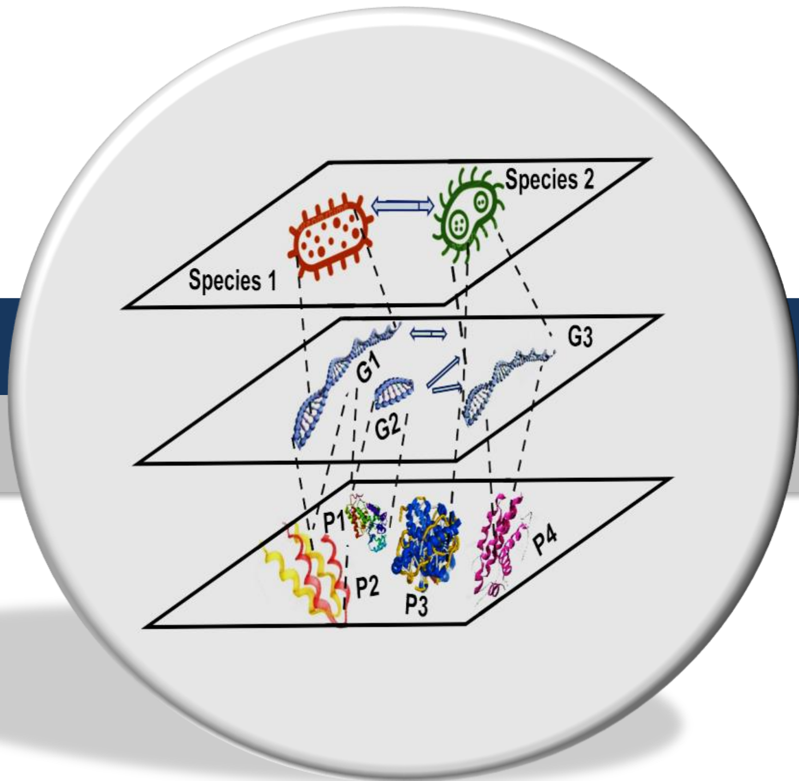
High-throughput sequencing techniques to analyze microbial communities in the gastrointestinal tract of broiler chickens



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HIGH-THROUGHPUT SEQUENCING TECHNIQUES TO ANALYZE MICROBIAL COMMUNITIES IN THE GASTROINTESTINAL TRACT OF BROILER CHICKENS

DISSERTATION
submitted in fulfilment of the requirements for the degree
"Doktor der Agrarwissenschaften" (Dr. sc. agr. / PhD in Agricultural Sciences)

to the
Faculty of Agricultural Sciences

presented by

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Stuttgart-Hohenheim, 2018

The presented studies were realized at the Institute of Animal Science and at the Agricultural Research Station Lindenhöfe (72800, Eningen), of the University of Hohenheim in Stuttgart, Germany. The animal experiments were performed in February 2015 and April 2016 under the supervision of Jun.-Prof. Dr. Amélia Camarinha Silva (University of Hohenheim, Head of the "Livestock Microbial Ecology") and the co-supervision of Prof. Dr. Markus Rodehutscord (University of Hohenheim, Head of the "Animal Nutrition").

University of Hohenheim, Faculty of Agricultural Science, December 2018

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MY FAMILY

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LIST OF ABBREVIATIONS

%	Percentage
~	Approximately
°C	Celsius degrees
µl	Microliter
µm	Micrometer
µM	Micromolar
16S rRNA gene	16S ribosomal ribonucleic acid gene
ABC transporter	ATP-binding cassette transporters
ATP	Adenosine triphosphate
B	Boron
BLAST	Basic local alignment search tool
bp	Base pairs
BW	Body Weight
Ca	Calcium
CBM	Carbohydrate-binding module
cDNA	Complementary deoxyribonucleic acid
cm	Centimeters
CO_2	Carbon dioxide
Cu	Cupper
d	Days
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphates
e.g.	For example
ENA	European Nucleotide Archive
FC	Feed consumption
FTU	Phytase unit
g	Gram
Gb	Gigabase
GH	Glycoside hydrolases
GIT	Gastrointestinal tract
GT	Glycosyltransferases
h	Hour
H’	Shannon-weaver index of diversity
InsP	Inositol Phosphate
IL	Interleukin
KEGG	Kyoto Encyclopedia of Genes and Genomes
kg	Kilograms

KO	KEGG Orthology
L	Liter
m	Meter
min	Minute
ml	Milliliter
mm	Millimeter
Mn	Manganese
mRNA	Messenger ribonucleic acid
N_2	Nitrogen
NADH	Nicotinamide adenine dinucleotide
NGS	Next Generation Sequencing
NMDS	Non-metric multidimensional scaling
NSPs	Non-starch polysaccharides
nt	Nucleotide
O_2	Oxygen
OTU	Operational taxonomic unit
P	Phosphorus
p	Probability value
p. m.	Post meridiem
PcoA	Principal coordinate analysis
PCR	Polymerase chain reaction
PERMANOVA	Permutational manova
pH	Potential of hydrogen
ppm	Parts per million
RDP	Ribosomal Database Project
RNA	Ribonucleic acid
rRNA	Ribosomal RNA
rpm	Revolutions per minute
s	Second
SCFA	Short-chain fatty acids
SEM	Standard error of the mean
SIMPER	Similarity percentage Analysis
spp.	Species
TCA	Trichloroacetic acid
U	Enzyme unit
VFA	Volatile fatty acid
wk	Week
Zn	Zinc
σ	Standard deviation

CHAPTER I

INTRODUCTION

1. INTRODUCTION

1.1 General introduction

In a world of continuous changes and increasing population, there is a need for efficient agriculture and food production with sustainable relation, among natural resources and human activities. The broiler chicken is a highly requested farming animal by reason of its very efficient feed conversion ratio, and its meat is an excellent source of protein, vitamins, and minerals (1). Currently, around one-third of the global crop cultures are used to produce feed for animals (2), and its production and transportation contributes to approximately 70% of the potential global warming in production systems (3), being this the significant environmental impact of poultry farming. Other environmental complications are associated with nitrogen emissions, litter management and energy consumption (3). Despite these matters, poultry production has the lowest impact on the environment when compared to other livestock. In 2020 is expected to be produced 120 million tons of chicken meat (OECD/FAO). This fast and massive production can only happen with proper strategies for disease prevention and control, which minimize the impact of bacterial, parasitic or viral infections in animals and subsequently humans, along with minimizing the production environmental effect.

Critical issues in modern production, are focused on nutrition, diet composition and dietary supplements (4). Cereals grains including corn, barley, wheat, and oat are the most common energy based diet and correspond to a considerable percentage in feedstuff (5, 6). Depending on the cereal, it can be expected different carbohydrate composition, a dissimilar linkage between nutrients and cell wall structure, and antinutritive compounds like non-starch polysaccharides (NSPs) (7, 8). The influence of the cereal grain is studied considering changes in physicochemical properties in the luminal content, changes in the morphometry of the mucosal morphology, and also fluctuations in microbial composition (9). For this reason, the addition of the enzymes to reduce viscosity and to dilute the antinutritional effects has become a more accepted practice in the chicken nutrition field (5). A positive side effect to add enzymes in the diet consists of diminishing the environmental impact, through the reduction of losses from macronutrients like nitrogen (N) and phosphorus (P).

Generally accepted enzymes in broiler chicken nutrition are the xylanases, which act on the NSPs and efficiently degrade fiber. The enzyme increases the flow of fermentable xylo-oligomers and minimizes the variation in apparent metabolizable energy (7, 10). Another commonly used enzyme is phytase that hydrolize the compound phytate. A significant

number of studies are carried out in phytase, due to the high presence in plant-based diets of myo-inositol phosphate as a storage form of P (11). The enzyme produces less phosphorylated compounds and additionally reduces the need for the supplementation of P and calcium (Ca) in the diet (12, 13). Alternatively, proteases are added to increase the digestibility of proteins and amino acids, and to reduce the putrefaction in the distal digestive tract, which enhances gut physiology, reduces the viscosity of luminal content and improves the retention of P and Ca (14). On the other hand, due to the higher digestibility of starch, dry matter, and organic matter, the inclusion of amylases in broiler chickens diets promotes body weight, feed intake and feed efficiency (15). Furthermore, cellulases and hemicellulases are enzymes which significantly increase the metabolizable energy, reduce the moisture in the digesta and improve ileal digestibility (16, 17). To further obtain a cumulative positive effect in digestibility of nutrients, a mixture of enzymes has also been applied in poultry nutrition. They can reduce the anti-nutritional properties of feed-based diets coming from corn, canola and soybean meal. Some examples of active mixtures comprise (amylase, xylanase, and protease (18)), (phytase, β-glucanase, α-amylase, cellulose, pectinase, xylanase and protease (19)) and (xylanase, cellulase, and β-D-glucanase (20)).

Diets should also be formulated to meet specific mineral requirements, and its concentration must be maintained to keep the functional and structural integrity of the animal tissues. Ingestion of diets that are imbalanced in minerals, either positively or negatively, may lead to animal health problems and can result in inefficient use of the natural resources involved in the production cycle. Minerals are structural components of organs and tissues, while in body fluids, they are present as electrolytes, maintaining the homeostasis and acting as catalyzers of many enzymatic reactions (21). Their concentrations differed but can represent 3 to 4% of the total weight, and since the chickens cannot synthesize them, they have to be supplemented (21). Ca must be provided in an adequate concentration in the feed because it is involved in the bone mineral content, muscle function, blood coagulation, enzymatic activity and hormone regulation (22). Different metabolic disorders such as leg weakness, lameness, rickets, can be the consequence of a deficiency in this element (23).

As an influence of dietary enzyme supplementation like phytase, the release of minerals Ca and P from phytate complex is expected (12). Furthermore, when adding Ca, the ratio with P is studied, being a general relation of 1:1 to 2:1 (23). This ratio is studied since it is found that dietary Ca concentrations can affect the P utilization (24). Phosphorus is essential in bone formation and is vital for the cellular membranes and cellular functions. Besides, this element is a structural component of nucleic acids and is involved in energy metabolism conforming the adenosine triphosphate (ATP) (21). Other minerals that support physiological needs and are shown to improve performance parameters in broiler chickens include the minor elements such as Boron (B) (25), Zinc (Zn) (26), Manganese

(Mn) and Cupper (Cu) (27). A performance improvement was found in broiler chickens with higher digestibility of minerals Mn, Cu and Zn and amino acids (18). Those trace nutrients correspondingly increase the ileal digestibility of dry matter, organic matter and crude protein (19), and enhance the feed conversion ratio (FCR) for starter and further growth periods (20).

In the last years, attention has been driven to microbiota studies because of its significant impact on metabolism. Understanding the ecology of the microbial communities distributed along the gastrointestinal tract (GIT) and their interaction with the host will extend the horizons from the actual state of knowledge. Many investigations are currently undergoing, to identify the role of feed substrates in the modulation of the microbial composition of the GIT. It is known that there is a close interaction between the chicken intestinal microbiome and the diet (1). Microbes hydrolyze indigestible carbohydrates and polysaccharides that cannot be absorbed by the broiler chickens but can further be fermented by other microbes in the GIT to produce short-chain fatty acids (SCFA). Thus, microbial enzymes have carbohydrate hydrolysis activity that makes substrates available and improves its assimilation (28). Therefore, the study of broilers gut microbiota is of great interest since they are responsible for the breakdown of complex substrates and further energy storage that is partly used by the host (5).

1.2 Recent techniques to investigate broiler chicken GIT microbiota

Advances in high-throughput next generation sequencing technologies (NGS) improve our knowledge of taxonomical and functional microbial characterization. The relatively short time in which the results can be obtained, the independence of microbial culture methods and the not necessary need for previous knowledge of the microorganism are some of the advantages of using NGS (29). Hence, these advances bring an opportunity for scientists, interested in the impact of animal nutrition in GIT microbes, to use them and bring innovative observations in diet microbiota interactions. NGS has been applied to characterize the bacterial communities and its function in the different GIT sections. The sequencing platforms used in chicken studies were mainly Roche 454 pyrophosphate genome sequencing and illumina/solexa (30). Recently, Illumina is the technology which is dominating the studies because of the good relation between sequence number and cost, and the lower error rate in the sequencing procedure that are estimated between 0.3% to 3.8% in datasets of 2.8 million 27mers reads, and 12.3 million 36 mers reads (31). These errors are mainly attributed to substitution errors, while the frequency of insertion and deletion errors are low (32, 33). The technologies allow us to get information at phylogenetic level through the universal genetic region in bacteria, the 16S r RNA gene; a complete cataloguing of the genes, through shotgun sequencing, by getting information of the total DNA in the sample (34); and at metatranscriptomic level insights into the functionality of the community focusing on RNA (35).

Sequencing of the 16S rRNA gene has the advantage of amplifying microbial communities without a significant sampling effort and underestimation of species (36). This approach has allowed to investigate the microbial diversity and to identify bacteria members in defined environments (37), while also shifts under different periods of time or even between different ecological niches are captured (38). Additionally with high confidence through this gene, taxonomy assignation can be done until the species level (38). The 16S rRNA gene comprises nine hypervariable regions, which determine diversity differences among microorganisms (39). In order to capture those regions the design of universal primers are done based on conserved stretches enabling their amplification (39). Previous broiler chicken studies used the V2 (39), V3 (40), and V4 (41) regions; however, the use of a single hypervariable region cannot distinguish among all bacteria. On the other hand, their combination can give a better resolution in sequence diversity (39), the regions V1-V2 (42), V1-V3 (43, 44), V4-6 (41) and V1-V5 (44) have been used in other chicken surveys. Through the taxonomic information obtained, the corresponding sequence generally designated as operational taxonomic unit (OTU) will be characterized and classified with an assigned nomenclature (36). The microbial diversity will then be seen under species turnover, which can be calculated based on indexes obtained for alpha-diversity, looking for characteristics within the sample, and the beta- diversity to establish differences between samples (45). Previous chicken studies described the GIT sections: crop, jejunum, ileum, caeca, the feces, as well as processed chicken products: the carcass (30). Some of the insights obtained were related to the microbial structure such as the increase of diversity indexes with age, influence of the diet, shifts in composition due to challenging experiment with potential pathogens, and even environmental factors such as the litter (40, 43, 46, 47). Some drawbacks of using these hypervariable regions are the different copy numbers of each bacterial strains that can overestimate the diversity (48), and the chosen variable region could indicate a different percentage of taxa recovery at lower taxonomical levels (36).

Metagenomics, a methodology used to assess functionality, avoid the use of sequencing primers and focuses on gene presence (49). Up to date, few metagenomic projects were done in chicken and only covering two environments, the caeca, and feces (40, 50, 51). The main focus of those studies was on deciphering the diversity and functionality of the caeca (51), to evaluate the responses to treatments with anticoccidial and growth promoting agents (40), to bring information about virulome in chickens (50) and to compare microbiome profiles between fat and lean lines (52). Even though more information can be obtained with metagenome sequencing over 16S rRNA studies, challenges are still present. Intensive computational analysis is required, there is lack of appropriate genome references for annotation, together with the need to obtain the sufficient depth and coverage in the sequencing (29).

In line with phenotypic analysis to investigate functionality, only two metaproteomic studies are found in the literature, one which investigates the crop and the caeca based on different diet supplementation (53) and another describing protein expression in feces (54). A significant effort can still be driven in the microbial community analysis of the chicken GIT, with more studies using the techniques mentioned earlier, and step forward into the transcriptional level, with studies covering the whole metatranscriptome to investigate further the genes expressed in the GIT.

1.3 Microbial colonization and the influence of digestive activities in the GIT

The microbiome of the chicken GIT reflects an evolutionary interaction, where speciation and specific functions have been developed due to the cross-talk between host and microorganisms (55). The total length of the digestive tract is 2.171 m., and the average passage rate of chymus in GIT is approximately 3.5 hours, being the caeca the section with the slowest passage rate (56, 57). These shifts in the passage rate, influence the establishment and development of the microbial communities. The first source of GIT colonization corresponds to the egg-shell that is exposed to the microbiota from the mother and surrounding environment (58). The colonization increases exponentially until the first week and afterward (approx. 30d) the number of bacterial cells remains stable (59). This activity is facilitated through the interaction between bacteria adhesion and their receptors, where the bacteria synthesized adherence factors like the BSP-A surface antigen (60). Another characteristic that supports bacteria adhesion is the bacterial motility with fimbria and pilus which help their movement to the mucosa (60). Carbohydrate-specific molecules like the lectins, present in the cellular surface of the microorganisms facilitate the adhesion to epithelial cells and the interaction with the host, and it is expected an increase of galactosylation in host cells (60). The broiler chicken influences the establishment of normal microbial flora with activities like continuous renewal process of the intestine and the synthesis of different compounds like mucilaginous glycoproteins, which act as a barrier that decreases the permeability of the mucosa (60).

In the public databases: GenBank, SILVA, Ribosomal Database Project, 117 genera are assigned to sequences of bacteria that belong to the GIT of chickens (61). Firmicutes is the predominant phylum with 70% of abundance (61). This phylum is found in high abundance in animals with excellent feed efficiency because they can accumulate energy from the diet (62). Representative genera include *Clostridium, Ruminococcus, Lactobacillus, Eubacterium, Faecalibacterium, Butyrivibrio, Butyriciccocus, Blautia, Roseburia, Megamonas* among others (61). The phylum Bacteroidetes represented 12.3% of the total community and included the genera *Bacteroides, Prevotella, Parabacteroides,* and *Allistipes*. Proteobacteria registered 9.3%, and the representative genus was

Desulfohalobium. Minor phyla include Cyanobacteria, Spirochaetes, Synergistetes, Fusobacteria, Tenericutes and Verrucomicrobia (61).

It is important to highlight that microbial composition is highly variable between individuals. One possible explanation is that in modern broiler production the eggs have more influence from the environment than from the mother (63). The diet is another influencing factor considering the different availability of nutrients in the luminal content, which can also modify the chemistry in that environment (64). The different feed additives have a specific impact on the community that is based on the mode of action of enzymes or antimicrobials (40, 64). Moreover, host-derived substrates might influence the establishment of the microbiota per individual, considering the presence of mucins, bile acids and immune responses (64).

In the chicken, the digestive activities such as the breakdown of starch and fermentation of lactate, initiate in the crop. Physiologically it consists of a ventral diverticulum of the esophagus with longitudinal folds on the inner surface with average retention times of 31 minutes (57). The number of bacteria in this section account for 10^8 – 10^9 bacteria/g, and includes mainly *Lactobacillus,* with lower presence *Bifidobacterium* and *Enterobacter* (49) (Figure 1). *Lactobacillus* is an excellent colonizer of the gut because of its abilities of adherence and synthesis of carbohydrate-specific molecules such as lectins (60). The Lactobacillus species present in the crop are *L. salivarius, L. acidophilus, L. reuteri, L. johnsonii, L. crispatus, L. gallinarum, L. amylovorus* and *L. gasseri* (65). These species are known to produce acids from fermentative metabolism including lactic acid, acetic acid and ethyl alcohol, which constitute more than 3% of crop content, and therefore this is the reason of the lower pH in this section (41, 66).

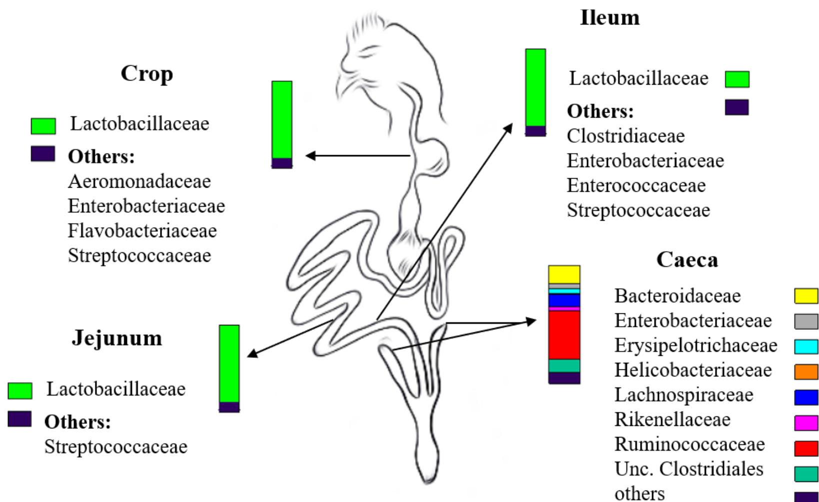

Figure 1. Bacterial families in the chicken digestive tract (data from Witzig *et al*., 2015 (65)).

The stomach in birds consists of two chambers, the proventriculus or glandular stomach and the gizzard defined as the muscular stomach (57) The latter is the place where the feed goes through a mechanical and chemical breakdown (4, 57). It comprises an inner surface lined with a cuticle to protect against acids and proteolytic enzymes secreted from the proventriculus (57). In this GIT section is found 10^7 - 10^8 bacteria/g of cells, with a high presence of *Lactobacillus* and *Enterococcus*. This section is important to regulate the GIT motility, control digesta flow, and refluxes, enhance secretions of hydrogen chloride, bile acids and endogenous enzymes (67).

The small intestine has four layers, the mucosal, submucosal, muscle tunic and serosal layer (57). It is divided into duodenum, jejunum, and ileum and contains an extensive innervation of the nervous system including the division in sympathetic and parasympathetic (57). Activities of digestion and absorption in the small intestine are facilitated by the addition of bile pigments, bile salts, amylase, and lipase. Furthermore, the pancreatic secretions include trypsin breakdown of proteins into peptides and amino acids. Also, enzymes like saccharase hydrolyze polysaccharides to glucose and fructose, while lipases are responsible for degrading fats to fatty acids and glycerol (4). Small intestine comprises bacteria in the range of 10^8 – 10^9 bacteria/g of cells (64). The jejunum has low pH and is mainly colonized by *Lactobacillus and Streptococcus* at lower percentages (49). The ileum is crucial for the digestion and nutrient absorption (66). This section is colonized by facultative and microaerophilic bacteria including the dominant *Lactobacillus* followed by the presence of *Streptococcus*, *Enterobacteriaceae,* and *Clostridiaceae* (64).

Thus, the crop and the different sections of the small intestine comprise microorganisms, which do not necessarily need oxygen (O_2) and its metabolism is mainly fermentative. Alongside, facultatively anaerobic bacteria are also present since lower concentrations of O_2 are present in the epithelium and proximal digesta (9).

The caeca have a combination of villi and musculature and are divided into three regions: the *basis ceci* closed to the ileocecal junction, the medial cecal region and the distal cecal region (57). This GIT section is considered as the place of crude fiber digestion, metabolism of complex polysaccharides and accumulation of amino acids (13, 57, 68). It possesses the higher bacterial concentration, accounting for 10^{10} – 10^{11} bacteria/g of cells, and register greater diversity than ileum and crop (49). The digesta has long transit times in the caeca, and the most fermentation activities are carried out here; however, this can influence the predisposition to be colonized by pathogens (69). Only strict anaerobic bacteria are found in the caeca, which are dominated by Ruminococcaceae, Clostridiaceae,

Lachnospiraceae, and in a lesser extent Lactobacillaceae (12, 70). Its metabolic activities are related to the production of SCFA, and a special focus has been given to butyrate production (62, 70, 71). *Bacteroides, Clostridium, Fusobacterium, Streptococcus,* and *Enterococcus* are responsible for proteolytic activities are listed genera (72). Also, in this section, members of the family Bifidobacteriaceae are linked to the production of lactic and acetic acid; while Coriobacteriaceae is associated with the metabolism of lipids and cholesterol (73). In small concentration are reported *Bacillus, Streptococcus, Enterococcus,* and *Flavobacteria* (61). *Methanobrevibacter* is present as the most dominant archaeal genus involved in methanogenic activities (74).

In the colon and the cloaca take place water and electrolytes absorption, transport of undigested components from the feed and the recovery of some secretions (4). A study focusing on the analysis of cloacal swabs found a clear dominance of *Firmicutes* and the dominant genus with 40% of abundance was *Lactobacillus* followed by *Enterococcus* (23,3%), while with less than 3% were detected *Clostridium, Faecalibacterium, Ruminococcus, Staphylococcus, Coprobacillus, Coprococcus and Sphingomonas* (75). In feces, a large presence of Firmicutes (abundance of 54%) followed by Bacteroidetes with 41% was revealed. Therein, feces core gut microbiota comprises the genera *Clostridium, Bacteroides, Lactobacillus, Subdoligranulum, Faecalibacterium, Roseburia* and *Eubacterium* (76).

As a result of the colonization with the above-described commensal bacteria, the proliferation of pathogens is reduced (60). The production of SCFA implies a bacteriostatic effect, due to decreasing in the pH, which causes resistance to the colonization (77). Additionally, *Lactobacillus* and *Bifidobacterium* are genera reported to synthesized bacteriocins with antimicrobial properties (77) and the presence of oligopeptides and homoserine lactones, molecules from bacterial signaling or quorum sensing which act against the pathogens (59, 78).

1.4 Effect of the age in the GIT of broiler chickens

The microbiota of the GIT of broiler chickens goes through adaptations during the lifespan of the birds (79). In the gut of younger birds, the phylum Proteobacteria and the family Enterobacteriaceae are more prevalent, while in older birds is more abundant Firmicutes and the families Lachnospiraceae, Ruminococcaceae, Clostridiaceae and Lactobacillaceae (79). Young birds were colonized by a diverse community with 19 phyla, while this proportion decreased in older birds due to the turnover in the colonization from facultative aerobes to anaerobes (79). These show that microbiota goes through a process of maturation until it becomes stable and more resistant to changes (80).

The age effect is directly observed in the crop, where the dominant class corresponds to *Bacilli* which registered 56% of abundance in chicks (0-5 weeks) and 73% in adult stage (>20 weeks). Negativicutes, Epsilon- and Gamma- proteobacteria, together with Clostridia registered an average abundance of 31% in younger chicks (0-5 weeks) however this number drop to 7% in older chickens (41). The opposite situation was observed with *Lactobacillus* that increases progressively from 40 to 70% at older stage (41).

In the gizzard, it was revealed that the presence of *Lactobacillus* decreased from day 15 (86%) to day 36 (58%) (81). The jejunum is colonized by Proteobacteria in the first day of life, and it shifts to Firmicutes on days 14 and 28 (79). Wise and Siragusa (2005) reported in the ileum, that seven days old chicks have an increase in Enterobacteria and Bifidobacteria and on day 14 *Clostridium leptum, Bacteroides* and *Campylobacter* spp. were also detected (82). Another study showed an increase in the relative abundance from day 8 to 36 of *Clostridium* (from 1% to 18%) and *Streptococcus* (from 1 to 5%) (81). Another survey established that Enterobacteriaceae abundance decreased from day 7 to day 35 while it increases Lactobacillaceae, Clostridiaceae, and Lachnospiraceae (69).

In the caeca, significant shifts at the genus level occur at days 1 and 2, and then on days 10-23 (75). In the first days, caeca are colonized mainly by the phylum Proteobacteria (70%) followed by Firmicutes (30%) and specifically OTUs were assigned to *E. coli, Enterococcus faecalis, Clostridium paraputrificum* and *Clostridium sartagoforme* (79). At day seven was detected a significant number of *Clostridium leptum, C. perfringens,* and Bifidobacteria. After day 14, Firmicutes is the dominant phylum (79) and genera such as *Enterococcus, Atopobium, Veilonella,* and *Lactobacillus* were detected. At day 21 it was found *Atopobium, Bifidobacterium,* and *Bacteroides* (82). *Lactobacillus* decreased its presence at later stages from 17% at day 15 to 2-3% of abundance in the following days (81). At later stages, there is an increase of *Blautia* (41) and *Faecalibacterium* (75).

One day old chickens have their feces colonized with Firmicutes (68%), Proteobacteria (26%) and Streptophyta (5%) while at day 35 the sample is mainly represented by Firmicutes. At the genus level, *Enterococcus* dominates with 52% of the assigned sequences, followed by *Escherichia* with 26% and *Clostridium* with 14%. The change at 35 days consisted of a high dominance of *Lactobacillus* with 72% compared to 2% in the first day, and a decrease of *Escherichia* (1%) (83).

1.5 Influence of the microbiota in broiler chicken metabolism

It is well recognized that microorganisms and the broiler chickens have co-evolved in a very interactive way, influencing the bird performance. Microorganisms can induce the maturation of the intestine through the synthesis of molecules. Their balance in ecological distribution imply better assimilation of nutrients for the host and influence the

proliferation of mucosa (84). The formation of the mucosa is regulated and promoted by the host, and its unbalanced development might infer a dysbiosis in the gut. On the other hand, a high proliferation of the mucosa could imply a pathogenic infection, triggering an energy disequilibrium (84). In the vascular system, microbes induce the transcription of angiogenin-3 that acts on the good function of the microvascular of the intestine (85). Some *Lactobacillus* species have been reported to inhibit the angiotensin-1 enzyme that is responsible for regulating blood pressure (86). The distribution of Firmicutes and Bacteroidetes affect the energy balance, and it is known that animals with a better metabolism efficiency are dominated by Firmicutes (84, 87). In line with arguments that microbiota affects the host, current investigations suggest that compounds produced by the microorganisms affect the responses of the central nervous system. These compounds include tryptophan, a precursor to the neurotransmitter serotonin, which gives a chemical stimulus that induces different behavior in the host like changing feed consumption, and cytokines (88, 89) (Figure 2).

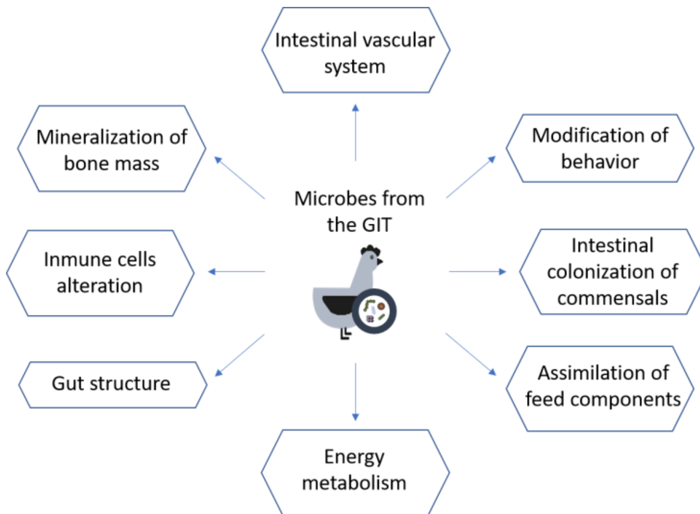

Figure 2. Main effects of the microbial communities on the intestinal tract of chickens (adapted from Lunedo and Pedroso 2017 (84)).

The cereals used in the feed are digested by the broiler chickens through excretion of enzymes mainly from the pancreas like pancreatic alpha-amylase which is stimulated with the ingestion of high contents of starch (90). However, it is also expected the presence of carboxypeptidases, proteases, and lipases which contribute to the hydrolysis of the compounds ingested (91). Other enzymatic activity of the host that contributes to the digestion process takes places in the glandular stomach (proventriculus) with the secretion

of hydrochloric acid that supports in the fragmentation of many peptides (91). Moreover, the small intestine is also responsible for the digestion due to many pancreatic enzymes that are active in that environment (91). Nevertheless, many complex polysaccharides cannot be hydrolysed, and therefore microorganisms have an important role of action.

Microorganisms have sizeable enzymatic machinery, which comprises the active carbohydrate metabolism in the GIT including the presence of glycoside hydrolases, polysaccharide lyases, and carbohydrate esterase enzymes (49). Moreover, there is evidence of modulation of the lipid metabolism, since the presence of genes related to that activity is found in metagenome sequences of chickens (50, 92). The caeca metagenome, obtained in one project from the MG-RAST website (93) (project: mgp19727), it was observed that high proportion of metabolism was assigned to the catalogue of genes (32%), being amino acid and carbohydrate metabolism the most represented categories (both 7%) (Figure 3). Additionally, it was revealed in that assignation that transport and catabolism registered an important proportion in the cellular processes (6%), being an indication of active metabolic communities (49). It is reported that synthesis of SCFAs in the caeca are a product of the microbial metabolic activities by *Allistipes* and *Bacteroides* and at great extent members of Clostridiales (49, 68). In the caeca, the production of SCFA increased gradually with age, and the most produced compounds are: acetic acid, lactic acid, butyric acid, propionic acid, branched volatile fatty acid (VFA) and valeric acid (94). Total concentrations of these SCFAs registered 20 mM at 11 days, 80 mM at 21 days and 100 mM at 42 days (94). On the last day of the measurement (42d), the proportion of acetic acid was 64%, butyric acid 23% propionic acid 8.4% and valeric acid 0.8% (94). SCFAs are essential for the enhancement of the muscular system in the colon together with the vascular system (45). From them, butyrate is the source of energy for the colon cells, as it maintains the homeostasis of colonocytes and influences the formation of villus in the GIT (62). Also, butyrate stimulates the activity of GLUT2, which influence the bidirectional transport of glucose in the GIT (84). The central metabolic pathways used by the microorganisms to produce butyrate, mostly includes the Acetyl-Coenzyme A, a product from carbohydrate fermentation of pyruvate; that can also be obtained from the glutarate, lysine, and succinate. Valerate, isobutyrate, and isovalerate are found in lower amounts (95). Furthermore, lactate produced from lactic acid bacteria is rapidly absorbed in the hindgut or is likely to be converted to butyrate and other VFAs by lactate-utilizing bacteria (58, 94).

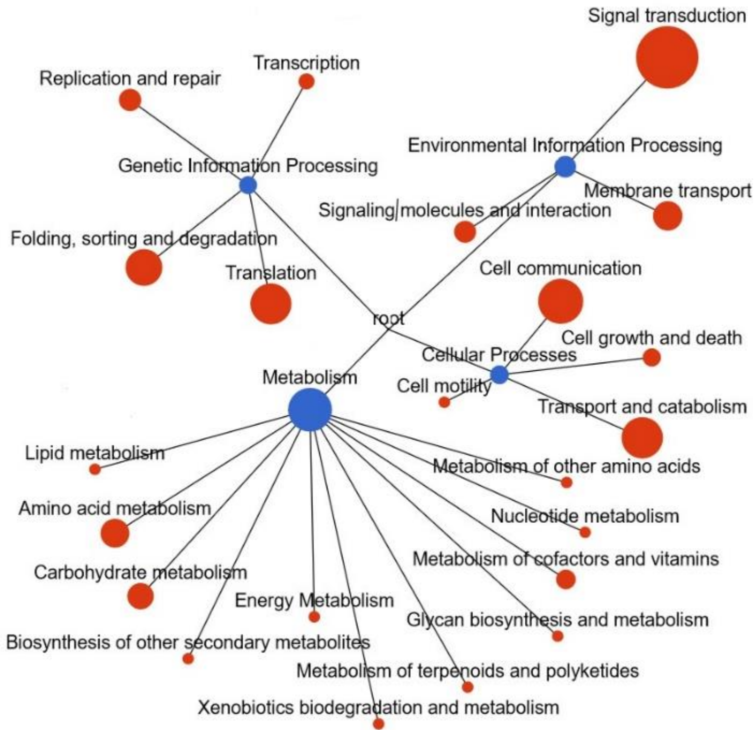

Figure 3. Metagenome assignation for gene abundance from the caeca digesta in chickens. The network was obtained using cytoscape (96), where the size of the circles represents the proportion of assigned genes, and the colors indicate the first level of classification in KEGG Orthology (blue) and second level (red). (source: https://www.mg-rast.org/linkin.cgi?project=mgp19727).

The metabolism of proteins, amino acids, and nitrogen derivatives have a significant influence on the nitrogen metabolism (68). Caeca bacteria use uric acid to produce ammonia, that is absorbed in the GIT to synthesize amino acids like glutamine (97). Microbiota metabolizes first the proteins and afterward occurs deamination and decarboxylation of amino acids. In this process, not only beneficial compounds are obtained including volatile fatty acids, branched chain fatty acids, lactate, and succinate but also, as a result from undigested nitrogen, putrefactive compounds such as indoles, phenols, sulfur compounds and amines (72, 97). The presence of this non-desired compounds is also influenced by the low concentration of carbohydrates, combined with high concentrations of undigested proteins that need to be further fermented (98).

Phytate or *myo*-inositol 1,2,3,4,5,6-hexakis dihydrogen phosphate ($InsP_6$) is the principal form of phosphorous in the plant-based diets (13). Many efforts have been made to study alternatives to increase the availability of P in the diet, through the hydrolysis of $InsP_6$ achieving less phosphorylated compounds (13, 99). Possible mechanisms in which the phytate is hydrolyzed implies the presence of endogenous mucosal phytase (not so efficient in non-ruminants), plant phytase, exogenous addition of synthetic phytase enzyme and gut microbial phytase (99). At this point must be considered that due to microbial activities, non-gnotobiotic broiler chickens display higher concentrations of $InsP_6$ when compared to gnotobiotic (11, 100). This fact might be a result of the higher microbial activity of intestinal alkaline phosphatases (11).

In regards to health and performance, microbes can boost the host immune system, stimulate the mucin production and epithelial cells (60). Commensal bacteria synthesized small peptide molecules with antimicrobial properties that maintain gut balance and reduce pathogens appearance (101). The interaction between the microorganisms and the immune system starts directly after hatching, where increased levels of interleukin-8 (IL8) are registered, being this cytokine related to a reduction in the inflammation levels. IL17, related to gut maturation and defense against fungal and bacterial infections also increases (102). Gnotobiotic chickens showed a decrease in the expression of T cells, considering that induction of this cells orchestrates an active local immune development its reduction might affect intestinal homeostasis development (103). Furthermore, the presence of IgY and IgA increased in colonized chickens when compared to gnotobiotic animals (103). In this sense, the microbial communication in the GIT is established in a natural and stable form leading to an undisturbed ecology, diminishing the possibility of pathogens to proliferate (58).

1.6 Diet components in broiler chicken nutrition, and its effects on microbiota

The search for suitable feeding alternatives is the main concern in poultry nutrition, being expected an increase in the nutritional value of the feed while achieving reductions in costs and no negative effects in the performance of the bird. As an example in chickens, high contents of NSPs infers villis fusion, thinner tunica muscularis, more aggregation of immune cells in the mucosa and higher rate of apoptosis from epithelial cells (104). Those are adverse effects which are being solved with the consequent addition of enzymes. Other alternatives which have gained importance in poultry nutrition comprises the addition of essential oils, probiotics, and prebiotics (105).

Proteases are supplemented in the diets to increase protein hydrolysis and further release of amino acids and peptides which can be absorbed by the chicken (106). The secretion of

trypsin from the pancreas does not entirely achieve crude protein digestibility since a concentration of proteins goes through the GIT without complete digestion (107). The increase of crude protein digestibility improves body weight and feed conversion ratio (108). Concerning microbial communities, it was reported that low levels of feed protein together with the supplementation of protease were not affecting the concentration of *Lactobacillus* spp. Also, the enzyme maintained low concentrations of *C. perfringens*, a pathogen that can lead to necrotic enteritis in the ileum and caeca and clostridial diahrrea (109). In a study with a combination of multiple enzymes, it was observed an increase of beneficial groups including *Bifidobacterium*, *Staphylococcus*, *Bacteroides*, and *Megamonas* (110).

Multicarbohydrases (exogenous enzymes) have an impact in the gut health of chickens because its addition to the feed depolymerizes many polysaccharides resulting in galacto-, gluco-, manno- or xylo-oligomers. The enzymes possess similar functions to prebiotics, which is reflected in the abundance of *Bifidobacterium* and *Lactobacillus* (111). This supplementation improves digestibility of fat, starch, nitrogen, and NSPs and reduces the viscosity and degradation of cell wall structure of the small intestine (112). Hemicellulase supplementation increases ileal digestibility of proteins by breaking the cell wall structures (113). Xylanases, when added to diets with high NSPs, diminishes digesta viscosity and improves nutrient digestibility and performance (114). With this supplementation, the carbohydrate degrading bacteria increase in the caeca, due to the higher concentration of xylo-oligosaccharides (113, 114). The combination of enzymes xylanase, amylase, and protease in the diet increase the retention of dietary Ca and P and also increase in energy availability (115). However, the type of cereal used affects the synergetic effect of the mixture of enzymes, where wheat and barley showed a better release of phytate compared to maize based-diet (116).

Environmental concerns encourage to reduce P excretion in the form of non-assimilated substrates (99). Therefore, phytases are provided to the feed, to support the digestibility of inositol-phosphate, due to the low activity of the endogenous enzyme present in chickens. Only up to 25% of phosphorus from wheat-corn-soybean meal diet is assimilated without this supplementation (7). The enzyme has an impact on the use of limestone and phosphates, which indeed increases the acid binding-buffer capacity and the values of pH in the content. Additionally, it improves performance, mineral retention, and amino acid digestibility (117). Also, the intake of digestible P increases because of the higher break-down of P from the phytate (115). It is reported in the crop, gizzard, and ileum that higher concentration of Ca increases the pH (118). Although, it is still not evident in the literature the effects of phytase and Ca:P ratio on the pH of the GIT (117). Different P supplementations with phytase affect the pH in jejunum digesta, but no effect was observed in the crop, stomach, ileum and caeca digesta (119). The positive effect of adding phytase is the reduction of the buffering capacity and pH, which preserves the integrity of the

intestine and promotes the presence of commensal bacteria (12). Ptak et al., 2015, showed that as a result of enzyme addition an increase of total counts of bacteria in *Lactobacillus* and *Enterococcus* species was registered. Diets supplemented with the enzyme were enriched with microbial sequences for carbohydrate metabolism, showing that these diets contain higher availability of polysaccharides and high expression of KEGG Orthology (KO´s) from glycolysis/gluconeogenesis, together with starch and sucrose metabolism (53). When the enzyme is supplemented, a higher concentration of *myo*-inositol is available which promotes feed conversion ratio and microorganisms. It is known that archaea can metabolize it and it serves as carbon source and energy source for *B. subtilis, Aerobacter aerogenes, Rhizobium leguminosarum, Sinorhizobium melilloti, Corynebacterium glutamicum* and *L. casei* (120). The metabolism of *myo*-inositol is initiated with a dehydrogenase enzyme and further a dehydratase. In this metabolic process is also involved the major facilitator superfamily, which imports the polyol, and the compound is suggested to be integrated as complex cellular glycolipids, such as lipomannans and lipoarabinomannans (121).

Butyrate could be included in the diet as a synthetic source since its production is limited in the small intestine. It is an energy source for intestinal cells, controlling the production of cytokines lymphocytes and macrophages, it alters the intestinal barrier positively and has an anti-inflammatory effect (62). The microbial modulation implies a decrease in the distribution of *Ruminococcaceae* with the addition to 6.4% without sodium butyrate; however, *Faecalibacterium prausnitzi* is promoted (62), and potential probiotic *Bifidobacterium* (122). *F. prausnitzi* has a positive impact on feed efficiency and performance of broilers (62, 123). Both, *F. prausnitzii* and *Bifidobacterium* produce choline metabolites and modulate lipid metabolism with further glucose homeostasis (122). Butyrate addition to the diet regulates the abundance of pathogens *Salmonella* and *Clostridium perfringens* while increasing the presence of *Lactobacillus* (89). Additionally, it was seen in the caeca a reduction, in 6-fold, of members from the Mollicutes class, which are mainly related to pathogenesis, supporting the beneficial effects of this addition (122). The addition of butyrate reduces the presence of *Subdoligranulum*, which is described to produce butyric and lactic acids. This feedback is implying a functional reduction as a result of active cross-talk between the host and its microbiota (122).

Probiotics have a positive influence on the immune status since they can be a source of microbial signals that reduce inflammation, decrease pathogen colonization and increase the digestibility of different substrates (5). Improvement in efficacy is observed if the administration is done *in ovo* or directly after eclosion, considering the non-well established commensal communities (84). Some of the strains used as probiotics are *L. acidophilus, Bifidobacterium bifidum,* and *Streptococcus faecalis*. They enhance the production of antibodies, through the stimulation of Th2 cytokine production, specifically IL-4 and IL-10 (124). Moreover, it was described that throughout the synthesis of SCFA,

mineral solubility from probiotics increases bone mass density. In mice and rats, *Lactobacillus* species increased calcium absorption and promoted bone mineralization and bone mass density (125, 126). Likewise, *Bifidobacterium* spp. exclude pathogen agents due to the production of bacteriocins, decrease the pH, synthesize vitamins and stimulate the immune system, which are benefits to the host (60).

The prebiotics cover feed ingredients which are not assimilated by the host and only specific microbial members can process them, resulting in benefits for the host (69). Some of these substrates comprise inulin and oligosaccharides like fructo- mannan- and xylo-oligosaccharides (127). Positive benefits include the exclusion of pathogens by preventing attachment to epithelial cells, production of antimicrobial compounds, stimulation of the immune system and improve gut structure. In humans, the addition of prebiotic into the diet has an influence on the microbial population of the large bowel and improves the production of SCFA (72). As a result of this supplementation, it was found in broilers an increase in the diversity of *Lactobacillus* species as well as *Bifidobacterium* and a reduction of *Salmonella* colonization in the ileum (128, 129).

Minerals are inorganic components of the feed because they become part of body tissues and serve as catalysts for different enzymes. They allow the development of primary functions like energy production, bone health, nerve and muscle function and immune status (4). Calcium and phosphorous are mainly present in the skeleton; potassium is essential for muscle functions; iron is important for the blood; and silicone for the feathers (4). Because the availability of minerals in feed ingredients is variable, there is need to include them in the diet. Special attention is given to the macrominerals calcium and phosphorus because its deficiency in growing animals has an impact in bone formation, which could trigger diseases like rickets or osteomalacia (130). In those cases, long bones can break easily and when the deficiency is severe paralysis could occur. Ca and P are studied due to their interaction before and after digestion. Thus, the ratio Ca:P has to be considered because cereals are deficient in Ca, but P is present in higher concentration (4). In pigs has been reported that metabolic activities of the microorganisms from the large intestine are dependent on the availability of P and Ca (131); while P is an essential condition for an optimal fermentation in ruminants (130). It is expected that intestinal microbiota from animals influence positively or negatively the bioavailability of minerals (132). The adherence of *L. salivarius* improves when there are available Ca^{++} ions, and a study with higher levels of P and Ca demonstrates that specific genera are promoted in the crop and the ileum (65), which shows an impact of the minerals on the microbiota (133). Supplementation of iron (Fe) in the diet implies the promotion of Firmicutes, which is associated with an increase of energy availability in the host, and also increases the abundance of butyrate-producing bacteria *F. prausnitzii*. Meanwhile, Fe did not increase pathogenic microbial load, and no alteration of genetic capacity was registered, meaning that this fortification was positively related with the host (134).

The use of essential oils is another alternative in broiler chicken nutrition. They have antimicrobial properties linked to the presence of phenols, alcohols, ketones, and aldehydes, that go through the permeability of cell membranes inhibiting the membrane electron flow and energy metabolism (135). Essential oils can reduce lipid peroxidation in the muscles because of their antioxidant properties. Furthermore, they have a positive effect on the stimulation of the immune system by promoting the presence of immunoglobulins, lymphocytes, and interferon-γ (1). However, they should be administered in proper concentrations, considering that high concentrations can cause lysis of membranes and cytoplasmic proteins (135). In the influence of microbes, conflicting results have been reported and is not clear if the reduction of pathogens with their supplementation is obtained (105). For instance, thymol and cinnamaldehyde might modulate the intestinal communities by increasing the profile of %G+C and decrease the presence of *E. coli* in the supplemented diets (136). *Campylobacter perfringens, Salmonella* and *Campylobacter,* are also negatively affected by this supplementation (105). On the other hand, the essential oil, lemon myrtle oil, did not reduce the concentration of potential pathogen *Campylobacter jejuni* (137) and rosemary oil, oregano oil, yarrow oil and thyme oil does not affect on the reduction of *Clostridium perfringens* and total coliforms (138). Therefore, the antimicrobial activity of essential oils needs to be further studied, because the mode of action is not consistent in the different challenging trials.

There is still a long way to describe the active cross-talk between host and microorganisms because many aspects have to be taken into consideration to get a balanced ecosystem. Health and wellness from the host, diet and supplements, the age, the GIT corresponding section and the influence of the environment, have a significant impact on the microbiota. Therefore, the understanding of the microbiome using holistic approaches can imply a better comprehension and possible modeling of the behavior of this ecosystem. Together these facts will impact the actual knowledge in chicken production and would bring us new approaches considering an efficient use of nutrients and a lesser impact on the environment.

1.7 Scope and work hypothesis

An appropriate diet formulation is one of the main issues in animal nutrition, where every change on it, has implications on the gut microbial community, health status, and production performance. The understanding of the ecological distribution of microbes, their interactions, and role in the active cross-talk with the host, gives an opportunity to look for alternatives that give better assimilation of nutrients which will reduce the loss of nutrients, a crucial environmental concern.

Sequencing technologies have taken us to a step in which extensive data information is obtained from the microbial ecosystem, but this is the beginning of the process, further interpretation to recognize which are the main contributors and its principal roles needs to be addressed. Therefore, the challenge is not only to give sense to this massive amount of information, but also to correlate it with other disciplines and to bring new developments and discoveries in the field of animal nutrition.

The general aim of this thesis is to describe the changes in bacterial community structure that occurred in chickens, in response to different experimental diets. The specific objectives consisted in an update state of the art in the chicken GIT microbiota, considering the technologies available, focusing on the concluding remarks from those studies, and bringing future perspectives (chapter 2). Furthermore, it was developed an extensive investigation of the microbiota composition in the digesta and mucosa of individual samples in animals supplemented with calcium, phosphorus, and phytase to understand if in the crop, ileum, and caecum the diet impacts the distribution of the microbial community (chapter 3). Additionally, it was assessed the effect of supplementing different proteases in the microbial community of the GIT of broilers (chapter 4). Finally, as part of the discussion, an outlook with metagenome sequencing will be presented, that further characterizes the result of feeding strategies in gut microbiota (chapter 5).

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CHAPTER II

FIRST MANUSCRIPT

CURRENT PERSPECTIVES OF THE CHICKEN GASTROINTESTINAL TRACT AND ITS MICROBIOME

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Published in:
Computational and Structural Biotechnology Journal (2018)
Volume 16, 2018, Pages 131-139
doi: 10.1016/j.csbj.2018.03.002
Accepted: March 12, 2018

Open access.
The original publication is available at https://doi.org/10.1016/j.csbj.2018.03.002.

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2.1 Abstract

The microbial communities inhabiting the gastrointestinal tract (GIT) of chickens are essential for the gut homeostasis, the host metabolism and affect the animals' physiology and health. They play an important role in nutrient digestion, pathogen inhibition and interact with the gut-associated immune system.

Throughout the last years high-throughput sequencing technologies have been used to analyze the bacterial communities that colonize the different sections of chickens' gut. The most common methodologies are targeted amplicon sequencing followed by metagenome shotgun sequencing as well as metaproteomics aiming at a broad range of topics such as dietary effects, animal diseases, bird performance and host genetics. However, the respective analyses are still at the beginning and currently there is a lack of information in regard to the activity and functional characterization of the gut microbial communities. In the future, the use of multi-omics approaches may enhance research related to chicken production, animal and also public health. Furthermore, combinations with other disciplines such as genomics, immunology and physiology may have the potential to elucidate the definition of a "healthy" gut microbiota.

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2.2 Introduction

The global population is increasing continuously and is estimated to comprise about 9.6 billion individuals by 2050. Correspondingly, poultry production has intensified during the last years and is predicted to produce about 130 million tons of chicken meat in 2020 (OECD/FAO) to match the demands of a growing world population. Such extreme growth is only feasible with proper strategies for disease control and prevention to minimize the impact of bacterial, parasitic or viral infections of the animals and simultaneously reduce associated ecological damage and waste of resources.

Chicken breeders focused on high performance, fast growth, breast meat yield, efficiency of feed conversion rates, skeletal quality, heart and lung functionality and as well on egg production and quality. Looking for the preferred phenotypic traits and selecting the most

superior individuals influenced the animals' genetics [1]. However, selection for a single trait may also affect other traits. For example, broiler chickens that were selected for meat production gained a higher body weight (~3 kg) within 42 days. On the other hand, ascites and/or lameness occurred in the animals [2]. Thus, a balanced selection across the different traits might improve the animals' well-being.

Besides breeding and selection, optimized nutrition of broiler chickens is a fundamental component of efficient poultry production. The animals' fodder accounts for 70% of the total costs in chicken production [3] and poultry diets are expensive since egg and meat production require high amounts of energy and protein sources. Diets contain energy and protein, mineral supplements, specific amino acids and vitamins in a defined formulation providing all nutrients necessary for the bird's health and adequate performance. Diets with imbalanced mineral supplementation may lead to health problems and result in inefficient use of the natural resources. Consequently, high amounts of valuable nutrients such as nitrogen, phosphorus (P), calcium (Ca) and zinc get lost by defecation and urination [4].

Gut microorganisms are mainly responsible for the degradation of complex substrates such as non-starch polysaccharides which requires highly specialized, hydrolytic enzymes [5]. The discovery of novel enzymatic tools depends on metagenomic data for instance from the broiler caeca. Recently, a xylanase gene from the chicken caecum has been isolated and overexpressed which emphasizes the potential for the development of new, optimized feed additives for industrial application [6]. Close interactions between the intestinal microbiome and the animals' diet are well established since dietary factors are known to alter the gut microbiota. Bacteria are able to hydrolyze indigestible carbohydrates and polysaccharides allowing further fermentation by other members of the gut ecosystem that produce short chain fatty acids (SCFA) which in turn become available for the host.

Moreover, microorganisms growing on poultry litter have an influence on the gut microbiome and may constitute a source of infection. Since the first day of life, chicks start pecking and ingesting litter materials including the adhered microorganisms that are usually detected in feces and soil. In this way, microbes of other habitats can be transferred to the gastrointestinal tract [7]. Previous studies have shown that *Salmonella* and *Clostridium perfringens* decrease in abundance in reused litter and *Campylobacter jejuni* and *Escherichia coli* become more prevalent [7]. Wang *et al.* compared the microbiota of fresh and reused litter and its effects on the chickens' gut microbiota finding an increase of halotolerant/alkaliphilic bacteria in reused litter and a stronger effect of the litter on the microbiota of the ileum in comparison to the caecal microbiota. Caecal samples of young birds raised in reused litter showed a higher bacterial diversity when compared to mature animals that were kept under the same conditions. The reuse of litter is a common practice in broiler production. Despite studies showing that reused litter does not exhibit higher

abundances of *C. perfringens* or *Salmonella* [8], chickens raised in fresh litter revealed an increasing colonization with beneficial *Lactobacillus* spp. [9]. Proper litter management may reduce pathogen activity, promote a balanced gut microbiome and improve the chickens' health status.

This review will focus on the methodologies that were used in the past years to characterize the microbial communities within the chickens' gut to provide insights into the effects of different feeding strategies and host genetics on the gut microbiome. New perspectives will elucidate yet unknown aspects of the chickens' gut microbiome.

2.3 Exploring the composition and function of the chicken gut microbiome

2.3.1 Targeted amplicon sequencing of the 16S rRNA gene

Next-generation sequencing revolutionized the characterization of microbial communities. The respective studies are mainly based on amplifying the small subunits of the 16S ribosomal gene of Bacteria and Archaea, the 18S rRNA gene of eukaryotic species and the nuclear ribosomal internal transcribed spacer (ITS) regions of Fungi [10]. In this way, deep characterization of microbial communities and quantification of relative abundances of the different microorganisms can be achieved. Most of the studies available aim at the bacterial 16S rRNA gene. Even though this method has been used in other scientific disciplines for several years, the first study characterizing the chickens' gastrointestinal microbiota was published in 2011 [11]. The 16S rRNA gene comprises nine hypervariable regions [12]. However, so far microbial studies of the chickens' gut have covered the V1–V3, V3–V4, V4–V5, V1, V3 or V4 regions [5, 7, 11, 13-18]. The sequencing technologies of choice are Roche 454-pyrosequencing, Illumina MiSeq, HiSeq and Ion PGM systems [19]. Bioinformatic processing of the generated sequences can be achieved by employing open sources platforms such as QIIME [20] and mothur [21] that, in order to perform taxonomic assignments, depend on public databases like GreenGenes [22], the ribosomal database project (RDP) [23] and SILVA [24]. The latter represents the most recent database. Functional prediction algorithms such as PICRUSt and Tax4Fun can be used to obtain further information from 16S rRNA gene sequencing data. PICRUSt is based on the GreenGenes database and uses an algorithm with proved accuracy regarding humans, soils and mammalian guts [25]. However, the GreenGenes database was last updated in 2013. Tax4Fun employs the SILVA database and claims to reach higher correlations regarding the functional predictions since the link association is based on the nearest neighbor with a minimum sequence similarity. Despite the promising information that can be obtained by functional prediction processing, caution is advised when drawing strong conclusions since there are large numbers of operational taxonomic units (OTUs) that cannot be assigned to a specific genus and not even to a family level [31]. Moreover, the respective

approaches should be validated thoroughly in particular for avian species since their deviating organism may imply different functions and associations between microorganisms and the host.

More than 900 bacterial species inhabit the GIT of broilers being involved in the digestion of food, breakdown of toxins, stimulation of the immune system, exclusion of pathogens and endocrine activity. Interactions between microorganisms and the GIT influence the stability of the microbial communities, the animals' health, growth and consequently also feed conversion rates [26]. As feed is ingested and moves through the GIT, different groups of microbes start the digestion. The chickens' GIT is divided into three parts: the upper segment, small intestine and large intestine that are colonized by microbes in their entire length. Due to the enormous diversification of each GIT section, they are commonly studied as independent ecosystems. However, it is known that the different sections are highly interconnected and thus also influence each other's community composition [27]. Variations regarding the protocols for DNA extraction, choice of the amplified 16S rRNA gene regions and overall microbial community characterization make comparison between studies difficult. The study design strongly influences the microbial profiles of each gut section due to the differences between individual birds, species, gender, age, genetics, diets and housing. Microbiota studies in individual chickens showed a high inter-individual variation, disregarding the identical diet composition or housing conditions [5,13,16].

In the crop, breakdown of starch and lactate fermentation are initiated by several Lactobacillus sp. and Bifidobacterium sp. as well as by members of the Enterobacteriaceae family that were also detected within this section [28]. Lactobacilli also appear in high abundances in the proventriculus and gizzard. Nutrient absorption occurs in the ileum which exhibits high numbers of Lactobacillus sp. and to a lesser extend bacteria with butyrate producing activities such has Clostridium, Streptococcus and Enterococcus [28]. Fermentation and digestion of complex substrates such as cellulose, starch and other polysaccharides occur in the caecum, which is the most diverse gut section characterized by the longest feed retention time (12–20 h). In contrast, only 2.5 h are required to pass through the upper parts of the intestine [36]. The most abundant families within the caecum are Clostridiaceae, Bacteroidaceae, Lactobacillaceae and butyrate producers like Lachnospiraceae. The caecum is highly dominated by not yet characterized bacteria and exhibits the highest concentrations of short chain fatty acids (SCFA) [28]. As broilers age, their caecal microbiota becomes more diverse. Out of 50 genera detected on day zero post-hatching the caecal genera increased to above 200 on day 42 post-hatching [29]. Temporal fluctuations occur particularly in the fecal microbiota due to the random emptying of the GIT section [30].

Previous studies of chicken broilers focused on lumen samples neglecting the mucosa that is mainly composed of mucins and glycans which promote colonization by distinct groups of microorganisms. Studies in humans, mice, rats, macaques, pigs and cows showed a divergence between lumen- and mucosa-associated microbiota structures [38-41]. In contrast to the continuous flux of nutrients in the lumen, the mucosa is expected to show a more stable balance of nutrients which may represent a selective criterion for certain bacterial species [39]. A recent comparison between lumen and mucosa associated microorganisms revealed a much greater microbial community richness in the mucosa, particularly in the ileum and caecum of broiler chickens [13]. Pseudomonas spp. were detected in the ileal mucosa but not in the lumen. These species have the ability to hydrolyze phytate, degrade starch and in soils they are known to improve plant phosphorus availability [31]. Species belonging to the genera of Clostridium XI and Ralstonia were present in higher abundance in mucosa samples, while Lactobacillus sp. were three times more abundant in the ileal lumen. High abundance of commensal Clostridium XI species might induce a greater bacterial translocation from the ileal mucosa to the lymph nodes triggering an inflammatory immune response in the lymphatic tissues as previously described for pigs [32]. The caecum is the most diverse gut section and distinct community structures were observed in the lumen and mucosa samples. While the genera *Anaeroplasma, Oscillibacter, Papillibacter, Peptococcus* and *Subdoligranulum* were more abundant in the lumen, *Lactobacillus, Ruminococcus, Turicibacter, Clostridium* XlVa and *Clostridium* XlVb were detected in higher abundances in the mucosa. These observations emphasized the importance of studying the variations between the bacterial communities of the lumen and mucosa throughout the different sections of the GIT to improve our understanding of host-microbe interactions.

The majority of studies based on targeted 16S rRNA gene sequencing demonstrated effects of specific diet supplementations on the microbiota: probiotics, prebiotics and synbiotics [14,33,34]; Ca, P, phytases [13,28,35] and sodium butyrate [17]. Other studies characterized the different sections of the GIT of broilers under varying conditions analyzing bird performance [36, 37, 38], antimicrobial feed additives [11,39,40], gender [41], disease [42], host genetics [18,41], spatial microbial diversity [30,43] and meat flavor [33]. However, this is only a sparse depiction of the complexity and variability that exists within the highly diverse feeding and management conditions in animal production. Moreover, these investigations could not access the functional profiles and the activity of the respective microbiotas.

2.3.2 Metagenomic Shotgun Sequencing

Metagenomics, as a procedure to describe the collection of genomes and corresponding genes of a given ecosystem, permits the characterization of the potential bacterial functionality in specific environments [44]. Only a few metagenomics studies made the effort to answer the question: What are microorganisms actually doing in the chickens' GIT? (Table 1). The respective studies employed Roche 454-pyrosequencing and Illumina MiSeq or HiSeq platforms [11,45] to obtain the respective sequence information. It is expected that in the future more studies will rely on the Illumina technology since it grants a more convenient treatment of sequencing errors through computational approaches [19] including a greater coverage and yield as well which decrease systematic errors and costs [46]. Bioinformatic analyses include sequence assembly using the Velvet assembly tool (CLC workbench, Newbler version 3.0, BaseSpace) or automatic annotation by MG-RAST. The basic local alignment search tool (BLAST) is used to define functional groups and bacterial taxa. Subsequently, gene functions may be analyzed using the Kyoto Encyclopedia of Genes and Genomes (KEGG) or cluster of orthologous genes (COG). Up to now, metagenomics studies of the chickens' GIT have focused on the functions of the caecum [5], the response mechanisms to challenge by pathogens [45], the prominent role of the microbiota regarding performance parameters [47], comparisons between fat and lean lines [15], depiction of the virulome [45,48,49] and of antibiotic resistance genes [50,51] (Table 1). To obtain information about the taxonomic distribution of the microbial communities, studies focused on the most prevalent phylotypes representing the functional gene composition of the metagenome. The most abundant caecal phylotypes belong to the phyla of Firmicutes (44–55%) and Bacteroidetes (22–42%) [45], followed by the low abundant phyla of Actinobacteria, Chlorobi, Deferribacteres, Fusobacteria, Proteobacteria and Verrucomicrobia [45]. Analysis of environmental gene tags (EGTs) revealed that approximately 1% of the sequences belong to Archaea, mostly to Euryarchaeota but as well as to Eukaryota, Fungi and Viridiplantae [45]. The caecal metagenome of chickens challenged by *Campylobacter jejuni* revealed that mobile elements were a contributing factor to the functional components of the microbiota and that these genes were associated with virulence clustering according to the environment [45].

Table 1. Summary of the studies investigating chicken microbiome in respect to the influence of feeding impact with metagenomics and metaproteomics methodologies.

Metagenome details	Study focus	Diet	GIT sections	Number of samples	Sampling time	Reference
GS-FLX sequencing Reads: 1.291.219 Av. length: 234–399 bp	Effects of subtherapeutic levels of virginia and tylosin and the coccidial monensin on bacteria composition from the chicken caecum (metagenomics and 16S)	7 d of basal diet followed by supplementation with: Monensin sodium, Monensin sodium + virginiamycin or tylosin phosphate	Caeca	Pooled samples per treatment	0 d, 7 d, 14 d and 35 d Ross × Ross chickens	[11]
Illumina MiSeq2000 Reads: 81.772.788 Av. length: 110 bp	Elucidation of the functions of the cecal microbiota and characterization of the community profile (metagenomics and 16S)	Wheat based diet with 5% maize	Caeca	20	42 d of Ross broilers	[5]
Illumina HiSeq2000 Reads: 52.485.882 Av. length: 100 bp	Study if variation of fatness is link to the composition of gut microbial metagenome. Lean and fat lines were employed.	Commercial diet	Feces	29	Fat and lean lines. Weeks 37 to 40	[54]
Illumina HiSeq2000 Reads: 37.9 million (per sample) Av. length: 100 bp	Comparison of two lines of chickens (fat and lean). Understand the influence of the host in the gut microbiota	Commercial diet	Feces	6	35 wks	[15]
Illumina HiSeq 2000 Av. length: 100 bp	Antibiotic resistance genes annotation from metagenome of pig, chicken and human and its co-occurrence with associated genetic elements	Commercial diet	Feces	8	20 d and 80 d	[51]
454 sequencing Reads: 24–30 million Av. length: 100 bp	Phylotype and functional gene content characterization before and after inoculation with *Campylobacter jejuni*	Commercial diet and 14 days post-hatching one group was challenged with 10^5 CFU of *C. jejuni*	Caeca	2	28 d (14 d of challenge)	[45]
GS-FLX sequencing Reads: 94.926 (low FCR); 63.891 (high FCR) Av. length: 227 bp	Characterization of poultry fecal microbiome of low and high feed conversion ratio (FCR) broilers	Commercial diet	Feces	Pooled samples for high and low FCR	49 d broiler strain MY	[53]
Illumina HiSeq 2000 Reads: 4.737.146	Investigate the occurrence, diversity and abundance of antibiotic resistance genes in feces of layers and broilers	Commercial diet	Feces	Pooled samples	6 wk broilers and 52 wk laying hens	[50]
Metaproteomics	Microbial composition in the healthy chicken gut	Attlee's nonmedicated poultry feed	Feces	Pooled samples	18 wk white leghorn chickens	[57]
	Dietary effect of mineral phosphorous and microbial phytase	3 diets with P from plant sources (BD−), 3 diets with P supplementation (BD+). BD− and BD+ supplemented with 0, 500 and 12,500 U/kg of *E. coli* phytase	Crop Caeca	Pooled samples per treatment	25 d broilers Ross 308	[58]

The caecum consists of two long anoxic blind sacs that harbor a microbiota dominated by carbohydrate metabolism with lower occurrence of respirational genes [45]. Fermentation pathways in this GIT section lead to the production of short chain fatty acids (SCFA), which are further absorbed and assimilated by the host [52]. Sergeant et al. [5] identified butyrate-producing genes for enzymes like 3-hydroxybutyril-CoA dehydrogenase, phosphate butyryltransferase and butyrate kinase. Moreover, acetate-CoA transferase responsible for acetate synthesis and gene clusters that encode for the beta, gamma and delta subunits of methylmalonil-CoA decarboxylase, which is involved in the formation of propionate, were found to be present [5]. Twelve putative uptake hydrogenases produced by *Megamonas, Helicobacter* and *Campylobacter* were also identified in the caeca. The authors speculated that the respective hydrogenases have the potential to serve as hydrogen sinks that facilitate succinate production [5]. High proportions of the metagenomic sequences encoded for glycosyl hydrolase domains of glucanases, which act on oligosaccharides and are produced by bacteria belonging to Negativicutes and Lentisphaera, and further of endoglucanases that degrade polymers like cellulose and xylan, synthesized by Actinobacteria, Clostridia and Bacteroidia [5]. Furthermore, genes involved in cell wall metabolism and virulence were found to be present [45]. Regarding supplementation with antibiotics, it was reported that diets containing monensin and antibiotic growth promoters have no influence on the broadest functional classification of the microbes present in the caeca when compared to control diets. However, a combination of monensin with virginiamycin and tylosin increased the presence of conjugative secretion systems, specifically for plasmid types commonly found in *E. coli*. However, antibiotic resistance genes were also present in control and treatment groups [11]. As experiments are usually carried out in standardized and controlled animal facilities, conclusions about antibiotic resistance should be carefully stated. A comparison of metagenomes from feces of chickens, pigs and humans showed a high homology to tetracycline genes (*tet*A) and the presence of gene combinations of individual resistance elements, which encode for resistance to beta-lactams, aminoglycosides, macrolides and multidrug [51]. These findings demonstrated that there is a potential risk in the dissemination of the antibiotic resistance between farming animals and humans, therefore these supplementations should be considered cautiously.

Metagenomic analyses of fecal samples found Proteobacteria to be the most abundant phylum (47–79%) followed by Firmicutes (12–28%) and Bacteroidetes (7–27%) [50,53]. Animals with a high feed conversion ratio (FCR) exhibited a higher abundance of the genera of *Acinetobacter, Bacteroides, Streptococcus, Clostridium* and *Lactobacillus* whereas in low FCR animals *Escherichia, Shigella* and *Salmonella* were more abundant [53]. Regarding lean lines, the same study revealed an enrichment of microbial functions in four classes of the category transport and metabolism of the clusters of orthologous groups: amino acid, nucleotide, coenzyme and lipids [54]. Another study supported that

lean lines exhibit an increase in lipid storage, including the peroxisome activated receptor (PPAR) and the citrate cycle, which unifies the carbohydrate, lipid and protein metabolism [15]. The same functions were detected in human studies that related the microbiome to the development and progression of obesity, besides the citrate synthase activity [15,55,56]. The limited amount of studies and samples that have been analyzed so far reveals that metagenomic approaches are still not affordable for a great percentage of groups studying the chickens' GIT. However, additional research is necessary, as microbial communities have an impact on the chickens' metabolism, immune homeostasis and colonization resistance.

2.3.3 Metaproteomics

Advances in DNA and RNA sequencing caused a boost in the discipline of metaproteomics. The increased availability of sequenced genomes and metagenomes promotes the identification and characterization of an increased number of proteins that are expressed by specific microorganisms in a given sample. Metaproteomic studies of the chicken's gut are scarcely available in the literature. Up to now, only two studies applied this technique to characterize the adaptation of the chickens' gastrointestinal microbiota to a specific challenge [57,58] (Table 1).

Another study by Polansky et al. investigated the chickens' caecal microbiome following inoculation with caecal extracts from chickens of different ages, in order to elucidate the colonization patterns and predict the most promising probiotic genera for caecal colonization of newly hatched chickens [59].

Tang et al. studied two fecal samples of 18-week-old white leghorn chickens [57] identifying 3673 proteins of 799 different genera. The most abundant bacterial genus was *Lactobacillus* (11% of total proteins) followed by *Clostridium* (4% of total proteins) and *Streptococcus* (2% of total proteins). The findings could not be correlated with the 16S rRNA gene sequencing analysis that exhibited higher abundances of Clostridiales (25% of total sequences), Bacteroidaceae (21% of total sequences) and Lactobacillaceae (19% of total sequences). GroEL, a stress-related protein, was the most abundant protein followed by glyceraldehyde-3-phosphate dehydrogenase which is a key enzyme in glycolysis and gluconeogenesis [57].

The second study by Tilocca et al. investigated the influence of supplementing inorganic phosphorous (P) and/or microbial phytases on the formation of inositol phosphates and the intestinal microbiome [58]. Crop and caeca contents of 48 animals were sampled and pooled per pen and dietary treatment resulting in 24 analyzed samples. A total of 381 bacterial proteins were identified in the crop with most identified proteins being assigned to the Lactobacillaceae family, disregarding the dietary treatments. In diets supplemented

with P, the number of proteins belonging to the Veillonellaceae family increased [58]. In the caeca, a total of 1719 proteins were identified. Proteins synthesized by species of the Eubacteriaceae family appeared in lower abundance in diets supplemented with P while proteins of the Bacteroidaceae family increased in abundance. The number of proteins of the Ruminococcaceae family was higher in diets with microbial phytase supplementation. A lack of P and microbial phytase supplementation caused a stressed microbial community with exclusive occurrence of COG categories at low relative abundances, while P and microbial phytase supplementation showed a prosperous microbiota assemblage. The authors identified a low number of host proteins in the crop (248) and in the caeca (405), emphasizing that an accurate sample preparation is essential to enrich proteins of prokaryotic microorganisms to improve the numbers of total proteins detected by mass spectrometry-based metaproteomics [58]. Figure 4 shows a comparison of the bacterial families detected in caecal samples from identical basal diets by targeted ampliconsequencing [13] and metaproteomics [58]. There was a great discrepancy in the relative abundance of identified families. Ruminococcaceae, Lachnospiraceae, Erysipelotrichaceae, Peptococcaceae, Anaeroplasmataceae and Carnobacteriaceae were detected in higher abundance by targeted amplicon sequencing, while Lactobacillaceae, Clostridiaceae, Eubacteriaceae, Streptococcaceae and Succinovibrionaceae were found to be more abundant in the metaproteomic study. Methodological biases such as varying numbers of 16S rRNA gene copies and a higher sensitivity of the targeted amplicon sequencing approach in regard to low abundant species as well as a lack of genomic sequences in databases required for proteomic approaches [57,58] could be an explanation for these results.

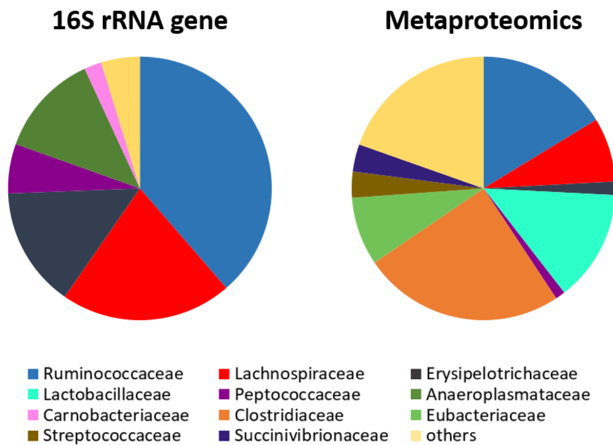

Figure 4. Families with more than 1% of abundance obtained from caeca content with 16S rRNA gene [13], and metaproteomic [58] analyses.

The advantage of metaproteomics and also metatranscriptomics is to gain more precise insights into the actual functions carried out by microorganisms of a microbiome, especially when compared to the rather vague predictions based on 16S rRNA genes or metagenomics. In addition, the co-extraction of host RNA or proteins may as well be beneficial to gain concomitant information about the host status, although a high quantity of these host biomolecules can clearly impair the analysis of the microbiome. Thus, a balanced methodological workflow has to be established for proper application of the respective meta-omic approaches.

2.4 Chicken feeding and its influence on the microbiota

The nutrition of chickens is based on plant diets that are supplemented with a variety of amino acids, minerals, vitamins, enzymes, pre-, pro- and anti-biotics to improve growth performance. The respective supplements may replace nutrients or improve the accessibility of nutrients that are not easily assimilated by the animals due to the varying digestibility of substrates. The use of a high percentage of animal protein is avoided in chicken diets because it increases the abundance of *Clostridium perfringens* in the GIT which is a predisposing factor for necrotic enteritis in chickens [60]. The ban of antibiotics as growth promoters by the European Union and its potential restriction in other countries [61] intensified the search for alternatives to improve growth performance and to avoid a raise in animal diseases such as necrotic enteritis, gut dysbiosis, diarrhea, loss of appetite and dysregulation of the immune system [62].

Poultry diets have a tremendous impact on the gut microbiome in regard to diversity and composition. Varying dietary compositions influence growth performance in the intensive growing period. Cereal types comprise different concentrations of soluble non-starch polysaccharides (NSP) such as arabinoxylans which occur in higher concentrations in wheat as when compared to maize [63]. Diets with high levels of NSP, such as barley-, rye- and wheat-based diets, improve lumen viscosity, increase the retention time of feed and reduce nutrient digestibility [64]. Short retention time selects for rather fast-growing bacteria which adhere to the epithelium [65]. Such conditions favor the colonization of *Clostridium perfringens* and prompt the occurrence of necrotic enteritis disease [65]. The inclusion of feed additives in the diet helps the modulation of gut microbiome by stimulating the growth of specific microorganisms that improve gut health. Particularly, the enzymes xylanaseand β-glucanase are known to foment the growth of lactic acid bacteria. Those bacteria have the ability to adhere to the gut epithelium and compete with pathogens for its colonization while decreasing lumen viscosity [65, 66].

High amounts of phytic acid in plant-based diets and derived feedstuffs and the limited presence of endogenous phytase in the GIT mucosa of chickens leads to the

supplementation with microbial phytases that are highly beneficial since catalyzing the hydrolysis of phosphate groups from the inositol ring [67]. In substrates like rapeseed cake, phytase supplementation improves the apparent total protein digestibility [68]. During the last years several studies have been designed to address the influence of phytase supplementation on the availability and interaction with P and Ca in regard to the microbial communities and to meet the animal requirements. Diets supplemented with microbial phytases increase the release of P and Ca from phytate and hence reduce the supplementation of inorganic phosphate and Ca required in poultry diets [35]. In the crop, phytase promotes the abundance of Aeromonadaceae and Flavobacteriaceae while reducing the dominance of *Lactobacillus* [69]. Furthermore, DAPI counts of bacteria revealed that the presence of phytase in the diet, with adequate or deficient levels of Ca and P, enhances the total number of bacteria [35]. Phytase supplementation increases the abundance of *Lactobacillus* sp., *Clostridium leptum* and *Enterococcus* sp. in the ileum [35]. Monocalcium phosphate, an inorganic compound generally added to diets, increases the presence of members of the Clostridiales order and the Bacteroidaceae family [69].

Organic acids, such as acetic acid, propionic acid and butyric acid [70], were used to selectively stimulate the permanence of beneficial bacterial species and various studies reported fluctuations regarding gain of weight, feed intake and feed conversion ratio [71-74]. Sodium butyrate is a common dietary supplement and is transformed to butyric acid by the chicken's metabolism. It affects the development of the gut epithelium and promotes the presence of symbiotic bacteria. A decreasing pH in crop and gizzard favors the establishment of lactic acid producing bacteria including *Lactobacillus* spp. and *Bifidobacterium* spp. [75,76], while reducing the colonization by harmful bacteria like *Salmonella enterica* and *Campylobacter jejuni* [77].

Prebiotics are non-digestible oligosaccharides that show a positive effect on the host by stimulating the growth of certain bacteria. They serve as a source of nutrients for commensal microbes and can mislead pathogenic bacteria to attach to the oligosaccharide and to be excreted before attaching to the mucosa and causing infections [78]. Xylo-oligosaccharides are products of the hydrolytic degradation of arabinoxylans and have been used in broiler diets as prebiotics. Their main functions are associated with the increment of villus length in the ileum and the promotion of beneficial microbial groups in the GIT. In the colon, xylo-oligosaccharides increase the presence of *Lactobacillus* and in the caeca the *Clostridium* cluster XIVa which is known to possess genes related to butyrate production such as the butyryl coenzyme A and acetate CoA transferase [79]. Another source of oligosaccharides includes the ones derived from palm kernel expeller. It is assumed that improves the immune responses due to the increase of IgA and IgM along with the promotion of *Bifidobacterium* and a reduction of *Salmonella* [80]. Alternatively, lactulose, a synthetic disaccharide prebiotic, can stimulate the growth of *Lactobacillus* and

Bifidobacterium and reduce pro-carcinogenic activity based on enzymes such as azoreductase or 7-alpha-dehydroxylase [81]. Prebiotics produced from yeast cells and cell walls are used due to the positive effect on gut health and microbiota modulation. Beta-D-glucan and mannan-oligosaccharides, components of this supplement, bind to the receptor mannose-specific type-1 fimbriae and prevent pathogen colonization while favoring the genus *Faecalibacterium* which is commonly associated with gut health [82].

Probiotics are living microorganisms that improve gut health and animal performance if added to the diets in adequate amounts. These microorganisms compete with pathogenic bacteria for adhesion sites at the intestinal epithelium [83]. Moreover, mechanisms of action from probiotics consist of the enhancement of activity of digestive enzymes like proteases, lipases and amylases [84], the improvement of mucosa ultrastructure, thus also increasing nutrient absorption [85]. The use of the probiotic *Lactobacillus plantarum* P-8 in broiler diets enhances the immune response, weight gain, feed efficiency and feed intake. Moreover, metabolic activity and nutrient utilization are improved and furthermore, the fecal microbial composition is modulated [62]. *Enterococcus faecium* supplementation (0.5% of the total diet) reduces the microbial counts of *Salmonella* and increases body weight gain and breast muscle yield [85]. *Bacillus* sp. can be delivered in pelleted feeds due to their stability and heat resistance which improves the production of enzymes like proteases, amylases and lipases positively influencing growth performance. In addition, *Bacillus* sp., also impact the small intestinal micro structure with an increase of villous height and *Lactobacillus* and *Bifidobacterium* counts in the caeca. Its supplementation decreases the presence of harmful bacteria such as *E. coli* and *Salmonella* sp. [86].

Synbiotics combine the effects of pre- and probiotics. Such mixtures improve the implantation and survival of the supplemented bacteria in the GIT [87]. Synbiotics showed a great efficacy in the reduction of *C. jejuni*, which causes zoonosis frequently and provokes a strong inflammatory response [88]. The combination of *Bifidobacterium longum* PCB133 with a xylo-oligosaccharide (XOS) successfully reduced the load of *Campylobacter* spp. and *C. jejuni* [89]. It has been demonstrated that the delivery of synbiotics by in ovo technology [90] can modulate gene expression levels in immune related tissues and gut structures. The inoculation of galacto-oligosaccharides and *L. salivarius* or raffinose and *L. plantarum* increased the absorbent surface of duodenum and jejunum [91,92].

Metabolites synthesized from probiotics are referred to as “postbiotics” and represent an alternative since exerting the positive effect of probiotics without applying living cells [93]. As an example, *Lactobacillus* sp., are able to produce organic acids and bacteriocins that promote the presence of lactic acid bacteria. Consequently, there is a decrease of pH and counts of enterobacteria, an intensification of mRNA IGF1 expression which is an

indicator for body composition, growth, fat deposition and metabolic activities, and mRNA GHR gene which plays a role as mediator of body size [93].

Innovative dietary supplements, announced as an environmentally friendly solution, appear in the market with a lower cost. Earthworm meal can positively affect the growth performance of chickens and increases the concentrations of Ca and P in the blood [94]. Another dietary intervention includes the addition of dry whey powder, a co-product of cheese industry, acting as a prebiotic for gut microflora due to its high content of lactose and protein quality, and exhibiting a positive influence on the bird performance from early to later growth stages [14]. Essential oils of oregano and laurel are being explored due to their antioxidant and antimicrobial characteristics and the enhanced digestibility based on the stimulation of endogenous enzymes, nitrogen absorption and inhibition of odor and ammonia [95]. These compounds were also shown to increase the body weight and FCR and exhibiting less mortality when compared to the control group. In ileum and caecum, they modulate the microbiota towards an increase of *Lactobacillus* and *Bifidobacteria* counts. Essential oils of oregano and laurel enhance villus height, antioxidant capacity of breast and thigh meat [95]. Moreover, a resin from the plant Boswellia serrata was approved as a safe additive in poultry production and exhibited therapeutic capabilities including anti-inflammatory and antibacterial effects which stabilize the intestinal functions. A better digestive efficiency was achieved considering dry and organic matter and an increase of the genus of *Lactobacillus* and *Enterococcus* [96] was observed.

2.5 Future Perspectives

The current state of knowledge about the chickens' intestinal microbiota is mainly based on the general inventory of the bacterial populations. Variations of the community structures were mainly investigated with respect to different feeding strategies and the influence of pathogenic species, but the question arises if the results obtained by numerous studies are comparable to each other. Although experiments are commonly standardized and based on identical breeds such as Ross 308 broilers, there is a lot of deviation concerning the subsequent processing like DNA extraction and selection of the variable region for amplification. Different laboratory protocols lead to incomparable results. Thus, a standardized protocol as it is available in human microbiota research should be established in chicken microbiome research to obtain comparable datasets. Another issue regarding the experimental design is the pooling of samples from different animals which concerns numerous studies. Borda-Molina et al. [13] reported a high individuality of the microbiota structure of single birds despite the fact that the animals originated from the same breeder and were housed under the same conditions. Consequently, pooling of samples can mimic changes in the microbiota composition which otherwise would not be visible. Regarding the sampling procedure itself, the study mentioned above also emphasized the importance of sampling mucosa and lumen digesta separately to obtain a more complete representation of the microbiota. A combination with a predictive functionality may depict the microbial processes that are running at the host interface and identify microorganisms which are most relevant to the host animal. This may represent a starting point to further study the interaction between microbiota and host.

So far studies of the chicken microbiota are mainly performed using 16S rRNA gene amplicon sequencing and metaproteomics. The use of metatranscriptomics and metabolomics, and the combination of all are still at the very beginning but have the potential to move from predictive analyses to more accurate descriptions of the actual microbial activities. Another important issue is the limited culture collection of strains inhabiting the GIT of chickens. An increase in bacterial cultures and their genetic and biochemical characterization would strongly support the Omics data evaluation. To reach this, cultivation strategies should be created which consider the demand of co-culturing or host-derived substrates as it was done for the mouse and humans [97] (Figure 5).

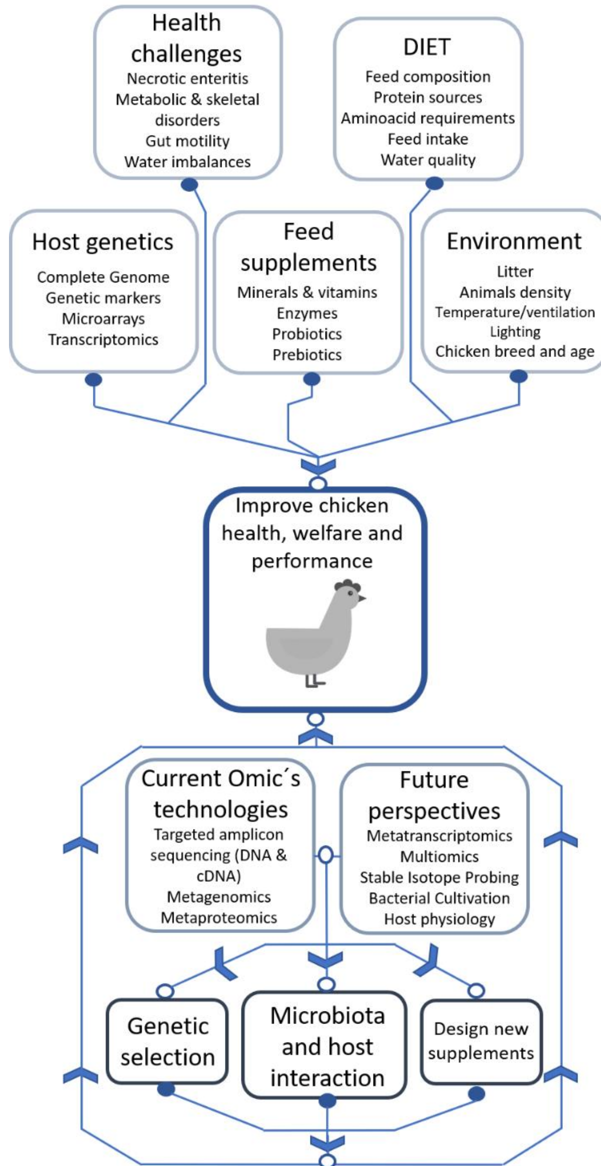

Figure 5. Overview of the factors affecting chicken health, welfare and performance and future perspectives in the analysis of the chicken microbiome.

So far, the main focus of microbiota research in chickens has been on understanding how the microbiota is changing under defined feeding strategies and how this influences the performance of the broilers and laying hens. Another focus is the control of pathogens under production conditions. For both interests and many others, gnotobiotic chickens could be of great importance. They are available and already used to study the expression of host enzymes [98]. Although the handling of gnotobiotic chickens is also challenging, including facts like faster growth, higher caloric intake, abnormal gut motility, thinner intestinal wall or high urea/uric acid ratio in feces and metabolism and recycling of bile acids [99-102], they should be used for infection and feed digestion studies with defined microbial cultures structures to gain more insights into the function of the microbiome and the interaction with the host in the future.

2.6 Acknowledgements

The authors would like to acknowledge the financial support of the Ellrichshausen'sche Stiftung, Carl Zeiss Stiftung and DFG Research Unit FOR2601. We would like to thank Simon Deusch for carefully reading the manuscript. ACS was supported by the European Social Fund and by the Ministry of Science, Research and Arts of Baden Württemberg.

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CHAPTER III

SECOND MANUSCRIPT

INSIGHTS INTO BROILERS' GUT MICROBIOTA FED WITH PHOSPHORUS, CALCIUM, AND PHYTASE SUPPLEMENTED DIETS

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Published in:
Frontiers in Microbiology | Microbial Symbioses (2016)
Volume 7, 2016, article2033
doi: 10.3389/fmicb.2016.02033
Accepted: December 02, 2016

Open access.
The original publication is available at https://doi.org/10.3389/fmicb.2016.02033

3. Insights into broilers' gut microbiota fed with phosphorus, calcium, and phytase supplemented diets

3.1 Abstract

Phytase supplementation in broiler diets is a common practice to improve phosphorus (P) availability and to reduce P loss by excretion. An enhanced P availability, and its concomitant supplementation with calcium (Ca), can affect the structure of the microbial community in the digestive tract of broiler chickens. Here, we aim to distinguish the effects of mineral P, Ca, and phytase on the composition of microbial communities present in the content and the mucosa layer of the gastrointestinal tract (GIT) of broiler chickens. Significant differences were observed between digesta and mucosa samples for the GIT sections studied ($p = 0.001$). The analyses of 56 individual birds showed a high microbial composition variability within the replicates of the same diet. The average similarity within replicates of digesta and mucosa samples across all diets ranged from 29 to 82% in crop, 19–49% in ileum, and 17–39% in caeca. Broilers fed with a diet only supplemented with Ca had the lowest body weight gain and feed conversion values while diets supplemented with P showed the best performance results. An effect of each diet on crop mucosa samples was observed, however, similar results were not obtained from digesta samples. Microbial communities colonizing the ileum mucosa samples were affected by P supplementation. Caeca-derived samples showed the highest microbial diversity when compared to the other GIT sections and the most prominent phylotypes were related to genus *Faecalibacterium* and *Pseudoflavonifractor*, known for their influence on gut health and as butyrate producers. Lower microbial diversity in crop digesta was linked to lower growth performance of birds fed with a diet only supplemented with Ca. Each diet affected microbial communities within individual sections, however, no diet showed a comprehensive effect across all GIT sections, which can primarily be attributed to the great variability among replicates. The substantial community differences between digesta and mucosa derived samples indicate that both habitats have to be considered when the influence of diet on the gut microbiota, broiler growth performance, and animal health is investigated.

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3.2 Introduction

Broiler chickens are one of the most used farm animals due to the efficient conversion of feed into body weight gain (Stanley et al., 2014). Phosphorus (P) supply with the diet plays an important role in skeletal system development and maintenance of chickens. P is, however, a non-renewable resource that is expected to be depleted in the next 100 years (Shastak and Rodehutscord, 2013). Phytate, an organic source of P contained in plant seeds and plant-based diets for broilers, is a principal source of P for the animal, but it has the disadvantage of not being easily accessible by broilers (Witzig et al., 2015; Zeller et al., 2015). The P availability of plant-based diets can be improved by supplementing the diets with phytase, an enzyme that increases P digestibility and reduces P excretion (Witzig et al., 2015). In consequence, the amount of calcium (Ca) and P required in diet formulation can be reduced following release of these two elements from phytate complexes (Zeller et al., 2015). Changes in Ca and P supplementation affected the composition and activity of the microbial community in the digestive tract of broilers (Ptak et al., 2015). Because the microbes are involved to a variable extent in enzymatic hydrolysis of nutrient fractions in the digestive tract, it is necessary to understand the role of the microbial community of the gut (Eeckhaut et al., 2011) and its interaction with the host, in order improve the utilization of nutrients such as phytate bound P by the bird.

The microbial community present in the broilers' gastrointestinal tract (GIT) has more than 900 bacterial species (Stanley et al., 2014). They play a crucial role in feed digestion, breakdown of toxins, exclusion of pathogens, stimulation of the immune system, and endocrine activity (Zhu et al., 2002). Several studies have analyzed the microbiota from specific sections of the GIT including the crop, ileum, and caeca (Sekelja et al., 2012; Sergeant et al., 2014; Ptak et al., 2015; Witzig et al., 2015), whereas only a few have focused on the whole GIT (Lu et al., 2003; Sekelja et al., 2012). Nonetheless, it is now known that they are highly connected and should influence up and down-stream the different GIT sections (Stanley et al., 2014). Most studies have focused on content of the GIT (digesta) samples only (Sekelja et al., 2012; Walugembe et al., 2015; Witzig et al., 2015), ignoring the mucosa communities, that are the closest to the host epithelium (Collado and Sanz, 2007). Epithelium attached microbial communities have biological roles that should be characterized. A high bacterial diversity was observed in the *Pars non glandularis* of the pig stomach (Mann et al., 2014) and previous reports in rats and humans have found differences between the microbial counts in the colonic mucosa and feces (Zoetendal et al., 2002; Haange et al., 2012).

The crop, the section where feed is temporally stored and fermentation activities initiate, is highly dominated by *Lactobacillus* species (Stanley et al., 2014; Witzig et al., 2015). The ileum, where nutrients are absorbed, is mainly colonized by *Lactobacillus* species and

also by partially characterized bacteria with butyrate producing activities, such as *Clostridium*, *Streptococcus*, and *Enterococcus* (Stanley et al., 2014). The caeca, where complex substrates such as cellulose, other polysaccharides, and phytate are fermented (Stanley et al., 2014; Choi et al., 2015; Zeller et al., 2015) is the most diverse section of the GIT and is highly dominated by unknown microbes. The most abundant families in caeca are Clostridiaceae, Bacteroidaceae, Lactobacillaceae, and butyrate producers (Stanley et al., 2014).

Considering the low availability of P in plant-based diets, and the effect of supplementing diets with phytase, Ca, and P on chickens' performance and phytate degradation in the digestive tract, this study aims to investigate the influence of these supplements, on the microbial communities of digesta and mucosa samples of three sections of the GIT of broiler chickens.

3.3 Materials and methods

3.3.1 Animal Sampling

The animal experiment was carried out in the Agricultural Experiment Station of Hohenheim University, location Lindenhöfe in Eningen (Germany). All procedures regarding animal handling and treatments were approved by the Regierungspräsidium Tübingen (approval number HOH33|14TE).

A total of 1064 broiler chickens (unsexed, strain Ross 308) were allocated to 56 floor pens. Animals were fed with a commercial starter diet (Table S1) until day 14 of age. On day 15 each pen was randomly assigned to one of eight different dietary treatments (seven pens per diet; Table 2). The diets were mixed based on corn and soybean meal (Table S1) with the supplementation of two levels of P (monosodium phosphate; 0 or 2 g P/kg), Ca (limestone; 0 or 3 g Ca/kg), and an *E. coli*-derived 6-phytase Quantum™ Blue, AB Vista (0 or 1500 FTU/kg; Table 2). The experiment followed a 2 × 2 × 2 factorial arrangement of treatments. On day 26 one animal per pen was euthanized by carbon dioxide asphyxiation following anesthesia in a gas mixture (35% CO_2, 35% N_2, and 30% O_2; Zeller et al., 2015). The GIT was dissected immediately after euthanization and crop, ileum (terminal two-thirds of the section between Meckel's diverticulum and 2 cm anterior to the ileo-ceco-colonic junction) and the two caeca, were opened longitudinally and digesta samples were collected with a sterile spoon. The mucosa was washed with sterile phosphate-buffered saline and scraped with a sterile glass slide. In some cases, the amount of digesta contained in a certain section was not sufficient, resulting in a total of 281 samples collected, which included 3–7 replicates per dietary treatment and sample type (mucosa and digesta; Table S2A). Samples were stored at −80°C.

Table 2. Phosphorus (P), calcium (Ca), and phytase concentration in the eight dietary treatments**.**

Diets	**A**	**B**	**C**	**D**	**E**	**F**	**G**	**H**
	P- Ca- Ph-	**P- Ca- Ph+**	**P- Ca+ Ph-**	**P- Ca+ Ph+**	**P+ Ca- Ph-**	**P+ Ca- Ph+**	**P+ Ca+ Ph-**	**P+ Ca+ Ph+**
Total-P (g/kg)	4.1	4.1	4.1	4.1	6.9	6.9	6.9	6.9
Ca (g/kg)	6.2	6.2	10.4	10.4	6.2	6.2	10.4	10.4
Phytase (FTU/kg)[a]	0	1500	0	1500	0	1500	0	1500

[a]*The calculated activity in the diet based on enzyme supplements; intrinsic enzyme activity is not included. -, without supplementation; +, with supplementation.*

3.3.2 Broiler performance analysis

Information regarding final body weight (BW), feed consumption (FC), BW gain and feed to gain ratio, was obtained from day 15 to 26 and analyzed with MIXED procedure of the software SAS (version 9.1.3, SAS Institute, Cary, NC). The statistical model was $y_{jjklm} = \mu + r_i + T_j + \beta_k + x_l + (T\beta)_{jk} + (Tx)_{jl} + (\beta x)_{kl} + (T\beta x)_{jkl} + e_{ijklm}$; where μ = general mean, r_i = effect of the block (random), T_j = effect of the P addition (fixed), β_k = effect of the Ca addition (fixed), x_l = effect of the phytase addition (fixed), $(T\beta)_{jk}$, $(Tx)_{il}$, $(\beta x)_{kl}$ are the two factor interactions, $(T\beta x)_{jkl}$ are the three factor interaction and e_{ijklm} = random error of the observations. Statistical significance was evaluated by one-way ANOVA. Differences between treatments were tested with a multiple *t*-test (LSD). A significance level of $p \leq 0.05$ was considered.

3.3.3 DNA extraction and illumina amplicon sequencing

DNA was extracted from 281 samples with FastDNA™ SPIN Kit for soil from MP Biomedicals (Solon, OH, USA), following the instructions of the manufacturer's protocol. DNA was quantified in a NanoDrop 2000 spectrophotometer (Thermo Scientific, Waltham, MA, USA) and stored at −20°C.

Illumina library preparation with PCR amplification of the V1-2 region of the 16S rRNA gene using PrimeSTAR HS DNA Polymerase (Clontech Laboratories, Mountain View, CA, USA) was performed according to Camarinha-Silva et al. (2014). Amplicons were verified by agarose gel electrophoresis, purified with Macherey-Nagel 96-well-plate (Macherey Nagel, Düren, Germany) and quantified using a QuantiFluor® dsDNA system (Promega, Madison, USA). Equimolar ratios of amplicons (30 ng) were pooled followed

by an ethanol precipitation in order to remove any contaminants. Correct size of the PCR product was obtained and purified with QIAquick gel extraction kit (Qiagen, Hilden, Germany). Libraries were sequenced using 250 bp paired-end sequencing chemistry on an Illumina MiSeq platform.

Bioinformatic processing of sequences was done according to Camarinha-Silva et al. (2014) with some modifications. Raw reads were assembled (Cole et al., 2014) and subsequently aligned using MOTHUR (gotoh algorithm with the SILVA reference database) prior to pre-clustering (diffs = 2). Sequences were clustered into operational taxonomic units (OTU) at ≥97% similarity. All OTUs with an average abundance lower than 0.001% across all the samples and with sequence length <250 bp were discarded from the analysis. Finally, 293,862 ± 1459 sequences were obtained per sample comprising a total of 1796 OTUs that were taxonomically assigned using the naïve Bayesian RDP classifier (Wang et al., 2007; Table S3). OTUs were then manually evaluated against the RDP database using Seqmatch function. Sequences are available at the European Nucleotide Archive (ENA) under accession number PRJEB14628 in http://www.ebi.ac.uk/ena/data/view/PRJEB14628.

3.3.4 Multivariate analysis

A multivariate dataset with the respective abundances of each OTU on each sample was analyzed using PRIMER (version 7.0.9, PRIMER-E, Plymouth Marine Laboratory, Plymouth, UK; Clarke and Warwick, 2001). Data was standardized and a sample similarity matrix was created using Bray-Curtis coefficient (Bray and Curtis, 1957). The community similarity structure was depicted through non-metric multidimensional scaling plots (nMDS) and shade plots were used to study species distributions between the diets and each section (Clarke and Warwick, 2001). Similarity percentages analysis (SIMPER) identified the species contribution to the Bray-Curtis similarity among samples within each diet (Clarke and Warwick, 2001). PERMANOVA routine was used to study the significant differences and interactions between factors [diet, type of sample (digesta or mucosa) and GIT section], and differences between the diets were studied based on the pair-wise tests using a permutation method under a reduced model. Pielou's evenness index and Shannon-weaver index of diversity (H′) were used to calculate OTUs evenness and diversity.

Differences in the abundance of OTUs of interest between diets were evaluated using the unpaired Welch's *t*-test that can handle unequal variances, unequal sample sizes and non-parametric data (Welch, 1947). OTUs abundances were considered significantly different if $p < 0.05$. Correlations were estimated with Pearson correlation coefficient (999 permutations) using PRISM 6 (GraphPad Software, CA). Correlations were considered significantly different if $p < 0.05$.

3.4 Results and discussion

3.4.1 Global overview of broiler performance and the microbial community in crop, ileum, and caeca

The growth performance of broiler chickens was significantly affected by the levels of P, Ca, phytase, and their corresponding interactions (Table 3). Final BW, FC, and BW gain increased in diets that included P supplementation (E, F, G, and H) and in diet B with only phytase supplementation (Tables 2, 3). The growth performance of birds on these diets was significantly different from the others. The lowest performance birds were those on diet C, with only supplementation of Ca.

Table 3. Broiler chickens performance data between day 15 and 26 for the eight dietary treatments**.**

Diets	A	B	C	D	E	F	G	H
	P- Ca- Ph-	P- Ca- Ph+	P- Ca+ Ph-	P- Ca+ Ph+	P+ Ca- Ph-	P+ Ca- Ph+	P+ Ca+ Ph-	P+ Ca+ Ph+
Final BW (g)	1433^{bc}	1527^{a}	1202^{d}	1420^{c}	1510^{a}	1539^{a}	1492^{ab}	1530^{a}
FC (g/d)	117^{b}	121^{ab}	96^{d}	112^{c}	124^{a}	123^{a}	119^{ab}	122^{a}
BW gain (g/d)	78^{b}	86^{a}	58^{c}	76^{b}	86^{a}	86^{a}	83^{a}	86^{a}
F:G (g/g)	1.49^{b}	1.41^{d}	1.66^{a}	1.47^{bc}	1.44^{cd}	1.42^{d}	1.44^{cd}	1.41^{d}
	p-value							
	Pooled *SD*	**P**	**Ca**	**Phy**	**P*Ca**	**P*phy**	**Ca*phy**	**P*Ca*phy**
Final BW (g)	21.02	<0.0001	<0.0001	<0.0001	<0.0001	0.0003	0.0383	0.0756
FC (g/d)	1.26	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	0.0006	0.0526
BW gain (g/d)	1.31	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	0.0007	0.0406
F:G (g/g)	0.012	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	0.0178	0.0407

Final body weight (BW), feed consumption (FC), BW gain and feed to gain (F:G) ratio of broiler chickens. Data are given as treatment means with respective SD (standard deviation); n = 7 blocks, 16-18 animals per block, and means without common superscript resulted being significantly different ($p < 0.05$).

Based on the taxonomic threshold defined by Yarza et al. (2014), which takes into consideration a hierarchical classification applied on both cultured and uncultured microorganisms, 16S rRNA gene sequences were taxonomically assigned with sequence identity of 82% to orders, 86.5% to family, 94.5% to genera (Yarza et al., 2014), and 97% identity was used for species identification (Konstantinidis and Tiedje, 2005). A total of 1796 OTUs were classified into class (78.5%), order (76.8%), family (63.4%), genera (22.8%), and species (4%). A total of 3.8% of the sequences could only be assigned to the phylum Firmicutes. This result confirmed previous findings, which stated that gastrointestinal microbiota of the chicken remains largely unexplored and <200 species are isolated from chicken gastrointestinal tract (Stanley et al., 2014; Waite and Taylor, 2015). Next generation sequencing techniques have exposed the hidden diversity of microorganisms, but its taxonomic classification is difficult because of the time consuming effort to isolate and biochemically characterize individual bacteria (Yarza et al., 2014).

High variability in the microbial composition was observed between individuals (3–7 birds) within each diet and section (Table S2B). The average similarity of individuals in the studied sections ranged in the crop digesta from 29 to 82% and crop mucosa from 29 to 73%. In the ileum digesta the observed similarity of individuals was between 19 and 49% and in the ileum mucosa 25–47%. The caeca showed the lowest similarity of individuals, namely 17–38% in digesta and 30–39% in mucosa samples. The crop is dominated by *Lactobacillus* (Hagen et al., 2005; Stanley et al., 2014; Witzig et al., 2015), explaining the higher values of similarity and its simple structured microbiota when compared to other sections of the GIT. In ileum and caeca sections, the more diverse microbial communities are responsible for phytate degrading activities (Palacios et al., 2008), degrading complex organic substrates, and to the production of short chain fatty acids (SCFA; Stanley et al., 2013b; Mann et al., 2014; Choi et al., 2015). The average similarity decreased in these sections, perhaps related to the presence of a higher number of OTUs. Taking as an example diet H (with all supplements) and diet A (without any supplement), a variation in the relative abundance of predominant families was observed between the replicates in each section (Figures S1A,B). The variability between individuals has been previously reported in two studies that characterized chicken caeca (Stanley et al., 2013b; Sergeant et al., 2014) and in cattle feces (Durso et al., 2010). Furthermore, human studies found inter-individual differences in mucosa associated microbiota from colon and rectum samples (Hong et al., 2011). These studies showed that, independently of the core microbiota colonization, there is a great variation in the relative abundance of the bacterial community between individuals. A possible explanation is that shifts in microbial composition are influenced by the initially colonizing microbiota, diet, and immune system of the host (Donaldson et al., 2015).

Exploring the bacterial community structure of the 281 samples, regardless of the diet, a great distinction between crop, ileum, and caeca was found to exist ($p = 0.001$; Figure 1A and Figure S2A). This confirms similar results from previous studies (Stanley et al., 2014; Witzig et al., 2015). For the first time, and in all three sections analyzed, a separation was observed between digesta and mucosa samples ($p = 0.001$; Figure 6B). Additionally, PERMANOVA results using the total number of OTUs indicated that two-way interactions, diet × section and section × type of sample, were significantly different ($p < 0.05$), showing that the type of community depends on the diet and section studied and on the interactive effect of section and type of sample.

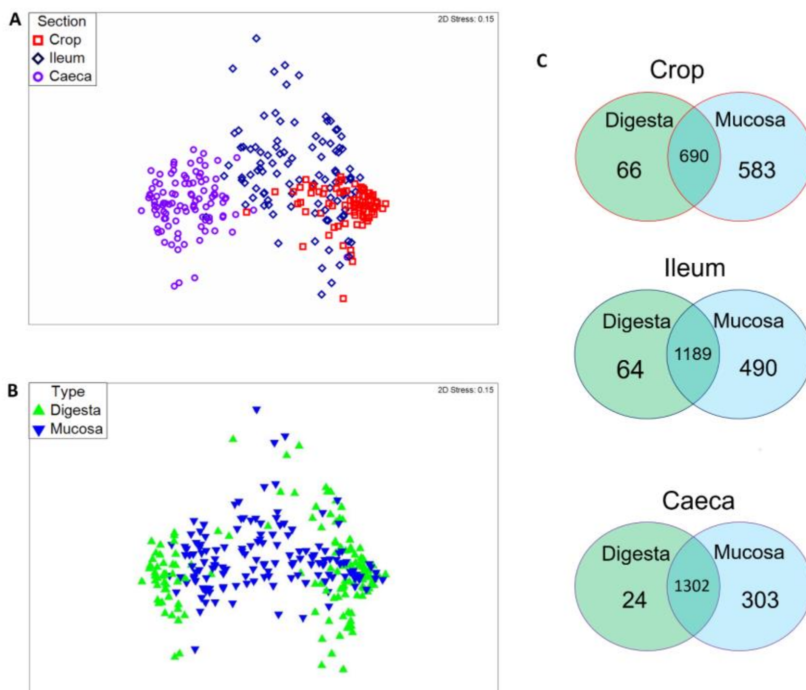

Figure 6. Global bacterial community structure of 281 samples. Sequencing data was standardised prior to the use of Bray-Curtis similarity algorithm. Non-metric multi-dimensional scaling (nMDS) plot illustrates: (A) crop, ileum and caeca samples, and (B) digesta and mucosa samples. The symbols represent a unique sample comprising all OTUs and its abundance information. (C) Venn diagram of the OTUs common/unique to each type of sample in the crop, ileum and caeca. Overlapping areas show the OTUs shared between digesta and mucosa samples.

Crop samples comprised 690 OTUs shared between digesta and mucosa, a further 66 OTUs were specific to digesta and 583 OTUs to mucosa samples (Figure 6C). The diversity indices showed on average the lowest Pielou's evenness and Shannon diversity for both digesta (0.33 and 1.47, respectively) and mucosa (0.35 and 1.88, respectively), which is in accordance with previous studies (Hagen et al., 2005; Witzig et al., 2015). A similar diversity was observed in ileum digesta; however, an increase in diversity was detected in the ileum mucosa (Pielou's evenness = 0.49 and Shannon diversity = 2.9). Specific OTUs belonging to ileum digesta and mucosa samples were 64 and 490, respectively, while 1189 OTUs were observed in both (Figure 6C). The higher microbial diversity could be attributed to more suitable physicochemical conditions that allow a better establishment of complex microbiota and influence their nutrient availability (Stanley et al., 2014). Caecal digesta and mucosa samples resulted in the highest OTUs evenness (0.68 and 0.73, respectively) and diversity (4.15 and 4.6, respectively), when compared with all other sections. In the caeca digesta and mucosa 1302 OTUs were detected. A total of 24 OTUs were only detected in the digesta and 303 in the mucosa of caeca (Figure 6C). Overall, mucosa samples shared more OTUs between the three sections than digesta samples (Figure S2B). Several studies have shown that this higher diversity in the caeca is due to the low passage rate, pH, and the presence of small and soluble particles, which enhance the role of the microorganisms in assimilation of nutrients from food, in producing vitamins, and amino acids (Zhu et al., 2002; Sergeant et al., 2014), and protecting the host against pathogens (Stanley et al., 2014). Mucosa samples showed higher species diversity than digesta in all GIT sections. Most of the studies characterizing chicken microbiota have focused on digesta of the different GIT sections (Deusch et al., 2015; Waite and Taylor, 2015). The mucosa or mucous layer, which is mainly composed by mucins and glycan, help the colonization of some groups of microorganisms in the gut (Donaldson et al., 2015).

The majority of the microorganisms colonizing the three GIT sections belonged to the phylum Firmicutes, as commonly described in previous studies that characterized the microbial communities of the chicken GIT (Stanley et al., 2013a; Deusch et al., 2015). In the crop, the most abundant family was Lactobacillaceae, which was previously reported as a dominant group in that environment (Sekelja et al., 2012; Witzig et al., 2015). Crop mucosa was additionally colonized with Lachnospiraceae, Burkholderiaceae, Ruminococcaceae, and Streptococcaceae (Figure 7). In the ileum, the dominance of Lactobacillaceae family decreased in comparison to the crop, showing 66% of abundance in digesta and 25% in the mucosa samples. The percentage of this family in the luminal content is in accordance to other broiler studies (Stanley et al., 2014; Witzig et al., 2015). However, special attention should be given to the lower abundance of Lactobacillaceae in the mucosa, which has not been reported before (Figure 2). The caeca showed higher

family diversity in both digesta and mucosa samples, with similar distribution of families Ruminococcaceae, Lachnospiraceae, Anaeroplasmataceae, Erysipelotrichaceae, Peptococcaceae, and Lactobacillaceae (Figure 7).

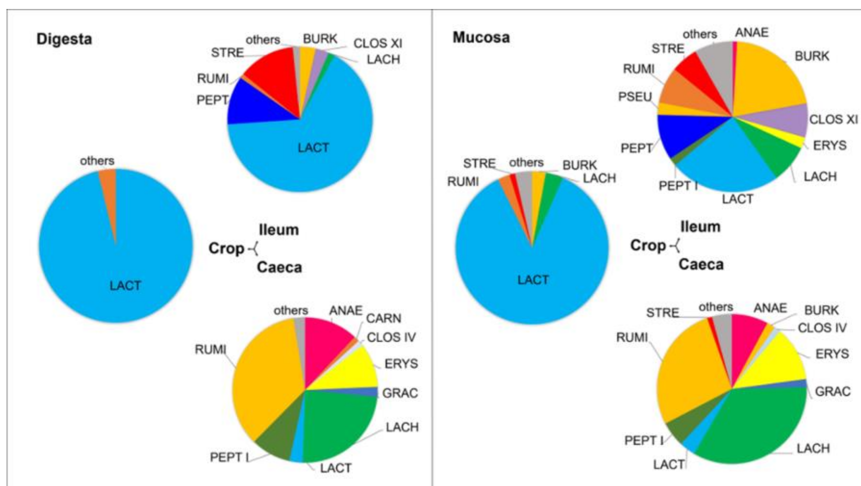

Figure 7. Family distribution of digesta and mucosa samples in the crop, ileum, and caeca**.** OTUs present in 281 samples were taxonomically assigned to a family and families present in abundances higher than 1% plotted. Abbreviations in the graph represent each family: ANAE, Anaeroplasmataceae; BURK, Burkholderiaceae; CARN, Carnobacteriaceae; CLOS IV, Clostridiales incertae sedis IV; CLOS XI, Clostridiales incertae sedis XI; ERYS, Erysipelotrichaceae; GRAC, Gracilibacteriaceae; LACH, Lachnospiraceae; LACT, Lactobacillus; PEPT I, Peptococcaceae I; PEPT, Peptostreptococcacaea; PSEU, Pseudomonadaceae; RUMI, Ruminococcaceae; STRE, Streptococcaceae, (Table S6).

3.4.2 Diet effect in the crop microbial community

The composition of the microbial community of crop mucosa was significantly affected by the diets ($p = 0.003$). Such effect was not found in digesta samples, highlighting the fact that both, digesta and mucosa samples, should be studied in regard to diet effects on gut homeostasis (Figure S3A). Pair-wise comparisons showed that microbial communities of crop digesta of birds fed with diet C were significantly distinct to those derived from other diets ($p < 0.05$), with the exception of diet D (Table S4). Lower values of Shannon diversity were observed in diet C (Figure S4). This reveals a diet effect in presence of only Ca supplementation, which could be related to the lower growth and feed consumption of

birds obtained with diet C (Table 3). High dietary calcium chelates part of the lipid fraction, which may reduce the energy value of the diet (Driver et al., 2005). Additionally, Ca forms insoluble complexes with phytate (Angel et al., 2002) and in the lumen interacts with inorganic phosphorus resulting in Ca-ortophosphate (Plumstead et al., 2008). Those complexes have a negative impact on the birds' performance due to the reduced solubility and availability of the P (Hamdi et al., 2015). High Ca diets have been associated with an increase of crop pH in chickens (Shafey et al., 1991) and in an higher attachment of *L. salivarius* to the GIT mucus of chickens when different *Lactobacillus* strains were studied *in vitro* (Craven and Williams, 1998), however in our study *L. taiwanensis* was the most abundant species in mucosa samples (Figures 8C,D and Table S5).

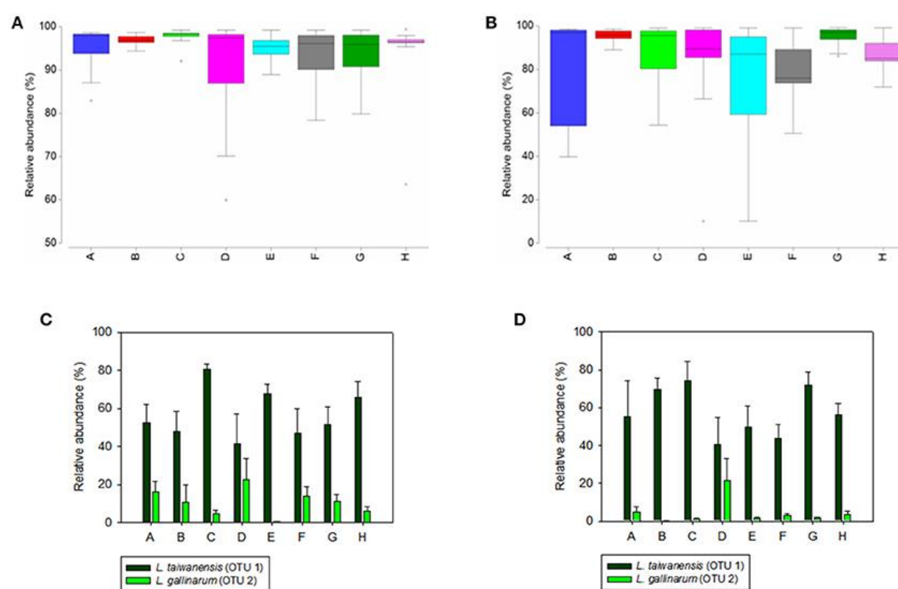

Figure 8. Box-plots showing the relative abundance of the genus *Lactobacillus* in crop digesta (A) and mucosa (B) across eight dietary treatments (Table 2). The box extends from the lower quartile (25%) to the higher quartile (75%). The line in the box is the median and the whiskers are the minimum and maximum values. The column charts include the relative abundances (Mean, SEM) of the two main species of *Lactobacillus, L. taiwanensis* (OTU 1), and *L. gallinarum* (OTU 2) detected in digesta (C) and mucosa (D) samples.

The abundance of *Lactobacillus* had the greatest fluctuation across all replicates when compared to other genera (Figures 8A,B), indicating a high variability between individuals

at genus level. *Lactobacillus* was the most predominant genus in crop digesta and mucosa (Figures 8A,B and Figure S3A). Bacteria belonging to this genus efficiently colonize the squamous lining of the crop and decrease the pH due to the production of organic acids (Abbas Hilmi et al., 2007). Its presence in the gut has several advantages such as inhibition of pathogens by colonization (Abbas Hilmi et al., 2007), production of salt base hydrolase (BSH), and reduction of cholesterol concentration (Ramasamy et al., 2009). *L. taiwanensis* was the most dominant OTU in digesta and mucosa samples (OTU 1; Table S5). Birds fed with diet C showed a higher tendency to be colonized more abundantly by this OTU (74%). This result suggests that the presence of Ca favors this species. This microorganism was previously observed in the GIT of chickens fed with diets supplemented with monocalcium phosphate (Witzig et al., 2015). OTU 1 was negatively correlated with other species of *Lactobacillus* ($p < 0.003$), and a negative correlation between *L. taiwanensis* and *L. crispatus* has been previously reported in the jejunum (Witzig et al., 2015). The second most abundant OTU in crop digesta and mucosa was *L. gallinarum* (OTU 2), a homofermentative lactic acid bacterium (Hagen et al., 2005). Its abundance in crop mucosa was lower in diet B supplemented with phytase when compared to diet E, F and G ($p < 0.05$). OTU 2 was found to be negatively correlated with *L. taiwanensis* ($p < 0.001$). The *Lactobacillus acidophilus* complex, also studied in the crop (Hagen et al., 2005), consists of *L. amylovorus* (OTU 9), *L. crispatus* (OTU 11), *L. mucosae* (OTU 38), and *L. vaginalis* (OTU 25). Those OTUs revealed a propensity to be detected in lower abundance in all diets.

3.4.3 Diet effect on the microbial community in the ileum

The ileum showed a higher diversity in the microbial communities when compared to the crop. Digesta samples belonging to diets C and H, that were both supplemented with Ca, were significantly different from samples derived from Ca-free diets E and F ($p < 0.05$; Table S4). It is known that higher doses of Ca in the diets can lead to an increase of the pH (Ptak et al., 2015) and low precaecal P digestibility (Adeola and Walk, 2013; Hamdi et al., 2015), which could possibly influence the presence or absence of some OTUs. An effect of P supplementation was observed in the microbial communities of the ileum mucosa. Statistical differences were obtained between diet A and F, G and H; B and F and G; diet C and F, G and H ($p < 0.05$; Table S4).

Lactobacillus, a genus widely present in crop, decreased in abundance in the ileum for most diets analyzed. The exception was for diets C and G, where it was detected at high abundances (>83%) in digesta samples. With regards to the mucosa, this genus was observed in higher abundance in diets F and G (32–37%; Figures 9A,B and Figure S3B) when compared to the other diets. Previous studies using mice and pigs have shown that diets supplemented with P and Ca, like diet G, increases *Lactobacillus* abundance (Ten

Bruggencate et al., 2004; Metzler-Zebeli et al., 2010). *L. taiwanensis* (OTU 1), highly abundant in the crop, decreased its abundance in ileum digesta samples of diets supplemented with Ca (C, G, and H; 27%), while in the mucosa the highest percentage was observed on diet G (17%). The second most abundant OTU was *L. gallinarum* (OTU 2), which showed a tendency to be more abundant in diets A, C, and F (27%) for digesta and 16% in mucosa samples of diet F.

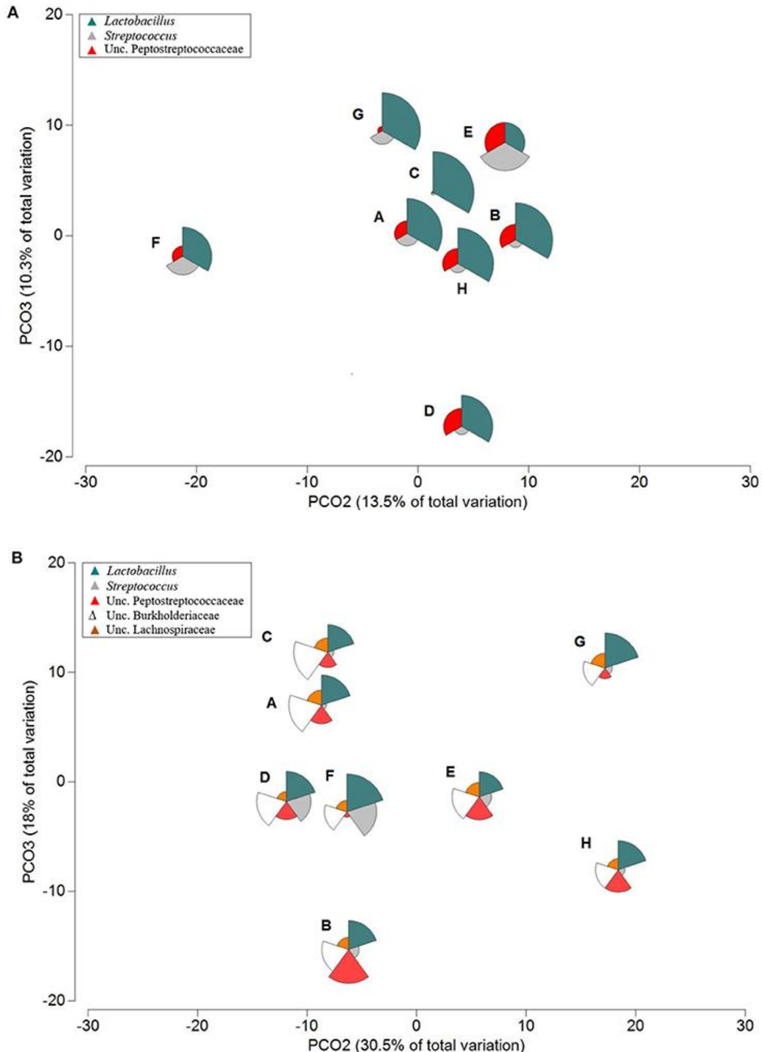

Figure 9. Principal coordinate analysis (PCoA) ordination of the global bacterial community structure of ileum (A) digesta and (B) mucosa samples across eight dietary treatments (A–H) (Table 2). Bubbles were superimposed to visualize the relative abundance of the most relevant genera, *Lactobacillus* and *Streptococcus* and families, Peptostreptococcaceae, Burkholderiaceae, and Lachnospiraceae (slice scale 1–100% abundance).

Diets E and F in digesta, and F in mucosa, both with P supplementation resulted in an increase of *Streptococcus* (44, 19, and 23%, respectively; Figures 9A,B). Lu et al. (2003) demonstrated that sequences of OTUs related to *Streptococcus* were more prevalent in the ileum digesta than in the caeca (Lu et al., 2003). In accordance with the study of Ptak et al. (2015), *Streptococcus* abundance was reduced in diets supplemented with Ca, P, and phytase (Ptak et al., 2015), represented in this study by diet H. *Streptococcus* abundance was even lower in diet C, with Ca supplementation only. OTUs assigned to uncultured Clostridium XI tended to be detected in digesta in higher abundances on diets D (18%) and E (23%) when compared to other diets, which accounted for <14%. Likewise, in the mucosa, colonization with this group mainly occurred with diet B (26%), E (14%), H (13%), and D (12%), while other diets showed abundances lower than 8%. In regard to ileum mucosa, OTUs belonging to Burkholderiaceae accounted for more than 12% of the total abundance in all dietary treatments, being detected in higher abundance in diet A and C (30%). This bacterial group showed moderate heritability in chickens, but it has not been attributed any function (Meng et al., 2014). OTUs assigned to Lachnospiraceae were commonly present in all treatments, with relative abundance ranging from 2.4 to 5.9% (Figure 9B). This family was reported to be associated with corn-based diets and is mainly composed by anaerobes and some *Clostridium* members (Munyaka et al., 2015).

Streptococcus alactolyticus (OTU 4) showed a tendency to be present in higher abundance in digesta samples of diets E and F with P addition (38 and 20%, respectively) and in mucosa samples of diets F and D, with phytase supplementation (22 and 13%, respectively). This lactic acid bacteria has been found in ileum samples of broilers fed with a commercial corn-soy diet (Lu et al., 2003). An uncultured Clostridium XI (OTU 7) was found with similar abundance in both digesta and mucosa samples, with the highest values observed when fed diet B (33 and 26%, respectively). Furthermore, diet B showed only 30% similarity to other diets with OTU 7 responsible for the dissimilarity. The closest relative sequence to OTU 7 was an uncultured Clostridium XI previously isolated from ileum and caeca of a conventional Ross 208 chickens grown under conditions of organic farming (Bjerrum et al., 2006). Uncultured *Ralstonia* (OTU 6), observed in the crop mucosa (<5%), showed a more prominent increase of abundance in mucosa samples for diets A and C (28 and 30%, respectively). Its abundance decreased in diets supplemented with P. A trend was detected in the increase of abundance of an OTU belonging to

Clostridiaceae 1 (OTU 21) in diet F digesta (15%) and diet H mucosa (30%); which have P and phytase supplementation in common.

3.4.4 Diet effect on the microbial community in the caeca

Caeca digesta and mucosa samples showed a more diverse community at genus level than observed in the other sections (Figure S3C). This fact was previously reported in chickens under standard commercial conditions (Stanley et al., 2013a; Sergeant et al., 2014; Mohd Shaufi et al., 2015) and in chickens exposed to different supplementation of monocalcium phosphate and phytase (Witzig et al., 2015). The highest OTU abundance detected in both type of samples was 14% (OTU8). Pair-wise comparison showed an effect of P in digesta samples of diet B contrasted to E, F, G, and H, but also between diet C and E (Table S4). This effect was also observed in the mucosa samples of diet B compared to F, G and H; diet D with E, F, and G; diet C with E and F, and lastly diet A and G. A high proportion of microorganisms belonging to order Clostridiales were detected in the caeca. This group is known to be an indicator of healthy chickens, due to its main role in the SCFA metabolism (Choi et al., 2015). SCFA have influence on host physiology through regulatory, immunomodulatory, and nutritional functions. They increase the growth of epithelial cells, stimulate mineral absorption and inhibit the growth and adherence of pathogenic microorganisms by decreasing the pH (Walugembe et al., 2015).

OTUs belonging to Lachnospiraceae are known to degrade complex polysaccharides to SCFA (Biddle et al., 2013). They were more abundant in digesta samples of diets supplemented with P (12–22%), while in the mucosa showed a similar distribution within all diets (17–28%; Figure 10 and Figure S3C). Ruminococcaceae is a common family reported in the chicken caeca (Bjerrum et al., 2006; Mohd Shaufi et al., 2015) and it was detected in both digesta (4–8%) and mucosa (3–13%) samples. Both families have been associated with the maintenance of gut health and have the enzymatic capability to degrade cellulose and hemicellulose (Biddle et al., 2013). Erysipelotrichaceae showed an abundance of 2% in the digesta samples of diets supplemented with P, however in the mucosa a higher abundance was detected (3–8%). In the caeca, protein sequences related to butyryl-CoA production enzymes have been previously detected on this family (Eeckhaut et al., 2011; De Maesschalck et al., 2014). One group of OTUs, closely related to the family Anaeroplasmataceae, were observed in all diets (Figure 10). This family has been reported in the chicken gastrointestinal microbiome (Oakley et al., 2014), but the exact role in chicken GIT remains unknown. A species belonging to Anaeroplasmataceae was previously described in rumen samples and related to bacteriolytic and non-bacteriolytic activities (Robinson et al., 1975). This can explain the negative correlation of OTU 8 (uncultured *Anaeroplasma*) with other OTUs in digesta and mucosa samples such

as OTU 394 (uncultured Lachnospiraceae), OTU 116 (uncultured Clostridium XIVa), OTU 390 (uncultured Ruminococcaceae), and OTU 93 (uncultured *Faecalibacterium*; $p < 0.05$).

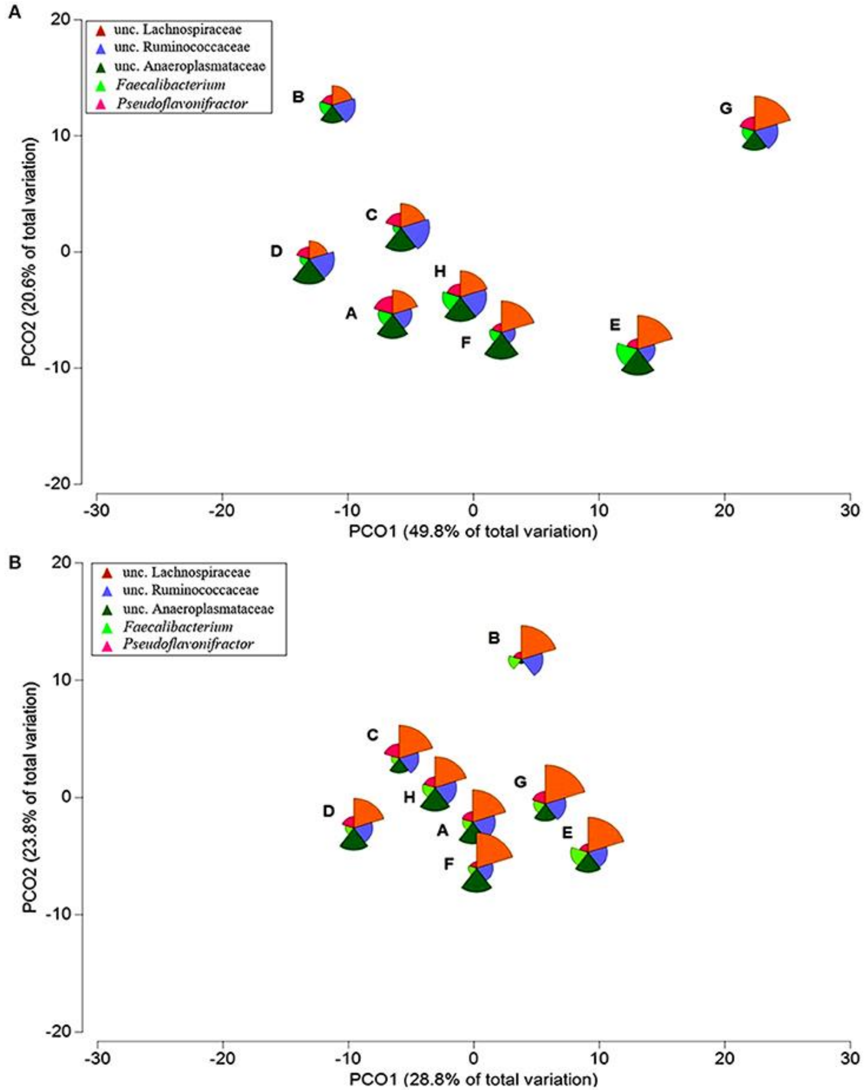

Figure 10. Principal coordinate analysis (PCoA) ordination of the global bacterial community structure of caeca (A) digesta and (B) mucosa samples across eight dietary treatments (A–H) (Table 2). Bubbles were superimposed to visualize the relative abundance of the most relevant genera, *Faecalibacterium* and *Pseudoflavonifractor* and families, Lachnospiraceae, Ruminococcaceae, and Anaeroplasmataceae (slice scale 1–30% abundance).

The OTUs in digesta samples related to *Lactobacillus* were more abundant when fed diet G (14.8%), with P and Ca additions (Figure S3C), with *L. gallinarum* (OTU 2; 12%) and *L. taiwanensis* (OTU 1; 2%) as the main colonizers. However, in the other diets, these OTUs were present in abundances lower than 2%. This is in accordance with a recent metagenomic study on the chicken caeca that showed *Lactobacillus* in low abundances (<4%; Mohd Shaufi et al., 2015). Diet E, with P supplementation, showed a group of OTUs closely related to *Faecalibacterium* in both type of samples. This genus is one of the most prominent butyrate producers, providing energy to the colonic mucosa and known to regulate gene expression, inflammation, differentiation, and apoptosis in host cells (Luo et al., 2013). *Pseudoflavonifractor*, detected in digesta and mucosa, is a common caeca colonizer that has a protein from class IV alcohol dehydrogenase that influences the final butyrate production pathway (Polansky et al., 2015). *Erysipelotrichaceae incertae sedis* previously reported in chicken caeca (Stanley et al., 2012) was detected more consistently throughout the diets in digesta samples and the same applied to *Streptococcus* in the mucosa.

Supplementation of Ca in diet C enhanced the presence of OTU 45 (5%) in caeca digesta. This OTU is related to an uncultured *Subdoligranulum* sp. that was previously found in the caeca of turkeys (Scupham, 2007) and is capable of producing butyric acid. OTU 37, an uncultured Ruminococcaceae, was detected in lower abundance (3%) in diets without P supplementation (A to D) or P with phytase supplementation (F) and has been previously detected in the intestinal microbiota of preadolescent turkeys (Scupham, 2007). In the caeca mucosa samples, an OTU with high similarity to an uncultured Bacillales (OTU 23) was found. This OTU was present in higher abundance on diet B (6.7%), with phytase supplementation, when compared to diets A, E, and F (4.5, 3.1, and 1.8%, respectively; $p < 0.05$). Particularly, this OTU was negatively correlated with OTU 31, related to an uncultured Lachnospiraceae, and OTU 91 related to an uncultured Ruminococcaceae ($p < 0.05$). Furthermore, OTU 4 identified as *Streptococcus alactolyticus* and highly abundant in some ileum samples, decreased its abundance in the caeca being 1.5% the highest value observed. This result contradicts a previous study on broilers fed diets including peas and organic acids where *S. alactolyticus* was a dominant species (Czerwiñski et al., 2010).

In mucosa samples, the abundance of OTUs belonging to the Clostridium XIVa and XIVb was higher than in digesta. The first family comprises some microorganisms that are butyrate producers while the second includes propionate producers and therefore may be linked to beneficial effects in the GIT (De Maesschalck et al., 2015). An uncultured Clostridium XIVb (OTU 56) previously found in caeca of preadolescent turkeys (Scupham, 2007), was present in birds fed diets B, C, D, E, and F (2.5–3%). OTU 81, similar to uncultured Clostridium XIVb, was positively correlated with OTU 56 ($p < 0.05$) and was previously reported to be present in the human ileum (Li et al., 2012). OTU 87, an uncultured Clostridium XIVa found in human feces (Turnbaugh et al., 2009), was more abundant on diet A and F, without calcium supplementation when compared to diet D, supplemented with Ca ($p < 0.05$).

It is known that non-ruminant animals are not efficient in utilizing phytate-P. In this study we have found, in the ileum and caeca, OTUs related to the genus *Clostridium*, which have been previously isolated and associated to the production of cysteine phytase (Gruninger et al., 2009). *Megasphaera elsdenii* (OTU 111) and *Mitsuokella* spp. (OTU 1501), common members of the rumen microbiota that have the ability to produce phytases (Yanke et al., 1998), were also detected in the ileum and caeca samples from birds on diets supplemented with Ca, P, or P with phytase.

3.5 Conclusions

Diet supplementation with P, Ca, or phytase has an effect on the microbial community that colonizes the GIT. However, a consistent effect of diet on the microbiota harbored in the different sections of the GIT was not observed. This was likely due to the high variability between individuals. Lower microbial diversity was associated with lower growth performance in animals fed with a diet only supplemented with Ca. Diets supplemented with P influenced the caeca microbiota and positively affected the growth of the broilers. For a better understanding of dietary effects on broiler performance, gut function and balance, and the microbial community, digesta and mucosa samples should be studied in separate as both showed different microbial communities.

3.6 Authors Contributions

Conceived and designed the experiment: AC, VS, MR. Performed the experiments: DB. Bioinformatics analysis: MV. OTUs annotation: DB. Data analysis: DB, AC. Performance data analysis: VS. Wrote the paper: DB, AC. Article revision and final approval: MV, VS, MR, AC.

3.7 Acknowledgments

The author would like to thank Maren Witzig for contributing to the design of the experiment, Bruno Tilocca for animal experiment set up and Silke Kahl for technical assistance.

3.8 Supplementary material

The Supplementary Material for this article can be found online at:

https://www.frontiersin.org/article/10.3389/fmicb.2016.02033/full#supplementary-material

Table S1. Dietary composition of the commercial starter diet fed until day 14 and basal diet for the corresponding treatments with P, Ca and phytase supplementation fed from days 15 to 26**.**

Table S2. Description of the three GIT sections in regard to (A) number of replicates per diet and type of sample and (B) average similarity of the replicates.

Table S3. OTUs abundances across the eight dietary treatments and the GIT sections**.**

Table S4. Statistical differences between the sections and the type of samples based on PERMANOVA results**.** Pairwise comparison results of the diets that showed a significant difference.

Table S5. Taxonomic assignment of the most relevant OTUs present in the chicken gastrointestinal tract**.** The assignment was performed in the Seqmatch function of the RDP database for type and non-type strain.

Table S6. Percentages of the families present in crop, ileum and caeca for digesta and mucosa**.**

Figure S1. Shade plot showing the relative abundance of each family present on each replicate of crop, ileum and caeca mucosa samples of (A) diet H (Ca, P, and phytase supplementation) and (B) diet A (no supplementation). The intensity of the color increases to black if the family was detected in higher abundance, while white indicates family absence.

Figure S2. (A) Non-metric multi-dimensional scaling (nMDS) plot to illustrates the three GITsections crop, ileum and caeca samples, splitted by the type of sample digesta and mucosa. The symbols represent a unique sample comprising all OTUs and its abundance information. (B) Venn diagrams of the OTUs common/unique to the type of samples digesta and mucosa in the three GIT sections: crop, ileum and caeca. Overlapping areas show the OTUs commonly shared.

Figure S3. Bar plots showing the relative abundance of the genus detected in digesta and mucosa samples in the eight dietary treatments (A) crop, (B) ileum, and (C) caeca.

Figure S4. Diversity observed across the three GIT sections studied: crop, ileum, and caeca and the two types of samples: digesta and mucosa, for the eight dietary treatments**.** Values are calculated based on the Shannon diversity index.

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CHAPTER IV

THIRD MANUSCRIPT

EFFECTS OF PROTEASE AND PHYTASE SUPPLEMENTS ON SMALL INTESTINAL MICROBIOTA AND AMINO ACID DIGESTIBILITY IN BROILER CHICKENS

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Published in:
Poultry Science | Metabolism and Nutirition (2019)
Volume 98, 2019, Pages 1-13
http://dx.doi.org/10.3382/ps/pez038
Accepted: January 23, 2019

Open access.
The original publication is available at http://dx.doi.org/10.3382/ps/pez038

4. Effects of protease and phytase supplements on small intestinal microbiota and amino acid digestibility in broiler chickens

4.1 Abstract

The objective of this study was to determine the effects of protease origin and dosage on the prececal (**pc**) amino acid (**AA**) digestibility and the influence on composition of the microbial community in the small intestine. In addition, the effects of phytase supplementation were investigated. A total of 8 dietary treatments were included. The basal diet contained mainly corn and soybean meal. Three protease products were added to the basal diet, each at the level recommended by the supplier and at an 8-fold level. Phytase was supplemented in another dietary treatment. Each dietary treatment was allocated to 8 replicates of 15 birds each. The experimental diets were offered from day 15 to 21 for ad libitum consumption. The effect of protease supplementation on the pc AA digestibility depended on the protease product type and the amount supplemented. The pc AA digestibility was significantly increased by 1 protease product when supplemented at high level and when phytase was supplemented. In all the other treatments, protease supplementation had no significant influence, or it decreased pc AA digestibility, when compared with the treatment with no enzymes added. In general, Firmicutes was the most abundant phylum among the ileal microbiota across all the treatments. Significant effects on microbiota composition were observed at the genus level for some but not all protease treatments and phytase supplementation. The genera *Streptococcus*, *Lactobacillus*, and uncultured Clostridiaceae were responsible for these differences. Furthermore, microbial networks established for each diet showed either high or low number of intergeneric interactions, but without a consistent enzyme effect. We conclude that enzyme supplementation effects were evident in the terminal small intestine microbiota composition, and to a lesser extent, in pc AA digestibility. However, the changes in microbiota composition and pc AA digestibility could not be correlated, indicating absence of a causal relationship.

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4.2 Introduction

Increasing the nutrient utilization efficiency in broiler chickens is an effective approach to minimize nutrient intake for growth and meat production, and reduce N excretion. The utilization of CP is subject of many studies because of its economic impact on the industry and detrimental effects of N excretion on the environment.

Protease supplements have been suggested to potentially achieve increased prececal (**pc**) amino acid (**AA**) digestibility in broiler chickens, and thereby reduce the dietary CP level. The effects of protease supplementation on pc CP and AA digestibility have been found to be inconsistent. Studies on broiler chickens and turkeys showed that the pc digestibility was increased for all AA (Vieira et al., 2013; Stefanello et al., 2016; Cowieson et al., 2017) or some AA (Bertechini et al., 2009; Angel et al., 2011; Vieira et al., 2013). Whereas, in some other studies, no effects (Boguhn et al., 2011; Kaczmarek et al., 2014; Rada et al., 2016; Erdaw et al., 2017) or decreasing effects (Walk et al., 2018) of protease supplementations on pc digestibility were described. Divergent results may be caused by differences in the composition of the experimental diet (Selle et al., 2016; Toghyani et al., 2017), supplementation level (Angel et al., 2011), or concurrent supplementation of other enzymes (Lee et al., 2018).

Characteristics of the supplemented protease likewise contributed to the divergent results as the efficacy of protease is influenced by the environment of the surrounding medium, including pH and temperature (Ghazi et al., 2002; Mahmood et al., 2017). Hence, differences in the efficacy of proteases have been reported. Ghazi et al. (2002) reported no effect of a protease isolated from a *Bacillus* species on pc CP digestibility, whereas it increased with the addition of a protease isolated from an *Aspergillus* species. In another study, supplementation of proteases derived from *Aspergillus niger* and *Bacillus subtilis* had no effect on the total tract CP digestibility (Mahmood et al., 2017). In a screening of several proteases derived from various bacteria and fungi, the effect on pc AA digestibility differed widely (Walk et al., 2018). These authors reported that protease supplementation in most cases had no effect on the pc AA digestibility. They found that pc digestibility of some AA was increased, whereas it was decreased for others, depending on the supplemented protease and the experiment. However, in their study all diets contained a phytase supplement (Walk et al., 2018).

Phytase is primarily used to increase degradation of phytate and utilization of phosphorus, and additionally has potential to increase pc AA digestibility. Similar to protease, the effects of phytase supplementation on pc AA digestibility are variable, with some studies reporting an increasing effect (Amerah et al., 2014; Sommerfeld et al., 2018) and others without any effect (Rodehutscord et al., 2004; Manangi et al., 2009). Should phytase have

a similar effect like protease and if such effects are not additive, then it is possible that the overall addition of phytase in the study by Walk et al. (2018) masked possible effects of protease supplementation. Furthermore, the study by Walk et al. (2018) did not include different dosages of protease, but doses necessary to achieve increased pc AA digestibility might vary between proteases.

The supplementation of enzymes can influence the microbiota composition in the intestine. Phytase supplementation increased the total number of microbial counts in the small intestine and increased relative abundance of bacteria such as *Lactobacillus* and *Enterococcus* (Ptak et al., 2015; Witzig et al., 2015). To the best of our knowledge, the effects of protease supplementation on microbial ecology have not been investigated using Next Generation Sequence (NGS) techniques. However, different scenarios of consequences of protease supplementation on the microbiota can be deduced from the literature. In a study that used qPCR methodology to target specific microbial groups, protease seemed to increase the presence of *Lactobacillus* spp. but decrease the presence of Clostridium perfringens in the ileum (Giannenas et al., 2017). In another study, protease supplemented in combination with α-amylase and glucoamylase increased the relative abundance of *Bifidobacterium*, *Staphylococcus*, *Bacteroides*, and *Megamonas* (Yin et al., 2018), which usually are considered to be beneficial bacteria. It also is possible that protease supplements alter the microbiota composition by modifying the substrates that the microorganisms access. For instance, higher availability of AA was shown to be either beneficial or harmful to the growth of certain microorganisms (Dahiya et al., 2007). Other metabolites like short chain fatty acids, amines, and AA derivatives were also shown to impact the microorganisms (Hemarajata and Versalovic, 2013). Therefore, effects of protease supplementation on pc digestibility might partly be explained by a shift in the microbial composition. To our knowledge, such a relationship has not been investigated to date.

Hence, our main objective was to investigate the effects of different proteases at 2 dosage levels on the pc AA digestibility and the composition of the microbiota in the terminal small intestine. We also aimed to examine whether effects on pc AA digestibility, if caused by proteases, could be found upon supplementation of phytase in a separate treatment. We hypothesized that enzymes affect the pc AA digestibility in a dose-dependent manner and that the microbiota composition in the terminal small intestine is altered due to enzyme supplementation.

4.3 Materials and methods

4.3.1 Experimental diets

The study comprised of 8 dietary treatments. The basal diet (**BD**) did not contain any enzyme supplement and was mainly based on corn and solvent-extracted soybean meal (Table 1). For other 6 treatments, the BD was supplemented with 3 different proteases at 3 levels each: *Aspergillus* Acid Protease (**Protease A**) (Meiji Seika Pharma Co., Ltd., Japan) produced from *A. niger* with a declared protease activity of not less than 950,000 U/g at pH 2.6, supplemented at 25 or 200 mg/kg of diet; CIBENZA DP100 (**Protease B**) (Novus International Inc., MO, USA) produced from *Bacillus licheniformis* with a declared minimum protease activity of 600,000 U/g supplemented at 500 or 4000 mg/kg of diet; RONOZYME PROACT (**Protease C**) (DSM Nutritional Products AG, Kaiseraugst, Switzerland) produced from a genetically engineered *B. licheniformis* strain with a declared minimum protease activity of 75,000 U/g, supplemented at 200 or 1600 mg/kg of diet. The lower dosage of the proteases was chosen based on supplier recommendations and the other dosage was set at 8 times the recommended dosage. For the eighth treatment, Natuphos E (**Phy**) (BASF SE, Germany) was supplemented to provide 1500 FTU/kg of diet. The calculated phytase level was verified by analysis (1410 FTU/kg). Titanium dioxide (TiO2) was included as an indigestible dietary marker (5 g/kg). All diets were adequate in phosphorus concentration. The CP concentration of the diets was uniform and ranged from 245 to 248 g/kg DM (Table 2). Diets were manufactured at Research Diet Services (Hoge Maat 10, 3961 NC Wijk bij Duurstede, Netherlands). Application of all enzymes in this experiment was approved by the Regierungspräsidium Tübingen, approval number 34/8302.31.

Table 4. Composition of the experimental diets and supplementation levels of the enzyme products (g/kg unless otherwise stated).

	Basal diet	Protease A		Protease B		Protease C		Phytase
Supplementation level[1]	No enzyme	25 mg/kg	200 mg/kg	500 mg/kg	4,000 mg/kg	200 mg/kg	1,600 mg/kg	1500 FTU/kg
Corn	560	560	560	560	560	560	560	560
Soybean meal[2]	371	371	371	371	371	371	371	371
Soybean oil	30	30	30	30	30	30	30	30
Calcium carbonate	19	19	19	19	19	19	19	19
Sodium chloride	4	4	4	4	4	4	4	4
Monocalcium phosphate	6	6	6	6	6	6	6	6
Choline chloride	2	2	2	2	2	2	2	2
Vitamin- and mineral mix[3]	3	3	3	3	3	3	3	3
TiO_2	5	5	5	5	5	5	5	5

[1] The respective amount of enzyme was added on top of the mixtures; the lower supplementation of each protease product complies with the recommendations of the suppliers, the other dosage is eightfold higher.

[2] Solvent-extracted soybean meal; trypsin inhibitor activity: 1.36 g/kg; urease activity: < 0.02 mg N/g (min, 30°C); nitrogen solubility index: 12%; Lys: 29.0 g/kg; reactive Lys: 24.4 g/kg (all on as-is basis).

[3] Provided per kg of mixed feed: vitamin A (retinyl acetate): 12,000 IE; vitamin D_3 (cholecalciferol): 2,500 IE; vitamin E (DL-α-tocopherol): 50 mg; vitamin K_3 (menadione): 1.5 mg; Vitamin B_1 (thiamine): 2.0 mg; vitamin B_2 (riboflavine): 7.5 mg; vitamin B_6 (pyridoxine): 3.5 mg; vitamin B_{12} (cyanocobalamin): 20 µg; niacine: 35 mg; pantothenic acid: 12 mg, choline chloride: 460 mg; folic acid: 1.0 mg, biotine: 0.2 mg, iron: 80 mg; copper: 12 mg; manganese: 85 mg; zinc: 60 mg; iodine: 0.8 mg; selenium: 0.15 mg, anti-oxidant: 125 mg.

Table 5. Analyzed chemical composition of the experimental diets (g/kg dry matter).

Supplementation level[1]	Basal diet	Protease A		Protease B		Protease C		Phytase
	No enzyme	25 mg/kg	200 mg/kg	500 mg/kg	4,000 mg/kg	200 mg/kg	1,600 mg/kg	1500 FTU/kg
CP	246	246	246	248	245	248	246	245
Ether extract	71	-[2]	-	-	-	-	-	70
Crude fiber	27	-	-	-	-	-	-	27
aNDFom	118	-	-	-	-	-	-	117
ADFom	44	-	-	-	-	-	-	45
Ala	12.2	12.4	12.3	12.1	12.1	12.2	12.3	12.4
Arg	16.7	16.8	16.7	16.6	16.3	16.7	16.6	16.8
Asp/Asn	26.4	26.8	26.5	26.1	26.2	26.5	26.5	26.9
Cys	3.7	3.8	3.7	3.7	3.7	3.7	3.9	3.8
Glu/Gln	46.0	46.5	46.1	45.5	45.5	45.9	46.0	46.6
Gly	10.3	10.5	10.4	10.3	10.2	10.4	10.4	10.5
His	7.2	7.3	7.3	7.1	7.3	7.3	7.4	7.5
Ile	10.6	10.5	10.3	10.4	9.8	10.4	10.3	10.2
Leu	21.4	21.6	21.4	21.2	21.0	21.2	21.3	21.5
Lys	13.4	13.5	13.3	13.3	13.1	13.4	13.3	13.6
Met	3.6	3.6	3.6	3.5	3.5	3.6	3.6	3.6
Phe	12.5	12.6	12.5	12.4	12.2	12.5	12.5	12.6
Pro	14.9	15.1	15.4	15.0	15.3	15.0	15.2	14.8
Ser	12.9	13.3	13.2	12.9	13.1	13.1	13.2	13.4
Thr	9.8	10.0	9.9	9.7	9.7	9.8	9.9	10.0
Tyr	8.5	8.6	8.6	8.5	8.5	8.7	8.5	8.6
Val	11.5	11.2	11.1	11.2	10.6	11.2	11.1	10.9
Calcium	10.9	-	-	-	-	-	-	11.0
Phosphorus	5.7	-	-	-	-	-	-	5.8

[1] The lower supplementation of each protease product complies with the recommendations of the suppliers, the other dosage is eightfold higher.
[2] - = Not analyzed.

4.3.2 Birds and experimental procedures

The experiment was conducted in the Agricultural Experiment Station of Hohenheim University, location Lindenhöfe, Eningen, Germany. All the animal procedures were in accordance with the German Animal Welfare Legislation and were approved by the Regierungspräsidium Tübingen (approval number HOH34-15TE).

A total of 960 unsexed broiler chicken hatchlings of the strain Ross 308 were allocated to 64 pens of 15 birds each on a wood shavings bedding. Lighting in the barn was permanent and the temperature was 34°C for the first 2 d of the experiment. Following this, the lighting schedule was adjusted to 18 h of light and 6 h of dark, and the temperature was decreased continuously to reach 19°C until day 21.

From day 1 to day 14 post-hatching, birds received a commercial starter diet that was calculated to be adequate in ME and all nutrients according to the recommendations of the Gesellschaft für Ernährungsphysiologie (1999) (Club Mastkükenstarter 4150020, Deutsche Tiernahrung Cremer GmbH & Co. KG, Germany; contained according to the manufacturer data sheet per kg 215 g CP, 10.5 g Ca, 5.5 g P, 12.5 MJ ME, 110 mg coccidiostat monensin sodium, 10 IU endo-1.4-β-xylanase (EC 3.2.1.8), and 750 FTU 6-phytase (EC 3.1.3.26)). On day 15, 8 pens were allocated to each of the dietary treatments in a randomized complete block design. The experimental diets were provided for 7 d in mash form for ad libitum consumption.

Bird weight and feed consumption were determined on day 14 and day 21 of the experiment. Dead birds were weighed and feed consumption up to the day of removal of the bird was recorded. Determination of ADFI for 1 pen with low level of Protease A supplementation and 2 pens with high level of Protease C supplementation did not deliver plausible results. The determined level of ADFI of these observations was untrustworthily low (18 g/d and 39 g/d) or high (92 g/d). These observations together with their related values of final BW, ADG, and G:F were excluded from the data evaluation to ensure the comparability of the results for all traits. On day 21, birds were euthanized by carbon dioxide asphyxiation following anesthesia in a gas mixture (Zeller et al., 2015b). The terminal two-thirds of a section of the small intestine between the Meckel's diverticulum and 2 cm anterior to the ileo-ceco-colonic junction were isolated. Approximately, 2 cm from this section (randomly taken) was dissected and longitudinally opened. The randomization was practiced in order to make sure that, on average of the pen, samples were from the same section as samples for AA analysis. Digesta from this 2-cm piece was aseptically collected with a sterile spoon, pooled on a pen-basis, and stored at −80°C for microbiota analysis. From the remaining part of the chosen intestine section, digesta was

flushed out using deionized water, pooled on a pen basis, and immediately frozen at -20°C until freeze-drying.

4.3.3 Chemical analyses

A vibrating disc mill (Fritsch Pulverisette 9, Fritsch GmbH, Germany) was used to grind the diet and digesta samples for AA and Ti analysis. Samples were ground using a centrifugal mill (Retsch ZM200, Retsch GmbH, Germany) and passed through a 0.5 mm sieve for all other analyses. All analyses were performed in duplicate.

The German official methods for nutrient analyses of the Verband Deutscher Landwirtschaftlicher Untersuchungs- und Forschungsanstalten (2007) were followed for the analyses of DM (no. 3.1), CP (no. 4.1.1), crude ash (no. 8.1), ether extract (no. 5.1.1), crude fiber (no. 6.1.1), neutral detergent fiber after pre-treatment with α-amylase without residual ash (aNDFom; no. 6.5.1), and acid detergent fiber without residual ash (ADFom; no. 6.5.2). Vadopest and Fibretherm analysis systems (C. Gerhardt GmbH & Co. KG, Germany) were used for Kjeldahl digestion and crude fiber, ADFom, and aNDFom analysis, respectively. Concentrations of Ti, phosphorus and calcium were analyzed using an ICP-OES following wet digestion as described by Zeller et al. (2015a). Amino acids were analyzed as described previously (Rodehutscord et al., 2004) with minor laboratory modifications (Zuber et al., 2016). Briefly, samples were oxidized in an ice bath with a mixture of hydrogen peroxide, phenolic formic acid solution, and phenol prior to hydrolysis in acidic conditions at 113°C for 24 h in a mixture containing hydrochloric acid and phenol. Norleucine was used as the external standard. Separation and detection of AA was done using the L-8900 AA analyzing system (VWR/Hitachi Ltd, Japan) after post-column derivatization using ninhydrin. The oxidation procedure might slightly affect the calculated concentration of His, Phe, and Tyr (Mason et al., 1980). Asn and Gln were determined together with Asp and Glu, respectively, due to the loss of amide residue from the side group of Asn and Gln during acid hydrolysis and convertion to Asp and Glu, respectively (Fontaine, 2003).

4.3.4 DNA Extraction, illumina amplicon sequencing and data analysis

DNA was extracted using the FastDNA SPIN Kit for soil (MP Biomedicals LLC, OH, USA), following the manufacturer's instructions. DNA was quantified with a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, MA, USA) and stored at −20°C. Illumina library was prepared according to Kaewtapee et al. (2017). In brief, the V1–2 region of the 16S rRNA gene was amplified in a 20 µl reaction. About 1 µl of the first PCR product was used as a template in the second PCR with multiplexing and indexing primers as described previously (Camarinha-Silva et al., 2014). Amplicons were verified by

agarose gel electrophoresis, purified, and normalized using the SequalPrep Normalization Kit (Invitrogen Inc., CA, USA). Samples were pooled and sequenced using the 250 bp paired-end sequencing chemistry on an Illumina MiSeq platform.

Raw reads were checked for quality, assembled, and aligned using Mothur pipeline tool (Kozich et al., 2013). A total of 28,151 ± 2,736 reads were obtained per sample. The UCHIME program included in Mothur pipeline was used to identify possible chimeras (Edgar et al., 2011). Reads were clustered at 97% identity into 1,021 operational taxonomic units (**OTU**). Only OTU with an average abundance higher than 0.0001% and a sequence length >250 bp were considered for further analysis. The closest representative was manually identified using seqmatch from the Ribosomal Database Project (RDP) (Wang et al., 2007). Sequences were submitted to the European Nucleotide Archive (accession number PRJEB26340).

4.3.5 Calculations and statistical analysis

The pc digestibility of CP and AA was calculated on a pen basis using the following equation:

$$\text{pc CP or AA digestibility (\%)} = 100 - [(TiO_{2Diet} \times CP/AA_{Digesta}) / (TiO_{2Digesta} \times CP/AA_{Diet})] \times 100 \quad [1]$$

where $CP_{Digesta}$ or $AA_{Digesta}$ and CP_{Diet} or AA_{Diet} are the concentrations of CP and AA in the digesta and diets, respectively, and TiO_{2Diet} and $TiO_{2Digesta}$ are the concentrations of TiO_2 in the diets and digesta, respectively. Statistical evaluation of growth performance, pc CP, and pc AA digestibility was done using the MIXED procedure of SAS for Windows (Version 9.3, SAS Institute, Cary, NC). Data were analyzed considering the fact that the observations recorded for some traits related to growth performance were unbalanced by one-way analysis of variance (**ANOVA**) using the following statistical model:

$$y_{ij} = T_i + b_j + e_{ij} \quad [2]$$

with y_{ij} as the dependent traits, T as the fixed effect of treatment *i*, b as the random effect of block *j*, and e_{ij} as the residual error. Treatment effects were considered significant if $P < 0.050$.

Illumina amplicon sequencing data were analyzed using PRIMER (PRIMER-E version 7.0.9, Plymouth Marine Laboratory, UK) as described by Clarke and Warwick (2001). Data were standardized, square-root transformed, and a sample similarity matrix was created using Bray–Curtis coefficient (Bray and Curtis, 1957). Alpha-diversity was calculated based on Shannon diversity index at 97% of identity (Paul et al., 2015). Beta-

diversity was studied based on community similarity structure and depicted through non-metric multi-dimensional scaling plots (nMDS) (Clarke and Warwick, 2001). Similarity percentage analysis (**SIMPER**) was used to identify the genera responsible for the differences observed between the treatments (Clarke and Warwick, 2001). PERMANOVA routine was used to study the significant differences observed when the dietary treatments were investigated using a permutation method under a reduced model. Pearson correlation was calculated using GraphPad Prism 6 (GraphPad Software Inc., CA, USA). Correlations were considered significantly different at $P < 0.050$.

Co-occurrence network analysis was done considering OTU with more than 0.1% abundance and clustered at the genus level as proposed (Manasson et al., 2018). Correlations were estimated based on the sparse correlation for compositional data approach (Friedman and Alm, 2012; Ramayo-Caldas et al., 2016), which determines the co-abundance and co-exclusion of bacteria present in the absolute abundance (Zhang et al., 2018). Two-sided pseudo P-values were obtained considering 10 iterations and 100 bootstraps. Non-significant correlations ($P > 0.050$) were ignored. Cytoscape software version 3.6.0 (Shannon et al., 2003) was used to build the network, with each node representing a genus and the edges denoting the strongest positive and negative association of all possible pairs (Ramayo-Caldas et al., 2016).

4.4 Results

4.4.1 Growth performance and prececal amino acid digestibility

The average initial BW on day 14 was 405 (SD 14.5) g/bird and was not significantly different between the treatments. Compared with the BD, protease supplementation did not significantly influence ADG, except for a significant reduction in ADG when fed the high level of Protease B supplementation (Table 3). When Phy was supplemented, ADG was significantly higher than that in all the other treatments. Supplementation of Phy and the higher level of Protease C significantly increased the G:F values. There was no significant difference in G:F of the other protease-supplemented treatments when compared with that of the BD.

Higher level of supplementation of Protease C and Phy significantly increased the pc digestibility of not only CP but also of all measured AA compared with that of the BD (Table 4). No significant differences in the pc CP and AA digestibility were found between supplementation of Protease C and Phy. The supplementation of Protease A and B had no significant influence on the pc CP and AA digestibility when compared with that of the BD in most cases. Exceptions include among others a significantly higher pc Ser digestibility observed for the lower supplementation level of Protease A and a significantly

lower pc Ile and Val digestibility for Protease B at both supplementation levels. Compared with BD, the lower supplementation level of Protease C significantly decreased the pc digestibility of AA except for His, Met, Pro, and Tyr.

Table 6. Growth performance of broiler chickens in the 7-d-experimental period (8 replicates per treatment unless otherwise stated).

	Basal diet	Protease A		Protease B		Protease C		Phytase		
Suppl. level	No enzyme	25 mg/kg[1]	200 mg/kg	500 mg/kg	4,000 mg/kg[2]	200 mg/kg	1,600 mg/kg	1,500 FTU/kg	Pooled SEM	*P*-value ANOVA
Final BW (g/bird)	692^{bc}	674^{c}	690^{bc}	689^{bc}	670^{c}	708^{b}	702^{b}	741^{a}	9.0	< 0.001
ADG (g/d)	41^{cd}	40^{de}	41^{cde}	41^{bcd}	39^{e}	42^{bc}	43^{b}	46^{a}	0.8	< 0.001
ADFI (g/d)	73^{bc}	73^{bc}	72^{cd}	73^{bc}	69^{d}	75^{ab}	71^{cd}	78^{a}	1.2	< 0.001
G:F (g/g)	0.56^{bc}	0.55^{c}	0.57^{b}	0.57^{b}	0.56^{bc}	0.56^{bc}	0.61^{a}	0.60^{a}	0.010	< 0.001

a-e Values without a common superscript within one row are significantly different ($P < 0.050$).
[1] 7 replicates.
[2] 6 replicates.

Table 7. Precaecal crude protein and amino acid digestibility (%) of the experimental diets (8 replicates per treatment).

	Basal diet	Protease A		Protease B		Protease C		Phytase		
Suppl. level	No enzyme	25 mg/kg	200 mg/kg	500 mg/kg	4,000 mg/kg	200 mg/kg	1,600 mg/kg	1,500 FTU/kg	Pooled SEM	*P*-value ANOVA
CP	80^{b}	80^{b}	80^{b}	79^{c}	80^{bc}	79^{d}	82^{a}	82^{a}	0.6	< 0.001
Ala	81^{b}	82^{b}	81^{b}	79^{c}	80^{bc}	79^{c}	83^{a}	84^{a}	0.7	< 0.001
Arg	88^{bc}	88^{b}	88^{bc}	87^{d}	87^{cd}	87^{d}	90^{a}	89^{a}	0.3	< 0.001
Asp/Asn	79^{bc}	80^{b}	79^{bc}	77^{d}	78^{cd}	77^{d}	81^{a}	81^{a}	0.5	< 0.001
Cys	67^{b}	67^{b}	68^{b}	65^{c}	67^{b}	65^{c}	72^{a}	71^{a}	0.9	< 0.001
Glu/Gln	86^{bc}	87^{b}	86^{bc}	85^{d}	86^{cd}	85^{d}	88^{a}	88^{a}	0.4	< 0.001
Gly	75^{bc}	76^{b}	76^{bc}	74^{de}	75^{cd}	74^{e}	78^{a}	78^{a}	0.7	< 0.001
His	80^{bc}	81^{b}	80^{b}	78^{d}	80^{b}	79^{cd}	83^{a}	83^{a}	0.7	< 0.001
Ile	82^{b}	83^{b}	82^{bc}	81^{d}	81^{d}	81^{cd}	84^{a}	85^{a}	0.6	< 0.001
Leu	83^{bc}	83^{b}	83^{bc}	81^{d}	82^{cd}	81^{d}	85^{a}	85^{a}	0.6	< 0.001
Lys	85^{bc}	85^{b}	84^{bcd}	83^{d}	84^{cd}	83^{d}	86^{a}	87^{a}	0.5	< 0.001
Met	84^{b}	85^{b}	84^{bc}	82^{c}	84^{bc}	83^{bc}	87^{a}	87^{a}	0.7	< 0.001
Phe	83^{bc}	84^{b}	83^{bc}	81^{d}	82^{bc}	82^{d}	86^{a}	86^{a}	0.6	< 0.001
Pro	80^{bc}	81^{b}	81^{b}	78^{d}	80^{b}	79^{cd}	83^{a}	82^{a}	0.7	< 0.001
Ser	78^{c}	79^{b}	78^{bc}	76^{d}	78^{bc}	76^{d}	81^{a}	82^{a}	0.6	< 0.001
Thr	73^{b}	74^{b}	73^{b}	71^{c}	73^{b}	71^{c}	76^{a}	77^{a}	0.8	< 0.001
Tyr	81^{bc}	81^{b}	81^{bc}	79^{d}	81^{bc}	80^{cd}	84^{a}	84^{a}	0.6	< 0.001
Val	81^{b}	80^{b}	80^{b}	78^{c}	79^{c}	78^{c}	82^{a}	82^{a}	0.7	< 0.001

a-e Values without a common superscript within one row are significantly different ($P < 0.050$).

4.4.2 Microbial communities in the terminal small intestine

A total of 1,021 OTU were identified from the entire dataset. Firmicutes were the most abundant phylum, commonly observed across all diet treatments (>98%). A significant difference between the bacterial profiles at the genus level was observed between treatments (P = 0.024) (Table S1). Regardless of dosage, the microbial communities in the terminal small intestine were significantly different between the treatments with Protease B and Protease C supplementation and between Protease C at low level and Phy ($P < 0.050$). The clustering of OTU in cases where Protease C was fed at both supplementation levels was influenced by a higher presence of the genus *Lactobacillus*, whereas supplementation of Phy and both dosages of Proteases B grouped further apart and may be caused by the abundance of *Enterococcus* and uncultured Clostridiaceae 1 (Figure 1). Additionally, diets supplemented with Phy and the lower level of Protease B resulted in the numerically highest diversity index among all diets. Significant differences in the diversity index were found between the supplemented Phy and Protease C, and between Protease B and Protease C both at low level (Figure 2, Table S1). *Lactobacillus* genus was the most abundant in all treatments (Figure 3). With Protease C supplementation at both levels, *Lactobacillus* accounted for 77% and 64% of the total community, whereas it was only 38% in the Phy diet, 43% in Protease B at low level, and 56% at the high level. The most relevant OTU identified were *Lactobacillus salivarius* (OTU 53, 77, and 40) and *Lactobacillus gallinarum* (OTU 86).

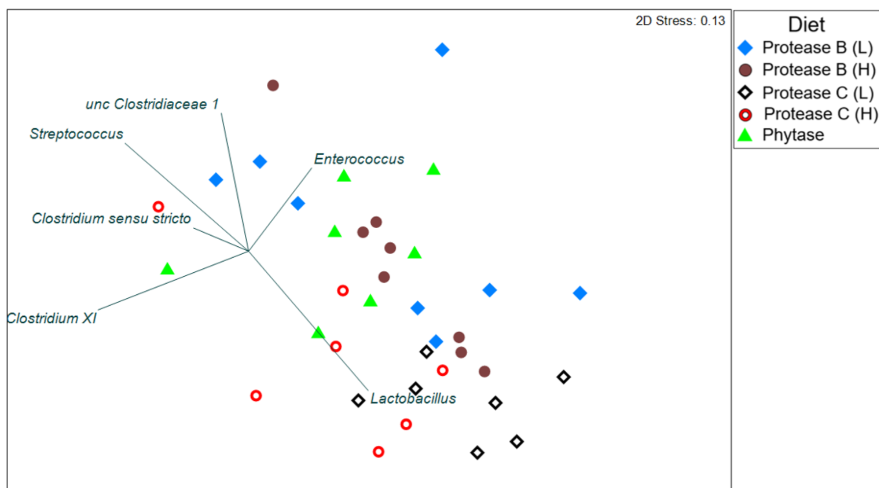

Figure 11. Non-metric multi-dimensional scaling plot illustrating the global bacterial community structure of dietary treatments that showed a statistical difference among each

other (low (L) and high (H) supplementation levels of protease). The symbols represent one pooled sample from each pen comprising all Operational Taxonomic Units clustered at genus level.

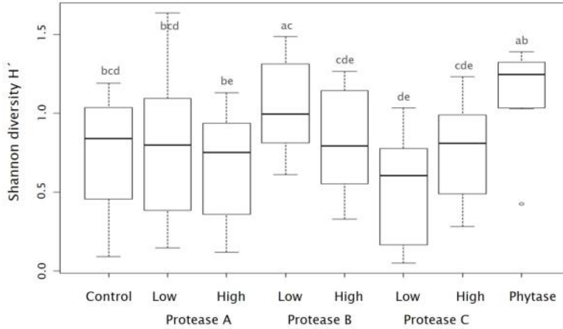

Figure 12. Shannon diversity obtained for the experimental diets at genus level. The plot indicates the second (box) and third quartiles (whiskers), and the median value is represented by the vertical line. Values without common letters are significantly different ($P > 0.050$).

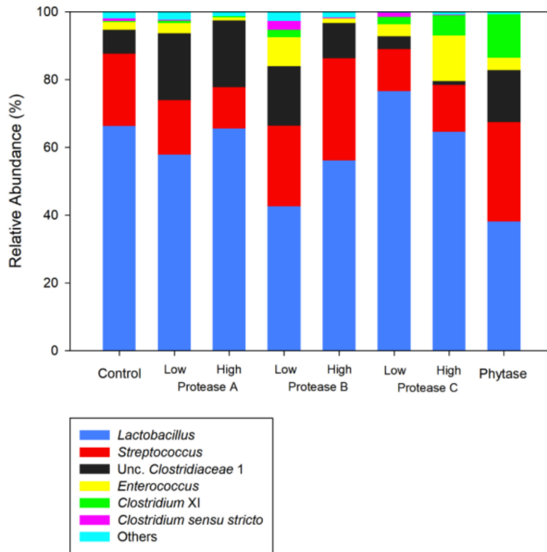

Figure 13. Relative abundance of microbes at the genus level detected in the terminal small intestine of broiler chickens for the experimental diets (8 replicates per treatment).

Streptococcus counts increased with both dosages of Protease B (24% and 30% for the low and high supplementation levels) when compared with 13% abundance in diets containing Protease C (Figure 3). The OTU 79, related to *Streptococcus alactolyticus* (Table S2), contributed to 20% of the total community in the low supplementation levels of Protease B and Protease C. In the Phy-supplemented diet, OTU 79 caused a significant difference with Protease C supplementation at low level ($P < 0.050$), where it accounted only for 30% abundance.

The genus *Clostridium* XI was more abundant when Phy or the higher level of Protease C was supplemented (13% and 6%, respectively) than in the other treatments (ranging from 0.4% to 3%). In the Phy treatment, OTU 13 (uncultured *Clostridium* XI) was negatively correlated with OTU related to the genus *Lactobacillus* (4, 57, 72, 90, and 98). Also, *Clostridium sensu stricto* was not highly abundant in this study. The *Enterococcus* genus was highly abundant when the high level of Protease C was supplemented (14%), being mainly represented by OTU 8 (*Enterococcus azikeevi*).

With special consideration to the 2 treatments, Protease C at the high level and Phy that had significantly higher AA digestibility than the other treatments, SIMPER analysis revealed that Phy supplementation increased the fold change (FC) of *S. alactolyticus* (OTU 79, FC = 1.9), uncultured *Clostridium* XI (OTU 13, FC = 2.2), and uncultured Clostridiaceae 1 (OTU 52, FC = 16) in comparison to the high supplementation level of Protease C. Upon supplementation of Protease C at the higher, level the principal OTU observed were the uncultured *Lactobacillus* (OTU 53, FC = 2.4), *L. salivarius* (OTU 40, FC = 2.7), *Lactobacillus taiwanensis* (OTU 77, FC = 1.4), and *E. azikeevi* (OTU 8, FC = 7).

Microbial networks revealed different levels of connectivity between the microbes as reflected by the significant interactions observed among them (Figure 4). The total number of negative correlations was found to be higher than the positive ones. The BD and Protease C at low level had fewest correlations (185 and 128, respectively) and a smaller number of genus (nodes) (15 and 20, respectively) (Figure 4). When Protease B was supplemented at the high level, we observed multiple correlations (1,187 edges) in the 43 genera. The other diets (both dosages of Protease A, Protease B at the low level, Protease C at the high level, and Phy addition) all yielded similar quantity of edges and nodes (on average 724 and 37, respectively). This co-occurrence analysis also showed that *Lactobacillus* was negatively correlated to other abundant genera in the small intestine

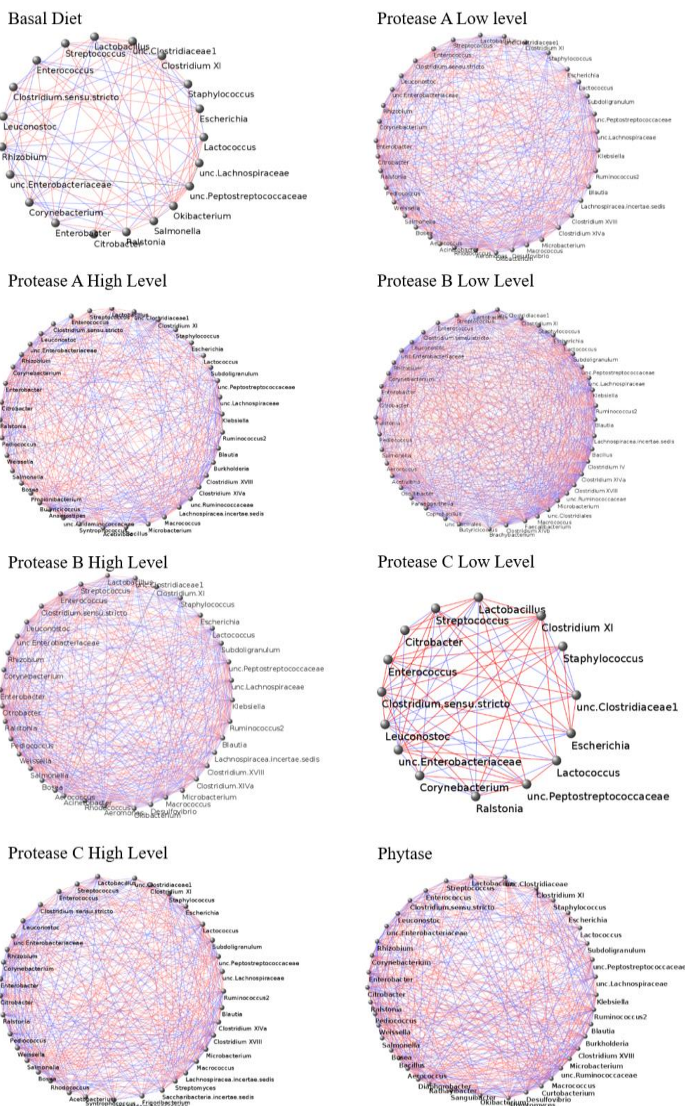

Figure 14. Microbial network at genus level for the experimental treatments (8 replicates per treatment). Significant interactions are indicated by the connective lines (edges) between genus (nodes). Negative and positive interactions are shown in red and blue, respectively.

4.5 Discussion

4.5.1 Prececal aminoacid digestibility

The results of this study show that the effect of protease supplementation on pc AA digestibility depended on the protease product and supplementation level. Protease A and B at both supplementation levels did not increase the pc AA digestibility. For some AA, supplementing these proteases even reduced pc digestibility. Protease C had no influence or decreased pc AA digestibility when supplemented at the recommended level. Supplementation of Protease C at an 8 times higher level, however, increased pc digestibility of all measured AA by an average of 2.6 percentage points. These results overall are in accordance with previous results, which showed that the supplementation of different proteases at a certain level can either decrease or increase the pc AA digestibility (Walk et al., 2018). However, classifying distinct protease products with respect to their effectiveness in increasing pc CP and AA digestibility is difficult. The protease products and concentrations used in this study did not increase pc AA digestibility, but had increasing effects on pc CP digestibility in other studies (Liu et al., 2013; Selle et al., 2013).

The present study also showed that the effect of protease supplementation was dose-dependent. Whereas, there was no effect of Protease B at the higher supplementation level, the lower supplementation level tended to decrease pc AA digestibility. Supplementation of 1,600 mg/kg of Protease C increased pc CP and AA digestibility, but no effect or a decreasing effect was observed at the dosage of 200 mg/kg. A dose-dependent effect of Protease C was also determined by Angel et al. (2011), who found the effect of protease supplementation to be fully expressed at the 200 mg/kg supplementation level. This shows that protease product and supplementation levels can explain divergent effects on pc CP and AA digestibility reported in the literature. Other possible influences on the efficacy of protease supplementation should be investigated to obtain more predictable results. Likewise, responses to protease supplementation can be affected by the choice of raw materials used, especially protein sources. For instance, effects due to diet composition were reported by Cowieson et al. (2016). For the present study, the diets used were based on corn and soybean meal, whereas sorghum, wheat bran, and canola meal have been used by Liu et al. (2013) and Selle et al. (2013). The diets used by Angel et al. (2011) were based on corn and soybean meal similar to the diets used in the present study, but the proportions of feedstuffs varied. Dietary composition may also contribute to the differences in the efficacy of protease supplementation between experiments observed by Walk et al. (2018). These authors used diets based on corn and soybean meal in one experiment, whereas a wheat-soybean-meal-based diet was used in another experiment. Such differences alter the substrate and might also modify gastro-intestinal conditions

relevant for enzyme activity, such as the pH in the digestive tract. In the present study, supplementation of Phy increased the pc AA digestibility by about the same extent achieved with Protease C supplementation at the higher level (2.7 percentage points on average).

Increase in pc AA digestibility due to protease supplementation has been attributed in part to a reduction of basal endogenous AA loss (Cowieson and Roos, 2016). Among the basal endogenous AA lost, the proportions of Asp/Asn, Cys, Glu/Gln, Pro, Ser, and Thr are relatively high (Kluth and Rodehutscord, 2009; Adedokun et al., 2011; Adeola et al., 2016). For some of these AA, namely Asp/Asn, Cys, and Thr the median of increase in pc AA digestibility by Protease C supplementation was above the median of the increase of all AA, whereas it was below the median value for others (Glu/Gln, Pro, and Ser). Therefore, based on the present results, the observed increase in pc AA digestibility cannot be simply explained by a reduction of basal endogenous AA loss. In regard to phytase supplementation, increased pc AA digestibility through reduced basal endogenous AA loss has also been described (Selle et al., 2016). Upon phytase supplementation in the present study, the increase in pc digestibility of those AA with a high concentration in basal endogenous losses was above the median increase of all AA for Asp/Asn, Cys, Pro, Ser, and Thr, but markedly below for Glu/Gln. Basal endogenous AA losses are affected by ADFI (Adedokun et al., 2011; Adeola et al., 2016), which was influenced by phytase supplementation in the present study. This means that basal endogenous AA losses may be affected by Phy, either directly by the enzyme or by feed intake, or both. Hence, our results do not clarify if phytase supplementation led to an increase in the pc AA digestibility through reduction of basal endogenous AA losses.

4.5.2 Microbial Communities in the Terminal Small Intestine

Protease supplements altered the overall microbial composition. This change was mostly observed with the higher diversity obtained for Phy and the low supplementation level of Protease B (Figure 2). A similar finding was reported in a study testing fecal protease activity in humans, in which higher protease activity was reported to result in a lower number of bacterial species and a decreasing diversity index (Carrol et al., 2013).

Streptococcus commonly found in the small intestine during the growth of broiler chickens (Han et al., 2016; Ranjitkar et al., 2016) was higher in abundance upon supplementation of Protease B when compared with that of Protease A and C. The presence of *Streptococcus* has been related to an increase in the density of CD8+ T cells influencing the immune functions in the intestine (Huang et al., 2013), and can be involved in the reduction of pathogens (Dahiya et al., 2007). *Enterococcus* also increased with high level of Protease C supplementation, and low level of Protease B supplementation. This genus is usually

found in low abundance in the small intestine of broiler chickens (Lu et al., 2003). Also, a probiotic mixture of *Enterococcus* and *Lactobacillus* increased the number of mucosal adherent bacteria in the terminal small intestine apart from increasing the goblet cells and mucous layer (Chichlowski et al., 2007). Furthermore, strains from this genus are able to synthesize bacteriocins that are active against pathogens like *Eimeria* spp., making these bacteria a potential probiotic candidate (Ivanova et al., 2004; Pan and Yu, 2014).

The uncultured Clostridiaceae 1 was the lowest in abundance for Protease C at both supplementation levels in comparison with that of the other treatments. The high percentage of sequences (around 11% of the total abundance) of this uncultured bacterium demonstrated that there is still a need for culturing and better characterizing the microbiota of the digestive tract of chickens. A better characterization offers a clearer view of the microbial abundance and the effects of supplementing diets with enzymes (Borda-Molina et al., 2018).

The microbial composition after providing the high level of Protease C and Phy that caused increased pc AA digestibility was different when compared with that of the other treatments. Most of quantified OTU in Protease C at low and high dosages belonged to *Lactobacillus* species. The high presence of *Lactobacillus* increased the production of extracellular proteins with adhesive properties in the study of Spivey et al. (2014). This adhesion influences gut health and the population dynamics in the gut through the synthesis of compounds such as bacteriocins that are active against Gram-positive bacteria (Fasina et al., 2016). Based on the high dominance, it was estimated that this genus assimilates 3% to 6% of the protein ingested by the chicken (Apajalahti and Vienola, 2016).

The *Clostridium* genus in the small intestine was reported to have less than 20% in abundance (Mohd Shaufi et al., 2015) similar to observations in the present study. Species belonging to clusters IV, XI, and XIVa are able to increase the growth of chickens due to butyrate production, which is an indispensable source of energy for the gut wall and mediator of immune responses (Pourabedin and Zhao, 2015; Sun et al., 2018). These clusters showed different significant interactions in the co-occurrence network and in the case of Phy, *Clostridium* XI was found to increase in abundance with potential benefits to the host.

Network co-occurrence analysis was performed to deduce significant interactions between the microorganisms. A higher diversity index observed in diets supplemented with Protease B at low level and with Phy may have influenced the higher presence of significant interactions visualized in the microbial network. Except for Protease C at low level, different supplementation of Protease enzymes and Phy increased the connectivity within microbiota in the terminal small intestine of broiler chickens. It is important to

highlight that with the approach applied it was not possible to identify a "hub" or dominant genus (Mandal et al., 2015). Perhaps the reason is a higher rate of absorption of substrates from the broiler chickens with Protease supplementation (except Protease C at low level) and Phy. This rate of absorption could be influenced by the action of the enzymes because they start to increase the AA digestibility even as early as in the proximal jejunum (Selle et al., 2016) and the distal jejunum (Liu et al., 2013). Also, this fact would imply possible consequences in the modification of the substrate before they arrive at the terminal small intestine.

Modified microbiota composition could be attributed to the different modes of action of the enzymes. Protease A is obtained from the fungi *A. niger*, whereas Protease B and C are derived from the bacteria *B. licheniformis*. Enzymes synthesized from different microorganisms catalyze precise reactions that are influenced by the case-specific evolution of the protein (López-Otín and Bond, 2008). An influence on pc AA digestibility probably is specific for certain sources of proteases. A study testing 2 proteases in degrading whey protein found that a more significant extent of protein hydrolysis occurred at higher concentrations of the enzymes (Pintado et al., 1999). Furthermore, a single type of protease action resulted in a hydrolysate richer in peptides, whereas in others it was richer in AA (Pintado et al., 1999). Another potential influencing factor on enzyme activity is the substrate concentration. A protease isolated from *B. licheniformis* had a reduced rate of hydrolysis and enzyme selectivity with increased substrate concentration (Butré et al., 2014). On the contrary, protease from *A. niger* revealed that at least 30% of the activity could be increased if optimal conditions are provided (Mandal et al., 2005). These facts can lead to the different availability of products that do not affect the measurements of digestibility but may impact the microbial composition.

In line with the effects on the microbiota from the protease supplementation, an antimicrobial effect could be speculated. A protease derived from *B. licheniformis* is capable of removing the biofilm produced from *Bacillus cereus* and *Pseudomonas aeruginosa* (Morvay et al., 2011). The mechanisms behind are related to the breakdown of extracellular polymeric substances that can be produced in the digestive tract by members of the genus *Lactobacillus*. Until now, there is no literature discussing antimicrobial activity of proteases from *A. niger*. Whether or not these effects were relevant to the present study and what they mean for pc AA digestibility cannot be answered at this time.

Connections between pc AA digestibility and microbiota composition in the terminal small intestine could not be clearly established in our study. It cannot be ruled out that closer connections exist in the other sections of the digestive tract or on the basis of functionality rather than abundance. Our study showed that protease effects in principle exist. Hence, it

should be considered as a pilot study that needs to be verified through other experiments and exploring deeper in phylogeny and functionality of the microbiota.

In conclusion, the effect of protease supplementation on pc AA digestibility in broiler chickens depended on protease product and supplementation level. Supplementation of Phy resulted in an increased pc AA digestibility. The microbiota composition and interactions between microbial groups were different between treatments. However, no clear relationship between pc AA digestibility and microbiota composition was detected.

4.6 Supplementary material

The Supplementary Material for this article can be found online at:
http://dx.doi.org/10.3382/ps/pez038

Table S1. One-way PERMANOVA analysis of the effects of diets based on enzyme supplementation, and one-way ANOVA for the difference across treatments considering the Shannon diversity index (8 replicates per treatment).
Table S2. Taxonomic assignment of the most relevant Operational Taxonomic Units present in the terminal small intestine of broiler chickens. The assignment was performed in the Seqmatch function of the Ribosomal Database Project (https://rdp.cme.msu.edu/) database for type and non-type strain.

4.7 Acknowledgements

The authors acknowledge the support by the State of Baden-Württemberg through bw-HPC. We would also like to thank Dr. Yuliaxis Ramayo-Caldas for assistance in constructing the microbial network.

4.8 Conflict of interest

The authors declare that there are no conflicts of interest.

4.9 Notes

Presented in part at the annual meeting of the Society of Nutrition Physiology, Göttingen, Germany, March 13–15, 2018. Borda-Molina, D., Zuber, T., Camarinha-Silva, A., Feuerstein, D., Rodehutscord, M. 2018. Protease and phytase supplementation effects on microbiota composition in the ileum and amino acid digestibility in broiler chickens. Proc. Soc. Nutr. Physiol. 27:46

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CHAPTER V

GENERAL DISCUSSION

5. General discussion

Broiler chickens are a crucial source of food worldwide, due to the lower environmental impact on its production and efficient feed-gain and feed-conversion ratio (1). This scenario is the consequence of many strategies in the breeding selection, and the well understanding of its physiological and nutritional needs (2, 3). Chicken is also an important model organism for non-mammalian species, and the publication of the first draft genome in 2004 represented a scientific lever to the genomic research (4). Studies carried out in this animal model for gene regulation, viruses, cancer, and also used as one of the first models for embryology and development (5), became broiler chickens an excellent case-study to further elucidate its interaction with the microbial communities. The host-associated microbial composition is affected by diverse conditions. The main influencing factors are the first source of colonization, age, gender, environment, and diet (6). The work presented in this thesis focus on the effects on the microbiota when adding enzymes (e.g., phytases and proteases) and mineral supplements to optimize substrate assimilation and reduce losses by excretion.

5.1 Methods standardization

Aware of the importance to establish a point of comparison between microbiota studies in broiler chickens, it was designed a standard operational procedure (SOP) to collect gastrointestinal samples and analyze the taxonomical composition, which will be a reference for other animal nutrition studies in the Institute of Animal Science at the University of Hohenheim and project partners. Different projects in microbial research have established reference documents, that have to be used by all partners. For instance, the human microbiome project has defined a manual operating procedures with a comprehensive screening, sampling, and establishment of clinical metadata and the further specimen processing and sequencing (7). Also, the earth microbiome project, interested in the analysis of the microbial diversity in different environments, made an effort to connect researchers on the field and proposed a standardized procedure for collection, curation, and analysis to have a better understanding of the ecological trends (8).

After the broiler is euthanized with a gas mixture and further opened, the gastrointestinal tract is removed to a disinfected working bench. All instruments (e.g., scissors, glass slides, tweezers) were disinfected with ethanol, each time a new sample arrives, and sterile latex gloves were used during all procedure. Each section of the gastrointestinal tract (GIT) was open longitudinally with sterile scissors, and a sterile plastic spoon was used to collect the digesta (or luminal content) and mixed it in a sterile container. After removing the digesta, the mucosa was washed with sodium phosphate buffer 1% (PBS), and one glass slide was

used to hold the GIT section while another one scratch the mucosa. Samples were stored in sterile containers and conserved at -80°C.

To avoid the influence of different proportion of DNA in the bacterial composition, mainly because of the diverse cell wall structure of gram-positive and gram-negative microorganisms, a DNA extraction kit, that was successfully used in pig GIT samples of digesta and mucosa (9), was also tested with broiler samples. A physical disruption step is included in the kit (bead-beating), which diminishes the bias generated with enzymatic cell disruption, where usually Gram-negative cells are efficiently lysed, and the recovery of Gram-positive microorganisms is reduced (10, 11). The experimental procedure on the pig study resulted in good DNA quality, concentration, and a similarity in the abundance of bacterial groups based in the V1-2 and V5-6 regions of the 16S rRNA gene (9). Similar results were obtained when the same test was performed in chickens GIT samples. The resulting DNA showed good concentration (minimum of 20 ng/ul) and quality (on average 1.8 for the 260/280 ratio) and has very good compatibility with further downstream analysis. Briefly, the 250 mg of digesta or mucosal sample were placed in a silica matrix with glass spheres and lysis buffer, that help in cell disruption. After a step of contaminant removal and DNA washing, high-quality DNA is obtained. It has been reported in soil studies that this kit improves the purity indices, suggesting that contaminants such as polysaccharides, carbohydrates humic acids and polyphenols are efficiently removed (12). These procedures were established to further validate with sequencing analysis the succession of profiles that communities undergo in the different sections of the GIT.

It is demonstrated that short read studies based on the 16S rRNA gene are very informative surveys, concerning the analysis of microbial communities in environments like human saliva and GIT, soils, wastewater treatments and different sections of GIT from various animal species (13–15). This genetic region serves as a chronological marker, due to the conserved function and structure, and additionally contains variable regions that allow the differentiation between most of the microorganisms (16). Chicken studies have been using different 16S rRNA regions to characterize the microbial community of the GIT. For example, a study, correlating the efficiency of energy extraction and caeca microbial population, 32 individual samples were pyro-sequenced with 454/Roche technology, using the regions V1-V3 of the 16S rRNA gene (17). Ileal and caeca content from broiler chickens at different ages were analyzed based on illumina sequences targeting the V3 region of the 16S rRNA gene (18). Fecal microbiota of high and low feed conversion ratio (FCR) broiler chickens was analyzed targeting the V1-V5 regions of the 16S rRNA gene (19). Moreover, impacts on the poultry house and litter were studied with 454 pyrosequencing considering the V1-V2 regions of the 16S rRNA gene (20). A study in 2007 validated the different 16S rRNA gene regions using a known dataset of pathogenic bacterial species and established that the hypervariable regions V2, V3, and V6, resulted

in the maximum nucleotide heterogeneity and the maximum discriminatory resolution (21). Furthermore, a study testing a mixture of DNA isolated from known bacteria established that the V1-V2 region performed better than other regions where the number of species assignation was two times higher than with other regions (22). Due to the difficulty in finding a common region for target amplicon sequencing and based on previous results it was decided that studies carried out in the institute will be sequenced based on the V1-V2 region of the 16S rRNA gene, in order to facilitate the comparison between different studies and to increase our knowledge in the chicken GIT.

Regarding the bioinformatics analysis of the sequencing data it is necessary to highlight that up to date, there are several bioinformatic pipelines available to work with amplicon sequencing datasets (eg., Mothur (23), QIIME (quantitative insights into microbial ecology) (24), pplacer (25), DADA2 (divising amplicon denoising algorithm) (26), MG-RAST (metagenomics- rapid annotation using subsystem technology) (27)). They all follow a similar procedure starting with the assemblage of forward and reverse reads, followed by the removal of low-quality reads, low abundant operational taxonomic units (OTU) and chimeras, followed by taxonomy assignation and OTU table generation (23, 24). The taxonomy assignation can be performed based on four databases (e.g., SILVA, RDP, greengenes (gg) or NCBI) and those rank the sequences into the domain, phylum, class, order, family, genus, and species (28). The majority of the pipelines are implemented with SILVA or greengenes. In a comparison study of shared taxonomic units, it was stated that the NCBI database shared more taxa with SILVA database. On the other hand, greengenes, which is not curated since 2013 and has the smallest amount of reference sequences, showed lower diversity in comparison with the others (28). Furthermore, mapping taxonomies onto each other established that SILVA performed better than RDP and greengenes (28). Therefore, using the most recent database, OTUs in this study were assigned to the SILVA repository, within the Mothur pipeline. This pipeline was chosen because it comprises several quality check procedures which lead to a better annotation of sequences (29); also it includes a unique dereplication, alignment to the SILVA database and clustering with Uclust (23). Along with the previous reasons and considering that the microbiota present in broiler chickens GIT is not yet well characterized, the most recent database would include more viable information to OTU assignation (15). Moreover, it was found in a human gut microbiota *in-silico* approach using three pipelines (Mothur, QIIME, and pplacer), that the ones using the Greengenes database gave a lower phylogeny assignation (15). Looking to specificity, sensitivity and percentage of amplicons dropped (outliers), the OTU performance was more accurate for Mothur compared to the other approaches (Figure 15) (15). In another evaluation done in babies gut microbiota, MG-RAST, QIIME, and Mothur resulted in comparable results for diversity measures and taxonomic classifications between the pipelines, where the last two resulted in the more powerful tools, concerning statistical capabilities and user freedom (14).

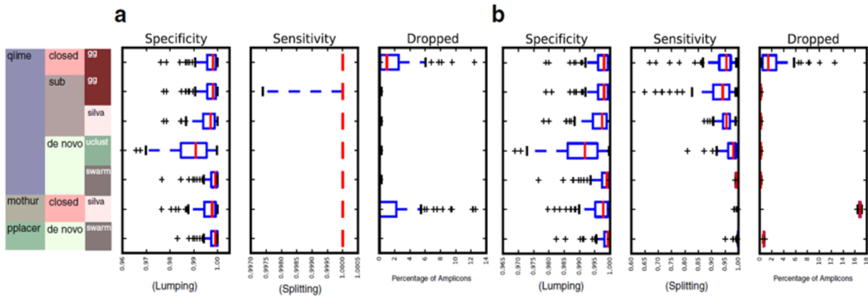


Figure 15. Assessment of OTU performance (A) No sequencing error, (B) simulated sequencing error. (source: Golob et al., 2017).

5.2 The chicken GIT microbiota from culturing to high-throughput sequencing

First efforts to characterize microbial communities in broiler chickens have focused on classical culturing using specific media (30). With the culturing approach, it was estimated that the chicken ileum is colonized with 10^9 bacteria/g and the caeca with 10^{11} bacteria/g (31). The groups identified described coliforms and lactic acid bacteria (30). Based on biochemical characterization other groups were recovered from the caeca and assigned to *Peptostreptococcus* spp., *Propionibacterium* spp., *Eubacterium* spp., *Bacteroides* spp., *Clostridium* spp., and also unknown species (32). Culturing is essential to characterize the contributors to the ecology of the GIT in broiler chickens. Despite the significant knowledge this methodology brings, it is well-recognized that underestimation of the microbial ecology is obtained with this approach. Strict anaerobic conditions and specific growth requirements are some of the challenges that a scientist has to overcome when using culturing methods (33). Moreover, the estimation of recovery percentage in the caeca is between 10 to 60% (32, 34, 35). To improve bacterial classification, DNA based techniques, targeting the 16S rRNA gene, became more popular and were seen as robust methodologies. Therefore, the first ecological studies using PCR amplification of 16S rRNA gene were addressed with molecular fingerprinting techniques such as temperature gradient gel electrophoresis (TGGE) (33), denaturing gradient gel electrophoresis (DGGE) (36), single-strand conformation polymorphism (SSCP) (37) and terminal restriction fragment length polymorphism (T-RFLP) (38). They gave a better overview of the microbial community and showed, for the first time, that age and GIT location have an effect on chicken microbiota (37). A DGGE study confirmed that a unique pattern is obtained in the crop, duodenum, ileum, and caeca, and also described inter-individual variability between samples (36). A TGGE study in feces also confirmed that patterns are

specific per individual, and the most dominant band was assigned to *Clostridium* clusters (39). TGGE coupled to a clone library, and further sequencing determined that there are specific phylogenetic groups which are shared in the caecum of broiler chickens. The random clone sequencing determined a high presence of *Clostridium* group (27%), followed by *Sporomusa* group (21.2%), *Clostridium leptum* group (20.2%), while enteric and relatives comprised 20.8% (33). However, even if a better overview of the dominant bacteria could be obtained in comparison to classical growth methods (36), the resolution is dependent on band separation in the gradient gel. Besides is required proper staining of the gels, which could lead to problems of reproducibility (37). Also, it is described that populations with less than 1% can be overlooked with PCR-DGGE technique, and gel reproducibility is usually not achievable (40).

Another study that monitored changes in the microbial ecology of chickens under different rearing conditions used capillary electrophoresis coupled with SSCP. The authors showed that pooling six chickens samples decrease microbial variability. Moreover, SSCP revealed that profiles from the ileum and cloaca were more similar than ileum and caeca. *Lactobacillus* was more abundant in the ileum and the cloaca, while *Clostridium* was more present in the caeca (37). T-RFLP is a rapid and cost-efficient technique to fingerprint the microbial community (41). The specificity to determine which microorganism are present in a sample increases with the use of multiple restriction enzymes, and if coupled with clone libraries. The fingerprint results show different diversity when compared to DGGE (40). With this technique, it has been identified as possible butyrate producers in the mucosa of the caeca, and it was confirmed that the diversity of culturable organisms is lower than non-culturable (38).

Supplementation of antimicrobial feed additives established that inter-bird variabilities were reduced with the antimicrobial agent, and GIT sections (ileum and caeca), age and diet had an influence on the T-RFLP pattern (42). In a recent study, with different dietary supplementations of phosphorous (P) and calcium (Ca), was found that regardless the supplementations, crop, jejunum, and ileum are mainly colonized by the family Lactobacillaceae, and the caeca are the most diverse GIT section (43). On the same study was determined that *Lactobacillus taiwanensis* and *Lactobacillus vaginalis* decrease their abundance from the crop to the ileum (43). Another study, using T-RFLP, determined that differences in the caeca microbiota are influenced not only by the high or low fiber diet, but also by the chicken line (44). The disadvantage of this approach is the incomplete or nonspecific restriction that might lead to overestimations. Additionally, sequence redundancy can be found since the cleavage sites generate fragment lengths which are similar for different species (45). Another disadvantage consists of the variation in the microbial diversity due to non-specificity in the phylogenetic composition at the species level (45). Also, even if strong correlations and similar results between T-RFLP and 454

pyrosequencing were observed (43), T-RFLP relies on published sequences and clone libraries that might elucidate new species of microorganisms.

Quantitative polymerase chain reaction (qPCR) studies have been used to calculate positive and negative correlations for performance parameters and microbial communities in broiler chickens (46). The method targets only specific bacterial groups that were previously reported, and mostly comprises the more abundant species (46). The use of these techniques could imply a bias with the non-well-defined microorganisms and the less abundant groups in broiler chickens. The study of Rubio *et al*., (2015) targeting bacterial groups in the crop such as *Clostridium coccoides*, Enterobacteria and *Escherichia/Shigella* is an example of this bias. It is known that those groups are not the most abundant species (it is generally accepted that *Lactobacillus* dominates this section) and therefore not contributing in high proportions to the differences in performance parameters. Additionally, in that study, the caeca microbiota was characterized, based on primers targeting Lactobacilli, Enterobacteria and *Escherichia/Shigella*, but there was no focus on *Clostridium* clusters, as well abundant in this GIT section. In the ileum has been reported primers for *Lactobacillus aviarius, Lactobacillus reuterii*, and *L. salivarius*, which showed negative correlations against *Clostridium perfringens* (47). However, in the same study is stated that is unknown the ecological and physiological significance of *L. aviarius* (47), demonstrating a disadvantage of qPCR methodology. Only targeting specific microbes cannot address the ecological meaning of the bacteria in a specific environment. qPCR is commonly used to detect pathogens in chicken samples such as the ones belonging to the genus *Salmonella, Campylobacter* and the species *Clostridium perfringens* (48). However not analyzing the microbiota as a whole, it leads us to weak conclusions about the influence of the intestinal microbiome.

Advances in technology allowed scientist to go deeper in the characterization of microbial communities. High throughput Next Generation Sequencing (NGS) techniques produce high amounts of sequencing data with costs that are continuously dropping (29). 454 pyrosequencing was one of the first technologies that appeared on the market and permitted with more confidence to analyze bacterial richness and diversity indexes (eg., Chao1 estimator, Shanon- Simpson index) (49). With this methodology, Firmicutes was revealed as the most abundant phylum across the different sections of the chicken GIT (gizzard, proventriculus, duodenum, jejunum, ileum, caeca, and cloaca) (83.2% $\pm$ 16.3) followed by Bacteroidetes (0.3 -14.3%) and Proteobacteria (1.0-3.7%) (43, 50). The core families were Lactobacillaceae (0.7-96%), Lachnospiraceae (0.1-20.7%), Bacteroidaceae (0.1-8.7%), Streptococcaceae (0.1-2.3%), Pseudomonadaceae (0.1-1.8%), Prevotellaceae (0.1-1.3%) and Enterobacteriaceae (0.2-1.0%) (50). Differences between the GIT sections were obtained with the high presence of lactic acid bacteria in the upper gut including *Lactobacillus, Enterococcus,* and *Streptococcus*, while the caeca and large intestine were

mainly represented by *Alistipes,* unclassified *Ruminococcaceae* and unclassified *Lachnospiraceae* (50). In a study with P and Ca supplementation differences between the diets were attributed to OTUs closely related to *Lactobacillus crispatus, L. salivarius, L. taiwanensis, L. aviarius, L. vaginalis, Bacteroides fragilis, Shigella flexneri* and *Aeromonas sharmana* (43). Despite the deep analysis achieved with this technology it also gives sequencing errors, sequence artifacts and chimeras (29, 51). Recently most projects use illumina MiSeq technology to perform target amplicon sequencing and characterize the microbial ecology. The reasons comprise the flexibility, high throughput, and sequence length sequencing with high confidence and accuracy (52). Other aspects which promote this technology as one of the most used are the lower costs and the possibility to obtain deep sequencing (53) with information of around 7.5Gb from 15 million of 250-base paired-end reads in short timing (e.g., three days) (54). In Chapter 3 and 4 this technique was used to deeply characterize different sections of the GIT (crop, ileum, and caeca) and to compare differences between dietary supplementations further.

Nevertheless, even if NGS is up to date a robust technique to study microbiota, it is expected that new technologies will solve some problems like: primer selection, being clear that this choice in amplicon sequencing have the major effect on the outcome (55); PCR conditions and template concentration (52); together with problems from the sequencing itself, considering technology chosen, errors and sequencing depth (52). It has been reported that technical issues with the PCR amplification of the 16S rRNA gene, led to biases in microbial studies and they were inherent in all sequencing platforms (56, 57). For instance, primer choice based on the length and the 16Sr RNA region can influence the richness and evenness of the samples (58). And even more, shorter amplicons (<400 bp) produce higher richness than longer amplicons (58). A study was testing different NGS technologies (illumina MiSeq, Hi-Seq, and Ion PGM), and it demonstrated that, despite the inter-individual variability, samples clustered according to technology or primer set (55). Furthermore, it has been proved that possible overestimation/underestimation can be obtained with High-Throughput sequencing platforms since results of the composition of microbial soil communities were different when compared to a quantitative microscope-based analysis, namely fluorescent *in situ* hybridization coupled to a catalyzed reporter deposition (CARD) (59).

5.3 Sample variability

Even though, standard procedures were followed in all experiments a high variability in the microbial composition between individual and pooled samples was observed (chapter 3 and 4). In the individual sampling study (chapter 3) the average similarity within the replicates was for the crop digesta between 29 to 82%, and crop mucosa 29 to 73%. The ileum showed a decrease in sample similarity, registering values between 19 to 49% in the

digesta and 25 to 47% in the mucosa. The caeca had the lowest sample similarity with 17-38% in digesta and 30-39% in the mucosa. In the pooled study (chapter 4) also, low similarity percentages were obtained, with values between 21 and 35%. This variability has been reported in a pooled sample survey testing the influence of antimicrobial feed additives; there T-RFLP registered similarities at OTU level in the ileum, which ranged from 29 to 61% and in the caeca (34 to 59%) (42). In a pyrosequencing study was also revealed a high individual variability in broiler chickens (207 individual caeca samples) allocated in the same flock (60). The chicken metaproteome of crop and caeca revealed a high inter-individual diversity seen in the unpaired distribution of the phylogenetical assignation for biological duplicates (61). This high variability was also observed in gut human microbial studies (stool, rectal swab, and mucosa), where higher variability was obtained between individuals in comparison to different sample points within the same individual (62, 63). A study with 207 pigs growing under the same conditions showed that only 35% similarity was shared in regards to the bacterial community (64). Additionally, in a rumen study with 16 lactating cows, the similarity percentage between individuals was 51% (65).

With the above-described findings could be affirmed that the association of the bacterial community to its host is individual-dependent, even if this can be classified as stable in a particular niche or environment (66). The genetic background, different rate of assimilation of substrates, behavior, and interactions with other individuals, are elements that may contribute to the variability (60). A possible solution to reduce the influence of significant variability in the data is the inclusion of more samples in the survey (62, 67); however, nowadays with strict ethical committees, it is more complicated to include more animals in the experiments. Another alternative would consist of the inclusion of technical replicates which might help to arise a better interpretation of the results (52).

5.4 Microbiota in the chicken GIT and the influence of dietary interventions

The gut microbiota of chickens has an impact on the nutrient intake and immune homeostasis which will directly influence bird performance (68). The chicken GIT is mainly colonized by Firmicutes, while Bacteroidetes is found in very low abundances. These results were observed in 26-day old birds from chapter 3 and 4, regardless of the type of diet or enzyme supplementation or gastrointestinal section. In other animal models like mice, rats, and pigs (69–71), alongside with human studies (72, 73), the Firmicutes/Bacteroidetes ratio is used as a marker. Those studies revealed that higher Firmicutes/Bacteroidetes ratio is found in obese individuals while a trend to decrease is seen when a loss of weight is registered (72, 74). The significant presence of Firmicutes is consistent with efficient feed conversion ratio of broiler chickens (6) since it is stated that

due to the promotion of more efficient absorption of calories, microorganisms from the phylum Firmicutes provide more energy when compared to Bacteroidetes (72).

To further contribute to the knowledge of the established microbiota on broiler chickens, chapter 3 included for the first time not only the analysis of digesta samples but also of mucosa, because it is the interface between the host and the intestinal bacteria. In chapter 3 it was characterized three different section of the GIT: crop, ileum, and caeca. Previous studies using PCR-DGGE, T-RFLP or high-throughput sequencing showed higher richness and diversity in mucosal samples of jejunum, ileum or caecum compared to luminal content (38, 75, 76). Other recent studies focused their microbial analysis in one environment or GIT section; either ileum mucosa and caeca digesta, but not digesta and mucosa at the same time (77). In contrast with the dynamic nutrient flux happening in the lumen, the mucosa is expected to have a more stable nutrient balance that can be selective for specific species (Donaldson et al., 2015). Genetic association with the host could drive possible conclusions on the influence of certain bacteria in the mucosa, due to the expression of specific carbohydrates in the epithelium and direct regulation of interaction between microorganism species (75, 78). Gnotobiotic animals demonstrate losses in the epithelial turnover, reduction in the smooth muscle function and motility, together with less local endocrine function and mucosal (79). Therefore, it can be seen that microbial communities in the mucosa establish direct communication with the host since they are attached to the epithelium, in comparison to the luminal content which has a continuous flow rate (80).

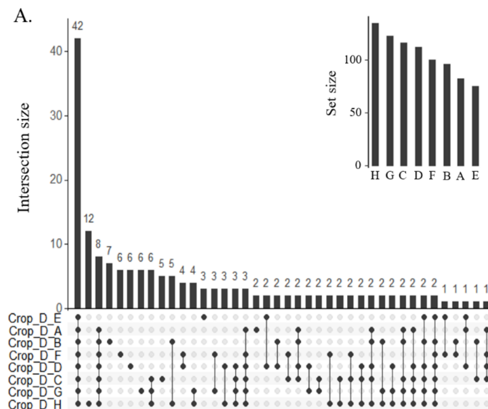
As a negative consequence of the disrupted communication in the equilibrium between the host and its microbial ecology, it was found in pigs that higher abundance in *Clostridium* XI is related to the induction of inflammatory responses in lymphatic tissues, attributed to bacterial translocation from the ileum to lymph nodes (81). Thus, it is crucial to consider the GIT as a whole and to study the digesta and mucosa microbial community, in as many sections as possible, in order to give complete information of the GIT under a specific condition and to provide more powerful conclusions regarding host-microbe interaction.

Dietary supplements such as minerals and enzymes are formulated to fulfill the requirements of broiler chickens (82). These supplements might modify the composition and metabolism of the intestinal microbiota. Enzymes have the capability to accelerate the hydrolysis of substrates and change the biochemical characteristics of the digesta while increasing the concentrations of products that can be accessible and metabolized (82). It has been demonstrated that mineral absorption of Ca is positively correlated with *Lactobacillus paracasei* and Bifidobacteria, and their presence increases the absorptive area and microbial biomass while reduce the turnover from the bones (83). Thus, the performance of broilers is affected by different mechanisms, combined with the influence

of the microbiota in the different parts of the GIT (84). Therefore, the following sections will discuss the supplementation of enzymes (e.g., phytase and proteases) and minerals (e.g., Ca and P), focused on three different sections (crop, ileum, and caeca) and taking into consideration both type of samples digesta and the mucosa.

5.4.1 Crop

The crop is defined as a temporary food storage site. Due to its characteristic low pH (value = 4.5) (85), mainly *Lactobacillus* species are found (chapter 3). However, at low abundance, there were different species which were also part of the crop microbial ecology, as *Streptococcus alactolyticus* and different unclassified microorganisms from the family Erysipelotrichaceae and the genera *Clostridium, Parvimonas,* and *Ralstonia*. The same findings were reported by several studies (34, 38, 86), and 454-pyrosequencing revealed that *Streptococcus* is present in a percentage below 1% (87). Based on the core microbiota, in chapter 3, a total of 208 species were observed in the digesta while 282 appeared in the mucosa samples. Regarding digesta, 42 species (Figure 16 A and B) were commonly shared by the eight dietary treatments, where 23 correspond to uncultured or unidentified species, and seven *Lactobacillus* species were assigned (*L. crispatus, L. gallinarum, L. helveticus, L. mucosae, L. salivarius L. taiwanensis* and *L. vaginalis*). In mucosa, a higher number of shared species were detected (59) and from those 40 corresponded to uncultured species and the same *Lactobacillus* species as in the digesta, except for *L. gallinarum* that was not detected. Shared by both environments were *Ralstonia picketii, R. solanacearum, Streptococcus alactolyticus, Anaerostipes butyraticus,* and *Pseudomonas mucidolens.* A possible explanation for the higher diversity in the mucosa is that polysaccharides present in that environment, allow the presence and establishment of more microorganisms when compared to the transient digesta (88).



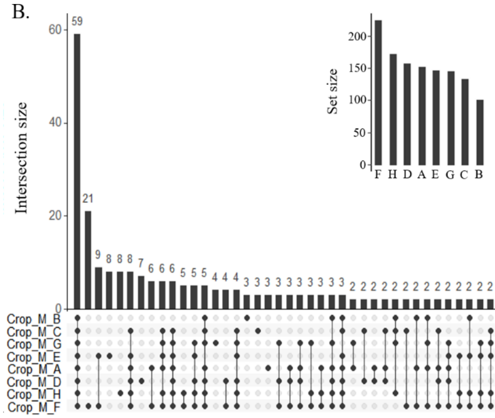


Figure 16. Intersection matrix for the core microbiota at species level found in the crop for digesta (A) (left side) and mucosa (B) (right side). This data corresponds to the eight dietary treatments described in chapter 3 (supplementations of Phosphorus, Calcium, and phytase).

A diet effect was observed in the crop mucosa when animals were fed with diet C (with Ca supplementation) (chapter 3). The core microbiota detected did not show a high number of unique species in comparison to the others. This fact is an indication that proportion of highly dominant species and the lower values of Shannon diversity (H´: C, B, G with 1.4; A, D, E with 2.1; H with 2.2; F with 2.5), should contribute more to the significant differences. About the Ca effect, an *in vitro* study concluded that presence of free Ca^{++} ions increases cellular adhesion of probiotic strains from *Lactobacillus* (89). A pig study also demonstrated that Ca-P diets influence the increase of the adherence of *Lactobacillus* to the mucosa (81). In mice is seen that Ca provide a favorable environment for the growth of potential prebiotics *Prevotella* and *Bifidobacterium* spp. (90). Supplementation of monocalcium phosphate in chickens led to a decrease in the crop of the family Flavobacteriaceae (43). Among the possible effects can be mentioned the protective consequence on permeability and maintenance of luminal buffering capacity together with the increment of microbial fermentation (90). Those facts agree with the assumption that large influence of mainly mineral supplementation could drive changes in the microbiota.

Despite at high taxonomical levels the crop environment including digesta and mucosa, showed no difference, a more in-depth analysis at species level highlight the fluctuations on the different species of *Lactobacillus* (Figure 17) which was emphasised in a previous study (91). Moreover, it reveals that the crop has particular conditions, where a couple of species are dominating and contributing for more than 60% of the total abundance. A high

abundance of *Lactobacillus* was observed by another study in crop digesta. However, the dominant species was *L. salivarius* (average abundance of 46%) followed by *L. crispatus* with an average abundance of 19% (43). On a wheat diet, three main species dominate in the crop: *L. reuteri* (33%), *L.crispatus* (18.7%) and *L. salivarius* (13.3%) (92). *Lactobacillus* species dominance is a well-known fact in the crop of broiler chickens, and together with the favorable pH conditions, the production of polysaccharides allows the genus, efficient colonization of the stratified squamous epithelium lining of the crop (93). Nevertheless, low abundant species like the ones described above should also be the focus of research considering that they interact with the host and they can also shape the gut environment (94).

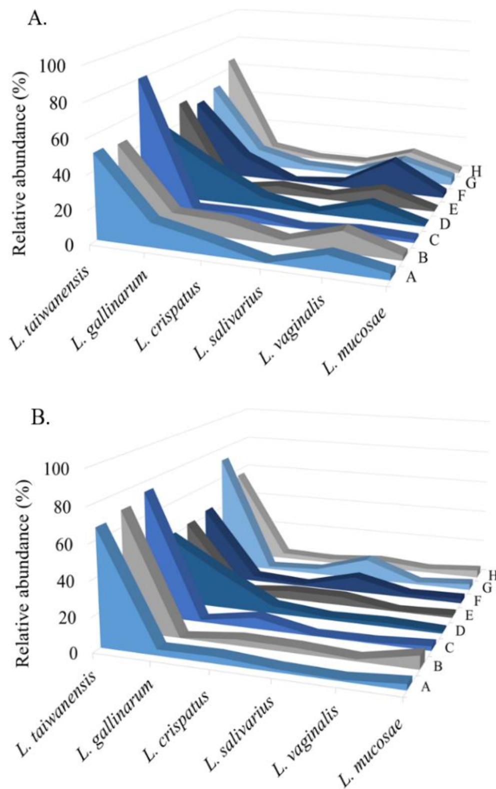


Figure 17. *Lactobacillus* fluctuations in the crop for digesta (A) (left side) and mucosa (B) (right side). This data corresponds to the eight dietary treatments described in chapter 3 (supplementations of Phosphorus, Calcium, and phytase).

Phytase enzymes are usually supplemented in the diet due to the high presence of phytate in plant-based diets, and that broiler chickens do not have the full capabilities to assimilate this compound (95, 96). It has been demonstrated that the presence of the enzyme increases the concentration of compounds assimilated in the GIT tract, like 6-phosphate groups and myo-inositol (96). Not only the release of the P from the phytate, but also Ca complexed in that substrate will be released, and therefore less concentration in the feed will be needed (97). Phytate breakdown activities begin in the crop, and the microbiota plays an important role in it (95). Due to the high dominance of *Lactobacillus* in this GIT section, it has been suggested that *Lactobacillus* species comprises phytase activities which helps in the phytate breakdown. One possible way in which *Lactobacillus* partially degrade the phytate is through the action of acid phosphatase found in *Lactobacillus plantarum* (98). However, further research established the presence of phytase activity in different strains from this genus. *L. salivarius* has been reported as a species with high levels of phytase production (99). Additionally, in the NCBI database, a coding region comprising the phytase gene (accession Nr: LSQY01000000) was found in *L. crispatus* isolated from humans. Species not reported in chapter 3 but tested in the harsh conditions of the intestine, *L. fermentum* produced a tyrosine phosphate like phytase (PTPLP) protein, which based on metagenome surveys is the principal form of phytase in the GIT (100). Other *Lactobacillus* species identified as phytase producers and verified with experimental approaches are *L. brevis* (101), *L. pentosus* (102, 103), and *L. plantarum* (104). This evidence demonstrates that more investigation has to be driven to *Lactobacillus* species of the broiler chicken GIT, to improve our knowledge of enzyme activity under different supplemented conditions. Besides, considering that microbial enzymes are generally more stable at different pH conditions and could react better in the harsh GIT environment, this source became a possible way to reduce the antinutritional effects of phytate through the establishment of bacteria active in phytate degradation (105).

5.4.1.1 Functional predictions in the crop

Several studies proposed the use of functional predictions to explore microbial activities and to obtain a predicted overview of the microbial community functionality (106). Because the reference database used in chapter 3 was SILVA, the only prediction program that could be used was "Tax4Fun" which implement the calculations based on the same database (106). The functional prediction showed that the metabolism comprises 60% of the predicted functions for both digesta and mucosa (Figure 18). Furthermore, carbohydrate metabolism with 16% of contribution was the most representative, followed by amino acid metabolism with 8%. It has been reported that in the crop activities of starch breakdown and lactate fermentation are carried out and facilitated by the presence of the *Lactobacillus* (34, 36, 86). In the predictions, it was found an average percentage of 2.4% starch and sucrose metabolism activities. Stanley *et al.*, (2014) proved that the crop in

contrast to the gizzard promote many fermentation activities which are in line with the great contribution of the carbohydrate metabolism on this study, considering that only 13 functions from 183 resulted in more than 2% contribution (chapter 3).

Particularly, glycolysis and gluconeogenesis, fructose and mannose metabolism, galactose metabolism and pyruvate metabolism were detected with more than 1% in the predictions. These functions are an indication of productive microbial communities in a harsh environment like the crop (107). In regards to environmental information processing (11% of abundance), the ABC transporters were detected which is an indicator of active transport of organic and inorganic molecules (108). In agreement with the communities of the crop in this survey; a metaproteome study revealed that in one supplemented condition with phosphorous, the family Lactobacillaceae was responsible for encoding for ABC transporters (61).

Microbial predictions in the digesta showed a statistical difference between diet C and the others, being the metabolic activities, less detected in diet C, the driven factor. When comparing mucosa and digesta samples, slight changes in the percentages showed significant differences between both ($p \leq 0.05$). These results could be attributed to the dominant presence of *Lactobacillus* species in the crop. Nevertheless, a more sensitive technique is needed to quantify metabolic changes considering that, in the overall percentage was not perceived a marked change and that metabolic events related to the lactic acid metabolism were not represented, being this one of the main activity in the crop (34, 87). A metaproteome study of the crop with supplemented diets with monocalcium phosphate and different concentrations of phytase revealed that proteins are actively represented by the families Lactobacillaceae, Veillonelaceae and Bradyrhizobiaceae (61), and the addition of phytase increases the KO´s ribosome (KO 03010), aminoacyl t-RNA biosynthesis (KO 00970) and ABC transporters (KO 00970) (61). The assumption behind this result was the increase of metabolic activities as a response to a maximized P uptake (61). However, with the current microbial prediction, even if those functions were as well present, no differences were registered confirming that precise techniques are required to more confidently drive conclusions of the impact of the microbiota in the chicken GIT.

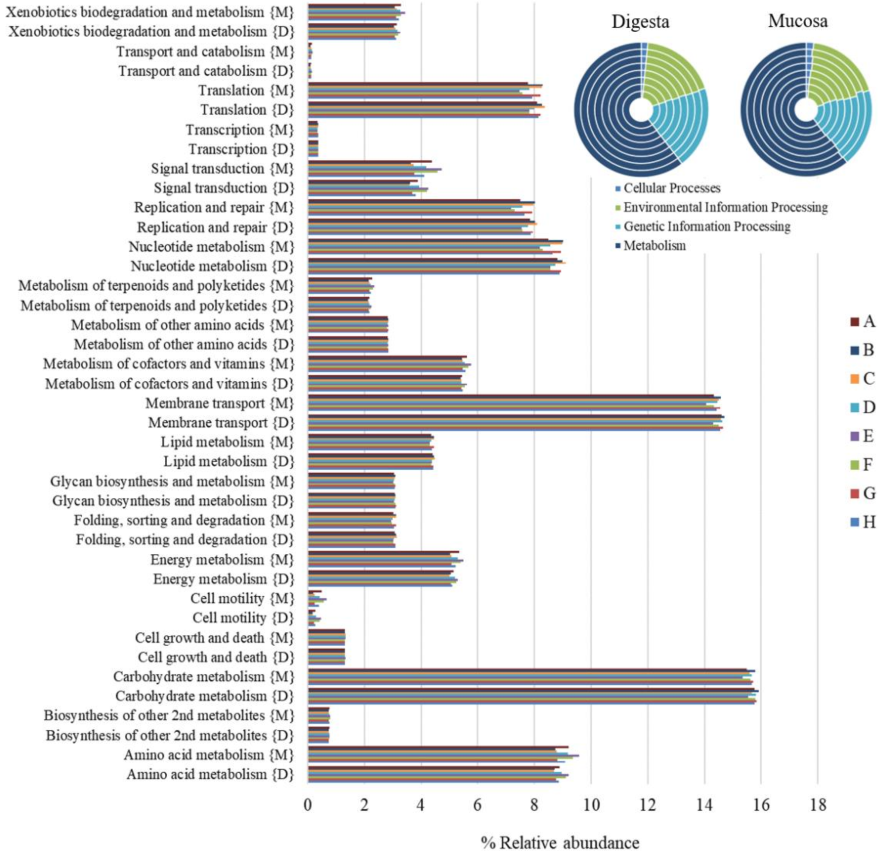


Figure 18. Functional predictions for crop digesta and mucosa based on KEGG Orthology. The first level is indicated with the pie chart (circles); the inner circle corresponds to diet A and following sequence until the outer circle which corresponds to diet H. Second level of classification corresponds to the bar chart and letter indicate D for digesta and M for mucosa.

5.4.2 Ileum

Studying the ileum is of particular interest since it has a direct influence on the immune responses and metabolism of broiler chickens and is where mainly activities of nutrient absorption, including carbohydrates, vitamins, and amino acids, take part (75, 84, 109). This GIT section registered near to 16% of the $InsP_6$ hydrolysis when there is no mineral or enzymatic supplementation to the feed (109). As described for the crop, ileum is also a less diverse microbial environment when compared to the caeca, being this an indicator of highly specialized microorganisms to promote energy acquisition to the host (94). Another characteristic of both environments (crop and ileum) is the presence of facultative anaerobes including the group of lactic acid bacteria (110). The microbial composition in the ileum is more complex when compared to the crop, due to the higher pH (pH 6.1 -6.5), higher availability of nutrients and the, therefore, better establishment of other microbial species (85). Ileum mucosa core microbiota comprises three times more species than the ones in digesta; 137 for the mucosa in comparison to 44 in the digesta (chapter 3) (Figure 19 A and B). Approximately 70% of the species in both digesta and mucosa were belonging to not yet classified bacteria, commonly classified as uncultured. From this percentage sequences assigned to the genera *Clostridium, Bacillus, Ruminococcus, Faecalibacterium,* and *Lactobacillus* were detected. The same *Lactobacillus* species described in the crop were present in the ileum digesta and mucosa. Species shared for both were *Citrobacter farmeri, Clostridium spiriforme, Enterococcus faecalis, Shigella alberti, R. picketti, R. solanacearum* and *Streptococcus alactolyticus*. However, ileum mucosa includes the presence of *Burkholderia ferrariae, B. ginsengisoli, Clostridium lactatifermentans Pseudomonas saccharophila, P. mucidolens, P. oleovorans, Salmonella enterica, Pseudomonas peli.* As an effect of dietary supplementation of phytase, Ptak *et al*., (2015) reported that this treatment increases the presence of *Lactobacillus* in the ileum. This effect was not observed either in the pooled or individual studies. However, the study of Ptak *et* al., (2015) found a reduction of some groups like *Clostridium* spp., and *Enterococcus* spp., and higher colonization of *Lactobacillus* (97). This effect was clearly seen in the digesta samples supplemented with only Ca, where a comparison with the other treatments revealed no presence of the genera *Streptococcus* or the uncultured Peptostreptococcaceae. The mechanisms behind this Ca influence are the higher complexed compounds with ion bonded, that can display antinutritional effects in the lumen, alongside with its influence in some bacterial groups to a better adhesion, having a direct effect in the microbial community resemblance (97, 111, 112). Moreover, the digesta being the source of higher concentration of supplemented Ca probably increased that effect when compared to the mucosa. In the case of P in the digesta, it is known that its availability in the lumen promotes *Bacteroides, Prevotella, Porphyromonas, Clostridium coccoides* and *Clostridium leptum* (113), which is in accordance with the results obtained in chapter 3 where an increase of *Streptococcus* was observed.

A.

B.

C.

Figure 19. Intersection matrix for the core microbiota at species level found in the ileum for study chapter 3 ((A) digesta (D); and (B) mucosa (M)) and ileum digesta from study chapter 4 (C). The nomenclature of the diets corresponds to the abbreviation used on those studies.

In a digesta study with pooled samples, the ileum from chickens fed a corn-soybean meal based diet was dominated by *Lactobacillus*, with approximately 99% of abundance (43). This complete dominance was not achieved with the outcome obtained for the protease study with pooled digesta samples (chapter 4); however, also higher percentage were obtained for *Lactobacillus* genus accounting for 77% of abundance. In the individual sample study (chapter 3) in digesta samples, *Lactobacillus* genus achieved in one diet high percentages (approx. 83%). Meanwhile, in the mucosa, this dominance was reduced with *Lactobacillus* achieving the maximum of 37% abundance. This result shows the importance of studying individual samples because, by pooling samples, the effect of diet supplementation is no longer observed (43). However, if a sample must be analyzed using different approaches, such as microbiota, performance, digestibility, bone mineralization, genetic traits, among others, and the results must be correlated, it is important to use the same homogenized sample.

Pooled studies are designed to address deductions based on the treatment effect. Therefore, the study in chapter 4 was done analyzing microbiota from pooled samples, so that was possible to establish if there was a correlation between the data coming from performance and amino acid digestibility. Nevertheless, due to the variability of the individuals previously stated, it is recommended that microbiota studies focus more on changes obtained with individual broiler chickens. It was proposed that sequencing of pooled bacterial samples can be considered as a cost-effective approach when studying population at the genetic level and their differences between bacterial strains (114). Even if Next Generation Sequencing (NGS) has reduced the costs significantly in the last decade, is still considered expensive in population-level surveys (114). Choosing a specific region such as the 16Sr RNA gene, which is well conserved, diminished the problem of pooling samples in limitations like loss of linkage information and sequencing errors (114). The proportion of unassigned species was closely similar for the individual sample study (chapter 3) with 68% and the pooled sample study (chapter 4) with 61%. Differences were seen based on pooling or not the sample, considering that significant differences were only detected until genus level in the study from chapter 4, whereas in chapter 3 differences at species level were possible to be calculated. The total number of species found in individual samples were 279 while in the pooled study it was reduced to 172. In the pooled study was not found a high presence of unique species like in diet A, C, D, F and G as it diminishes the individual effect (Figure 19 A and C). As expected with this finding twice of the sequence number was assigned to the core microbiome of the pooled study (31%),

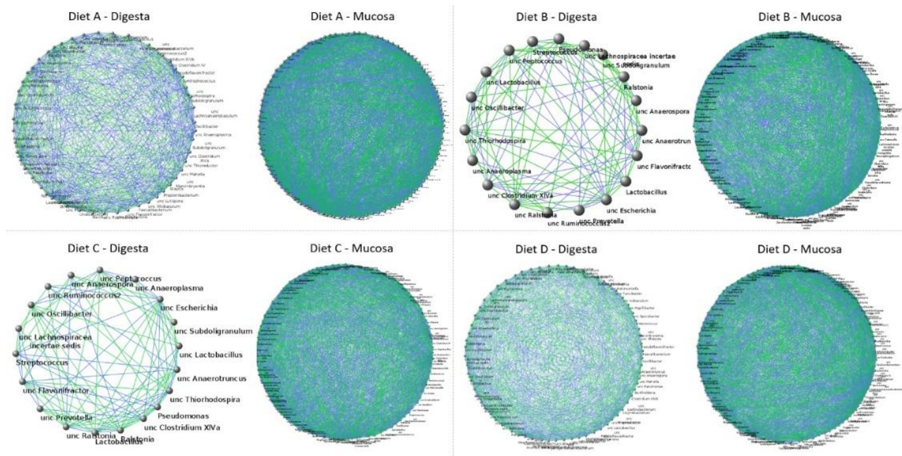
while only 16% of the sequences were considered as core microbiome in the individual study. A study using T-RFLP also reported that the number of T-RFs for individual samples was nearly twice as high as for the pooled samples (115). Thus, care should be taken when addressing conclusions of diversity in pooled studies because a loss of variation among samples is obtained, which only leads to a description of the overall community (115).

Proteases are included in the diets to increase energy and protein digestibility in broiler chicken production, through the reduction of the anti-nutritive effects of non-starch polysaccharides (NSP) present in the diet (116). Furthermore, this enzyme could improve the passage rate and the nutrition digestion rate (117). The ileum has been studied due to the high impact of the enzyme supplementation on this GIT section where an increase in protein and amino acid digestibility are registered (116, 118). As a result of the modifications in nutrients availability and probably biochemical changes in the lumen, direct effects of the proteases in the microbiota are expected (116). Unfortunately, not many studies have addressed these impacts. A study in ileum and caecum demonstrated that the addition of protease might increase the presence of *Lactobacillus* spp. at the expense of *Clostridium perfringens*; still, only specific groups of bacteria were targeted (119). Feed supplemented with a mix of the protease with amylase, cellulase, xylanase, and glucoamylase, showed an increase of beneficial bacteria in the caeca, such as *Megamonas,* with genes encoding for carbohydrate degrading enzymes and *Bacteroides* with enzymes that degrade cellobiose and xylan (117). In chapter 4 was confirmed the dominance of *Lactobacillus*, ranging from 38 to 77%. *Streptococcus* another common genus found in the ileum at mature ages in broiler chickens is described with abundances between 6 to 14% (97, 120, 121) and the same diets with low abundance of *Lactobacillus* are the ones promoting its presence. Those lactic acid bacteria are reported to resist the presence of gastric acid and to adhere to the colonic mucosa while reducing the putrefactive fermentation-like products and the colonization of pathogenic species (122). The source of the enzyme influences not only the protein and amino acid digestibility but also the interaction with the microbiota (chapter 4). Indeed, feed enzymes can reduce microbial activities in the ileum, probably interfering with the concentration of substrate accessible for bacterial fermentation (123).

5.4.2.1 Microbial networks in the ileum

Microbial networks were built based on co-occurrence patterns to evaluate if the level of connectivity could be influenced by the addition of the different protease enzymes. Usually, microbial community studies involve the alpha-diversity analysis, focusing on the total number of taxa in an environment or the beta-diversity, studying relative abundance among different environments (124). However not much attention has been addressed to

document interactions between taxa and since microbes do not stay alone in complex environments like the GIT, many complex ecological networks are expected (125). The co-occurrence patterns could reproduce processes in the GIT like co-existence and maintenance within the microbes (126). Each node of the network represents a specific genus and neighborhood connectivity is lower when a few nodes are presented. With the exclusion of protease C at low level, there was higher connectivity in the diets when compared to the basal condition (chapter 4). From the data of chapter 3 was observed less connectivity in the digesta supplemented only with either phytase – diet B, calcium – diet C or phosphorous – diet E (Figure 20). Therefore, in the digesta at both individual or pooled samples were possible to determine how dietary supplementation influence interactions between microbial communities. In the mucosa similar network topology was observed across all diets, showing a high presence of different genera which leads to a more stable community where more interactions are possible (127). In environments where many microbial taxa remain unknown inter-taxa associations or direct symbioses can be elucidated through network analysis (124); indeed too many unassigned genera were giving the highest values in network parameters calculations like: neighborhood connectivity, betweenness, and topological coefficients, established with assigned genera such as *Lactobacillus, Streptococcus, Clostridium, Bacillus, Ruminococcus* among others. Despite the high dominance in the relative abundance, *Lactobacillus* did not show to be a keystone in the co-occurrence network which is in line with the observations of a human study where low dominant taxa served as hubs in the gut bacterial network (128).



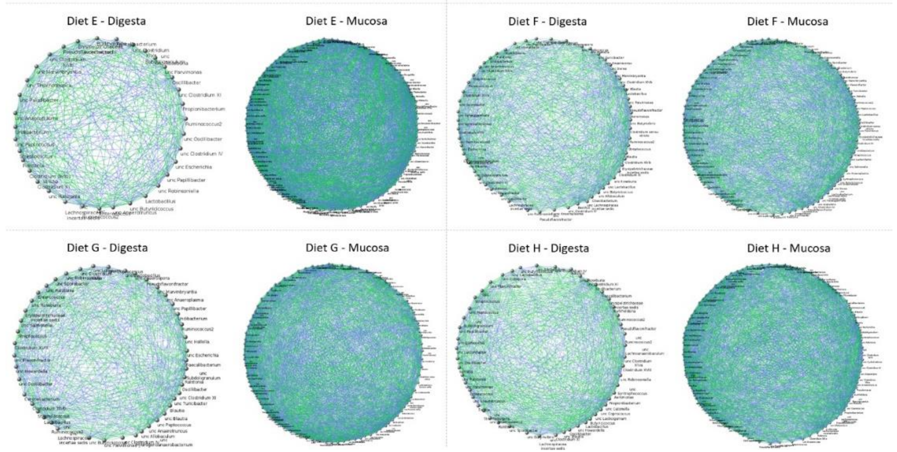


Figure 20. Microbial network at genus level for digesta and mucosa samples in the eight dietary treatments (chapter 3). Significant interactions are indicated by the connective lines (edges) between genus (nodes). Negative and positive interactions are shown in green and blue, respectively.

5.4.2.2 Functional predictions in the ileum

Understanding the metabolic activities of the active microbiota caused by changes in the diet could have a meaningful impact on the health and performance of broiler chickens (84). The predicted functional assignation showed that 60% of the information was related to metabolism activities followed by 20% for information related to environmental information processing. Some differences were detected between digesta and mucosa. While digesta showed 20% of genetic information processing, in the mucosa it decreased to 12%. In mucosa were found two times more of cellular processes. Only ileum mucosa registered shifts between the diets regarding the cellular processes and genetic information processes. The profile of ileum digesta in chapter 3 and chapter 4 studies was similar, but no statistical differences were found in the diets pairwise comparison of protease supplementation.

As shown with abundance data of digesta samples, functional predictions revealed that diet E was significantly different from diets B, G and H. The functions responsible for this separation were at first classification level, amino acid metabolism, glycan biosynthesis, and a more in-depth classification includes the two-component regulatory system. Mainly, this regulatory system allows a response to environmental conditions due to the presence of a sensor kinase and a response regulator, which are in charge to modulate gene

expression (18); therefore supplementation of only P in the diet might serve as a potent stimulus to change the system. More information for the biosynthesis of phenylalanine, tyrosine, and tryptophan, together with glycan degradation was also detected showing the significant influence of P in the diet. Ca supplemented diets promote the lipid metabolism, nucleotide metabolism, and carbohydrate metabolism, and, at the deepest classification level, pyrimidine and purine metabolism, glycolysis and gluconeogenesis and glycerophospholipid metabolism. Together with phosphotransferase system showed evidence of altered status influenced by the dietary treatment (110, 129). The higher presence of *Lactobacillus* species in diet C and G showed the effect of this genus in the metabolic activities of the ileum content. The presence of P in the diet E promotes the establishment of other microorganisms such as *S. alactolyticus* and *Clostridium* XI, also previously reported in the ileum (18, 97).

The predicted functions of mucosal samples revealed a trend in the comparison between the diets (p=0.06) (Figure 21). In this case, the pairwise comparison showed that the presence of P in the diet caused the significant difference, where E, F, and G were different from not P supplemented diets (A and C) and this effect was seen in the microbial community. From the relative abundance information, diets F and G showed a high presence of *L. taiwanensis* and *L. gallinarum*, while E comprised more *S. alactolyticus*. In contrast, diets A and C included more information on OTUs from the family *Burkholderiaceae* specifically an OTU assigned to an uncultured *Ralstonia*. This change in abundance was reflected in the predicted functions where diets without P increased the cell motility, energy metabolism and signal transduction and metabolism of amino acids. Mainly amino acid metabolism has been related to the downstream synthesis of short chain fatty acids (110, 130), and exploring the values of butanoate metabolism, diet F and G had lower values (1% diets with P vs. 0.8% in F and G), probably causing less activity in the amino acids. Meanwhile, diets F and G, with P addition, were more abundant for carbohydrate metabolism, membrane transport, nucleotide metabolism, and translation. Phosphotransferase system, ABC transporters were more detected and are referred to alterations in the diet and the energy metabolism (110, 129). Moreover, F and G also had an increase in glycolysis and gluconeogenesis, galactose, starch, and sucrose metabolism. These related functions suggest a higher metabolic activity coupled to energy production when there is the presence of phosphorous in the diet.

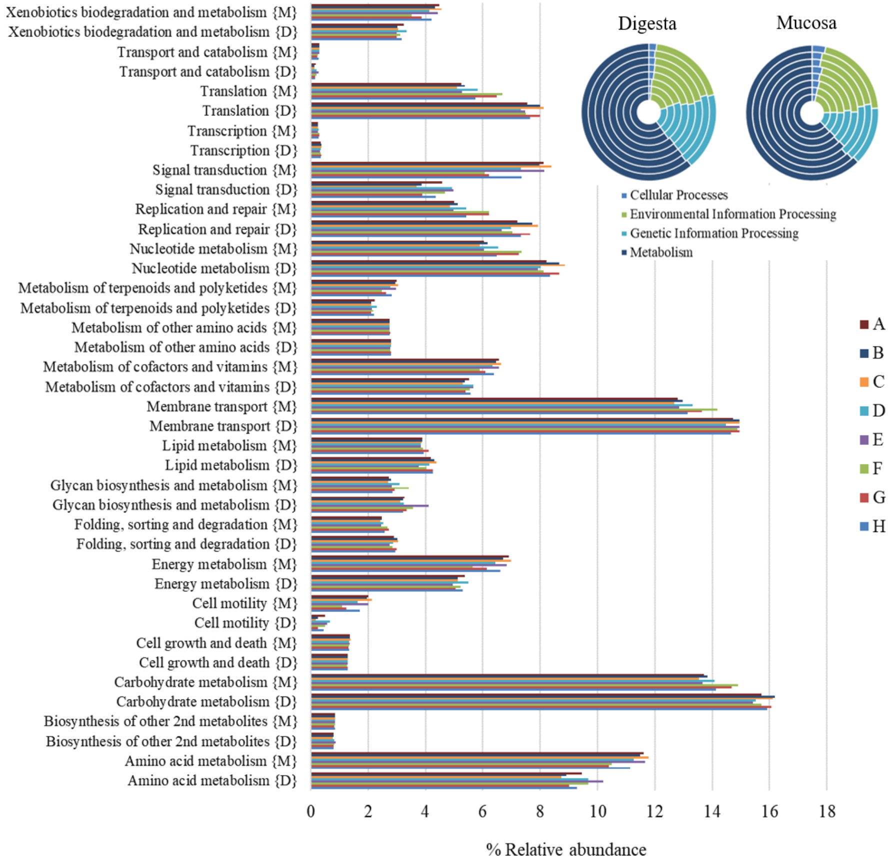


Figure 21. Functional predictions for ileum digesta and mucosa based on KEGG Orthology. The First level is indicated with the pie chart (circles); the inner circle corresponds to diet A and following sequence until the outer circle which corresponds to diet H. Second level of classification corresponds to the bar chart and letter indicate D for digesta and M for mucosa.

5.4.3 Caeca

In the caeca, 144 species were detected in the mucosa and 109 in the digesta (Figure 22 A and B), a higher number compared to crop and ileum. This result is in agreement with other studies that showed the caeca as the most extensive reservoir of bacteria, where it is found the lowest similarity consistency when compared with other GIT sections either with DNA based approaches (29, 34, 86) or metaproteomic (61). In the caeca, more species are shared within the diets than with the other environments. The higher diversity could be due to higher pH (5.7 to 7.0) and extended retention times (119 min in the caeca, 97 min in the ileum and 31 min in the crop) (131). A possible implication of less diversity in the crop and ileum could be related to the promotion of energy to the host, while higher species richness in the caeca could be related to an efficient assimilation of limiting resources and better enzymatic capabilities to degrade complex polysaccharides, uric acid, starch and cellulose (18, 94). So, it is suggested that caeca being a more diverse environment could have more characteristics to be resilient, where influences due to environmental stress will not affect the equilibrium state (110).

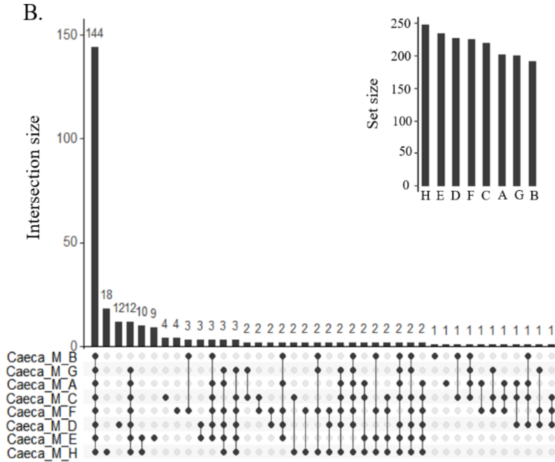


Figure 22. Intersection matrix for the core microbiota at species level found in the caeca for digesta (A) and mucosa (B). This data corresponds to the eight dietary treatments described in chapter 3 (supplementations of Phosphorus, Calcium, and phytase).

Firmicutes was a dominant phylum in the caeca digesta and mucosa, on average abundance around 70%, which is lower than previously published in other studies (17, 18, 86, 110). Though, it is reported in those studies that the following most abundant phyla were either Proteobacteria or Bacteroidetes with abundances between 0.5 to 20%. In chapter 3 the second most abundant phylum was Tenericutes with 29% in the digesta and in 22% in the mucosa, except on the mucosa of the phytase treatment where it was less abundant, followed by Proteobacteria 0.82% in digesta and 4% in the mucosa. Tenericutes has been detected in sequences of avian genome survey where chicken, turkey, and penguin were the related hosts (132). Also, it was present in the digesta of chicken supplemented with monocalcium phosphate and phytase with an abundance of 1-5% (43), and in less than 2% of total abundance in chicken with or without supplementation of mannan oligosaccharide (133). Anaeroplasmataceae was the most representative family of this phylum; however, no information regarding the specific function of it in the metabolism of chicken can be found in the literature. In rats, a diet rich in pectin promotes its presence (134), and in the rumen is associated to bacteriolytic activities (121); meanwhile, in chapter three it was described to have a negative correlation among other families.

Another characteristic of the data of chapter 3 was the higher percentage of sequences related to uncultured species (approx. 75%) and those mainly representing strict anaerobes (50). These sequences were assigned to several genera: *Acetivibrio, Anaerococcus, Bacillus, Blautia, Butyricicoccus, Clostridium, Lactobacillus, Oscillibacter,*

Paenibacillus, Ralstonia, Ruminococcus, and *Syntrophomonas*. Six species of *Lactobacillus* were detected (*L. crispatus, L. helveticus, L. taiwanensis, L. mucosae L. vaginalis* and *L. salivarius*); however, this genus was less abundant than in crop and ileum which is in accordance with other studies (43, 110) (18). One impact of the reduction of *Lactobacillus* in the caeca is the production of more SCFA since this genus is negatively correlated with that activity (94).

Only two species of *Clostridium* were confidently assigned to a species name (*C. lactatifermentans* and *C. spiriforme*) may be due to a weak characterization in the databases of this genus and the difficulty of isolating the strains, resulting in the high amount of sequences identified as uncultured or unclassified *Clostridium*. The essential features from this microorganisms rely on metabolic cross-feeding fermentation of metabolites that impact the presence of other species (94). Besides those, other common species were *R. torques, S. enterica,* and *S. alactolyticus. Ruminococcus* promotes feed efficiency and has many metabolic capabilities such as the assimilation of complex carbohydrates (94). Synthesis of butyrate, an important energy source of colonocytes, is attributed to the presence of *Ruminococcus* and *Clostridium* (86, 94). A high proportion of uncultured Ruminococcaceae and uncultured Lachnospiraceae were found in the caeca digesta and mucosa; however, the former family was higher in digesta and the latter in the mucosa. Besides, an increase of uncultured Lachnospiraceae was reported in the digesta, when P was added. Those two families have been found with positive effects to the broiler chickens since they stimulate the production of fatty acids, amino acid, and vitamins (94). Due to the production of mucosal polysaccharides species such as *Pseudomonas mucidolens* and *P. peli* were detected in the caeca. Unique species present in the mucosa were *Burkholderia ginsengisoli, Citrobacter farmer, Shigella disenteriae, Pantoea ali* and *Undibacterium oligocarboniphilum*. Other studies revealed different genera in lower abundance. As an example, one survey performed on chickens from different geographical regions detected *Turicibacter* in low abundance (94). Also, broilers with 42 days housed in standard commercial conditions showed a high prevalence of the genus *Megamonas* (135). Therefore, house conditions and probably the lack of maternal microbiota transfer could be essential factors determining shifts in general microbial community distribution (60, 94).

5.4.3.1 Functional predictions in the caeca

The functional prediction revealed a similar pattern as in the other two GIT sections (ileum and crop). Metabolism registered 60% of abundance, followed by environmental information processing, genetic information with approximately 20%, and cellular processes with approximately 4% (Figure 23). Moreover, as shown in luminal contents between ileum and caeca by Mohd-Shaufi *et al*., (2015), significant differences in the GIT

sections were also obtained in the present study. A significant difference between both types of sample (digesta and mucosa) were revealed, due to the higher abundance in the digesta of the ABC transporters, phosphotransferase system, aminoacyl-tRNA biosynthesis, glycolysis and gluconeogenesis, galactose metabolism and amino-sugar metabolism. The amino-sugar metabolism is related to the breaking down activities in the feed (probably due to the direct contact with the substrate in digesta) and to produce amino acids and peptides (18). On the other hand, in the mucosa were more abundant the two-component systems, probably due to higher sensing of stimuli for global responses in this environment (18, 136), arginine and proline metabolism, nitrogen metabolism, glyoxylate and dicarboxylate metabolism and lipopolysaccharide biosynthesis.

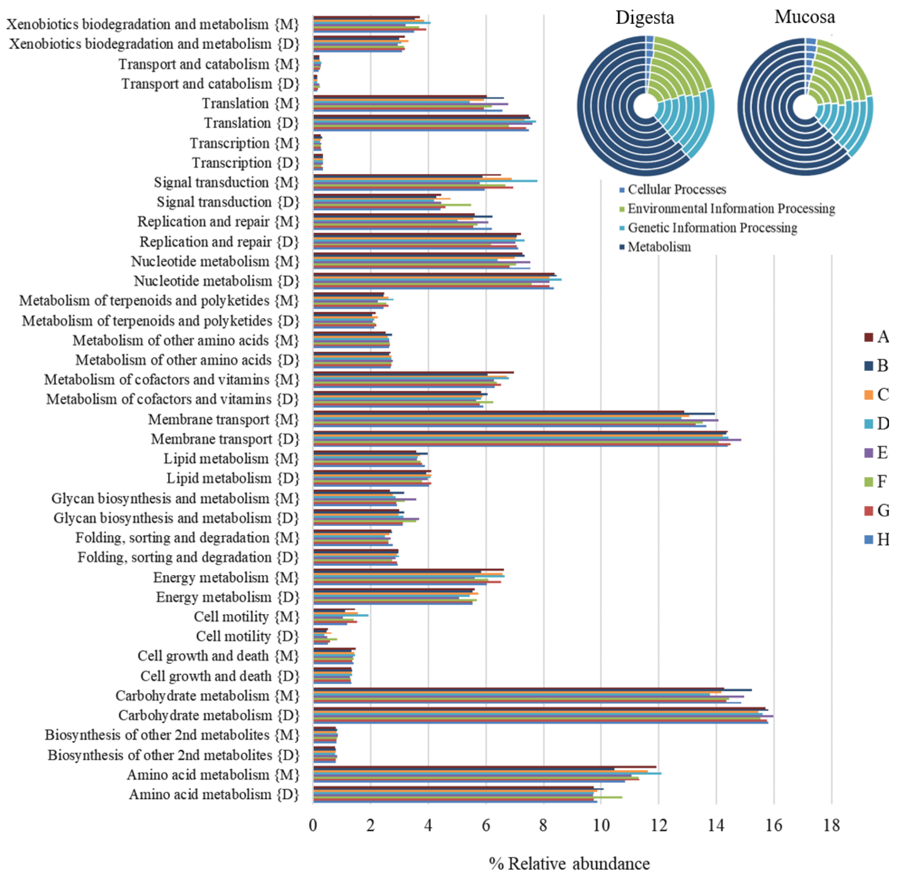


Figure 23. Functional predictions for Caeca digesta and mucosa based on KEGG Orthology. The first level is indicated with the pie chart (circles); the inner circle corresponds to diet A and following sequence until the outer circle which corresponds to diet H. Second level of classification corresponds to the bar chart and letter indicate D for digesta and M for mucosa.

Specifically, in the digesta only diets B and D (not supplemented with P) were different from diet F (with P), as observed with the sequencing dataset where this supplementation was causing a different distribution in the microbial communities. In both types of samples, diet F was more abundant in two-component system information, crucial as well for increasing the colonization in the GIT (18), and in the phenylalanine, tyrosine and tryptophan metabolism, nitrogen metabolism and lipopolysaccharide biosynthesis. Diets B and D increase the presence of ABC transporters, indicative of an altered status of diet and energy metabolism (110), purine metabolism, influencing the presence of the substrate for deoxyribonucleic acid derivatives (18), aminoacyl-tRNA biosynthesis, starch and sucrose metabolism and glycolysis and gluconeogenesis. Probably this difference was caused by the presence of OTUs assigned to Lachnospiraceae and Erysipelotrichaceae that were more abundant with phosphorous supplementation. This phosphorous effect was confirmed in the mucosa samples with the functional prediction with significant differences obtained between diets C and D (not supplemented) in comparison to the diet E (supplemented). Diet E promotes the functions ABC transporters, purine metabolism, aminoacyl-tRNA biosynthesis, starch and sucrose metabolism and glycolysis and gluconeogenesis which is differing from the digesta profile. Moreover, with sequencing data, the main difference was observed with the high abundance of OTUs assigned to the family Erysipelotrichaceae and OTUs related to *Faecalibacterium.* The referred *Faecalibacterium* is commonly found in the caeca of broiler chickens and is suggested to improve the immune status of the host protection against pathogens (137). In line with the influence of P on this study, a proteomic study revealed that mineral P supplemented in the diet caused a grouping in the overall profile of proteins that are separated from treatments without P (61).

Thus, a pattern was observed, where changes in the diet modify taxonomic composition, but also potential functions. Such a result was also obtained in a chicken trial supplemented with mannan-oligosaccharides when compared to the basal condition (133) and in association with residual feed intake with chickens from different geographical locations (94). Even if some associations can be predicted, it is difficult to interpret functional capabilities in the three GIT section and the two environments (digesta and mucosa). Small changes in assigned predictions do not elucidate if there is a high or low impact in the addition of supplementation. This fact might be related to the high amount of unclassified sequences in the three environments which underestimate the metabolic potential (2). Not specific functions are obtained through prediction methodology, meaning that only the

three levels of the KEGG orthology are given as output. This fact could lead in this study to similar profiles in the three GIT sections. Moreover, it must be considered that most cited prediction algorithms, PICRUSt (138) and Tax4Fun (106), they are validated in human microbiome, mammalian guts, and environments like soil and hypersaline microbial mat. However, in chicken, there is still not a predictive tool, and no validation is carried out in this animal model; thus, it is expected that prediction implies bias and not accurate information. As a consequence also differences in ABC transporters and DNA repair, and recombination proteins, the two-component system, purine metabolism and ribosome are obtained in completely different environments like the intestine of shrimp (139) or catfish (140).

5.5 Outlook: An eye on the metagenomic information

Caeca mucosa and digesta samples derived from the animal experiment of chapter 3 were analyzed with metagenomics approach. The covered treatments were: control with no supplementation (diet A), supplementation with only calcium (diet C) and with only phosphorous (diet E). It is important to highlight that up to date only three studies have been published based on the chicken caeca digesta metagenome (2). Metagenome sequencing gives information about the alteration in gene abundance promoted by for example an altered diet and can support information obtained by 16S rRNA gene regarding taxonomy while validating if functional predictions correspond to reality. Regarding the methodology, the DNA was fragmented by enzymatic tagmentation, and metagenome library was prepared with Illumina Nextera and further sequenced in the Illumina NextSeq platform, with sequencing length of 150 base pairs. The bioinformatic analysis included a quality trimming and length filtering with PRINSEQ (141), and the taxonomy comparison was against the non-redundant database (nrDB) from the NCBI. Two filters were considered due to the expected high information from host sequences. First, a taxonomic and functional classification was done based on the diamond algorithm (142) with a taxonomic and functional placement using the LCA algorithm visualized in MEGAN6 (143). With this information reads assigned to Bacteria domain were extracted and submitted to the MG-RAST pipeline for taxonomy classification with RefSeq and KEGG Orthology (KO). In the mucosa sequenced samples, the number of workable reads was very low, which shows how challenging is to work with samples with cellular content from the host. Filtering steps have to be included during DNA extraction and in the bioinformatics analysis because background noise with host information is considerably high compared to the desired microbial information.

The resulting data were standardized for statistical comparison, and multivariate analysis was done with Primer7. Significant differences at functional categorization and based on KOs were observed between digesta and mucosa samples ($p = 0.001$), and the samples

were clustering based on the source of the sample, one group with digesta samples and another with mucosa samples (Figure 24 A and B). In the mucosa was observed the less percentage of similarity, where both supplemented diets shared 73% with the control while the most similar values were found in the digesta samples with 93% similarity within the supplemented diets.

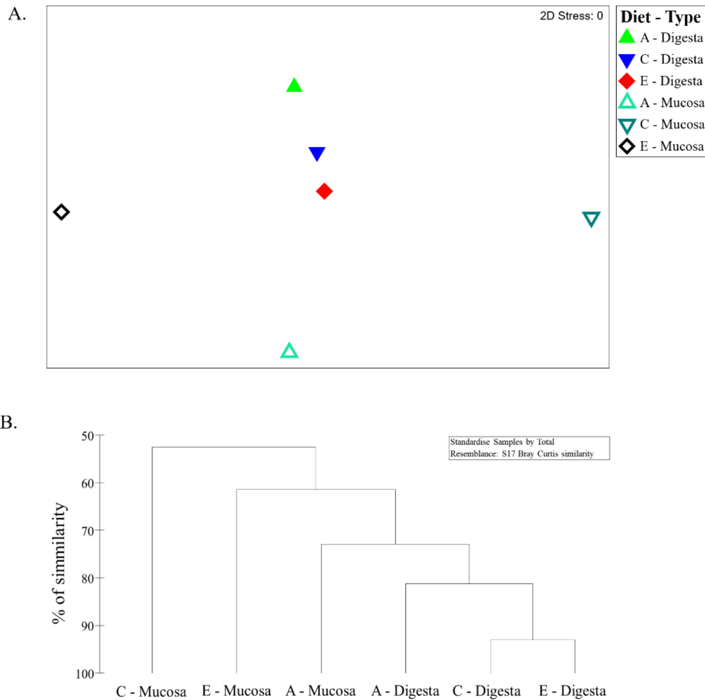


Figure 24. Clustering visualization for digesta and mucosa samples based on metagenomic information assigned with KEGG Orthology groups. (A) Non-Metrical Multidimensional Scaling (nMDS) (B) cluster analysis based on similarity percentage.

The taxonomical composition of the metagenomes revealed a dominance of the phylum Firmicutes with abundance between 75-84%, followed by Proteobacteria and Bacteroidetes with average percentages of 6 and 5% respectively and in lower abundance was Actinobacteria, Fusobacteria, and Tenericutes (3%) (Figure 25). This data confirms the results obtained with target amplicon sequencing in chapter 3. In abundance lower than 1%, there were found reads representative of Eukaryota, Archaea, and viruses as seen in other metagenome studies (135). At phylum level, a low percentage of unknown reads for

both environments was obtained which is in line with other caeca metagenome studies (144), (145); however, the same result was not demonstrated by Sergeant et al., (2014) (135). Additionally, at both phylum and genus level, significant differences were revealed between digesta and mucosa samples ($p < 0.05$) being also in accordance with chapter 3 and findings from human studies, where distinct profiles of bacterial taxa result from the two environments (146). The further statistical difference was inferred, with 10% of confidence, regarding the diets in digesta samples ($p = 0.099$). *Ruminococcus, Bacteroides, Eubacterium, Faecalibacterium*, and *Subdoligranulum* were more abundant in the digesta of animal fed with supplemented diets; while in the control increased the presence of *Bacillus* and *Streptococcus*. Concerning the mucosa samples, clear differences were established only at phylum level ($p = 0.04$). As shown in Figure 25 (A), the differences were mainly represented by the increase of abundance in diet C of the phylum Proteobacteria (11% vs. 5% in diet A and E) and higher percentage of genera accounting for less than 1% of abundance (46% diet C vs. 40% diet A and 36% diet E). A possible reason for the higher presence of Proteobacteria is that higher availability of Ca could enhance the presence of the protein domain cadherin (147). This domain has been described with adhesion functionality, and therefore, attachment to the mucosa could be improved (147). In the colonic mucosa of pigs fed with high calcium diet, it was observed changes in gene expression and their correlation with the phyla Bacteroidetes, Firmicutes, and Proteobacteria (148).

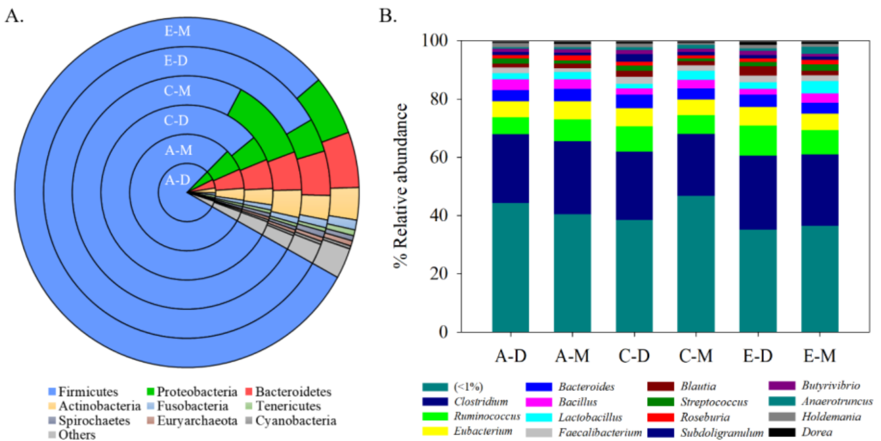


Figure 25. Taxonomical composition based on RefSeq assignation, for the metagenomes in digesta and mucosa samples including diet A (control), diet C (Ca supplementation) and diet E (P supplementation). (A) phylum level (the first letter corresponds to the diet and second letter to D for digesta and M for mucosa) and (B) genera level.

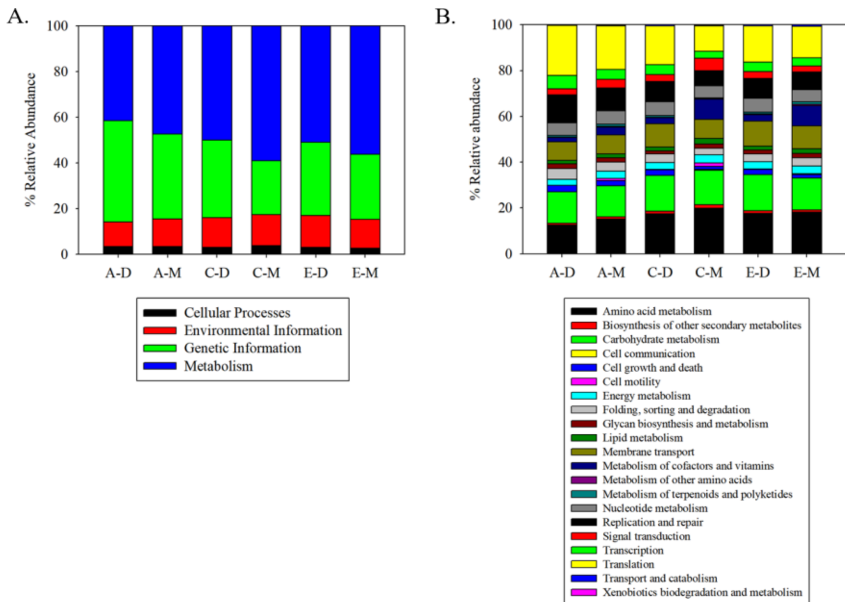
At the functional level, there was a statistical difference between digesta and mucosa (p = 0.0001). In the control the metabolism increased in the mucosa 47% vs. 40%, while genetic information processing is more represented in the digesta (Figure 26). Only one study in lambs was found comparing functional information from metagenome sequencing between both types of samples, and from the top ten most abundant microbial functions were displayed different capabilities (149). In accordance with them, in the caeca digesta were registered more activity of glycolysis (18% in digesta vs. 15% in mucosa). However, differences were seen in butanoate metabolism, wherein lambs the digesta showed higher abundance; while chickens obtained 1% in mucosa vs. 0.3% in digesta. Starch degradation was present in both types of samples in chickens but only depicted in the digesta of lambs.

Figure 26. Visualization with Krona tool of metagenome sequencing from control diets based on KEGG Orthology (KO) assignation (150).

Within the digesta samples, a significant difference was also obtained between the three diets (p = 0.0331). Metabolism information, essential to microbial activities because it promotes the building blocks and the energy source to the cells (151), registered 50% of abundant genes in the supplemented diets while in control is reduced to 40% (Figure 27). Furthermore, the control comprised more information for genetic and information processing (44%) when compared to the supplemented diets (10%). A possible explanation for more genes encoding for the metabolism in the supplemented diets might be that higher concentration of minerals activates the metabolic flux; while the absence, represented by the control, improve the gene expression of different transcription factors which increases the abundance of genes related to genetic information (151). Similar abundances were

observed in all the diets regarding the environmental and information processing and cellular processes. Deeper in the classification, diet C, and E registered an increase of amino acid metabolism (17%) and carbohydrate metabolism (15.7%), compared to the control (12.4% and 13.7% respectively).

The caeca microbiota is crucial in the polysaccharide metabolism because it improves the chicken metabolism, and it has been reported in abundances around 20% of the total genes (135, 144). Membrane transports and metabolism of cofactor and vitamins were detected in higher abundance in diets C and E, which is an indication of a higher stimulus for extracellular and intracellular signals in the presence of the minerals (151). The control had more information for replication and repair, translation and transcription. The most represented genes in the last level of KOs classification were involved in the catalysis of the transcription of DNA into RNA, and those were highly represented in the control diet, which could be an indication of greater bacterial turnover and replication (146). The carbohydrate metabolism included genes related to the pyruvate metabolism, starch and sucrose metabolism, pentose phosphate pathway and amino sugar and nucleotide metabolism, all with similar abundances across the diets. Regarding the amino acid metabolism more genes encoding for tyrosine, alanine, aspartate, glutamate, glycine-serine were detected, and threonine and cysteine methionine metabolism were predominantly present in the supplemented diets.



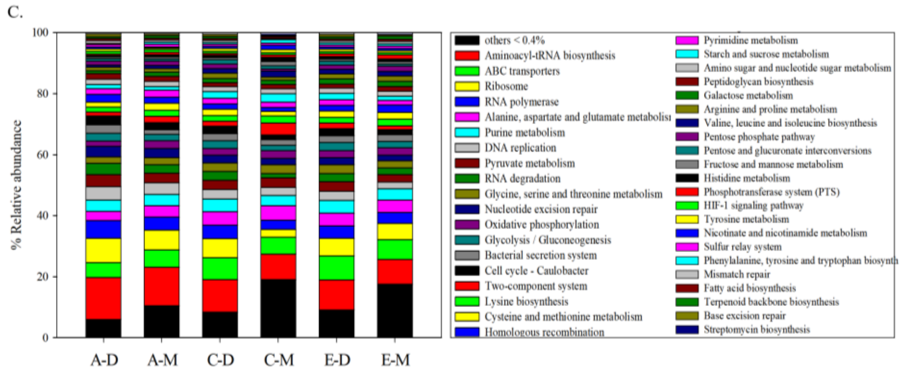


Figure 27. Metagenome sequencing data for caeca digesta (D) and mucosa (M) based on KEGG Orthology (KO) assignations. Diets A (control), C (supplemented with Calcium) and E (supplemented with Phosphorus). Figures (A) first level; (B) second level (C) third level.

Phosphatases appeared in relative abundance between 0.3 to $2x10^{-5}$ %, and most of the assigned functions were present in the phosphorous supplemented diet. Additionally, these enzymes were involved in the energy metabolism, glycan biosynthesis and metabolism, carbohydrate metabolism and nitrogen metabolism. Only one phytase enzyme was found in low abundance in diet C, with calcium supplementation ($7,71x10^{-5}$%), and it was identified as 4-phytase/acid phosphatase [EC:3.1.3.26 3.1.3.2]. Calcium signaling pathway involved in the inner presence of Ca inside the cell (152), was highly present in the control diet (0.007%) in comparison to diet C and E (approximately 0.004%). The nitrogen metabolism was more abundant in the supplemented diets (C: 0.023% and E: 0.014%) than in control (0.008%). This overview shows the impact of supplementing minerals in the diets, as they affect the interaction between the microbial communities in the digesta and increase the metabolic activities.

The pathway of Crotonoyl-CoA to Butyryl-CoA, the enzyme 3-hidroxybutyril CoA dehydrogenase (135), related to butyrate production was more present in supplemented diets (0.054%) in comparison to control diet (0.035%). The enzyme butyrate kinase involved in the reaction of Butyryl-CoA to butyrate was also predominant in the supplemented diets (0.02% vs. 0.009% in the control diet), as well as the enzyme phosphate butyryl transferase (C and E with 0.01% vs. 0.005% in control). Only two genes involved in the acetogenesis of acetyl-CoA synthase, known to reduce CO_2 to acetyl- CoA, were detected (135). Sergeant et al., 2014 identify a possible hydrogen sink in the caecal

metagenome; however, this large subunit was not detected on this study, possibly due to the methodological approach including the prediction of functional profiles based on Hidden Markov Models or the chosen sequencing platform. Also, the study from Sergeant and colleagues (2014) determined antibiotic resistance genes against tetracycline and bacitracin in the caeca metagenome, however in this study only an antibiotic transport system and bacitracin transport system was found in very low abundance.

In the metagenomics sequences of the mucosa, a clear significant difference was not observed in the data at the functional level. Only considering 10% of confidence, at the third level of classification of the KOs, a p-value of 0.084 was obtained (Figure 27). Similar to digesta samples, genetic information processing is more abundant in diet A with 37%, when compared to 23% in diet C and 28% in diet E. Cellular processes and environmental information processing were similar in all the supplementations, with average values of 12.7% and 3.2% respectively. Although no significant difference was determined between the diets in the second level of KOs classification, it was observed an increase of information for diet A in cellular processes including; translation, replication and repair, transcription, folding and sorting and degradation. In the diet C increased the presence of metabolism of cofactors and vitamins, amino acid metabolism and lipid metabolism, exposing the high metabolic activities which were also observed in the digesta.

Moreover, at the second level of KO´s comparing between digesta and mucosa, it was observed, with more than 10% of abundance, a similar distribution of amino acid metabolism, carbohydrate metabolism, and translation is obtained. With more than 5% of abundance, it is found similarities in digesta and mucosa for the classified functions: membrane transport, nucleotide metabolism, replication, end repair. Different from the digesta, the supplemented diets from the mucosa (C and E), had more information related to metabolism of cofactors and vitamins.

With the metagenomic approach, it can be concluded that mineral phosphorous and calcium affect the distribution of microbial communities and gene abundance. This finding was also obtained in a chicken metaproteome study where mineral phosphorus supplementation was driving more an effect rather than phytase enzyme addition (61). As future perspectives could be expected that other phenotypic approaches including metatranscriptomics and metabolomics bring new information to further proceed in the data interpretation and improve bird health and performance.

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CHAPTER VI

SUMMARY

6. SUMMARY

Broiler chicken, as an established model organism for non-mammalian species with a sequenced and publicly available genome, represents an excellent case-study to elucidate the inter-communication between the host and its microbial communities. The understanding of microbial ecology alongside with the study of the interactions among the microbes and their leading roles provides the opportunity to look for alternatives to increase efficiency of nutrient assimilation in chickens. Sequencing technologies have taken us to a step in which obtaining information from the microbial ecosystem is the beginning of a process to elucidate which are the main contributors and their primary functions. Now, the challenge is to give sense to this massive amount of information and to establish appropriate pipelines to treat the obtained sequences quickly and precisely and to interpret the results in a biological context. Therefore, the general aim of this thesis was to describe the changes in bacterial community structure that occurred in chickens, in response to different experimental diets.

Thus, an update of the state of the art research of the chicken gastrointestinal (GIT) microbiota was done in chapter 2. The composition and functionality are described through the most recent technologies that provide taxonomic information at DNA level using 16S rRNA genes. Gene catalogues and their abundance are deciphered through shotgun metagenome sequencing, which is still at its infancy and only eight publications have been published so far. At the protein level, only two studies were found that contribute metaproteomic information. Thanks to these technologies many studies were able to focus on answering how feed supplementations altered the microbes in GIT sections, including crop, proventriculus, gizzard, jejunum, ileum, caeca, and feces. Feed additives comprise enzymes or mixtures of them, as well as organic acids, minerals, prebiotics, probiotics and synbiotics (as the combined effects from pre- and probiotics).

The second part presented in chapter 3 comprises an extensive investigation of the broiler chicken microbiota composition in digesta and mucosa of individual samples under varying supplementation of calcium (Ca), phosphorus (P), and phytase. The dietary impact on the distribution of the microbial communities was studied in the crop, ileum, and caecum through illumina sequencing of the 16S rRNA gene, amplifying the V1-2 region. One important outcome was the high variability in the microbial composition between individual samples. Significant differences were observed between the digesta and mucosa samples, supporting the hypothesis that being close to the host, mucosa associated communities show a different composition. A calcium effect on the performance was observed, where values for body weight gain and feed conversion were lower in comparison to the other treatments. Microbial communities in the crop mucosa revealed a

dietary effect, while in the digesta samples no significant changes were seen. Regarding the ileum mucosa, there was an effect of P addition on the microbial distribution. As expected, caeca-derived samples showed an increase in the diversity indexes when compared to the ileum and crop and butyrate producers were detected in higher abundance.

A lower microbial diversity in the crop was linked to lower growth performance regarding the supplementation of Ca. Hence, each dietary treatment affected the microbial communities; nevertheless, none of the dietary treatments displayed a consistent effect across the studied gut sections.

Additionally, the effects of supplementing different proteases and one phytase on the microbial community of the ileum of broiler chickens was assessed. Thus, the specific aim of chapter 4 was to determine how enzyme supplementation affects the microbiota composition in the ileum of broilers and whether these effects were related to differences in pre-caecal (pc) AA digestibility. Three different protease sources at a low and high level were included: protease A (Meiji), protease B (Cibenza), protease C (Ronozyme ProAct), and one phytase (Natuphos E). The microbial taxonomy was assessed through 16S rRNA gene Illumina amplicon sequencing. Performance results revealed a significant increase in growth and feed efficiency in broilers fed with phytase only and the high dosage of protease C, in comparison to the control. Most of the AA showed a significant difference between the control diet and protease C at high dosage and phytase diets. Effects on microbiota composition were observed at the genus level for some protease and phytase supplementations. The genera *Streptococcus*, *Lactobacillus*, and uncultured Clostridiaceae were responsible for these differences. This study demonstrates that effects of enzyme supplementation were evident in the terminal small intestine microbiota composition, and, to a lesser extent, in pc AA digestibility. However, the changes in microbiota composition and pc AA digestibility could not be correlated which may indicate the absence of a causal relationship.

Finally, an outlook with metagenome sequencing is presented in chapter 5, to further characterize the result of feeding strategies. Caeca samples from chapter 3 were analyzed including mucosa and digesta from control treatments and mineral supplemented diets only with Ca, and P. Significant differences in functional categorization were observed between digesta and mucosa samples. Metabolism information, essential to microbial activities registered 50% of abundant genes in the supplemented diets while being reduced to 40% in the control samples Phosphatases pathways and butyrate production increased in the supplemented diets while calcium signaling pathway was higher in the control.

In conclusion, within this project a method of standardization to study the microbiota along the gastrointestinal tract of broiler chickens was successfully established. The obtained results revealed a significant impact of both, enzyme and mineral supplementation in the individual sections of the GIT. Also, it was proved that even if the GIT works as an interconnected system, its compartmentalization creates different environmental conditions which influence the microbiota. This study provides insights into the responses of the bacteria and their functionality which were stimulated by the feed supplementations.

CHAPTER VII

ZUSAMMENFASSUNG

7. ZUSAMMENFASSUNG

Broiler, die als Modellorganismus für Nicht-Säugetierarten etabliert sind und deren Genom seit 2004 sequenziert und verfügbar ist, stellen eine ausgezeichnete Fallstudie dar, um die Kommunikation zwischen dem Wirt und seinen intestinalen Mikrobengemeinschaften aufzuklären. Das Verständnis der mikrobiellen Ökologie und die Untersuchung der Wechselwirkungen zwischen den Mikroorganismen und ihrem Wirt, bieten eine Möglichkeit um nach Alternativen zu suchen, die die Aufnahme von Nährstoffen in Hühnern effizienter gestalten. Moderne Sequenzierungstechnologien haben uns an einem Punkt geführt, bei dem die Gewinnung von Informationen aus mikrobiellen Ökosystemen nur den Beginn eines Prozesses darstellt, bei dem es schlussendlich darum geht relevante Mikroorganismen und ihre Rolle innerhalb des Ökosystems zu erfassen. Die Herausforderung besteht dabei darin, dieser enormen Menge an Information einen Sinn zu geben und geeignete bioinformatische Pipelines zu etablieren, um die Sequenzen schnell und präzise zu verarbeiten und in angemessenem biologischen Kontext auszuwerten. Das Hauptziel dieser Arbeit bestand darin, die Veränderungen in der bakteriellen Gemeinschaftsstruktur von Hühnern, als Reaktion auf verschiedene experimentelle Diäten, zu beschreiben.

Der erste Teil dieser Arbeit beinhaltet eine Zusammenfassung des aktuellen Stands der Forschung bzgl. der Mikrobiota des Magendarmtraktes von Hühnern. Die Zusammensetzung und Funktionalität werden dabei mit modernsten Methoden untersucht, welche taxonomische Information auf DNA-Ebene unter Verwendung des 16S-rRNA-Gens bereitstellen. Genkataloge werden via Schrotflinten-Metagenom-Sequenzierung erstellt, eine Technik, die noch in den Kinderschuhen steckt, was über die limitierte Verfügbarkeit von nur acht veröffentlichten Publikationen nochmals verdeutlicht wird. Auf Proteinebene wurden nur zwei Studien gefunden, die metaproteomische Daten beinhalten. Aufgrund des technologischen Fortschritts, haben sich viele Forschungsgruppen darauf konzentriert, die Auswirkungen von Futterergänzungen auf die Mikroben in den verschiedenen Sektionen des Magendarmtraktes wie dem Kropf, Proventrikulus, Muskelmagen, Jejunum, Ileum, Caeca und dem Kot zu untersuchen. Zu den häufigsten Futteradditiven zählen einzelne Enzyme, aber auch komplexer Enzymmischungen sowie organische Säuren, Mineralien, Präbiotika, Probiotika und Synbiotika (wie die kombinierten Wirkungen von Prä- und Probiotika).

Der zweite Teil, der in Kapitel 3 vorgestellt wird, beinhaltet eine umfassende Untersuchung der Mikrobiota in Proben von Digesta und Schleimhäuten individueller Broiler unter Einfluss von variierender Futterzusätze in Form von Calcium (Ca), Phosphor (P) und Phytase. Der Einfluß der Futterzusammensetzung auf die Struktur der mikrobiellen

Gemeinschaften wurde im Kropf, Ileum und Caecum via Illumina-Sequenzierung der V1-2-Region des 16S-rRNA-Gens untersucht. Dabei stellte die hohe individuelle Variabilität der mikrobiellen Zusammensetzung zwischen den einzelnen Tieren eine grundlegende Erkenntnis dar. Signifikante Unterschiede wurden zwischen den Digesta- und Mucosaproben beobachtet, was die Hypothese stützt, dass Mucosa-assoziierte Gemeinschaften, in engerem Verbund mit dem Wirt, eine abweichende Struktur aufweisen. Des Weiteren wurde ein Effekt der Calciumsupplementierung auf die Tierleistung beobachtet, wobei die Werte für die Körpergewichtszunahme und die Futterumsetzung im Vergleich zu den anderen Behandlungen abnahmen. Die mikrobielle Gemeinschaften der Kropfschleimhaut wurden ebenfalls von der Diät beeinflusst, während in den Digestaproben keine signifikanten Veränderungen ersichtlich waren. Die Zugabe von Phosphor zeigte einen signifikanten Einfluss auf die mikrobielle Gemeinschaftstruktur in Proben der Ileummukosa. Im Vergleich mit den Proben des Ileums und des Kropfes, wiesen die Blinddarmproben eine höhere Diversität auf und zeigten ebenfalls eine höhere Abundanz von Buttersäure-produzierenden Bakterien. Eine geringere mikrobielle Diversität im Kropf war mit einer geringeren Wachstumsleistung bei der Supplementierung von Ca verbunden. Alle angewandten Futtermittelzusammensetzungen beeinflussten die mikrobielle Gemeinschaftsstruktur. Jedoch zeigt keine der diätetischen Behandlungen eine konsistente Wirkung über die untersuchten Magendarmabschnitte hinweg.

Zusätzlich wurde der Effekt verschiedener Proteasen und einer Phytase auf die mikrobielle Gemeinschaft des Ileums von Masthühnern untersucht. Kapitel 4 behandelt den Einfluss verschiedener Enzymzusätze auf die Zusammensetzung der Mikrobiota im Ileum von Masthühnern und untersucht, ob diese Effekte mit Unterschieden in der praecaecalen Verdaulichkeit von AA zusammenhingen. Der Zusatz von drei verschiedene Proteasen, in niedriger und hoher Konzentration wurde untersucht: Protease A (Meiji), Protease B (Cibenza) und Protease C (Ronozyme ProAct). Außerdem wurde eine Phytase (Natuphos E) zugesetz. Die mikrobielle Taxonomie wurde durch 16S-rRNA-Gen-Illumina-Amplikon-Sequenzierung untersucht. Die Leistungsergebnisse zeigten eine signifikante Zunahme des Wachstums und der Futtereffizienz bei Broilern, die mit Phytase gefüttert wurden oder hohen Dosierungen der Protease C erhielten. Der Großteil der AA zeigte einen signifikanten Unterschied zwischen der Kontrolldiät und der Supplementierung mit Protease C in hohen Dosierungen sowie einen Effekt der Phytasezugabe. Effekte auf die Mikrobiotazusammensetzung wurden auf Gattungsniveau für einige Protease- und Phytase-Ergänzungen beobachtet. Die Genera *Streptococcus, Lactobacillus* und unbekannte Clostridiaceae waren für diese Unterschiede verantwortlich. Diese Studie zeigt, dass Enzymsupplementierungseffekte die Zusammensetzung der Enddarmdarmmikrobiota und in geringerem Ausmaß auch die praecaecalen Verdaulichkeit von AA bedingte. Die Veränderungen in der Zusammensetzung der

Mikrobiota und der praecaecalen Verdaulichkeit von AA konnten jedoch nicht korreliert werden, was auf das Fehlen einer kausalen Beziehung hinweist.

Schließlich wird in Kapitel 5 ein Ausblick bzgl. Metagenomsequenzierung vorgestellt, um das Ergebnis der Fütterungsstrategien detaillierter zu beschreiben. Die Caeca-Proben aus Kapitel 3, einschließlich Mucosa und Digesta, aus der Kontrollbehandlung und Mineral ergänzten Diäten wurden untersucht. Es wurden signifikante Unterschiede in den Funktionsprofilen zwischen Digesta und Mucosa Proben beobachtet. Daten des Wirtsmetabolismus, die für mikrobielle Aktivitäten essentiell sind, zeigten 50% der relevaten Gene in den ergänzten Diäten, während die Kontrollgruppen nur 40% aufzeigten. Die ergänzten Futtermittel zeigten einen erhöhten Aktivität des Phosphatase-Stoffwechselweg und der Butyratproduktion, während der Calcium-Signalweg in den Kontrollen aktiver war.

Zusammenfassend wurde im Rahmen dieses Projekts eine Standardisierungsmethode zur Untersuchung der Mikrobiota im Magen-Darm-Trakt von Broilern etabliert. Die Ergebnisse zeigten einen signifikanten Einfluss von Enzym- und Mineralsupplementation auf die Zusammensetzung der Mikrobiota in den einzelnen Abschnitten des GIT. Es konnte auch gezeigt werden, dass selbst wenn der Madendarmtrakt als verbundenes System funktioniert, seine Abschnitte verschiedene Umweltbedingungen darstellen, die wiederum die Mikrobiota beeinflussen. Diese Studie liefert Einblicke in die Reaktionen der Bakterien und deren Funktionalität auf verschiedene Futterergänzungen.

CHAPTER IX

APPENDIX

ACKNOWLEDGEMENTS

To follow scientific career is a challenge, and only being up to date, aware of the recent advances, and surrounded by highly skilled professionals, it is possible to succeed. In this sense, my first thanks go to Jun.-Prof. Dr. rer. Nat. Amélia Camarinha Silva, for giving me the opportunity to be part of her group. During these years, I could learn and refine my abilities and expertise not only in wet-lab, but also *in silico* working, and this was true, thanks to her guidance. Her continuous trust in this project, the positive attitude to get the best outcomes and the willingness to give a meaningful sense from the data obtained, encourage me to always go further in the interpretation of my results.

I would like to thank Prof. Dr. Markus Rodehutscord for the commitment and passion in which he oriented this work, always he keeps me aware of looking at different perspectives in order to answer critical concerns. His wise advices were not only crucial for this work, but also on the influence of my professional progress. Also, I thank Jun.-Prof. Dr. Jana Seifert for her open doors to scientific discussion, her advices and always motivating attitude.

My special thanks to Dr. Diego Chaves for his support and always meaningful opinion in the bioinformatic work. Also, I thank Dr. Carolina Pineda for the positive attitude to the collaboration with our group. And Dr. Simon Deusch for its great input with many discussions and parts of this work. It was a pleasure to work and have advices from all of you.

To my colleagues in the “Livestock Microbial Ecology” and the “Feed-Gut Microbiota Interaction,” I feel a lot of gratitude to share life moments which made me grow as a person. To them the best wishes of success. Also, I feel grateful to Birgit Sprott and Ingrid Neff for her support in all administrative work and all other members of the Institute of Animal Science at the University of Hohenheim.

From the “Salvi lab” in Colombia, first I have much special gratitude to Dr. José Salvador Montaña for showing me the route to become a scientist and to mentor many important friends and scientist, that are examples to follow. Dr. Diego Jiménez always an inspiring microbiologist. Paola Palacios and Lynda Prieto always special friends. To Dr. Sandra Baena also many thanks for trust and confidence during my master studies. And to Dr. Howard Junca also thanks for the confidence in my work and take me close to the path to the enrollment in my doctoral studies.

To my family, my father, my mother, my sister and my little brother, always present and supporting my professional and life achievements, you are always in my thoughts. My unique and deepest thanks. And won't be the same without you, Grace, my lovely wife, thanks for taking this risk with me and dream together.

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Affidavit

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

1. I hereby declare that I independently completed the doctoral thesis submitted on the topic "*High-throughput sequencing techniques to analyze microbial communities in the gastrointestinal tract of broiler chickens"*.

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 paraphasing - 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, Germany 11th July 2018

……

(Place, date) (Signature)

Daniel Enrique, Borda Molina

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).

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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.

The German original version of this affidavit is solely valid; all other versions are merely informative.

I have taken note of the information on the affidavit.

Stuttgart, Germany 11th July 2018
…………………………………………………………………………………………………

(Place, date) (Signature)
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