EFFECTS OF DIETS WITH DIFFERENT PHOSPHORUS AVAILABILITY ON THE INTESTINAL MICROBIOTA OF CHICKENS AND PIGS

Bruno Tilocca

2019

Institute of Animal Science

University of Hohenheim

Feed-Gut Microbiota Interaction

Jun. Prof. Dr. Jana Seifert

EFFECTS OF DIETS WITH DIFFERENT PHOSPHORUS AVAILABILITY ON THE INTESTINAL MICROBIOTA OF CHICKENS AND PIGS

INAUGURAL DISSERTATION

submitted in fulfillment of the requirements for the degree

Doctor of Agricultural Science (Doctor scientiarum agriculturae)

to the

Faculty of Agricultural Science

presented by

Bruno Tilocca

Born on 13/03/1988 in Sassari, Italy

2018

The Faculty of Agricultural Sciences at the University of Hohenheim accepted this thesis as a doctoral thesis (Dissertation) in fulfillment of the regulations to acquire the doctoral degree "Doktor der Agrarwissenschaften" on April 25, 2018.

Dean of the faculty	Prof. Dr. Ralf Vögele
Date of the oral examination:	June 22, 2018
Chairperson of the oral examination:	Prof. Dr. Jörn Bennewitz
Dissertation supervisor:	Prof. Dr. Jana Seifert
External reviewer:	Prof. Dr. Sergio Uzzau
Third reviewer:	Prof. Dr. Dr. h.c. Rainer Mosenthin

In memory of the friends

that are looking down on us

The research projects presented in this work were funded by Carl Zeiss Stiftung and the Ministry of Science, Research and the Arts of Baden Württemberg-Germany (microP and METAPHOR projects) which are sincerely acknowledged.

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LIST OF SYMBOLS AND ABBREVIATIONS

%	Percentage
°C	Degree Celsius
2-DE	bi-dimensional electrophoresis
2D-DIGE	Bi-dimensional-Differential In Gel Electrophoresis
bp	Base pairs
BW	Body Weight
CaP	Calcium-phosphorus
COG	Cluster of Orthologous Groups
Da	Dalton
dl	Deciliter
DM	Dry matter
DNA	Deoxyribonucleic acid
e.g.	For example
EBI	European Bioinformatic Institute
et al./et al.	et alii/et aliae/et alia
g	gram
GIT	Gastrointestinal tract
HCD MS/MS	Higher-energy Collisional Dissociation Mass Spectrometry
HPLC	High-performance liquid chromatography
i.e.	That is
IDs	Identities
IMG/M	Integrated Microbial Genomes and Metagenomes
InsP	Inositol phosphate
InsP1	Myo-inositol monophosphate
InsP2	Myo-inositol biphosphate
InsP3	Myo-inositol triphosphate
InsP4	Myo-inositol tetrakisphosphate
InsP5	Myo-inositol pentakisphosphate
InsP6	Myo-inositol 1,2,3,4,5,6-hexakisphosphate
KEGG	Kyoto Encyclopedia of Genes and Genomes

kg	Kilogram
LC-MS/MS	Liquid Chromatography-tandem Mass Spectrometry
M	Molar
m	Meter
m/z	mass-to-charge ratio
MCP	Monocalcium Phosphate
MEGAN	MEtaGenome ANalyzer
mg	Milligram
MG-RAST	Metagenomics-Rapid Annotation using Subsystem Technology
min	Minute
ml	Milliliter
mm	Millimeter
mmol	Millimole
mol	mole
MP	Microbial Phytases
ms	Millisecond
MS	Mass Spectrometry
MS/MS	Tandem Mass Spectrometry
MS^1	First mass spectrometry measurement
MS^2	Tandem mass spectrometry measurememt
NCBI	National Center for Biotechnology Information
nl	Nanoliter
nMDS	non-Metric Multidimensional Scaling
P	Phosphorus
p\P	Probability
PCoA	Principal Coordinate Analysis
PCR	Polymerase Chain Reaction
pH	PH-Value
PO_4^{3-}	Phosphate

rRNA	ribosomal Ribonucleic acid
S	Svedberg
SCFA	Short-chain fatty
SCFAs	Short-chain fatty acids
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
sec	Second
SEM	Standard error of the mean
spp.	Species
t-RFLP	Terminal Restriction Fragment Length Polymorphism
UHPLC	Ultra High-Performance Liquid Chromatography
µl	Microliter

CHAPTER 1

INTRODUCTION

1.1 Introduction

Phosphorus (P) is the second most abundant mineral in the animal body after calcium (Ca). Approximately 80% of the body P is mineralized as hydroxyapatite, the main mineral composing bones and teeth. The remaining 20% of P is commonly found in the internal organs and body fluids where it is involved in a variety of physiological functions of vital importance. [1]. Indeed, P is a building block for the nucleic acids and regulatory phosphoproteins, hence, of essential importance for the cell growth and differentiation, besides the transmission of the genetic code. Phospholipids are involved in maintaining the integrity and fluidity of the cellular membranes, other than participating to the nerve myelination. Inorganic phosphate (PO_4^{3-}) contributes to maintaining osmotic pressure and the acid-base balance. Moreover, P plays a pivotal role in the energetic metabolism and energy transfer via AMP, ADP and ATP with strong implications both at cellular (*i.e.* glycolysis/gluconeogenesis, protein synthesis, fatty acid transport, Na^+/K^+ pump) and systemic level (*i.e.* control of appetite, efficiency of feed utilisation) [1-4].

Chickens and pigs diets are mainly based on cereals assumption. In cereals, P is mostly present in the seed coat, where 70-80% of the total phosphorus is stored in the form of phytate (myo-inositol 1,2,3,4,5,6-hexakisphosphate, InsP6) [5, 6]. The structure of phytate consists of a poly-phosphorylated *myo*-inositol ring where each of the six carbon is esterified with a phosphoric group [7, 8]. Plants and cereals are rich in phytases and acid phosphatases, the enzymes involved in the hydrolysis of phytate to low-molecular inositol phosphates (InsP5, InsP4, and InsP3), with the consequent release of one, two, and three PO_4^{3-} molecules, respectively [9]. Diverse classes of phytases are distinguished depending on their pH optima, temperature, stomach stability, and substrate specificity, with most of the enzymes preferring to hydrolyse equatorial rather than axial PO_4 groups. Also, phytase activity is strongly influenced by the diet composition [10]. Natural sources of phytases are plants, gut microbes and intestinal epithelial cells. However, abundance and activity of phytase in the latter two is rather reduced in monogastric animals (including chickens and pigs), leading to a reduced bioavailability of the organic phosphorus content in the feedstuff [11]. This, in turn, results in a direct excretion of the bound phosphorus through the kidneys along with other bivalent cations complexing the phytate [10, 12]. To date, several strategies have been developed in the attempt to overcome this issue. Of these, supplementation of microbial phytases in the diet composition is among the most promising strategies, enabling both for increased availability of phosphorus and its reduced renal excretion [10]. Microbial phytases (MP) are produced intracellularly by Gram-negative bacteria, whereas Gram-positive bacteria and fungi produce these enzymes

extracellularly [9]. Microbial phytases commonly supplemented in poultry and swine diets arise from fungi (mainly *Aspergillus niger* and *Peniophora lycii*) and Gram-negative bacteria (*Escherichia coli*), demonstrating a higher efficiency than plant-derived phytases (*e.g.* wheat and rye) [9, 11].

Diet is one of the major environmental factors shaping the composition and activity of the microbial community harboured in the host intestine [13, 14]. Hence, the introduction of new ingredients in the feed formulation, as well as over/under loading of the dietary constituents, may impair the gut bacterial composition and the complex network of interactions occurring between the intestinal microbial specimens and their host. In this context, a large number of diets have been optimized in order to improve diverse aspects of animal husbandry such as stimulation of a beneficial bacterial community and enhance of digestive functions [15]. Modulation of the dietary calcium-phosphorus (CaP) and supplemented MP, represent a valuable strategy to functionally characterize the bacterial specimens involved in the P accessibility and uptake. This allows a comprehensive understanding of how the intestinal bacteria adapt to a new diet and the metabolic routes affected by changing levels of supplemented P and/or MP. Resulting information could be integrated for a future diet optimization, enabling for a targeted stimulation of the intestinal bacteria aimed to improve P bioavailability and reduce P excretion.

1.2 The gut microbiota of chickens

The old notion of "organism" intended as a singular entity is nowadays being outclassed, to accept the novel view of the "superorganism" or "metaorganism". This innovative perspective integrates the functional districts of the body along with its associated microbial community [16, 17]. The ensemble of microorganisms harbored in a given ecological niche (*e.g.* the gut) is referred as "microbiota"; whereas, it is defined "microbiome" the microbial community inhabiting a given ecological niche along with their genetic elements and the relationships occurring among members and the environmental habitat where they survive [18]. Such an overall vision opens new avenues in understanding the physiologic processes orchestrating the functions of diverse districts of the organisms.

At birth, animals are essentially sterile but immediately after birth all body surfaces (*i.e.* skin, oral cavity, vagina) and the gastrointestinal tract (GIT) are colonized by an impressive number of microorganisms, including bacteria, archaea, fungi and viruses [19, 20]. The onset of the animal microbiota starts as a dynamic ecosystem where the microbial composition increases both its diversity and richness until achievement of the highest complexity in the adult animals

[21]. At this stage, the diverse physico-chemical features of each anatomical site enable the maturation of a site-specific microbiota, which composition is the result of the environmental influence and the continuous competition and coexistence between microbial specimens [17].

The gastrointestinal tract (GIT) microbiota is the most investigated microbial community in both humans and animals. The gut microbiota of adult animals is mainly composed of bacteria, but also virus, fungi, archaea and protozoa are included. It has been estimated that, in humans, bacterial cells harbored in the intestine outnumber of approximatively ten times the number of host cells, comprising a microbiome that consists of more than five million genes, outnumbering the host´s genetic potential of about 100-fold [22, 23]. This huge potential of gene products enables for a wide array of metabolic activities of the intestinal microbiome. Commensal microorganisms colonizing the intestine establish mutualistic relationships that concern a variety of function and physiological processes, including organ development and morphology [24, 25], modulation of the host's immune system [26, 27] and shaping of the metabolic and digestive activity [28, 29]. The figure listed below (Figure 1) provides a schematic view of the major physiological processes where the gut microbiota is known to be involved in.

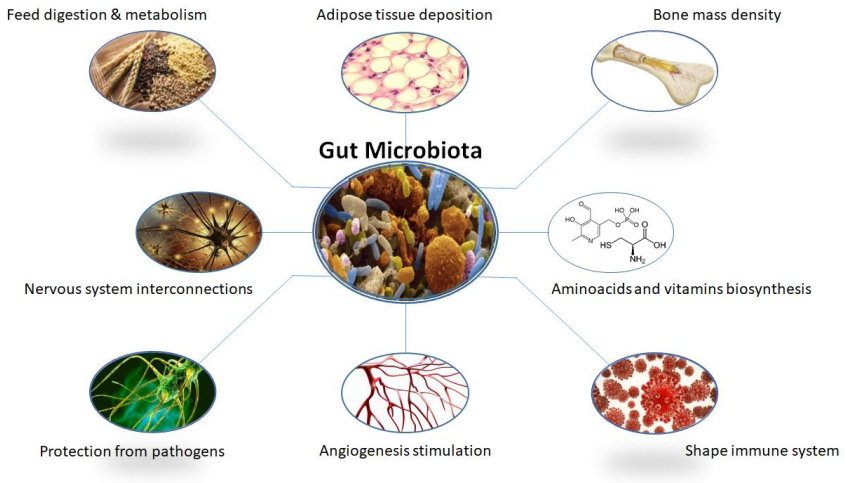

Fig. 1. The gut microbiota and the major physiological processes where it is involved in.

The chicken GIT comprises three upper segments (*i.e.* crop, proventriculus and gizzard) and three lower segments (*i.e.* small intestine, ceca and large intestine). Crop represent the food

storage segment and fermentation processes initiate in this organ. Feed digestion continues in the proventriculus, also known as the glandular stomach, following the addition of hydrochloric acid and digestive enzymes to the feed. Gizzard is the mechanical stomach providing the mashing and mixing of the feed particles. The small intestine is further divided into three main sections. Duodenum represents the first section; here, the acidic secretion from the upper gastrointestinal tract are mixed with the bile and pancreatic juices, leading to a sudden alkylation of the environment that marks the beginning of the digestive processes. It has been estimated that 95% of the fat digestion occurs in the duodenum [30, 31]. Jejunal segment extends from the duodenal loop until the Meckel's diverticulum. Main feature of this section is represented by its large size, enabling the digestion and adsorption of the major nutrients, despite a reduced retention time of 40-60 minutes [32-34]. Ileum is the third segment of the small intestine, it ends at ileo-cecal junction and plays a pivotal role in the absorption of water and minerals. Only a minor activity has been observed in digestion and absorption of fat, carbohydrates and proteins [35]. Ceca in chickens are two blind sacs that mark the joint point between the small and the large intestine. In these segments, materials of ileal and renal origin are subjected to long retention times, enabling for most of the electrolytes and water absorption [36]. Moreover, the ceca are an important site for the fermentation of any residual raw material with the consequent production of SCFA and B-group vitamins. Ceca empty their content two to three times per day, producing cecal droppings, whose color and texture are used to evaluate the normal functionality of the chicken´s digestive tract [36, 37]. The large intestine represents the last small segment of the digestive tract where occur the reabsorption of the last water content in the fecal material and the digestive wastes are mixed along with the wastes from the urinary system.

Although GIT sections are shown to be strongly interconnected among each other [38], a certain degree of variability occur between the microbiota of the diverse GIT segments (Figure 2). This poses the need to consider them as independent ecosystems. Crop and gizzard share a very similar microbiota, dominated by *Lactobacillus* spp. and *Clostridiaceae*. Slight differences are imputable to the reduced fermentation activity of the gizzard reflected as a reduced amount of *Enterococcus* spp. and *Bifidobacteria* spp. in its microbiota composition [39]. Duodenal microbiota is almost exclusively composed of *Lactobacillus* spp. (up to 99% of the total diversity), but Streptococcus spp. and *Enterobacteriaceae* may also be abundantly identified [40]. Despite being dominated by *Lactobacillus* spp., the ileum is considered as a source of novel bacteria, including butyrate-producing species and other novel bacteria involved in nutrient availability and absorption; *Clostridium* spp., *Streptococcus* spp. and *Enterococcus*

spp. are also found to dominate the ileal environment [39, 41, 42]. Cecal microbiota is the most investigated GIT segment since it is an important site of fermentation, enabling the digestion of food rich in cellulose, starch, and resistant polysaccharides. Moreover, the cecum is also the principal site of water reabsorption and nutrient transport and absorption. Therefore, elucidation on the microbial profile harbored in this section provide insights on the mechanisms of food utilization and open new frontiers for the animal husbandry optimization. Ceca is the most colonized section and is featured by a very high bacterial diversity. Here, the most abundant bacterial specimens are *Clostridiaceae*, *Bacteroidaceae*, *Lactobacillus* spp., Proteobacteria, and unknown Firmicutes [39].

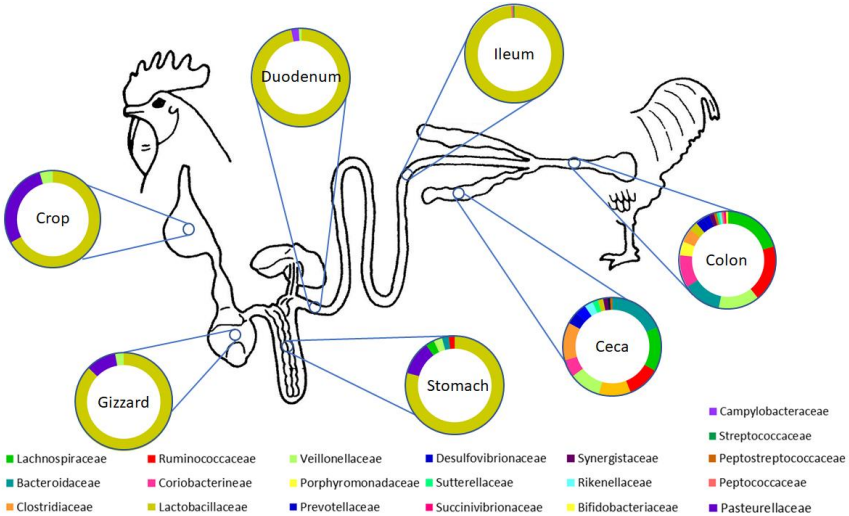

Fig. 2. Distribution of the bacterial families dominating the chicken GIT sections. Section-specific composition of the microbiota has been drawn according to the data published by Videnska et al. [43].

Owing the importance of the chicken GIT microbiota and its implication in host physiology, several studies have been so far performed on the attempt to elucidate the mechanisms employed by the GIT microbiota under diverse circumstances. A handful of studies focused on the characterization of the chicken gut microbiota both in terms of composition and functions. Sergeant and colleagues recently investigated the cecal microbiota using a deep microbial community profiling technique; whilst, information on the potential functions were obtained through metagenomic investigation [44]. Similarly, Tang et al. (2014) investigated the chicken fecal microbiota via 16S rRNA gene sequencing and metaproteomics, elucidating the bacterial

phylogenetic distribution and the ongoing metabolic processes in the gut of a healthy chicken [45]. Chicken gut microbiota has been also extensively studied in regard to improving animal health and husbandry strategies. A previous study from Singh et al. (2014) investigated, in a comparative manner, the fecal microbiome of low- and high- feed conversion rate chickens, underlying that genes involved in stress, sulphur assimilation and flagellar motility are over-represented in low-feed conversion rate animals [46]. Danzeisen et al. (2011) investigated the chicken cecal microbiome to evaluate the effect of the coccidiostatic monesin and the growth promoters tylosin and virginiamycin. The study provided a first insight on the cecum-associated microbial community and how it is modulated by growth promoters, which is of great importance while developing alternative approaches for animal growth promotion [47]. Another recent study performed by Polansky and colleagues investigated the chicken cecal microbiome following inoculation with cecal extracts from chickens of different ages, in order to elucidate the colonization patterns and predict the most promising probiotic genera for cecal colonization of newly hatched chickens [48]. Besides the above-mentioned studies, the chicken GIT microbiota is also studied in relation to other contexts such as the influence of the dietary intake [49, 50], host genetics [51, 52], gender [51], age [53-55], and spatial microbial diversity [49, 55-57].

1.3 The gut microbiota of pigs

The porcine digestive system shows both anatomical and physiological similarities to humans. It is composed of several organs (mouth, esophagous, stomach, small- and large- intestine) connected in a continuous musculo-membranous tube expanding from mouth to anus [58]. Similarly to humans, each of the porcine GIT section is featured by specific functions and micro-environment, hence, harbor a specific microbial community [58]. Microbiota associated to the porcine intestine is a very dynamic ecosystem with a microbial density that increases in a cranio-caudal manner. Although all types of microorganisms are harbored, bacteria represent the principal intestine colonizers, which amount tends to increase from the proximal small intestine (10^3 to 10^4 cells per g of digesta) until the cecum and colon (large intestine), where over 500 bacterial species are estimates in a density of 10^{10}-10^{11} cells per g of digesta [59, 60]. In accordance with its reduced bacterial abundance, ileum shows the lowest bacterial diversity with Firmicutes and Proteobacteria being the principal phyla colonizing this section. Moving towards the large intestine, a progressive increase in the bacterial abundance and diversity is observed. This is most likely due to the favorable environmental conditions (*e.g.* pH increase), enabling the growth of a variety of other bacterial phyla such as Bacteroidetes and Spirochaetes.

Other bacterial phyla such as Tenericutes, Fibrobacteres, Planctomycetes, Actinobacteria, and Synergistetes can also be detected even though with a minor contribution to the total bacterial diversity [59]. A schematic overview of the composition of the bacterial community inhabiting the most investigated pig gut sections is displayed in figure 3.

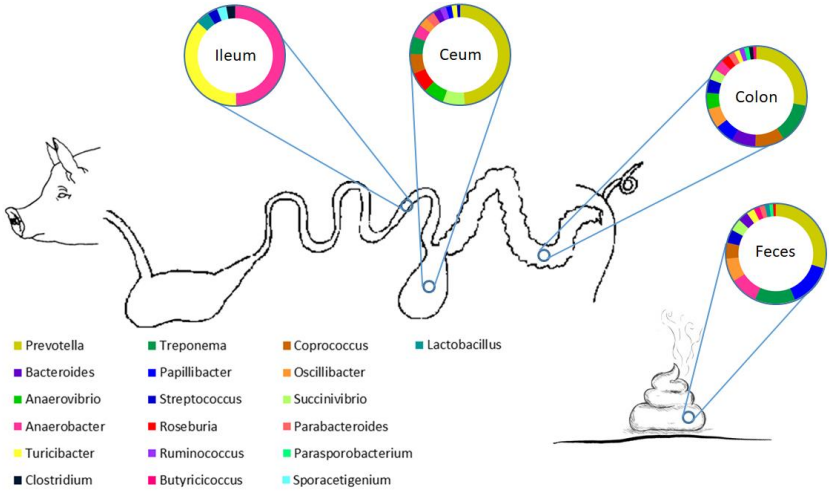

Fig. 3. Distribution of the bacterial genera dominating the porcine GIT sections. Section-specific composition of the microbiota has been drawn according to the data published by Looft et al. [59].

Due to the strong relationships occurring between the gut microbiota and the host metabolism, and the high anatomical and physiological similarities with human beings, several studies are performed on porcine microbiota primarily intended as a model for human translational studies [58]. Specifically, porcine microbiota is carefully investigated in relation to metabolic disorders of human concern such as diet-induced obesity [61], necrotizing enterocolitis [62] and amino acid metabolism [63, 64]. Other studies aim to investigate the pigs microbiota in the attempt to develop innovative [65], ecologic [66] and cost-effective feeding strategies [67]. To this purpose, particular interest is being attributed to the problem of P resources depletion and the issue of water eutrophication due to the fecal excretion of P excess. Recent studies demonstrated an important implication of the gut microbiota in the phosphorus uptake and maintaining of the calcium-phosphorus (CaP) homeostatic balance (reviewed in [68]). Besides being important for the host physiology, phosphorus has a strong impact on microbiota structure and metabolic processes. Bacteria might store P as polyphosphate and it may be used as an energy and P source

to involve in metabolic processes and for bacterial proliferation. Previous *in vitro* studies demonstrated that sufficient P levels are required in the culture medium for the bacterial synthesis of fibrolytic enzymes and P act also as a coenzyme of essential importance for the bacterial degradation of fibers [69]. Phosphorus concentration of the culture media was also positively correlated with the growth yield of *Bacteroides amylophilus* [70]. Metzler and colleagues performed a study on ileally-fistulated pigs kept at diets with low P levels and differently fermentable carbohydrates. Obtained results indicate that carbohydrate fermentation is associated to a higher ileal uptake of P and a reduced excretion through the feces, whereas the bacterial fermentation of the carbohydrates was strongly dependent on the fermentable carbohydrate supplied [71].

The effect of variations of dietary P levels and carbohydrate sources on the porcine microbiota have been investigated in a handful of studies and elegantly reviewed in [68]. A previous study of Metzler-Zebeli et al. (2013), conducted on growing pigs over an experimental time of 14 days, showed that feeding diets with a high CaP content stimulate an increased amount of gastric *Enterobacteriaceae* as well as increase in the ileum-associated *Bacteroides* spp, *Prevotella* spp, *Porphyromonas* spp, *Enterococcus* spp and *Campylobacter* spp. Although the dietary impact on specific bacterial groups was shown, the authors conclude that the intestinal microbiota of piglets is rather resistant to dietary modulation such as the changes in the CaP content and carbohydrate sources [66, 68]. Similar results were obtained in a study focused on the effect of dietary P and inulin supplementation on the ileal digesta microbiota composition and activity. Here, authors could not observe any effect attributable to the dietary CaP content neither concerning the microbiota composition, nor on its activity [72]. Further studies demonstrated that increased P level (either supplemented as CaP or phytase-derived) is associated to an increased gene copy number of Clostridium cluster XIVa both in the distal ileum and cecum [73, 74]. Also, high CaP levels are linked to a reduction of lactate and propionate concentration in the stomach, as well as a decreased *Streptococcus* spp. gene copy number [73].

A study conducted on ileally-fistulated pigs to investigate the effect of dietary Ca, P and MP supplementation, shows that a high monocalcium phosphate (MCP) dietary supplementation is associated to an enhanced Ca and P uptake both at ileal and fecal level. A bacterial incorporation of Ca and P in the mixed bacterial mass have also been observed as a result of the increased P availability [75]. On the other hand, dietary supplementation of MP resulted in a decreased P recovery in the mixed bacterial mass at ileal and fecal level. Moreover, the reduced P content in the mixed bacterial mass indicates a decreased P availability for the bacteria, leading to a

limited fermentation activity as already observed in ruminant model [76]. Effects of the MCP supplementation in the ileal microbiota include a lowering of the gene copy numbers of *Enterococcus faecium* and *Clostridium leptum*; whereas, the dietary supplementation of MP stimulate the growth of *Bacteroides* spp, *Prevotella* spp, *Porphyromonas* spp, *Clostridium leptum* and *Clostridium coccoides* [74]. Although associated to the increased P availability, some of the changes due to the MP supplementation were not observed in the case of the diet supplemented with MCP. In this view, authors suggest that the high level of Ca co-administered in the MCP diet may inhibit the proliferation of specific bacterial groups, hence leading to the differences observed while feeding MCP- or MP-supplemented diets.

Inositol phosphates (InsPs) and P can also challenge bacterial properties such as their metabolism and/or virulence [68]. Several pathobionts and pathogenic microorganisms evolved to use the host´s InsP metabolism to ensure their replication and optimal survival conditions along the GIT sections [77, 78]. Enteropathogens such as *Enterococcus fecalis*, *Listeria monocytogenes*, and *Bacillus cereus* are capable of *myo*-inositol utilization as carbon and energy source. Other mechanisms adopted by human (*e.g. Vibrio cholerae*) and porcine (*e.g. Clostridium perfringens*) pathogens include $InsP_6$-induced auto processing of toxins for toxin activation and delivery to the target cells [79, 80]. Nevertheless, it is still not clear to what extend the dietary P and InsP can modulate the metabolism and virulence properties of the intestinal pathogens, for which further researches are required.

1.4. Methods to characterize the intestinal microorganisms

1.4.1 The microbiota investigation in the "pre-omics era": Culture-based methods

The comprehensive understanding of the complex biological network featuring a given organism (or consortia) requires a reciprocal integration of the genome-derived facts along with the environmental variables.

The gut microbiota is recognized as one of the most important environmental factor and through its metabolic output, interaction with diet, lifestyle, and xenobiotics, it appears to strongly impact the whole biology of the living systems of diverse organization levels [81, 82]. Initial investigations of the gut-related microbial community were performed through traditional microbiological methodologies, mostly based on pure cultures. Single strains of microorganisms were cultured in selective medium under controlled environmental conditions (temperature, pH, humidity, % CO_2 etc.) to ensure an optimal bacterial growth. Cultured microorganisms were subsequently characterized on the basis of genetic and biochemical assays, enabling a broad evaluation of their metabolic capability. This allows an estimation of

the physiological importance of given microorganism in the context of the gut-associated microbial community. In this view, cultivation strategies have had a pivotal role in providing a knowledge base on single microorganism strains and, still today, cultivation attempts are needed to improve our knowledge to both known and unknown specimens. Although it has been recently demonstrated the potential possibility to cultivate more than 90% of the major gut colonizer through multiple culture conditions [83, 84], these strategies still suffer of the long times required by the microorganisms to growth and their inapplicability to the quantitative assessment of the microbiota composition, due to the cultivation step. Moreover, phenotypic characterization of the cultured fraction encounters the additional problem that many bacteria, even phylogenetically diverse, share many phenotypic traits, besides their capability of growing in the same selective media and growing conditions [81]. On the basis of these reasons, the traditional microbiological culture approaches are commonly considered as unsuitable for the study of microbial consortia, such as the case of the gut microbiota, although still in use either as a self-standing method or flanked with other culture-independent methods.

1.4.2 Culture-independent methods for studying the gut microbiota

The first alternative to the culture-based techniques is represented by the community fingerprinting techniques, aimed to provide an overall depict (*i.e.* fingerprint) of the investigated microbiota [85]. These techniques include denaturing gradient gel electrophoresis (DGGE), temperature gradient gel electrophoresis (TGGE), and terminal restriction fragment length polymorphism (T-RFLP). Although based on different principles, all the above-mentioned techniques share the initial step of 16S rRNA gene amplification [86-88].

The 16S rRNA gene is composed of highly conserved regions flanking hypervariable regions that provide a species-specific sequence, exploitable for bacterial identification. After Woese pioneered the bacterial identification via 16S rRNA gene analysis in the mid-'70s [89], a number of molecular tools facilitating the bacterial identification were developed and, still today, the 16S rRNA gene-based identification of bacteria is adopted for reclassifying previously cultured bacteria or even describe new bacterial species that have never been cultured [90]. 16S rRNA gene analysis is also extensively used in the investigation of the gut microbiota. Here, the microbial composition is determined through a direct extraction of the bacterial DNA from the intestinal environment, avoiding the cultivation step, thus the loss of the uncultivable specimens. Universal primers are subsequently adopted to amplify regions of the 16S rRNA gene, creating a clone library of the amplified fragments [81].

In DGGE studies, 16S rRNA amplicons are separated through a gradient of DNA denaturant (*e.g.* urea), enabling the separation of DNA fragments of similar size on the basis of their sequence properties. Assuming that every sequence variant represents a different bacterial specimen, the profile produced by this technique indicates the overall taxonomic diversity of the investigated microbiota in a rapid and cost-effective manner [87]. Similar information are also provided by TGGE technique although profile produced by this technique depends on the application of a gradient of temperature (instead of urea) to discriminate 16S rRNA fragments according to their sequence properties [91].

Terminal restriction fragment length polymorphism (T-RFLP) utilize marked (generally fluorescent) DNA fragments to produce the community profile. To this purpose, one of the primer adopted in the 16S rRNA gene amplification must be fluorescently labelled. Marked 16S rDNA fragments are subsequently subjected to restriction through restriction enzymes that cut the DNA sequence at definite recognition sites. Assuming that each bacterial specimen is featured by a specific DNA sequence, the restriction enzymes will produce an array of restriction fragments of different lengths. The community profile is finally depicted through a size-by separation of the terminal restriction fragments (*i.e.* the fluorescently labelled restriction fragments) through capillary electrophoresis. The laser detection acquires the size of the terminal fragments along with the relative fluorescence intensity providing a comprehensive snapshot of the microbiota composition [88, 92].

Although community fingerprinting techniques allow for a time- and cost- effective evaluation of the microbiota composition, their outcome is limited to portray an overall picture of the microbial diversity, and identification of individual microbial species is not supported [82]. These methods are therefore replaced by other thorough investigation approaches such as 16S rRNA sequencing. Here, the hypervariable regions carried in the 16S rRNA clones are sequenced to depict the composition of the microbial community up to strain level; moreover, abundance of each clone mirrors the abundance of the bacterial species, enabling for a relative quantification of the identified specimens [81, 82].

1.4.3 The microbiota investigation in the "omics era".

Technological progress over the past decade enabled a shift in the methodology adopted for microbiota investigation, moving from the traditional culture-based approach to the state-of-art omics sciences. Omics sciences employ a holistic view of the biomolecules responsible for the structure, function and dynamics of a given organism or consortia of organisms. Their main objective is, therefore, the detection and quantification of genomic DNA (genomics), RNA

(transcriptomics), proteins (proteomics) and metabolites (metabolomics) of the studied organism. In the context of the microbiota investigation, omics sciences deal with the same biomolecules produced, instead, by the totality of microorganisms composing the microbiota. These community-based approaches are generally referred as metagenomics, metatranscriptomics, metaproteomics, and meta-metabolomics.

Although considered as single disciplines, none of the single omics science is able to provide a comprehensive and detailed understanding of the biology of the investigated consortia, for which purpose an integrative approach of all these omics sciences is required [82]. Following sections provide an overview of the Omics sciences in the context of the microbiota investigation, in order to provide guidance on the adopted methods and its results.

Metagenomics

Metagenomics is a community-based approach that employs the study of the microbial genomes collected from the environmental niche where the microorganisms coexist, enabling the study of their phylogenetical, physical and functional features in a culture-independent manner. Key step of metagenomics is the extraction and fragmentation of the metagenomic DNA. The metagenomic fragments can subsequently be used for sequence driven- or function driven-analysis [93, 94]. The first approach describes the overall genetic diversity featuring the investigated microbial community by creating a catalogue of the identified genes and genetic elements (*e.g.* mobile genetic elements). Among these information, insights on the taxonomic diversity are also included. The functional analysis of the metagenomic library provides a deep knowledge on the potential function of the microbial community as assessed through the investigation of the functions attributable to the identified genes. Moreover, this approach enables discovering of new classes of genes responsible for specific functions and infer novel functions to unannotated genes via sequence homology-based approaches [17, 93, 95].

Metagenomic sequencing is based on the so-called Next Generation Sequencing (NGS) techniques. Nowadays, 454 Roche pyrosequencing and Illumina sequencing by synthesis are the most frequently adopted techniques [94, 96].

In the 454-sequencing platform, the extracted DNA is fragmented and bounded to microscopic beads that are then subjected to an emulsion PCR to produce a clonal expansion of the attached fragments. Beads are subsequently deposited into a picotitre plate where the attached DNA fragments will be individually and in parallel sequenced. The pyrosequencing process involves the sequential administration of the four nucleotides, which incorporation is stoichiometrically related to the release of a pyrophosphate (PPi) molecule. PPi Production is detected through

two enzymatic reactions that produce light in a proportional manner. Emitted light is detected and recorded by a charge coupled device camera and the recorded signal is finally translated into the template sequence. The average reads length obtained with this technology span from 600-800 bp. The current technology allows multiplexing up to 12 samples in a single run. Major drawbacks related to this technology concern the bias introduced with the PCR-mediated DNA amplification, and the difficulty in detecting light intensity when sequencing homopolymeric regions, sometimes resulting in deletions or insertions that may lead to frameshift while reading the coding sequences [97-99].

The Illumina technology anchors the DNA fragments to the internal surface of the flow cell's channel. Clusters of identical DNA fragments are obtained following the bridge PCR. These are subsequently sequenced according to the principle of synthesis by synthesis. Briefly, the complementary chain of the anchored fragment is synthesized through the use of reversible dye terminators, resulting in one-by-one nucleotide incorporation and, since the nucleotide incorporation is featured by a nucleotide-specific light emission, enabling the identification of every single nucleotide as soon as it is included in the DNA strand. The read length currently achieved with this technology is around 150 bp and nearly 300 bp in the case of the paired-end approach. Multiplexing of the sample is strongly supported by this technology [94, 100, 101]. Error rate reported in this technology is far smaller than this of 454 Roche. However, the reduced reads length of the Illumina technology resulted in a greater proportion of unassembled reads with important consequences for the functional annotation and/or the neglect of low abundant specimens [102].

Besides these two major platforms, other sequencing technologies are currently available even though less frequently applied in metagenomics or limited to restricted investigation areas [94]. The Applied Biosystem SOLiD technology, for instance, have been extensively used in genome resequencing studies. Although featured by the lowest error rate among all NGS technologies, the provided reads cannot exceed 50bp making difficult and unreliable the functional annotation of the reads, but providing interesting results while adopted in resequencing studies [103]. Other important sequencing technologies are Pac Bio from Pacific Biosciences and Ion Torrent from Life Technologies [94, 96].

NGS, are nowadays defined Second Generation Sequencing (SGS), supporting the emergence of a new-NGS technology defined as Third Generation Sequencing (TGS). Features of the emerging technology rely on the sequencing of entire single molecules of DNA, avoiding the biases introduced by the step of DNA fragmentation, PCR-based construction of cluster and phased sequencing. Moreover, TGS exploit the high catalytic rates and processivity of the DNA

polymerase, enabling the sequencing of longer reads in a shorter time, when compared with the "previous" SGS technology. TGS technologies are grouped into three main categories depending on the principle exploited to inspect the DNA molecule [104, 105]. Single Molecule Real Time sequencing (SMRT, Pacific Bioscience) is the first TGS technology; it is based on the observance of a DNA polymerase while it synthesizes a ssDNA molecule, using labelled nucleotides [106]. Nanopore sequencing technology inspects the DNA molecule sequence by measuring the translocation of nucleotides across a nanopores-containing membrane. Nucleotides are cleaved by the ssDNA molecule and driven through the nanopore via differential salt concentration [107]. Third category of sequencing technologies relies on the direct observation of the DNA throughout the use of advanced atomic microscopy techniques that enable the visual observation and chemical detection of the DNA molecule building blocks, simultaneously [108].

Metatranscriptomics

Metatranscriptomics is an emerging discipline based on the NGS platforms (mainly Roche and Illumina), aimed to investigate the gene expression of the whole microbial community [93]. Indeed, metagenomics provides information about all the potential functions a given microbial community is capable of, but does not provide indication on the effective gene expression (or gene dynamics) of the microbial community under determined circumstances [94, 109]. Moreover, following mRNA- and gene- sequence comparison, metatranscriptomics enable a comprehensive annotation of the structure of the transcribed genes, highlighting their start- and stop-site, and assessing the potential splicing variants (or protein isoforms) per each of the expressed gene [110].

Despite the wide choice of techniques, the most used one in the metatranscriptomics studies is the RNA sequencing. Although the sequencing technology is the same adopted in metagenomics, key differences are represented by the selective removal of the interfering nucleic acids (*e.g.* DNA, t-RNA, rRNA) and m-RNA to cDNA transcription prior to library production and its subsequent sequencing [111]. A comprehensive review on the RNA-seq technology is provided in [112] and [113].

Metaproteomics

Rapid advances in high-throughput genome sequencing and the continuous improvement in quantitative mass spectrometry enabled the analysis of the protein complement of a single organism (proteome) and consortia (metaproteome) [19, 114].

Although of enormous importance, metatranscriptomics lack to consider the cellular organization and regulation levels governing the process of mRNA translation and protein maturation (*e.g.* controlled proteolysis, protein turnover, post-translational modification etc.), which greatly impact the final expression of the genome itself. On this basis, one could argue that metaproteomics and metabolomics (addressing the small molecules/metabolites) provide the most realistic picture of the key effectors that directly mediate the metabolic functions operated by the organisms at the specific moment of sampling [115].

Early proteomics investigations were performed through 2DE, often complemented by spot detection and MS characterization [114]. An improvement of this technology is represented by 2D-DIGE which employs diverse dyes in a single electrophoresis run, overcoming the issue of the scarce reproducibility among multiple 2DE runs, and enabling a direct comparison of several samples in a high-fidelity manner [114]. Although these techniques are successful for microbial isolates, interest in the investigation of more complex samples, such as the microbiota, leads to the advent of high-performance MS platforms. MS is currently the dominant approach for almost all proteomics investigations. It fulfils the technological requirements for a proteomic investigation that include high throughput processing, sensitive analyte detection, wide dynamic range, mass accuracy, ability to characterise and resolve peptide sequences. Improvements of the actual MS technologies enable proteomics measurements that provide a wide array of information such as differential proteins expression (*e.g.* overtime or following experimental treatments), investigation of sub-proteomes (*i.e.* protein profile of the subcellular structures), post-translational modification (PTM) pattern, protein-protein interactions, and absolute protein quantitation [115].

MS-based proteomics protocols are mainly distinct into two major branches: top-down- and bottom-up-proteomics. The first is a straightforward strategy, where the complex proteins mixture is separated via liquid chromatography (LC) prior the mass spectrometry (MS) or tandem mass spectrometry (MS/MS) measurements. This protocol is particularly useful when interested in the protein identity but also in its whole molecular form and the presence of isoforms. However, analysis might be severely complicated by the range of possible protein modification (*e.g.* truncation, SNPs, PTM) affecting the protein mass as predicted from the genome sequence. In addition, the protocol still suffers from drawbacks related to separation and detection of large proteins (>50 kDa). These limitations, coupled with the intrinsic challenge of the metaproteomes (*e.g.* increased sample complexity, species variability and protein homology), preclude the adoption of the top-down proteomics for the investigation of complex samples such as the microbiota [115, 116].

Bottom-up (or shotgun) proteomics includes additional processing steps that enable far deeper proteomics measurements. Here, proteins are primarily digested in an enzyme-specific manner (*e.g.* trypsin) to peptides. Peptides are subsequently separated via LC prior to their MS/MS measurements. Fragmentation spectrum resulting from the first MS measurement (MS^1) indicate the parent ion mass spectrum of the peptides directly eluting from the LC system; whereas the tandem mass spectrum (MS^2) provide insights on the fragmentation pattern and sequence information of the previous parent ions. Altogether, mass spectra are acquired and serve as a bar-code that univocally identify a given peptide [117].

Key point in the experimental proteomics protocols is represented by the efficient protein separation prior to their measurements, either via top-down or bottom-up proteomics. In this context, gelbased and gel-free proteomics approaches can be distinguished, depending on the use of an intermediate, gel-mediated, pre-fractionation step of the complex proteins mixture. Gel-based proteomics is also known as the classical proteomics workflow, expecting a pre-separation of the protein mixture through SDS-PAGE or 2DE. Although featured by a reduced dynamic range and a general underestimation of the membrane proteins, this approach enables to deal with intact proteins allowing for further downstream analysis such as selective staining and the PTM analysis [118, 119]. The gel-free approach does not expect a gel-mediated pre-fraction step but rather profit of the LC system (online coupled with the MS/MS instrument, LC-MS/MS) to separate proteins and peptides mixture [115, 118, 120, 121]. Nowadays, a variety of offline LC systems, simplifying the complex mixtures upfront the LC-MS/MS instrument, are also employed. These perform the protein separation according to one or multiple dimensions (*e.g.* isoelectric point, charge, hydrophobicity, size) and are usually compatible with the advanced online separation technologies such as the ultra-high pressure liquid chromatography (UHPLC), providing a better separation of the complex proteins mixture, thus enabling improved protein detection rate [115, 122, 123].

Meta-metabolomics

Metabolomics provides a snapshot of the metabolites suite (metabolome) produced by a given cell, or consortia, at the moment of sampling, enabling their link back to the ongoing physiological processes [124]. In the context of metabolomics, it is defined “metabolite” any molecule smaller than 1 kDa [125]; this includes intermediates and end-products of metabolic processes, hormones, and other signaling molecules. These, all together, form the metabolic fingerprint of the organism or consortia. Similarly to transcriptome and proteome, metabolome is very dynamic; therefore, its investigation under diverse circumstances provide precious

information on the causality of a given phenotype or, inversely, the systemic effects of specific substances (*e.g.* drugs) and other experimental treatments on the targeted cell lines or even the microbiota [126].

Main steps in metabolome investigation include the pre-separation of the complex metabolites mixture, following the cell lysis and purification of the extracted metabolites. Metabolites separation is generally accomplished through HPLC, enabling for a wide range of metabolites to be analyzed at a high sensitivity. Gold standard for the metabolites detection is represented by MS, where, similarly to proteomics, information on the metabolite IDs and subsequent quantitation is computed back on the basis of the mass spectral fingerprint and the fragmentation pattern. Although HPLC-LC-MS is the most used approach metabolomics, nuclear magnetic resonance (NMR) spectroscopy is also widely used in analysis that specifically targets a reduced number of metabolites [124].

Bioinformatics

Progress in the field of NGS has a tremendous impact on the omics technologies currently adopted for the characterization of the microbial communities. Metatranscriptomics and metaproteomics are strongly dependent on the quality and availability of the (meta-) genomes, used as a blueprint for the mRNA and protein identification, respectively. Although the huge advances in the sequencing technologies, it is still difficult to obtain complete strain-specific genomes and, in the context of the microbiota evaluation, only partial population genomes are generally available. Despite this limitation, a wide number of bioinformatics tools is nowadays dealing with metagenomics sequences, enabling an integrative approach between *omics* data. Commonly used metagenomics tools are MEGAN (MEtaGenome ANalyzer), IMG/M (Integrated Microbial Genomes/Metagenomes), and MG-RAST (MetaGenome-Rapid Annotation using Subsystem Technology). These, although based on different algorithms and a slightly different statistics, enable the taxonomic and functional annotation of the metagenomic sequences, and allow the comparative evaluation of multiple datasets. In addition, MG-RAST and IMG/M are also used as data repositories and, along with NCBI (National Center for Biotechnological Information) and EBI (European Bioinformatics Institute) represent the biggest data repositories currently available [127, 128].

A typical LC-MS/MS experiments generate hundreds of thousands of fragmentation spectra. Here, the MS^1 provide information on the mass-to-charge (m/z) of the intact peptide along with its amino-acidic composition whereas the MS^2, providing information of the fragmentation pattern of each parent ion, determine the correct amino-acidic order. The peptide sequences, in

turn, are assigned to specific protein IDs through a protein database-dependent search, where the measured peptides are compared with the peptides profile deriving from the *in silico* digestion of user-defined protein database, designated as the reference for the investigated organism or consortia. Clearly, this enormous amount of data cannot be manually elaborated and a wide variety of bioinformatics software and tools is nowadays available to perform the computational operations, required to translate the myriad of spectra into a meaningful output that is both concise and informative [115]. To this purpose, commonly used search engines are OMSSA (Open Mass Spectrometry Search Algorithm), X!Tandem, MASCOT, Andromeda and SEQUEST. Major tasks of these tools include quality filtering of the raw MS spectra, peptide-spectrum matching and scoring, protein database searching, data mining, and graphical representation of the obtained results [129]. Similarly to metagenomics tools, proteomics/metaproteomics-committed tools are based on diverse algorithms and statistics, resulting in varying performance that, for each tool, depends on several factors such as sample complexity, separation efficiency and quality of the reference database [127].

In light of the above, it is easily understandable that the quality of the metaproteome data is strongly dependent on the availability and quality of the metagenomics sequence. Three main strategies can be employed to obtain a suitable reference database. The first option is to use the metagenome sequence of the exact same sample or arising from a comparable community/habitat. This is considered the best option since has been associated with an improved identification rate. A second choice is to create an artificial, *in silico* metagenome (pseudometagenome) that includes genome sequences of microbial species supposed to be present in the microbial community of interest. This strategy has been demonstrated to be effective [130], but *a priori* knowledge of the taxonomic complexity is required. The third option expects a survey search before the "official" main search. The survey is conducted against a publicly available database (*e.g.* NCBInr) and the list of identified proteins is exported either unfiltered or weakly filtered, in order to obtain a comprehensive database to use in the later main search. This approach enables the use of small-sized database (leading to a higher identification rate) and avoids the bias introduced with the manual selection of the microorganisms. Further taxonomic levels may also be implemented from publicly available repositories [127].

On the attempt to overcome the total dependency of metaproteomics from the metagenomics sequence availability, bioinformatics tools are currently being developed to perform a new bioinformatics approach termed "de novo sequencing "of the uncharacterized proteins. The rationale behind this promising approach expect the *de novo* calculation of the peptide

sequences on the basis of the parental ion masses and their fragmentation pattern provided respectively from the MS^1 and MS^2 of the tandem mass spectrum. This will provide the peptide sequence of peptides that are not included in the current metagenomes as well as enable insights on the PTM and protein polymorphisms. Although of great interest, the accuracy and speed of this groundbreaking approach still need substantial improvements to facilitate its widespread implementation in the "routinely" practice [115, 131].

Detailed metaproteomic characterization of a given microbial community requires a quantitative assessment of the identified protein repertoire. The methods applied for protein quantification are distinguished as label-based- or label-free approach depending on the need to label the target proteins to gain quantitative information. Because of the independence of any cultivation step and the ability to estimate protein amount without any labelling procedure, the label-free quantification is the most suitable approach for metaproteomic studies [127, 132, 133]. However, this requires high reproducibility LC-MS/MS data. Indeed, quantification is based on either spectral counting (SC) or signal intensity (SI). Quantification through SC relies on the assumption that the number of fragment spectra mirrors the abundance of the peptide the spectra belong to. Normalized Spectra Abundance Factor (NSAF) is considered an improved version of the SC, since it estimates the protein abundance considering the effect of the protein size, thus avoiding the overestimation of larger proteins. Main software adopted for SC and NSAF quantification are Scaffold, Proteo IQ, APEX, CENSUS etc; a comprehensive review of these tools is provided in [127, 132, 133].

The SI-based quantification relies on the peptide ion abundance at specific retention times to estimate protein abundance. Although it is performed by a variety of software (*e.g.* MaxQuant, Elucidator, Open MS, SIEVE), the label-free quantification provided by MaxQuant is currently the most used, enabling an accurate detection and comparison of changes in the abundance of both high- and low- abundant proteins [127]. Key points of the MaxQuant software reside in the integrated algorithms performing the process of "ID transfer" and "peak normalization" leading to improved peptide ID and accurate relative protein quantification, respectively [134].

Another important task of the bioinformatics in the field of metaproteomics concern the annotation of identified proteins into predicted functional classes and clustering of the metaproteome dataset into functional groups. Data repositories can be distinguished on the basis of the annotation type. UniProt KB, for instance, is further divided into Swiss-Prot and TrEMBL. The first comprises the only entries whose annotation has been reviewed and manually annotated; whereas, TrEMBL entries are not reviewed and "only" automatically annotated. Other data repositories commonly used are Kyoto Encyclopedia of Genes and

Genomes (KEGG), Gene Ontology (GO), BioCarta. These provide information concerning the sub-cellular localization, biological process and molecular function for each of the listed proteins. Moreover, KEGG integrates all these information and organize them as biochemical pathways that fall into seven main groups, namely metabolism, genetic information processing, environmental information processing, cellular process, organismal system, human diseases and drug development. Besides being a comprehensive data repository, KEGG has also a common use as a web-based tool for the functional annotation of the metaproteomics dataset. Diverse other software and web-based application are nowadays available for this purpose, such as Web MGA, Cytoscape, IPath, DAVID and others (as reviewed in [127]) that retrieve and integrate functional annotation from diverse sequence repositories to provide a more comprehensive functional annotation of the queried metaproteomics dataset.

1.5 Project overview

Despite the extensive and heterogeneous ensemble of studies performed on chickens and pigs, in-depth investigations of the possible functional changes of the GIT-associated microbial community challenged with diets varying in the levels of P and/or MP are missing.

The microbial community harboring the GIT of chickens has been investigated mainly in relation to its spatial microbial diversity [56, 135, 136]. A variety of studies is also focused on the investigation of the avian gut microbiota following dietary supplementation of antimicrobials [47, 137], probiotics [50, 138, 139], and high-energy feed formulations [140].

Only a handful of studies investigated the effect of Ca, P and MP supplementation on the chicken gut microbiota composition [141, 142].

The bacterial community inhabiting the pig intestine is investigated in relation to a variety of applicative fields, such as human translational research [143, 144] and animal welfare. Several studies aimed to animal husbandry optimization and animal health improvement are nowadays being performed [14, 145, 146].

Although a variety of studies investigate the dietary-induced modulation of the pigs gut microbiota, it is still being discussed how and how long the microbiota adaptation process lasts, which is of a great importance for all nutritional studies, including the translational research for human health. Recently, Kim and colleagues investigated the natural, age-dependent shift of the fecal microbiota composition of commercial swine, underlying the importance of animal´s age intended as a factor shaping the pigs gut microbiota [147]. Even though, investigation on how the intestinal microbial community progressively adapts to a new diet composition and the dynamic linked to P supplementation are still missing.

Owing this lack of available information, our project seeks to investigate the functional changes of the bacterial community harboring the intestine of the above-mentioned monogastric animals (chickens and pigs) with the following hypotheses.

- Feeding experimental diets supplemented with varying amounts of Ca:P and/or MP impairs the composition and function of the microbial community inhabiting the diverse GIT sections of monogastric animals (chickens and pigs).
- Diet-derived alterations of the gut microbiota are mostly observable on the protein repertoire of both microbial community and its host.
- Experimental diets fed do not stimulate a sudden shift in the architecture and function of the gut microbiota. Instead, a gradual adaptation occurs to shape a balanced microbial community that stays functionally tuned with the host physiology to maintain the homeostatic balance.

Two experimental trials were designed to answer the postulated hypotheses.

In the first study, diets varying in the amount of supplemented CaP and/or MP were fed to growing broilers, aimed to investigate whether the differences in the diet composition are also reflected in the gut-associated bacterial community, both in terms of composition and activity of the microbial community. A metaproteomic approach was used to assess the dietary-induced changes in the composition of the bacterial fraction of the chickens crop and ceca. Depicted bacterial community was compared with the total bacterial community designated, for the same sections and dietary treatments, via 16S rRNA gene sequencing. Functional features of the microbial community of chickens crop and ceca identified on protein level, at diverse dietary treatments, were assigned through the functional categorization of the identified protein profiles into bioinformatic data repositories.

The second experimental trial expects a long-term feeding of weaned pigs with experimental diets composed of different protein sources and a varying amount of CaP. This enables for a stepwise description of the events featuring the gut microbiota restore after its homeostatic balance has been altered. Similarly to chickens, 16S rRNA gene sequencing and metaproteomics were used to investigate the potential time- and diet-induced changes in the composition of the bacterial community content in the pig feces. Investigation of the pigs feces is commonly used in literature as an indicator of the pigs gut microbiota. Functional features of the gut-related bacterial community were qualitatively and quantitatively investigated on a protein repertoire basis, on the attempt to elucidate the molecular mechanisms responsible for the progressive adaptation of the intestinal microbiota to a new dietary composition. Moreover, the production of SCFAs was both predicted from metaproteomics and assessed through direct

measurements of the metabolites, in order to investigate the fermentative ability of the intestinal microbiota when challenged with a new diet, other than evaluating metaproteomics as a predictive tool for SCFAs production.

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CHAPTER 2

NEWS IN LIVESTOCK RESEARCH – THE USE OF OMICS TECHNOLOGIES TO STUDY THE MICROBIOTA IN THE GASTROINTESTINAL TRACT OF FARM ANIMALS

Simon Deusch[1], Bruno Tilocca[1], Amélia Camarinha-Silva, Jana Seifert[*]

Institute of Animal Nutrition, University of Hohenheim, 70593 Stuttgart, Germany

[1] Authors contributed equally

[*] Corresponding author

Published in Computational and Structural Biotechnology Journal 13 (2015) 55-63

The original publication is available at http://dx.doi.org/10.1016/j.csbj.2014.12.005

2.1 Abstract

Technical progress in the field of next-generation sequencing, mass spectrometry and bioinformatics facilitates the study of highly complex biological samples such as taxonomic and functional characterization of microbial communities that virtually colonize all present ecological niches. Compared to the structural information obtained by metagenomic analyses, metaproteomic approaches provide, in addition, functional data about the investigated microbiota. In general, integration of the main Omics-technologies (genomics, transcriptomics, proteomics and metabolomics) in live science promises highly detailed information about the specific research object and helps to understand molecular changes in response to internal and external environmental factors.

The microbial communities settled in the mammalian gastrointestinal tract are essential for the host metabolism and have a major impact on its physiology and health. The microbiotas of livestock like chicken, pig and ruminants are becoming a focus of interest for veterinaries, animal nutritionists and microbiologists. While pig is more often used as an animal model for human-related studies, the rumen microbiota harbors a diversity of enzymes converting complex carbohydrates into monomers which bears high potential for biotechnological applications.

This review will provide a general overview about the recent Omics-based research of the microbiota in livestock including its major findings. Differences concerning the results of pre-Omics-approaches in livestock as well as the perspectives of this relatively new Omics-platform will be highlighted.

2.2. Introduction

The methodology to study the microbial communities (microbiota) inhabiting the gastrointestinal tract (GIT) of livestock was changing from classic cultivation techniques and pure culture characterization to state of the art Omics-approaches (Fig. 1). Despite cultivation being a sound technique to characterize the physiological properties of microorganisms [1], there are severe drawbacks in using this as a tool for characterizing bacterial communities. Typically, the culture media do not resemble *in situ* conditions and in some cases the carbon richness is higher than the substrates found *in situ*, allowing the growth of only a small fraction of the community while suppressing other members [2]. In the past, cultivation studies have contributed to our understanding of the gut microbiota, but the limits of these methods directed us to an inaccurate and incomplete knowledge of a niche where most microbiota still remain unknown. The inconsistency between *in situ* and cultivable diversity has resulted in the widespread use of culture-independent molecular approaches [3, 4]. Microbial community profiling methods (16S ribosomal RNA gene based approaches) have become important tools to characterize microbial communities and the interactions between the microorganisms present in the GIT. In addition, the complexity of the microbial processes harbors new enzymatic functions, which are of interest for biotechnological applications. Overall, the analysis of the microbiota is important to improve animal nutrition strategies and animal health. This knowledge can be used to modulate the microbiota to reduce antibiotic treatments and, in the case of ruminants, to inhibit the formation of emission gases. Thus, the progress of Omics-technologies and the availability of bioinformatic tools to evaluate big datasets demand their use in these fields of research.

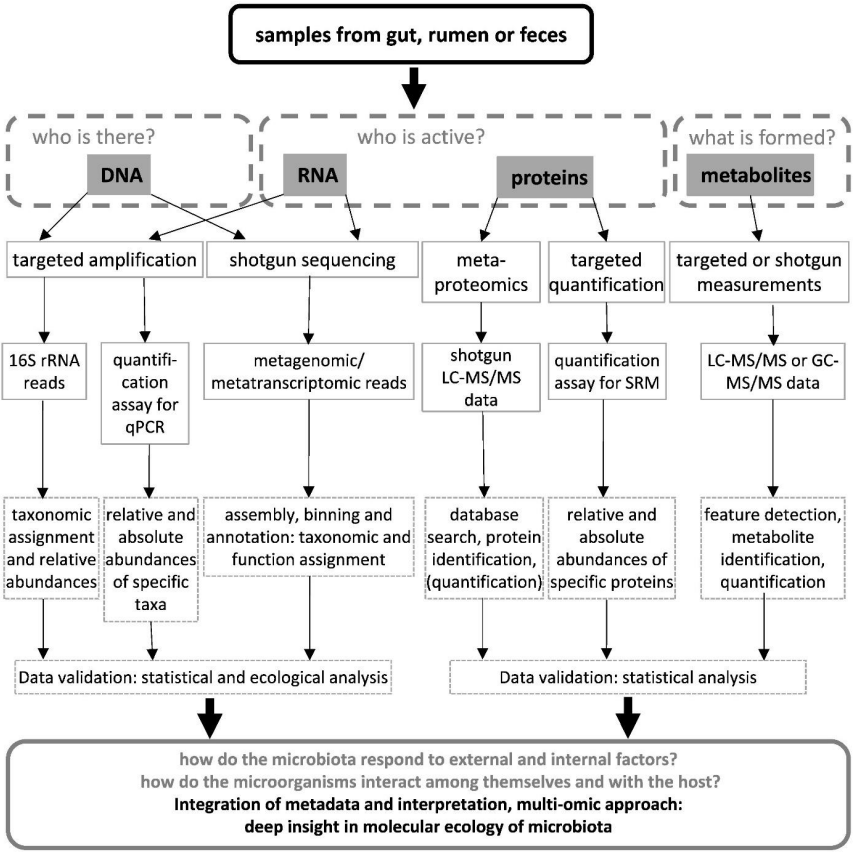

Fig. 1. Workflow of possible methods to study the structure and function of the microbiota in farm animals.

Two pyrosequencing techniques, 454 (Roche) and sequencing by synthesis (Illumina), are mainly used for (meta-) genomic and (meta-) transcriptomic projects. Both systems have unique features, such as short paired-end reads (max. 2 × 300 bp) with Illumina vs. long read length (600–800 bp) with 454. The latter one is more feasible in terms of shotgun sequencing studies (see below) [5, 6], while Illumina provides barcoding strategies and bigger data sets that are more favorable to analyze hundreds of samples in targeted sequencing projects [6]. Two other techniques, that were not frequently applied for metagenomic studies of animal microbiota, are the Ion Torrent (Life Technologies) and the PacBio (Pacific biosciences). All techniques are continuously improving and a state of the art overview is given by C. Knief [6] or can be found at the respective company webpages.

The gene of choice to analyze the phylogenetic composition of a microbial community is the 16S rRNA gene, a ribosomal gene in prokaryotes characterized by conserved and variable sequence regions, which is used to calculate evolutionary relationships and similarities between the species [7]. There are a couple of techniques in molecular ecology, such as fingerprinting methods, microarrays and fluorescence *in situ* hybridization which use the 16S rRNA gene as a target molecule. In this review, we focus mainly on next-generation sequencing methods to describe the microbial community structure. Nowadays the total diversity of a microbiological sample is analyzed preferably by pyrosequencing of the 16S rRNA gene, obtained by amplification of extracted DNA. The active fraction of the community is analyzed using mRNA/cDNA. Subsequent to pyrosequencing, quality filtering and denoising processes have to be applied. The reads should be checked for chimeras and clustered to operational taxonomic units (OTU) in order to assign the respective taxonomies to the sequences. There is a diverse range of bioinformatic tools available in free software platforms such as Mothur, QIIME, RDP pipeline, LIBSHUFF, UniFrac and MEGAN that support data analysis and convert data to formats that can later be used in statistical packages like R, Metastats or Primer-E. A detailed overview of the methods can be found in several review papers [5, 8]. These pipelines should be used with special care as it is not only important to make sense of all the raw data, but also to ensure that the final picture is a direct reflection of the original raw data collected and thus of the original community structure of the sample. The output data reveal ecological indices, relative abundance values of the identified taxa and enable a pre-selection for a targeted quantitative PCR (real-time PCR) approaches if necessary.

In addition to the phylogenetic structure of the community, the analysis of encoded and expressed metabolic pathways is the second objective. Metagenomic or metatranscriptomic data are obtained by shotgun pyrosequencing of the total DNA and cDNA, respectively. Reads have to be quality filtered, assembled to contigs, binned and assigned to taxonomies and possible gene functions. As the assembly requires sequence reads with appropriate length, so far 454 pyrosequencing was the method of choice as it produces reads up to 800 bp (see above). Due to the progress in data generation and bioinformatic processing Illumina pyrosequencing is recently used as well. Several tools are available for the annotation of open reading frames on the contigs, MG-RAST [9], MEGAN5 [10], IMG/M [11], Metarep [12] and MicroScope [13]. CAMERA portal [14] was shut down in July 2014. These tools can also be used for metabolic pathway reconstruction. This is usually done based on the KEGG database [15] or the subsystem classification of SEED [16].

In addition to metatranscriptomic studies, the community activity can be assessed based on expressed proteins and formed metabolites. Metaproteomic studies investigate the protein inventory of a specific sample at a certain point of time [17]. This allows the identification of the active microbial fraction and their expressed metabolic pathways. The first key step is to find an optimized sample preparation protocol to avoid co-extraction of eukaryotic proteins and to get a purified protein sample. The following workflow depends on the available technical equipment [18]. In a gel-based approach, proteins are separated and proteolytically digested into peptides followed by a one-dimensional liquid chromatography directly coupled to the mass spectrometric analysis (LC–MS/MS). In a gel-free approach, peptides are prepared by in-solution digestion directly in the protein mix. Peptides are separated by two-dimensional LC and measured by MS/MS analysis. The protein identification is the second big challenge as it is highly depending on the available sequence database which can either be used from public resources or sample-specific sequences. An overview of available bioinformatic tools and workflows are given in [18, 19]. The coverage of metaproteomic studies of complex microbial samples, such as feces or rumen contents, is still low. Since there is a high species diversity and cell density in these types of samples, only abundant proteins are identified while rare species, that may have important metabolic functions, are missed. Targeted proteomic approaches, like selective reaction monitoring (SRM) can be used to specifically detect and quantify proteins of interest [20]. Metabolomic approaches are becoming more interesting for microbial ecology studies as the technical progress allows a comprehensive analysis of hundreds to thousands of metabolites. NMR- and MS-based methods are available and their application to detect defined groups of metabolites is reviewed by Xie et al. [21].

The following sections will provide an insight into the ongoing research of the microbiota of the gastrointestinal tract of livestock animals with special emphasis to the use of Omics-technologies and their importance for the understanding of these niches.

2.3 The Microbiota of chicken

The chicken intestinal environment comprises a vast and diverse assemblage of microorganisms living not as single species populations, but rather in complex communities comprising multiple species that include animal and human pathogens. Intricate networks of interactions between the microorganisms and their environment shape the respective communities and are important for animal welfare and food safety reasons. The chicken GIT consists of more than 900 species of bacteria. This diverse microbiota helps not only the breakdown and digestion of food but also plays an important role concerning the growth and health of the host [22].

In the past, the chicken GIT microbial community was studied by culture-based methods. These studies discovered that 10–60% cecal bacteria can be cultured [23, 24] and about 45% could be assigned to the genus level [22]. The profiles of the different gut sections are nowadays studied using cultivation-independent methods like clone libraries [25-27], denaturing gradient gel electrophoresis (DGGE) [28], temperature gradient electrophoresis (TGGE) [27], terminal restriction fragment length polymorphism (T-RFLP) [4, 29-31], quantitative PCR (qPCR) [32], microarrays [33], next-generation sequencing [34-39] and metaproteomics [40].

Regarding the research in microbial ecology of the chicken GIT, several studies focused on the influence of diet [25, 31, 32, 41], antimicrobial feed additives [29, 34] host genotype [38, 42], gender [38], spatial microbial diversity [25, 28, 31, 43], age [28], development and temporal microbial variations [22, 26, 28]. It is important to take into consideration that all these factors may change the bacterial community of each section. Sklan et al. showed that the different sections of the chicken GIT are highly inter-connected [44]. However, because of the high diversity within each section, it has been suggested to analyze them as separate ecosystems [28]. It was demonstrated that the microbial communities colonizing the GIT of chicken benefit the host [29, 31, 36]. Nevertheless, two recent studies revealed that this colonization can also harm the host [35, 45].

After hatching, the colonization of the chicken GIT begins. This is a moment of great importance regarding the establishment of the microbial communities. Although the colonization of the chickens by maternally derived bacteria is low, some studies postulated that the microbial community structure of the small intestine settles within two weeks. Older studies showed that cecal bacteria need longer time to develop [40, 46]. The gut is colonized by commensal, transient and pathogenic microorganisms. Commensal microorganisms are beneficial to the host as they provide amino acids, short-chain fatty acids and vitamins [40]. Stanley et al. observed inter-individual GIT variation between microbial groups and also differences between groups of birds from replicate trials. It was suggested that the hygiene levels of the new hatcheries might cause highly variable gut microbial community [37].

The chicken gut is divided in three upper segments: crop, proventriculus and gizzard. The crop is a food storage muscular pouch related to the breakdown of starch and the fermentation of lactate. Digestion starts in the proventriculus while the gizzard grinds food. Because of its lower pH and fermentation activity, the gizzard functions as microbial barrier. Similar microbial communities were found in the crop and gizzard. Lactobacilli, facultative and microaerophilic bacteria are the most dominant bacteria present in this two segments. Other abundant species belonged to *Clostridiaceae*, Enterococcus and in the case of the crop also Bifidobacterium and

Enterobacteriaceae (Fig. 2) [25, 46, 47]. The small intestine is relatively long and has a constant diameter. It consists of three parts: the duodenum, jejunum and ileum where the nutrient absorption and food digestion occurs. Due to the low pH, pancreatic and bile secretions, the bacterial density in the duodenum is comparably low. Besides Lactobacillus as the main colonizer of the jejunum (reaching coverage of up to 99%), Streptococcus was identified as well. Amit-Romach et al. has shown that the relative proportion of Lactobacillus spp. in duodenum and jejunum increases within age [48]. The chicken's ileum harbors Lactobacillus in higher abundance (> 68%) and in lower abundances Streptococcus, *Enterobacteriaceae* and *Clostridiaceae* [28, 43]. Lu et al. demonstrated that during all different stages of microbial community development in the ileum Lactobacilli were dominant [26]. This gut section is also known to be colonized by novel butyrate producing bacteria that may play an important role regarding the availability of nutrients, absorption rate and chicken performance [47].

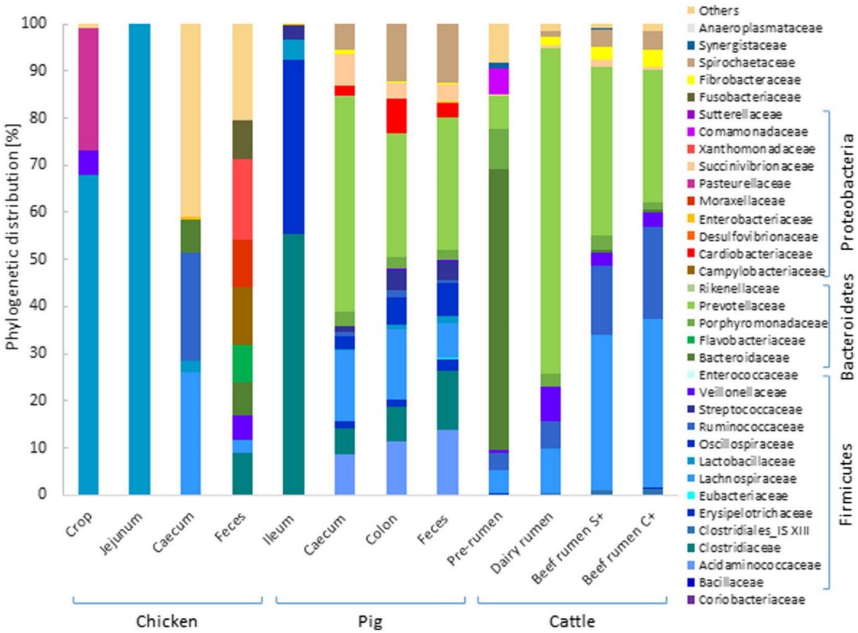

Fig. 2. Phylogenetic distribution of bacterial families in different GIT sections of chickens, pigs and cows. Chicken's crop, jejunum and caecum data arise from the analysis of V1–V3 16S rRNA region as performed by Videnska et al. [51], Stanley et al. [36] and Sergeant et al. [39], respectively. All pig's data arise from the study performed by Looft et al. [57] on V1–V3 16S rRNA region. Cow's data derive from the work performed by Wu et al. [89] on V3–V5 16S rRNA region.

Chickens have two caeca which are important for recycling urea, the absorption of water, and digestion of cellulose, starch and polysaccharides. These two fermentation chambers have the highest bacterial density and are colonized by obligate anaerobes like Clostridium, Bacteroidetes, and Bifidobacterium (Fig.2) [42]. Recently, 16S rDNA amplicon pyrosequencing studies estimated a bacterial population of about 700 species [39]. This wealth of microorganisms makes the caeca an important study site and a reservoir rich in unknown and uncultured microorganisms and pathogens [30, 39, 46, 47]. Qu et al. proved that mobile DNA elements are the cause of functional microbiome evolution and that horizontal gene transfers and the metavirulomes of cecal microbiomes were related to the host environment [49]. A metagenomic analysis of the chicken caecum using the Illumina MiSeq 2000 system revealed a relatively high proportion of sequences encoding glycosyl hydrolases that were identified by sequence comparison with carbohydrate active enzymes (CAZY) database (Fig. 3) [39]. More than 200 genes of non-starch polysaccharide degrading enzymes were identified indicating a great potential for xylane degradation compared to a lower cellulolytic potential in the caeca. This is also congruent to the comparative study of Waite and Taylor describing an abundance of β-xylosidase and β-glucosidase in grain-fed chickens [50]. Both studies also described the presence of genes involved in propionate and lactate production [39, 50].

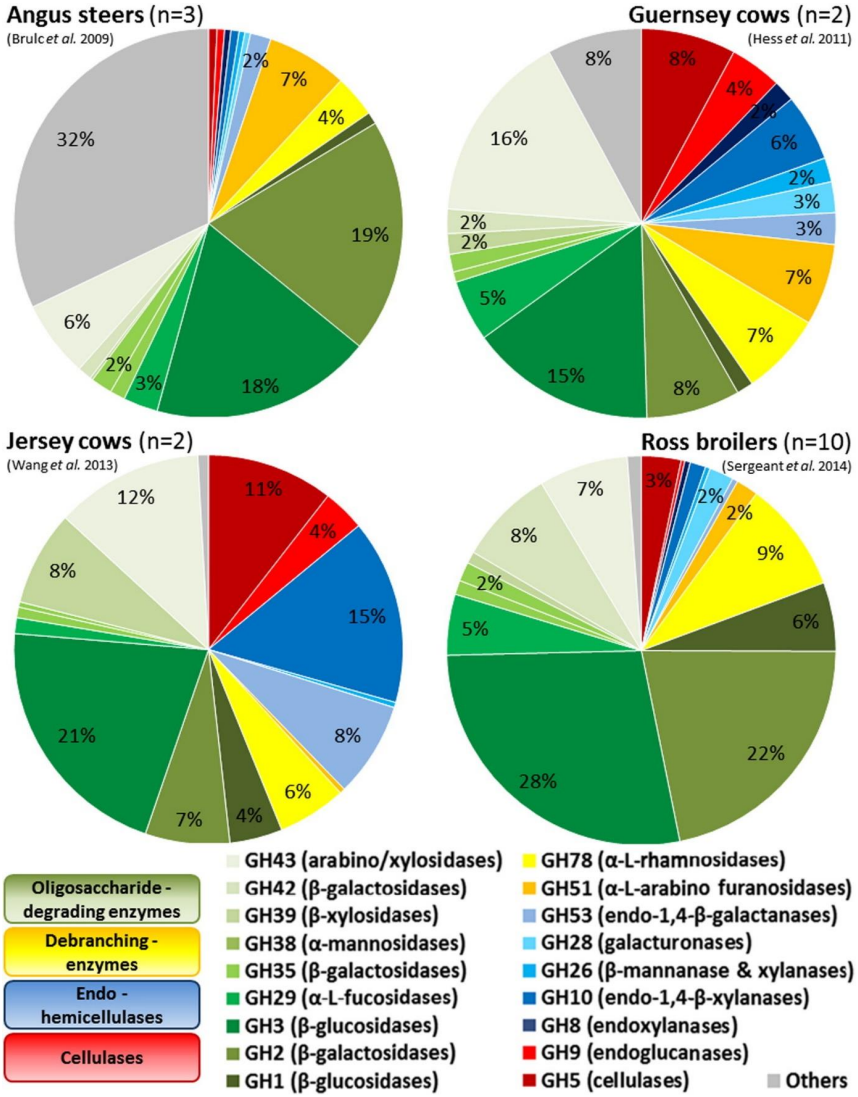

Fig. 3. Abundance of glycoside hydrolase (GH)-families in metagenomes of bovine rumen and chicken caecum. The percentage of each GH-group relative to the total number of GH-families identified in each metagenomic dataset is shown grouped according to major activity [90]. Brulc et al. [72] [Angus steers] — Pyrosequencing data (shotgun sequencing using GS20 from 454 Life Science) of 4 metagenomic samples; the mean of three fiber-adherent and one pooled liquid sample is shown. The average size of the metagenomes was 0.026 Gb. The samples were obtained from three 5 year old Angus Simmental Cross steers maintained on grass-legume hay. Hess et al. [70] [Guernsey cows] — Massively parallel shotgun sequencing using Illumina GAIIx and HiSeq 2000 was

applied on metagenomic samples of the fiber-adherent rumen microbiota of two Guernsey cows kept on a mixed diet containing 60% fiber. The total metagenome size was 268 Gb. Wang et al. [91] [Jersey cows] — All samples were pooled at equal amount and pyrosequenced with the Roche GS FLX Titanium system. Average size of metagenomes was 0.49 Gb. Rumen digesta samples were collected from two Jersey cows fed mainly Timothy grass hay ad libitum. Sergeant et al. [39] [Ross broilers] — Cecal samples were collected from 10 Ross broilers consuming a wheat based diet with 5% maize which contained ionophores but no antibiotics. Sequencing was carried out on the Illumina Miseq 2000 system.

Chicken feces samples are colonized by Lactobacillus, Clostridium, Faecalibacterium, Ruminococcus, Bacillus, Eubacterium, and Fusobacterium (Fig. 2). Here the microbiota is not stable and it has been proposed that these fluctuations are related to the emptying of the previous gut sections [25]. A recent study in meta-analysis of the avian gut microbiota showed that genes related to cytokine receptors and cell adhesion grouping into “signaling molecules and interaction” were less present in fecal samples indicating a lower potential of host/bacteria interactions [50]. The only metaproteomic study using a chicken fecal sample identified about 3487 proteins in total [40]. Bacterial proteins mainly belonged to Lactobacillus and Clostridium. Gene ontology analyses showed that the majority encodes for stress-related proteins like chaperons and proteases as well as enzymes involved in glycolysis [40].

Antibiotic growth promoters improve chicken growth performance and health status. The inclusion of penicillin in diets increases the body weight of chickens and also the Firmicutes to Bacteroidetes ratio in caeca. These effects might be caused by a reduction of the weight of the small intestine and the thickness of the gut wall, increasing the absorption of nutrients. The addition of the antibiotics tetracycline and streptomycin also induces a rapid shift in microbial community, increasing the prevalence of Lactobacillales and Enterobacteriales in fecal samples. The restoration of the microbial community after usage of these antibiotics was observed after removing the therapy [51].

In the era of next-generation sequencing, high-throughput technologies have brought an immense contribution in characterizing the poultry microbiota, bridging genomics, immunology, physiology, host and environmental factors to give a precious insight into animal production, food safety and public health.

2.4 The Microbiota of pig

Pigs harbor a complex gut-microbiota which establishes strong and complex interactions with the host. Since the importance of these interactions and their implication in nutritional, immunological and physiological functions became more relevant, several research groups

started to focus on the characterization of the porcine gut microbiota by using different methods. In the past, members of the porcine gut microbiota were investigated by cultivation attempts that are limited to a small fraction as it is difficult to achieve optimal growth conditions *in vitro* [52]. However, cultural methods are still used and flanked with cultivation-independent techniques. Furthermore, isolation attempts of novel species are still necessary to describe novel metabolic functions by physiological tests. Disadvantages of the culture-based methods triggered a wider use of cultivation-independent methods for the investigation of gut microbiota in the last two decades [4]. QPCR [53-55], T-RFLP [53, 55] and microarrays [56] were used to study the porcine microbiota. A comparison between culture-based and fluorescence *in situ* hybridization combined with flow cytometry detection (FCM-FISH) methods were performed by Collado and Sanz [52] and revealed a better sensitivity with the FCM-FISH technique. Currently, several studies applied Omics-technologies such as metagenomics [57-62] and metabolomics [63-66]. To our knowledge, no metaproteomic and metatranscriptomic study on pig's gut microbiota was published so far.

Most investigated sections within pig's GIT are ileum (small intestine), caecum and colon (large intestine) (Fig. 2). Phylogenetic characterization, based on amplification of the V1–V3 region of 16S rRNA gene and pyrosequencing of the amplicons, showed both longitudinal and radial differences along the GIT [57]. The ileum lumen samples, for example, revealed a lower diversity in terms of richness and abundance when compared with other gut sections. This comprises almost exclusively Firmicutes and Proteobacteria, whereas the phylum-level profiles of the caecum and mid-colon are highly congruent and include mainly Firmicutes, Proteobacteria, Bacteroidetes and Spirochetes. Other phyla such as Fibrobacteres, Actinobacteria, Tenericutes, Synergistetes and Planctomycetes are present but their sequences constitute less than 1% of total rRNA gene sequences [57]. Interestingly, mucosa-associated bacterial communities along GIT are different from those present in the lumen. However, statistically significant differences were found solely in the ileum between the mucosal and luminal communities and most lumen-associated bacteria were also found at mucosal level. Total DNA sequencing using 454 pyrosequencing and a subsequent SEED subsystem annotation of metagenomic sequences from GIT sections showed that unlike samples from the large intestine, the ileum microbiota was completely devoid of enzymes for pectin and hemicelluloses degradation [57]. By contrast, all sites encode starch-degrading enzymes. Members of Bacteroidetes represented about half of the microbiome in large intestine sections and harbored enzymes for polysaccharide degradation. The ileum was enriched in Firmicutes associated genes of numerous bacterial ABC transporters for monosaccharides and amino-acid

uptake and bacterial carbohydrate transport phosphotransferase systems showing a preference for the metabolization of easily accessible low molecular weight molecules by Firmicutes species. Therefore, a clear separation of the carbohydrate degradation steps based on the phylogenetic level in the pig GIT can be made, starting with the conversion of polysaccharides to oligosaccharides by pathways encoded in Bacteroidetes and followed by the uptake and fermentation of monosaccharides by metabolic processes encoded in Firmicutes.

Concerning fecal-associated microbiota, shotgun metagenomic analysis followed by sequence annotation using both MG-RAST and JGI IMG/M-ER pipelines [59] showed that metagenomic swine fecal datasets were dominated by the phyla Firmicutes and Bacteroidetes. Numerically-abundant bacterial orders revealed that Clostridiales, unclassified Firmicutes, Bacteroidales, Spirochaetales, unclassified Gammaproteobacteria, and Lactobacillales were the top six most abundant bacterial orders. Archaeal sequences constituted less than 1% of total 16S rRNA gene sequences, and were dominated by the Methanomicrobia and Thermococci [59]. Annotation pipelines used by Lamendella and co-workers have shown that carbohydrate metabolism was the most abundant SEED subsystem, representing 13% of swine fecal metagenomes [59]. Other abundant functional genes were associated with the subsystem cell wall and capsule, stress, and virulence. Additionally, 75% to 90% of metagenomic reads could not be assigned to subsystems, suggesting the need for improving binning and coding region prediction algorithms to annotate these unknown sequences [59].

Structure and activity of GIT microbiota can differ significantly between animals depending on the breed, diet, health status, age and environment [56-58]; suggesting the investigation of pig's gut microbiota as a powerful and versatile tool to predict effects of new feeding/breeding strategies and also perform studies on animal welfare. A study investigating diet-induced obesity in pigs identified an increase in proportion of the phyla Firmicutes compared to Bacteroidetes by T-RFLP and qPCR approaches [55]. This study also points towards high fat/high caloric diets as a main factor changing the gut microbial community composition. In addition, non-targeted metabolite profiling approaches used by Hanhineva et al. discovered that metabolic effects of high fat diets causing obesity were observed in all examined biofluids (plasma, urine, and bile) [66]. 16S rRNA sequencing investigations were performed to observe possible effects of genetically modified maize on the intestinal microbiota either in short [67] or long-term [60] pig-feeding studies. Similar levels of overall biodiversity for both treatments (isogenic vs. Bt-maize) were determined; moreover no statistical differences occurred in microbiota composition except for the genus Holdemania that was more abundant in isogenic group. However, the authors argued that this difference may be related to the changing of the

maize source during the animal's early life, when the gut microbiota has not completely developed [60].

Several other studies investigated how different diet composition can affect porcine gut microbiota in order to draw either a balanced diet able to ensure a higher animal growth rate [53, 61, 63, 64], or cost-effective [60] and environmental friendly diets [54, 61]. Another point of interest is the potential of the intestinal microbiota to improve the animal's health status by stimulating the growth of beneficial commensal on the expense to opportunistic pathogens [53, 54].

Since the importance of gut microbiota in animal production was clarified, the study of in-feed antibiotic (AB) effects on porcine gut microbiota is now of great importance. Nowadays various groups focus on understanding how the use of antibiotics promotes animal growth and how it affects the gut microbiota in short- [58] and long-term treatments [56]. It is also of interest if different effects occur depending on genetic background, age, and/or environment where the animal is bred [58]. Particular attention is attributed to the investigation of gut microbiota development of AB-treated saw's offspring in order to understand how imprinting mechanisms can be impaired in AB-treated pregnant saws [56]. However, more investigation in this field is required, not only due to its importance to human health. Further studies to analyze the active fraction of the microbiota in the porcine gut by using metatranscriptomics and metaproteomics have to be done in the future.

2.5 The Microbiota in the rumen

Over 3.5 billion domesticated ruminants worldwide including cattle, sheep and goats (http://faostat.fao.org/) constitute a highly significant source of food products to humans. These animals host a complex gut-microbiome (comprising about 1010 bacteria, 107 archaea, 108 protozoa and 103 fungal spores per ml rumen fluid [68]) which in exchange provides various enzymes essential for the breakdown of plant fibers into volatile fatty acids and microbial crude protein. The microbial community composition and the active metabolic pathways involved in ruminal microbial metabolism were studied intensively during the last years and are of great interest to animal nutrition [69], biotechnology [70] and climatology [71].

In cell numbers bacteria are most abundant representing over 95% of microorganisms within the rumen ecosystem [72] and were first described using classical microbiology methods [73]. Over 200 bacterial species from the rumen were cultivated and most of them have been described physiologically [74]. Nevertheless, nucleic acid based approaches revealed that culture-dependent methods can only detect around 11% of the present bacterial phylogeny, thus

yielding imprecise and incomplete datasets [75]. For example, the cultivable genus Ruminococcus was believed to play a major role in ruminal cellulose degradation but actually appeared only below quantities of 2% [76].

Combinations of high throughput Omics-technologies in rumen microbial ecology provide a deeper insight into the symbiotic host–microbe relationship and the impact of nutritional strategies on the animal performance [77]. Comparisons between studies are challenging due to numerous analysis steps, varying methods and sampling strategies. Additionally the structure of the rumen microbiota differs significantly across individual animals [78] and depends on the substrates provided by specific diets [75].

Investigations of the rumen biology usually focus on bacterial or archaeal communities neglecting eukaryotic microorganisms. In order to characterize the entire rumen community, barcoded amplicons from all three domains of life were mixed and analyzed via Multiplex 454 Titanium pyrosequencing [79]. Twelve DNA samples from 11 ruminants out of three different species kept on various diets were processed revealing potential relationships between microorganisms as they indicated positive associations of Methanobrevibacter ruminatium and the *Fibrobacteraceae* family. The phylogenetic distribution was determined considering 257,485 bacterial, 125,052 archaeal, 45,231 protozoal and 186,485 fungal sequencing reads using the QIIME software package [79].

A comparable high-throughput approach analyzed the gut bacteria, archaea and fungi of 12 beef cows via 454 pyrosequencing concluding that in comparison with the bacterial community, archaea and fungi were more consistent during dietary alteration in liquid and solid fractions [80]. DNA sequences were processed using Mothur and CD-HIT suite. Observed species richness based on the V1–V3 region of the 16S rRNA gene accounted for 1903 to 2432 bacterial OTUs and between 8 and 13 archaeal OTUs per sample. Fungal OTUs based on 18S rRNA gene ranged from 21 to 40 [80].

Similar species richness was determined, with more than 1000 OTUs, by a pyrotag sequencing approach of DNA extracts from plant fiber material placed in the rumen for 72 h. The same material was used for a deep sequencing approach of the total DNA detecting a huge number of CAZymes (Fig. 3) and allowing the assembly of 15 genomes of uncultured bacteria [70].

The diversity of the bacterial community structure was analyzed in liquid and solid fractions of the rumen via metagenomic approaches [72, 81] and confirmed the previous findings of a DGGE-ARISA study [82]. Bacteria more abundant in solid fractions, as Ruminococcus spp., Fibrobacter succinogenes and Selenomonas ruminatium, are more likely to be involved in the degradation of polysaccharides. The average number of identified sequences per animal within

diet and fraction ranged from 1822 in the Bermuda grass liquid fraction to 3675 in the wheat solid fraction [81].

A PCR-DGGE fingerprint study indicated that the bacterial community structure of three Holstein cows did not change among five different gut sampling locations and three daily time points. Anyhow, a greater community shift was observed between individuals fed the same diet concluding that the deviation between animals is greater than the differences between fractions or time points [83].

An Illumina GAIIx-based study applied massively parallel sequencing to establish quantitative rumen microbiome profiles [84]. Eleven rumen fluid samples of three dairy cows resulted in more than 6 million reads of 146 bp length in each library. Commonly applied freeware was used to process the obtained sequence data. It was confirmed that the variation in rumen microbial metagenomes of different animals was greater within samples of the same rumen [84].

Furthermore, differences in rumen microbial ecology of 16 Holstein Friesian dairy cows kept on an equal diet were determined by bacterial tag-encoded amplicon pyrosequencing from the V2 and V3 regions of the 16S rRNA gene. In total 162,000 sequencing reads were filtered using the QIIME pipeline yielding 4986 OTUs overall. The samples had an average of 1800 OTUs but shared only 154 OTUs out of 32 genera. This comparably small core microbiome suggests a high functional similarity between individuals despite the actually observed phylogenetic differences [78].

The rumen microbiotas of three steers consuming a common diet were investigated by a full-length 16S rDNA clone library approach and 454 pyrosequencing of the total DNA [72]. Most sequences (64%) aligned to 59 OTUs are present in all libraries, whereas 273 OTUs containing 10% of sequences belonged to a single library. Besides, a wide range of unique glycoside hydrolase catalytic modules with 3800 sequences belonging to 35 glycoside hydrolase families were found to be present in the bovine microbiomes [72].

The rumen microbiome represents an important source of novel enzymes promising for biotechnological applications (Fig. 3). A deep sequencing approach using paired-end Illumina sequencing of DNA extracts obtained from plant fiber-adherent bacteria of a cow rumen yielded in 268 Gb of metagenomic DNA [70]. 27,755 putative CAZY genes were identified after sequence analyses showing a sequence similarity of less than 95% for 99% of the sequences. To discover new enzyme activities 90 ORFs were selected for protein expression studies and 57 of the expressed proteins showed clear cellulolytic activities. This study demonstrated for

the first time the benefit of deep metagenomic sequencing and activity screenings in the discovery of novel enzymes from the cow rumen [70].

Ferrer et al. used metagenomic libraries and functional screening assays for the detection of novel glycosyl hydrolases (GH) [85]. They discovered a multifunctional enzyme of GH family 43 belonging to Clostridiales and showing unusually broad substrate specificity. The 3D structure of the enzyme was modeled to determine the substrate binding sites and catalytic domains. These activity-based screening studies showed clear benefits to discover new metabolic functions besides the sole sequence analyses of DNA or RNA extracts.

Along with the microbial community composition two studies analyzed the rumen microbial metabolic profile via NMR [86, 87]. Thereby Lee et al. [86] suggested that the bovine host breeds are overlaying specific diets as major factor in determining the bacterial community structure and their metabolite profiles. Zhao et al. [87] was able to associate several metabolites with specific diets containing different types of roughages.

One study providing valuable information for milk production investigated the bacterial communities of 15 dairy cows via pyrosequencing and compared to production parameters and milk composition [88]. 141,344 reads averaging 338 bp in length were obtained detecting 17 bacterial phyla in total of which only 7 were present in all cows. The results indicated that the ratio of Firmicutes to Bacteroidetes was clearly associated with milk fat content, but most other taxa were rather related to the residual feed intake phenotype. Elucidating the role of rumen microbiota in shaping host physiological parameters may promote better agricultural yield through modulation of bacterial community structure [88].

2.6 Concluding remarks

The most extensive surface in the animal body is the GIT that harbors an immense variety and amount of microorganisms. Internal and external factors can unbalance this dynamic and complex niche and thereby, also disturb or improve the animal's health status.

Until recently, comparative studies of the microbiota were done between a few points of time and samples, sometimes even pooled samples were used. The results are often contradictory depending on the used animal (breed, age, gender etc.), the experimental setup (feeding and sampling), and used DNA extraction and sequencing method (target region of the 16S rDNA gene sequence). Therefore, it is hard to compare those studies and correlate them with each other. Nowadays, Omics-methods offer the advantage of being able to reliably measure and compare hundreds of samples simultaneously with low costs per sample. The millions of sequence reads available through pyrosequencing methods exceed the depths necessary to

describe microbial community compositions of a few samples by far. Therefore, inter- and intra-population similarities, temporal dynamics and effects of external factors on the GIT community of livestock should be addressed with the comparison of a broad array of samples. Requirements to deeply cover the phylogenetic diversity are optimized nucleic acid extraction methods and amplification strategies, especially the choice of the amplification region within the 16S rRNA.

Metagenomic sequencing and genome assemblies of uncultured prokaryotes already allows the detection of potential functions of the microbiota, but the analysis of the active fraction of the microbiota in the GIT of animals is still in their infancy. Metatranscriptomic and metaproteomic analysis should gain more importance within the next years to grant deeper insights into the expressed pathways and community interaction mechanisms. Labeling and imaging techniques will support the description on the *in vivo* activity of the communities and of single members. Combination of the collected data will support modeling approaches to detect microbial response mechanisms towards different feeding strategies, pathogens, antibiotics or environmental changes. When compared to the human gut, the analysis of livestock GIT was clearly neglected in the past years, but mainly due to the functional diversity, it should become of interest for future analyses.

2.7 Acknowledgments

We greatly acknowledge the financial support by the Carl-Zeiss-Stiftung and the Ellrichshausen'sche Stiftung.

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CHAPTER 3

VARIATIONS OF PHOSPHORUS ACCESSIBILITY CAUSING CHANGES IN MICROBIOME FUNCTIONS IN THE GASTROINTESTINAL TRACT OF CHICKENS

Bruno Tilocca[1], Maren Witzig, Markus Rodehutscord, Jana Seifert[*]

Institute of Animal Nutrition, University of Hohenheim, 70593 Stuttgart, Germany

[*] Corresponding author

Published in PLoS One 11(10): e0164735

The original publication is available under DOI:10.1371/journal.pone.0164735

3.1 Abstract

The chicken gastrointestinal tract (GIT) harbours a complex microbial community, involved in several physiological processes such as host immunomodulation and feed digestion. For the first time, the present study analysed dietary effects on the protein inventory of the microbiome in crop and ceca of broilers. We performed quantitative label-free metaproteomics by using 1-D-gel electrophoresis coupled with LC-MS/MS to identify the structural and functional changes triggered by diets supplied with varying amount of mineral phosphorous (P) and microbial phytase (MP). Phylogenetic assessment based on label-free quantification (LFQ) values of the proteins identified *Lactobacillaceae* as the major family in the crop section regardless of the diet, whereas proteins belonging to the family *Veillonellaceae* increased with the P supplementation. Within the ceca section, proteins of *Bacteroidaceae* were more abundant in the P-supplied diets, whereas proteins of *Eubacteriaceae* decreased with the P-addition. Proteins of the *Ruminococcaceae* increased with the amount of MP while proteins of *Lactobacillaceae* were more abundant in the MP-lacking diets. Classification of the identified proteins indicated a thriving microbial community in the case of P and MP supplementation, and stressed microbial community when no P and MP were supplied. Data are available via ProteomeXchange with identifier PXD003805.

3.2 Introduction

The chicken gastrointestinal tract (GIT) microbiome comprises more than 900 species of microorganisms [1]. This diverse microbiome establishes a complex network of interactions with the host playing an important role for the animal growth and health, since it is involved in several physiological processes such as modulation of the host immune system as well as breakdown and digestion of the feedstuff [1, 2]. Microbial composition changes longitudinally and radially along the gastrointestinal tract, since each intestinal section has its own characteristic "microenvironment" suitable for a better colonization by specific microbial species [2, 3]. In addition, changes of the phylogenetic structure are also caused by animal genetics, dietary composition and other environmental factors [2, 4].

To date, several studies investigated the chicken microbiota based on cultivation and 16S rRNA gene analysis (for reviews see [2, 4-6]). The potential microbial functions were described in only a handful of studies using shotgun sequencing to analyze the metagenome in either cecal or fecal samples from chickens [7-10]. In a recent study, Sergeant *et al*. [9] analyzed the metagenome of the cecal content from a single bird in order to assess the bacterial phylogenetic distribution and its potential activity. Another recent study investigated the microbiome of two pooled fecal samples based on 16S rRNA gene sequencing and metaproteomics in order to understand the metabolic processes in the gut of a healthy chicken [11]. Polansky et al. investigated the cecal microbiome of chickens at different ages, including 1-week-old chickens after inoculation with cecal extracts from hens of different ages, on the attempt to explain colonization patterns and predict the most promising probiotic genera for cecal colonization of newly hatched chickens [12]. Despite the extensive and heterogeneous ensemble of published studies, in-depth investigations about the possible functional changes of the microbiome of the chicken gut challenged with different dietary treatments are missing.

Phosphorous (P) is an essential macro element involved in a multitude of physiological processes such as bone development, growth and productivity of livestock. In plant seeds, organic phosphorous is mainly present as phytate, the salt of *myo*-inositol 1,2,3,4,5,6-hexakis (dihydrogen phosphate) (phytic acid; $InsP_6$). $InsP_6$ is only partially digestible for many monogastric animals such as chicken [13, 14]. Here, P bioavailability can be improved by phytases, phosphatases responsible for phytic acid dephosphorylation and release of P, available for the animal absorption in the intestine. Since endogenous phytases in chicken are less efficient and their activity depends on dietary phosphorous and cholecalciferol [15, 16], standard chicken diet formulation requires supplementation of mineral P and microbial phytase (MP) in order to reduce the problem of low endogenous phytase activity and ensure adequate P

provision to the animal [17]. Additional phytase dosage also helps to reduce the need for supplementation of mineral P in the feed formulation. This leads to a consequent reduction of P excretion which has a great significance for the ecological problem of water eutrophication and saving of P, a limited resource of global importance. Feeding diets with mineral levels under or above the optimal requirement may trigger alterations in microbial activity and composition, on the attempt to fulfil the nutritional requirements or by the alteration of the physico-chemical environment in the gut lumen [17-19].

To the best of our knowledge, no studies are published so far investigating the functional changes of the bacterial community inhabiting different GIT sections and correlating it to the different effects of broiler diets either supplemented with mineral P and/or MP. Witzig and colleagues (2015) characterized changes in bacterial phylogenetic compositions along the GIT of broilers fed with different mineral P and MP supplemented diets by T-RFLP and 16S rRNA gene sequencing [20]. They showed an effect of mineral P and MP towards the abundance of certain *Lactobacillus* spp. especially in the crop and a possible influence of MP in the cecal community. Here, we used this feeding experiment to analyze proteins extracted from crop and cecal bacterial communities to obtain indications for possible functional changes in the microbiome of these gut sections. A label-free quantitative (LFQ) metaproteomic approach was used for the assessment of the protein phylogenetic composition and abundance. Discussion on the detected functional pathways and the change of abundance of certain proteins in the diverse dietary treatments is given.

3.3 Material and methods

3.3.1 Animal experiment

Animal handling and treatments of the present study were approved by the animal welfare commissioner of the University of Hohenheim (internal experiment number T98/12 TE) in accordance with the German welfare regulations (documents are included in the supplementary information as S1 File Ethical approval document). A master of poultry farming did the animal experiment and euthanasia as regulated by the German law.

Samples were obtained from an animal experiment described in detail by Zeller et al. [14]. A schematic overview of the experimental workflow is provided in Figure A in S2 file. A total of 1,140 unsexed broiler hatchlings (Ross 308) were obtained from a local hatchery (Brüterei Süd GmbH and Company KG, Regenstauf, Germany). One hundred and eighty birds were housed in 18 pens (10 animals each) to investigate tibia mineralization, details on [14]. The remaining 960 animals were housed in 48 pens (20 animals each). On day 15 of age, broiler chickens were

assigned to six experimental treatments (8 pens/treatment) until day 25 of age. On day 25, birds were sacrificed by carbon dioxide asphyxiation following anesthesia in a gas mixture (35% CO_2, 35% N_2, and 30% O_2). From six pens per treatment, four animal each, were chosen for DNA-based investigation of the chicken GIT microbiota as detailed in [20]. Out of these, 4 animals each from two pens per treatment (48 animals in total) were chosen for the metaproteomic investigations. Remaining animals were used for investigation on inositol phosphate degradation [14]. The contents of crop as well as of cecal sections were separately collected and homogenized on a pen basis, yielding two pooled cecal and crop samples, respectively, per dietary treatment, for a total of twenty-four samples (Figure A in S2 file).

3.3.2 Experimental diets

All animals were fed a commercial broiler starter diet until day 14 of age. At day 15 the experimental diets were fed, all consisting of a basal diet [21] containing adequate levels of all nutrients, according to the recommendations of the German Society for Nutritional Physiology (GfE), with the exception of mineral P and calcium. Three diets contained P exclusively deriving from plant sources (BD–), whereas in another three diets additional P was supplied as mono-calcium phosphate (BD+). Diets of BD – and BD+ groups were further supplemented with 0, 500 and 12500 U/kg respectively of an *Escherichia coli* microbial phytase product (Quantum Blue, EC 3.1.3.26, supplied by AB Vista, Marlborough, UK), allowing a further distinction between MP0 and MP+ (MP500 and MP12500) diets.
Insights on the manufacturing of the diets as well as details on the diets composition and analysis performed on the experimental diets, are provided in the S1 Table and in reference [14].

3.3.3 Sample preparation

Twenty four pooled samples were kept on dry ice during their transport to the laboratory and stored at -80°C until their analysis. After thawing at 4°C, bacterial cells were separated by using a previously described method [22] with modifications. Briefly, aliquots of 0.5 g of pooled samples were resuspended by vortexing in 15 mL washing buffer (50 mM Na_2HPO_4, 0.1% Tween 80, [pH 8.0]). Samples were then incubated for 10 min in a sonication bath (amplitude 50%, 0.5 cycle), shaken for 20 min in a reciprocal shaker at 100 oscillations/min and centrifuged at low speed (200 x g, for 15 min, 4°C). Supernatant containing the bacterial cells was collected in a 50 mL tube and the remaining pellet was subjected to further 4 rounds of the whole protocol, for a total of five rounds. Bacterial cells in the pooled supernatant were pelleted by centrifugation at 15,000 x g for 30 min at 4°C and subjected to protein extraction.

3.3.4 Protein extraction, quantitation, digestion

Recovered cells were resuspended by vortexing with 100 µL extraction buffer (2% SDS, 20 mM Tris-HCl [pH 7.5]) and mixing at 1400 rpm for 10 min at 60°C. Each sample was then mixed with 1 mL Tris-HCl buffer (20 mM Tris-HCl [pH7.5], 0.1 mg/mL $MgCl_2$, 1 mM phenylmethanesulfonyl fluoride , 1 µL/mL benzonase (Novagen, 99% 25 U/ µL). Cell lysis was ensured by 5 rounds of 1 min ultra-sonication using a sonication probe (amplitude 50%, cycle 0.5) and 1 min rest on ice. After 10 min shaking at 1400 rpm, 37°C, samples were centrifuged at 10,000 x g for 15 min at 4°C. Extracted proteins contained in the supernatant were quantified with the Quick Start™ Bradford protein assay (Bio-Rad, Hercules, USA) following the manufacturer's instructions. Approximately 50 µg of the extracted proteins were precipitated by incubation (30 min at 4°C) with precooled 20% trichloroacetic acid (TCA). Protein pellet was resuspended in 25 µL Laemmli-buffer for 5 min at 95°C before being purified on a one-dimensional sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE, 4% stacking gel, 20 mA; 12% running gel, 40 mA). Proteins were trapped in the first centimeter of the separation gel and an overnight in-gel digestion using in-gel trypsin (Promega) was done on the complete part of the protein-loaded gel piece [23, 24]. Recovered peptides were purified and desalted by using Zip-Tip C18 tips (Millipore, Billerica, USA), dried at the SpeedVac and resuspended in 5% acetonitrile (ACN) / 0.1% trifluoroacetic acid (TFA) before the LC-MS/MS measurement.

3.3.5 LC-MS/MS analysis

Five microliters of the resuspended peptide mixture were measured in three technical replicates by using Q-Exactive Plus mass spectrometer (Thermo Fisher Scientific, Darmstadt, Germany) faced with EASY-nLC 1000 (Thermo Fisher Scientific) equipped with an EASY-Spray PEPmap column (50 cm x 75 µm inner diameter) packed with C18 resin, 2 µm particles, 100 Å pore size (Thermo Fisher Scientific). Peptides were loaded onto the HPLC column through solvent A (0.1% formic acid) at a flow rate of 500 nl/min and eluted over a solvent B (80% ACN in 0.1% formic acid) gradient ranging from 5% to 35% in the first 200 min and from 35% to 45% in the following 40 min.

MS/MS instrument was set to positive ion mode. Full scan was acquired in the mass range from m/z 300 to 1650 in the Orbitrap mass analyzer 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 $3*10^6$ charges for the MS scans and $1*10^5$ charges for the MS/MS scans with a maximum fill time of 25 ms and 45 ms,

respectively. Fragmented masses were excluded for 30 s after MS/MS. Spectra de-noising was performed prior to peptide identification by considering the only top 12 peaks in a window of 100 Da width.

3.3.6 Bioinformatic data analysis

To reduce the false discovery rate of peptide identification and for a better evaluation of protein abundance a two-step approach for bioinformatics data analysis was chosen. Briefly, proteins identified in the first database-dependent search are used to build a second sample-specific database, used for the second database-dependent search. The smaller size of the latter database enabled for a high coverage of the metaproteome as well as a reduced number of false positive inference [25, 26].

Acquired raw data were at first processed using Thermo Proteome Discoverer software (v.1.4.1.14), Mascot (v. 2.4) and searched against NCBI-nr bacteria and chicken databases (release July 12th, 2014) in order to export a protein fasta database. Methionine oxidation was set as variable modification and carbamidomethylation of cysteine as fixed modification. Default settings of the software were kept, these include 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%. In the second process, exported protein fasta file from the first search was subsequently used as *in-house* database for the peaks alignment and mass re-calibration in the first step of the MaxQuant search. MaxQuant software (v.1.5.1.2) set on LFQ modality was used for peptide identification and protein IDs inference. In the second step of MaxQuant analysis, raw data were independently searched against UniProtKB databases (release October 2014) bacteria (UniProt ID 2, 18976242 entries) and chicken (UniProt ID 9031, 82439 entries). In the database search, cysteine carbamidomethylation was set as fixed modification and methionine oxidation as variable modification. Two missed cleavage sites were allowed for 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.

The mass spectrometry proteomics data are publicly available in the ProteomeXchange Consortium via the PRIDE [27] partner repository with the dataset identifier PXD003805.

Further insights on the identified peptides and their implication for protein IDs inference, are provided in the S2 and S3 Tables.

Phylogenetic information were inferred on basis of the protein description outputted from the MaxQuant searches. These, in turn, are gathered from the protein annotation of the chosen database (*i.e.* UniProt KB).

LFQ abundances from MaxQuant´s output results were subjected to statistical analysis by using Primer6 v.6 statistical software (PRIMER-E, Plymouth, UK). Principal coordinate analysis (PCoA) was calculated on the basis of the Bray-Curtis similarity matrix [28].

Statistical difference between diet treatments was calculated by performing analysis of variance with permutations (PERMANOVA). Statistically different treatments were then subjected to SIMPER analysis in order to isolate proteins responsible for dissimilarity between pairs of groups with a cut-off threshold of 99.99% [29]. Selected proteins were functionally classified into COG and KEGG categories by using WebMGA on-line tool [30] with an e-value cutoff of 10^{-3} and exclusively considering the best hit.

Cladograms visualizing the dietary effects on the structure of the chicken´s crop and cecal microbiome were drawn using the Galaxy on-line tool. It implements the computation of the Linear Discriminant Analysis [21] between the technical triplicates of each experimental diet group ($n = 2$). Here, the Kruskal-Wallis test is performed to check whether differences between the experimental diets are statistically significant ($p < 0.05$).Only bacterial families showing discriminative effects with respect to the diets were considered and ranked according to the effect size with which they differentiate the diets [31].

Heat-Maps for phylogenetic composition across the different diet treatments and functional classification of the identified proteins were drawn using heatmap.2 provided by the gplots package [32] implemented in R v.3.1.2 software (http://www.R-project.org).

3.4 Results and discussion

3.4.1 Chicken proteome

Despite using a protocol to analyze microbial proteins, it is a common phenomenon that eukaryotic proteins are always co-extracted and processed during the metaproteomics workflow [33-35]. In this study, a total of 248 and 405 host proteins were identified in the crop and cecal section, respectively, which were investigated in order to highlight the possible presence of specific transporters or any other activity related to the adaptation to the changing dietary conditions. A complete list of the identified host proteins is given in S4 Table. Functional classification of the identified proteins was performed by categorization of their abundance intensity into COG classes and KEGG pathways. In addition, subcellular classification [36] of the chicken proteins reveals that only a small fraction of the proteomes were plasma membrane

proteins (6.9 and 14.8% on average for crop and ceca respectively, data not shown). No statistical differences in chicken protein abundances were observed between the diets in both GIT sections. The reason may be found in the relatively low protein numbers, which were obtained by the sample preparation protocol, which favored prokaryotic cells [22].

3.4.2 Analysis of the bacterial metaproteome

The used sample preparation protocols yielded a total of 381 and 1,719 bacterial proteins for crop and ceca sections respectively, with 3.1% of the total proteins shared among both sections. The relatively low identification rate in the crop section is probably explained by the low bacterial abundance typical of this section and the high amount of feed residues, which were co-extracted [37]. Details on the number of identified proteins and peptides in each sample, as well as a general overview of the abundance intensities of proteins in both GIT sections along with their grouping into KEGG biochemical pathways, are summarized in Figure B in S2 file and S5 Table.

Out of the overall dataset, PCoA analysis was performed to ordinate the samples depending on the different dietary treatments (Fig 1). In the crop section, 38.9% of the total variation was observed in the PCoA1, where metaproteomes of the samples without mineral P supplementation clustered together and drift apart from the samples supplemented with mineral P ($p = 0.043$, Fig 1A). PCoA analysis of the cecal samples showed a clustering of the microbial proteins from birds fed with mineral P- supplied diets and a separation from treatments without mineral P supplementation, which was not significant ($p > 0.05$, Fig 1B). A very clear distinction was shown between the MP-containing diets that clustered apart from the MP-lacking diets ($p = 0.008$).

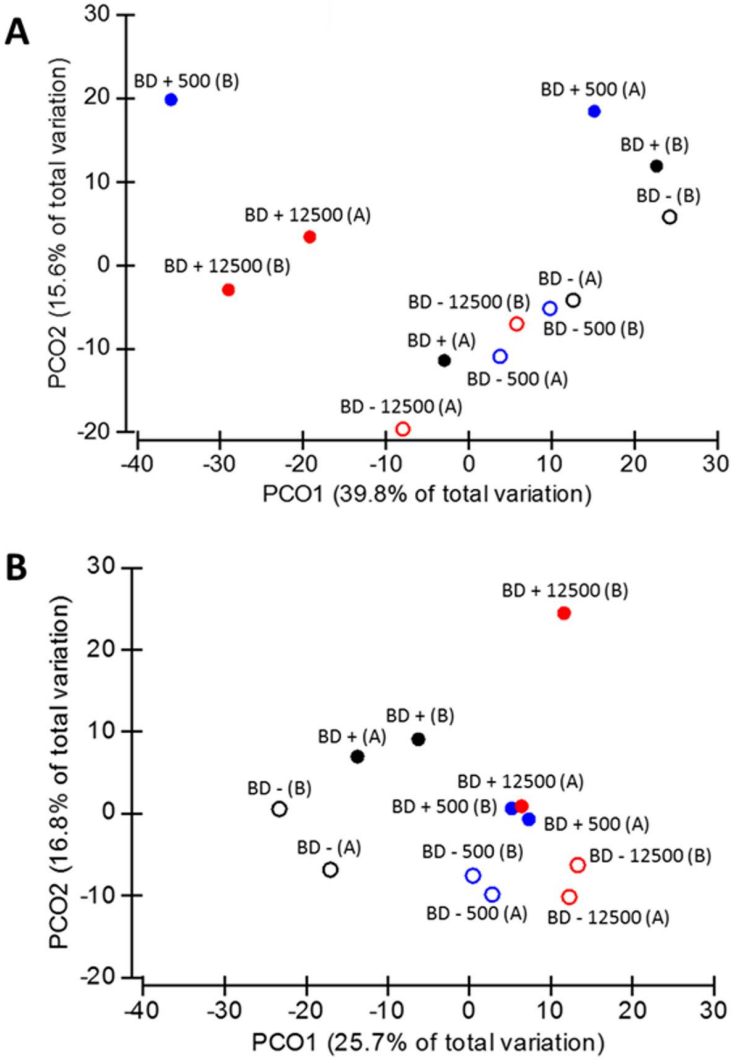

Fig 1. Principal coordinate analysis of the crop (A) and ceca (B) microbiome at different dietary treatments. Open shapes refer to diet without P supplementation, full shapes concern diets with P addition. Black, blue and red colors refer to MP0, MP500, and MP12500, respectively.

Experimental error due to the possible variability of the analytical techniques was controlled by averaging the technical triplicates measured for each sample. The PCoA analysis of cecal samples (Fig 1B) also showed that the pairs of biological duplicates were close together, with an average similarity of 72.2% (ranging from 69.0% to 84.5%), meaning that observed differences represent a "true" biological difference induced by our experimental treatments.

3.4.3 Bacterial taxonomy of the proteins

The phylogenetic composition of the bacterial proteins extracted from crop and ceca was determined based on the abundance values of the proteins belonging to each bacterial family. These values were summarized and only families with a cumulative abundance greater than 3% or 1% of the total were considered for phylogenetic analysis of crop and ceca samples, respectively (Fig 2, Figure C in S2 file). The abundance of each bacterial family in respect to the experimental diets is shown for the crop (Fig 2A) and cecal (Fig 2B) samples. Only bacterial families showing a discriminative effect ($p < 0.05$) with respect to the diets were considered and ranked according to their contribution in the different experimental treatments. This rank is graphically displayed as width of the cladogram portion attributed to each bacterial family. In addition, a phylogenetic assessment was done considering only families expressing at least one phylogenetic marker protein (*i.e.* highly conserved proteins employed as marker for phylogenetic analysis) [38-41] (S6 Table). This assessment reflected qualitatively the phylogenetic distribution of the total proteins.

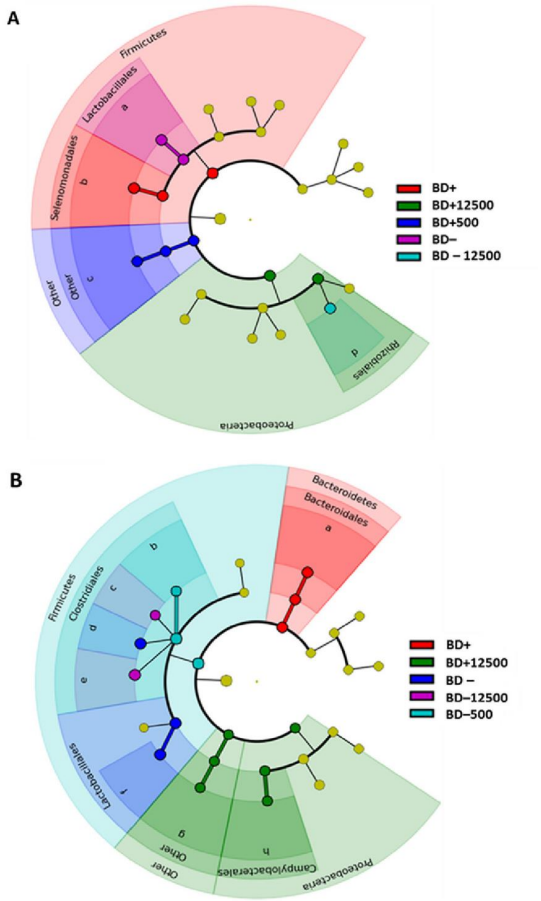

Fig 2. Dietary effect on phylogenetic composition of the chicken´s crop (A) and cecal (B) microbiome. Cladograms of both sections show a comparative evaluation of the experimental treatments effects on the structure of the chicken´s GIT microbiome. Effects are calculated through LDA Effect Size [42], a two-module algorithm. In the first module, technical triplicates of each dietary group (n= 2) were subjected to non-parametric Kruskal-Wallis test to detect features with significant differential abundance with respect to the experimental treatments. In the second module, tabular abundance data formatted in the previous module are subjected to Linear Discriminant Analysis [21] to estimate the effect size of each differentially abundant feature. The only diets and bacterial families showing statistical significance ($p<0.05$) in the previous statistical tests are visualized in the figures. Yellow dots refer to bacterial specimens whose protein pattern and abundance did not score a statistical significant effect ($p>0.05$) in any of the experimental diets.

Bacterial families legend:

(A) a: *Lactobacillaceae*; b: *Veillonellaceae*; c: *Other families*; d: *Bradyrhizobiaceae*.

(B) a: *Bacteroidaceae*; b: *Clostridiaceae*; c: *Eubacteriaceae*; d: *Lachnospiraceae*; e: *Ruminococcaceae*; f: *Lactobacillaceae*; g: *Other families*; h: *Helicobacteraceae*.

In general, phylogenetic distribution of the crop metaproteomes showed a reduced bacterial diversity and a high inter-individual diversity among all dietary treatments causing the unpaired scattering of the biological duplicates in the PCoA analysis (Fig 1A). In accordance with other studies on the chicken´s GIT microbiota composition (for reviews see [2, 4, 6]), proteins belonging to *Lactobacillaceae* were the most abundant regardless of the diets (Figure C in S2 file). The number of proteins belonging to *Veillonellaceae* increased on average in BD+ diets (36%) when compared with the BD– diets (23%; Figure C panel A in S2 file, Fig 2A). *Veillonellaceae* has been often associated to fiber digestion and short chain fatty acid production [43, 44], therefore its increase in P-supplied diets suggests a potential beneficial effect for the animal growth and performance [45]. With the exception of *Veillonellaceae* and a few other families commonly found in the chicken´s upper GIT (*Propionibacteriaceae, Erysipelotrichaceae, Eubacteriaceae, Clostridiaceae*) [46], other minor bacterial families identified in this study such as *Nocardiaceae*, *Gordoniaceae*, *Bradyrhizobiaceae*, *Rhizobiaceae*, *Moraxellaceae*, *Desulfovibrionaceae* and *Pseudomonadaceae* are more likely to be found in environmental samples [47, 48]. However, all these families were also found in the gut microbiome of humans or other animals [46, 49-51], therefore their presence in the crop microbiome may be either attributed to an intake from the environment (*e.g.* with the feed) or such bacterial families can be considered as common members of the crop´s microbial fraction. The DNA-based phylogenetic analysis of the same crop samples analyzed by Witzig et al. resembled also the predominance of *Lactobacillaceae* and a decrease of them concomitant to MP supplementation [20]. Other minor bacterial families were either not detected in the DNA-based study or in the present one.

Phylogenetic distribution of the cecal microbial community (Fig 2B) showed a higher phylogenetic diversity of the identified proteins in all dietary treatments and a change in the composition depending on the diets. Proteins belonging to *Bacteroidaceae* showed an average abundance of 14.9% in mineral P-supplied diets regardless of the presence of MP compared to 8.4% abundance in the samples from the diets without mineral P supplementation. Conversely, proteins belonging to *Eubacteriaceae* were more abundant in all diets without mineral P supplementation (4.6%) than with mineral P supplementation (3.9%). Proteins belonging to *Ruminococcaceae* were more abundant in the diets with increasing amounts of MP (25.4% in the MP+ diets *vs.* 11.5% in the MP0 diets) whereas proteins of *Lactobacillaceae* showed a

contrary abundance (5.2% in MP+ *vs.* 27.8% in MP0 diets; Fig 2B, Figure C panel B in S2 file). The increased abundance of *Bacteroidaceae* in the BD+ diets as well as the decrease of *Lactobacillaceae* due to the MP supplementation is in line with the results of the DNA-based analysis performed by Witzig et al. [20]. However, no increased abundance of *Bacteroidaceae* due to the dietary MP addition was observed and no *Erysipelotrichaceae* members were found in the present metaproteomic investigation. Nevertheless, a higher bacterial diversity in the microbiome of crop and ceca sections was highlighted with the present approach. The discrepancy in the results from the same samples is imputable to the different methods. The amplification steps of T-RFLP and amplicon pyrosequencing analyses, including a possible primer bias and the presumed overestimation of taxa with a higher number of 16S rRNA genes, are probably the reason of the reduced heterogeneity found in the microbiota composition. On the other hand, this step allows a higher sensitivity to target minor bacterial families which are missing in the metaproteomic approach due to the lack of genomic sequences. Besides these technical issues, the greater number of changes in the bacterial composition highlighted in the present work may be due to the point that a change of abundance of the expressed proteins is detected earlier than change in the number of DNA copies. Consistently, other studies observed this phenomenon. Haange et.al described a higher number of phyla and classes in their metaproteomic dataset than in 16S rDNA sequencing data, while investigating colon mucosa and fecal rats microbiota [34]. Similarly, inconsistencies between DNA- and protein-based microbiota assessment was described by Tang et al. [11]. They also showed that the correlation between the potential and active bacterial community is not always possible since the species identification in proteomics was different to that of 16S rRNA gene sequencing. Contrariwise, comparable results were observed by Polansky and colleagues while considering the cecal microbiota composition as determined by 16S rRNA gene sequencing and through protein mass spectrometry [12].

3.4.4 Abundance of metabolic functions varying between the dietary treatments

A general overview of the global chicken crop and cecal metaproteomes and abundance intensities of the identified proteins grouped into KEGG biochemical pathways and COG categories is shown in the Figure B and D in S2 file, respectively. PERMANOVA analysis of the total datasets of both sections was done to check for significant differences between diets. Crop samples showed that the only pair of treatments MP0 – MP12500 across P factor were statistically significant (p = 0.048). Within ceca section, statistical significance was shown by the experimental treatments with and without mineral P supplementation across the MP factor

(BD– /BD+; p = 0.037), whereas among the MP-containing diets, the pairs MP0/MP500 and MP0/MP12500 showed statistical significance (p = 0.025 and p = 0.031, respectively).

The entries of the statistically different treatments were subjected to SIMPER analysis, using a strict cut-off threshold (99.99%), to identify single proteins that caused the dissimilarity between treatment groups. These proteins were functionally classified by grouping them into COG categories (Fig 3). Concerning crop section, the comparison between MP0 and MP12500 treatments showed that the MP0 metaproteome include some unique COG categories expressed at a low relative percentage (Figure E in S2 file). In MP12500 metaproteome, lipid metabolism (I) was uniquely identified and three categories were more abundant in comparison with MP0 such as translation, ribosomal structure and biogenesis (J), carbohydrate transport and metabolism (G) and amino acid transport and metabolism (E) (Figure E in S2 file).

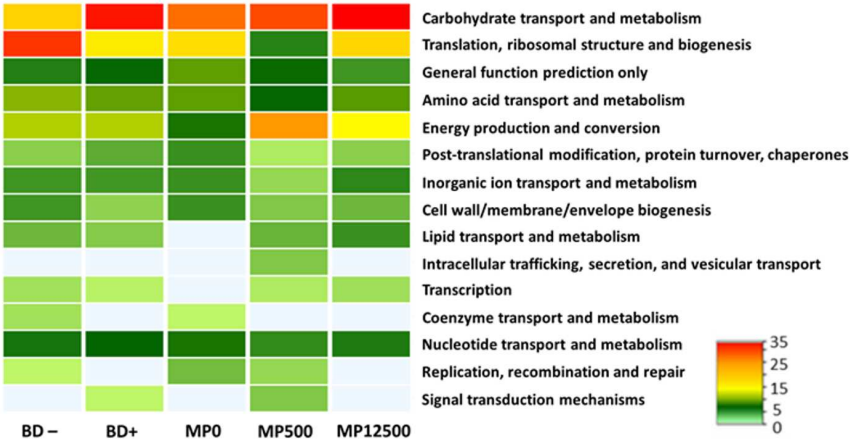

Fig 3. Functional classification of cecal proteins into COG categories.

Heat-Map is drawn on the basis of the relative percentages of the proteins of each statistically different treatment. COG classification of crop samples proteins is available in Figure D in S2 file.

Protein data of the cecal samples showed that proteins belonging to translation, ribosomal structure and biogenesis (J) were more abundant (23%) in the BD- samples than in the BD+ ones (14%) (Fig 3). In contrast, proteins of the carbohydrate transport and metabolism (G) group were more abundant, increasing from 16% in the BD– to 24% in the BD+ diets. Among the MP-containing treatments, some COG categories were found to increase with the addition of MP such as carbohydrate transport and metabolism (G) and energy production and

conversion (C); while the lipid transport and metabolism (I) category was solely identified in the MP-containing diets. The post-translational modification, protein turnover, chaperones (O), by contrast, was found to decrease with the MP-addition. This last, together with the increasing category carbohydrate transport and metabolism (G) and lipid transport and metabolism (I) are in agreement with the results obtained in the crop section (Fig 3), suggesting that these changes are particularly triggered by the MP addition. Based on these results, our initial hypotheses that the diets will affect the functional profile of the bacterial communities was accepted. Specifically, mineral P-available diets (BD+; MP+) stimulate a "productive bacterial community" where bacterial resources are focused on complex anabolic functions; while the microbial community present at low mineral P diets (BD– ; MP0) is concerned in the stress response mechanisms, suggesting that the mineral P limitation affecting the host health status is also reflected as stress factor for the gut microbiota.

This idea seems to be in accordance with a study of Tang et al., which correlated the expression of proteins involved in metabolic processes of carbohydrate, alcohol and proteins to a thriving microbial community [11]. Protein folding has been linked to the microbial stress response to the high temperature of the chicken body. In our results, the thriving condition is maintained by the adequate nutrients supplementation, while the main stress factor is represented by the lack of P. The great expression of proteins involved in carbohydrate metabolic processes (BD+, MP+) is also supported by another work providing the genetic evidence of numerous enzymes involved in polysaccharide degradation and sugar transport and utilization [9]. Qu et al. observed an enrichment in the "carbohydrate metabolism" SEED subsystem in the cecal microbiota of the control bird when compared to the metabolic potential of a chicken challenged with *Campylobacter jejuni* infection [8]. The metabolic potential of the chicken´s fecal microbiota as assessed by Singh et al. [52] shows that the abundance of the "carbohydrate metabolism" SEED subsystem was stable between the metagenomes of low and high feed conversion rate chickens whereas SEED subsystems related to sulphur metabolism and motility/chemotaxis were statistically different.

3.4.5 Single proteins and pathways highlighting the functional differences

The abundance intensities of the proteins, responsible for significant dissimilarities between experimental treatments in the two GIT sections, were additionally grouped according to KEGG biochemical pathways (Figure F in S2 file). In both sections, major biochemical pathways were identified in all experimental treatments, but only a low number of proteins within these pathways was shared between the different treatments, suggesting a diverse overall activity of

the microbial communities depending on the fed diets (Figure F in S2 file, S7 Table). Additionally, differences in the abundance level of the common proteins across the diets were observed, indicating a probable modulation of the highly conserved functions of the microbiota on the attempt to shape an adequate response to the changing environment. Specifically, the higher abundance of the KEGG Orthologous system [53] for "ribosome" (KO 03010) and "aminoacyl-t-RNA biosynthesis" (KO 00970) observed in crop MP12500 samples, as well as the higher abundance of "ABC transporters" (KO 02010) registered in crop MP0 samples, suggest an increased metabolic activity, and an overall effort of the bacterial community to maximize the P uptake respectively for the MP+ and MP0 samples (Figure F in S2 file). Similar trend was observed in the cecal samples. The high-expression of several KOs such as "glycolysis/gluconeogenesis" (KO 00010, BD+ samples) and "starch and sucrose metabolism" (KO 00500, MP+ samples) indicate a productive bacterial community and a coexistence of the bacterial members in a thriving microenvironment. Microbial communities of BD- and MP0 samples by contrast, are more focused on diverse degradation pathways and "ABC transporters", suggesting harder survival conditions for the bacterial members that must effort on the attempt to maintain the level of required phosphorous above the least threshold (Figure F in S2 file).

A comparative evaluation on how the abundances of the KOs [53] vary in the different diets is shown in Fig 4. In the proteins of the ceca, the majority of pathways are overrepresented in the BD+ diets (Fig 4A). Several KOs were found to be overrepresented in the MP0 treatments such as 'pyrimidine metabolism' and 'arginine and proline metabolism' (KO 00330). Their abundances decreased with the addition of MP, resulting in a more favorable ratio for the MP0 treatment (Fig 4 panel B and C). Conversely, 'lysine biosynthesis' as well as 'oxidative phosphorylation' registered an overrepresentation in MP0 in the only pair MP0/MP500, while a further MP addition (MP0/MP12500) led to a slight overrepresentation in the MP12500 sector.

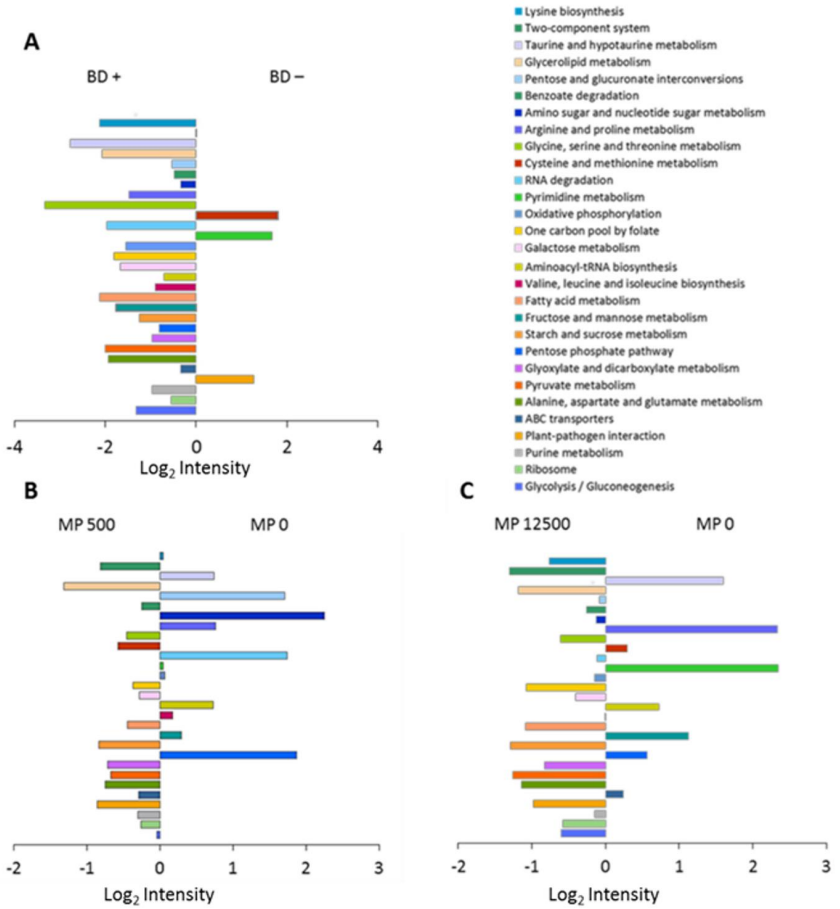

Fig 4. Relevant biochemical pathways between experimental treatments.

Comparison of different dietary treatments: (A) BD+ and BD–. (B) MP500 and MP0. (C) MP12500 and MP0 based on the log2 of the ratios between the cumulative intensities of the statistically significant pairs of KOs. Each of the graph´s bars represent a KEGG biochemical pathway. Only pathways with a cumulative abundance greater than 1% of the total are considered in the graph.

Identified proteins were sorted into KEGG metabolic maps (S7 Table) in order to confirm the previous COG results and give further support to our hypothesis that experimental diets affect the protein expression of the microbial community. The BD+ metaproteomes differ from the BD– counterpart basically in the pyrimidine metabolism, where the BD+ microbial community is involved in the production of carbamoyl phosphate, an intermediate in the biosynthesis of

arginine and the pyrimidine nucleotides. In addition, differences were observed in the two component system (KO 02020) pathway. The bacteria induced by feeding of the mineral P-supplied diets expressed PhoP, an OmpR family regulator involved in P assimilation, whereas samples of the BD– microbiota showed a higher abundance of outer membrane proteins OmpC and OmpF, involved in the passive diffusion of small molecules across the outer membrane. This suggests an attempt of the microbial community to save and/or improve the uptake of the limited available P. A study of the potential metabolic activity of the cecal microbiome showed a significant enrichment of the 'transporters in models' SEED subsystem in the chickens treated with coccidiostats/growth promoters when compared to the control animals [7]. Predicted proteins of this group are involved in several biological processes such as amino acid, potassium, calcium and heavy metals transport. A closer look within KOs abundant in the MP samples underline that the MP+ microbiota was active in the *de novo* synthesis of nucleotides as suggested by the identification of dihydroorotate dehydrogenase, carbamoyl-phosphate synthase and UMP kinase. MP0 microbiota by contrast, seemed to be more active in the stress response mechanisms as supported by the identification of D-proline reductase, the enzyme involved in the production of intermediate products that will enter the lysine degradation pathway. Both MP0 and MP+ metaproteomes are involved in P assimilation, but only the microbiota of the MP+ diets was concerned in energy production to support the biosynthetic metabolism. This is supported by the exclusive detection of proteins involved in aerobic/anaerobic respiration and other enzymes (beta-galactosidase, galactokinase, galT, PTS-Aga-EIIA, tagatose 1,6-diphosphate aldolase) indicating an enhanced activity on sugars digestion oriented to energy production. Additional activity of the MP+ microbiota included amino acids biosynthesis as suggested by the identification of several enzymes such as argininosuccinate lyase, glutamate synthase, phosphoglycerate dehydrogenase, glycine hydroxymethyltransferase, tryptophan synthase, glycine acetyltransferase and threonine dehydrogenase.

The differences in the abundances of certain functional pathways depending on the diet was strongly linked to changes of the microbial community composition (S8 Table). In the cecal BD+ diets for example, *Bacteroidaceae* was among the most abundant families in many pathways, in particular the glycolysis/gluconeogenesis (KO 00010), ribosome, fructose and mannose metabolism (KO 00051), and the uniquely expressed pyruvate metabolism (KO 00620) KEGG pathway. Results of the BD– diet metaproteome showed *Eubacteriaceae* as the major family, together with other bacterial families, encoding for plant-pathogen interaction and glyoxylate and dicarboxylate metabolism (KO 00630). *Lactobacillaceae* was the principal

family in the MP0 treatment encoding the ABC transporter and RNA degradation (KO 03018) as the most abundant KEGG pathways. In contrast, *Ruminococcaceae* was the main family to the most abundant pathways in the MP+ diets. Comparing results of MP0 and MP+ diets, the family *Lactobacillaceae* tends to reduce its proportion with the addition of MP in the shared pathways between MP0 and MP+ diets, the opposite was found for the *Ruminococcaceae* family.

In conclusion, this is the first study describing the metaproteome of the crop and ceca bacterial communities of broilers fed with different dietary treatments. Our results proved that changes in the bacterial protein inventory were triggered by the experimental diets. The bacterial community was focused on complex and productive functions in the case of P-available diets (BD+; MP500; MP12500), which was contrary to the overall direction towards stress response in the case of P-deficient diets (BD–; MP0). The data provide key findings for further investigations aimed to design innovative poultry husbandry strategies to reduce supplementation of mineral P in the diet and to maintain a balanced microbial activity in the GIT. Nevertheless, further studies are needed to draw a complete picture of the complex activities of all GIT sections and the changes of the microbiota due to different dietary regimen. Moreover, investigation of the mucosa-associated microbiota as well as the specific bacteria involved in the $InsP_6$ turnover are needed for an overall description of the chicken´s GIT microbiota and its changes triggered by the diet.

3.5 Acknowledgement

The authors wish to thank Prof. Dr. Hans-Peter Piepho for the precious suggestions for the statistical data analysis and the team of the mass spectrometry group at the University of Hohenheim for the LC-MS/MS analysis.

Supporting Information

The supporting information listed below are freely available at the following link:

http://journals.plos.org/plosone/article?id=10.1371/journal.pone.0164735#sec015

S1 File Ethical approval document

Ethical approval document of the animal experiment

S2 File Supplementary Figures

This file includes all supplementary figures (Figure A – Figure F, see below)

Figure A Experimental workflow.

Diagram shows a general overview of the experimental workflow of this study. Orange part of the diagram highlight the main steps of the workflow. Briefly, animal experiment consists of 1140 Ross 308 broiler chicken. Out of these, 960 animals were housed in 48 pens, 20 birds each. Pens were assigned to six different experimental diets (8 pens/ diet). For the whole microbiome analyses, 6 pens/diet were first chosen. All other animals were used for other investigations. For metaproteomic analyses, 4 animals each from 2 pens/diets were randomly selected. Collected content of crop and ceca was homogenized on a pen basis yielding two crop and cecal samples per diet (biological duplicates of crop and ceca, respectively). Obtained samples were subjected to the protocols for sample preparation for tandem mass spectrometry measurements, obtained raw files were finally subjected to bioinformatics data analysis as detailed in material and methods section.

Figure B Crop and cecal metaproteome overview.

The heatmaps show Log2 intensities of the identified proteins for crop **(panel 1)** and ceca **(panel 2)** samples.
Biological duplicates of each dietary treatments are distinct with A and B letters.
Identified proteins of both sections are functionally categorized into KEGG biochemical pathways.
KEGG pathways abbrevaiations:
Gl. : Glycolysis/gluconeogenesis; Rib. : Ribosome; Pur. : Purine metabolism; Pl.-pat. : Plant-pathogen interaction; ABC : ABC transporters; Ala, Asp, Glu : Alanine, aspartate and glutamate metabolism; Pyruv. : Pyruvate metabolism; Glyox. : Glyoxylate and dicarboxylate metabolism; PentP : Pentose phosphate pathway; Fruc. : Fructose and mannose metabolism; Starch : starch

and sucrose metabolism; Amin. t-RNA : Aminoacil-t-RNA biosynthesis; Galac : Galactose metabolism; One C : One carbon pool by folate; Gly, Ser, Thr : Glycine, serine and threonine metabolism; FA : Fatty acid metabolism; Pyr. : Pyrimidine metabolism; Val, Leu, Ile : Valine, leucine and isoleucine biosynthesis; Amino & nucleotide sugar : Amino sugar and nucleotide sugar metabolism; Cys Met : Cysteine and Methionine metabolism; Ox.ph. : Oxidative phosphorylation; RNA deg. : RNA degradation; Prot ER : Protein processing in endoplasmic reticulum.

Figure C Taxonomic composition at family level of the chicken´s GIT microbiota.

(A) Crop active microbiota. **(B)** Cecal active microbiota. Grey background is assigned to non-detected families.

Figure D Functional classification of the whole metaproteomes into COG categories.

Bar´s height is calculated on the basis of the Log2 abundance intensites of the identified proteins for crop **(A, B)** and ceca **(C,D)**. Biological duplicates are shown for crop **(A)** and cecal **(C)** samples. **(B and D)** charts refer to the COG categorization of the averaged duplicates of crop and ceca respectively.
COG categories abbreviations:
C : Energy production and conversion; E : Amino acid transport and metabolism; F : Nucleotide transport and metabolism; G : Carbohydrate transport and metabolism; H : Coenzyme transport and metabolism; I : Lipid transport and metabolism; J : Translation, ribosomal structure and biogenesis; K : Transcription; L : Replication, recombination and repair; M : Cell wall/membrane/envelope biogenesis; N : Cell motility; O : Posttranslational modification, protein turnover, chaperones; P : Inorganic ion transport and metabolism; Q : Secondary metabolites biosynthesis, transport and catabolism; R : General function prediction only; T : Signal transduction mechanisms; U : Intracellular trafficking, secretion, and vesicular transport; V: Defense mechanisms.

Figure E Functional classification of crop proteins into COG categories.

Heat-Map is drawn on the basis of the relative percentages of the proteins of each statistically different treatment. COG categories abbreviations: G: Carbohydrate transport and metabolism; J: Translation, ribosomal structure and biogenesis; R: General function prediction only; E: Amino acid transport and metabolism; C: Energy production and conversion; O: Post-translational modification, protein turnover, chaperones; P: Inorganic ion transport and metabolism; M: Cell wall/membrane/envelope biogenesis; I: Lipid transport and metabolism;

U: Intracellular trafficking, secretion, and vesicular transport; K: Transcription; H: Coenzyme transport and metabolism; S: Function unknown; V: Defense mechanisms; Q: Secondary metabolites biosynthesis, transport and catabolism.

Figure F Functional characterization of the crop and cecal metaproteome.

Heat maps show the LFQ abundances of the proteins responsible of significant dissimilarities between experimental treatments in crop **(A)** and ceca **(B)**. Proteins are categorized into KEGG biochemical pathways: Gl.: Glycolysis/gluconeogenesis; Rib.: Ribosome; Pur.: Purine metabolism; Pl.-pat.: Plant-pathogen interaction; ABC: ABC transporters; Ala, Asp, Glu: Alanine, aspartate and glutamate metabolism; Pyruv.: Pyruvate metabolism; Glyox.: Glyoxylate and dicarboxylate metabolism; PentP: Pentose phosphate pathway; Fruc.: Fructose and mannose metabolism; Starch: starch and sucrose metabolism; Amin. t-RNA: Aminoacil-t-RNA biosynthesis; Galac: Galactose metabolism; One C: One carbon pool by folate; FA: Fatty acid metabolism; Pyr.: Pyrimidine metabolism; Val, Leu, Ile: Valine, leucine and isoleucine biosynthesis; Cys Met: Cysteine and Methionine metabolism; Ox.ph.: Oxidative phosphorylation; RNA deg.: RNA degradation.

S1 Table. Study diet composition.

The two basal diets were formulated to contain adequate levels of all nutrients according to the recommendations of the German Society for Nutritional Physiology (GfE), with the exception of P and Ca. The table below show the ingredient composition and the concentration of the analyzed nutrients of the two basal diets.
Table adapted from Zeller E, Schollenberger M, Witzig M, Shastak Y, Kuhn I, Hoelzle LE and Rodehutscord M. 2015. Poult Sci 94:1018-1029. doi: 10.3382/ps/pev087.

S2 Table. Peptide/protein inference overview.

(A) Table report a summary of information on the peptides/proteins identified in crop section.
(B) Table include further insights on peptide identification and their implication in protein IDs inference

S3 Table. Peptide/protein inference overview.

(A) Table report a summary of information on the peptides/proteins identified in ceca section

S4 Table. **Chicken´s proteome overview in the different diet treatments.**

The green section show an averaged estimation of the proteins and peptides number across the experimental treatments. Yellow section details the list of all proteins identified, and their respective abundance, in each replicate (A), (B) of all treatments.

S5 Table. Metaproteome overview in the different diet treatments.

A) The green section show an averaged estimation of the proteins and peptides number across the experimental treatments. Yellow section details the list of all proteins identified, and their respective abundance, in each replicate of all treatments. Blue section refer to the number of peptides identified per protein in every treatment.
B) The table summarize the effective number of proteins and peptides identified in the repliactes of all experimental treatments.

S6 Table. Marker proteins.

Table below list the phylogenetic marker proteins which this work refer to for the phylogenetic assessment of the microbial community in the different dietary treatments in the ceca samples.

S7 Table. Proteins mapping into KEGG biochemical maps.

KO number of the proteins belonging to the selected pathways are mapped into KEGG biochemical maps in order to obtain detailed informations on the direction of each biochemical pathway undertaken by the micrbiota kept at differnt dietary treatments. Colors of the table specify whether a given protein is found exclusively in a GIT section: crop (red), ceca (blue) or in both (green) sections or in a specific diet: MP+ (red), MP0 (blue),both diets (green), ; P+ (red), P- (blue) or both diets (green)

S8 Table. Bacterial families involved in the identified KEGG pathways.

The table summarize the bacterial families active in the crop (panel A) and cecal (panel B) microbiota. Percentual contribution of the specimens for every KEGG biochemical pathway was calculated on the basis of the proteins counting. Only KEGG pathways with a cumulative number of proteins greater than 1% of the total were considered.

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CHAPTER 4

DIETARY CHANGES IN NUTRITIONAL STUDIES SHAPE THE STRUCTURAL AND FUNCTIONAL COMPOSITION OF THE PIG'S 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], Jana Seifert[*]

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

[2] Department of Agricultural, Food and Nutritional Science, University of Alberta, T6G2R3 Edmonton, Canada

[*] Corresponding author

Published in Microbiome (2017) 5:144

The original publication is available under DOI 10.1186/s40168-017-0362-

4.1 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.

4.2 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.

4.3 Methods

4.3.1 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 1.

Table 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.

4.3.2 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).

4.3.3 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.

4.3.4 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.

4.3.5 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).

4.3.6 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).

4.4 Results

4.4.1 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 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 S2.

Both datasets, based on DNA and metaproteomic investigation, were depicted in a PCoA plot on a sample basis (Fig. 1). The samples ordination revealed a highly comparable clustering on a time point dependent manner along the PCO1 axis (Fig. 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 ($p = 0.001$) (Fig. 1b, d, Additional file 2: Figure 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 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 S1B).

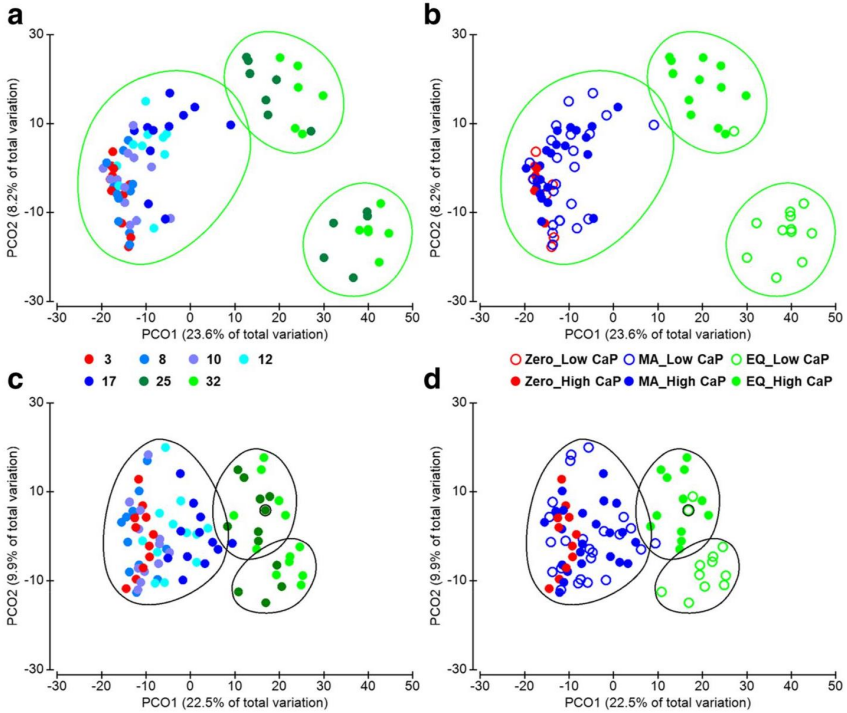

Fig. 1 Samples ordination reveals three adaptation periods.

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).

4.4.2 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 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 (Fig. 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 (Fig. 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 (Fig. 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 (Fig. 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 (Fig. 2).

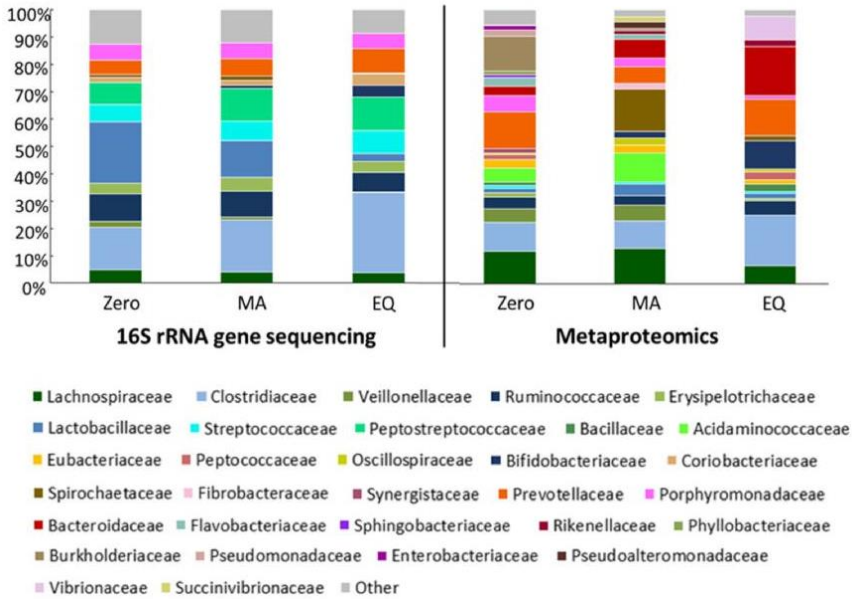

Fig. 2 Gut microbiota composition changes in a time point-dependent manner.

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.

4.4.3 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 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 S3), therefore, their consideration hinders a clear visualisation of the hypothesised gradual functional shift of the microbiota (Additional file 2: Figure 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 S5 and 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 S6).

The heat map shown in Fig. 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 (Figs. 3 and 4).

A further focus on the carbohydrate metabolic pathways and the related bacterial families is summarised in Fig. 4 and Additional file 2: Figure 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 (Fig. 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 (Fig. 4b).

Investigation of proteins of the pentose phosphate pathway highlighted that diverse bacterial proteins are involved in common functions (Fig. 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 (Fig. 4d). The bacterial community of the Zero samples, in line with the previous observation (Fig. 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].

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 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 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 S3).

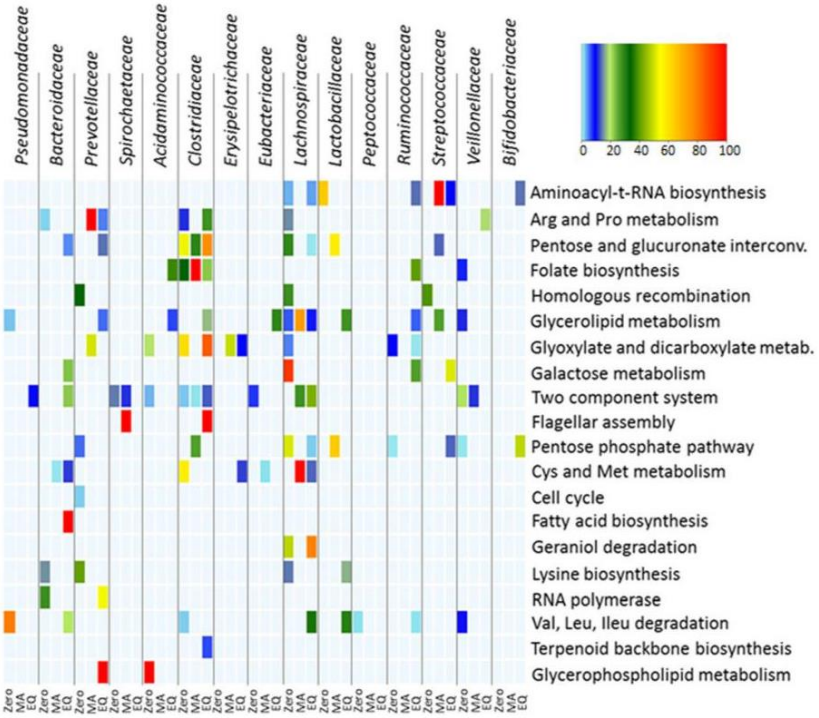

Fig. 3 Microbiota members are involved in a variety of diverse biochemical pathways.

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.

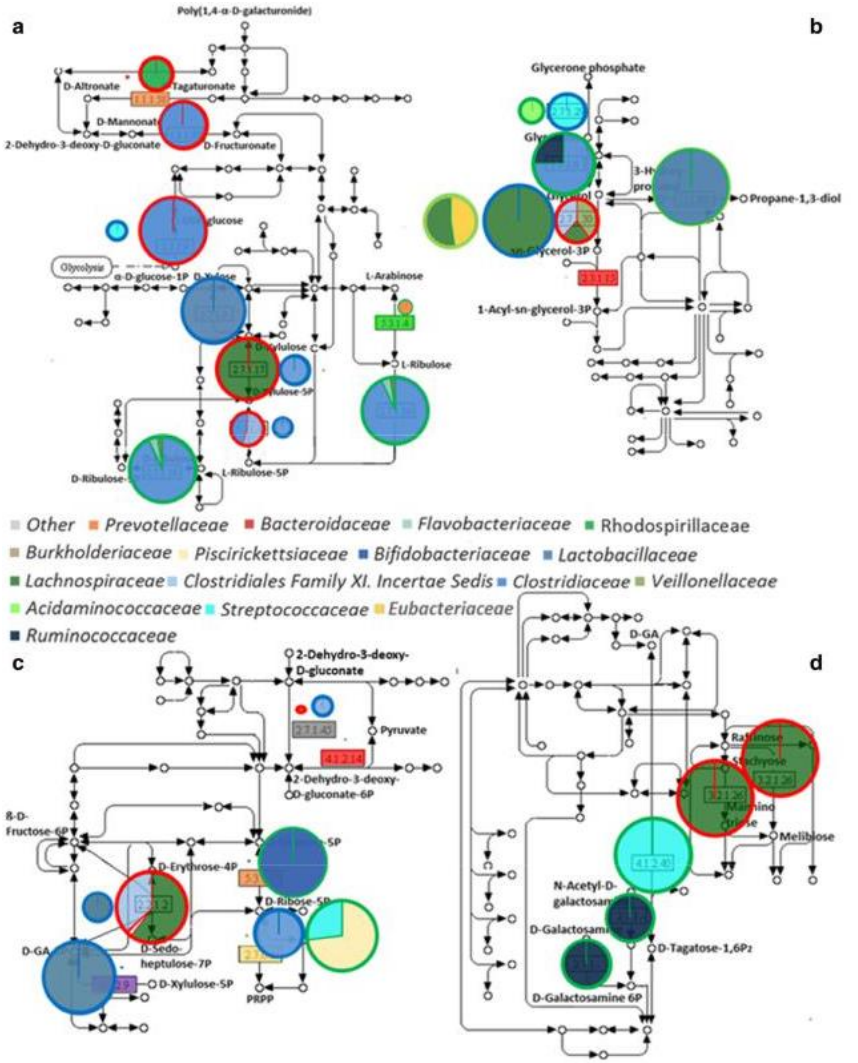

Fig. 4 Microbial community exhibit a varying concern in selected carbohydrates pathways.

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.

4.4.4 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 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 S7).

Details on the bacterial specimen involved in the SCFA production are provided in Additional file 2: Figure 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 S4 and Additional file 2: Figures S7 and 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 S4 and Additional file 2: Figures 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 S9).

4.4.5 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 Fig. 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 ($p = 0.001$, Fig. 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.

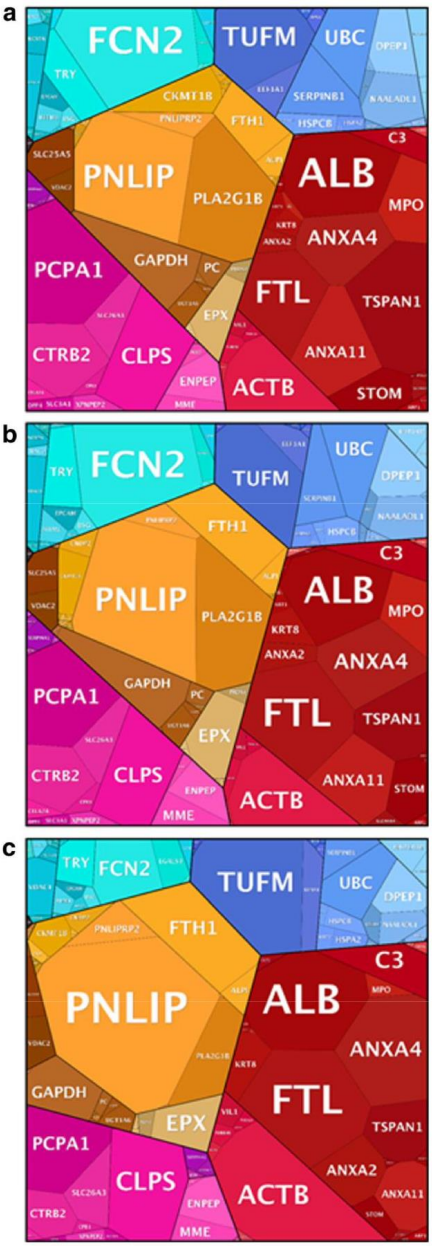

Fig. 5 Host proteome changes along with the remodelling of its gut metaproteome.
Voronoi diagrams show the host proteome of Zero „a“, MA „b“ and EQ „c“ samples. Identified proteins are visualised as polygons, whose area reflect their relative abundance. Gene IDs are detailed for each polygon.

4.5 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 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 divergence 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.

4.6 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.

4.7 Acknowledgements

We would like to thank the Proteome Center in Tübingen for their excellent support.

Funding

JS gratefully acknowledge the financial support of the Carl Zeiss Stiftung and the microP and METAPHOR project funded by the Ministry of Science, Research and the Arts Baden Württemberg. ACS was supported by the European Social Fund and by the Ministry of Science, Research and the Arts Baden Württemberg.

Additional Files

Additional file 1: Table S1**.** Table report the fast sequence of each OTU. Table 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 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 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 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 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 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 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 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 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).

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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.

Received: 11 April 2017 Accepted: 17 October 2017

CHAPTER 5

DISCUSSION

5.1 The gut microbiota and its importance in animal nutrition

Over the last decade, once unveiled the importance of understanding the synergistic interactions between host and its microbiota, studies aimed to characterise the microbiota composition and activity received a worldwide increasing interest under several applicative fields such as energy production [1], bioremediation [2], agriculture [3] and human [4] and animal health [5].

The importance of the microbiota and its interactions with the host organism for the maintenance of the homeostatic balance became clear immediately after the groundbreaking publication of the first human genome sequence, in 2001 [6]. In this regard, has been argued that purposes of the human genome project would not be considered completely fulfilled until the synergistic functions between the microbiota and its host are understood [7]. Recent researches concerning the GIT microbiota have remarked the importance of the intestinal flora and its implications in orchestrating development, structure and function of both "proximal" and "distal" body sites. A mini review of Clarke and colleagues [8] depict the gut microbiota as a neglected endogenous organ since its hormones, peptides and microbial metabolites regulate diverse physiological processes of the liver (*i.e.* gut-liver axis), skin (*i.e.* gut-skin axis), brain (*i.e.* gut-brain axis), and many other internal organs and glands. Gut microbiota composition and functions are also associated to organ and tissue development as well as inflammation and immune system modulation [9].

In the context of animal nutrition, the GIT microbiota plays a pivotal role in regulating the uptake, storage and expenditure of the nutrients obtained from the dietary formulations. Experimental evidences show that conventionally-raised mices are more prone to obesity and other metabolic disorders compared to germ-free littermates, suggesting a central role of the gut microbiota in the absorption and storage of the dietary-derived energy and nutrients. Moreover, the structure of the gut microbiota changes significantly between lean and obese animals and it is strongly affected by diet composition [9].

5.2 Phosphorus and animal nutrition

Phosphorus contained in the feedstuffs fed to livestock is mainly stored as InsP6, a scarcely digestible form for non-ruminant animals; thus, external supplementation of easily absorbable P is required in order to ensure adequate P levels [10, 11]. Moreover, the negatively-charged phosphate moieties of the InsP6 molecule, confer a high affinity to metallic cations such as Ca, Fe, K, Mg, Mn and Zn, making them unavailable as nutritional factors [12]. Also, InsP6 could affect the digestion and absorption of the other feedstuff's ingredients such as proteins and carbohydrates [13, 14].

Owing the limited P resources and the problem of water eutrophication, new animal breeding strategies have been adopted, in the field of animal nutrition, to reduce and/or safe the P usage and excretion [10]. Among these, the use of diets supplemented with phytases of microbial origin registered up to 50% improvement in P digestibility with no side effects for the animal health, enabling a reduced supplementation of easily absorbable P with a consequent reduction of P excretion [10].

Absorption of dietary-derived P is supposed to occur mainly in the small intestine [15], whereas controversial results have been produced for the large intestine since both absorptive and secretive mechanisms were demonstrated for the large intestine in regard to P [16-18]. Phosphorus homeostasis depends on several factors such as the diet formulation (*e.g.* InsP6, Ca and P content), feeding level and the amount of externally-supplemented P [19-21]. In the present work, P homeostasis was modulated through feeding diets with different levels of mineral Ca, P and MP. We hypothesized that diet-driven imbalance is also reflected on both composition and function of the host's intestinal microbiota. Specifically, we postulated that alterations deriving from the challenging diets are principally visible in the gut microbiota protein repertoire. Moreover, it was also supposed a gradual adaptation of the gut-associated microbial community, in order to configure a balanced microbial community that preserves the complex network of interconnections with the host body and ensure the overall homeostatic balance.

5.3 Chicken gut microbiota is affected by the experimental diets fed

Investigations performed on broilers (chapter 3) highlighted a typical composition of the crop and cecal microbiota. Nevertheless, we observed some differences with previously studied crop and ceca-associated microbial community which are most likely due to the diverse animals age and/or breed. Differences were also observed among the taxonomic assessment of the same microbiota as inferred through metaproteomics or DNA-based methods. These discrepancies are strongly dependent on the adopted investigation method. The vast majority of taxonomic assignment methods are nowadays based on the metagenomics and 16S rRNA gene sequencing. Here, a strong influence is attributed to the datasets and algorithms adopted for the binning, trimming and retrieving of the raw data obtained by the sequencers. At this purpose, Liu et. al [22] demonstrated already in the 2008 how diverse primers pairs (*e.g.* targeting diverse hypervariable regions), sequencing chemistry, datasets and algorithms may produce largely divergent results. In a similar manner, Tanca and colleagues described how the choice of diverse sample preparation protocols, data repositories, and algorithms for protein ID inference and

functional annotation influence the results of the metaproteomics investigations of similar samples [23-25]. In our specific case, the differences registered while comparing our results with the other studies available in literature and/or among investigation methods concern the fraction of low abundant bacteria and the weakly expressed functional features; whereas, major bacterial specimens and functional features are highly congruent with similar studies available in literature. Therefore, to a good approximation, we retain that the observed results discrepancies are mainly imputable to the diverse investigation strategies. Indeed, the lack of standardized methods for the gut microbiota investigation leads to a hard comparison of the results obtained from similar investigations. In this view, it is recommendable to establish standardized investigation protocols throughout the whole analytical workflow, ranging from sample preparation until bioinformatics data analysis. This would benefit in terms of an easier data interpretation, specifically when a meta-analysis among results from diverse studies and authors is needed.

Feeding the experimental diets reported a shift in the microbial community composition. This confirms our first hypothesis stating that experimental diets fed impair the gut-associated microbial community. Phylogenetic assessment performed through DNA and metaproteomics datasets reveals that a higher bacterial diversity is provided by the metaproteomics dataset, supporting our hypothesis that microbiota alterations are primarily visible in its protein repertoire. Nevertheless, some bacterial specimens were uniquely identified by the DNA-based approach and *vice versa*. This encourages the integration of both metaproteomics and DNA-based methods for the gut microbiota investigation, although metaproteomics is "autonomously" capable of detecting the functional feature and the main phylogenetic shift of the gut-associated microbial community.

Noteworthy, diet-driven structural changes concern mainly the less abundant bacterial specimens. We assume the presence of a core microbiota that differ slightly between diets, most likely because of its involvement in the central metabolism and the various housekeeping functions. A similar interpretation has also been done by Dawson and colleagues [26] in a recent work describing the rhizosphere bacteria. Here, only a small fraction of low abundant bacteria is found to be involved in the stress response. A similar concept is also supported by Hajishengallis and colleagues [27] in a closer study where it is demonstrated that, despite the presence of a core microbiota composed of highly abundant specimens, low-abundant bacteria may be responsible for the alteration of host-microorganisms homeostasis, and favor the onset of pathological conditions.

Diet supplemented with P and/or MP stimulate a thriving bacterial community; whose architecture increase the levels of bacterial specimens that are commonly associated to prosperous conditions. Functional annotation of their protein inventory highlighted that the activities of the changing bacterial specimens are involved in complex anabolic functions which confirm the overall good health status of the animals as ensured by the adequate micronutrient availability. Contrariwise, P and/or MP lacking diets are associated to a generalized stress status of the animal which is also mirrored in its crop and cecal microbiota. Here, we registered an increase of the bacterial specimens that are commonly found in the stress condition and their main activities concern the optimization of the P uptake and catabolism of macromolecules aimed to P recycling, on the attempt to mitigate the systemic effect deriving by the mineral lack. Several studies already demonstrated, in diverse animal models, the effects of the diet in the microbiota composition and activity. In this context, the dietary effects are generally studied by focusing on the physicochemical composition of the "substrate" where the microbiota members are growing and the changes triggered by the diet in the ecological niche colonized by the microbiota members. The experimental diets fed in our studies differ in the amount of supplemented P and/or MP, thus are probably unable of drastic physicochemical change in the surrounding environment. This open new avenue in the results interpretation, leading to look beyond the direct effect that the diet composition exerts on the microbiota but rather focus on the effect that such a tiny compositional change has at a systemic level on the animal body and that is subsequently mirrored, in an amplified manner, on its microbiota. Indeed, investigation of the host proteome revealed a diverse protein profile of the chickens depending on the experimental diets fed. However, no statistical differences were observed among animals. This is most likely due to the sample preparation protocols adopted. Crop and ceca of chickens are very complex sections comprising a wide array of substances and molecules deriving from the feed intake and the surrounding environment where the animals are bred, other than the host molecules and the bacterial commensals. This huge diversity of molecules poses the needs to develop enrichment protocols aimed to favor the identification of the molecules of our interest (*i.e.* host and bacterial commensals cells). Several tests have been performed in our laboratory in order to avoid as much as possible the feed particles and other environmental "contaminations" (data not shown). However, the reduction of feed particles resulted unavoidably coupled to the loss of host molecules, leading to a compromise that underestimate the host protein repertoire to highlight the microbiota-associated one. In this view, a future investigation using diverse and/or multiple sample preparation procedures would be desirable,

in order to draw a more comprehensive picture of the microbial commensals and their interaction with the host.

5.4 Porcine gut microbiota adapts to challenging diets through a stepwise process

The second part of the project (chapter 4) points to investigate the dynamics featuring the microbiota rearrangement following the feeding of the experimental diets, composed of different protein sources and varying amount of CaP. In this context, the mechanisms behind the restore of the microbiota homeostatic balance as well as the duration of the microbiota adaptation process are still object of discussion in diverse application field, including the human microbiota [28]. Owing the similarities occurring between pigs and humans in terms of anatomy, physiology, dietary regimen and gut microbiota composition, we adopted pig as the animal model of our experimental trial. Specifically, our investigation focused on fecal samples since it is, nowadays, commonly accepted to refer to the fecal microbiota as an index for the gut microbiota composition and activity, although the microbial community harboring the diverse GIT sections are featured by a specific architecture and activity [29].

Ordination of 16S rRNA gene sequencing- and metaproteomics- datasets according to statistical methods aimed to evaluate the overall sample similarities, indicate the presence of a stepwise adaptation process. Specifically, samples clustered overtime into three adaptation phases that mark the microbiota evolution from the "basal" homeostatic balance (*i.e.* Zero, including samples prior the feeding of the experimental trial) to the newly achieved homeostatic balance (*i.e.* EQ, terminal phase, where a new microbiota is suited). The intermediate step is represented by the MA phase (*i.e.* metabolic adaptation), which show intermediate features both in terms of microbiota composition and function since it included samples in the stage of "transition" between the two homeostatic conditions: the Zero homeostatic balance and the freshly obtained EQ balance. The statistical evidence of a stepwise adaptation processes undertaken by the gut microbiota support the third hypothesis of our project, postulating a progressive and finely-orchestrated adaptation process in order to ensure the achievement and maintenance of the homeostatic balance, even though a stress factor (*e.g.* feeding of the experimental diets) challenge both the host physiology and its intestinal microbiota. As expected for the chicken trial (chapter3), the structural composition of the gut microbiota differs in dependence of the adopted method. Taxonomic composition as assessed on the basis of the protein repertoire depict a heterogeneous bacterial community which structure changes strongly in the diverse adaptation phases. Here, the emergence and disappearance of proteins affiliated to diverse bacterial specimens give the idea of a "super-organ", where bacterial commensals in concert

with their host respond in an ordered and finely-tuned manner to the challenging stressor leading to the achievement of a freshly restored equilibrium.

Functional annotation of the whole protein repertoire did not prove any statistical difference among the adaptation phases. Functional dynamics across the adaptation phases have been studied in a quantitative manner via comparison of the abundance factor for each of the identified proteins, and attributing zero as abundance factor to the proteins that were not identified in one or more phases. Although this evaluation method has been successfully used in several other studies, abundance factors of the proteins involved in housekeeping functions were far higher than those of the proteins featuring the functional peculiarities of each adaptation phase (Fig. 4). That means that housekeeping proteins have a masking effect against other less abundant ones.

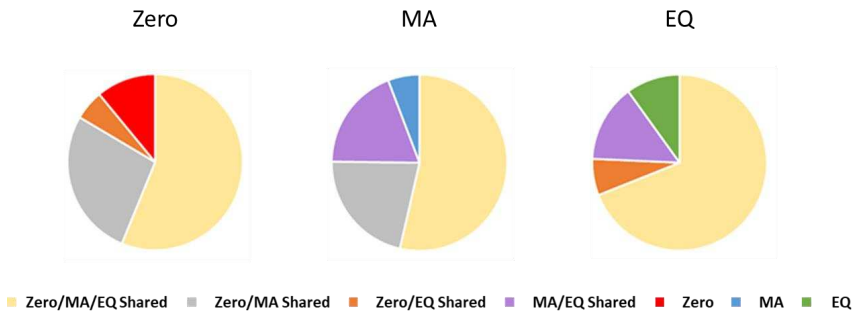

Fig. 4. Distribution of the protein abundance index (LFQ) across the Zero, MA and EQ adaptation periods. Pie charts depict the total LFQ and detail the portion of abundance relative to shared and uniquely identified proteins.

This issue has been overridden by exclusively focusing on the phase-specific protein profile. Functional annotation of these proteins confirms the phase-dependent shift also on a functional perspective. Noteworthy, the bacterial specimens driving the functional shift are also those involved in the microbiota architecture rearrangement, providing further support to our previous conclusion that changes in the microbiota architecture is a key step in the adaptation to the challenging experimental diet fed. Almost all of the changing bacterial families were involved in carbohydrate metabolism regardless the adaptation phase. One might interpret this data as a conserved function during the whole experimental time frame. However, a deeper investigation performed at the level of single proteins reveals that different routes of the same pathways are concerned in a phase-specific manner. In addition, adaptation phases registered quantitative differences in the expression of the commonly expressed metabolic routes of each

biochemical pathway. These suggest a global alteration of the microbiota metabolism, in the attempt to achieve a new homeostatic balance that provide a mutual beneficial effect: for the host and the bacterial commensals.

Similarly to the chicken experimental trial, the adopted sample preparation protocol did not enable for a comprehensive investigation of the host proteome and the complex interconnections existing with the intestinal microbiota. Anyhow, on the basis of the host protein repertoire, we can speculate that the alteration of the gut microbiota is also reflected in the host proteome. Proteins of Zero and particularly MA samples reveal an active host participation in restructuring the gut microbiota composition in order to better face the new challenging diet. EQ samples are instead active in maintaining the balance of the microbiota and, in concert with the microbial community itself, prevent from pathogen colonization. Analysis of Zero and EQ host proteome also highlight how the two diverse homeostatic conditions are featured by specific microbiota structure and activity, leading to the establishment of a diverse network of interactions with its host, mirrored by a diverse host proteome even though regarding similar functions.

In the context of our trial we also used metaproteomics as a predictive tool for the quantitation of the production of SCFA. The rationale behind was to quantitatively target the proteins involved in the SCFA production, enabling for a comparative evaluation of the SCFA potentially produced by the microbiota in the diverse adaptation phases. Nevertheless, only one of the targeted SCFA positively correlated with the measurements performed with conventional techniques. Diverse metabolic routes can be employed by bacteria in the production of the SCFA; it is probable that the list of protein indicators adopted in our study was not complete. The fact that one of the targeted SCFA correlates with the measurements performed with the conventional techniques prevent us to reject our initial idea. However, a more comprehensive list of indicators/target proteins must be employed to use metaproteomics as a predictive technique for the SCFA production.

5.5 Conclusion

Investigation of the gut microbiota is becoming an increasingly promising tool to prevent and/or overcome a variety of issues that concern animal production. Our research focused on the study of the intestinal microbiota and the evaluation of its dynamic architecture and range of functions enabled in the diverse environments, obtained following the feeding of experimental diets. To achieve our goals, we split the whole project into two separate experimental trials. The first one was performed on broiler chickens kept at diets with varying levels of Ca, P and MP. This

enabled to investigate whether the stress due to the altered P homeostasis is also reflected in the intestinal microbiota structure and functions. Hence, shed light on the microbial species and the biochemical pathways that may be involved in P digestion and uptake.

The second trial of our project seek to define how and how long the intestinal microbiota requires to adapt to a challenging diet composed of varying amount of Ca and P. Moreover, we also investigated whether the influence on the protein and carbohydrates metabolism is mirrored on the intestinal microbiota. Altogether, data from both trials provide useful information applicable for future studies aimed to design effective breeding strategies *e.g.* based on the use of probiotics and/or prebiotics, finalized to reduce the P supplementation in the routinely breeding of livestock. This would also benefit the "ecological side" of animal production, enabling for a reduced P excretion and a consequent reduction of water eutrophication.

Investigation of the time wise adaptation of the porcine microbiota provides precise instructions on the minimal exposure time required from the intestinal microbiota to adapt to the new dietary composition. This is of fundamental importance for the design of future experimental studies aimed to confirm and/or continue our results. Moreover, owing the anatomical and physiological similarities occurring between humans and pigs, obtained findings are also of interest for future human nutritional studies, where the mechanisms and lasts of the microbiota adaptation process is still object of discussion.

5.6 Future perspectives

The studies performed in the present project represent a starting point for the design of further investigations aimed to indicate alternative animal production strategies. Exploiting the complex functions of the gut-associated microbial community is a key factor for an efficient P digestion and uptake from the feedstuffs InsP6. This will prevent the dietary supplementation of inorganic P with consequent benefits both in terms of a reduced water pollution and animal breeding costs.

Additional studies are needed to achieve these main objectives. A comprehensive evaluation of the animal physiology (*e.g.* InsP6 hydrolysis pattern, InsPs residuals in organs and body fluids *etc.*), the host biochemical routes undertaken to modulate P homeostasis, and its linkage to the intestinal microbiota (composition and activity) is desirable. This will provide a more detailed picture and a direct cause-effect scheme, highlighting the influence of each bacterial specimen on the host metabolism and *vice versa*.

As already mentioned, the analysis of the results obtained in the above-described studies reveals the emergence of some methodological issues. These rely mainly in the reduced coverage of

the host proteome and the "masking effect" of the highly abundant proteins. The first issue is responsible of an underestimation of the host metabolism, making hard the connection between the host physiology and the gut microbiota functions. The second issue concern the underestimation of the low-abundant bacterial proteins which are involved in the gut microbiota adaptation process. On the basis of these evidences, care should be taken in the design of future studies. Specifically, the adoption multi-step sample preparation protocols that enrich the samples in the host- and bacterial- proteins, as well as emphasize the less abundant proteins, is strongly recommended to provide a more exhaustive protein repertoire.

In regard to the chicken trial, presented results concern the crop and ceca sections. Complementary investigations focusing the microbiota associated to the other GIT sections, are desirable. Moreover, we retain that structural and functional featuring of the mucosa-associated microbial community will largely improve the knowledge on the bacterial specimens along with their functional traits involved in the P uptake and InsP6 turnover.

Pig trial is focused on the analysis of the fecal microbiota. Although feces-associated bacteria are widely used as an index of the entire gut microbiota, we believe that focus on the microbial community harboring each GIT section provides complementary information of a great relevance while drawing a comprehensive overview of the porcine microbiota and its response against a given challenging factor such as the feeding of experimental diets.

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SUMMARY

Phosphorus is a macronutrient involved in a variety of physiological processes of a vital importance such as the energetic metabolism and energy transfer. Also, P and PO_4^{3-} are known to be the active moieties of many macromolecules that are, in turn, responsible of several other functions with strong implications both at cellular and systemic level. Owing these peculiarities of P, it is crucial for the animal nutritionists to provide adequate levels of P in the livestock's diets, since a P-deficiency may result in severe alteration of the animal production and welfare. Chicken and pig diets are mainly based on cereal assumption. Here, P is almost exclusively stored as phytate, an insoluble form of P storage that requires the activity of specific enzymes (*i.e.* phytases) to hydrolyze the phytate to low-molecular inositol phosphates, with the consequent release of bioavailable PO_4^{3-} molecules. Nevertheless, abundance and activity of the phytases in monogastric animals (including chickens and pigs) is rather reduced, leading to a reduced bioavailability of the P content in the feedstuffs. Moreover, phytate is excreted in urine complexed with other cations, resulting in an antinutrient effect of the undigested P.

Several strategies are nowadays explored in the attempt to overcome these issues. Among these, dietary supplementation of microbial phytases (MP) appears to be a promising solution, since enhancing the load of bioavailable P and reduce its renal excretion. However, alteration of the diet composition may affect the intestinal microbiota composition and the wide array of interactions that are finely tuned with the host metabolism.

In the research works of the present thesis, 16S rRNA gene sequencing and metaproteomics were employed to investigate the gut microbiota of chickens and pigs kept at experimental diets with varying amount of calcium-phosphorus (CaP) and supplemented MP. This represents a valuable approach to investigate the bacterial specimens involved in the P absorption, allowing for a comprehensive understanding of how the intestinal bacteria adapt to a new diet and which metabolic routes are affected by changing levels of supplemented P and/or MP.

Two major experimental trials were performed during the investigation. The first one was conducted on chickens operating a modulation in the dietary levels of Ca, P and MP. This trial highlighted a shift in the composition of the crop and ceca-associated microbial community depending on the composition of the diet fed. Also, investigated protein inventory revealed that the stress condition due to the reduced P availability is mirrored in the gastrointestinal tract (GIT)-associated microbiota. Marked differences were observed in the functions of the bacterial community in the case of P-available diets versus P-deficient ones. Protein repertoire of the first case draws a thriving microbial community focused on complex and anabolic functions.

Contrariwise, the bacterial community in the case of P-lacking diets appears to deal with catabolic functions and stress response.

The second trial was conducted on pigs and attempts to define the dynamics featuring the microbiota adaptation to a new challenging diet composed of different protein sources and varying levels of Ca and P. Statistical evidences reveal a stepwise adaptation of the fecal microbiota to the experimental diets fed. Both DNA-based approach and metaproteomics independently reveal three main adaptation phases: -before the feeding of the experimental trial (*i.e.* Zero), -the response of the microbial community to the challenging factor (i.e. MA) and, finally, - the newly achieved homeostatic balance (*i.e.* EQ).

As observed in the first trial, feeding of the experimental diets impairs the overall fecal microbiota composition, stimulating the presence of phase-specific bacterial specimens and a characteristic relative abundance of the shared ones. Bacterial families responsible for the phase-specific architecture of the fecal microbiota are also active in the biochemical pathways driving the functional peculiarities of each adaptation phase. A deeper investigation of the identified protein repertoire revealed that the observed statistical differences among the adaptation phases are uniquely due to the Ca and P composition of the diets fed. None of the observed effects can be attributed to the diverse protein sources supplemented with the diets.

Functional categorization of the identified protein inventory depicts three diverse functional assets of the microbial community. Specifically, prior the feeding of the experimental diets, bacteria are hypothesized to live under homeostatic condition, since they appear to be involved in complex and highly-specialized functions. Following the administration of the experimental diets microbial community changes its functional priority and reduce the expression of highly specialized functions to focus on more essential ones. Proteins involved in complex functions such as widening the substrates array and facing complex sugars tend to increase in abundance while the new homeostatic balance is achieved.

Altogether, data from both trials provide useful information for future studies aimed to design effective breeding strategies finalized to reduce the P supplementation in the routinely breeding of livestock and maintain a balanced microbial activity in the animal GIT.

Investigation of the dynamics of the porcine microbiota provides instructions on the minimal exposure time required from the intestinal microbiota to adapt to a new dietary composition. This is of fundamental importance for the design of future studies aimed to confirm and/or continue our results. Moreover, the anatomical and physiological similarities occurring between humans and pigs, make our findings of interest for future human nutritional studies, where the mechanisms and lasts of the microbiota adaptation process is still object of discussion.

ZUSAMMENFASSUNG

Phosphor ist ein Makronährstoff, der in einer Vielzahl von lebenswichtigen physiologischen Prozessen involviert ist, wie dem Energiestoffwechsel und der Energieübertragung. Phosphor (P) und die Verbindung PO_4^{3-} sind aktive Bausteine vieler Makromoleküle, die wiederum für vielfältige Funktionen verantwortlich sind, die sich sowohl auf zellulärer als auch auf systemischer Ebene auswirken. Aufgrund dieser besonderen Bedeutung von P ist es äußerst wichtig, dass Ernährungsbeauftragte angemessene Mengen an P Nutztieren in der Nahrung zur Verfügung stellen, denn ein P-Mangel kann zu einer starken Beeinträchtigung der Tierzucht und der Gesundheit der Tiere führen.

Die Nahrung von Hühnern und Schweinen besteht hauptsächlich aus Getreide. Dort ist P fast ausschließlich als Phytat vorhanden, eine unlösliche Form von P. Die Hydrolyse von Phytat in niedermolekulare Inositolphosphate erfolgt unter der Einwirkung spezifischer Enzyme (d.h. Phytasen) und setzt wiederum bioverfügbare PO_4^{3-}-Moleküle frei. Allerdings ist die Häufigkeit und Aktivität von Phytasen in monogastrischen Tieren (wozu auch Hühner und Schweine gehören) recht gering, was zu einer verminderten Bioverfügbarkeit des P-Gehalts in den Futtermitteln führt. Darüber hinaus wird Phytat an andere Kationen gebunden und im Urin ausgeschieden, was zu einer antinutritiven Wirkung des unverdauten P führt.

Heutzutage werden verschiedene Alternativen untersucht, die es ermöglichen sollen, diese Probleme zu überwinden. Darunter scheint eine Nahrungsergänzung mit mikrobiellen Phytasen (MP) eine vielversprechende Lösung zu sein, da der Anteil von bioverfügbarem P erhöht und die renale Ausscheidung verringert wird. Eine Veränderung der Nahrungszusammensetzung kann jedoch die Zusammensetzung der Darmmikrobiota und die vielfältigen Wechselwirkungen, die mit dem Metabolismus des Wirts fein abgestimmt sind, beeinflussen.

In den Forschungsprojekten der vorliegenden Arbeit wurden die 16S rRNA Gensequenzierung und Metaproteomik zur Untersuchung der Darmmikrobiota von Hühnern und Schweinen verwendet. Dabei wurde der Einfluss von Versuchsdiäten mit unterschiedlichen Mengen an Calcium-Phosphor (CaP) und ergänzten MP untersucht. Dies ist ein wertvoller Ansatz zur Identifizierung von Bakterienspezies, welche an der P-Absorption beteiligt sind, und ermöglicht es ein umfassendes Verständnis darüber zugewinnen, wie sich die Darmbakterien an eine neue Ernährung und an Stoffwechselwege, die von sich verändernden Mengen an ergänztem P und / oder MP beeinflusst werden, anpassen können.

Im Rahmen dieser Arbeit wurden zwei große experimentelle Studien durchgeführt. Die erste Studie wurde an Hühnern durchgeführt und basierte auf der Verabreichung unterschiedlicher Mengen an Ca, P und MP in der Nahrung. Die Ergebnisse dieser Studie zeigten eine Nahrungs-

abhängige Verschiebung in der Zusammensetzung der mikrobiellen Gemeinschaft in Kropf und Blinddarm. Außerdem zeigte das untersuchte Proteinrepertoire, dass der Stresszustand, aufgrund der reduzierten P-Verfügbarkeit, sich in der Mikrobiota im Gastrointestinaltrakt (GIT) widergespiegelt. Der Vergleich von Diäten mit verfügbarem P mit P-armen Diäten zeigte deutliche Unterschiede in den Funktionen der Bakteriengemeinschaft. Bei einer Diät mit verfügbarem P in der Nahrung, deutet die Analyse des Proteinrepertoires auf eine florierende mikrobielle Gemeinschaft hin, in der komplexe und anabole Funktionen vorherrschen. Im Gegensatz dazu scheinen bei Diäten mit P-Mangel katabole Funktionen und Stressreaktionen in der bakteriellen Gemeinschaft zu überwiegen.

Die zweite Studie wurde an Schweinen durchgeführt und versuchte die Dynamik zu definieren, mit der sich die Mikrobiota an eine neue, herausfordernde, aus verschiedenen Proteinquellen und unterschiedlichen Mengen an Ca und P bestehende Nahrung, anpasst. Statistische Beweise zeigen eine stufenweise Anpassung der fäkalen Mikrobiota an die verabreichten Versuchsnahrung. Sowohl DNA- als auch metaproteomische Analysen, die getrennt voneinander durchgeführt wurden, zeigen drei Hauptanpassungsphasen, welche die folgenden experimentalen Adaptationsgruppen definieren: - vor der Verabreichung der Versuchsdiät (d.h. Null), - die Reaktion der mikrobiellen Gemeinschaft auf den herausfordernden Faktor (d.h. MA) und schließlich, - das Stadium des neu erreichten homöostatischen Gleichgewichts (d.h. EQ).

Wie im ersten Versuch beobachtet wurde, beeinträchtigt die Fütterung der Versuchsnahrung die gesamte Zusammensetzung der fäkalen Mikrobiota, sie stimuliert das Vorhandensein von phasenspezifischen Bakterienarten und eine charakteristische relative Häufigkeit der phasenunspezifischen Bakterien. Bakterielle Familien, die für die phasenspezifische Struktur der fäkalen Mikrobiota verantwortlich sind, sind auch in den biochemischen Pfaden aktiv und sind im Wesentlichen für die funktionellen Besonderheiten jeder Anpassungsphase verantwortlich. Eine tiefere Untersuchung des identifizierten Protein repertoires ergab, dass die beobachteten statistischen Unterschiede zwischen den Adaptationsphasen eindeutig auf die Ca- und P-Gehalte der gefütterten Diäten zurückzuführen sind. Keine der beobachteten Effekten kann den verschiedenen gleichzeitig verabreichten Proteinquellen zugeschrieben werden.

Die funktionelle Kategorisierung des identifizierten Proteinbestands zeigt drei verschiedene funktionelle Aspekte der mikrobiellen Gemeinschaft. Insbesondere wird angenommen, dass vor dem Füttern der experimentellen Diäten, die Bakterien unter homöostatischen Bedingungen leben, da sie in komplexe und hochspezialisierte Funktionen involviert zu sein scheinen. Nach der Verabreichung der experimentellen Diäten ändert die mikrobielle Gemeinschaft ihre

funktionelle Priorität und reduziert die Ausübung hochspezialisierter Funktionen, um sich auf die wesentlichen zu konzentrieren. Während das neue homöostatische Gleichgewicht erreicht wird, nimmt die Menge an Proteinen, die an komplexen Funktionen beteiligt sind, wie z. B. die Verarbeitung einer großen Substratvielfalt sowie die Metabolisierung von komplexen Zuckern, zu.

Zusammengefasst, liefern die aus beiden Experimente gewonnenen Daten nützliche Informationen für zukünftige Studien mit dem Ziel, effektive Zuchtstrategien zu entwickeln, um die P-Supplementierung bei der routinemäßigen Viehzucht zu reduzieren und eine ausgewogene mikrobielle Aktivität im Gastrointestinaltrakt des Tieres aufrechtzuerhalten.

Die Untersuchung der Anpassungsdynamik der Schweinemikrobiota liefert genaue Informationen darüber, wie lange die intestinale Mikrobiota benötigt, um sich an eine neue Nahrungszusammensetzung anzupassen. Dies ist von zentraler Bedeutung für die Gestaltung zukünftiger experimenteller Studien, die darauf abzielen, unsere Ergebnisse zu bestätigen und/oder unsere Forschung fortzusetzen. Aufgrund der anatomischen und physiologischen Ähnlichkeiten, die zwischen Menschen und Schweinen auftreten, sind die erhaltenen Befunde auch für zukünftige Ernährungsstudien am Menschen von Interesse, bei denen die Mechanismen und die Dauer der Adaptationsprozesse der Mikrobiota noch untersucht werden.

ACKNOWLEDGEMENT

Running a PhD project requires strong moral and professional efforts. These requirements are fully met by each of the faculty members, to which goes my sincere gratitude.

First, I want to express my very sincere thanks to Jun. Prof. Dr. Jana Seifert for giving me the opportunity to run this project and supervise me throughout all phases of my doctorate. Her expertise, patience and guidance have been fundamental for my professional and personal growth.

Moreover, I wish to acknowledge Prof. Dr. Sergio Uzzau for his invaluable support throughout my scientific career. He encouraged me to apply for this PhD project at the University of Hohenheim. Also, he always provided me his excellent support from a scientific and moral point of view.

I am very grateful to Dr. Amelia Camarinha-Silva for her precious help, encouragement and scientific suggestions. Also, I wish to thank Prof. Dr. Markus Rodehutscord and Prof. Dr. Rainer Mosenthin for providing me valuable insights on animal physiology.

I would like to thank Dr. Simon Deusch for his extreme kindness and help in the solution of all the "bureaucratic issues" I encountered during my permanence in Germany.

I am very grateful to the members of the "Feed-Gut Microbiota Interaction" group and the young investigator group "Microbial Ecology" for the continuous sharing of scientific opinions and advices. They made special my permanence, providing a friendly environment and a cheerfully atmosphere.

I am sincerely grateful to Jan and Valeria for having shared unforgettable experiences and a solid friendship, as well as all the Italian fiends (fortunately too many for being mentioned). I retain myself being very lucky to meet such nice persons.

I would like to intensely thank my parents and my sister Mariella for always being my reference point. They constantly believed in me and pushed to go further. This work will probably not exist without their support.

Finally, my deepest acknowledge is to Laura, my wife. She gave me the biggest happiness of being the father of a wonderful baby, Rebecca. Moreover, she played the hardest role of experiencing all positive and negative outcomes of my research project.

BRUNO TILOCCA

CURRICULUM VITAE ET STUDIORUM

Personal Data

Name	Bruno Tilocca
Date of birth	13.03.1988
Place of birth	Sassari, Italy
Nationality	Italian

Education

Since 07/2013	PhD candidate, Institute of Animal Science, University of Hohenheim, Stuttgart, Germany
10/2010 – 10/2012	M.Sc. Medical and Veterinary Biotechnology, Department of Veterinary Medicine, University of Sassari, Italy
10/2007 – 07/2010	B.Sc. Biotechnology, Department of Mathematical Physical and Natural Sciences, University of Sassari, Italy

Professional Career

Since 11/2017	Research activity, Department of Agricultural Science, University of Sassari, Italy
05 – 07/2017	Teacher at Istituto Tecnico Industriale GM.Angioy, Sassari, Italy
07/2013 – 03/2017	Scientific staff and PhD student, Institute of Animal Science, University of Hohenheim, Stuttgart, Germany
03 – 06/2013	Biotechnologist fellow, Virology section, Istituto Zooprofilattico Sperimentale della Sardegna “G.Pegreffi”, Sassari, Italy
06 – 11/2011	Biotechnologist fellow, Food inspection, Istituto Zooprofilattico Sperimentale della Sardegna “G.Pegreffi”, Sassari, Italy

Practical experience

04 – 10/2012	Porto Conte Ricerche, Alghero, Italy
03 – 07/2010	Institute of Pathological Anatomy, University of Sassari, Italy

Stuttgart, 19.03.2018

Place, Date — Tilocca Bruno

LIST OF PUBBLICATIONS

Deusch S[1], Tilocca B[1], Camarinha-Silva A, Seifert J (2015) News in livestock research - use of Omics-technologies to study the microbiota in the gastrointestinal tract of farm animals. Computational and Structural Biotechnology Journal 13, 55-63
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https://doi.org/10.1186/s40168-017-0362-7

AFFIDAVIT

pursuant to Sec. 8(2) of the University of Hohenheim's doctoral degree regulations for Dr.sc.agr.

1. For the dissertation submitted on the topic

"Effects of diets with different phosphorus availability on the intestinal microbiota of chickens and pigs"

I hereby declare that I independently completed the work.

2. I only used the sources and aids documented and only made use of permissible assistance by third parties. In particular, I properly documented any contents which I used - either by directly quoting or paraphrasing - from other works.

3. I did not accept any assistance from a commercial doctoral agency or consulting firm.

4. I am aware of the meaning of this affidavit and the criminal penalties of an incorrect or incomplete affidavit.

I hereby confirm the correctness of the above declaration. I hereby affirm in lieu of oath that I have, to the best of my knowledge, declared nothing but the truth and have not omitted any information.

Stuttgart, 19.03.2018

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Tilocca Bruno

AFFIDAVIT

Information

The University of Hohenheim requires an affidavit declaring that the academic work was done independently in order to credibly claim that the doctoral candidate independently completed the academic work.

Because the legislative authorities place particular importance on affidavits, and because affidavits can have serious consequences, the legislative authorities have placed criminal penalties on the issuance of a false affidavit. In the case of wilful (that is, with the knowledge of the person issuing the affidavit) issuance of a false affidavit, the criminal penalty includes a term of imprisonment for up to three years or a fine.

A negligent issuance (that is, an issuance although you should have known that the affidavit was false) is punishable by a term of imprisonment for up to one year or a fine.

The respective regulations can be found in Sec. 156 StGB (Criminal Code) (false affidavit) and in Sec. 161 StGB (negligent false oath, negligent false affidavit).

Sec. 156 StGB: False Affidavit

Issuing a false affidavit to an authority body responsible for accepting affidavits or perjury under reference to such an affidavit shall be punishable with a term of imprisonment up to three years or with a fine.

Sec. 161 StGB: Negligent False Oath, Negligent False Affidavit:

Subsection 1: If one of the actions described in Secs. 154 and 156 is done negligently, the action shall be punishable by a term of imprisonment of up to one year or a fine.

Subsection 2: Impunity shall apply if the perpetrator corrects the false information in a timely manner. The regulations in Sec. 158 (2) and (3) apply mutatis mutandis.

I have taken note of the information on the affidavit.

Stuttgart, 19.03.2018

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Tilocca Bruno