences was done as previously published and using MOTHUR (Camarinha-Silva et al. 2014). Samples of low coverage, with less than 10000 sequences, were removed from dataset. In addition, low abundant operational taxonomical units (OTUs), with less than 50 sequences per sample, were excluded from data analysis. Finally, the microbiota analysis was based on 18,122,491 sequences in total. Sequences will be submitted to the European Nucleotide Archive (ENA).

A venn diagram representing commonly present OTUs was generated using an online tool (http://bioinformatics.psb.ugent.be/webtools/Venn).

The OTU's closest taxonomical representative was manually assigned considering previously suggested thresholds by Yarza et al. and the Seqmatch tool from Ribosomal Database Project (Wang et al. 2007; Yarza et al. 2014).

The relative abundance values are reported as mean ± standard error of the mean (sem).

Statistical analysis

The sequencing generated a multivariate data set, which was statistically evaluated by using PRIMER 6 (v 6.1.16) & PERMANOVA + (v 1.0.6) (Clarke and Warwick 2001). Samples were standardized by total abundance and a resemblance matrix was created using Bray-Curtis index. The microbiota structure between samples, β-diversity, was explored by principal coordinate analysis (PCoA). With the RELATE routine, a Mantel-type test, the relations between multivariate patterns along GIT sampling sites were quantified. Permanova test was performed on samples of defined a priori groups (sample type, sampling site, diet) and differences were considered to be significant if $P < 0.05$.

Shannon index was used to calculate α-diversity of the microbiota. Here a Permanova test was based on Euclidean distance matrix.

In addition, linear discriminant effect size (LEfSe) analysis was performed to identify genera, which were significantly enriched either by sampling site or by dietary group. The LEfSe algorithm was performed online within a Galaxy workflow framework using the default settings (https://huttenhower.sph.harvard.edu/galaxy). This statistical approach is based on nonparametric factorial Wilcoxon sum-rank in combination with a linear discriminant analysis to assess the effect size of differentially abundant genera (Segata et al. 2011).

2.6.3 Results and Discussion

Piglets fed with control diet (CON) and diets including *Hermetia* larvae meal (HERM-2.5, HERM-5, HERM-10) showed no differences in performance parameter during a 35 day-rearing period (Jeanette Kluess, personal communication, February 2018).

Assessing the microbiota's specificity within the GIT

A total of 476 samples, collected from 15 different sites, was included in the microbiota analysis and reflected both a cross-section and a longitudinal- section of the piglets' GIT and, in addition, feces (Table 2.6-1).

Illumina amplicon sequencing of the V1-2 region of the 16S rRNA gene yielded to a sequencing depth of 38,072 ± 1154 sequences per sample. Sequences clustered into 1420

OTUs with 1319 OTUs being commonly present in the GIT, Figure 2.6-1A. Despite this high number of common OTUs, the Shannon-diversity, considering both presence and abundance of OTUs, was significantly reduced in samples of chymus compared to mucosal and fecal samples (P = 0.001, Figure 2.6-S1). In accordance with a previous study, chymus of the small intestine revealed the lowest diversity index compared to other sampling sites along the GIT (Looft et al. 2014).

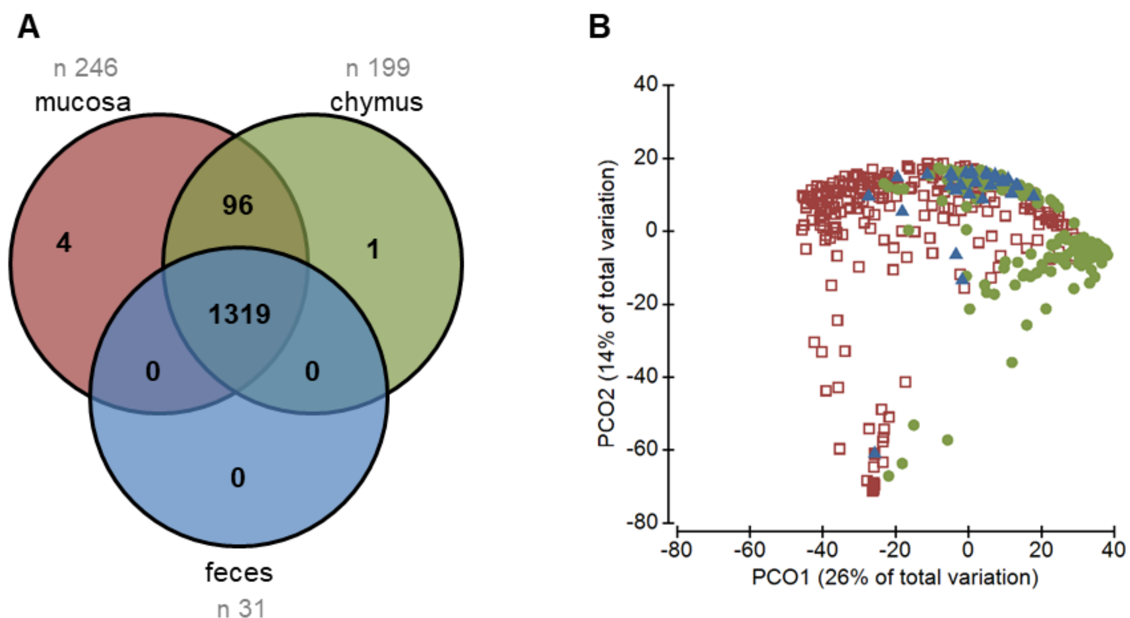

Figure 2.6- 1 Overall microbiota structure of the piglet's gastrointestinal tract. A) Venn diagram highlighting shared and unique OTUs detected in different types sample origin, n represents the sample number. B) PCoA plot depicting the similarity structure of samples from mucosa (red squares), chymus (green circles) and feces (blue triangle).

Similar, the overall β-diversity structure of the microbiota of mucosa, chymus and feces was significantly different (P = 0.001; Figure 2.6-1B). A closer look at fecal microbiota structure by a Mantel test revealed significant correlations to several sites across and along the GIT. Fecal microbiota showed the strongest relation to microbiota of the colonic chymus (ρ = 0.538, P = 0.001), Table 2.6-S1. So, microbiota studies restricted to fecal samples provide limited insights into overall microbiota structure of piglet's GIT.

The taxonomical assignment of the OTU sequences yielded into 13 phyla. Firmicutes, Bacteroidetes, Actinobacteria, and Proteobacteria were the dominant phyla and accounted cumulatively to 99% of the total abundance. Each sampling site was predominated by Firmicutes (total abundance 63% ± 0.9%). In comparison to chymus samples, mucosa was enriched by Bacteroidetes (29% ± 1% versus 8% ± 0.7%) and Proteobacteria (12% ± 0.7%

versus 3% ± 0.2%). Whereas, the proportion of Actinobacteria was higher in chymus than in mucosa (14% ± 0.8% versus 5% ± 0.4%).

On the family level, 14 families were detected with an average abundance > 1% and represent a cumulative abundance of 92 %. Among the predominant phyla, the main representative families were *Lactobacillaceae* (Firmicutes), *Prevotellaceae* (Bacteroidetes), *Bifidobacteriaceae* (Actinobacteria) and *Campylobacteraceae* (Proteobacteria). Family composition varied by sampling site. *Lactobacillaceae* showed the highest abundance in chymus of the distal small intestine (68% ± 2.6%), *Prevotellaceae* in colonic mucosa (CO.m, 36% ± 1.9%), *Bifidobacteriaceae* in chymus of the proximal small intestine (SIp.c, 19% ± 2.0%) and *Campylobacteraceae* in caecal mucosa (CA.m, 16% ± 1.9%).

Deeper in taxonomy, 15 main genera with a total average >1% were identified and cumulatively represented 77% of the total abundance across all sampling sites. The observation of site-specificity in taxonomical microbiota composition was strengthened by the identification of enriched genera within different sampling sites. *Bacteroidetes* was enriched in the mucosa of proximal jejunum, here with 4% ± 3.3% relative abundance. *Campylobacter*, *Alloprevotella*, and *Dialister* were enriched in the mucosa of caecum (CA.m, 16% ± 1.9%; 6% ± 0.6%; 2% ± 0.3%) and *Prevotella* in mucosa of colon (CO.m, 30% ± 1.8%). Whereas *Lactobacillus* was enriched in chymus of the proximal third of the small intestine (SIp.c, 68% ± 2.6%), unclassified *Succinivibrionaceae* in chymus of caecum (CA.c, 4% ± 0.3%), and unclassified *Ruminococcaceae* in fecal microbiota (FE, 4% ± 0.3%). Similarly, a recent meta-analysis of a porcine GIT microbiota identified *Alloprevotella* and *Prevotella* to be significantly enriched in the mucosa of caecum and colon, respectively (Holman et al. 2017). Previously, *Campylobacter* was identified as a common genus in the mucosal microbiota of piglets with a prevalent abundance in mucosal duodenum and jejunum (Kelly et al. 2017).

Effects of Hermetia inclusion to diet composition

The overall GIT microbiota of piglets fed with a dietary inclusion of *H. illucens* larvae meal did not significantly differ from those of piglets fed with the control diet (Permanova $P>0.05$).

Considering the high site-specificity of GIT's microbiota, a pairwise comparison of treatment groups within the sampling sites was performed, Table 2.6-S2. The dietary groups affected significantly different the microbiota structure at OTU level in chymus samples of the small intestine ($P < 0.05$), collected either from the proximal, middle or distal part, Figure 2.6-2.

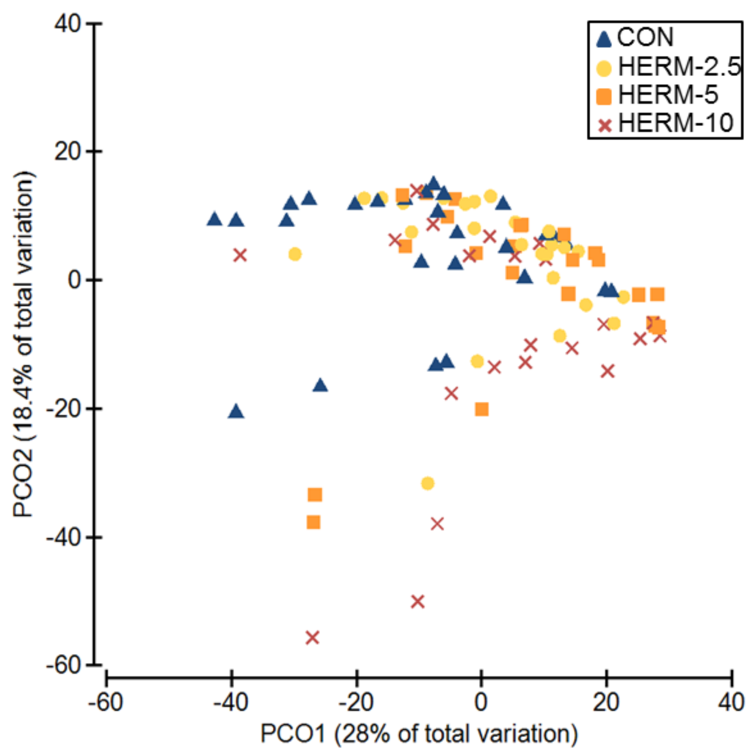

Figure 2.6-2 Dietary inclusion of *H. illucens* shows minor effects on the microbiota from chymus of the small intestine. PCoA plot of Bray-Curtis matrix on OTU level including all chymus samples from small intestine indicated by dietary group: CON: blue triangle, HERM-2.5%: yellow circle, HERM-5%: orange square, HERM-10%: red cross.

The dietary inclusion of *Hermetia illucens* larvae meal is an innovative approach in piglets' nutrition, and results of this study present no alarming impact on the animal performance or the overall microbiota structure of the GIT. Minor changes were observed in the microbiota associated with chymus of the small intestine, which indicated promoted abundances of saccharolytic bacteria, like *Lactobacillus* and *Clostridium* sensu stricto.

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2.6.5 Supporting information

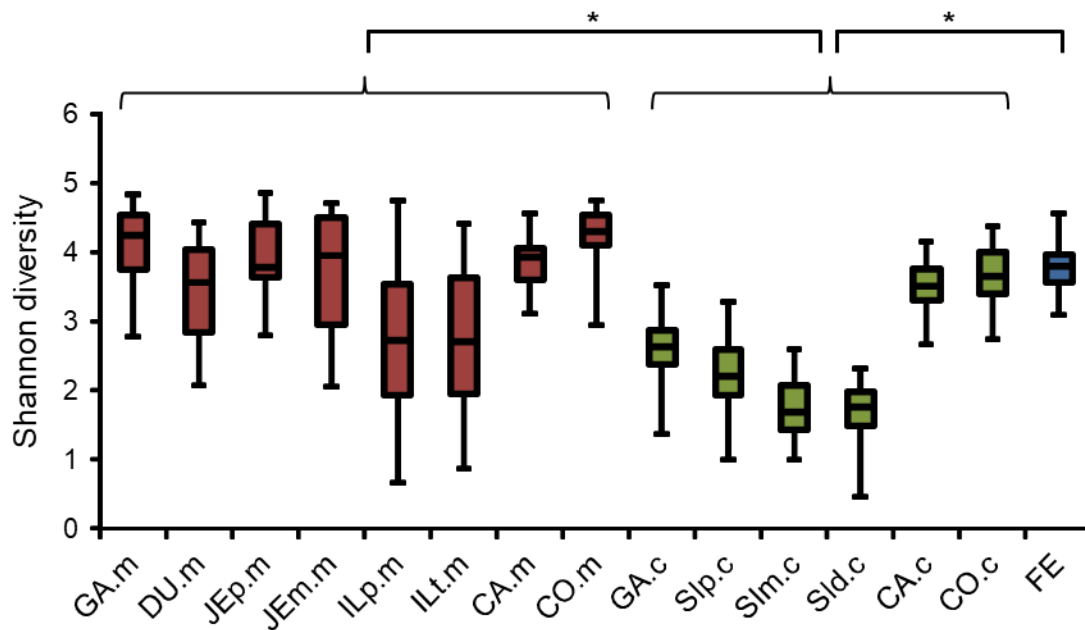


Figure 2.6-S1 Shannon diversity indices along the sampling sites. The boxplots display the variation in the α-diversity within sequencing results derived from different sampling sites. Each box ranges values from the first to the third quartile, with the median indicated by a horizontal line, and the whiskers represent the lowest and highest index-value. Asterisks indicate significant differences, with $P < 0.05$, as was assessed by Permanova test on samples grouped by mucosal, chymus or fecal origin.

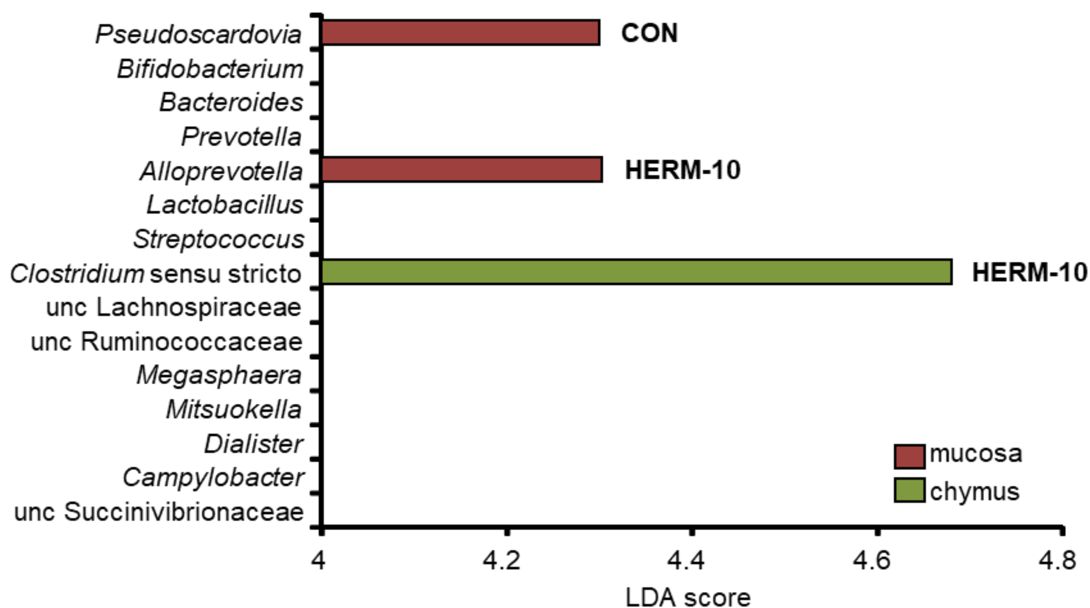


Figure 2.6-S2 Dietary effect on genera abundance in the GIT cross-section. Differentially abundant genera were identified by LEfSe analysis and the LDA score is displayed according to the affected GIT cross-section, mucosa: red, chymus: green.

Table 2.6-S1 Spearman correlation coefficients ρ between sequencing results from fecal and other sampling sites. Significant P values with P ≤ 0.05 are highlighted in bold and P≤ 0.001 in bold italic.

	ρ	P
GA.m	0.131	0.150
DU.m	-0.076	0.703
JEp.m	0.074	0.270
JEm.m	0.077	0.292
ILp.m	0.201	0.073
ILt.m	0.587	0.009
CA.m	0.249	0.051
CO.m	0.412	0.005
GA.c	0.428	0.009
SIp.c	0.126	0.181
SIm.c	0.324	0.026
SId.c	0.198	0.107
CA.c	0.514	0.002
CO.c	0.538	*0.001*

Table 2.6-S2 Permanova pairwise test revealed dietary effects in chymus samples from the small intestine. P-values < 0.05 are considered to be significant and are highlighted in bold.

	CON; H. 2.5%	**CON; H. 5%**	**CON; H. 10%**	**H. 2.5%; H. 5%**	**H. 2.5%; H. 10%**	**H. 5%; H. 10%**
GA.m	0.468	0.551	0.795	0.956	0.547	0.441
DU.m	0.685	0.859	0.138	0.552	0.308	0.405
JEp.m	0.991	0.585	0.611	0.704	0.596	0.958
JEm.m	0.057	0.614	0.263	0.307	0.589	0.642
ILp.m	0.451	0.832	0.318	0.668	0.458	0.266
ILt.m	0.210	0.396	0.192	0.604	0.087	0.304
CA.m	0.569	0.285	0.355	0.664	0.793	0.928
CO.m	0.581	0.568	0.136	0.727	0.797	0.454
GA.c	0.875	0.983	0.418	0.884	0.085	0.650
SIp.c	**0.024**	**0.008**	0.124	0.649	0.315	0.907
SIm.c	0.264	0.309	**0.032**	0.776	0.184	0.419
SId.c	0.243	0.060	0.147	0.207	0.115	**0.041**
CA.c	0.282	0.309	0.146	0.132	0.084	0.399
CO.c	0.332	0.399	0.168	0.108	0.228	0.564
FE	0.849	0.309	0.100	0.239	0.346	0.862

Chapter 3 Discussion

The introduction of high-throughput DNA sequencing methodologies, to the scientific field of microbiota analysis, increased our knowledge of bacteria inhabiting challenging environments, like the GIT of animals. Within the ecosystem of the GIT, mutual interactions exist between the host, feed, and microbiota. Furthermore, the intestinal microbiota is commonly acknowledged to be essential for maintaining health and growth performance of its host. Deciphering the microbiota composition and its function in livestock is of increasing interest. An increase in the world's population is closely linked to an increased demand for livestock products. This is directly connected to an improved animal health and welfare, including a healthy gut and a balanced intestinal microbiota. The here presented studies aimed to characterize the diversity of the porcine GIT microbiota with a closer look to dietary influences. The analyses were based on molecular-genetic methodologies targeting the 16S rRNA gene sequence as a phylogenetic marker.

3.1 Demand for standardization efforts within methodological diversity

The microbiota of the porcine GIT shares a lot of similarities with the microbiota of human GIT, so the pig is considered as a model for human (Heinritz et al. 2013; Zhang et al. 2013). In the field of human microbiota research, standard operating procedures have been suggested by large-scale projects (https://www.hmpdacc.org/). Such a methodological standardization is so far not common in studies of the porcine microbiota but is promoted by the COST-action PiGutNet, a recent European network, and findings from Chapter 2.1 are therefore taken into consideration. This network includes expertizes of scientific and applied porcine research and addresses factors affecting the microbial balance in the GIT and aims to improve the risk management associated with the spread of antibiotic resistance (Trevisi et al. 2015).

In conclusion of the evaluation of 19 commercial DNA extraction kits and sequencing of two regions of 16S rRNA gene, the FastDNA SPIN kit for Soil by MP Biomedicals as the DNA extraction kit of choice for microbiota analysis of samples from the porcine GIT, Chapter 2.1 (Burbach et al. 2016). This suggestion was based on high quantity and purity values of extracted DNA both for ileal digesta and fecal sample material, as well as on the positive correlation of microbiota detection by T-RFLP and Illumina sequencing.

At about the same time, a comparative study on DNA profiling of the porcine microbiota was published (Lu et al. 2015). In contrast to the own evaluation, the work by Lu and colleagues is only based on three DNA extraction protocols and misses explanatory power on the suitability

for sample material collected from the upper GIT, like ileal digesta. In accordance, the highest quantity and purity of extracted DNA was reported for the FastDNA SPIN kit for Soil. However, for this DNA extraction kit, Lu et al. 2015 reported a biased ratio of recovered Gram-positive and Gram-negative bacteria, as they determined with regard to 454-pyrosequencing of the V6-V8 region of the 16S rRNA gene. This bias against bacteria cell wall types might have been caused by an inappropriate cell lysis condition, as Lu et al. 2015 reported a bead-beating step exceeding the 40 seconds recommended by the manufacturer's instruction.

Lu and colleagues recommended the PowerSoil® DNA Isolation kit by Mo Bio for microbiota profiling of porcine fecal samples (Lu et al. 2015). Within the own study, DNA extraction using this kit resulted in a low yield of DNA and the purity results indicated protein contamination, both for fecal and ileal digesta samples, see Chapter 2.1. Samples were obtained from a pig fed a diet of high soybean proportion, and this protein-rich feed matrix probably prevented an efficient DNA extraction.

Further variations between the two studies might be affected by the use of different sequencing platforms, Illumina MiSeq versus 454 GS FLX, and different variable regions of the 16S rRNA gene, V1-V2, and V5-V6 versus V6-V8. To discuss this aspect and the associated challenge by the establishment of standardization, briefly, the principle of the 16S rRNA amplicon sequencing approach is repeated.

Taxonomical classification of bacteria is enabled by variations in sequences of the nine variable regions of the 16S rRNA gene. Universal primers, which match conserved sequences flanking the variable regions, are used in sequencing approaches. So by NGS, not the full 16SrRNA gene sequence but partial read lengths are produced. And subsequently, these sequence reads are used as query in taxonomical assignment using a database like RDP. However, the choice of primers and to some extent as well the sequencing platform is known to influence 16S rRNA gene based analyses and restrict the transferability of results (Claesson et al. 2010). So for example, the microbiota structure recovered from samples included in the human microbiome project differed by targeting the V1-V2 and V3-V5 region (Lozupone et al. 2013). Sequencing of the V1-V2 region yielded into enriched proportions of Erysipelotrichi and Verrucomicrobia, whereas the proportions of Actinobacteria and γ-Proteobacteria were enriched by sequencing the V3-V5 region (Lozupone et al. 2013). Further, the variable regions of the 16S rRNA gene differ in taxonomical accuracy. So for example by sequencing the V4 region, members within the groups of Enterobacteriaceae and

Clostridiales cannot further be differentiated due to high sequence similarities (Jovel et al. 2016).

In Chapter 2.1, Illumina amplicon sequencing of V1-V2 and V5-V6 region demonstrated a high correlation of microbiota at the genus level, which supports a general good comparability between studies targeting these variable regions (Burbach et al. 2016). However, with regard to an underestimation of Actinobacteria by universal primers targeting the V1-V2 region, a forward primer including degenerated nucleotides was used for subsequent studies. This improved the general amplification of Actinobacteria sequences and in particular provided insights on dietary effects on Bifidobacterium, which are considered to be enriched in a healthy intestinal microbiota.

In addition to comparability between studies, methodological standardization is as well linked to an increased reproducibility of results. As the DNA extraction procedures, reported in Chapter 2.1, were performed only once from a single ileal digesta and a fecal sample, no insights in the reproducibility are provided. However, according to findings by others, the intra-subject variation is expected to be lower than the inter-subject variation in DNA-based microbiota analysis (Lu et al. 2015; Wagner Mackenzie et al. 2015; Hiergeist et al. 2016).

An additional approach for methodological evaluation of microbiota analysis is the implementation of internal standards. So for example, sample material from the GIT can be spiked by known quantities of atypical species, like bacteria adapted to extreme halophilic environments (Hiergeist et al. 2016). Consistent recovery of these known bacteria will enable qualitative and quantitative validation of the processed workflow and improve comparability between different studies. Further, the implementation of negative controls in DNA extraction and 16S rRNA gene sequencing encourages the detection of contaminations.

In conclusion, even if 16S rRNA gene based analysis is a common approach studying the porcine microbiota, a comprehensive transfer of knowledge is impaired by methodological diversity and by suggesting a suitable DNA extraction protocol the present thesis aims to improve this transfer.

3.2 A core microbiota among different dietary studies

Besides the evaluation of suitable methods, the present thesis includes four studies on the dietary influence on porcine GIT microbiota, which produced sequence data sets with thousands of OTUs, see Table 3-1. These data sets were now used to define a bacterial core community of the porcine GIT. By a highly strict definition, only taxa being present within

each analyzed sample count to a core microbiota. However, a comparison of varying core-definitions revealed unexpected robustness in the overall beta-diversity as was reported for the microbiota within marine sponges (Astudillo-García et al. 2017).

In DNA sequencing data sets from the human microbiome project, members of the core microbiota have been defined as being present in at least 95% of all samples from a certain body site (Huse et al. 2012). Thereby, across all samples, no core microbiota was identified at OTU level. This absence of common bacterial OTUs along the whole human body emphasizes high environmental variations of different body sites as well as highly individual variations. Similar, no common core OTUs were identified in the microbiota from porcine GIT, as investigated recently by a meta-analysis (Holman et al. 2017). Here, 16S rRNA gene sequencing data from 20 studies were downloaded from public databases and consistently bioinformatically processed using the QIIME software package. This meta-analysis defined a core microbiota by bacterial taxa being present in at least 90% of all GIT samples. Even if, at OTU level, no core microbiota has been identified, taxonomical classification into phyla and genera recovered a core community in more than 900 analyzed samples from porcine GIT (Holman et al. 2017).

On purpose of a comprehensive evaluation of porcine GIT microbiota, the core definition by Holman et al. 2017 was applied on the here presented studies, see Table 3-1. So, bacteria phyla and genera with a prevalence of 90% among all 868 samples were defined. Samples represented a longitudinal and a cross-section of porcine GIT. At phyla level, members of Firmicutes, Bacteroidetes, Proteobacteria, and Actinobacteria account to the core microbiota. The first three were also identified as core phyla by Holman et al. 2017, and equally Firmicutes and

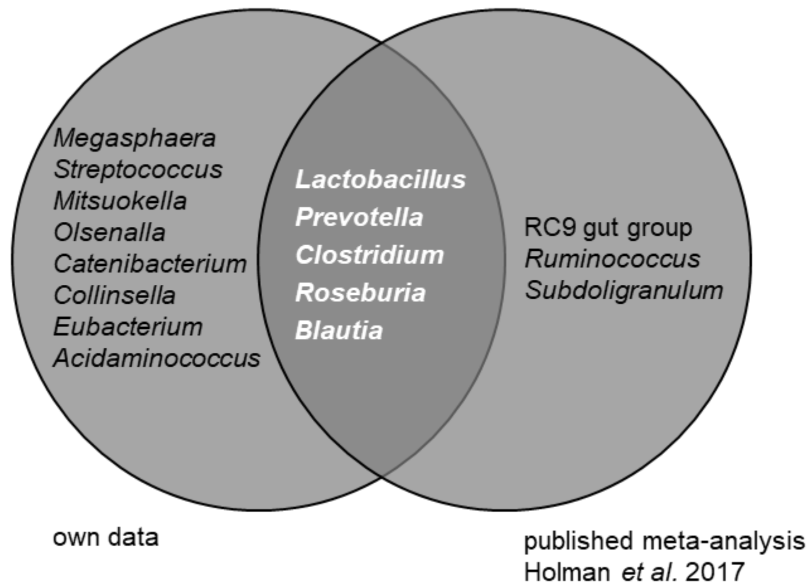
Table 3-1 Overview of amplicon sequencing projects, studying a dietary impact on porcine GIT microbiota. The column “Dietary effect (overall)” shows results of PERMANOVA result for the factor of experimental diet and significance is indicated by $P < 0.05$.

Project	Initial age of pigs	Initial body weight	Study duration	Dietary groups	GIT sites sampled	No. of samples	Sequence reads per sample	No. of OTUs	Dietary effect (overall)
Chapter 2.2	12 weeks	27.3 kg ± 1.0 kg	8 weeks	n = 2, cereal grain types	ileal content (cannula), feces	247	46396 ± 1470	2493	$P < 0.05$
Chapter 2.3	13 weeks	28.5 kg ± 0.64 kg	6 weeks	n = 4 + starter diet; Protein level (high, low) x Probioticum (-, +)	ileal content (cannula), feces	64	43643 ± 2070	2601	$P > 0.05$
Chapter 2.5	13 weeks	54.7 kg ± 4.1 kg	4 weeks	n= 4; Protein source (highly, low digestible) x CaP level (high, low)	feces	81	57901 ± 2139	3497	$P < 0.05$
Chapter 2.6	4 weeks	7.0 ± 0.4 kg	5 weeks	n = 4; Control diet supplemented with Hermetia larvae meal	gastric content, gastric mucosa, 1_3 small intestine content, duodenal mucosa, 2_3 small intestine content, jejunal proximal mucosa, jejunal middle mucosa, 3_3 small intestine, ileal proximal mucosa, ileal terminal mucosa, caecal content, caecal mucosa, colonic content, colonic mucosa, feces	476	38072 ± 1154	1420	$P > 0.05$

Bacteroidetes account together to nearly 85% of 16S rRNA gene sequences among all GIT samples. In contrast to the published meta-analysis, an increased weighting of Actinobacteria within the own data might refer to the choice of a degenerated primer.

Deeper in taxonomical composition, fourteen genera define the core microbiota in the own data sets, with five genera contributing as well to the core microbiota defined by Holman et al. 2017, see Figure 3-1. As discussed in Chapter 2.1, methodological variations cause variations in microbiota and might explain the varying cores at genus level. The meta-analysis by Holman et al. 2017 combined sequencing results derived from three sequencing platforms (454 FLX, Illumina MiSeq and Illumina HiSeq 2000), various variable regions of the 16S rRNA gene (V1-V3, V3, V3-V4 and V4) and twelve various DNA extraction methods. Whereas the own data sets derived from a consistent methodological workflow.

Taking a closer look at the common core genera, deviations are determined for the most abundant and prevalent genus, which was *Prevotella* in the core microbiota by Holman et al. 2017 and *Lactobacillus* in the own studies. However, the other one was respectively the second one, emphasizing the general importance of *Lactobacillus* and *Prevotella* in the porcine GIT microbiota. Properties of these two main bacteria represent the generally diverse properties of the GIT microbiota. So *Lactobacillus* is Gram-positive and facultative anaerobic, whereas *Prevotella* is Gram-negative and obligate anaerobically. According to oxygen sensitivity, *Lactobacillus* is found in higher abundance in the upper GIT, more precisely in the gastric mucosa. In contrast, *Prevotella* is found in the lower GIT, especially in the colonic mucosa (Holman et al. 2017). Within the own data sets, *Lactobacillus amylovorus* was a prevalent species, which is in accordance with results of a clone library analysis (Leser et al. 2002). This species is characterized by amylolytic activity and bile tolerance (Nakamura 1981; Kim et al. 2007). In contrast, only weak similarities have been identified between *Prevotella*-OTU sequences and known cultured species, with the highest similarity to the nonruminant supercluster and human oral isolates (Leser et al. 2002). *Prevotella* is known for its moderately saccharolytic activity and bile sensitivity, which probably supports its general high prevalence within porcine GIT microbiota (Shah and Collins 1990). Furthermore, a comparative study revealed the functional dominance of *Prevotella* in porcine fecal metagenome (Lamendella et al. 2011).



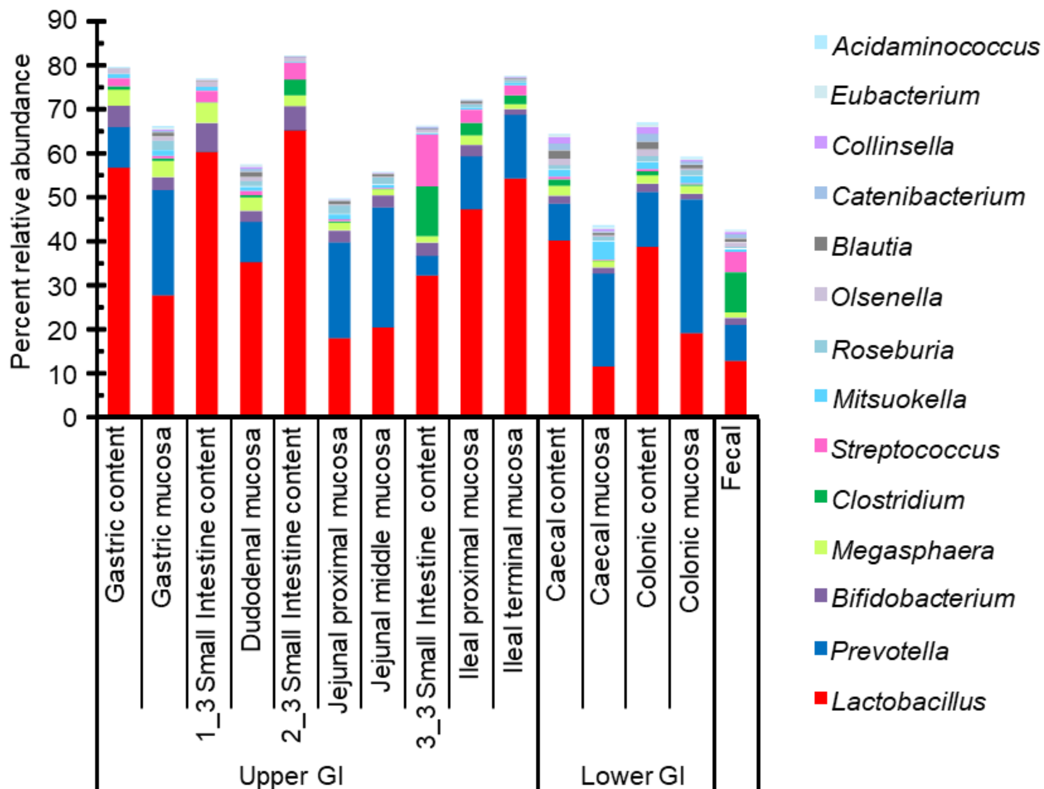


Figure 3-1 Core microbiota within studies of porcine GIT. A) Venn diagram depicting genera, identified in at least 90% of analyzed samples from porcine GIT, both in here presented studies and by a recently published meta-analysis. B) Relative abundance of core genera along the porcine GIT as defined by the here presented studies.

Among the other common core genera, *Roseburia* and *Blautia* are represented by a negligibly low abundance with less than 1%. Both genera belong to the family of *Lachnospiraceae*. Further, *Clostridium*, more precisely *Clostridium* sensu stricto, belongs to the family of *Clostridiaceae*. Members from *Lachnospiraceae* and *Clostridiaceae* encode genes of butyrate

synthesis pathways, so their presence in the core microbiota emphasizes the importance of butyrate-producing bacteria along the porcine GIT (Vital et al. 2014).

3.3 Factors affecting porcine GIT microbiota

In comparison to ruminants, the nutrition of monogastric animals places a higher requirement on the dietary composition. Corn and soybean meal represent the quantitative main components in diets for pigs, providing energy and easily digestible nutrients (Lindberg 2014). However, to maintain the health and growth of pigs, mutual interactions among host cells, diet and microbiota are considered throughout feeding strategies. The analysis of porcine GIT microbiota was the main focus in the here presented work and findings were considered together with factors of the diet and the host, see Figure 3-2.

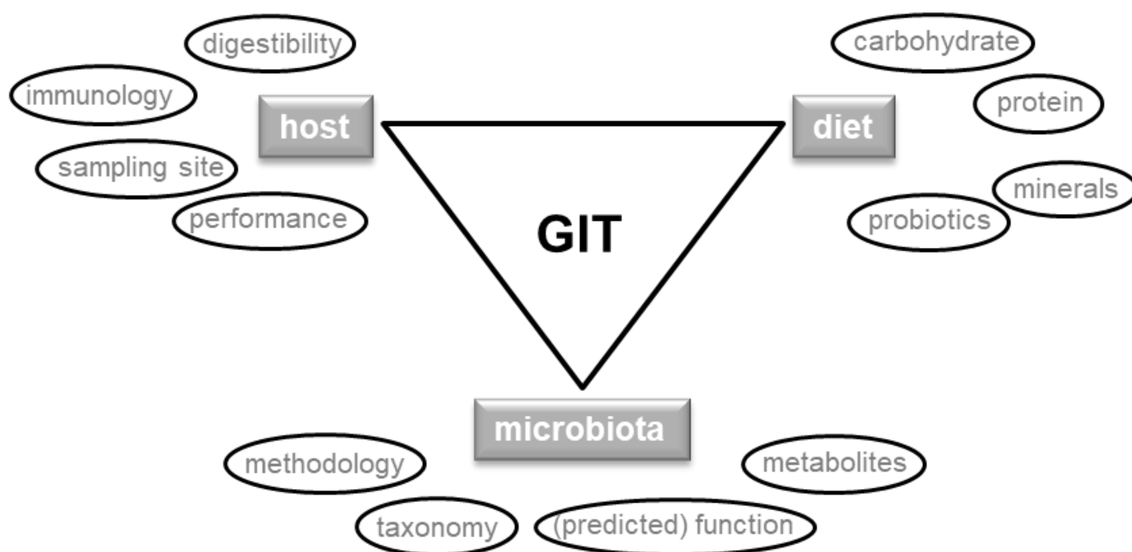


Figure 3-2 Schematic representation of the GIT ecosystem. The three components host, diet, and microbiota are displayed in frames and form interactions with each other. Each component combines further aspects with an impact on the GIT, and aspects, which are addressed by the here presented studies are highlighted by circles.

Microbiota structure is affected by varying chemical composition of the diet

In Chapter 2.2., a significant different microbiota structure was reported in ileal digesta and feces from pigs fed either rye or triticale. So far, these two cereal grains are of a relatively low relevance in the dietary formulation for pigs, probably due to a high content of indigestible, dietary fibers (Rodehutscord et al. 2016). However, attributes of cultivation and efforts in breeding make these cereal grains attractive for livestock nutrition. Studies on pig performance and carcass characteristics supported the suitability of triticale and rye (Zofia et al. 2011; Schwarz et al. 2015). The here analyzed samples derived from studies conducted to

compare eight genotypes of rye and triticale (Strang et al. 2016; Strang et al. 2017). Within the genotypes of each grain type, the protein and amino acid digestibility was not significantly different in growing pigs. According to these findings, varying samples of different genotypes were considered as replicates for rye and triticale in the published microbiota study (Burbach et al. 2017). Considering an above-described core microbiota, feeding pigs with rye promotes increased proportions of *Lactobacillus* and *Prevotella*, bacteria known for saccharolytic activities. Whereas triticale promotes the abundance of *Clostridium*, which indicates a more cellulolytic activity of the microbiota. To gain significant insights in functional profiles within the porcine GIT a meta-transcriptomic and proteomic approach will be needed. So, the results achieved by the correlation of bacterial OTUs abundance and SCFA concentration, as well as the predicted functions have to be considered as preliminary results. Even if significance is low, such tools give valuable insights in the complex mechanisms within the GIT, as was shown for butyrate. When comparing the dietary impact on bacterial metabolite butyrate, the gene prediction indicated a slight promoted butyrate production by triticale, whereas the fecal butyrate concentration was higher by rye. Such a discrepancy in metabolite production and detected concentration might be caused by the absorption by the host. By gene and protein expression analyses of monocarboxylate transporter MCT1 in colonic tissue samples, further insights in the host's butyrate absorption will be gained (Ritzhaupt et al. 1998; Metzler-Zebeli et al. 2012). Results of such analysis might strengthen the evaluating of health-promoting diets, as butyrate is an important energy source for colonocytes, induces cell differentiation and regulates growth of colonic mucosa (Kien et al. 2007). Beside interaction between the microbiota and host, butyrate is further associated with interactions among microbiota members, like the metabolic cross-feeding of bifidobacteria and butyrate-producing bacteria, mentioned above in Chapter 2.2.

Further dietary minerals like the calcium and phosphorus content showed effects on the porcine GIT microbiota. In contrast to varying cereal grain types, no significant discriminating variables were identified by the varying levels of calcium and phosphorus. Meta-proteomic analysis of samples from both pigs and chickens fed with varying calcium and phosphorus content indicated impaired nutrient availability probably to a physicochemical influence by the minerals (Tilocca et al. 2017; Tilocca et al. 2016).

The overall microbiota structure is not affected by dietary protein

In previous studies, porcine GIT microbiota was influenced by the source of dietary protein and additional by the levels of protein (Rist et al. 2014; Zhou et al. 2016). Dietary protein may

serve as substrate for bacterial protein fermentation yielding in the production of metabolites like ammonia and amines, see Figure 1-4. Increase in such metabolites has detrimental health effect and in addition, gives a strong smell to the manure. Therefore, there is a need to design feeding strategies which aim to use adequate amounts of dietary protein.

The diets analyzed in Chapter 2.3 and 2.4 expected to drive variations in porcine GIT microbiota due to previous studies using protein sources of varying digestibility (Rist et al. 2014). However, this reference study was conducted with pigs of younger age, which might have supported various observations. Further, the experimental diets were formulated with a relatively high amount of corn-starch, which might, in general, promote a microbiota of increased amylolytic and carbohydrate fermenting potential covering differences by protein (Rist et al. 2014). According to this aspect, the observed high abundance of Lactobacillus when the level of dietary crude protein is increased might rather be driven by available carbohydrate substrate than dietary protein (Rist et al. 2014; Kaewtapee et al. 2017).

A future perspective to meet protein requirement in pig nutrition might lie in feeding insects. So far in the European Union, insects as feedstocks are only allowed for fish but not yet for pig production (van Huis 2016). A dietary supplementation of *Hermetia illucens* larvae meal represents an innovative research approach, Chapter 2.6. Putative interactions with the porcine microbiota have been analyzed along the GIT and across varying micro-environments of digesta and mucosa. After an experimental duration of five weeks, the dietary supplementation of Hermetia revealed no overall differences compared to the control diet, both with regard to GIT microbiota and host's performance. So, it might be assumed, that feeding Hermetia has no effect on the ecosystem of the porcine GIT. However, an immunological challenge study indicated a dose-dependent response, when Hermetia was fed to piglets (personal communication, J. Klüss, FLI Braunschweig). As in conventional production sites living pigs are rarely in contact with insects, this innovative protein source might be recognized as dietary antigen and lead to an increased immune response. Similar to an immunological dose response, the bacterial α-diversity of some samples from the small intestine were influenced by feeding 2.5% and 10%, but not when feeding 5% of Hermetia-supplement. Further investigations are needed to fully ensure the utilization of *Hermetia illucens* larvae meal as feed-additive in pig nutrition. As dietary treatment showed mainly an effect within the upper GIT, the so far more studied fecal microbiota revealed only a restricted view on the relations between diet and porcine GIT microbiota, which should be considered in future studies. Certainly, the access to digesta samples from the upper GIT is more challenging than simply collecting feces. Next to a single sampling after euthanasia, repeated

samplings are enabled by a surgical insertion of a cannula into the animal's GIT. From the outside, the cannula is closed by a cap and for the sampling procedure, a plastic bag is attached to the barrel of the cannula. The ileal cannulation of pigs is a commonly established method to determine nutrient digestibility. Such cannulated pigs have been used in studies of Chapter 2.1, 2.2 and 2.3. The cannulation approach benefits of a randomization feeding of low animal numbers as well as longitudinal studies on digesta. However, it is worth noting that cannulated pigs demand an increased animal caretaker task.

Potential experimental drawbacks in assessing dietary effects on microbiota

The experimental diet in Chapter 2.2 contained to 95% solely rye or triticale, so here the dietary impact on the microbiota refers to variations in chemical composition of these cereal grains. In practice, pigs are more fed with compound feed, like it was used by the other studies in Chapter 2.1, 2.3 – 2.6. Here, relatively weak variations in dietary composition might be responsible for weak impact on the overall microbiota structure, see Table 3-1. However, even if not statistically proven, the relative composition of microbiota showed trends of variations due to the dietary treatment.

Next to minor differences in dietary composition, a putative dietary impact might be influenced by the experimental adaptation time, which was reported by the study in Chapter 6. Here, changes in the microbiota structure, both on taxonomy and functional level, were investigated along four weeks. Significant dietary impact on fecal microbiota was detected for the dietary factor of calcium-phosphorus (CaP), but only in samples collected at the third and fourth week of the experiment. Further, the microbiota analysis revealed a gradual adaptation process. Similar, a gradual adaptation by the host was demonstrated by the meta-proteomic results. So, it was suggested, that differences in microbiota structure might rather be driven by an altered host's metabolism than being directly influenced by dietary CaP content (Tilocca et al. 2017). This aspect highlights again a mutual interaction potential within the GIT ecosystem. The combination of 16S rRNA gene based and meta-proteomic analyses strengthen the study's significance. With regard to these findings, a prolonged adaptation time of three to four weeks was recommended for nutritional studies on porcine GIT microbiota, which might be transferred to studies on human GIT microbiota, as well (Tilocca et al. 2017).

In conclusion, results of the present thesis strongly promote the advantageous application of standardization in studies of intestinal microbiota. A unified analysis approach will improve knowledge on the actual diversity of the microbiota. So next to consistent DNA extraction and use of 16S rRNA gene region, it is suggested to conduct dietary studies with individuals

of the same age and weight and thus strengthen conditions of a consistent environment of the GIT. In addition, presented results support a prolonged adaptation time to the diet. In pig research, such standardization has not yet been announced. The analysis of samples derived from various sites of the GIT is considered to be advantageous to enhance insights into dietary effects on the microbiota. Furthermore, presented studies emphasize 16S rRNA gene based studies as a valuable component of both complementary and multidisciplinary approaches for challenging elucidation of the porcine GIT.

3.4 References (Discussion)

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Curriculum vitae

Personal data

Name	Katharina Burbach
Date of birth	20 August 1987
Place of birth	Esslingen am Neckar
Address	Stöckachstraße 3, 70190 Stuttgart, Germany
Email	katharina.burbach@uni-hohenheim.de; kburbach@gmx.de

Work experience

5/ 2014 – 3/2018	**Doctoral student** at the subinstitution ”Feed-Gut Microbiota Interaction”, Institute of Animal Science, University of Hohenheim, Stuttgart
6/ 2013 - 2/ 2014	**Student research assistant** at Institute for Ophthalmic Research, University Hospital Tübingen, Tübingen
12/ 2012, 12/ 2013	**Student assistant** for a botanical-microscopy course ‶Bau und Funktion der Pflanzen”, Department of Plant Physiological Ecology, University of Tübingen, Tübingen

Education

5/ 2014 – 3/2018	**Doctoral student** at University of Hohenheim, Stuttgart Topic of PhD thesis**:** “*The porcine intestinal microbiota: studies on diversity and dietary impact*”
10/ 2010 - 8/ 2013	**Master of Science in Biology** at University of Tübingen, Tübingen Area of specialization: Microbiology Topic of the M.Sc. thesis: “Studies on the topology and biogenesis of the autotransporter-adhesins Invasin and Intimin”; grade: 1,6 (good)
10/ 2007 - 8/ 2010	**Bachelor of Science in Biologie** at University of Tübingen, Tübingen Topic of the B.Sc. thesis “Physiological characterization of *amiC*2-mutante of *Nostoc punctiforme* ATCC29133“; grade: 2,4 (good)
9/ 1998 - 6/ 2007	**Abitur** at Schenk-von-Limpurg Gymnasium, Gaildorf grade: 2,1

Stuttgart, March 7, 2018

Katharina Burbach

Eidesstattliche Versicherung

gemäß § 7 Absatz 7 der Promotionsordnung der Universität Hohenheim zum Dr. rer. nat.

Bei der eingereichten Dissertation zum Thema

The porcine intestinal microbiota: studies on diversity and dietary impact

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____________________________ ____________________________

Ort und Datum Unterschrift

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