DISSERTATION
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Comprehensive characterization of microbiota in the gastrointestinal tract of quails and two high yielding laying hen breeds

Comprehensive characterization of microbiota in the gastrointestinal tract of quails and two high yielding laying hen breeds

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LIST OF ABBREVIATIONS

~	approximately
16S	rRNA gene 16S ribosomal ribonucleic acid gene
ASV	amplicon sequencing variant
ATP	adenosine Triphosphate
Av. abu	average abundance
BLAST	basic local alignment search tool
bp	base pairs
Br	breed
BW	body weight
BWG	body weight gain
Ca	calcium
Cae	caeca
CaU	calcium utilization
cDNA	complementary deoxyribonucleic acid
CE	capillary electrophoresis
CFU	colony forming unit
COG	cluster of orthologous groups of proteins
Cr	crop
D	duodenum
DNA	deoxyribonucleic acid
FC	feed consumption
FDR	false discovery rate
FI	feed intake
G	gizzard
GC	gas chromatography
GIT	gastrointestinal tract
GO	gene orthology
GS	gastrointestinal section
H’	shannon-weaver index of diversity
I	ileum
InsP	inositol phosphate
KEGG	Kyoto Encyclopedia of Genes and Genomes
KO	KEGG Orthology
LB	lohmann brown-classic
LC	liquid chromatography
LDA	linear discriminant anaylsis
LSL	lohmann LSL-classic
mRNA	messenger ribonucleic acid
NMR	nuclear magnetic resonance
MS	mass spectrometry
NGS	next generation sequencing
NMDS	non-metric multidimensional scaling
OTU	operational taxonomic unit
P	phosphorus
p	probability value
pH	potential of hydrogen
PCoA	principal coordinate analysis
PCR	polymerase chain reaction
PERMANOVA	permutational manova

pH	potential of hydrogen
PU	phosphor utilization
RDP	ribosomal Database Project
RNA	ribonucleic acid
rRNA	ribosomal RNA
SCFA	short-chain fatty acids
SEM	standard error of the mean
SIMPER	similarity percentage Analysis
spp.	species

CHAPTER I

General introduction

1. General introduction

In the last decades, the world population almost doubled from 1974 (4 billion) to 2021 (7.9 billion) [1]. Further predictions on the future global population expect an increase within the next three decades to 9.7 billion people in 2050 [2]. According to the rise in the world population, the demand for food, especially meat, will increase accordingly. Poultry meat production rose from about 9 million tons in 1961 to 127 million tons in 2018. Additionally, total meat production increased from 12% in 1961 to 35% in 2018 [3]. Further, global egg production has increased by over 100% since 1990 and reached a production volume of more than 87 million metric tons in 2020, with China being the country with the highest production amount (> 35 million tons (41%)), while Germany produces less than 1% of the production volume [4].

With the growing world population and the resulting need for animal products, the limited agriculture areas might inhibit further meat production. Moreover, essential food ingredients or supplements are necessary depending on the species and the growing parameters to sustain animal health and well-being. Especially the limited resources and the various ways of nutrient uptake depending on the species will be a major task to solve in the following decades. Currently, 70% of the potential global warming in production systems is caused by animal production and transportation [5] despite the environmental effects of nitrogen emissions, litter management systems and energy consumption in animal housing. Therefore, for the increase in animal production, proper strategies of promoting the animal's performance are necessary like the prevention of diseases and control of the health and hygienic standard to minimize bacterial, parasitic or viral infectious impacts on the animals or humans while reducing environmental effects of the production.

In poultry, the fed diet differs as it is grain-based compared to the e.g. crude fiber-rich diet in ruminants. The feed includes proteins, carbohydrates, fats and oils, minerals, vitamins, and additives like probiotics or enzymes. The modern production focuses on nutrition, with specialized diet compositions according to the animals' age, gender (sex), householding and breed [6]. Moreover, it is more known about poultry nutrition than most other species, which led to the poultry industry's success. For chicken meat production, nutritional research focuses on adjusted growth and development of the animals' body, while the aim in laying hen nutrition research is on egg production and quality. Further, the animal health and welfare are a major player for high animal performances [7]. Especially with the introduction of animal welfare aspects, the

housing conditions for laying hens changed [8] and feed formulations were adapted. This included applying research knowledge about the interactions of the animals in the field of nutrition, feeding, housing systems, temperature, stress, gut microbiota, and water quality, which enabled additional low-cost production in combination with high quality products (Figure 1.1).

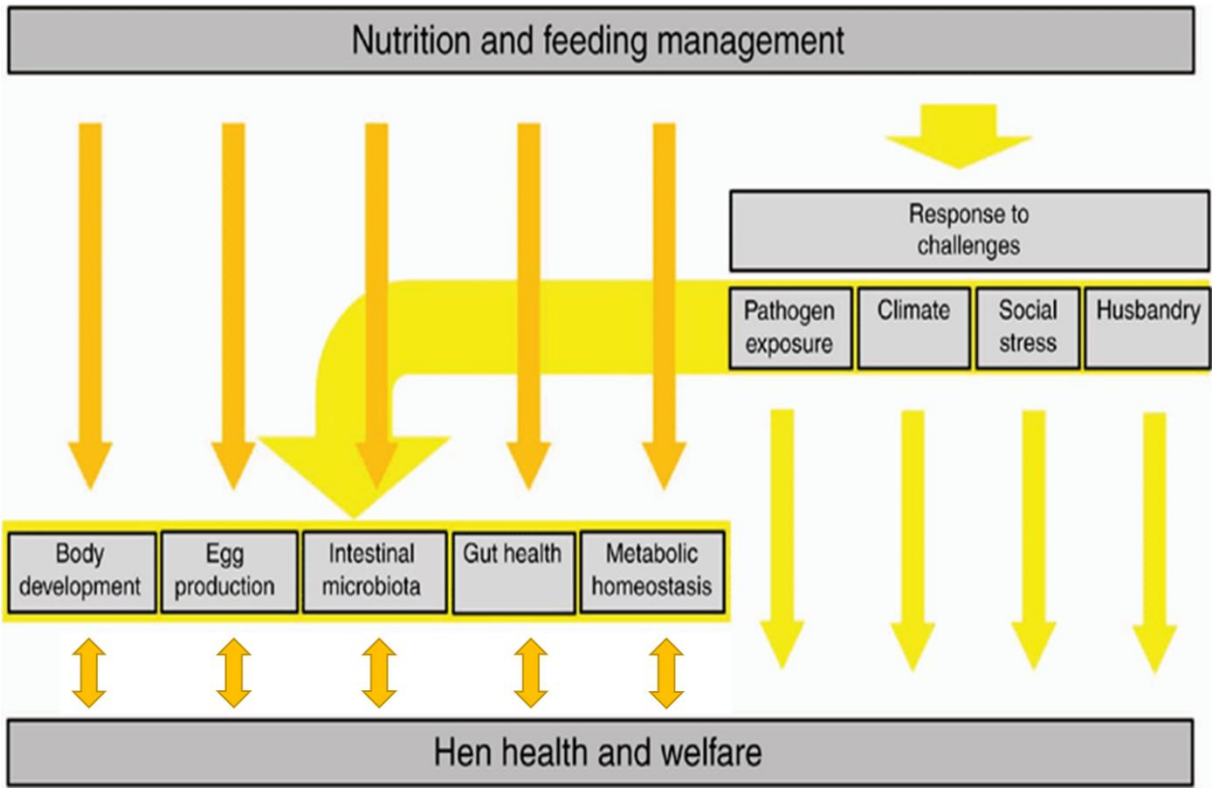

Figure 1.1: Direct and indirect effects of nutrition and feeding management on hen welfare. Feed and nutrients have a direct impact (orange arrows) on body development, egg production, intestinal microbiota, overall gastrointestinal health and metabolic homeostasis. Indirectly (yellow arrows), feed provides support to mitigate challenges such as pathogen exposure, adverse climate conditions, social stress and husbandry procedures, including vaccination, beak trimming and relocation. Together, the direct and indirect effects of nutrition and feed management are central to laying hen welfare, health and productivity (adapted from Bryden et al. 2021 [7]).

Overall, the basal diet is based on different cereal grains, including corn, wheat, oat and barley, and soya as the most important protein source [7, 9]. It is also known that the body development during the rearing and laying phase is strongly correlated with the ability to extend the laying period beyond 80 weeks [10]. Therefore, within the rearing of the pullets, it is mandatory to ensure to reach age specific body weights, flock uniformity, through a well-developed gastrointestinal tract (GIT) and the establishment of good feeding behavior.

The energy requirements of layers remain relatively constant during the production stages until the demand for body maintenance increases and egg production decreases [7]. The layers feed changes during the animal lifespan from a starter, then a grower /developer and finally a layer diet, which vary in many terms like energy content, percentage of recommended minerals or amino acids, crude protein or mineral nutrient content. A starter diet has a relatively high energy content (> 12.1 MJ/kg) to promote skeletal growth [11]. Also, the crude protein content is higher in the starter feed (20%), and reduced in the grower (15.5%) to diminish feather pecking. The lower nutrient density of the feed increases the amount of feed ingested, leading to a larger GIT volume [12]. Additionally, diets with lower energy concentrations (11.5 MJ/kg) promote the increase in feed intake, which stimulates gizzard activity and digestive enzyme secretion [13]. Especially in the following developer diet, raw materials with lower density and higher crude fiber concentrations with a concentration of 5.5% in the feed is recommended to train the feed intake behavior and prepare the animals for the egg-laying period [10]. Further, the crude protein concentration increases again in the layer feed (18%) [12]. The calcium (Ca) supplementation in the young birds feed is lower (1%) than in the layer feed (~4%). This increase is necessary to fulfill Ca needs regarding the development of the eggshell [12]. Further, an adequate Ca concentration is necessary due to the necessity in the overall bone mineral content, muscle function, blood coagulation, enzymatic activity and hormone regulation [14]. Additionally, it was reported that the hen age positively correlates with egg mass and weight, reducing the eggshell thickness and the potential number of sellable eggs in older laying hen flocks [15], indicating a maximum of Ca assimilation in the hens. Other minerals that support the animals' physiological needs and improve the performance parameters include phosphorus (P) or trace minerals like zinc, manganese and cupper [16]. P is a structural component of nucleic acids and is involved in energy metabolism in the form of adenosine triphosphate (ATP), and essential in bone formation, cell membrane and cell functions vitality [17].

Especially the balance of the essential amino acids is crucial in diet formulation as protein is a critical component for layers [18]. The sulfur amino acids (methionine and cysteine) are the first limiting amino acids in the most commonly used laying hen diets, and lysine is used as the reference amino acid [19]. However, the reported and recommended ideal amino acid profile varies across studies [7]. It has been observed, that each 0.05% increase in sulfur-containing amino acids higher than 0.23%

increased the egg weight by 0.7g, besides an additional linear increase in egg weight by supplementing methionine [20, 21]. On the other hand, if the amino acid concentration is at the lower limit of the requirement, a lower number of eggs were pulled. Therefore, hens increase the overall feed intake to maintain amino acid requirements, which coheres with higher total energy consumption [22, 23]. Consequently, modern poultry production in recent decades has significantly improved the outcome of meat and egg products.

Many investigations are currently performed to understand the role and interaction of microorganisms with the host and how feed substrates modulate the microbial community in the gastrointestinal tract (GIT) of animals. The GIT of laying hens and quails is densely colonized by complex microbial communities comprising bacteria, fungi, archaea, protozoa and viruses [24]. The microbiota colonizing the epithelial surfaces forms a protective barrier and reduces the colonization of pathogenic bacteria in the GIT [25]. Also, these microbes hydrolyze indigestible carbohydrates and polysaccharides that the fowls could not absorb. Moreover, microbial colonizers of the GIT produce vitamins, short-chain fatty acids (SCFA) such as acetic acid, butyric acid, propionic acid, organic acids (lactic acid), anti-microbial compounds, and lower triglycerides. Bacteria play an essential role in inducing the non-pathogenic immune response to ensure nutrition and protection for the animal [25–28]. On the other hand, the GIT is also a possible source of potential bacterial pathogens such as *Escherichia* and *Salmonella,* which can be spread to humans or lead to antibiotic resistance and threaten public health [29–31].

1.1 Tools to characterize the fowl gastrointestinal tract microbiome

With the possibility of performing high-throughput next-generation sequencing technologies (NGS) from 2005 on, an increase in knowledge about taxonomical and functional microbial composition could be obtained (Figure 1.2).

The commonly available NGS technologies are based on sequencing a large number of DNA fragments in parallel machine runs. The nucleic acids like RNA, total DNA or genomic DNA will be converted to sequencable fragments, after the extraction and purification, by performing the library preparation [32]. NGS is performed on different sequencing platforms such as Roche GS FLX+, Illumina MiSeq, and Ion Torrent PGM, which are all capable of generating comparable data of high quality [33, 34]. Especially with Illumina, the number of sequences and the costs combined with a low error rate

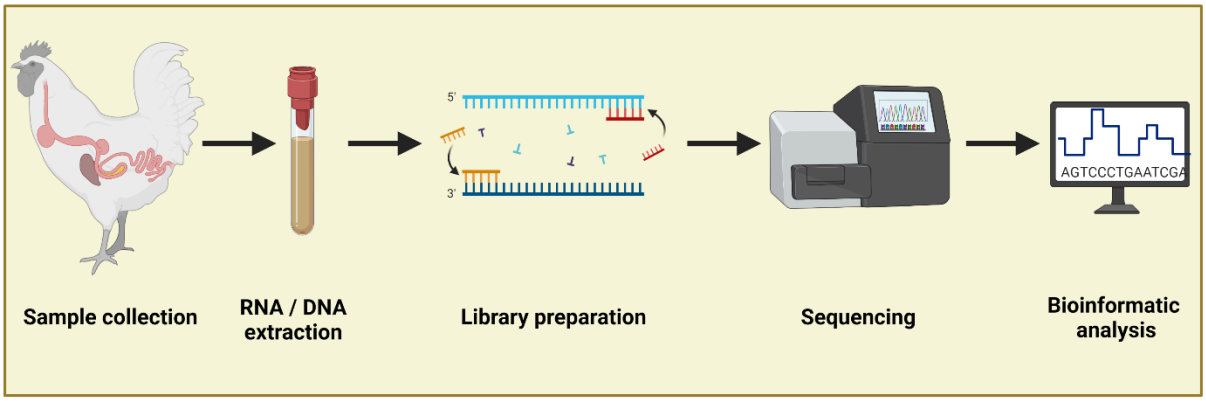

Figure 1.2: Standard procedure from sample collection to sequencing analysis in poultry gut *(created with BioRender.com)*.

during the sequencing procedure (Quality score 30: 99.9% inferred base call accuracy [35]) makes it a reliable platform. Further, obtaining the taxonomical and functional microbial characterization results is relatively short. These benefits improve the formulation of the new hypothesis. Therefore, scientists enhance their knowledge of the effects of animal nutrition and genetics, besides additional endo- and exogenic factors on the GIT microbiota and the corresponding responses from the host, which can lead to innovative observations in the field. Further differences on the microbial composition can be expected due to various DNA extraction protocols, primers and sequencing approaches.

The 16S ribosomal RNA (rRNA) gene is commonly used to identify the archaeal and bacterial community members. The amplification of this gene, or part of it, is used to analyze the microbial composition across various niches from soil, to ocean and human sites [36]. This gene has nine hypervariable regions, and the V1-V2 [37], V1-V3 [38], V3-V5 [38], V3-V4 [39] and V4-6 [40] regions have been used in recent chicken studies. However, the resolution of especially lower-rank taxa (genera and species) varies, depending on the chosen variable region [41]. A wide range of primers can target these regions and amplify them with a polymerase chain reaction (PCR) [42]. By sequencing the amplified products (amplicons), the discrimination among bacteria to the genus or species level and the relative abundance of each sequence gives an overview of the microbial community per sample [43, 44]. This overview is provided by the bioinformatic processing of the generated sequences performed by open platform pipelines such as Mothur [45], QIIME [46] or QIIME2 [47]. Based on public databases used within the pipeline, as the ribosomal database project (RDP) [48], SILVA [49] or GreenGenes [50], the taxonomical assignment can be performed. The corresponding

sequences will finally be linked to operational taxonomic units (OTU) [51] or amplicon sequencing variants (ASV) [52]. OTUs reflect a clustering of reads at a specific identity. The 3% value is the most chosen one and clusters the sequences sharing 97% of similarity [53]. ASV can resolve sequence differences to a single nucleotide change and represent a finer distinction between sequences [53].

Depending on the research goals, we can investigate the active or total (dead and live microbes) bacterial community, by extracting the RNA or DNA of the samples. While DNA sequences help identify the genomic content and which microbes are present within a community, RNA sequences can be used to study the diversity of active genes within the same community and identify the expression levels in regards to varying factors of the experiment [54]. Consequently, the microbiome composition differs within the same sample depending on the use of RNA and DNA extractions [55]. Both extraction types should be combined to maintain and quantify transcriptional activity and stratify bacterial taxa [55]. As adequate quality control is needed, the development of other high throughput omics- technologies to investigate the fields of epigenome, genome, metabolome, transcriptome, and proteome are a benefit for the overall research, and the complexity of a microbial community can be untangled by the usage of omics- technologies.

The high-throughput sequencing approach shotgun metagenomics allows the investigation of the related microbes' taxonomic composition and functional potential [55]. A benefit of shotgun metagenomics is the higher accuracy at the species level by performing unbiased microbial profiling [56]. After sequencing, the low-quality bases and chimeras are removed from the primary dataset and the sequences of the host, viruses, archaeas and protozoas. The filtered data will further be compared to available datasets (e.g. GenBank, Kyoto Encyclopedia of Genes and Genomes (KEGG) or Basic Local Alignment Search Tool (BLAST)) [57] and based on that, the identified microbial community with the linked functionality can be statistically analyzed and plotted in graphs according to the alpha- and beta- diversity [58]. In general, metagenomics avoids the overestimation of the community diversity due to horizontal gene transfer in the analysis of 16S sequences [59]. Nevertheless, this approach can have the same limitations like target amplicon sequencing, the limited sensitivity for less abundant taxa and the often missing reference- and cultivated sequences [60].

Metabolomics provides quantitative and qualitative determination of microorganism metabolites associated with various metabolic pathways included in many

physiological processes like energy and amino acid metabolism [61, 62]. This information on poultry is still scarce to set up a full description of the animals' metabolome. Methods to study the metabolites use gas chromatography - mass spectrometry (GC-MS), liquid chromatography - mass spectrometry (LC-MS), nuclear magnetic resonance spectroscopy NMR, Fourier-transform ion cyclotron resonance mass spectrometry, and capillary electrophoresis - mass spectrometry (CE-MS) [63]. Metabolomic data analysis can be divided into processing (denoising, quality control), statistical analysis, and machine learning techniques for pattern recognition [64]. After establishing the peak pattern, the comparison against spectral databases such as BioMagResBank [65], METLIN metabolite database [66], MassBank [67], Madison-Qingdao Metabolomics Consortium Database [68] can identify the metabolites in the extracted sample. Each differs in functionality and serves spectral data linked to biological interpretations [69]. As expected, previous experiments reported highly influences of endo- and exogenic factors on the metabolite profile in laying hens [70]. However, more information is needed as the metabolomics research is still in the starting phase. Moreover, the interpretation especially of untargeted MS is not trivial due to differences in the sampling method, extraction, sample preparation protocols as well as the data acquisition and the choice of the analyzing tool [71, 72]. Standard protocols can enable the comparability and evaluate the data in regards to already existing data.

Metatranscriptomics is the study of the expressed RNA with information on the specific regulation and expression profiles [54]. Moreover, the active functional profile can be achieved through the expression of the microbial community [54]. While metagenomics helps to understand the microbial composition under different conditions, metatranscriptomics help to investigate the genes being collectively expressed under different conditions. Recently, this approach helped to understand the chickens GIT resistome response to phytogenetic feed additives instead of antibiotics in poultry farming [73]. The bioinformatics pipeline is similar to those used for metagenomic data. Reads are aligned to specialized databases with aligning tools like Bowtie2 [74], BLAST [75], or MEGAN [76], which allow to perform contig assembly to possibly full-length transcripts and annotated to databases such as gene orthology (GO), KEGG, or clusters of orthologous genes (COG) [69, 74, 77]. However, most of the RNA comes from ribosomal RNA and reduces the coverage of mRNA, as mRNA is notoriously unstable and the differentiation between host and microbial RNA can be challenging

[78, 79]. These drawbacks must be considered, and until now, transcriptome reference databases have been limited in terms of their coverage [69].

Metaproteomics identifies and quantifies proteins in a specific sample e.g. cells, tissue, feces, at a given time. Proteins can be considered as the backbone of metabolism, homeostasis, cell division, nutrient transport, cell-cell communication, protein synthesis, and the construction of cellular and extracellular structures [80]. Compared to metatranscriptomics and metagenomics, the metaproteomes advance our understanding of microbiome functionality by assessing the spatio-temporal expression of microbial genes and the dynamics within a microbial consortium [80]. Most proteomics and metaproteomics use liquid chromatography coupled with tandem mass spectrometry (LC–MS/MS). The complex peptide mixture's resulting from MS/MS results are further assigned to peptide sequence databases with pipeline tools like STRING [81] or Mascot [82]. Until now, customized databases composed of a series of unknown gut microbial genomes are often used instead of high-sequence coverage databases (e.g. NCBI) [83] because of their low sensitive peptide identification [84]. The absence of available wide-scale and updated peptide spectra matching databases is one of the main limitations in analyzing gut metaproteome and limits the comparability [69]. Difficulties can arise due to posttranslational modifications and insensitivity for low copy proteins [85]. Nevertheless, metaproteomics can provide new knowledge and strengthen the linkage of the microbiome to animal performance and health parameters e.g. identifying biomarker candidates for selection for higher feed utilization [86] or infections in the gut microbiota [87] in chickens.

These different ‘omic’ approaches provide valuable information for understanding microbiomes and their interaction with the host. However, they are still expensive and not affordable for most research groups. Currently, omics approaches are starting to be integrated into poultry research, while the majority focus only on a single omics approach [88], specific GIT sections, or experimental conditions. One individual omics approach cannot predict this complex biological system. A holistic approach can assist in overcoming future challenges in fields such as breeding, nutrition, and animal health. The future goal will be more precise sequencing (minimizing error rates and artifacts) by using fewer DNA / RNA input and lowering the related costs. Moreover, protocol standardization will enable accurate comparability and improve the holistic view of the microbiome.

1.2 Microbial composition of quails and laying hens gastrointestinal tract

The GIT of fowl has a diverse bacterial community, and each bacterium is adapted to its specific ecological niche and lives in synergy with other bacterial species within the same GIT section. The microbial composition is affected by various exo- and endogenous factors such as age, stress, genotype, or diet [89]. Therefore, changes in microbial composition and diversity are the leading indicators used to infer variations in microbe activity regarding affecting factors. Nevertheless, a few bacterial groups can carry out many typical microbiome functions [90, 91]. However, there is a natural progression in the microbial community over time in terms of presence, absence and prevalence of bacteria, while an imbalanced microbial composition can cause a dysbiosis. The dysbiosis itself, commensal bacteria become opportunistic pathogens due to an overgrowth which causes an immune response of the gut [92]. Therefore, understanding the effects on taxonomic composition due to treatments of the animal may lack a complete understanding of the effects of the bacteria on the healthy and diseased gastrointestinal tract, or the appropriate therapy in terms of the predominant cause of gut dysbiosis. Additionally, the housing condition plays an important role in the microbial composition. It was reported, free-range laying chickens had, compared to cage-laying chickens, different abundance levels of the dominating bacterial groups [93]. The overall fowl GIT consists of the crop, proventriculus, gizzard, duodenum, jejunum, ileum, pair of caeca, large intestine, and cloaca [94] (Figure 1.3). The total GIT length is around 253-269 cm with an average digesta passage rate of 2,25-4,67 hours in chickens [95, 96] with the caeca being the GIT section with the slowest passage rate. Due to these shifts in the passage rate, the development and establishment of the microbial community is influenced. Each section has a role in feed digestion and has a specific microbial community with different metabolic functions. The intestinal microbiota can be described as dynamic due to interaction with the host, diet, environment, and bedding material [7, 97]. Further, the dietary components are the main modulators.

The crop is a blind sac, connected to the mouth and the proventriculus. Overall, the crop provides the capacity to hold and store the food until further digestion. It is the first site for feed fermentation, with digestive enzymes present there [98]. In microbiology, the colony-forming unit (CFU) is a unit to estimate the number of microbial cells like bacteria, fungi or viruses in a sample [99]. The number of bacteria

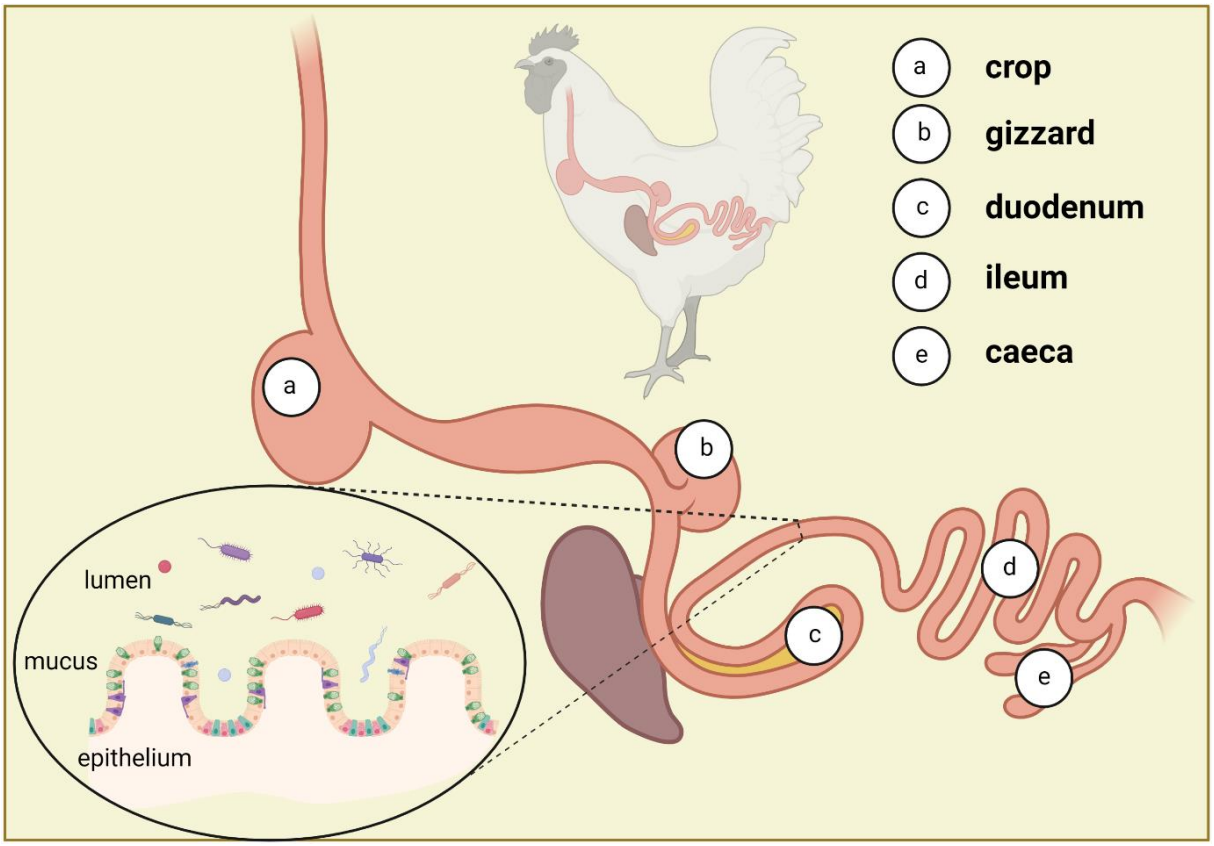

Figure 1.3: The gastrointestinal tract sections of fowl: crop (a), gizzard (b), duodenum (c), ileum (d), caeca (e) (created with BioRender.com).

in the crop account for 10^3-10^5 CFU/g, including mainly *Lactobacillus*, *Bifidobacterium* and *Enterobacter* [100, 101]. *Lactobacilli* are the most prevalent bacteria in the crop [102, 103] and the species *L. salivarius, L. acidophilus, L. reuteri, L. johnsonii, L. crispatus, L. gallinarum, L. amylovorus* and *L. gasseri* are known acid producers from fermentative metabolisms, including lactic and acetic acid, which cause a low pH in the crop [104–106].

The gizzard or muscular stomach is covered by red muscles and contains, besides the food, gravel or other grit for disintegrating the food. The gizzard contractions are rhythmic activities, and the number of muscle cells largely changes from hatch to further development stages [107]. It is known that the feed can often reflux back to the proventriculus for enzymatic digestion [108]. The concentration of bacteria is similar to the crop (10^3-10^4 CFU/g), but the fermentation activity is lower, due to the inhibiting lower pH in the gizzard [101]. The predominant genera are *Lactobacillus* and *Enterococcus* and the GIT section enhances secretions of hydrogen chloride, bile acids and endogenous enzymes [103].

The small intestine comprises the duodenum, jejunum, and ileum and is characterized by an extensive innervation of the nervous system, including division into sympathetic and parasympathetic nervous systems [109]. It is the GIT part with the major chemical digestion and the main place of absorption of nutrients, as a result of a rising pH after the crop and gizzard [105]. Furthermore, fat is primarily digested in the duodenum and this section is known as the most developed intestinal section due to rapid cell renewal as it is the first part of chemical, physical and hormonal interaction with the lumen [110]. The bacteria density in the small intestine ranges from 10^5-10^9 CFU/g of cells [101] and among the small intestinal compartments, the initial part duodenum has the lowest bacterial density due to a relatively short transition time and the secretion of bile [111]. The mainly harbored bacteria in the duodenum are *Lactobacillus* followed by *Enterococcus* [112].

The ileum is crucial for overall digestion and nutrition absorption and mainly involved in starch digestion and absorption, with a slower passage rate than in the previous sections [105]. It was reported as a habitat for novel bacteria with mainly butyrate producers that might impact the birds' performance by influencing the absorption rate and nutrient availability [105]. Therefore, the ileum is one of the most studied GIT sections of the small intestine. Facultative and microaerophilic bacteria colonize the ileum with a reported bacterial density in the range of 10^5-10^9 CFU/g and a dominance of the genera *Lactobacillus* followed by *Megamonas* [37, 101].

The pair of caeca have the most diverse and stable (in regards to endo- and exotherm factors) microbial composition than other GIT sections, and it includes mainly anaerobe bacteria [113, 114]. The caeca have a significant role in electrolyte and water absorption as well as in recycling renal nitrogen besides fermentation and digestion of starch, indigestible carbohydrates and cellulose [105, 108, 115]. Moreover, the size of the caeca is affected by the diet, which increases by feeding a high amount of fermentable, fiber-rich material [116, 117]. The passage rate in the caeca is slow in comparison to other sections (24 to 48 hours [116]) increasing the overall feed fermentation, which results in the production of higher amounts of SCFA concentrations [105, 108]. Moreover, the caeca are usually considered the most important part of the GIT regarding health status and major pathogen reservoirs [117]. They are also the most diverse section harboring the highest number of bacteria with 10^{11} - 10^{12} CFU/g [37, 101]. A popular diversity calculation method in ecological literature is the Shannon index (H'), also known as Shannon's diversity index or

Shannon-Wiener index [118]. The index is related to the weighted geometric mean of the proportional abundances, a quantitative biodiversity measurement. In a study with laying hens, the caeca samples were those across the GIT with the greatest Shannon diversity [37], which is in line with a previous longitudinal study at an index of approximately 6 [119] (Figure 1.4). This significant difference was consistent among

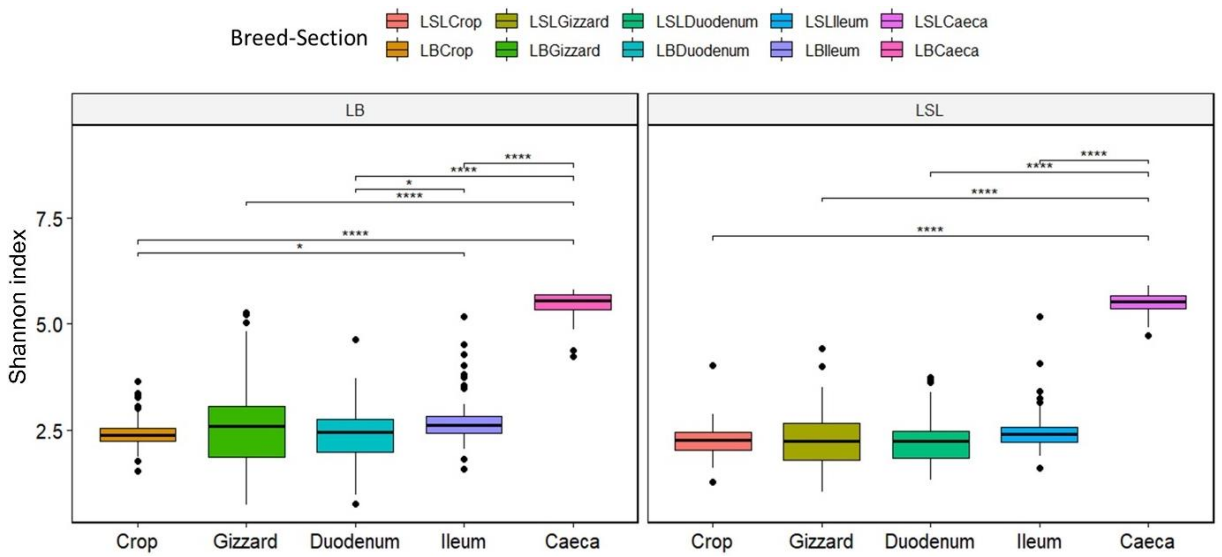

Figure 1.4: Boxplot of Shannon diversity index in laying hens separated by the breed, section (color) and Ca / P combination of the diet (* $p < 0.02$; **** $p < 0.001$) [37].

two different breeds of laying hens and on average, the index was doubled compared to the crop, gizzard, duodenum and ileum. The strict anaerobic bacteria in the caeca of chicken belong to families Ruminococcaceae, Clostridiaceae Lachnospiraceae, and less abundant Lactobacillaceae [120]. Members of these families are *Bacteroides, Clostridium, Fusobacterium, Streptococcus,* and *Enterococcus,* which are linked to proteolytic activities [121].

Likewise, the samples can be distinguished into two different sample types within the GIT compartments, the digesta / lumen / chyme and the mucus layer / mucosa of the intestine (Figure 1.3). Microbiota not only varies between GIT sections, but also within digesta and mucosa of the same GIT section [122, 123]. Moreover, the mucosa harbors a more diverse microbial community than the digesta within GIT sections [123]. Therefore, studying the mucosa-associated intestinal bacterial composition is essential regarding host mucosal response due to alterations in mucosal immunity by their implication on animal health [123].

Besides the variations of the microbial composition, the fowls' species also plays an important role. For example, the Japanese quail *Coturnix japonica* was domesticated between 1910 and 1941 and has been used as an animal model for poultry research

in recent decades [124]. However, unlike most studied broiler chickens, the quails' GIT is still poorly studied [125]. Regarding the quail microbial composition, Wilkinson et al. [125] analyzed for the first time the gastrointestinal tract from mouth, esophagus, crop, proventriculus, gizzard, duodenum, ileum, caeca, large intestine and the feces between males and females. Based on extracted DNA of the 16S V3-V4 region, the quails' crop, gizzard, duodenum and ileum were dominated by the genus *Lactobacillus* and depending on the GIT section, *Lactobacillus* comprised for up to 70% of the total community [125]. Although gender affected microbial abundance differences, the pH restricted the overall bacterial growth [125]. However, the pH limits pathogen growth, and the proximal GIT sections prefer an acid-tolerant bacteria composition [126]. The genus *Bacteroides* dominated the caeca, followed by *Ruminococcus*, *Faecalibacterium*, *Enterococcus*, and *Clostridium* [125]. Further, the highest diversity in quails was found in the caeca compared to the other GIT sections, aligning with the laying hens result [37].

The microbiota varies across GIT sections due to the physiochemical environment, while the section underlies its niche for specific bacteria, depending on the pH, redox potential, growth substrates, antibacterial secretions, and metabolites from host and microbiota [127]. There is still a need for knowledge about the effects of the microbiome regarding changes on endo- or exogenic influences and the corresponding metabolic changes. Animal variations can be expected under identical raising- and experimental conditions [37, 128]. The individual variation of intestinal microbiota might also result in individual changes in nutrient metabolism or feed utilization [129].

1.3 Effects of age on the intestinal microbiome in fowl

The GIT microbiota underlies adaptations and changes during the lifespan of the birds [127]. As mentioned, these changes can be endo- or exogenic and influence the bird before hatching until later life stages. The GIT microbiota takes part in the regulation of bone formation and growth [130], development and homeostasis of the immune system, maintenance of barrier functions, metabolites influencing energy sources, and cell to cell communication [127]. Moreover, interactions between microorganisms are essential for gut homeostasis by promoting the development of the intestinal mucus layer, host metabolism and affecting animal physiology and health [131]. Body weight increases with age [132], and all these regulations must be adjusted during aging. Overall, the microbiota evolves from hatching on, and major shifts are also observed

in layers with the transition to the layering period, which occurs with a dietary change from developer to layer diet [133]. However, the birds age has a more significant effect on the microbial population than the dietary treatment [134]. The first contact with microbes corresponds to the egg-shell microbial composition exposed to the mothers' microbiota and environment. From day 1 to day 3 the gut is a stabilized environment consisting of most bacteria detected in adult laying hens [134], but not on the same relative abundance level. The colonization increases exponentially within the first week, and the bacterial density stabilizes after day 30 [42]. Especially the relatively low diversity post-hatch, dominated by Gram-negative bacteria changes within the first week of life towards higher diversity of Gram-positive bacteria of the *Clostridiales* group [134]. Moreover, the diverse community colonization shifts from a facultative aerobic one to an anaerobic colonization [135].

In general, younger chickens have higher abundances of Proteobacteria, while adults have Firmicutes as their most prevalent phylum, and the according dominant families are Lachnospiraceae, Lactobacillaceae, Clostridiaceae, and Ruminococcaceae [135, 136]. Four microbial development stages from hatching until week 60 of age were identified in a study characterizing the caeca [137]. The first stage at 1 week of age, Enterobacteriaceae (Proteobacteria) dominated the caeca. The second stage from 2-4 weeks was characterized by a high prevalence of families belonging to the Firmicutes phylum e.g. Lachnospiraceae and Ruminococcaceae (genera *Ruminococcus* and *Oscillospira* [134]). The stage 3, ranging from the second to the sixth month, mainly Firmicutes was colonizing the caeca. From the seventh month on (stage 4), a constant ratio of Firmicutes and Bacteroidetes was established, with families from both phyla colonizing the caeca [137].

Similar variations were reported in other GIT sections. In the crop, *Lactobacillus* relative abundance increased progressively from 40 to 70% at later stages, while the opposite was observed for Clostridia, Negativicutes and Gamma-proteobacteria, as the summed abundance level decreased from 31% in young chickens (0-5 weeks) to 7% in later stages [104]. In the gizzard, the *Lactobacillus* presence decreased within the first weeks from over 80% to less than 60% and the ileum was characterized by an increase of *Clostridium* (1-18%), *Streptococcus* (1-5%) and members of the families Lactobacillaceae, Clostridiaceae, and Lachnospiraceae [138, 139]. The age affected changes in the intestinal microbiome can further be observed in the feces. The feces of one-day-old chickens were colonized by Firmicutes (68%) followed by

Proteobacteria (26%) and Streptophyla (5%), while the abundance level of Firmicutes increased further from day 35 on. The major shifts observed between both timepoints on genus level were an increase in *Lactobacillus* (2 to 72%) and a decrease in *Escherichia* (26 to 1%) [140].

Nevertheless, after a fluctuating microbiota in the early days of the birds, the microbial composition stabilizes in the later stages of the birds' life due to maturation [141]. Moreover, the microbiome complexity and richness increase during the lifespan [134] while variability between samples decreases [142]. However, shared microbial members will be consistent across animals within the same habitats and despite of the complex microbial assemblage and build the core microbiota [143]. Understanding the shared core microbiota is needed to gather knowledge about the functions of these microorganisms to the community and how dietary or environmental treatments affect the core bacteria. Especially observations of organisms present over time can be linked to their functional role as the animal faces biological challenges at different timepoints [143]. The core microbiota is known to shift in laying hens, and the relative abundance of the core bacteria changes significantly over the lifetime (weeks 1 to 51) and within the GIT section (ileum and caeca) [133] (Figure 1.5). In regards to the microbial community variation, the layer stage clearly separated from the pre-layer stage, which was most prevalent in the caeca [133]. Especially certain species of the genus *Lactobacillus* like *L. crispatus, L. gasseri* or *L. reuteri* were found to appear as core. However, whereas the core in the ileum comprised for more than 50% of the total abundance of bacteria, the core in the caeca comprised for 10 to 35%, which might include a better competitive exclusion of other bacteria in the ileum [133]. Age-dependent microbial changes in other GIT sections across extended periods have not been investigated deeply. Despite consistency of the core microbiota over time, a core microbiota of five bacteria (uncl. *Lactobacillus*, *L. helveticus*, *Megamonas funiformis, L. salivarius,* uncl. *Fusicatenibacter)* could be described across two different laying hen breeds, five GIT sections and four dietary treatments in 97% of the samples [37].

Still, it is known that the microbiome changes due to the diet adaptations to fulfill the requirements for starting the laying phase anatomically and consequently questions rise in regards to the core microbiota affecting the bacterial composition or vice versa. Nevertheless, it can be assumed that the resulting microbiota composition, which changes during the bird's life, can modulate the growth performance, egg production

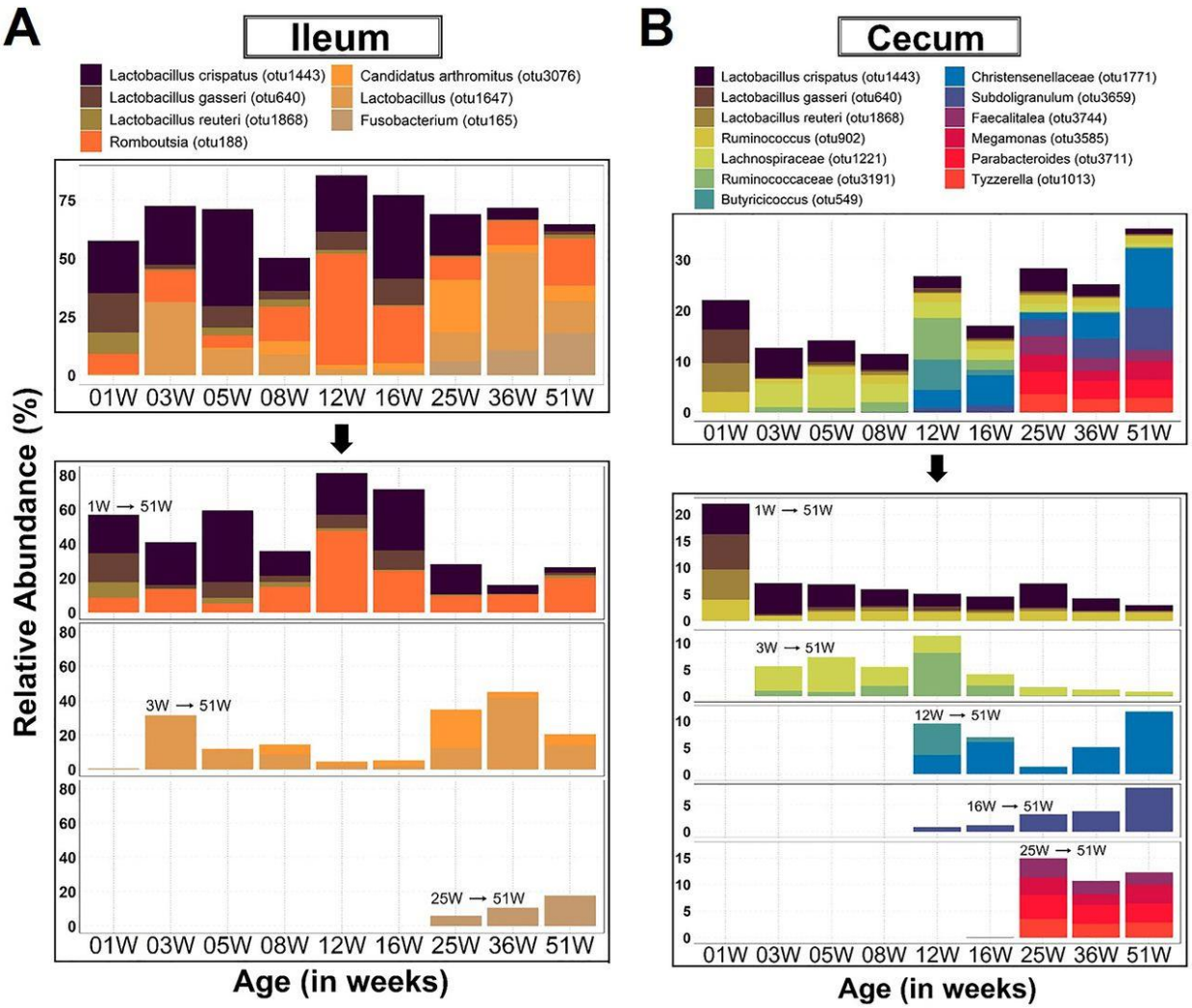

Figure 1.5: Simplified dynamics of core gut microbiota across age groups. OTUs were identified as core based on ≥75% occurrence in chickens sampled within a given age, 100% occurrence across all subsequent ages, and at least 3.5% relative abundance in one of the ages. 1W→51W, etc. within the chart indicates the time point at which an OTU emerged as core and its persistence over time. Note that some OTUs emerged earlier and persisted below core abundance levels but became core at the indicated time points [133].

and overall health status. A modulation and adaptation of the microbiota composition is needed, as the birds' ability to digest food reaches the maximum metabolizable energy level of the diet at 50 days of age [144]. From that day on, the animal requires a further establishing microbiome for a high feed efficiency and utilization leading to high animal performances. Additionally, these age affected microbiota variations show interactions with the environmental conditions with greater variations being observed in outdoor housing systems [145]. Regardless of the housing system, the egg and yolk weight increased with the age, whereas the albumen weight decreased, causing an increasing yolk:albumen ratio indicating metabolism and performance changes. Overall, greater differences and variations were observed at the later age [145]. This

was also reported in quails, as age-dependent effects were also found on the egg weight, yolk and albumen, shell weight and thickness [146]. Screening the bacterial community linked to these variations can help to find possible ways to modulate the microbiota to ensure animal health and performance.

The knowledge to describe the active host and microbiota cross-talk is still scarce, as many aspects must be considered to maintain a balanced ecosystem. For example, host health and well-being, diet and supplements, age, the appropriate section of the GIT, and the influence of the environment have a significant impact on the microbiota. In addition, there is still a breach in knowledge regarding host morphological development and the related functional properties of the GIT microbiota of the aging fowls. Overall, the use of holistic approaches can help to understand the microbiome and target to improve the necessary understanding of the whole ecosystem regarding the animals' productive stages.

1.4 The role of phosphorus, calcium and phytase in fowl

The poultry industry is a fast-growing and essential global food supply chain. Therefore, in the case of economic and environmental issues, feed and nutrient efficiency became a major role in poultry research. This has to be considered, especially with the knowledge about the close interaction of the chicken intestinal microbiota with the fed diet [147]. One of the primary components of poultry feed are plant seeds. Within the plant seeds, phosphorus (P) is primarily stored in phytic acid (myo-inositol 1,2,3,4,5,6-hexakis (dihydrogen phosphate); InsP6) as well as in its salts (phytate) (Figure 1.6).

In total, these P storage forms sum up to two-thirds of the total P in plant material but are not easily accessible to the animal [106, 149, 150]. Furthermore, mineral P produced from mined phosphate rock is included in the poultry feed and remains an industry standard. However, this P source is a finite resource and will, depending on the modelling and calculation, approximately last for the next 300 up to 400 years to create fertilizer [151]. This is crucial to produce sufficient food and feed to ensure the needs of a growing world population. On the other hand, the correlation and effects between GIT microbiome, host and diet have to be investigated to decrease the non-assimilated excretion of P, so there is a potential for a more efficient P utilization by the gut microbiota. Further, the environmental impacts of rising poultry production in

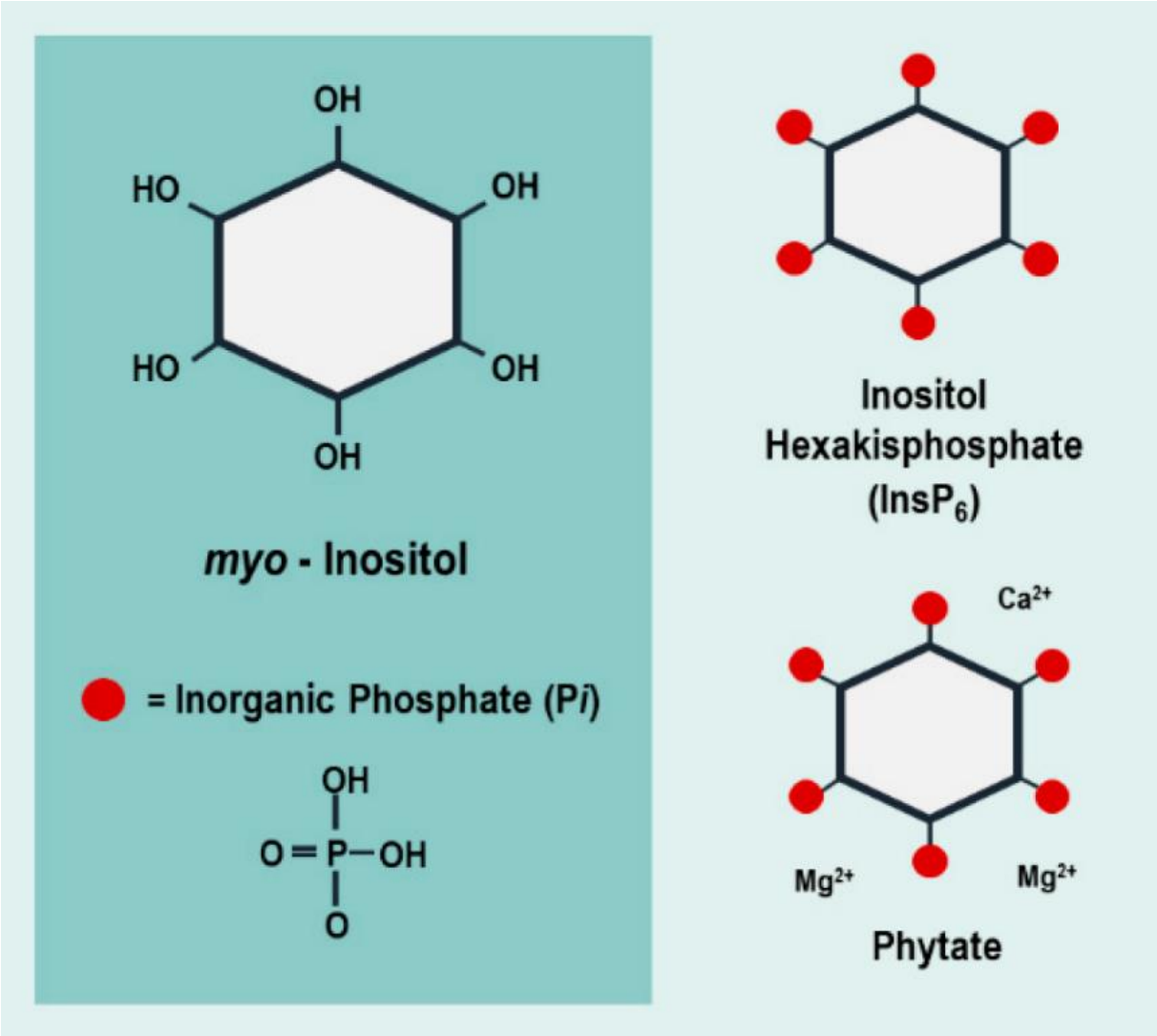

Figure 1.6: Simplified structures of myo-inositol, InsP6, phytate Inorganic phosphate (Pi) groups, covalently bound to the myo-inositol ring by an oxygen molecule, are represented in red. For simplification purposes, this figure does not take into account the axial and equatorial positions of the moieties (adapted from Freed, Adepoju et al. 2020 [148]).

the following decades have to be diminished as the depletion of the mineral P resource [152]. Therefore, to maintain health, optimal growth, and animal performance, accessible inorganic P in the feed is needed. Consequently, phytases are supplemented to the feed, to support the low endogenous enzyme activity in fowls to digest inositol-phosphate. Without the supplementation, approximately 25% of the total P stored in wheat-corn-soybean based diet can be assimilated while the enzyme impacts limestone and phosphate digestibility [153].

The catalyzation by the enzymes phytase and phosphatase of these forms are needed to catalyze the cleavage of P to enable absorption in the digestive tract [150, 154]. The phytate hydrolysis in the GIT of poultry is performed by the enzyme phytase (myo-

inositol hexaphosphate phosphohydrolase), which leads to less-phosphorylated inositol phosphates (InsP5, InsP4, InsP3, InsP2, InsP1), and inorganic P [155]. The InsP6 hydrolysis is catalyzed by phytases to myo-inositol and orthophosphates and an additional catalyzation by other phosphatases may be involved for InsP1 to 5 isomers. Archaea are known metabolizers of myo-inositol, and it serves as carbon and energy source for e.g. *B. subtilis, Aerobacter aerogenes, Corynebacterium glutamicum* or *L. casei* while the metabolism is initiated by the enzyme dehydrogenase and further dehydratase [156]. Non-Ruminants have often been assumed as unable to catalyze $InsP_6$-P due to a lack of enzymes and the low expressed phytase activity in the brush border membrane of the GIT [157]. However, recent studies could report InsP6 degradation in the GIT of broiler chickens without phytase supplementation in the diet [150, 158, 159].

Phytate is a polyanionic molecule capable of chelating divalent cations, including Ca to form mineral-phytate complexes. These Ca-phytate complexes resist the enzymatic hydrolysis by phytase and limit the phytate degradation in poultry [160, 161]. Therefore, several studies were performed to improve the P availability by adding phytase in the poultry feed; investigating different Ca:P ratios and the resulting pH variations, which can increase the Ca-complex solubility [160, 162, 163].

Consequently, the benefits of phytase supplementation are a possible increase in feed efficiency, improved mineral uptake with a better overall poultry performance and reduced amount of Ca and P required in the formulated diet due to phytate complex releases [106]. Additionally, adding phytase can occur with a reduction in the buffering capacity and pH to ensure intestine integrity and promote the presence of commensal bacteria [164].

Overall, the effect and ability to improve the nutrition of ingredients in the diet by phytase depend on the amount of P and Ca [165]. However, supplementing both minerals in the diet and the efficient absorption from the feed is important for bird development. Laying hens need Ca through the egg shell formation [166]. In addition, Ca is necessary for bone and nutrient metabolism [166]. Nevertheless, the requirement of Ca depends on factors like the overall Ca/P concentration in the diet, the strain/ breed, age, and the temperature the animals are exposed [166]. Moreover, the animals need depend on the overall laying hen weight and the egg weight, which leads to the recommended P and Ca concentrations in the diets of laying hens of 3.65 g/day/hen Ca and 0.35 g/day/hen P for a 1.8 kg weighting hen with an egg weight of 55g [167]. It

is known that the lack of P can inhibit the availability of minerals and nutrients, proteins, or necessary amino acids [168, 169]. Recent studies showed that an increase in Ca decreases animal growth and bone formation in the early life stages. Non-phytate P modified the digestive CaxP interactions, possibly due to calcium phosphate formation or Ca-phytate complexes by increased gut pH and Ca/P concentration [170].

Due to the recent findings of Jing et al. [171], a reduction of both nutrients compared to the recommended amounts should be discussed. The authors fed animals a diet with a reduction of 0.15% P and revealed no effect on growth, productive performance, or mRNA expression of P transporters in hens [171]. Additionally, the reduction to approximately 20% of P and Ca amounts in the feed has not significantly impacted laying hens' microbiota development [37]. On the other hand, diets supplemented with the enzyme phytase resulted in higher numbers of microbial sequences for carbohydrate metabolism, indicating higher availabilities of polysaccharides and glycolysis/gluconeogenesis expression together with starch and sucrose metabolism [172]. Understanding the different P and Ca pathways and the knowledge about animal-adjusted feeding in terms of age, sex, housing conditions and overall performance is needed to have healthier animals and reduce non-assimilated nutrient excess in the feces.

1.5 Scope and work hypothesis

This thesis aimed to comprehensively characterize the microbiota in the gastrointestinal tract of quails and across the lifespan of two high-yielding laying hen breeds and investigations of the microbiota by feeding different levels of Ca and P.

To achieve this main goal, specific aims were proposed:

- **The gastrointestinal microbial community of quails drives P utilization and is affected by genetic traits.**

  Therefore, the effect of the individual P utilization, Ca utilization, and the overall bird performance (feed intake, feed conversion, body weight gain) was investigated on the modulation of the intestinal ileum microbiota in male and female quails of an existing large dataset of F_2 design of the Japanese quail (Chapter II).

- **Different diet inclusion of P and Ca affects the gut microbial community of laying hens regarding bacterial structure and pathways involved in InsP6 degradation.**

  A study was performed using two breeds of high-yielding laying hens to explore the effects of different Ca / P supplementation levels on the active microbiota composition in five GIT sections (Chapter III).

- **The gut active microbial community of laying hens' changes during the bird productive life span.**

  The study aimed to characterize the active GIT microbiota composition during the lifespan of two high-yielding laying hens held under the same conditions and diet to gain insights into microbiota shifts from week 10 to week 60 (Chapter IV).

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CHAPTER II

Effects on the ileal microbiota of phosphorus and calcium utilization, bird performance, and gender in Japanese Quail

2. Effects on the ileal microbiota of phosphorus and calcium utilization, bird performance, and gender in Japanese Quail [1]

2.1 Simple Summary

The Japanese quail is an animal model for nutritional and biological studies in poultry. Diet assimilation is influenced not only by external factors, but also by the host, including its microbiota. The gut microbiota is involved in the digestion of feed constituents, facilitating the breakdown of polymers to compounds from which the animal can benefit. This study elucidates the influence of the ileal microbiota in the content of the intestine (digesta) from a large cohort of Japanese quail fed the same diet and offered identical environmental conditions. Phosphorus utilization (PU), calcium utilization, feed intake, feed conversion, and body weight gain were parameters evaluated in the birds to understand the microbial influences. A core microbial community of five bacterial species, Unc. *Lactobacillus*, Unc. Clostridaceae 1, *Clostridium* sensu stricto, *Escherichia coli*, and *Streptococcus alactolyticus*, colonized the ileum of all animals and contributed to more than 70% of the total community. Gender had a significant effect on the ileum microbial community. Even though birds were offered the same diet and housed in standardized conditions, it remains unclear if microbiota composition followed the mechanisms that caused different PU or if the change in microbiota composition and function caused the differences in PU.

2.2 Abstract

In this study, we aimed to investigate the ileum digesta of a large cohort of Japanese quail fed the same diet, with similar environmental conditions. We also address how P utilization (PU), Ca utilization (CaU), and bird performance (feed intake (FI), feed conversion (FC), and body weight gain (BWG)) modify intestinal microbiota of male and female quail. Despite the great number of samples analyzed (760), a core microbiome was composed of five bacteria.

[1] This chapter was published as:

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The Unc. *Lactobacillus*, Unc. Clostridaceae 1, *Clostridium* sensu stricto, *Escherichia coli*, and *Streptococcus alactolyticus* were detected in all samples and contributed to more than 70% of the total community. Depending on the bird predisposition for PU, CaU, FI, BWG, and FC, those species were present in higher or lower abundances. There was a significant gender effect on the ileal microbial community. While females had higher abundances of *Lactobacillus*, males were more colonized by *Streptococcus alactolyticus*. The entire cohort was highly colonized by *Escherichia coli* (8%–15%), an enteropathogenic bacteria. It remains unclear, if microbiota composition followed the mechanisms that caused different PU, CaU, FI, FC, and BWG or if the change in microbiota composition and function caused the differences in PU, CaU, and performance traits.

Keywords: Japanese quail; ileal microbiota; phosphorus utilization; calcium utilization; gender; performance traits

2.3 Introduction

The Japanese quail (*Coturnix japonica)* is an indigenous species to Japan, China, and Korea, and it has been used as an animal model in numerous fields of poultry research in the last 60 years [1]. It was introduced as a laboratory animal in the 1960s [2] and proved to be useful in many areas of biomedical, genetics, behavior, and nutritional studies [1,3–5]. The short developmental period makes *C. japonica* a convenient model for biological studies. Contrarily to the broiler chicken, the quail gastrointestinal tract (GIT) has been poorly studied [6].

The microbial communities detected in the GIT of quail provide several nutritional functions to the host and play an important role in the health status of the animal [7]. Kohl et al. (2014) have described the responses of the gut microbial community to prolonged fasting in quail. Samples from colon and caeca were collected in four fasting stages (nourished, early-, mid-, and late-fasting), and the phylogenetic diversity was characterized. Fasting affected colon and cecal microbial diversity by decreasing the abundance of *Prevotella*, *Lactobacillus,* and *Faecalibacterium* [7]. Another study identified an effect of host genotype and diet on ceca microbiota [8]. Wilkinson et al. (2016) characterized the microbial community of the mouth, esophagus, crop, proventriculus, gizzard, duodenum, ileum, ceca, large intestine, and feces of eight-week-old quail (10 males and 12 females). Different microbial

assemblages were observed in males and females, and ceca samples showed the highest community richness.

The dominant number of sequences found in the large intestine could not be assigned to any genera, while other detected operational taxonomic units (OTUs) belonged to the genera *Lactobacillus*, *Bacteroides*, *Ruminococcus,* and *Clostridium* [6]. In broiler chickens, gender had an influence on the microbiota composition [9].

The function of microbes in the avian gut can be distinguished into nutritional uptake, detoxification, immune-related, and the competitive exclusion of pathogens [10]. The gut microbiota is mainly involved in the digestion of feed constituents, facilitating the breakdown of polysaccharides and other molecules from which the animal can benefit. Diet composition can have a strong effect on the GIT microbiome. Variations in calcium (Ca) and phosphorus (P) supplementation altered the activity and composition of the birds' gut microbiota [11] and performance [12].

In this study, we aimed to investigate how P utilization, Ca utilization, and bird performance (feed intake, feed conversion, and body weight gain) can modulate intestinal microbiota in male and female quail.

2.4 Materials and methods

2.4.1 Ethical statement

This experiment was performed in congruence with the relevant national and international laws along with the institutional guidelines. The study was approved by the animal welfare commissioner of the University of Hohenheim (approval number S371/13TE) and conducted following animal welfare regulations.

2.4.2 Sample collection, DNA extraction, and illumina library preparation

Ileum digesta samples from 760 quail were obtained from a previous study that used an F2 design [13]. The experimental design is fully described by Beck et al. (2016). Briefly, the quails were fed with a starter diet from 1 d to 5 d (Supplementary Table S2.1) and then with an experimental diet (Supplementary Table S2.1) until the end of the experiment (15 d). Diets were designed based on the nutritional recommendations for young turkeys (Gesellschaft für Ernährungsphysiologie, 2004) [14], except for P and Ca concentration. The main feeding ingredients of the starter diet were corn, wheat, and soybean, while the experimental diet ingredients were corn, soybean, and potato protein. All information regarding phosphorus utilization (PU), calcium utilization

(CaU), feed intake (FI), body weight gain (BWG), feed conversion (FC), and gender for each animal is shown in Supplementary Table S2.2. On day 15 of age, birds were sacrificed [15]. The ileum was longitudinally opened and digesta collected with a sterile spoon and stored in RNA later at −80 °C until further analysis. DNA was extracted using Trizol (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions with a preliminary step of bead beating (30 s, 5.5 m/s) in a FastPrep instrument (MP Biomedicals, Santa Ana, CA, USA).

Library preparation was performed according to the Illumina protocol described by [16]. Briefly, primers 27F (slight modification) and 338R reported by [17,18] were used to target the V1–2 region of the 16S rRNA gene. A three-step PCR was performed using PrimeSTAR® HS DNA Polymerase kit (TaKaRa, Beijing, China). The first two PCRs were prepared in a total volume of 25 µL using 1 µL of DNA template, 0.2 µM of primer, and 0.5U Taq prime start HS DNA, and the third PCR was prepared in a total volume of 50 µL. An initial denaturation at 95 °C for 3 minutes was followed by 10 cycles (pre and first PCR) or 20 cycles (third PCR) of denaturation at 98 °C for 10 s, annealing at 55 °C for 10 s, and an extension at 72 °C for 45 s, and then a final extension of 72 °C for 2 min. Libraries were pooled by index, standardized and purified using SequalPrep Normalization Kit (Invitrogen Inc., Carlsbad, CA, USA), and sequenced using 250 bp paired-end sequencing chemistry on an Illumina MiSeq platform.

2.4.3 Samples grouping

The analysis of the dataset was divided into two sections, one covering the effect of PU, CaU, and animal performance on the microbial distribution (Section 1), and another on gender effects on microbiota, PU, CaU, FI, BWG, and FC (Section 2).

In the first section, three groups were created, depending on high, medium, or low predisposition for PU, CaU, FI, BWG, and FC. The high group comprised the top 50 animals, the low group contained the bottom 50 animals, and the remaining birds were grouped as medium. The groups were independently analyzed and animals may not correspond to the same birds in the different traits.

In the second section, groups were established based on the top 50 male and 50 female birds (male high and female high, respectively) and the bottom 50 male and 50 female birds (male low and female low, respectively) for PU, CaU, FI, BWG, and FC, while the remaining birds were grouped as the male or female medium. Each trait has its specific groups of males and females that may not correspond to the same birds in other traits.

2.4.4 Bioinformatics and statistical analysis

Raw sequence reads obtained from Illumina MiSeq system (Illumina, Inc., San Diego, CA, USA) were analyzed using QIIME v1.9.1 pipeline (http://qiime.org/) [19], following a subsampled open reference OTUs (operational taxonomic units) calling approach [20]. Demultiplexing and trimming of sequencing reads were done using the default parameters of the pipeline [16], with a maximum sequence length of 360 bp. The reads were merged into one fasta file and aligned using the SILVA Database (Release 132) (https://www.arb-silva.de/) [21]. Chimeras were identified and removed using usearch [22]. Reads were clustered at 97% identity into OTUs. Only OTUs present on average abundance higher than 0.0001% and with a sequence length > 250 bp were considered for further analysis. The closest representative was manually identified with the seqmatch function of RDP (Ribosomal Database Project—https://rdp.cme.msu.edu/). Sequences were submitted to European Nucleotide Archive under the accession number PREJB37544.

The cut-off for bacterial taxonomy classification followed the recommendations of Yarza et al. (2014) [23]. Sample reads were standardized, and the Bray–Curtis similarity coefficient [24] was used to create a sample-similarity matrix using the (Primer 7—https://www.primer-e.com/) [25]. Permutational Multivariate Analysis of Variance (PERMANOVA) routine was used to study the significant differences and interactions between groups and PU, CaU, FI, BWG, FC, and gender ($p < 0.05$) [25].

A total of 36 birds that could not be assigned to any gender were removed from further analysis. For the visual hierarchical clustering and ordination of the community structures, a two-dimensional principal coordinate analysis (PCoA) was created, whereby the centroids representing the average plotting position of each group (high, medium, and low) of each trait PU, CaU, FI, BWG, and FC were ordinated. The differences in the microbial community structure between the different groups were identified using analysis of similarities (ANOSIM) and pair-wise comparison test [25]. Groups of samples were considered significantly different if p-value < 0.05. The similarity percentage analysis (SIMPER) was used to calculate the similarity between and within the groups and to identify the OTUs contributing to the observed dissimilarities [25]. The statistical differences in the abundance of specific OTUs between the groups were determined with the unpaired Welch's t-test with a cut-off p-value < 0.05. Shannon diversity was calculated with Primer 7 software. Correlations between OTUs and traits were estimated with the Spearman coefficient using PRISM

6 (GraphPad Software, San Diego, CA, USA) and were considered significantly different if *p*-value < 0.05.

2.5 Results and discussion

2.5.1 Effect of PU, CaU, and animal performance on microbial distribution

For the first time, ileum samples from a large cohort of Japanese quail (760 samples) were characterized regarding their microbial composition. Ileum was chosen owing to its role as the gut section of nutrient absorption and high metabolic microbial activities [6,26]. Moreover, it has been hypothesized that ileum can seed other gut sections in terms of microbial composition [6]. After removing singletons, the total number of sequences obtained from the ileum digesta of quail was 39.914.727. Sequences were clustered into 1188 OTUs and taxonomically assigned. The most abundant phylum was Firmicutes (on average (av.) 83%), followed by Proteobacteria (on av. 14%). The dominance of Firmicutes confirms previous findings from 16S rRNA gene surveys in quail ileal samples with 12 animals [6] and 160 animals [6,27]. Bacteria belonging to the Firmicutes phylum synthesize short-chain fatty acids, an energy source that is directly absorbed in the intestine [10]. Other phyla with less than 2% of relative abundance were Actinobacteria, Bacteroidetes, Epsilonproteobacteria, and Tenericutes. A total of 45 genera were detected. The six most dominant included unclassified Clostridiaceae1 (on av. 29.6%), *Lactobacillus* (on av. 24%), *Escherichia-Shigella* (on av. 14%), *Clostridium* sensu stricto (on av. 14%), *Streptococcus* (on av. 8.2%), and *Enterococcus* (on av. 3.7%). These genera are known colonizers of the ileum of quail and other avian species [6,28].

The microbial community of the quail´s gastrointestinal tract has not yet been deeply analyzed, and this leads to a lack of sequencing information in the databases. As previously reported by Wilkinson et al. (2016) and other avian studies, some of the most abundant OTUs detected in the ileum could not be taxonomically classified [6,28,29]. The most abundant OTU, assigned to an unclassified Clostridiaceae1, correlated positively with PU, CaU, FI, and BWG (Supplementary Table S2.3). This OTU belongs to the order Clostridiales, which are known to degrade plant components, which are further fermented to short-chain fatty acids [30]. FC was negatively correlated with unclassified *Clostridium* sensu stricto (on av. 22.8%); BWG with *Streptococcus alactolyticus* (on av. 10.7%) and *Enterococcus faecium* (on av. 1.5%); PU, CaU, and FI with *Escherichia coli* (on av. 13.1%) and BWG; and FI with

unclassified *Lactobacillus* (on av. 29.3%) (Supplementary Table S2.3). Previously positive correlations for *Lactobacillus* species with egg production and feed conversion have been reported [31]. However, in the present study, only one negative correlation was observed between a high abundant unclassified *Lactobacillus* (on av. 29.3%) and FI. The presence of *Lactobacillus* species is considered to be beneficial for the bird because they transform carbohydrates to lactic acid, inhibit pathogen adhesion to the epithelium, and decrease the pH in the ileum [12]. The pH was not measured in this study, but one hypothesis for the high abundance of *E. coli* (on av. 13%) is the increasing presence of one member of Clostridiales (unclassified Clostridiaceae1) and the non-dominance of *Lactobacillus* as indicators of a higher pH. The lower dominance of *Lactobacillus* differs from previous reports on quail [6] and broiler chicken [12]. The negative correlation between *E. faecium* and BWG contradicts the results of a previous study in broilers [32]. *E. faecium* can exert probiotic effects and enlarge the villus height in the ileum of broilers [32]. In quails, it reduced the presence of pathogens like *Salmonella* owing to the production of a bacteriocin [33].

In order to better understand the effects of P and Ca utilization and other performance parameters (BWG, FI, and FC), a priori groups based on high, low, or medium bird predisposition for each trait were established. PERMANOVA test based on those a priori groups confirmed an influence of the single factors PU, CaU, and FI on the ileal microbial community (Supplementary Table S2.4a), while a trend was shown for the interaction BWG × FC (p-value < 0.10) (Supplementary Table S2.4b). The abundance of Candidatus *Arthromitus* was higher within birds with higher PU (Figure 7). These segmented filamentous bacteria attach to the intestine and have been previously isolated from the terminal ileum of chickens [34] and turkeys [35]. Moreover, at an early age, they have been found to positively correlate to bird performance, probably owing to its immunomodulatory capabilities [35,36]. Other genera promoted in the birds with higher PU were *Bacillus* and *Leuconostoc* (Figure 2.1).

Bacillus is considered as a probiotic in chickens; may improve bird performance [37]; exerts different enzymatic activities like amylase, xylanase, and pectinase [38]; and phosphatase activity can be expected from this genus, as previously reported in soils [39,40].

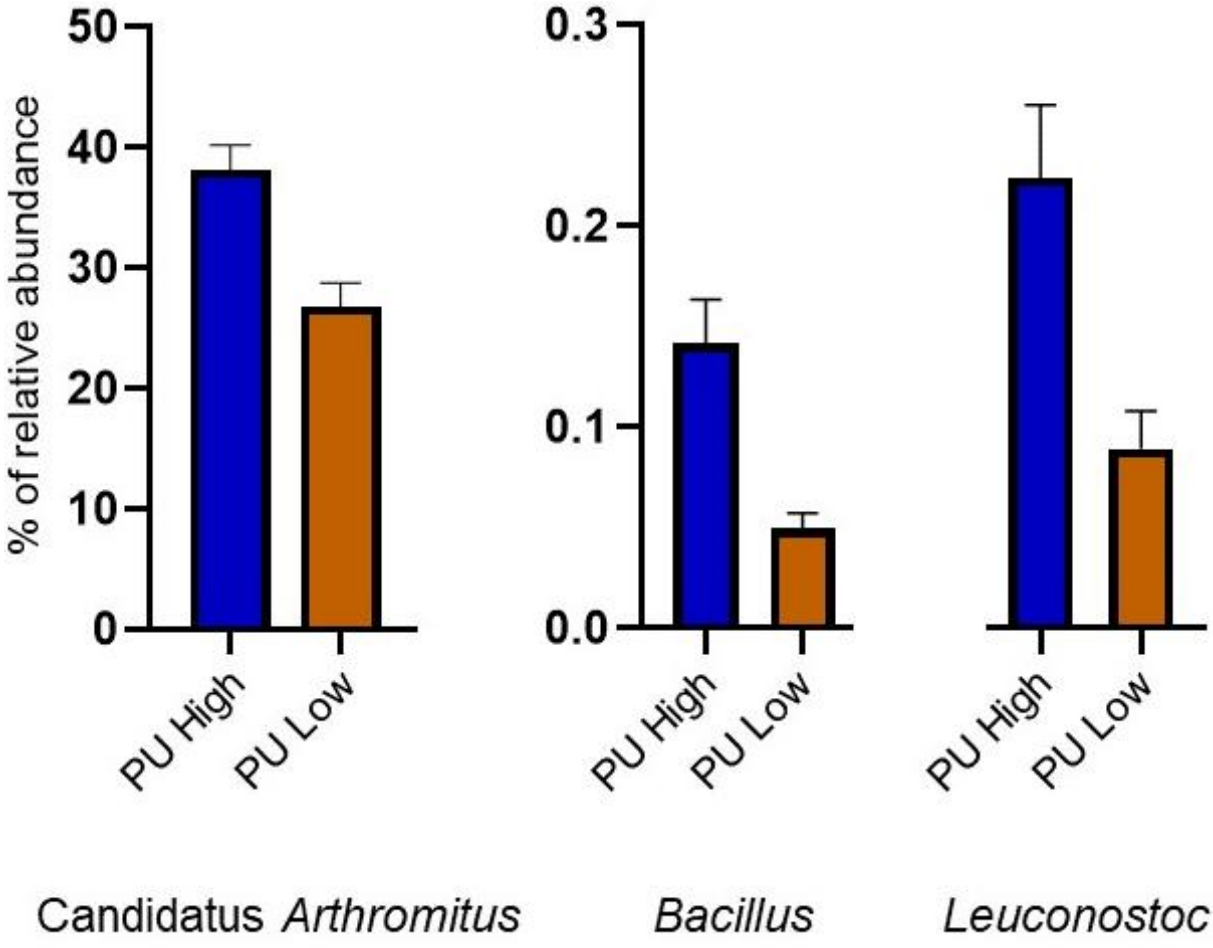

Figure 2.1: Relative abundance of the genera influenced by the P utilization (PU) in the high and low groups.

Gender had a statistically significant effect on the ileal microbial diversity of the present dataset (Supplementary Table S2.4c). Correspondingly, the Shannon diversity index significantly differed between males and females (Supplementary Figure S2.2). Previous studies demonstrated that gender differences exist in the presence of specific bacterial groups, such as *Lactobacillus* in quail [6]. In the present dataset, *Lactobacillus* was more abundant in females (26% vs. 22% in males), while the abundance of *Streptococcus* tended to be the opposite (7.3% in females vs. 9.3% in males) (Supplementary Figure S2.3).

Considering that all birds received the same diet and were housed under the same conditions, a possible explanation for the range of performance values observed can be attributed to individual differences for diet assimilation and the presence of indigestible dietary polysaccharides [41,42]. The percentage of dissimilarity between the high, low, and medium groups for the PU, CaU, FI, BWG, and FC ranged between 52.1% and 60.9% (Supplementary Table S2.5). Taking into account a high individual

variability not only in performance values, but also in microbial composition, it is expected that the microbial metabolic activities changed. It is possible that even bird behavior was affected as it has been demonstrated that gut microbiota affects emotional reactivity in Japanese quail [43,44].

2.5.2 Gender effects on microbiota, PU, CaU, FI, BWG, and FC

Female quail are physiologically different from males [45]; thus, it is expected to comprise different microbial resemblance. To evaluate whether gender variation exists and has an impact on PU, CaU, FI, BWG, and FC, centroids that compute the average plotting position of an a priori group of samples were calculated and ordinated using principal coordinate analysis (PCoA) (Figure 8). Gender affected the grouping of the high, medium, and low levels of PU, CaU, FI, BWG, and FC (p-value < 0.05). A previous study using only 200 quail observed an effect of gender on PU and CaU only as a trend [42]. It is important to highlight that, in the present study, PU ranged from 21% to 86% and CaU from 11% to 84%, a higher variation compared with that observed by Beck et al. (2014). The same study did not observe any effect of gender on FI, BWG, and FC, unlike what we observed in the present study. This discrepancy might be owing to the higher number of birds used in this study originating from an F2 design and the microbiota of the GIT being used to determine these observations.

For PU, CaU, and FI, the PCoA plots depicted three clusters comprising male/female low and medium, male high, and female high (Figure 2.2A–C). The two principal component axes accounted for 80% (PU), 83% (CaU), and 95% (FI) of variation among groups, thus providing a good ordination of the samples. ANOSIM pair-wise comparison tests showed a significant difference between female high versus male high, female high versus female low, and male high versus male low groups for the three traits (p-value < 0.05), except for the CaU between female high versus male high where a trend was observed (p-value = 0.06) (Supplementary Table S2.6). The same was not observed for female low versus male low and female medium versus male medium groups. An effect of gender in the medium group was also observed (Supplementary Table S2.6).

Regarding FC and BWG, the PCoA plots showed separation between low, medium, and high birds (Figure 2.2D and E). The two principal component axes accounted for

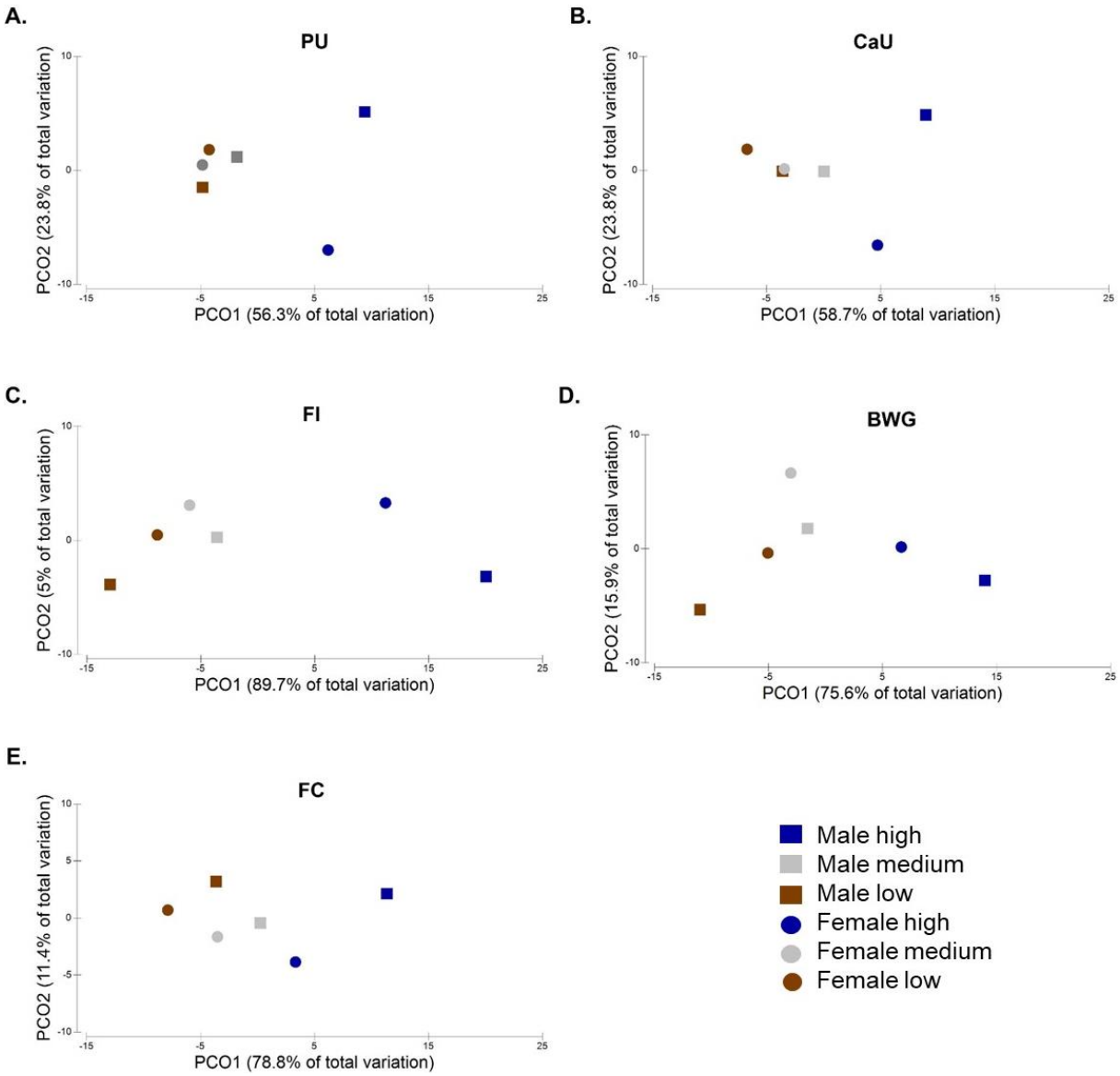

Figure 2.2: Principal coordinates analysis (PCoA) plots depicting the gender effect on (A) phosphorous utilization (PU), (B) calcium utilization (CaU), (C) feed intake (FI), (D) body weight gain (BWG), and (E) feed conversion (FC) in the high, medium, and low groups.

high coverage of the total microbial variation (90% for FC and 92% for BWG). ANOSIM pairwise tests showed no statistical significance between the gender for the higher and lower group, but between high and lower groups within the same gender (*p*-value < 0.05). Regarding BWG, the female medium group was statistically different from the male medium group, while a trend was observed between the two groups for FC (*p*-value = 0.1) (Supplementary Table S2.6).

A group of five bacteria was responsible for the separation observed between the groups in all traits. Unclassified *Clostridiaceae1*, unclassified *Lactobacillus*, *Streptococcus alactolyticus*, unclassified *Clostridium* sensu stricto, and *Escherichia*

coli contributed to more than 70% of the total community. Female and male groups were colonized by the same microorganisms, but relative abundances of microorganisms were different between genders. The average dissimilarity between the groups ranged from 51% to 62%, and the average similarity within the groups was between 37% and 50% (Supplementary Table S2.7).

Pair-wise comparisons for each of the performance measurements revealed that those five bacteria abundances significantly changed based either on gender or within the gender between the high, medium, and low groups (Supplementary Table S2.8). Unclassified *Clostridiaceae*1 was highly abundant in the high male and female groups of all traits, with an average abundance between 32% and 49% in males and 30% and 41% in females (Figure 2.3 and Supplementary Table S2.8).

In the low female and male groups, the average abundance ranged from 20% to 28%. A significant difference in the abundance of unclassified Clostridiaceae1 was observed for PU between the groups female high versus male high (36% vs. 40%), female high versus female low (36% vs. 27%), and male high versus male low (40% vs. 26%) (p-value < 0.05) (Supplementary Table S2.8A). For the CaU, a trend was observed between the female high versus female low group (32% vs. 25%) (p-value < 0.06) and a statistical significance between male high and low (37% vs. 28%) (p-value < 0.05) (Supplementary Table S2.8B). In regards to feed intake, an effect was detected between female versus male high (41% vs. 49%), female high versus female low (41% vs. 24%), and male high versus male low (49% vs. 20%) (p-value < 0.05) (Supplementary Table S2.8C) and in the case of BWG between female versus male high (36% vs. 43%), female high versus female low (36% vs. 26%), and male high versus male low (43% vs. 22%) (p-value < 0.05) (Supplementary Table S2.8D). This microorganism belongs to the Clostridiales order, and it was previously detected in the gastrointestinal tract of broilers [12]. Clostridia are common colonizers of broiler and quail GIT [46] and are responsible for plant material degradation [30]. Generally, they are not the most dominant group, as observed in this study, but are detected in lower relative abundance [6,47]. Corn favored the abundance of clostridia in the avian GIT [48]. The quail of this study were fed with a corn-based diet [13], which might explain the higher abundance of the unclassified Clostridiaceae1 in the samples. Bird age has a remarkable impact on microbiota composition and diversity, gut modulation, and metabolic functions [46]. All previous studies characterizing quail GIT have worked

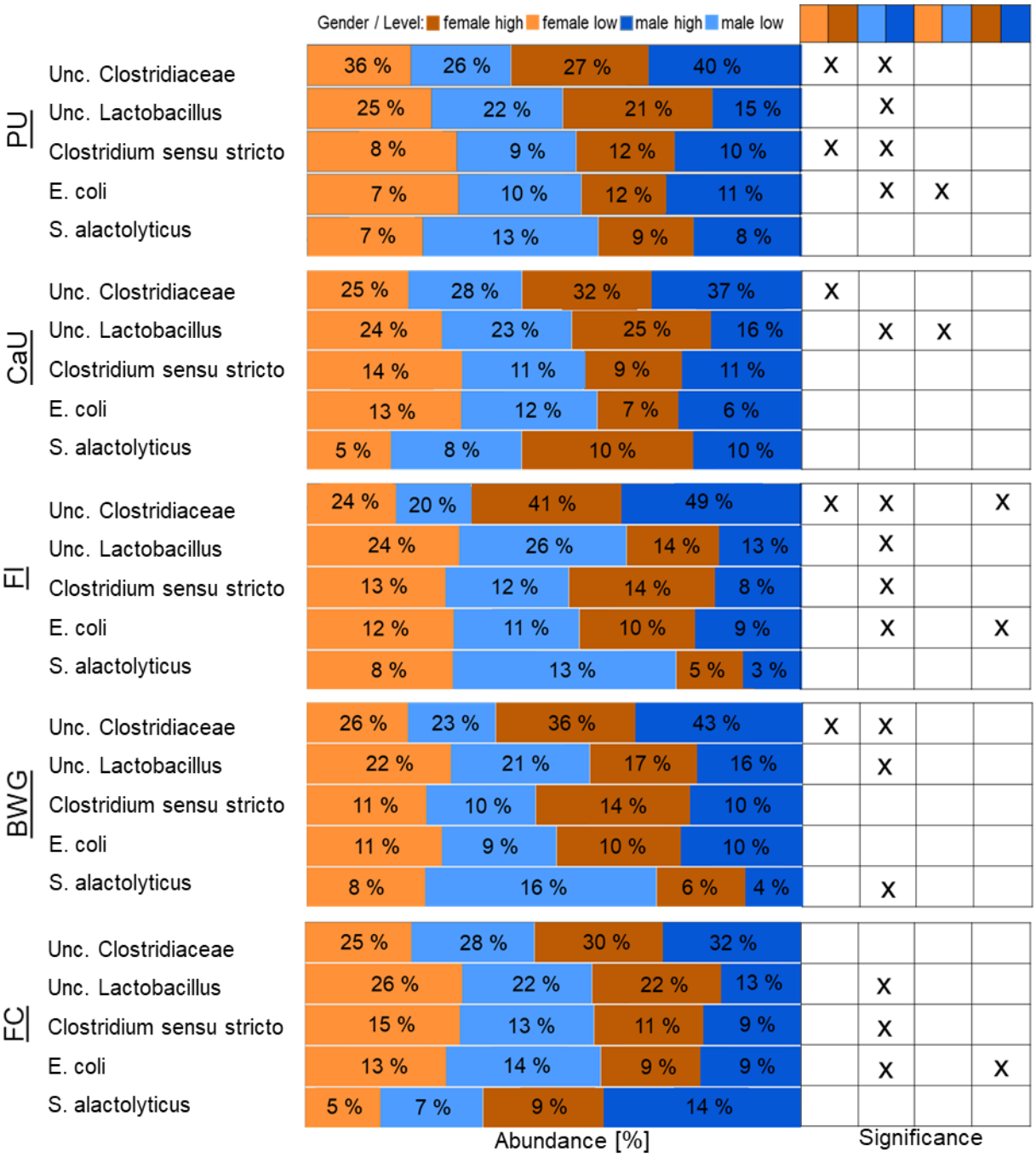

Figure 2.3: Abundance variation of the five operational taxonomic units (OTUs) that contribute to 70% of total bacterial community of females and males considering phosphorous utilization (PU), calcium utilization (CaU), feed intake (FI), body weight gain (BWG), and feed conversion (FC). Statistical significances between the groups are depicted on the graph (p-value < 0.05).

with animals at the age of 4–8 weeks [6,47,49]. This impairs the comparison between those and the present study (two weeks old). In broiler chicken, bacterial changes during their lifespan are known to exist, with an establishment of more stable communities in older animals [46]. Regarding the quails' GIT, there is still no knowledge of how the GIT evolves during lifespan.

Lactobacillus are common colonizers of the ileum of broilers and quail. They are known to improve bird health, inhibit pathogen adhesion, and maintain bacterial stability [47]. They are usually considered in the literature as beneficial; however, care should be taken because they colonize the GIT together with other species and are not independent of them. They interact either positively or negatively [12,50], and thus may have an impact on gut health. In the present study, an unclassified *Lactobacillus* was present in all traits in higher relative abundance in the low female and male groups (21%–26%) in comparison with the high groups (13%–25%) (Figure 9 and Supplementary Table S2.8). The female high group showed higher relative abundances (14%–25%) compared with the male group (13%–16%), while in the lower groups, the males showed higher bacterial abundance for the traits PU (22% vs. 21%) and FI (26% vs. 24%), and the females in the traits CaU (24% vs. 23%), FC (26% vs. 22%), and BWG (22% vs. 21%) (Figure 3 and Supplementary Table S2.8). The higher abundance of *Lactobacillus* in female birds is consistent with results by Wilkinson et al. (2016) [6], and a significant difference between gender was obtained for PU, CaU, and FCR for high and medium groups and in the medium group for FI and BWG.

Lactobacillus and *Streptococcus* are gram-positive lactic acid bacteria present in the GIT. Most of them are non-pathogenic and associated with host well-being. *S. alactolyticus* is a commensal bacterium that was isolated from pig intestine and chicken feces and can ferment glucose, fructose, and cellobiose [51]. *S. alactolyticus* was detected in low relative abundance in all high and low groups across all traits (3%–14% and 5%–16%, respectively). Differences between gender were detected for FC (high groups) and BWG (low groups), and within gender for PU, CaU, FI, BWG, and FC (p-value < 0.1). It is known that *Streptococcus* species are affected by host genotype and diet [27], but no study correlated its abundance with gender, PU, CaU, and performance traits.

Members of *Clostridium* sensu stricto are usually associated with pathogenesis and are indicators of imbalanced gut microbiota [52]. *Clostridium* sensu stricto was detected in higher abundance in the low female/male samples (9%–15%) in comparison with high female/male (8%–14%) (Figure 2.3 and Supplementary Table S2.8). An effect of gender on the abundance of *Clostridium* sensu stricto was observed for the medium groups of PU, CaU, and FC (Supplementary Table S2.8), where higher abundance was found in females. Despite the high abundance of this member of

Clostridium sensu stricto, the birds of this experiment were healthy, and there was no effect on BWG, as previously suggested by (Apajalahti and Kettunen 2006).
Escherichia coli is an enteropathogenic bacteria that can be responsible for disease. It is a common colonizer of the avian digestive tract with no principal effect on the health status of the birds. However, it can be a potential carrier of disease to other animals and humans [53]. In this study, it was detected in a range from 10%–14% abundance in low female/male and 7%–11% in high female/male birds (Figure 2.3 and Supplementary Table S2.8). Thus, it can be hypothesized that, in comparison with chicken surveys [11,12], quail may be particularly predisposed to harbor members of the family Enterobacteriaceae, as has been reported in other studies [47]. Despite the close relative abundance between the high and low groups, statistical significance ($0.05 < p\text{-value} < 0.1$) was denoted between gender for PU (high group) and CaU (high group), with being males more colonized. Within gender, PU (female high vs. low), CaU (female high vs. low), FC (female high vs. low), and FC (male high vs. low) showed statistical significance (Supplementary Table S2.8).

2.6 Conclusions

Even though birds were offered the same diet and housed in similar conditions, it remains unclear if microbiota composition followed the mechanisms that caused different PU, CaU, FI, BWG, and FC, or if the change in microbiota composition and function caused the differences in PU, CaU, and performance traits. Gender affects quail gastrointestinal microbial composition and affects the distribution of specific bacterial groups. Further studies in the interplay between microbiome functionality, host physiology, gender, and genetics are necessary to uncover the real effect of minerals' utilization and performance on microbiome distribution.

2.7 Supplementary materials

The following are available online at www.mdpi.com/2076-2615/10/5/885/s1, Supplementary Figure S2.1: Distance-based redundancy analysis (dbRDA) for A. Ca utilization (CaU), B. Feed intake (FI), C. Body weight gain (BWG), and D. Feed conversion (FC). Vectors indicate the direction of each performance trait and its relation to the groups high, medium, and low. Supplementary Figure S2.2: Shannon diversity index [H'] for the overall data, based on microbial ecology resemblance for female and male Japanese quails, Supplementary Figure S2.3: Percentage of relative

abundance of the genera detected in the ileum of female and male Japanese quails, Table S2.1. Ingredient composition and analyzed concentrations of the diets (Adapted from Beck et al. 2014), Table S2.2 (excel file): Information regarding phosphorous utilization (PU), calcium utilization (CaU), feed intake (FI), body weight gain (BWG), feed conversion (FC), and gender for each animal, Table S2.3: Pearson correlation and its corresponding significance value of the most abundant operational taxonomic units (OTUs) against phosphorus utilization (PU), calcium utilization (CaU), feed intake (FI), body weight gain (BWG), and feed conversion (FC), Table S2.4: Multivariate statistical analysis for the overall data at OTU level. A. PERMANOVA analysis for P and Ca utilization. B. PERMANOVA analysis for BWG, FC, and FI. C. ANOSIM to test gender effect, Table S2.5: Average dissimilarity (%) between high, medium, and low groups for phosphorus utilization (PU), calcium utilization (CaU), feed intake (FI) body weight gain (BWG), and feed conversion (FC) by males and females, Table S2.6: ANOSIM pairwise tests by groups: phosphorus utilization (PU), calcium utilization (CaU), feed intake (FI), body weight gain (BWG), and feed conversion (FC) by males and females, Table S2.7 (excel file): Average similarity and dissimilarity (%) between high, medium, and low groups for phosphorus utilization (PU), calcium utilization (CaU), feed intake (FI) body weight gain (BWG), and feed conversion (FC) by males and females, Table S2.8: Pairwise comparison based on t-test for phosphorus utilization, calcium utilization, feed intake, body weight gain, and feed conversion and the most abundant OTUs (unclassified Clostridiaceae1; unclassified *Lactobacillus*; unclassified *Clostridium sensu stricto* 1; *Escherichia coli*; *Streptococcus alactolyticus*; *Enterococcus faecium*). A. Phosphorus utilization. B. Calcium utilization. C. Feed intake. D. Body weight gain. E. Feed conversion.

2.8 Author contributions

Conceptualization, M.R., J.B., and A.C.-S.; Funding Acquisition, J.B. and A.C.-S.; methodology, D.B.-M., A.H.-A, C.R., S.V., and D.R.; writing—original draft preparation, D.B.-M., C.R., and A.C.S.; writing—review and editing, D.B.-M., C.R., and A.C.-S. All authors have read and agreed to the published version of the manuscript.

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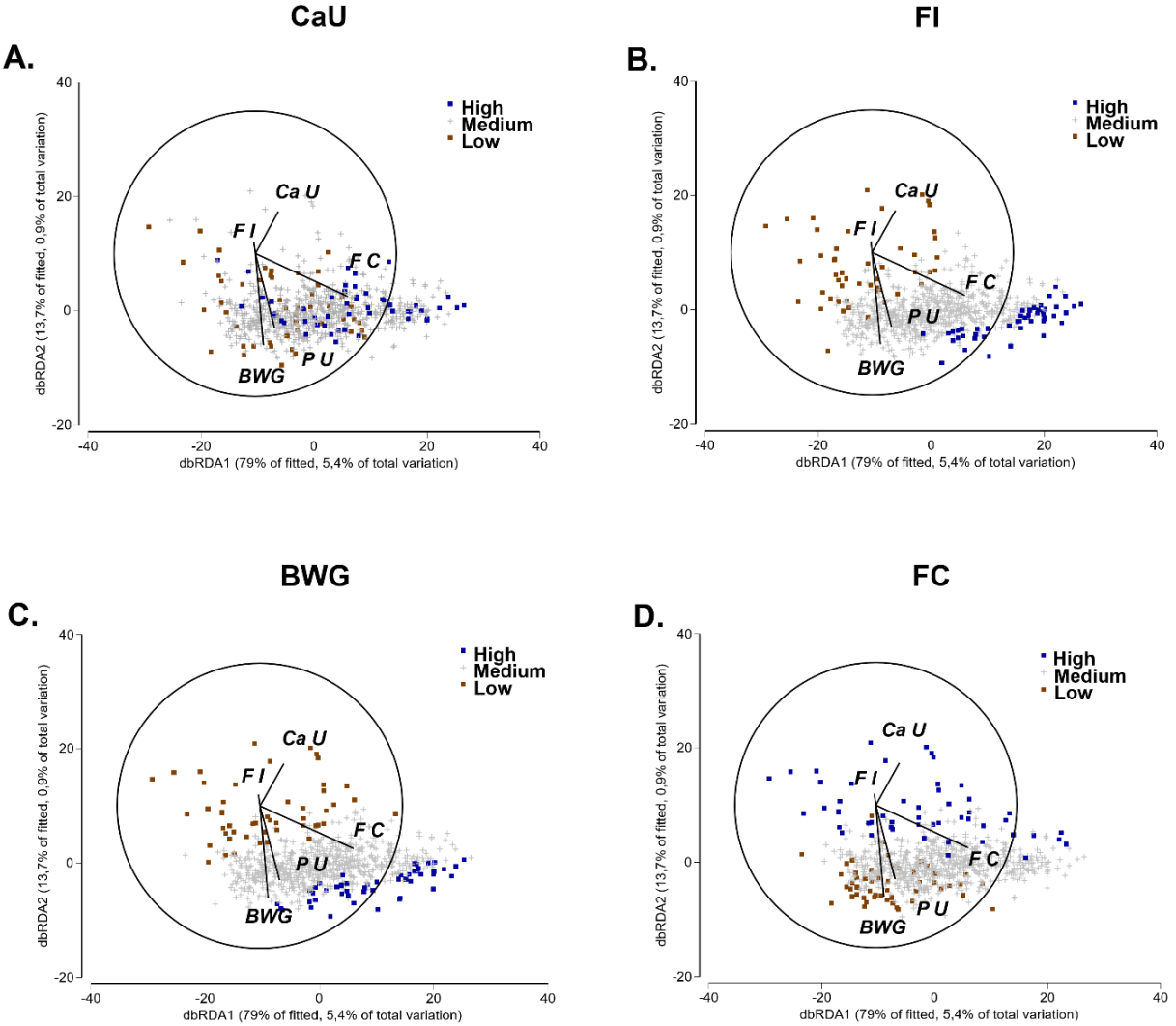
2.11 Conflicts of interest

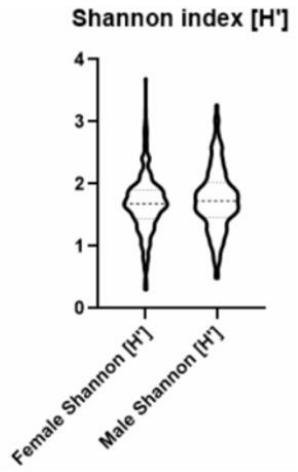
The authors declare no conflict of interest.

2.12 Supplementary material

Supplementary Figure S2.1: Distance-based redundancy analysis (dbRDA) for A. Ca utilization (CaU), B. Feed intake (FI), C. Body weight gain (BWG), and D. Feed conversion (FC). Vectors indicate the direction of each performance trait and its relation to the groups high, medium, and low.



Supplementary Figure S2.2: Shannon diversity index [H'] for the overall data, based on microbial ecology resemblance for female and male Japanese quails.



t test Shannon [H']	
P value	0,0434
P value summary	*
Significantly different (P < 0.05)?	Yes
One- or two-tailed P value?	Two-tailed
t, df	t=2,024, df=722
How big is the difference?	
Mean of column A	1,746
Mean of column B	1,674
Difference between means (B - A) ± SEM	-0,07179 ± 0,03547
95% confidence interval	-0,1414 to -0,002148
R squared (eta squared)	0,005641

Supplementary Figure S2.3. Percentage of relative abundance of the genera detected in the ileum of female and male Japanese quails.

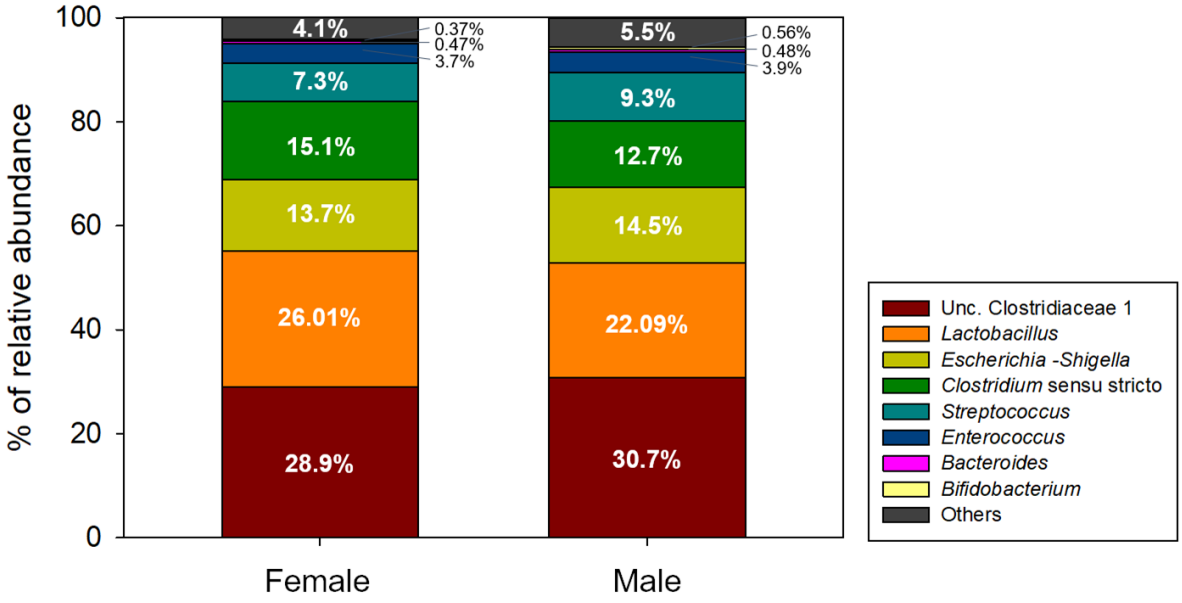


Table S2.1 (excel file): Information regarding phosphorous utilization (PU), calcium utilization (CaU), feed intake (FI), body weight gain (BWG) and feed conversion (FC), and gender for each animal.

Table S2.2: Pearson correlation and its corresponding significance value of the most abundant operational taxonomic units (OTUs) against phosphorus utilization (PU), calcium utilization (CaU), feed intake (FI), body weight gain (BWG), and Feed conversion (FC).

		PU	CaU	FI	BWG	FC
Unclassified Clostridiaceae 1	r	0.176	0.136	0.341	0.258	-0.037
	P-value	1.06E-06	0.000	0.000	0.000	0.304
Unclassified *Lactobacillus*	r	-0.018	-0.047	-0.16	-0.07	-0.05
	P-value	0.610	0.187	0.000	0.032	0.139
Unclassified *Clostridium* senso stricto 1	r	-0.004	-0.03	-0.02	0.033	-0.09
	P-value	0.891	0.396	0.560	0.349	0.008
Escherichia coli	r	-0.07	-0.08	-0.08	-0.011	-0.06
	P-value	0.041	0.033	0.031	0.760	0.067
Streptococcus alactolyticus	r	-0.06	0.01	-0.06	-0.104	0.086
	P-value	0.082	0.666	0.077	0.004	0.016
Enterococcus faecium	r	-0.03	-0.014	-0.06	-0.08	0.05
	P-value	0.356	0.684	0.073	0.016	0.126

Table S2.3: Distance-based linear model (DistLM) for defined environmental data and the microbial communities of 760 samples.

DistLM
Distance based linear models

VARIABLES

1	P Utilization	Trial
2	Ca Utilization	Trial
3	F I	Trial
4	BWG	Trial
5	F C	Trial

Total SS(trace): 1.2848E+06

MARGINAL TESTS

Variable	SS(trace)	Pseudo-F	P	Prop.
P Utilization	10822	6.4385	0.0001	0.0084226
Ca Utilization	8322.9	4.9422	0.0002	0.0064778
F I	40751	24.829	0.0001	0.031717
BWG	23757	14.28	0.0001	0.01849
FC	8172.8	4.8525	0.0003	0.006361

res.df: 758

Table S2.4: Multivariate statistical analysis for the overall data at OTU level. A. PERMANOVA analysis for P and Ca utilization. B. PERMANOVA analysis for BWG, FC and FI. C. ANOSIM to test gender effect

A. PERMANOVA analysis for P and Ca utilization

Source	df	SS	MS	Pseudo-F	P(perm)	Unique perms
P	4	15906	3976.4	2.3723	0.0013	9911
Ca	4	11154	2788.6	1.6637	0.0287	9903
P x Ca**	4	11763	2940.7	1.7544	0.024	9915
Res	710	1.1901E+06	1676.2			
Total	723	1.2364E+06				

B. PERMANOVA analysis for BWG, FC and FI

Source	df	SS	MS	Pseudo-F	P(perm)	Unique perms
BWG	4	7263.7	1815.9	1.1096	0.3369	9916
Feed intake	4	13694	3423.6	2.092	0.003	9912
Feed Conversion	4	6772.9	1693.2	1.0346	0.4132	9904
BWG x Feed intake	5	9381.2	1876.2	1.1465	0.2746	9893
BWG x Feed Conversion	7	15596	2228	1.3614	0.0755	9881
Feed intake x Feed Conversion	8	13081	1635.2	0.99918	0.4699	9883
BWG x Feed int. x Feed Conv.	0	0		No test		
Residuals	690	1.1292E+06		1636.5		
Total	723	1.2364E+06				

C. ANOSIM to test gender effect

Analysis of Similarities
One-Way - A

Tests for differences between unordered Gender groups
Global Test
Sample statistic (R): 0,005
Significance level of sample statistic: 1,3%
Number of permutations: 9999 (Random sample from a large number)
Number of permuted statistics greater than or equal to R: 131

Table S2.5: Average dissimilarity (%) between high, medium and low groups for phosphorus utilization (PU), calcium utilization (CaU), feed intake (FI) body weight gain (BWG) and feed conversion (FC) by males and females

	High vs. Medium	Low vs. Medium	High vs. Low
PU	54.6	57.9	58.6
CaU	55.2	54.6	54.6
FI	56.4	57.3	60.9
BWG	55.4	58.9	60.4
FC	60.3	52.1	58.4

Table S2.6: ANOSIM pairwise tests by groups: phosphorus utilization (PU), calcium utilization (CaU), feed intake (FI), body weight gain (BWG), and feed conversion (FC)) by males and females

Anosim		male high - male low	male high - female high	male medium - female medium	male low - female low	female high - female low
PU	R-statistic	0.048	0.032	0.006	-0.009	0.03
	p-value	0.005	0.024	0.026	0.8	0.023
CaU	R-statistic	0.038	0.024	0.005	0.002	0.03
	p-value	0.01	0.06	0.028	0.342	0.027
FI	R-statistic	0.255	0.028	0.007	-0.009	0.092
	p-value	0.0001	0.035	0.012	0.762	0.0001
BWG	R-statistic	0.133	0.011	0.007	0	0.029
	p-value	0.0001	0.156	0.018	0.43	0.021
FC	R-statistic	0.06	0.004	0.003	-0.002	0.027
	p-value	0.002	0.305	0.1	0.497	0.027

Table S2.7 (excel file): Average- similarity and dissimilarity (%) between high, medium and low groups for phosphorus utilization (PU), calcium utilization (CaU), feed intake (FI) body weight gain (BWG) and feed conversion (FC) by males and females

Table S2.8: Pairwise comparison based on t-test for phosphorus utilization, calcium utilization, feed intake, body weight gain, and feed conversion and the most abundant OTUs (Unclassified Clostridiaceae1; Unclassified Lactobacillus; Unclassified Clostridium sensu stricto 1; Escherichia coli; Streptococcus alactolyticus; Enterococcus faecium). A. Phosphorus utilization. B. Calcium utilization. C. Feed intake. D. Body weight gain. E. Feed conversion.

A- Phosphorus utilization

Unclassified Clostridiaceae1

PU Gender	Estimate	Std Error	DF	Lower 95%	Upper 95%	Arithmetic Mean Estimate	N
PU_female_high	35.894688	2.5983536	753	30.793809	40.995566	35.894688	50
PU_female_low	27.124174	2.5983536	753	22.023296	32.225053	27.124174	50
PU_female_medium	27.587116	1.1372685	753	25.354522	29.819710	27.587116	261
PU_male_high	40.086446	2.5983536	753	34.985568	45.187325	40.086446	50
PU_male_low	26.092141	2.5983536	753	20.991263	31.193019	26.092141	50
PU_male_medium	29.512019	1.1329360	753	27.287931	31.736108	29.512019	263

PU Gender	-PU Gender	Difference	Std Error	t Ratio	Prob>|t|	Lower 95%	Upper 95%
PU_female_high	PU_female_low	8.7705	3.674627	2.39	0.0172*	1.5568	15.9842
PU_female_high	PU_female_medium	8.3076	2.836339	2.93	0.0035*	2.7395	13.8756
PU_female_high	PU_male_high	-4.1918	3.674627	-1.14	0.2543	-11.4055	3.0220

PU Gender	-PU Gender	Difference	Std Error	t Ratio	Prob>|t|	Lower 95%	Upper 95%
PU_female_high	PU_male_low	9.8025	3.674627	2.67	0.0078*	2.5888	17.0163
PU_female_high	PU_male_medium	6.3827	2.834605	2.25	0.0246*	0.8180	11.9473
PU_female_low	PU_female_medium	-0.4629	2.836339	-0.16	0.8704	-6.0310	5.1051
PU_female_low	PU_male_high	-12.9623	3.674627	-3.53	0.0004*	-20.1760	-5.7485
PU_female_low	PU_male_low	1.0320	3.674627	0.28	0.7789	-6.1817	8.2458
PU_female_low	PU_male_medium	-2.3878	2.834605	-0.84	0.3998	-7.9525	3.1768
PU_female_medium	PU_male_high	-12.4993	2.836339	-4.41	<.0001*	-18.0674	-6.9313
PU_female_medium	PU_male_low	1.4950	2.836339	0.53	0.5983	-4.0731	7.0630
PU_female_medium	PU_male_medium	-1.9249	1.605280	-1.20	0.2309	-5.0763	1.2265
PU_male_high	PU_male_low	13.9943	3.674627	3.81	0.0002*	6.7806	21.2080
PU_male_high	PU_male_medium	10.5744	2.834605	3.73	0.0002*	5.0098	16.1391
PU_male_low	PU_male_medium	-3.4199	2.834605	-1.21	0.2280	-8.9845	2.1448

Unclassified *Lactobacillus*

PU Gender	Estimate	Std Error	DF	Lower 95%	Upper 95%	Arithmetic Mean Estimate	N
PU_female_high	25.129815	2.8503812	753	19.534177	30.725454	25.129815	50
PU_female_low	20.887317	2.8503812	753	15.291678	26.482955	20.887317	50
PU_female_medium	25.101431	1.2475780	753	22.652287	27.550576	25.101431	261
PU_male_high	15.042881	2.8503812	753	9.447243	20.638520	15.042881	50
PU_male_low	21.999348	2.8503812	753	16.403710	27.594987	21.999348	50
PU_male_medium	21.416683	1.2428253	753	18.976869	23.856497	21.416683	263

PU Gender	-PU Gender	Difference	Std Error	t Ratio	Prob>|t|	Lower 95%	Upper 95%
PU_female_high	PU_female_low	4.2425	4.031048	1.05	0.2929	-3.6709	12.1559
PU_female_high	PU_female_medium	0.0284	3.111450	0.01	0.9927	-6.0798	6.1365
PU_female_high	PU_male_high	10.0869	4.031048	2.50	0.0125*	2.1735	18.0004
PU_female_high	PU_male_low	3.1305	4.031048	0.78	0.4376	-4.7830	11.0439
PU_female_high	PU_male_medium	3.7131	3.109548	1.19	0.2328	-2.3913	9.8175
PU_female_low	PU_female_medium	-4.2141	3.111450	-1.35	0.1760	-10.3223	1.8940
PU_female_low	PU_male_high	5.8444	4.031048	1.45	0.1475	-2.0690	13.7579
PU_female_low	PU_male_low	-1.1120	4.031048	-0.28	0.7827	-9.0255	6.8014
PU_female_low	PU_male_medium	-0.5294	3.109548	-0.17	0.8649	-6.6338	5.5750
PU_female_medium	PU_male_high	10.0586	3.111450	3.23	0.0013*	3.9504	16.1667
PU_female_medium	PU_male_low	3.1021	3.111450	1.00	0.3191	-3.0061	9.2102
PU_female_medium	PU_male_medium	3.6847	1.760984	2.09	0.0367*	0.2277	7.1418
PU_male_high	PU_male_low	-6.9565	4.031048	-1.73	0.0848	-14.8699	0.9570
PU_male_high	PU_male_medium	-6.3738	3.109548	-2.05	0.0407*	-12.4782	-0.2694
PU_male_low	PU_male_medium	0.5827	3.109548	0.19	0.8514	-5.5217	6.6871

Unclassified *Clostridium* sensu stricto1

PU Gender	Estimate	Std Error	DF	Lower 95%	Upper 95%	Arithmetic Mean Estimate	N
PU_female_high	7.797617	2.1370915	753	3.602252	11.992983	7.797617	50
PU_female_low	11.851688	2.1370915	753	7.656322	16.047054	11.851688	50
PU_female_medium	15.896603	0.9353796	753	14.060341	17.732865	15.896603	261
PU_male_high	10.060631	2.1370915	753	5.865265	14.255997	10.060631	50
PU_male_low	9.409131	2.1370915	753	5.213765	13.604497	9.409131	50
PU_male_medium	12.598227	0.9318162	753	10.768960	14.427493	12.598227	263

PU Gender	-PU Gender	Difference	Std Error	t Ratio	Prob>|t|	Lower 95%	Upper 95%
PU_female_high	PU_female_low	-4.05407	3.022304	-1.34	0.1802	-9.9872	1.8791
PU_female_high	PU_female_medium	-8.09899	2.332830	-3.47	0.0005*	-12.6786	-3.5194
PU_female_high	PU_male_high	-2.26301	3.022304	-0.75	0.4542	-8.1962	3.6701
PU_female_high	PU_male_low	-1.61151	3.022304	-0.53	0.5940	-7.5447	4.3216
PU_female_high	PU_male_medium	-4.80061	2.331403	-2.06	0.0398*	-9.3774	-0.2238
PU_female_low	PU_female_medium	-4.04492	2.332830	-1.73	0.0833	-8.6245	0.5347
PU_female_low	PU_male_high	1.79106	3.022304	0.59	0.5536	-4.1421	7.7242
PU_female_low	PU_male_low	2.44256	3.022304	0.81	0.4192	-3.4906	8.3757
PU_female_low	PU_male_medium	-0.74654	2.331403	-0.32	0.7489	-5.3234	3.8303
PU_female_medium	PU_male_high	5.83597	2.332830	2.50	0.0126*	1.2563	10.4156
PU_female_medium	PU_male_low	6.48747	2.332830	2.78	0.0056*	1.9078	11.0671
PU_female_medium	PU_male_medium	3.29838	1.320309	2.50	0.0127*	0.7065	5.8903
PU_male_high	PU_male_low	0.65150	3.022304	0.22	0.8294	-5.2816	6.5846
PU_male_high	PU_male_medium	-2.53760	2.331403	-1.09	0.2767	-7.1144	2.0392
PU_male_low	PU_male_medium	-3.18910	2.331403	-1.37	0.1718	-7.7659	1.3877

Escherichia coli

PU Gender	Estimate	Std Error	DF	Lower 95%	Upper 95%	Arithmetic Mean Estimate	N
PU_female_high	6.723986	1.3516429	753	4.0705491	9.377422	6.723986	50
PU_female_low	12.137077	1.3516429	753	9.4836409	14.790514	12.137077	50
PU_female_medium	10.959678	0.5915980	753	9.7983004	12.121056	10.959678	261
PU_male_high	10.967929	1.3516429	753	8.3144924	13.621365	10.967929	50
PU_male_low	9.824519	1.3516429	753	7.1710828	12.477956	9.824519	50
PU_male_medium	10.886050	0.5893443	753	9.7290971	12.043004	10.886050	263

PU Gender	-PU Gender	Difference	Std Error	t Ratio	Prob>|t|	Lower 95%	Upper 95%
PU_female_high	PU_female_low	-5.41309	1.911512	-2.83	0.0048*	-9.16562	-1.66057
PU_female_high	PU_female_medium	-4.23569	1.475441	-2.87	0.0042*	-7.13216	-1.33923
PU_female_high	PU_male_high	-4.24394	1.911512	-2.22	0.0267*	-7.99647	-0.49142
PU_female_high	PU_male_low	-3.10053	1.911512	-1.62	0.1052	-6.85306	0.65199
PU_female_high	PU_male_medium	-4.16206	1.474539	-2.82	0.0049*	-7.05676	-1.26737
PU_female_low	PU_female_medium	1.17740	1.475441	0.80	0.4251	-1.71907	4.07387

PU Gender	-PU Gender	Difference	Std Error	t Ratio	Prob>|t|	Lower 95%	Upper 95%
PU_female_low	PU_male_high	1.16915	1.911512	0.61	0.5410	-2.58338	4.92167
PU_female_low	PU_male_low	2.31256	1.911512	1.21	0.2267	-1.43997	6.06508
PU_female_low	PU_male_medium	1.25103	1.474539	0.85	0.3965	-1.64367	4.14572
PU_female_medium	PU_male_high	-0.00825	1.475441	-0.01	0.9955	-2.90472	2.88822
PU_female_medium	PU_male_low	1.13516	1.475441	0.77	0.4419	-1.76131	4.03163
PU_female_medium	PU_male_medium	0.07363	0.835054	0.09	0.9298	-1.56568	1.71294
PU_male_high	PU_male_low	1.14341	1.911512	0.60	0.5499	-2.60912	4.89594
PU_male_high	PU_male_medium	0.08188	1.474539	0.06	0.9557	-2.81282	2.97657
PU_male_low	PU_male_medium	-1.06153	1.474539	-0.72	0.4718	-3.95623	1.83317

Streptococcus alactolyticus

PU Gender	Estimate	Std Error	DF	Lower 95%	Upper 95%	Arithmetic Mean Estimate	N
PU_female_high	7.045891	2.0804632	753	2.9616928	11.130088	7.045891	50
PU_female_low	8.537856	2.0804632	753	4.4536581	12.622054	8.537856	50
PU_female_medium	6.234464	0.9105940	753	4.4468588	8.022068	6.234464	261
PU_male_high	8.002447	2.0804632	753	3.9182496	12.086645	8.002447	50
PU_male_low	12.931992	2.0804632	753	8.8477944	17.016190	12.931992	50
PU_male_medium	8.211126	0.9071251	753	6.4303307	9.991920	8.211126	263

PU Gender	-PU Gender	Difference	Std Error	t Ratio	Prob>|t|	Lower 95%	Upper 95%
PU_female_high	PU_female_low	-1.49197	2.942219	-0.51	0.6122	-7.2679	4.28396
PU_female_high	PU_female_medium	0.81143	2.271015	0.36	0.7210	-3.6468	5.26970
PU_female_high	PU_male_high	-0.95656	2.942219	-0.33	0.7452	-6.7325	4.81937
PU_female_high	PU_male_low	-5.88610	2.942219	-2.00	0.0458*	-11.6620	-0.11017
PU_female_high	PU_male_medium	-1.16523	2.269626	-0.51	0.6078	-5.6208	3.29031
PU_female_low	PU_female_medium	2.30339	2.271015	1.01	0.3108	-2.1549	6.76167
PU_female_low	PU_male_high	0.53541	2.942219	0.18	0.8557	-5.2405	6.31134
PU_female_low	PU_male_low	-4.39414	2.942219	-1.49	0.1357	-10.1701	1.38179
PU_female_low	PU_male_medium	0.32673	2.269626	0.14	0.8856	-4.1288	4.78228
PU_female_medium	PU_male_high	-1.76798	2.271015	-0.78	0.4365	-6.2263	2.69029
PU_female_medium	PU_male_low	-6.69753	2.271015	-2.95	0.0033*	-11.1558	-2.23926
PU_female_medium	PU_male_medium	-1.97666	1.285324	-1.54	0.1245	-4.4999	0.54658
PU_male_high	PU_male_low	-4.92954	2.942219	-1.68	0.0943	-10.7055	0.84638
PU_male_high	PU_male_medium	-0.20868	2.269626	-0.09	0.9268	-4.6642	4.24687
PU_male_low	PU_male_medium	4.72087	2.269626	2.08	0.0379*	0.2653	9.17641

Enterococcus faecium

PU Gender	Estimate	Std Error	DF	Lower 95%	Upper 95%	Arithmetic Mean Estimate	N
PU_female_high	0.87315828	0.48805053	753	-0.0849432	1.8312597	0.87315828	50
PU_female_low	0.90883386	0.48805053	753	-0.0492676	1.8669353	0.90883386	50
PU_female_medium	0.96652190	0.21361391	753	0.5471723	1.3858715	0.96652190	261
PU_male_high	0.66023534	0.48805053	753	-0.2978661	1.6183368	0.66023534	50
PU_male_low	0.66618125	0.48805053	753	-0.2919202	1.6242827	0.66618125	50
PU_male_medium	0.63829015	0.21280014	753	0.2205381	1.0560422	0.63829015	263

PU Gender	-PU Gender	Difference	Std Error	t Ratio	Prob>|t|	Lower 95%	Upper 95%
PU_female_high	PU_female_low	-0.035676	0.6902077	-0.05	0.9588	-1.39064	1.319285
PU_female_high	PU_female_medium	-0.093364	0.5327516	-0.18	0.8609	-1.13922	0.952491
PU_female_high	PU_male_high	0.212923	0.6902077	0.31	0.7578	-1.14204	1.567883
PU_female_high	PU_male_low	0.206977	0.6902077	0.30	0.7644	-1.14798	1.561937
PU_female_high	PU_male_medium	0.234868	0.5324258	0.44	0.6592	-0.81035	1.280084
PU_female_low	PU_female_medium	-0.057688	0.5327516	-0.11	0.9138	-1.10354	0.988167
PU_female_low	PU_male_high	0.248599	0.6902077	0.36	0.7188	-1.10636	1.603559
PU_female_low	PU_male_low	0.242653	0.6902077	0.35	0.7253	-1.11231	1.597613
PU_female_low	PU_male_medium	0.270544	0.5324258	0.51	0.6115	-0.77467	1.315759
PU_female_medium	PU_male_high	0.306287	0.5327516	0.57	0.5655	-0.73957	1.352141
PU_female_medium	PU_male_low	0.300341	0.5327516	0.56	0.5731	-0.74551	1.346196
PU_female_medium	PU_male_medium	0.328232	0.3015208	1.09	0.2767	-0.26369	0.920153
PU_male_high	PU_male_low	-0.005946	0.6902077	-0.01	0.9931	-1.36091	1.349014
PU_male_high	PU_male_medium	0.021945	0.5324258	0.04	0.9671	-1.02327	1.067161
PU_male_low	PU_male_medium	0.027891	0.5324258	0.05	0.9582	-1.01732	1.073106

B- Calcium utilization

Unclassified Clostridiaceae1

CaU Gender	Estimate	Std Error	DF	Lower 95%	Upper 95%	Arithmetic Mean Estimate	N
CaU_female_high	32.489594	2.6223129	753	27.341681	37.637507	32.489594	50
CaU_female_low	25.387795	2.6223129	753	20.239881	30.535708	25.387795	50
CaU_female_medium	28.572073	1.1477552	753	26.318892	30.825253	28.572073	261
CaU_male_high	37.474924	2.6223129	753	32.327011	42.622837	37.474924	50
CaU_male_low	28.417672	2.6223129	753	23.269759	33.565585	28.417672	50
CaU_male_medium	29.566390	1.1433827	753	27.321793	31.810987	29.566390	263

CaU Gender	-CaU Gender	Difference	Std Error	t Ratio	Prob>|t|	Lower 95%	Upper 95%
CaU_female_high	CaU_female_low	7.1018	3.708510	1.92	0.0559	-0.1784	14.3820
CaU_female_high	CaU_female_medium	3.9175	2.862493	1.37	0.1715	-1.7019	9.5369
CaU_female_high	CaU_male_high	-4.9853	3.708510	-1.34	0.1793	-12.2656	2.2949
CaU_female_high	CaU_male_low	4.0719	3.708510	1.10	0.2726	-3.2083	11.3522

CaU Gender	-CaU Gender	Difference	Std Error	t Ratio	Prob>|t|	Lower 95%	Upper 95%
CaU_female_high	CaU_male_medium	2.9232	2.860743	1.02	0.3072	-2.6928	8.5392
CaU_female_low	CaU_female_medium	-3.1843	2.862493	-1.11	0.2663	-8.8037	2.4351
CaU_female_low	CaU_male_high	-12.0871	3.708510	-3.26	0.0012*	-19.3674	-4.8069
CaU_female_low	CaU_male_low	-3.0299	3.708510	-0.82	0.4142	-10.3101	4.2504
CaU_female_low	CaU_male_medium	-4.1786	2.860743	-1.46	0.1445	-9.7946	1.4374
CaU_female_medium	CaU_male_high	-8.9029	2.862493	-3.11	0.0019*	-14.5223	-3.2834
CaU_female_medium	CaU_male_low	0.1544	2.862493	0.05	0.9570	-5.4650	5.7738
CaU_female_medium	CaU_male_medium	-0.9943	1.620082	-0.61	0.5396	-4.1747	2.1861
CaU_male_high	CaU_male_low	9.0573	3.708510	2.44	0.0148*	1.7770	16.3375
CaU_male_high	CaU_male_medium	7.9085	2.860743	2.76	0.0058*	2.2926	13.5245
CaU_male_low	CaU_male_medium	-1.1487	2.860743	-0.40	0.6881	-6.7647	4.4673

Unclassified *Lactobacillus*

CaU Gender	Estimate	Std Error	DF	Lower 95%	Upper 95%	Arithmetic Mean Estimate	N
CaU_female_high	25.108605	2.8561456	753	19.501650	30.715560	25.108605	50
CaU_female_low	24.313484	2.8561456	753	18.706529	29.920439	24.313484	50
CaU_female_medium	24.449141	1.2501010	753	21.995043	26.903238	24.449141	261
CaU_male_high	16.320415	2.8561456	753	10.713461	21.927370	16.320415	50
CaU_male_low	23.368407	2.8561456	753	17.761452	28.975362	23.368407	50
CaU_male_medium	20.913529	1.2453387	753	18.468780	23.358277	20.913529	263

CaU Gender	-CaU Gender	Difference	Std Error	t Ratio	Prob>|t|	Lower 95%	Upper 95%
CaU_female_high	CaU_female_low	0.7951	4.039200	0.20	0.8440	-7.1343	8.7246
CaU_female_high	CaU_female_medium	0.6595	3.117743	0.21	0.8325	-5.4610	6.7800
CaU_female_high	CaU_male_high	8.7882	4.039200	2.18	0.0299*	0.8588	16.7176
CaU_female_high	CaU_male_low	1.7402	4.039200	0.43	0.6667	-6.1892	9.6696
CaU_female_high	CaU_male_medium	4.1951	3.115836	1.35	0.1786	-1.9217	10.3118
CaU_female_low	CaU_female_medium	-0.1357	3.117743	-0.04	0.9653	-6.2562	5.9848
CaU_female_low	CaU_male_high	7.9931	4.039200	1.98	0.0482*	0.0636	15.9225
CaU_female_low	CaU_male_low	0.9451	4.039200	0.23	0.8151	-6.9844	8.8745
CaU_female_low	CaU_male_medium	3.4000	3.115836	1.09	0.2755	-2.7168	9.5167
CaU_female_medium	CaU_male_high	8.1287	3.117743	2.61	0.0093*	2.0082	14.2492
CaU_female_medium	CaU_male_low	1.0807	3.117743	0.35	0.7290	-5.0398	7.2012
CaU_female_medium	CaU_male_medium	3.5356	1.764546	2.00	0.0455*	0.0716	6.9996
CaU_male_high	CaU_male_low	-7.0480	4.039200	-1.74	0.0814	-14.9774	0.8814
CaU_male_high	CaU_male_medium	-4.5931	3.115836	-1.47	0.1409	-10.7099	1.5236
CaU_male_low	CaU_male_medium	2.4549	3.115836	0.79	0.4310	-3.6619	8.5716

Unclassified *Clostridium* sensu stricto 1

CaU Gender	Estimate	Std Error	DF	Lower 95%	Upper 95%	Arithmetic Mean Estimate	N
CaU_female_high	9.058401	2.1496329	753	4.838415	13.278387	9.058401	50
CaU_female_low	14.274486	2.1496329	753	10.054500	18.494472	14.274486	50
CaU_female_medium	15.190936	0.9408688	753	13.343899	17.037974	15.190936	261
CaU_male_high	10.990355	2.1496329	753	6.770369	15.210341	10.990355	50
CaU_male_low	11.122698	2.1496329	753	6.902712	15.342684	11.122698	50
CaU_male_medium	12.095700	0.9372845	753	10.255699	13.935701	12.095700	263

CaU Gender	-CaU Gender	Difference	Std Error	t Ratio	Prob>|t|	Lower 95%	Upper 95%
CaU_female_high	CaU_female_low	-5.21609	3.040040	-1.72	0.0866	-11.1840	0.75188
CaU_female_high	CaU_female_medium	-6.13254	2.346520	-2.61	0.0091*	-10.7390	-1.52604
CaU_female_high	CaU_male_high	-1.93195	3.040040	-0.64	0.5253	-7.8999	4.03601
CaU_female_high	CaU_male_low	-2.06430	3.040040	-0.68	0.4973	-8.0323	3.90366
CaU_female_high	CaU_male_medium	-3.03730	2.345085	-1.30	0.1957	-7.6410	1.56638
CaU_female_low	CaU_female_medium	-0.91645	2.346520	-0.39	0.6962	-5.5229	3.69005
CaU_female_low	CaU_male_high	3.28413	3.040040	1.08	0.2804	-2.6838	9.25209
CaU_female_low	CaU_male_low	3.15179	3.040040	1.04	0.3002	-2.8162	9.11975
CaU_female_low	CaU_male_medium	2.17879	2.345085	0.93	0.3531	-2.4249	6.78247
CaU_female_medium	CaU_male_high	4.20058	2.346520	1.79	0.0738	-0.4059	8.80708
CaU_female_medium	CaU_male_low	4.06824	2.346520	1.73	0.0834	-0.5383	8.67474
CaU_female_medium	CaU_male_medium	3.09524	1.328057	2.33	0.0200*	0.4881	5.70237
CaU_male_high	CaU_male_low	-0.13234	3.040040	-0.04	0.9653	-6.1003	5.83562
CaU_male_high	CaU_male_medium	-1.10534	2.345085	-0.47	0.6375	-5.7090	3.49834
CaU_male_low	CaU_male_medium	-0.97300	2.345085	-0.41	0.6783	-5.5767	3.63068

Escherichia coli

CaU Gender	Estimate	Std Error	DF	Lower 95%	Upper 95%	Arithmetic Mean Estimate	N
CaU_female_high	6.910912	1.3506962	753	4.259334	9.562490	6.910912	50
CaU_female_low	13.151423	1.3506962	753	10.499845	15.803001	13.151423	50
CaU_female_medium	10.729549	0.5911837	753	9.568985	11.890113	10.729549	261
CaU_male_high	10.474913	1.3506962	753	7.823335	13.126491	10.474913	50
CaU_male_low	11.558874	1.3506962	753	8.907296	14.210452	11.558874	50
CaU_male_medium	10.650054	0.5889315	753	9.493911	11.806197	10.650054	263

CaU Gender	-CaU Gender	Difference	Std Error	t Ratio	Prob>|t|	Lower 95%	Upper 95%
CaU_female_high	CaU_female_low	-6.24051	1.910173	-3.27	0.0011*	-9.99041	-2.49061
CaU_female_high	CaU_female_medium	-3.81864	1.474408	-2.59	0.0098*	-6.71308	-0.92420
CaU_female_high	CaU_male_high	-3.56400	1.910173	-1.87	0.0625	-7.31390	0.18590
CaU_female_high	CaU_male_low	-4.64796	1.910173	-2.43	0.0152*	-8.39786	-0.89806
CaU_female_high	CaU_male_medium	-3.73914	1.473506	-2.54	0.0114*	-6.63181	-0.84647
CaU_female_low	CaU_female_medium	2.42187	1.474408	1.64	0.1009	-0.47257	5.31631
CaU_female_low	CaU_male_high	2.67651	1.910173	1.40	0.1616	-1.07339	6.42641
CaU_female_low	CaU_male_low	1.59255	1.910173	0.83	0.4047	-2.15735	5.34245
CaU_female_low	CaU_male_medium	2.50137	1.473506	1.70	0.0900	-0.39130	5.39404
CaU_female_medium	CaU_male_high	0.25464	1.474408	0.17	0.8629	-2.63980	3.14907
CaU_female_medium	CaU_male_low	-0.82932	1.474408	-0.56	0.5740	-3.72376	2.06511
CaU_female_medium	CaU_male_medium	0.07949	0.834469	0.10	0.9241	-1.55867	1.71766
CaU_male_high	CaU_male_low	-1.08396	1.910173	-0.57	0.5706	-4.83386	2.66594
CaU_male_high	CaU_male_medium	-0.17514	1.473506	-0.12	0.9054	-3.06781	2.71753
CaU_male_low	CaU_male_medium	0.90882	1.473506	0.62	0.5376	-1.98385	3.80149

Streptococcus alactolyticus

CaU Gender	Estimate	Std Error	DF	Lower 95%	Upper 95%	Arithmetic Mean Estimate	N
CaU_female_high	10.208999	2.0801208	753	6.1254739	14.292525	10.208999	50
CaU_female_low	4.991869	2.0801208	753	0.9083441	9.075395	4.991869	50
CaU_female_medium	6.307812	0.9104441	753	4.5205013	8.095122	6.307812	261
CaU_male_high	6.425196	2.0801208	753	2.3416706	10.508721	6.425196	50
CaU_male_low	7.775978	2.0801208	753	3.6924523	11.859503	7.775978	50
CaU_male_medium	9.491214	0.9069757	753	7.7107124	11.271716	9.491214	263

CaU Gender	-CaU Gender	Difference	Std Error	t Ratio	Prob>|t|	Lower 95%	Upper 95%
CaU_female_high	CaU_female_low	5.21713	2.941735	1.77	0.0766	-0.55785	10.9921
CaU_female_high	CaU_female_medium	3.90119	2.270641	1.72	0.0862	-0.55635	8.3587
CaU_female_high	CaU_male_high	3.78380	2.941735	1.29	0.1988	-1.99117	9.5588
CaU_female_high	CaU_male_low	2.43302	2.941735	0.83	0.4085	-3.34196	8.2080
CaU_female_high	CaU_male_medium	0.71779	2.269253	0.32	0.7519	-3.73703	5.1726
CaU_female_low	CaU_female_medium	-1.31594	2.270641	-0.58	0.5624	-5.77348	3.1416
CaU_female_low	CaU_male_high	-1.43333	2.941735	-0.49	0.6262	-7.20830	4.3417
CaU_female_low	CaU_male_low	-2.78411	2.941735	-0.95	0.3442	-8.55909	2.9909
CaU_female_low	CaU_male_medium	-4.49934	2.269253	-1.98	0.0478*	-8.95416	-0.0445
CaU_female_medium	CaU_male_high	-0.11738	2.270641	-0.05	0.9588	-4.57492	4.3402
CaU_female_medium	CaU_male_low	-1.46817	2.270641	-0.65	0.5181	-5.92571	2.9894
CaU_female_medium	CaU_male_medium	-3.18340	1.285112	-2.48	0.0135*	-5.70623	-0.6606
CaU_male_high	CaU_male_low	-1.35078	2.941735	-0.46	0.6462	-7.12576	4.4242
CaU_male_high	CaU_male_medium	-3.06602	2.269253	-1.35	0.1771	-7.52083	1.3888
CaU_male_low	CaU_male_medium	-1.71524	2.269253	-0.76	0.4500	-6.17005	2.7396

Enterococcus faecium

CaU Gender	Estimate	Std Error	DF	Lower 95%	Upper 95%	Arithmetic Mean Estimate	N
CaU_female_high	1.0514501	0.76519097	753	-0.450711	2.5536113	1.0514501	50
CaU_female_low	2.3583525	0.76519097	753	0.856191	3.8605137	2.3583525	50
CaU_female_medium	1.1321759	0.33491499	753	0.474698	1.7896540	1.1321759	261
CaU_male_high	2.1941066	0.76519097	753	0.691945	3.6962679	2.1941066	50
CaU_male_low	1.1413537	0.76519097	753	-0.360808	2.6435149	1.1413537	50
CaU_male_medium	1.8026372	0.33363912	753	1.147664	2.4576106	1.8026372	263

CaU Gender	-CaU Gender	Difference	Std Error	t Ratio	Prob>|t|	Lower 95%	Upper 95%
CaU_female_high	CaU_female_low	-1.30690	1.082143	-1.21	0.2275	-3.43128	0.817474
CaU_female_high	CaU_female_medium	-0.08073	0.835276	-0.10	0.9230	-1.72047	1.559020
CaU_female_high	CaU_male_high	-1.14266	1.082143	-1.06	0.2913	-3.26703	0.981720
CaU_female_high	CaU_male_low	-0.08990	1.082143	-0.08	0.9338	-2.21428	2.034473
CaU_female_high	CaU_male_medium	-0.75119	0.834765	-0.90	0.3685	-2.38993	0.887556
CaU_female_low	CaU_female_medium	1.22618	0.835276	1.47	0.1425	-0.41357	2.865922
CaU_female_low	CaU_male_high	0.16425	1.082143	0.15	0.8794	-1.96013	2.288623
CaU_female_low	CaU_male_low	1.21700	1.082143	1.12	0.2611	-0.90738	3.341376
CaU_female_low	CaU_male_medium	0.55572	0.834765	0.67	0.5058	-1.08303	2.194458
CaU_female_medium	CaU_male_high	-1.06193	0.835276	-1.27	0.2040	-2.70168	0.577815

CaU Gender	-CaU Gender	Difference	Std Error	t Ratio	Prob>|t|	Lower 95%	Upper 95%
CaU_female_medium	CaU_male_low	-0.00918	0.835276	-0.01	0.9912	-1.64892	1.630568
CaU_female_medium	CaU_male_medium	-0.67046	0.472740	-1.42	0.1565	-1.59851	0.257584
CaU_male_high	CaU_male_low	1.05275	1.082143	0.97	0.3309	-1.07162	3.177130
CaU_male_high	CaU_male_medium	0.39147	0.834765	0.47	0.6392	-1.24727	2.030212
CaU_male_low	CaU_male_medium	-0.66128	0.834765	-0.79	0.4285	-2.30003	0.977459

C- Feed intake

Unclassified Clostridiaceae1

Feed intake Gender	Estimate	Std Error	DF	Lower 95%	Upper 95%	Arithmetic Mean Estimate	N
FI_female_high	40.894289	2.4762081	753	36.033197	45.755382	40.894289	50
FI_female_low	24.278384	2.4762081	753	19.417292	29.139476	24.278384	50
FI_female_medium	27.174509	1.0838069	753	25.046866	29.302151	27.174509	261
FI_male_high	49.159110	2.4762081	753	44.298017	54.020202	49.159110	50
FI_male_low	20.438314	2.4762081	753	15.577221	25.299406	20.438314	50
FI_male_medium	28.862051	1.0796781	753	26.742514	30.981587	28.862051	263

Feed intake Gender	-Feed intake Gender	Difference	Std Error	t Ratio	Prob>|t|	Lower 95%	Upper 95%
FI_female_high	FI_female_low	16.6159	3.501887	4.74	<.0001*	9.7413	23.4905
FI_female_high	FI_female_medium	13.7198	2.703006	5.08	<.0001*	8.4135	19.0261
FI_female_high	FI_male_high	-8.2648	3.501887	-2.36	0.0185*	-15.1394	-1.3902
FI_female_high	FI_male_low	20.4560	3.501887	5.84	<.0001*	13.5814	27.3306
FI_female_high	FI_male_medium	12.0322	2.701354	4.45	<.0001*	6.7292	17.3353
FI_female_low	FI_female_medium	-2.8961	2.703006	-1.07	0.2843	-8.2024	2.4102
FI_female_low	FI_male_high	-24.8807	3.501887	-7.10	<.0001*	-31.7553	-18.0061
FI_female_low	FI_male_low	3.8401	3.501887	1.10	0.2732	-3.0346	10.7147
FI_female_low	FI_male_medium	-4.5837	2.701354	-1.70	0.0901	-9.8867	0.7194
FI_female_medium	FI_male_high	-21.9846	2.703006	-8.13	<.0001*	-27.2909	-16.6783
FI_female_medium	FI_male_low	6.7362	2.703006	2.49	0.0129*	1.4299	12.0425
FI_female_medium	FI_male_medium	-1.6875	1.529818	-1.10	0.2703	-4.6908	1.3157
FI_male_high	FI_male_low	28.7208	3.501887	8.20	<.0001*	21.8462	35.5954
FI_male_high	FI_male_medium	20.2971	2.701354	7.51	<.0001*	14.9940	25.6001
FI_male_low	FI_male_medium	-8.4237	2.701354	-3.12	0.0019*	-13.7268	-3.1207

Unclassified *Lactobacillus*

Feed intake Gender	Estimate	Std Error	DF	Lower 95%	Upper 95%	Arithmetic Mean Estimate	N
FI_female_high	14.440172	2.8123477	753	8.919197	19.961146	14.440172	50
FI_female_low	23.726048	2.8123477	753	18.205073	29.247022	23.726048	50
FI_female_medium	26.605438	1.2309312	753	24.188973	29.021902	26.605438	261
FI_male_high	12.927782	2.8123477	753	7.406808	18.448757	12.927782	50
FI_male_low	25.979150	2.8123477	753	20.458176	31.500124	25.979150	50
FI_male_medium	21.062177	1.2262419	753	18.654918	23.469436	21.062177	263

Feed intake Gender	-Feed intake Gender	Difference	Std Error	t Ratio	Prob>|t|	Lower 95%	Upper 95%
FI_female_high	FI_female_low	-9.2859	3.977260	-2.33	0.0198*	-17.0937	-1.4780
FI_female_high	FI_female_medium	-12.1653	3.069933	-3.96	<.0001*	-18.1919	-6.1386
FI_female_high	FI_male_high	1.5124	3.977260	0.38	0.7039	-6.2954	9.3202
FI_female_high	FI_male_low	-11.5390	3.977260	-2.90	0.0038*	-19.3468	-3.7311
FI_female_high	FI_male_medium	-6.6220	3.068056	-2.16	0.0312*	-12.6450	-0.5990
FI_female_low	FI_female_medium	-2.8794	3.069933	-0.94	0.3486	-8.9060	3.1473
FI_female_low	FI_male_high	10.7983	3.977260	2.72	0.0068*	2.9904	18.6061
FI_female_low	FI_male_low	-2.2531	3.977260	-0.57	0.5712	-10.0609	5.5547
FI_female_low	FI_male_medium	2.6639	3.068056	0.87	0.3855	-3.3591	8.6868
FI_female_medium	FI_male_high	13.6777	3.069933	4.46	<.0001*	7.6510	19.7043
FI_female_medium	FI_male_low	0.6263	3.069933	0.20	0.8384	-5.4004	6.6529
FI_female_medium	FI_male_medium	5.5433	1.737487	3.19	0.0015*	2.1324	8.9542
FI_male_high	FI_male_low	-13.0514	3.977260	-3.28	0.0011*	-20.8592	-5.2435
FI_male_high	FI_male_medium	-8.1344	3.068056	-2.65	0.0082*	-14.1574	-2.1114
FI_male_low	FI_male_medium	4.9170	3.068056	1.60	0.1094	-1.1060	10.9399

Unclassified *Clostridium* sensu stricto 1

Feed intake Gender	Estimate	Std Error	DF	Lower 95%	Upper 95%	Arithmetic Mean Estimate	N
FI_female_high	13.881817	2.1547856	753	9.651716	18.111918	13.881817	50
FI_female_low	13.180275	2.1547856	753	8.950173	17.410376	13.180275	50
FI_female_medium	14.476529	0.9431240	753	12.625064	16.327994	14.476529	261
FI_male_high	8.285485	2.1547856	753	4.055384	12.515586	8.285485	50
FI_male_low	11.725654	2.1547856	753	7.495553	15.955755	11.725654	50
FI_male_medium	12.495303	0.9395312	753	10.650892	14.339715	12.495303	263

Feed intake Gender	-Feed intake Gender	Difference	Std Error	t Ratio	Prob>|t|	Lower 95%	Upper 95%
FI_female_high	FI_female_low	0.70154	3.047327	0.23	0.8180	-5.2807	6.6838
FI_female_high	FI_female_medium	-0.59471	2.352145	-0.25	0.8005	-5.2123	4.0228
FI_female_high	FI_male_high	5.59633	3.047327	1.84	0.0667	-0.3859	11.5786
FI_female_high	FI_male_low	2.15616	3.047327	0.71	0.4794	-3.8261	8.1384
FI_female_high	FI_male_medium	1.38651	2.350706	0.59	0.5555	-3.2282	6.0012
FI_female_low	FI_female_medium	-1.29625	2.352145	-0.55	0.5817	-5.9138	3.3213
FI_female_low	FI_male_high	4.89479	3.047327	1.61	0.1086	-1.0875	10.8771
FI_female_low	FI_male_low	1.45462	3.047327	0.48	0.6333	-4.5276	7.4369
FI_female_low	FI_male_medium	0.68497	2.350706	0.29	0.7708	-3.9297	5.2997
FI_female_medium	FI_male_high	6.19104	2.352145	2.63	0.0087*	1.5735	10.8086
FI_female_medium	FI_male_low	2.75088	2.352145	1.17	0.2426	-1.8667	7.3684
FI_female_medium	FI_male_medium	1.98123	1.331241	1.49	0.1371	-0.6322	4.5946
FI_male_high	FI_male_low	-3.44017	3.047327	-1.13	0.2593	-9.4224	2.5421
FI_male_high	FI_male_medium	-4.20982	2.350706	-1.79	0.0737	-8.8245	0.4049
FI_male_low	FI_male_medium	-0.76965	2.350706	-0.33	0.7434	-5.3844	3.8451

Escherichia coli

Feed intake Gender	Estimate	Std Error	DF	Lower 95%	Upper 95%	Arithmetic Mean Estimate	N
FI_female_high	9.746420	1.3570771	753	7.0823160	12.410525	9.746420	50
FI_female_low	12.350082	1.3570771	753	9.6859780	15.014187	12.350082	50

Feed intake Gender	Estimate	Std Error	DF	Lower 95%	Upper 95%	Arithmetic Mean Estimate	N
FI_female_medium	10.339862	0.5939765	753	9.1738151	11.505909	10.339862	261
FI_male_high	8.998504	1.3570771	753	6.3343997	11.662608	8.998504	50
FI_male_low	10.553040	1.3570771	753	7.8889358	13.217144	10.553040	50
FI_male_medium	11.121964	0.5917137	753	9.9603589	12.283568	11.121964	263

Feed intake Gender	-Feed intake Gender	Difference	Std Error	t Ratio	Prob>|t|	Lower 95%	Upper 95%
FI_female_high	FI_female_low	-2.60366	1.919197	-1.36	0.1753	-6.37127	1.163951
FI_female_high	FI_female_medium	-0.59344	1.481373	-0.40	0.6888	-3.50155	2.314671
FI_female_high	FI_male_high	0.74792	1.919197	0.39	0.6969	-3.01970	4.515529
FI_female_high	FI_male_low	-0.80662	1.919197	-0.42	0.6744	-4.57423	2.960993
FI_female_high	FI_male_medium	-1.37554	1.480467	-0.93	0.3531	-4.28188	1.530791
FI_female_low	FI_female_medium	2.01022	1.481373	1.36	0.1752	-0.89789	4.918333
FI_female_low	FI_male_high	3.35158	1.919197	1.75	0.0812	-0.41603	7.119191
FI_female_low	FI_male_low	1.79704	1.919197	0.94	0.3494	-1.97057	5.564655
FI_female_low	FI_male_medium	1.22812	1.480467	0.83	0.4071	-1.67822	4.134453
FI_female_medium	FI_male_high	1.34136	1.481373	0.91	0.3655	-1.56675	4.249470
FI_female_medium	FI_male_low	-0.21318	1.481373	-0.14	0.8856	-3.12129	2.694934
FI_female_medium	FI_male_medium	-0.78210	0.838411	-0.93	0.3512	-2.42800	0.863799
FI_male_high	FI_male_low	-1.55454	1.919197	-0.81	0.4182	-5.32215	2.213076
FI_male_high	FI_male_medium	-2.12346	1.480467	-1.43	0.1519	-5.02979	0.782874
FI_male_low	FI_male_medium	-0.56892	1.480467	-0.38	0.7009	-3.47526	2.337411

Streptococcus alactolyticus

Feed intake Gender	Estimate	Std Error	DF	Lower 95%	Upper 95%	Arithmetic Mean Estimate	N
FI_female_high	4.506045	2.0684554	753	0.445420	8.566670	4.506045	50
FI_female_low	8.392691	2.0684554	753	4.332066	12.453315	8.392691	50
FI_female_medium	6.748834	0.9053383	753	4.971546	8.526121	6.748834	261
FI_male_high	2.742499	2.0684554	753	-1.318126	6.803124	2.742499	50
FI_male_low	12.840554	2.0684554	753	8.779929	16.901179	12.840554	50
FI_male_medium	9.228499	0.9018894	753	7.457983	10.999016	9.228499	263

Feed intake Gender	-Feed intake Gender	Difference	Std Error	t Ratio	Prob>|t|	Lower 95%	Upper 95%
FI_female_high	FI_female_low	-3.8866	2.925238	-1.33	0.1844	-9.6292	1.8559
FI_female_high	FI_female_medium	-2.2428	2.257907	-0.99	0.3209	-6.6753	2.1898
FI_female_high	FI_male_high	1.7635	2.925238	0.60	0.5468	-3.9790	7.5061
FI_female_high	FI_male_low	-8.3345	2.925238	-2.85	0.0045*	-14.0771	-2.5919
FI_female_high	FI_male_medium	-4.7225	2.256527	-2.09	0.0367*	-9.1523	-0.2926
FI_female_low	FI_female_medium	1.6439	2.257907	0.73	0.4668	-2.7887	6.0764
FI_female_low	FI_male_high	5.6502	2.925238	1.93	0.0538	-0.0924	11.3928
FI_female_low	FI_male_low	-4.4479	2.925238	-1.52	0.1288	-10.1905	1.2947
FI_female_low	FI_male_medium	-0.8358	2.256527	-0.37	0.7112	-5.2656	3.5940
FI_female_medium	FI_male_high	4.0063	2.257907	1.77	0.0764	-0.4262	8.4389
FI_female_medium	FI_male_low	-6.0917	2.257907	-2.70	0.0071*	-10.5243	-1.6592
FI_female_medium	FI_male_medium	-2.4797	1.277905	-1.94	0.0527	-4.9883	0.0290
FI_male_high	FI_male_low	-10.0981	2.925238	-3.45	0.0006*	-15.8406	-4.3555
FI_male_high	FI_male_medium	-6.4860	2.256527	-2.87	0.0042*	-10.9158	-2.0562
FI_male_low	FI_male_medium	3.6121	2.256527	1.60	0.1099	-0.8178	8.0419

Enterococcus faecium

Feed intake Gender	Estimate	Std Error	DF	Lower 95%	Upper 95%	Arithmetic Mean Estimate	N
FI_female_high	1.0165278	0.76222781	753	-0.479816	2.5128720	1.0165278	50
FI_female_low	3.3210065	0.76222781	753	1.824662	4.8173507	3.3210065	50
FI_female_medium	0.9544495	0.33361805	753	0.299517	1.6093815	0.9544495	261
FI_male_high	1.5457418	0.76222781	753	0.049398	3.0420860	1.5457418	50
FI_male_low	2.4312563	0.76222781	753	0.934912	3.9276005	2.4312563	50
FI_male_medium	1.6806718	0.33234712	753	1.028235	2.3331089	1.6806718	263

Feed intake Gender	-Feed intake Gender	Difference	Std Error	t Ratio	Prob>|t|	Lower 95%	Upper 95%
FI_female_high	FI_female_low	-2.30448	1.077953	-2.14	0.0329*	-4.42063	-0.18833
FI_female_high	FI_female_medium	0.06208	0.832041	0.07	0.9405	-1.57132	1.69547
FI_female_high	FI_male_high	-0.52921	1.077953	-0.49	0.6236	-2.64536	1.58694
FI_female_high	FI_male_low	-1.41473	1.077953	-1.31	0.1898	-3.53088	0.70142
FI_female_high	FI_male_medium	-0.66414	0.831532	-0.80	0.4247	-2.29654	0.96825
FI_female_low	FI_female_medium	2.36656	0.832041	2.84	0.0046*	0.73316	3.99995
FI_female_low	FI_male_high	1.77526	1.077953	1.65	0.1000	-0.34089	3.89141
FI_female_low	FI_male_low	0.88975	1.077953	0.83	0.4094	-1.22640	3.00590
FI_female_low	FI_male_medium	1.64033	0.831532	1.97	0.0489*	0.00794	3.27273
FI_female_medium	FI_male_high	-0.59129	0.832041	-0.71	0.4775	-2.22469	1.04210
FI_female_medium	FI_male_low	-1.47681	0.832041	-1.77	0.0763	-3.11020	0.15659
FI_female_medium	FI_male_medium	-0.72622	0.470909	-1.54	0.1235	-1.65067	0.19823
FI_male_high	FI_male_low	-0.88551	1.077953	-0.82	0.4116	-3.00166	1.23064
FI_male_high	FI_male_medium	-0.13493	0.831532	-0.16	0.8711	-1.76733	1.49747
FI_male_low	FI_male_medium	0.75058	0.831532	0.90	0.3670	-0.88181	2.38298

D- Body weight gain

Unclassified Clostridiaceae1

BWG Gender	Estimate	Std Error	DF	Lower 95%	Upper 95%	Arithmetic Mean Estimate	N
BWG_female_high	36.052649	2.5686635	753	31.010056	41.095242	36.052649	50
BWG_female_low	26.244438	2.5686635	753	21.201845	31.287031	26.244438	50
BWG_female_medium	27.725387	1.1242735	753	25.518304	29.932470	27.725387	261
BWG_male_high	43.160369	2.5686635	753	38.117776	48.202962	43.160369	50
BWG_male_low	22.556769	2.5686635	753	17.514176	27.599362	22.556769	50
BWG_male_medium	29.599747	1.1199905	753	27.401072	31.798422	29.599747	263

BWG Gender	-BWG Gender	Difference	Std Error	t Ratio	Prob>|t|	Lower 95%	Upper 95%
BWG_female_high	BWG_female_low	9.8082	3.632639	2.70	0.0071*	2.6769	16.9395
BWG_female_high	BWG_female_medium	8.3273	2.803930	2.97	0.0031*	2.8228	13.8317
BWG_female_high	BWG_male_high	-7.1077	3.632639	-1.96	0.0508	-14.2390	0.0236
BWG_female_high	BWG_male_low	13.4959	3.632639	3.72	0.0002*	6.3646	20.6272
BWG_female_high	BWG_male_medium	6.4529	2.802215	2.30	0.0216*	0.9518	11.9540
BWG_female_low	BWG_female_medium	-1.4809	2.803930	-0.53	0.5975	-6.9854	4.0235
BWG_female_low	BWG_male_high	-16.9159	3.632639	-4.66	<.0001*	-24.0472	-9.7846
BWG_female_low	BWG_male_low	3.6877	3.632639	1.02	0.3104	-3.4436	10.8190
BWG_female_low	BWG_male_medium	-3.3553	2.802215	-1.20	0.2315	-8.8564	2.1458
BWG_female_medium	BWG_male_high	-15.4350	2.803930	-5.50	<.0001*	-20.9394	-9.9305
BWG_female_medium	BWG_male_low	5.1686	2.803930	1.84	0.0657	-0.3358	10.6731
BWG_female_medium	BWG_male_medium	-1.8744	1.586937	-1.18	0.2379	-4.9897	1.2410
BWG_male_high	BWG_male_low	20.6036	3.632639	5.67	<.0001*	13.4723	27.7349
BWG_male_high	BWG_male_medium	13.5606	2.802215	4.84	<.0001*	8.0595	19.0617
BWG_male_low	BWG_male_medium	-7.0430	2.802215	-2.51	0.0122*	-12.5441	-1.5419

Unclassified *Lactobacillus*

BWG Gender	Estimate	Std Error	DF	Lower 95%	Upper 95%	Arithmetic Mean Estimate	N
BWG_female_high	16.594142	2.8372794	753	11.024224	22.164060	16.594142	50
BWG_female_low	22.386865	2.8372794	753	16.816947	27.956784	22.386865	50
BWG_female_medium	26.449348	1.2418434	753	24.011461	28.887235	26.449348	261
BWG_male_high	16.367436	2.8372794	753	10.797518	21.937355	16.367436	50
BWG_male_low	21.449781	2.8372794	753	15.879863	27.019700	21.449781	50
BWG_male_medium	21.269347	1.2371126	753	18.840747	23.697947	21.269347	263

BWG Gender	-BWG Gender	Difference	Std Error	t Ratio	Prob>|t|	Lower 95%	Upper 95%
BWG_female_high	BWG_female_low	-5.7927	4.012519	-1.44	0.1492	-13.6698	2.0843
BWG_female_high	BWG_female_medium	-9.8552	3.097149	-3.18	0.0015*	-15.9353	-3.7751
BWG_female_high	BWG_male_high	0.2267	4.012519	0.06	0.9550	-7.6503	8.1038
BWG_female_high	BWG_male_low	-4.8556	4.012519	-1.21	0.2266	-12.7327	3.0214
BWG_female_high	BWG_male_medium	-4.6752	3.095255	-1.51	0.1314	-10.7516	1.4011
BWG_female_low	BWG_female_medium	-4.0625	3.097149	-1.31	0.1900	-10.1426	2.0176
BWG_female_low	BWG_male_high	6.0194	4.012519	1.50	0.1340	-1.8576	13.8965
BWG_female_low	BWG_male_low	0.9371	4.012519	0.23	0.8154	-6.9400	8.8141
BWG_female_low	BWG_male_medium	1.1175	3.095255	0.36	0.7182	-4.9588	7.1939
BWG_female_medium	BWG_male_high	10.0819	3.097149	3.26	0.0012*	4.0018	16.1620
BWG_female_medium	BWG_male_low	4.9996	3.097149	1.61	0.1069	-1.0805	11.0796
BWG_female_medium	BWG_male_medium	5.1800	1.752890	2.96	0.0032*	1.7389	8.6211
BWG_male_high	BWG_male_low	-5.0823	4.012519	-1.27	0.2057	-12.9594	2.7947
BWG_male_high	BWG_male_medium	-4.9019	3.095255	-1.58	0.1137	-10.9783	1.1744
BWG_male_low	BWG_male_medium	0.1804	3.095255	0.06	0.9535	-5.8959	6.2568

Unclassified *Clostridium* sensu stricto 1

BWG Gender	Estimate	Std Error	DF	Lower 95%	Upper 95%	Arithmetic Mean Estimate	N
BWG_female_high	13.620069	2.1511890	753	9.397028	17.843110	13.620069	50
BWG_female_low	10.588467	2.1511890	753	6.365426	14.811508	10.588467	50
BWG_female_medium	15.023187	0.9415499	753	13.174813	16.871562	15.023187	261
BWG_male_high	9.974806	2.1511890	753	5.751765	14.197847	9.974806	50
BWG_male_low	9.629042	2.1511890	753	5.406001	13.852083	9.629042	50
BWG_male_medium	12.572735	0.9379630	753	10.731402	14.414069	12.572735	263

BWG Gender	-BWG Gender	Difference	Std Error	t Ratio	Prob>|t|	Lower 95%	Upper 95%
BWG_female_high	BWG_female_low	3.03160	3.042241	1.00	0.3193	-2.9407	9.0039
BWG_female_high	BWG_female_medium	-1.40312	2.348219	-0.60	0.5503	-6.0130	3.2067
BWG_female_high	BWG_male_high	3.64526	3.042241	1.20	0.2312	-2.3270	9.6175
BWG_female_high	BWG_male_low	3.99103	3.042241	1.31	0.1900	-1.9813	9.9633
BWG_female_high	BWG_male_medium	1.04733	2.346783	0.45	0.6555	-3.5597	5.6543
BWG_female_low	BWG_female_medium	-4.43472	2.348219	-1.89	0.0593	-9.0446	0.1751
BWG_female_low	BWG_male_high	0.61366	3.042241	0.20	0.8402	-5.3586	6.5859
BWG_female_low	BWG_male_low	0.95942	3.042241	0.32	0.7526	-5.0129	6.9317
BWG_female_low	BWG_male_medium	-1.98427	2.346783	-0.85	0.3981	-6.5913	2.6227
BWG_female_medium	BWG_male_high	5.04838	2.348219	2.15	0.0319*	0.4385	9.6582
BWG_female_medium	BWG_male_low	5.39415	2.348219	2.30	0.0219*	0.7843	10.0040
BWG_female_medium	BWG_male_medium	2.45045	1.329019	1.84	0.0656	-0.1586	5.0595
BWG_male_high	BWG_male_low	0.34576	3.042241	0.11	0.9095	-5.6265	6.3180
BWG_male_high	BWG_male_medium	-2.59793	2.346783	-1.11	0.2686	-7.2049	2.0091
BWG_male_low	BWG_male_medium	-2.94369	2.346783	-1.25	0.2101	-7.5507	1.6633

Escherichia coli

BWG Gender	Estimate	Std Error	DF	Lower 95%	Upper 95%	Arithmetic Mean Estimate	N
BWG_female_high	10.230986	1.3592326	753	7.5626500	12.899322	10.230986	50
BWG_female_low	11.165203	1.3592326	753	8.4968669	13.833539	11.165203	50
BWG_female_medium	10.474022	0.5949199	753	9.3061229	11.641921	10.474022	261

BWG Gender	Estimate	Std Error	DF	Lower 95%	Upper 95%	Arithmetic Mean Estimate	N
BWG_male_high	10.062772	1.3592326	753	7.3944360	12.731108	10.062772	50
BWG_male_low	9.416888	1.3592326	753	6.7485526	12.085224	9.416888	50
BWG_male_medium	11.135630	0.5926536	753	9.9721801	12.299080	11.135630	263

BWG Gender	-BWG Gender	Difference	Std Error	t Ratio	Prob>|t|	Lower 95%	Upper 95%
BWG_female_high	BWG_female_low	-0.93422	1.922245	-0.49	0.6271	-4.70781	2.839380
BWG_female_high	BWG_female_medium	-0.24304	1.483726	-0.16	0.8699	-3.15577	2.669695
BWG_female_high	BWG_male_high	0.16821	1.922245	0.09	0.9303	-3.60538	3.941811
BWG_female_high	BWG_male_low	0.81410	1.922245	0.42	0.6720	-2.95950	4.587694
BWG_female_high	BWG_male_medium	-0.90464	1.482819	-0.61	0.5420	-3.81559	2.006306
BWG_female_low	BWG_female_medium	0.69118	1.483726	0.47	0.6415	-2.22155	3.603912
BWG_female_low	BWG_male_high	1.10243	1.922245	0.57	0.5665	-2.67117	4.876028
BWG_female_low	BWG_male_low	1.74831	1.922245	0.91	0.3634	-2.02528	5.521911
BWG_female_low	BWG_male_medium	0.02957	1.482819	0.02	0.9841	-2.88138	2.940523
BWG_female_medium	BWG_male_high	0.41125	1.483726	0.28	0.7817	-2.50148	3.323981
BWG_female_medium	BWG_male_low	1.05713	1.483726	0.71	0.4764	-1.85560	3.969865
BWG_female_medium	BWG_male_medium	-0.66161	0.839743	-0.79	0.4310	-2.31012	0.986907
BWG_male_high	BWG_male_low	0.64588	1.922245	0.34	0.7370	-3.12771	4.419480
BWG_male_high	BWG_male_medium	-1.07286	1.482819	-0.72	0.4696	-3.98381	1.838092
BWG_male_low	BWG_male_medium	-1.71874	1.482819	-1.16	0.2468	-4.62969	1.192209

Streptococcus alactolyticus

BWG Gender	Estimate	Std Error	DF	Lower 95%	Upper 95%	Arithmetic Mean Estimate	N
BWG_female_high	6.312642	2.0611330	753	2.266392	10.358892	6.312642	50
BWG_female_low	8.417384	2.0611330	753	4.371134	12.463634	8.417384	50
BWG_female_medium	6.398012	0.9021334	753	4.627016	8.169007	6.398012	261
BWG_male_high	4.069952	2.0611330	753	0.023701	8.116202	4.069952	50
BWG_male_low	16.364045	2.0611330	753	12.317795	20.410295	16.364045	50
BWG_male_medium	8.306267	0.8986967	753	6.542018	10.070516	8.306267	263

BWG Gender	-BWG Gender	Difference	Std Error	t Ratio	Prob>|t|	Lower 95%	Upper 95%
BWG_female_high	BWG_female_low	-2.1047	2.914882	-0.72	0.4705	-7.8270	3.6175
BWG_female_high	BWG_female_medium	-0.0854	2.249914	-0.04	0.9697	-4.5022	4.3315
BWG_female_high	BWG_male_high	2.2427	2.914882	0.77	0.4419	-3.4796	7.9650
BWG_female_high	BWG_male_low	-10.0514	2.914882	-3.45	0.0006*	-15.7737	-4.3291
BWG_female_high	BWG_male_medium	-1.9936	2.248538	-0.89	0.3756	-6.4078	2.4205
BWG_female_low	BWG_female_medium	2.0194	2.249914	0.90	0.3697	-2.3975	6.4362
BWG_female_low	BWG_male_high	4.3474	2.914882	1.49	0.1363	-1.3748	10.0697
BWG_female_low	BWG_male_low	-7.9467	2.914882	-2.73	0.0066*	-13.6689	-2.2244
BWG_female_low	BWG_male_medium	0.1111	2.248538	0.05	0.9606	-4.3030	4.5253
BWG_female_medium	BWG_male_high	2.3281	2.249914	1.03	0.3011	-2.0888	6.7449
BWG_female_medium	BWG_male_low	-9.9660	2.249914	-4.43	<.0001*	-14.3829	-5.5492
BWG_female_medium	BWG_male_medium	-1.9083	1.273381	-1.50	0.1344	-4.4081	0.5915
BWG_male_high	BWG_male_low	-12.2941	2.914882	-4.22	<.0001*	-18.0164	-6.5718
BWG_male_high	BWG_male_medium	-4.2363	2.248538	-1.88	0.0599	-8.6505	0.1778
BWG_male_low	BWG_male_medium	8.0578	2.248538	3.58	0.0004*	3.6436	12.4719

Enterococcus faecium

BWG Gender	Estimate	Std Error	DF	Lower 95%	Upper 95%	Arithmetic Mean Estimate	N
BWG_female_high	1.0074827	0.76302966	753	-0.490436	2.5054010	1.0074827	50
BWG_female_low	2.7617785	0.76302966	753	1.263860	4.2596968	2.7617785	50
BWG_female_medium	1.0633141	0.33396901	753	0.407693	1.7189351	1.0633141	261
BWG_male_high	2.5964289	0.76302966	753	1.098511	4.0943472	2.5964289	50
BWG_male_low	2.5857747	0.76302966	753	1.087856	4.0836930	2.5857747	50
BWG_male_medium	1.4515453	0.33269674	753	0.798422	2.1046687	1.4515453	263

BWG Gender	-BWG Gender	Difference	Std Error	t Ratio	Prob>|t|	Lower 95%	Upper 95%
BWG_female_high	BWG_female_low	-1.75430	1.079087	-1.63	0.1044	-3.87267	0.364081
BWG_female_high	BWG_female_medium	-0.05583	0.832916	-0.07	0.9466	-1.69095	1.579283
BWG_female_high	BWG_male_high	-1.58895	1.079087	-1.47	0.1413	-3.70732	0.529430
BWG_female_high	BWG_male_low	-1.57829	1.079087	-1.46	0.1440	-3.69667	0.540084
BWG_female_high	BWG_male_medium	-0.44406	0.832407	-0.53	0.5939	-2.07818	1.190052
BWG_female_low	BWG_female_medium	1.69846	0.832916	2.04	0.0418*	0.06335	3.333579
BWG_female_low	BWG_male_high	0.16535	1.079087	0.15	0.8783	-1.95303	2.283726
BWG_female_low	BWG_male_low	0.17600	1.079087	0.16	0.8705	-1.94237	2.294380
BWG_female_low	BWG_male_medium	1.31023	0.832407	1.57	0.1159	-0.32388	2.944348
BWG_female_medium	BWG_male_high	-1.53311	0.832916	-1.84	0.0661	-3.16823	0.101999
BWG_female_medium	BWG_male_low	-1.52246	0.832916	-1.83	0.0680	-3.15757	0.112654
BWG_female_medium	BWG_male_medium	-0.38823	0.471405	-0.82	0.4104	-1.31366	0.537193
BWG_male_high	BWG_male_low	0.01065	1.079087	0.01	0.9921	-2.10772	2.129031
BWG_male_high	BWG_male_medium	1.14488	0.832407	1.38	0.1694	-0.48923	2.778998
BWG_male_low	BWG_male_medium	1.13423	0.832407	1.36	0.1734	-0.49988	2.768344

E- Feed Conversion

Unclassified Clostridiaceae1

Feed conversion Gender	Estimate	Std Error	DF	Lower 95%	Upper 95%	Arithmetic Mean Estimate	N
FC_female_high	29.785504	2.6370708	753	24.608619	34.962388	29.785504	50
FC_female_low	24.718322	2.6370708	753	19.541437	29.895207	24.718322	50
FC_female_medium	29.218349	1.1542145	753	26.952488	31.484210	29.218349	261
FC_male_high	31.859148	2.6370708	753	26.682263	37.036033	31.859148	50
FC_male_low	28.456622	2.6370708	753	23.279737	33.633507	28.456622	50
FC_male_medium	30.626623	1.1498175	753	28.369394	32.883852	30.626623	263

Feed conversion Gender	-Feed conversion Gender	Difference	Std Error	t Ratio	Prob>|t|	Lower 95%	Upper 95%
FC_female_high	FC_female_low	5.06718	3.729381	1.36	0.1746	-2.2540	12.3884
FC_female_high	FC_female_medium	0.56715	2.878603	0.20	0.8439	-5.0839	6.2182
FC_female_high	FC_male_high	-2.07364	3.729381	-0.56	0.5784	-9.3949	5.2476

Feed conversion Gender	-Feed conversion Gender	Difference	Std Error	t Ratio	Prob>|t|	Lower 95%	Upper 95%
FC_female_high	FC_male_low	1.32888	3.729381	0.36	0.7217	-5.9923	8.6501
FC_female_high	FC_male_medium	-0.84112	2.876842	-0.29	0.7701	-6.4887	4.8065
FC_female_low	FC_female_medium	-4.50003	2.878603	-1.56	0.1184	-10.1511	1.1510
FC_female_low	FC_male_high	-7.14083	3.729381	-1.91	0.0559	-14.4620	0.1804
FC_female_low	FC_male_low	-3.73830	3.729381	-1.00	0.3165	-11.0595	3.5829
FC_female_low	FC_male_medium	-5.90830	2.876842	-2.05	0.0403*	-11.5559	-0.2607
FC_female_medium	FC_male_high	-2.64080	2.878603	-0.92	0.3592	-8.2918	3.0102
FC_female_medium	FC_male_low	0.76173	2.878603	0.26	0.7914	-4.8893	6.4128
FC_female_medium	FC_male_medium	-1.40827	1.629200	-0.86	0.3876	-4.6066	1.7900
FC_male_high	FC_male_low	3.40253	3.729381	0.91	0.3619	-3.9187	10.7237
FC_male_high	FC_male_medium	1.23252	2.876842	0.43	0.6685	-4.4151	6.8801
FC_male_low	FC_male_medium	-2.17000	2.876842	-0.75	0.4509	-7.8176	3.4776

Unclassified *Lactobacillus*

Feed conversion Gender	Estimate	Std Error	DF	Lower 95%	Upper 95%	Arithmetic Mean Estimate	N
FC_female_high	21.725422	2.8453779	753	16.139605	27.311238	21.725422	50
FC_female_low	26.430286	2.8453779	753	20.844469	32.016102	26.430286	50
FC_female_medium	24.691743	1.2453881	753	22.246897	27.136588	24.691743	261
FC_male_high	13.335501	2.8453779	753	7.749685	18.921318	13.335501	50
FC_male_low	21.761135	2.8453779	753	16.175319	27.346952	21.761135	50
FC_male_medium	21.786568	1.2406437	753	19.351036	24.222099	21.786568	263

Feed conversion Gender	-Feed conversion Gender	Difference	Std Error	t Ratio	Prob>|t|	Lower 95%	Upper 95%
FC_female_high	FC_female_low	-4.7049	4.023972	-1.17	0.2427	-12.6044	3.1947
FC_female_high	FC_female_medium	-2.9663	3.105989	-0.96	0.3399	-9.0637	3.1311
FC_female_high	FC_male_high	8.3899	4.023972	2.08	0.0374*	0.4904	16.2895
FC_female_high	FC_male_low	-0.0357	4.023972	-0.01	0.9929	-7.9353	7.8638
FC_female_high	FC_male_medium	-0.0611	3.104090	-0.02	0.9843	-6.1548	6.0326
FC_female_low	FC_female_medium	1.7385	3.105989	0.56	0.5758	-4.3589	7.8360
FC_female_low	FC_male_high	13.0948	4.023972	3.25	0.0012*	5.1952	20.9943
FC_female_low	FC_male_low	4.6692	4.023972	1.16	0.2463	-3.2304	12.5687
FC_female_low	FC_male_medium	4.6437	3.104090	1.50	0.1351	-1.4500	10.7374
FC_female_medium	FC_male_high	11.3562	3.105989	3.66	0.0003*	5.2588	17.4537
FC_female_medium	FC_male_low	2.9306	3.105989	0.94	0.3457	-3.1668	9.0280
FC_female_medium	FC_male_medium	2.9052	1.757893	1.65	0.0988	-0.5458	6.3561
FC_male_high	FC_male_low	-8.4256	4.023972	-2.09	0.0366*	-16.3252	-0.5261
FC_male_high	FC_male_medium	-8.4511	3.104090	-2.72	0.0066*	-14.5448	-2.3574
FC_male_low	FC_male_medium	-0.0254	3.104090	-0.01	0.9935	-6.1191	6.0683

Unclassified *Clostridium* sensu stricto 1

Feed conversion Gender	Estimate	Std Error	DF	Lower 95%	Upper 95%	Arithmetic Mean Estimate	N
FC_female_high	10.549844	2.1527127	753	6.323812	14.775876	10.549844	50
FC_female_low	14.964459	2.1527127	753	10.738427	19.190491	14.964459	50
FC_female_medium	14.773041	0.9422168	753	12.923357	16.622725	14.773041	261
FC_male_high	9.365294	2.1527127	753	5.139262	13.591326	9.365294	50
FC_male_low	12.917624	2.1527127	753	8.691592	17.143656	12.917624	50
FC_male_medium	12.063406	0.9386273	753	10.220769	13.906044	12.063406	263

Feed conversion Gender	-Feed conversion Gender	Difference	Std Error	t Ratio	Prob>|t|	Lower 95%	Upper 95%
FC_female_high	FC_female_low	-4.41461	3.044396	-1.45	0.1475	-10.3911	1.5619
FC_female_high	FC_female_medium	-4.22320	2.349882	-1.80	0.0727	-8.8363	0.3899
FC_female_high	FC_male_high	1.18455	3.044396	0.39	0.6973	-4.7920	7.1611
FC_female_high	FC_male_low	-2.36778	3.044396	-0.78	0.4370	-8.3443	3.6087
FC_female_high	FC_male_medium	-1.51356	2.348445	-0.64	0.5195	-6.1238	3.0967
FC_female_low	FC_female_medium	0.19142	2.349882	0.08	0.9351	-4.4217	4.8045
FC_female_low	FC_male_high	5.59916	3.044396	1.84	0.0663	-0.3773	11.5757
FC_female_low	FC_male_low	2.04683	3.044396	0.67	0.5016	-3.9297	8.0233
FC_female_low	FC_male_medium	2.90105	2.348445	1.24	0.2171	-1.7092	7.5113
FC_female_medium	FC_male_high	5.40775	2.349882	2.30	0.0216*	0.7946	10.0208
FC_female_medium	FC_male_low	1.85542	2.349882	0.79	0.4300	-2.7577	6.4685
FC_female_medium	FC_male_medium	2.70963	1.329960	2.04	0.0420*	0.0988	5.3205
FC_male_high	FC_male_low	-3.55233	3.044396	-1.17	0.2436	-9.5288	2.4242
FC_male_high	FC_male_medium	-2.69811	2.348445	-1.15	0.2510	-7.3084	1.9122
FC_male_low	FC_male_medium	0.85422	2.348445	0.36	0.7162	-3.7561	5.4645

Escherichia coli

Feed conversion Gender	Estimate	Std Error	DF	Lower 95%	Upper 95%	Arithmetic Mean Estimate	N
FC_female_high	9.016991	1.3506857	753	6.365434	11.668548	9.016991	50
FC_female_low	12.771244	1.3506857	753	10.119687	15.422802	12.771244	50
FC_female_medium	10.398917	0.5911791	753	9.238362	11.559472	10.398917	261
FC_male_high	9.071459	1.3506857	753	6.419901	11.723016	9.071459	50
FC_male_low	13.962373	1.3506857	753	11.310816	16.613931	13.962373	50
FC_male_medium	10.459932	0.5889269	753	9.303798	11.616066	10.459932	263

Feed conversion Gender	-Feed conversion Gender	Difference	Std Error	t Ratio	Prob>|t|	Lower 95%	Upper 95%
FC_female_high	FC_female_low	-3.75425	1.910158	-1.97	0.0497*	-7.50412	-0.00439
FC_female_high	FC_female_medium	-1.38193	1.474396	-0.94	0.3489	-4.27634	1.51249
FC_female_high	FC_male_high	-0.05447	1.910158	-0.03	0.9773	-3.80434	3.69540
FC_female_high	FC_male_low	-4.94538	1.910158	-2.59	0.0098*	-8.69525	-1.19551
FC_female_high	FC_male_medium	-1.44294	1.473495	-0.98	0.3278	-4.33559	1.44971
FC_female_low	FC_female_medium	2.37233	1.474396	1.61	0.1080	-0.52209	5.26674
FC_female_low	FC_male_high	3.69979	1.910158	1.94	0.0531	-0.05008	7.44965
FC_female_low	FC_male_low	-1.19113	1.910158	-0.62	0.5331	-4.94100	2.55874
FC_female_low	FC_male_medium	2.31131	1.473495	1.57	0.1172	-0.58133	5.20396
FC_female_medium	FC_male_high	1.32746	1.474396	0.90	0.3682	-1.56696	4.22187
FC_female_medium	FC_male_low	-3.56346	1.474396	-2.42	0.0159*	-6.45787	-0.66904
FC_female_medium	FC_male_medium	-0.06101	0.834462	-0.07	0.9417	-1.69916	1.57713
FC_male_high	FC_male_low	-4.89091	1.910158	-2.56	0.0106*	-8.64078	-1.14105
FC_male_high	FC_male_medium	-1.38847	1.473495	-0.94	0.3463	-4.28112	1.50417
FC_male_low	FC_male_medium	3.50244	1.473495	2.38	0.0177*	0.60980	6.39509

Streptococcus alactolyticus

Feed conversion Gender	Estimate	Std Error	DF	Lower 95%	Upper 95%	Arithmetic Mean Estimate	N
FC_female_high	8.581803	2.0763884	753	4.5056050	12.658002	8.581803	50
FC_female_low	5.367097	2.0763884	753	1.2908986	9.443295	5.367097	50

Feed conversion Gender	Estimate	Std Error	DF	Lower 95%	Upper 95%	Arithmetic Mean Estimate	N
FC_female_medium	6.547652	0.9088105	753	4.7635489	8.331756	6.547652	261
FC_male_high	13.989286	2.0763884	753	9.9130880	18.065485	13.989286	50
FC_male_low	7.280618	2.0763884	753	3.2044199	11.356817	7.280618	50
FC_male_medium	8.147349	0.9053484	753	6.3700419	9.924656	8.147349	263

Feed conversion Gender	-Feed conversion Gender	Difference	Std Error	t Ratio	Prob>|t|	Lower 95%	Upper 95%
FC_female_high	FC_female_low	3.21471	2.936457	1.09	0.2740	-2.5499	8.9793
FC_female_high	FC_female_medium	2.03415	2.266567	0.90	0.3698	-2.4154	6.4837
FC_female_high	FC_male_high	-5.40748	2.936457	-1.84	0.0659	-11.1721	0.3571
FC_female_high	FC_male_low	1.30119	2.936457	0.44	0.6578	-4.4634	7.0658
FC_female_high	FC_male_medium	0.43445	2.265181	0.19	0.8480	-4.0124	4.8813
FC_female_low	FC_female_medium	-1.18056	2.266567	-0.52	0.6026	-5.6301	3.2690
FC_female_low	FC_male_high	-8.62219	2.936457	-2.94	0.0034*	-14.3868	-2.8576
FC_female_low	FC_male_low	-1.91352	2.936457	-0.65	0.5148	-7.6781	3.8511
FC_female_low	FC_male_medium	-2.78025	2.265181	-1.23	0.2201	-7.2271	1.6666
FC_female_medium	FC_male_high	-7.44163	2.266567	-3.28	0.0011*	-11.8912	-2.9921
FC_female_medium	FC_male_low	-0.73297	2.266567	-0.32	0.7465	-5.1825	3.7166
FC_female_medium	FC_male_medium	-1.59970	1.282806	-1.25	0.2128	-4.1180	0.9186
FC_male_high	FC_male_low	6.70867	2.936457	2.28	0.0226*	0.9441	12.4733
FC_male_high	FC_male_medium	5.84194	2.265181	2.58	0.0101*	1.3951	10.2888
FC_male_low	FC_male_medium	-0.86673	2.265181	-0.38	0.7021	-5.3136	3.5801

Enterococcus faecium

Feed conversion Gender	Estimate	Std Error	DF	Lower 95%	Upper 95%	Arithmetic Mean Estimate	N
FC_female_high	2.9013568	0.76313182	753	1.403238	4.3994757	2.9013568	50
FC_female_low	0.2713622	0.76313182	753	-1.226757	1.7694810	0.2713622	50
FC_female_medium	1.1775942	0.33401372	753	0.521885	1.8333030	1.1775942	261
FC_male_high	2.3984280	0.76313182	753	0.900309	3.8965469	2.3984280	50
FC_male_low	1.3550441	0.76313182	753	-0.143075	2.8531629	1.3550441	50
FC_male_medium	1.7231673	0.33274128	753	1.069956	2.3763781	1.7231673	263

Feed conversion Gender	-Feed conversion Gender	Difference	Std Error	t Ratio	Prob>|t|	Lower 95%	Upper 95%
FC_female_high	FC_female_low	2.62999	1.079231	2.44	0.0150*	0.51133	4.74865
FC_female_high	FC_female_medium	1.72376	0.833028	2.07	0.0389*	0.08843	3.35910
FC_female_high	FC_male_high	0.50293	1.079231	0.47	0.6413	-1.61573	2.62159
FC_female_high	FC_male_low	1.54631	1.079231	1.43	0.1523	-0.57235	3.66497
FC_female_high	FC_male_medium	1.17819	0.832518	1.42	0.1574	-0.45614	2.81252
FC_female_low	FC_female_medium	-0.90623	0.833028	-1.09	0.2770	-2.54157	0.72910
FC_female_low	FC_male_high	-2.12707	1.079231	-1.97	0.0491*	-4.24573	-0.00841
FC_female_low	FC_male_low	-1.08368	1.079231	-1.00	0.3156	-3.20234	1.03498
FC_female_low	FC_male_medium	-1.45181	0.832518	-1.74	0.0816	-3.08614	0.18253

Feed conversion Gender	-Feed conversion Gender	Difference	Std Error	t Ratio	Prob>|t|	Lower 95%	Upper 95%
FC_female_medium	FC_male_high	-1.22083	0.833028	-1.47	0.1432	-2.85617	0.41450
FC_female_medium	FC_male_low	-0.17745	0.833028	-0.21	0.8314	-1.81278	1.45788
FC_female_medium	FC_male_medium	-0.54557	0.471468	-1.16	0.2476	-1.47112	0.37997
FC_male_high	FC_male_low	1.04338	1.079231	0.97	0.3340	-1.07528	3.16204
FC_male_high	FC_male_medium	0.67526	0.832518	0.81	0.4176	-0.95907	2.30959
FC_male_low	FC_male_medium	-0.36812	0.832518	-0.44	0.6585	-2.00246	1.26621

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CHAPTER III

The active core microbiota of two high-yielding laying hen breeds fed with different levels of calcium and phosphorus

3. The active core microbiota of two high-yielding laying hen breeds fed with different levels of calcium and phosphorus [2]

3.1 Abstract

The nutrient availability and supplementation of dietary phosphorus (P) and calcium (Ca) in avian feed, especially in laying hens, plays a vital role in phytase degradation and mineral utilization during the laying phase. The required concentration of P and Ca peaks during the laying phase, and the direct interaction between Ca and P concentration shrinks the availability of both supplements in the feed. Our goal was to characterize the active microbiota of the entire gastrointestinal tract (GIT) (crop, gizzard, duodenum, ileum, caeca), including digesta- and mucosa-associated communities of two contrasting high-yielding breeds of laying hens (Lohmann Brown Classic, LB; Lohmann LSL Classic, LSL) under different P and Ca supplementation levels. Statistical significances were observed for breed, GIT section, Ca, and the interaction of GIT section x breed, P x Ca, Ca x breed and P x Ca x breed ($p < 0.05$). A core microbiota of five species was detected in more than 97% of all samples. They were represented by an uncl. *Lactobacillus* (average relative abundance (av. abu.) 12.1%), *Lactobacillus helveticus* (av. abu. 10.8%), *Megamonas funiformis* (av. abu. 6.8%), *Ligilactobacillus salivarius* (av. abu. 4.5%), and an uncl. *Fusicatenibacter* (av. abu. 1.1%). Our findings indicated that Ca and P supplementation levels 20% below the recommendation have a minor effect on the microbiota compared to the strong impact of the bird's genetic background. Moreover, a core active microbiota across the GIT of two high yielding laying hen breeds was revealed for the first time.

3.2 Introduction

The laying hen gastrointestinal tract (GIT) microbiota consists of a complex community of diverse microorganisms. The host influences the composition of the microbial community, which may have effects on the immune system, nutrient digestion, and regulation of intestinal physiology [62; 3; 36]. Depending on the diet and nutrient

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supplementation, variations in microbial composition can be observed (39). Moreover, it is essential to understand the interrelation between diet, microbiota, and host when investigating how they contribute to animal health.

Diets are formulated to fulfil the needs of the animals, and the specifically required nutrient concentrations are dependent on the host age, physiological status, and level of performance. Among required minerals, phosphorus (P) and calcium (Ca) are vital because of their function in avian biochemical pathways and bone and eggshell development [55]. However, P supplements are costly and negatively impact the environment when accumulated in the excreta of the animals. This has stimulated research on hydrolysis of phytate, which is the main binding form of P in plants, in poultry's digestive tract and variation in the level of P supplementation [49]. The influence of age, genotype and experimental design variations affect the results' comparability [34; 4; 17; 19]. The Ca concentration of the feed is related to P, and in laying hens, the highest Ca requirement is during the laying period [34; 4]. In this phase, the animal requirements must be fulfilled to maintain animal health and performance. Digested and undigested dietary compounds influence the microbial population in the GIT, which modifies the host intestinal integrity and improves pathogen resistance [19]. Moreover, there is a microbial distinction between mucosa and digesta samples [17; 68]. Mucosa samples of the gastrointestinal tract have shown higher microbial diversity than digesta samples [10]. The complex microbial diversity in both sample types consists of hundreds of species across different phyla, inhibiting a clear understanding of GIT variations [10].

Little is known about the dynamics and influence of common active bacteria on the GIT of laying hens. Therefore, the microbiota's response to a specific challenge and environment by targeting the active community has to be reflected. Despite showing similar diversity to total communities, the microbial taxa composition is significantly different [6]. Shade and Handelsman (2012) defined that the core microbiome consists of shared microbial members within similar habitats and across complex microbial assemblages. Furthermore, a core microbiome is present and interacts in the entire GIT. In addition, transient or resident bacteria can be considered a core microbiome. It is an approach to understanding, adjusting, and optimizing microbial functions in individuals or complete ecosystems [27; 28]. Knowledge about microbial changes across different GIT sections can help understand specific processes, e.g., food fermentation or predicting and controlling the microbiome [25; 63; 7].

This study aimed to evaluate the impact of different concentrations of P and Ca on the active microbiota of the GIT (crop, gizzard, duodenum, ileum, caeca) of two high yielding laying hen breeds and determine how the host genetic background and dietary changes influence the resident core microbiota.

3.3 Materials and methods

3.3.1 Sample collection, DNA extraction, and illumina library preparation

This research complements and extends recent publications [60; 27; 28]. Samples originated from an animal trial fully described by Sommerfeld et al. (2020). The study was approved by the Regierungspräsidium Tübingen (approval number HOH50/17 TE) and conducted following animal welfare regulations. Animals were housed at the University's Agricultural Experimental Station (Unterer Lindenhof, Eningen, Germany). A total of 80 laying hens of the breeds Lohmann brown-classic (LB) and Lohmann LSL-classic (LSL) were used in this study. Upon the arrival of the hatchlings at the farm, birds were raised together under the same conditions (floor pens, deep litter bedding on wood shavings, and diets). At 27 weeks, ten hens per breed were allocated to four dietary treatments in a randomized design and kept individually in metabolism units. The individuals received water and feed for ad libitum consumption for 3 weeks. Soybean meal and corn-based diets were supplemented to reach a standard (5.3 g/kg dry matter (DM); P^+) or reduced (4.7 g/kg DM; P^-) P concentration and a standard (39.6 g/kg DM; Ca^+) or reduced (33.9 g/kg DM; Ca^-) Ca concentration. Diets ingredient compositions are fully described in Sommerfeld et al. (2020).

At 31 weeks of life, birds were stunned with a gas mixture of 35% CO_2, 35% N_2, and 30% O_2 and sacrificed by decapitation. The crop (Cr), gizzard (G), duodenum (D), ileum (I) and caeca (Cae) were longitudinally opened, digesta was obtained with a sterile spoon, and after a cleaning step with sterile phosphate buffered saline solution, the mucosa was collected by scratching it with a sterile glass slide. Collected samples were immediately stored in RNA later at −80°C until further analysis. RNA of a total of 800 samples were extracted using Trizol (Invitrogen Inc., Waltham, United States) according to the manufacturer's instructions with a preliminary step of bead beating (30 s, 5.5 m/s) in a FastPrep instrument (MP Biomedicals, Eschwege, Germany). RNA was quantified with Nanodrop (ThermoFisher Scientific, Waltham, United States) and stored at −80°C until further analysis. RNA samples were treated with the DNase kit

(Invitrogen), and cDNA synthesis was performed using SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen).

Sequencing libraries were made according to the protocol described by Borda-Molina et al. [2020]. All PCR reactions were done with PrimeSTAR® HS DNA Polymerase kit (TaKaRa, Beijing, China). The first two PCR were prepared in a total volume of 25 µl using 1 µl of cDNA template, 0.2 µM of each primer and 0.5 U Taq prime start HS DNA and the third PCR was set up in a total volume of 50 µl. An initial denaturation at 95˚C for 3 min was followed by ten cycles (first and second PCR) or 20 cycles (third PCR) of denaturation at 98˚C for 10 s, annealing at 55˚C for 10 s and an extension at 72˚C for 45 s and a final extension of 72˚C for 2 min. PCR products were purified and standardized using SequalPrep Normalization Kit (Invitrogen Inc., Waltham, United States) and sequenced using 250 bp paired-end sequencing chemistry on Illumina Novaseq 6000.

3.3.2 Bioinformatics and statistical analysis

The bioinformatic analysis was performed with Mothur v1.44.3 [52]. Raw reads (forward and reverse fastq file) were assembled with make.contigs function. Reads with ambiguous bases, with homopolymers (> 8) and longer than 354 bp were removed. A total of 678 samples passed this filtering and were used for downstream-analysis. Sequences were aligned to the silva.seed v1.38.1 [48]. Chimeras were identified using vsearch [50] and removed from the dataset. Sequences were classified using the Bayesian classifier and the Silva reference and taxonomy set silva.seed v1.38.1. The output was filtered to get the amplicon sequencing variants (ASVs) with a minimum of 50 reads across all samples resulting in 6179 ASVs. An average of 34.566 ± 17.567 reads was obtained per sample. The cut-off for bacterial taxonomy classification followed the recommendations of Yarza et al. (2014). Digesta and mucosa samples have been merged for further analysis per section and considered gastrointestinal tract sections. Sample reads were standardized, and a sample-similarity matrix based on the Bray-Curtis similarity coefficient [11] was created using Primer6 [14]. PERMANOVA routine was used to study the significant differences and interactions between groups and diets [14]. Steel-Dwass test was performed to compare means of relative abundance data between genera and breed (Br), gastrointestinal tract section (GS), and Ca/P level combinations using JMP®Pro (Version 16.1 SAS Institute Inc., Cary, NC, 1989–2021). P-values based on ANOSIM

results were adjusted using the Benjamin-Hochberg correction (FDR). The core microbiota across all samples was identified with the phyloseq and microbiome library in R v4.1 [40; 37]. ASV table, taxonomy information, and metadata were combined in a phyloseq file. Groups were subset according to the metadata (diet, GS and Br) to create a phyloseq file for each combination of the three factors. All phyloseq files of all groups were standardized by ASVs. The detection level of core members was set to 0.01% of abundance and a prevalence of 97% across all samples. The output ASV list was compared between all groups to determine the common ASVs, and venn diagrams were drawn with the InteractiVenn tool [26].

The Shannon diversity index and richness were calculated using the phyloseq library in R v4.1. LDA scores were analyzed with microbiomeAnalyst [13]. Data filter and normalization were set to default. P-values threshold was set to $p = 0.05$ and the FDR correction was applied. LEfSe-graphs were built with the build-in graph builder [54].

Functional prediction was performed in R with the latest version of Tax4Fun2 v1.1.5 [https://github.com/bwemheu/ Tax4Fun2]. Bacterial genomes detected on the microbiota dataset were downloaded from the NCBI database, and a reference database was created to improve functional accuracy. Functional predictions were then performed using the reference file and the ASV table of all samples. The threshold for clustering (uclast) was set to 100%, and the number of 16S rRNA copies were normalized and calculated for each ASV.

3.4 Results

3.4.1 Experiment evaluation

The overall microbiota consisted of 6179 ASVs, where 2272 ASVs were shared by all GIT sections, breeds, and dietary treatments. LSL samples shared 2868 and the LB 2970 (Figure 3.1). The number of unique ASVs varied from 61 to 284, depending on

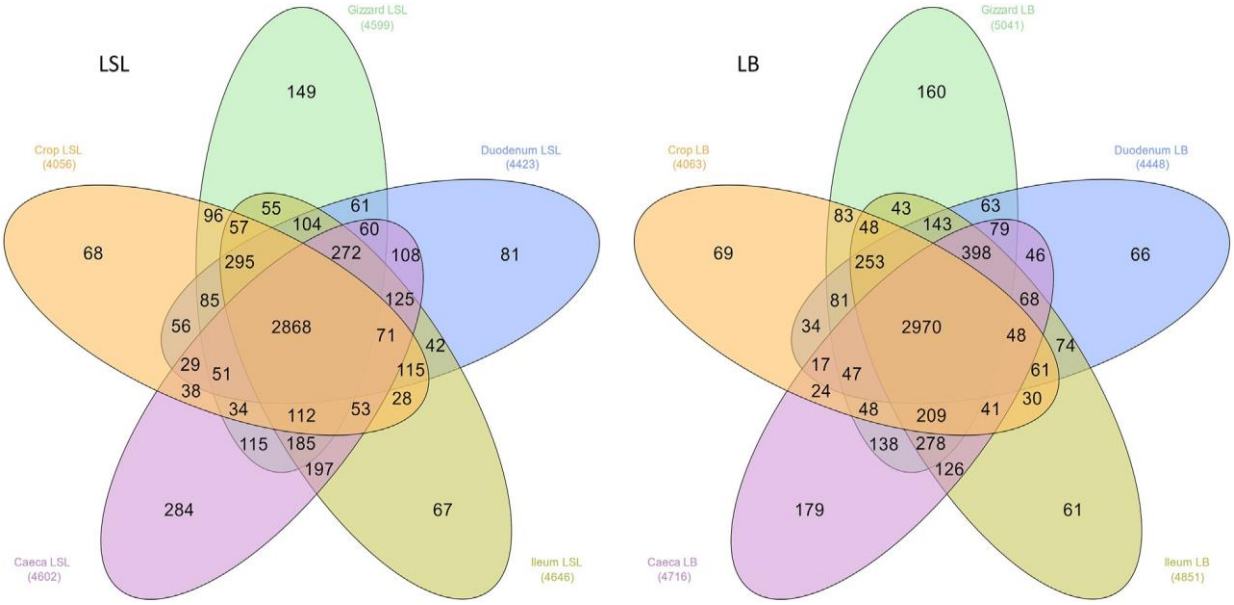

Figure 3.1: Distribution of the total number of ASVs among GIT sections across all samples in both breeds. The number in parenthesis is the observed number of ASVs in each group.

the breed and GIT section. Moreover, the breed comparison of each GIT section revealed that many ASVs were unique for each breed (Supplementary Figure S3.1). According to the sequencing data, the microbiota of all samples consisted of Firmicutes (average relative abundance [av. abu]) of 84.5% in LSL and 76.7% in LB ($p < 0.05$), followed by Bacteriodetes, which was more abundant in LB (18.2%) in comparison to LSL (10.7%) ($p < 0.05$) (Supplementary Figure S3.2A). The most abundant genera were *Lactobacillus* (25.1% LSL; 17.4% LB), followed by uncl. Lactobacillaceae (21.2% LSL, 8.2% LB), uncl. Lachnospiracea (10.8% LSL, 13.5% LB), and *Ligilactobacillus* (7.9% LSL, 12.5% LB). These genera reached an average relative abundance of more than 50% across all samples (Supplementary Figure S3.2B). Additionally, significant differences were found between breeds and GIT sections within the breeds (Supplementary Table S3.1). PERMANOVA routine was used to study the overall significant differences and interactions between GIT sections, laying hen breeds, P and

Ca supplementation. A statistical significance on ASV level was reached for each factor alone ($p < 0.03$) and the interactions between Br x GS, Br x Ca, P x Ca, P x Ca x Br ($p < 0.03$). A trend was observed for Br x P ($p = 0.09$) (Supplementary Table S3.2). The principal coordinates analysis plot revealed three clusters (Figure 3.2), one comprising

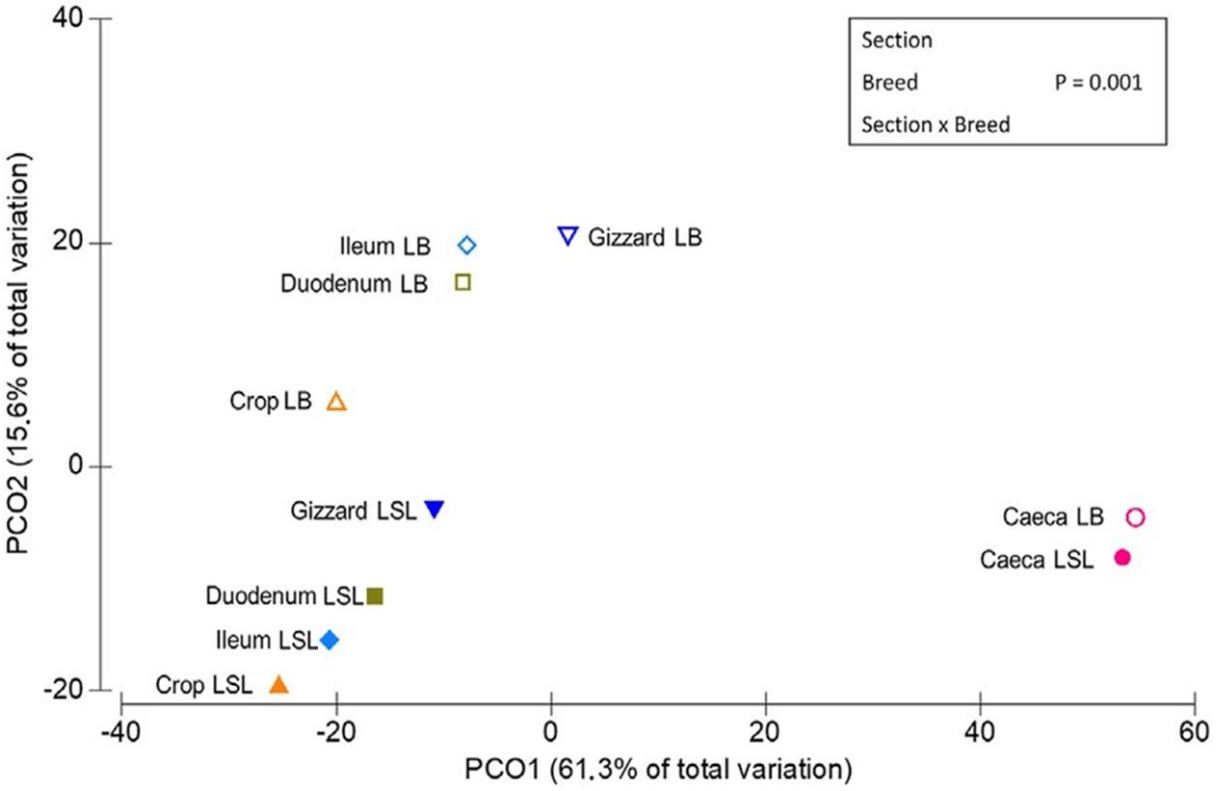

Figure 3.2: Multidimensional scaling of centroids showing the similarities among the sample types derived from sample combinations of GIT section.

the LSL samples of crop, gizzard, duodenum and ileum, another with those same samples but for the LB breed and a third one with the caeca samples of both breeds.

In crop samples, significant effects of the breed and Ca and a trend for the interactions of Br x Ca ($p < 0.08$) were observed. The gizzard, duodenum and ileum microbiota were significantly affected by the breed ($p < 0.05$). In the caeca, significant effects of the breed, P/Ca supplementation, the interactions of Br x Ca, Ca x Br, P x Ca x Br ($p < 0.03$) and a trend for P x Br were detected ($p < 0.08$). All significant interactions are provided in Supplementary Table S3.2.

Pairwise comparisons evaluating the Ca and P supplementation effects on the breed and GIT section, exhibited significant effects, depending on the GIT section. For an overview, see Supplementary Table S3.3. A significant difference was detected regarding P supplementation for LB caeca P^+ vs. P^- ($p < 0.01$). An effect of the Ca supplementation was observed in both breeds. In LB, a significant difference was

identified in crop Ca^+ vs Ca^- ($p = 0.02$) and caeca Ca + vs Ca- ($p < 0.01$) was revealed. For LSL, significant differences were observed in caeca Ca^+ vs Ca^- ($p < 0.01$). However, the strongest effect was driven by the breed rather than GIT section, Ca or P supplementation levels. The breed effect is clearly shown in caeca samples (Supplementary Figure S3.3), and all significant p-values are shown in Supplementary Table S3.3.

The LB showed significantly higher overall Shannon diversity (3.09) than LSL (2.93). A statistical significance between caeca and all GIT sections was observed for both breeds ($p < 0.05$). For the LB additional significances were observed between ileum and crop and ileum and duodenum. ($p < 0.03$) (Figure 3.3). Regarding the diet, the

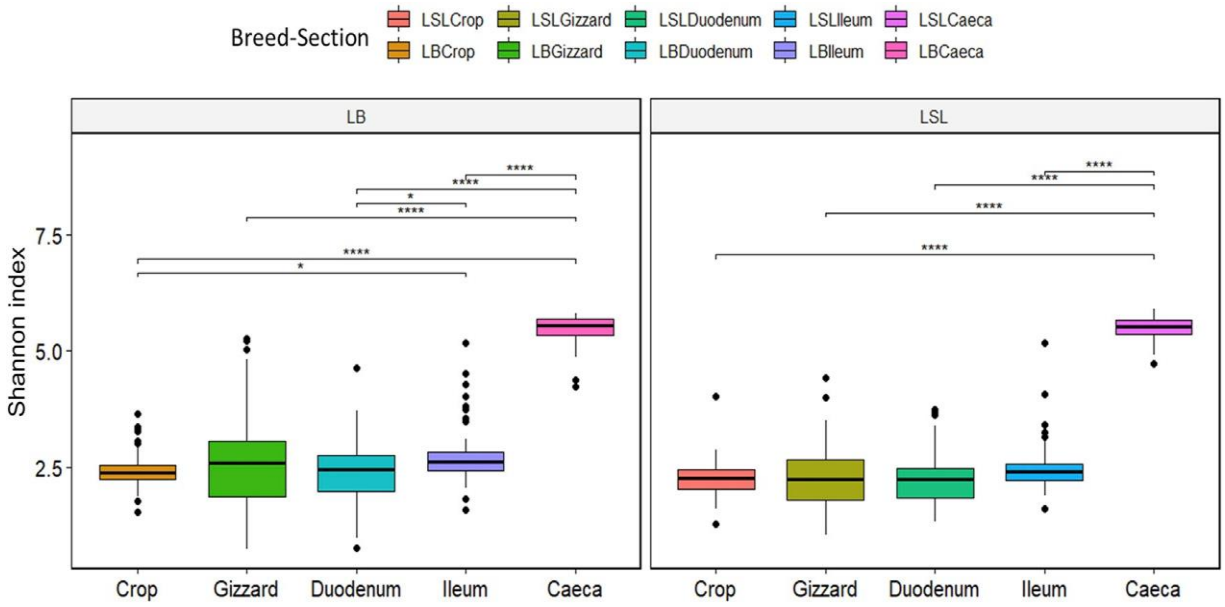

Figure 3.3: Boxplot of Shannon diversity index separated by the breed, section (color) and Ca/P combination of the diet (**p < 0.02; **** p < 0.001).

Shannon index differed depending on the GIT section and breed combination. Still, no statistical significance was observed between diets, with the highest index observed in caeca (Supplementary Figure S3.4).

3.4.2 Functional prediction

A total of 322 pathways and 7516 functions were assigned to the samples. Thirty KEGG pathways contributed to more than 50% of the total pathways across all samples and revealed significant differences between breeds and/or GIT sections of the same breed. These thirty KEGG pathways belonged to twelve second-level KEGG functional categories. The global/overview metabolism map was the most enriched function,

followed by membrane transport metabolism and signal transduction. Significant effects in the caeca were observed for the breed and the interaction of Br x P ($p < 0.05$) (Supplementary Table S3.4). Two of the top 30 pathways [ko02010 (ABC transporters) and ko00190 (oxidative phosphorylation)] showed significant breed effects ($p < 0.05$). Despite the significance of breed × P interaction, only one inositol related individual function [K06607 (myo-inositol catabolism protein IolS)] showed differences in LSL (Supplementary Table S3.4). Regarding Ca supplementation and its effect on the caeca, a significant difference was detected for the myo-inositol catabolism protein IolS (K06607, $p = 0.01$) in LSL, and scyllo-inositol 2-dehydrogenase ($NADP^+$) (K22230, $p < 0.05$) in LSL and LB. In addition, five other inositol related functions show breed effects (Supplementary Table S3.4).

3.4.3 Core Microbiota

A total of five ASVs were present in 97% of all samples (Figure 3.4). The core microbiota was represented by an uncl. *Lactobacillus* (ASV62, av. abu. 12.1%), *Megamonas funiformis* (ASV63, av. abu. 6.8%), *Ligilactobacillus salivarius* (ASV 137, av. abu. 4.5%), *Lactobacillus helveticus* (ASV197, av. abu. 10.8%) and uncl. *Fusicatenibacter* (ASV 561, av. abu. 1.1%). Except for the gizzard of LB and caeca of both breeds, the five bacteria accounted for 25%–71% of the total community (Supplementary Table S3.5). Uncl. *Lactobacillus* was more abundant in LSL compared to LB in all GIT sections (Supplementary Table S3.5). The highest abundance of *Megamonas funiformis* (ASV63) was observed in the crop of both breeds (Supplementary Table S3.5). *Ligilactobacillus salivarius* (ASV137) had the highest abundance in the crop and the lowest in the caeca. Furthermore, it was present in higher abundance in LB than LSL (Supplementary Table S3.5). Also, significant differences were shown between breeds in crop and between GIT sections within the breeds ($p < 0.05$, Supplementary Table S3.5). *Lactobacillus helveticus* (ASV197) was more abundant in all GIT sections of LSL, with the highest average relative abundance in the ileum, followed by duodenum and crop (Supplementary Table S3.5). Additionally, significant differences between breeds in all GIT sections ($p < 0.05$, Supplementary Table S3.5). Uncl. *Fusicatenibacter* (ASV561) was detected in very low abundances across the gastrointestinal tract (Supplementary Table S3.5). Moreover, significant differences existed between breeds and GIT sections within the breeds ($p < 0.05$, Supplementary Table S3.5).

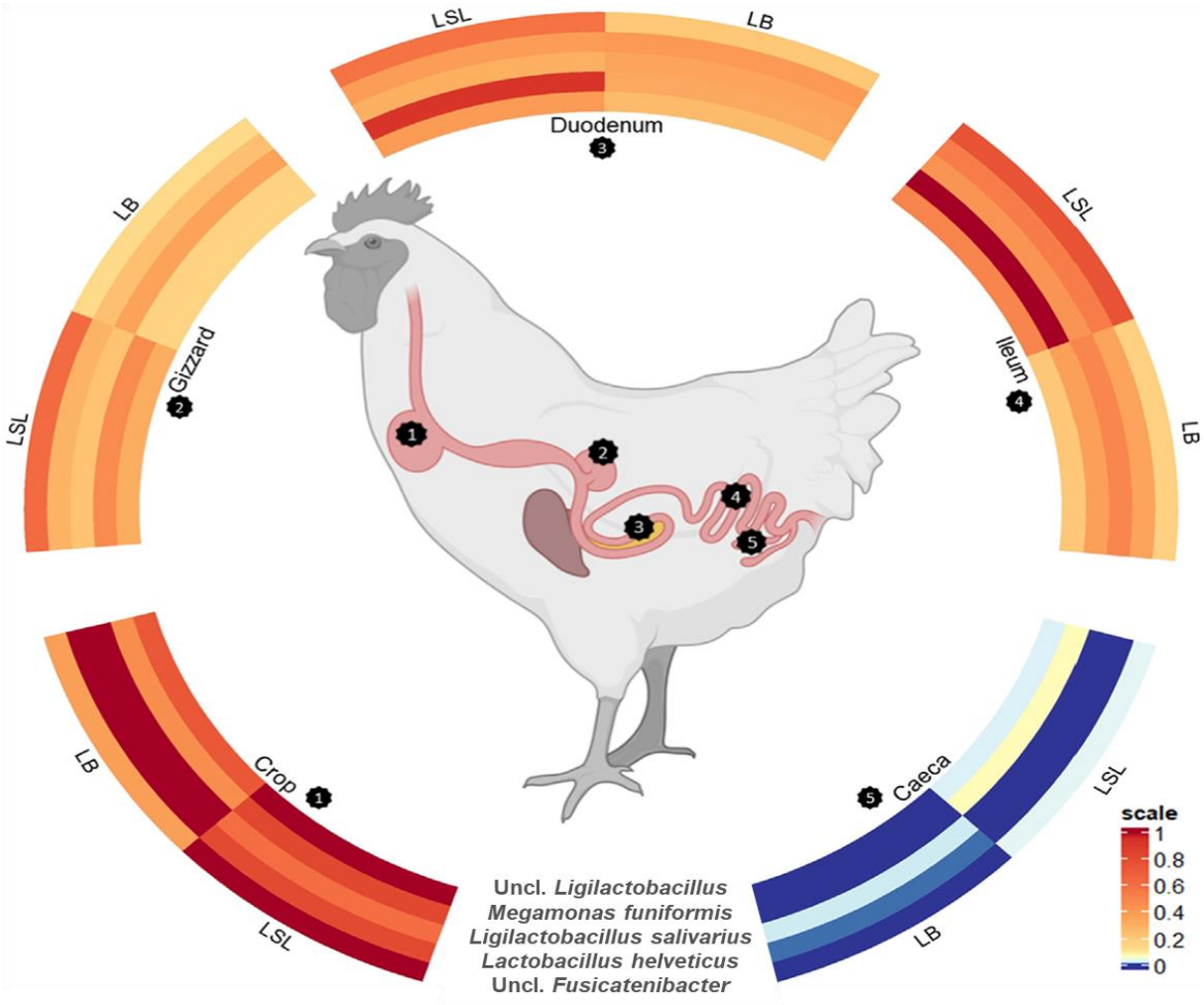

Figure 3.4: Scaled circulized heatmap of the five core microbiota separated by the GIT sections (crop, gizzard, duodenum, ileum, and caeca) and breed (LSL, LB).

3.4.4 The effect of P and Ca supplementation on the genera distribution and the core microbiome across the gastrointestinal tract

The Ca supplementation affected the microbial composition in LB crop ($p < 0.05$), and significant effects were found for the genus uncl. Lactobacillaceae and Streptococcus ($p < 0.01$) (Supplementary Figure S3.5). Further, the average relative abundance of uncl. Lactobacillaceae increased while *Streptococcus* decreased with Ca supplementation in the diet. Despite the higher diversity of the caeca, fewer differences at genus level were observed for Ca supplementation. Significant changes in LSL were observed for uncl. Bacteroides, uncl. Lachnospiraceae, *Ligilactobacillus* and *Megasphaera* in LB ($p < 0.10$) (Supplementary Figure S3.5). The average abundance of all genera increased by supplementing Ca except for uncl. Lachnospiraceae. Significant shifts in the genera *Helicobacter*, uncl. Gammaproteobacteria, and uncl. Prevotellaceae and the trends for *Lachnoclostridium* and *Megasphaera* supported the

significant P effect in LB caeca (Supplementary Figure S3.5). In addition, P supplementation increased the average abundance of uncl. Prevotellaceae, *Helicobacter*, and *Lachnoclostridium* while decreasing *Megasphaera* and uncl. Gammaproteobacteria.

LEfSe-analysis revealed the 25 most significant discriminant ASVs for breed and diet based on the average abundance across the factor's combination (breed x diet). Even if no significance for those ASVs was revealed by comparing the dietary groups within the breeds, the average relative abundance changes across the breed x diet combinations. Eleven ASVs were assigned to a species (*Lactobacillus kitasatonis, Ligilactobacillus aviarius, Lactobacillus helveticus, Ligilactobacillus agilis, Megamonas funiformis*, *Bifidobacterium longum, Sutterella timonensis and Negativibacillus massiliensis*) and additional eight were assigned to a genus, the rest remained unclassified at lower taxonomic levels (Figure 3.5). Additionally, two ASVs belong to

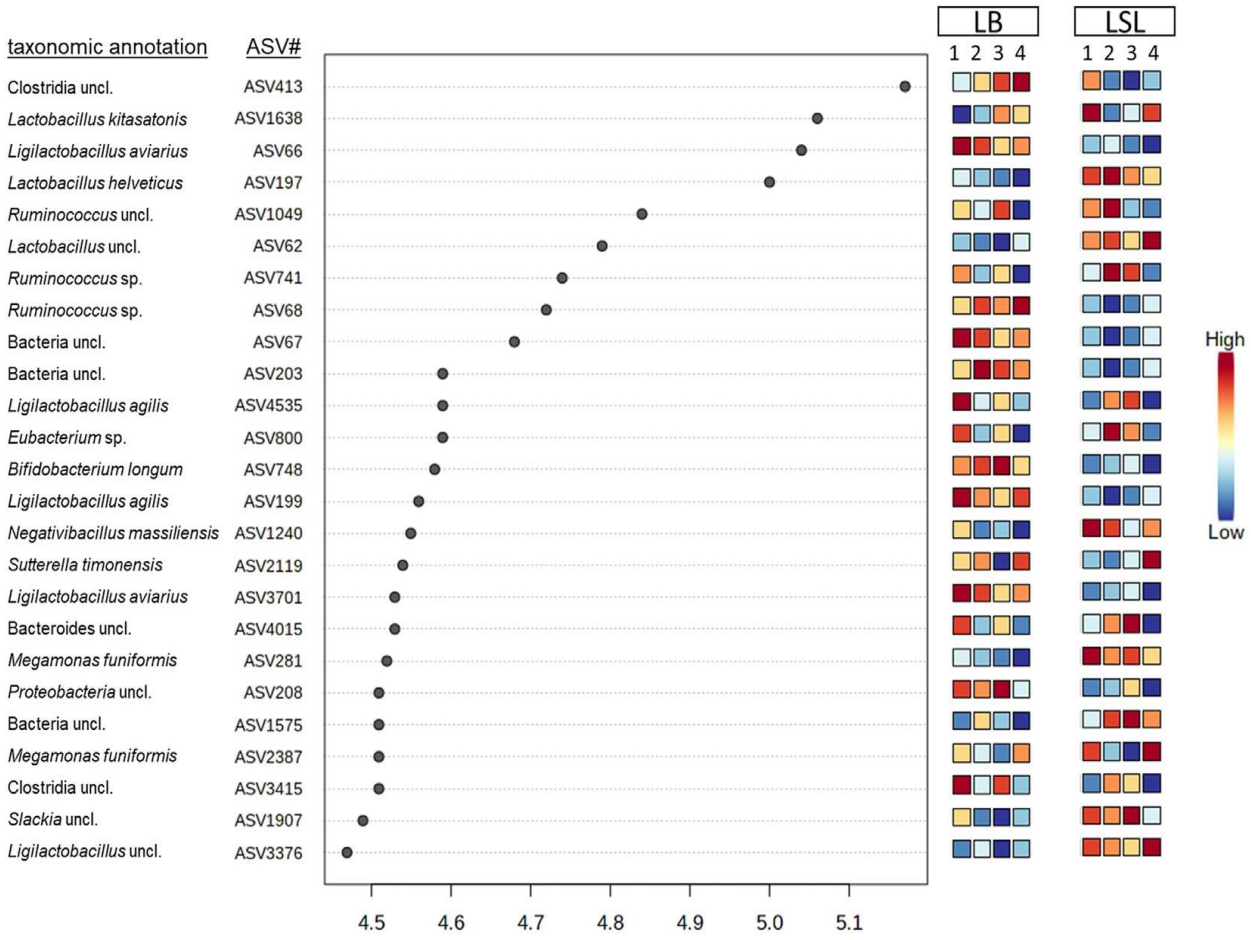

Figure 3.5: Discriminant analyses of the 25 most significant ASVs in caecal samples based on a LEfSe analysis showing the impact per diet (1: P+Ca+, 2: P-Ca-, 3: P+Ca-, 4: P-Ca+) and breed. The scale indicates the relative abundance in comparison to the average across the eight groups consisting of both breeds and the four diets.

the core microbiota (ASV62, ASV197) and were more abundant in LSL compared to

LB. Bacterial shifts were revealed across diets for each breed, either increasing or decreasing abundance and between the breeds, where some ASVs show higher relative abundance in one breed compared to the other. These results showed that the breed is the primary driver of microbial composition, followed by the GIT section and Ca/P supplementation.

3.5 Discussion

GIT microbiota in poultry is influenced by many exo- and endogenous factors such as animal age, stress, genotype, or diet [69]. Whereas the microbiome in broilers is extensively researched, knowledge about laying hens is scarce, especially the microbiota description along the whole GIT. Microbiota stimulates the immune system, contributes to host nutrition and pathogen inhibition, synthesizes amino acids and vitamins, and has a role in breaking down complex molecules and potential toxic feed components [10]. Changes in microbiota composition, either by feed, disease or other external factors, can affect these functions; thus, its understanding and characterization are of primary importance. Therefore, this study aimed to identify differences in the active microbiota composition along the GIT including digesta and mucosa in two commercial breeds of laying hens fed diets with dietary Ca and P concentrations 20% below the recommended levels.

Among the factors studied in the present work, the breed had the most significant effect on the microbial community, leading to fluctuations in relative abundance on every taxonomic level across the complete GIT. Consistently, breed disparities have been reported in caecal samples of a recent study comparing HyLine W36 and Hy-Line Brown [2]. Depending on the diet, such breed-related changes might be due to differences in body weight and average daily feed intake between breeds. Moreover, both breeds have different mechanisms regarding P absorption [1] and the significantly higher concentrations of inositol-6 phosphate and inositol-5 phosphate in LB gizzard and caeca [60] might be due to breed-dependent impacts of P, which results in changes in the GIT microbial community.

Previous studies have only characterized the microbiota of single sections of the GIT or feces and showed similar results at phylum and genus levels, as reported here [2; 18; 35; 53; 59; 61; 64; 70]. The use of different breeds also didn’t affect the overall picture of the microbiota, being the main bacterial groups detected across all studied breeds [18; 29; 65]. There is still a discussion on whether richness in microbiome

composition is positively [61; 62; 71] or negatively [58] correlated to animal health. The present study found the highest diversity in the caeca, followed by the duodenum and ileum, with statistical differences between breeds. The highest diversity in caeca is consistent with previous studies [10; 22].

Besides the differences in diversity index, the animal breed affected phyla abundance and species distribution, which was previously reported in broilers [47]. We detected fewer Firmicutes and higher levels of Bacteroidetes in LB than in LSL. Khan et al. [2021] reported that a lower abundance of Firmicutes in laying hens is associated with a decrease in certain bacteria, including *Peptostreptococcus* [35] which is contrary to the recent study, where LB with lower abundances of Firmicutes compared to LSL showed no decrease in *Peptostreptococcus*. On the other hand, Bacteroidetes was significantly higher in LB and an increased abundance of Bacteroidetes has been associated with later stages of the laying phase, where the abundance of Firmicutes decreases and Bacteroidetes overtakes [32].

One of our aims was to identify the effect of lower supplementation of Ca and P in the GIT, because an insufficient supply of one or both minerals might reduce animal growth and bone mineralization due to interference with homeostasis [57] and change the microbial community of the laying hens. Members of *Ligilactobacillus, Megasphaera*, Lachnospiraceae, Bacteroides, *Helicobacter*, Prevotellaceae, Lachnoclostridium, Streptococcus and Lactobacillaceae were affected by the diets. The relative abundance of Lachnospiraceae decreased with Ca supplementation, which might have a negative impact to gut health as members of Lachnospiraceae are related to the production of butyrate, crucial for the metabolism of the epithelial tissue [8]. The genus *Megasphaera* is known to be part of the SCFA production in the caeca of laying hens [23]. In our study, the higher Ca supplementation was causing a decrease in this genus's abundance and might have reduced the SCFA production in LSL. *Ligilactobacillus* and other members of the family Lactobacillaceae are known colonizers of the GIT of laying hens [20]. In this study, their prevalence changed depending on Ca and P supplementation, breed and GIT section. Members of these genera are usually associated with improved GIT health, productive performance and regulators of the immune system [16; 20]. In addition, Streptococcus is closely related to productive performance with negative correlations to feed conversion ratio [23]. Higher levels of ASVs belonging to this genus were observed in LB hens supplemented with higher Ca levels and that had probably led to the reduced average daily feed intake

under the same conditions in this breed [60]. Moreover, in a companion study that used the same hens, P^{-} affected the immune system by increasing immune cell numbers and mitogen-induced response of innate and adaptive immune cells [28]. In contrast, the relative abundance of potential pathogen Helicobacter increased with higher levels of P in the diet, which could have indicated some effect on the immune system [21; 42]; however, the numbers of T cells and $CD4^{+}$ increased in the same hens [28].

Most of the top 25 discriminant ASVs had higher relative abundances in LB compared to LSL, depending on the feature and the fed diet. Finally, the impact of the diet on the microbial composition showed that the offered diets were not challenging the laying hens GIT microbiota. Jing et al. [2018] reported that a reduction to 0.15% available P in the feed was not affecting growth, productive performance, and mRNA expression of P transporters in hens. It was assumed that a lower P and Ca supplementation might lead to functional shifts, as this was observed in a study with probiotic supplementation compared to a standard diet [30]. But, the predicted functional pathways revealed no overall direct influence of P and Ca in the present study.

Previous studies in layers revealed that members of Lactobacillaceae, Bacteroidaceae, Lachnospiraceae, Ruminococcaceae, Veilonellaceae, Prevotellaceae, Clostridiaceae, Rickenellaceae, or Enterobacteriaceae account for the core microbiota [66; 46]. However, none of the studies combined the information across the complete GIT or targeted the active microbiota. In the present study, five core bacteria were detected across 97% of the samples; uncl. *Lactobacillus, Megamonas funiformis, Ligilactobacillus salivarius, Lactobacillus helveticus* and uncl. *Fusicatenibacter*. Considering the high number of samples (n = 678) and the microbiota variation across the GIT, with common colonizers appearing or not in each GIT section digesta and mucosa, the likelihood of finding a core microbiota across all samples decreases [33; 38; 15]. In addition, the detection limit to classify a bacterium as a core member was set to its presence in more than 97% of the total sample number. This percentage is higher than the 50% coverage in Clavijo et al. [2022] and the 75% in Ngunjiri et al. [2019].

All core members are associated with animal health improvement and gut homeostasis. The genus *Lactobacillus* involves host-adapted lactic acid bacteria that colonize the digestive tract of humans and animals [73] and is part of the core microbiome in the ileum and caeca of laying hens [66; 46]. A beneficial effect on egg size and weight induced by *Lactobacillus* cultures as probiotics was reported [67];

however, in this study, LSL layers colonized with higher abundances of *Lactobacillus* had lighter egg weights [60]. Previous studies have reported *M. funiformis* as a hydrogen consumer in laying hen's caecal microbiome [73; 67]. It is a characteristic bacterium in adult hens [67] and accounted for the core microbiota in a recent broiler study [15]. In our study, *M. funiformis* was found in higher abundance in crop, ileum, duodenum and gizzard samples and almost disappeared in the caeca, which is partially in contrast to the findings of Gan et al. [2020] as they observed the genus *Megamonas* in higher abundances in caeca. The genus *Megamonas* has been previously described in ducks and humans as an important fermenter of glucose into acetate and propionate, which provide health benefits to the host [12; 51]. It can be postulated that *M. funiformis* fermented glucose mainly in the upper digestive sections and was displaced in the caeca by other SCFA producing bacteria. Further, *L. salivarius* is commonly isolated from the intestine or faeces of birds and was part of the core microbiome in a recent laying hen study [46]. Their response to food-borne pathogens by an antibacterial activity influences the host immune system and the microbial composition [41]. The LSL hens had a higher abundance of L. salivarius, and higher amounts of leukocytes, thrombocytes, monocytes, T cells, T helper cells, and cytotoxic T than LB [28], which might be a response of the host system to potential pathogens or a breed-dependent reaction to the housing conditions [43]. *L. helveticus* is an early colonizer of the broiler GIT [16]. Besides the function in pathogen reduction, this bacterium correlated positively with Ca absorption and bone metabolism in vitro [44]. Overall, *L. helveticus* was less abundant in the crop than duodenum and ileum, with main differences between the GIT section of each breed, specifically in LSL. Moreover, LSL might be more sensitive to stress, resulting in a more intense immune response and increased blood components [28] and the potential pathogen reduction and a decrease in stress-induced symptoms can be a breed-related effect. Uncl. *Fusicatenibacter* belongs to the family Lachnospiraceae and was previously associated with host GIT health [8], and detected in the ileum and caeca of laying hens [65] with a constant presence from day 1 to week 40 [5]. A recent study, using metagenomic analysis, showed several protologues for new candidatus *Fusicatenibacter* [24], this bacterial group was more abundant in crop and might be involved in the first steps of feed digestion together with *M. funiformis.*

The taxonomic core microbiota are microorganisms of a dataset that are postulated to indicate inherent functional relationships with the host. They have the potential to be

targeted for culturing and other omics analyses and can be used towards understanding the functional meaning of the core to the laying hen [45]. The knowledge of the active core microbiota further develops hypotheses about their role within the microbiome.

For the first time, the current study presents data on the active microbiota associated with the whole GIT of two high yielding laying hen breeds and the core active microorganisms detected in more than 97% of the samples. Significant differences in the microbiota composition were observed between the breeds which was unexpected to such an extent as hens were housed in the same stable, under the same conditions at the same time. Furthermore, we showed that a reduction of circa 20% of Ca and P concentration in the feed compared to the current standard had no effect on microbiota distribution and predicted functions.

3.6 Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ebi.ac.uk/ena, PRJEB52942

3.7 Ethics statement

The animal study was reviewed and approved by the Regierungspräsidium Tübingen, Germany (approval number HOH50/17 TE).

3.8 Author contributions

MR, JS, and AC-S conceived and designed the experiments, TS and CR performed laboratory analysis, CR and AC-S analyzed the data and wrote the paper. All authors reviewed the manuscript.

3.9 Funding

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3.10 Acknowledgments

We would like to thank all P-Fowl members for the support during the animal experimental trial and sample collection.

3.11 Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

3.12 Publisher’s note

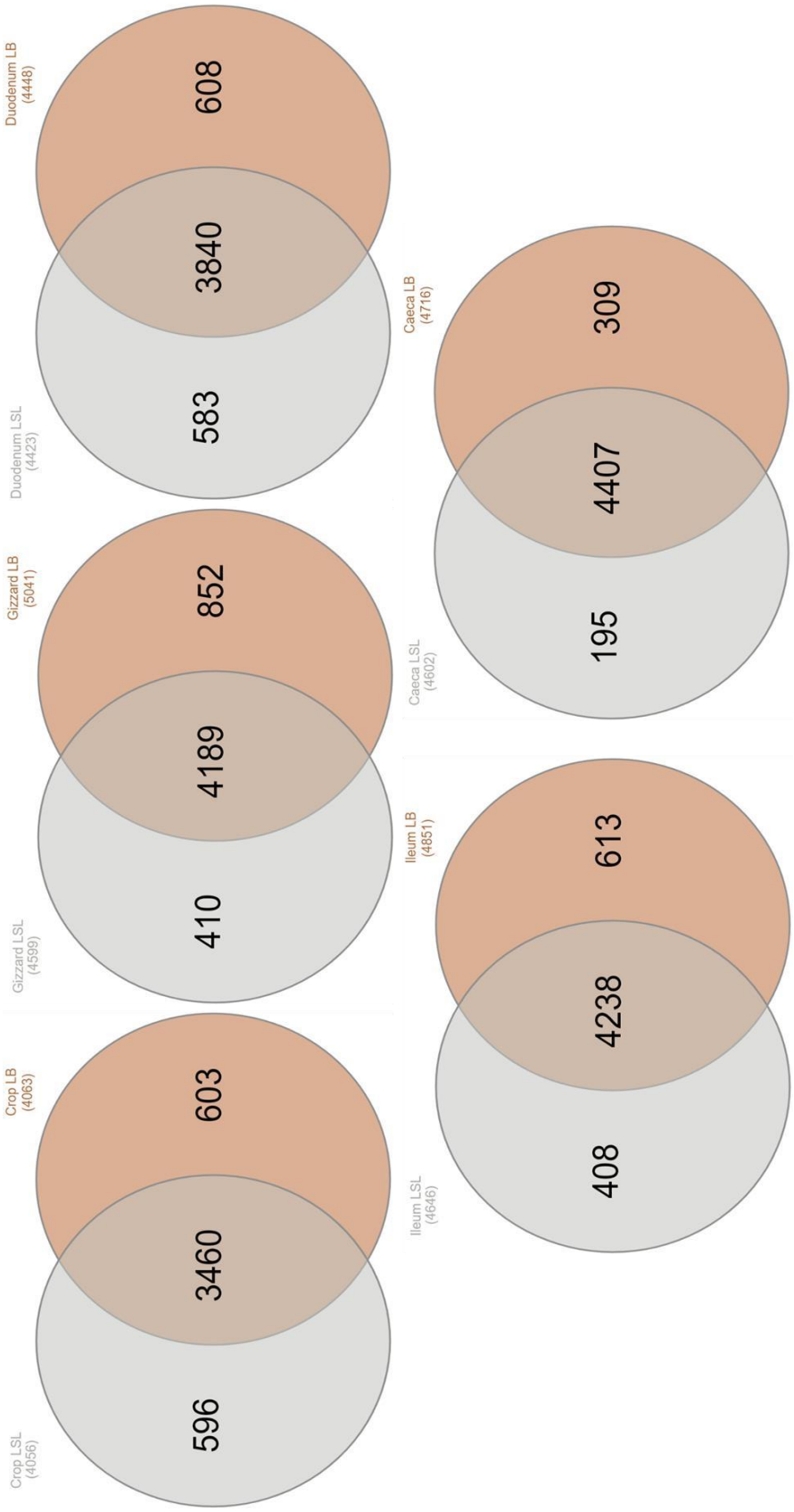
All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

3.13 Supplementary material

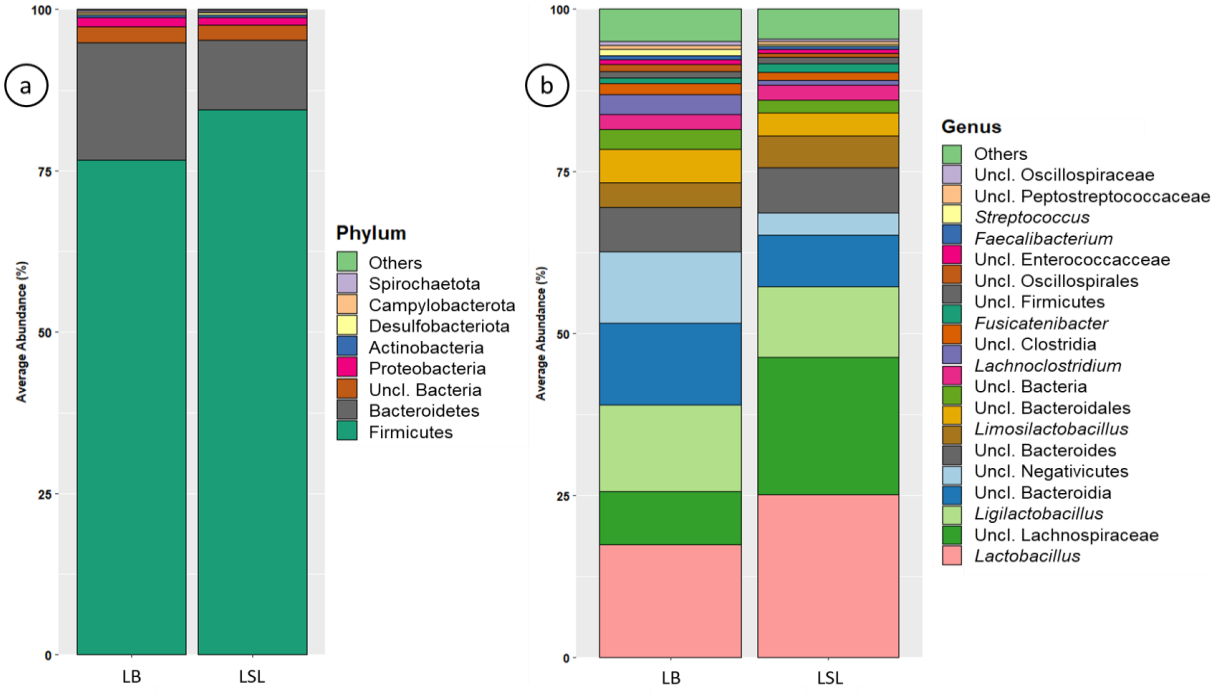
The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2022.951350/full#supplementary-material

3.13.1 Supplementary Figures

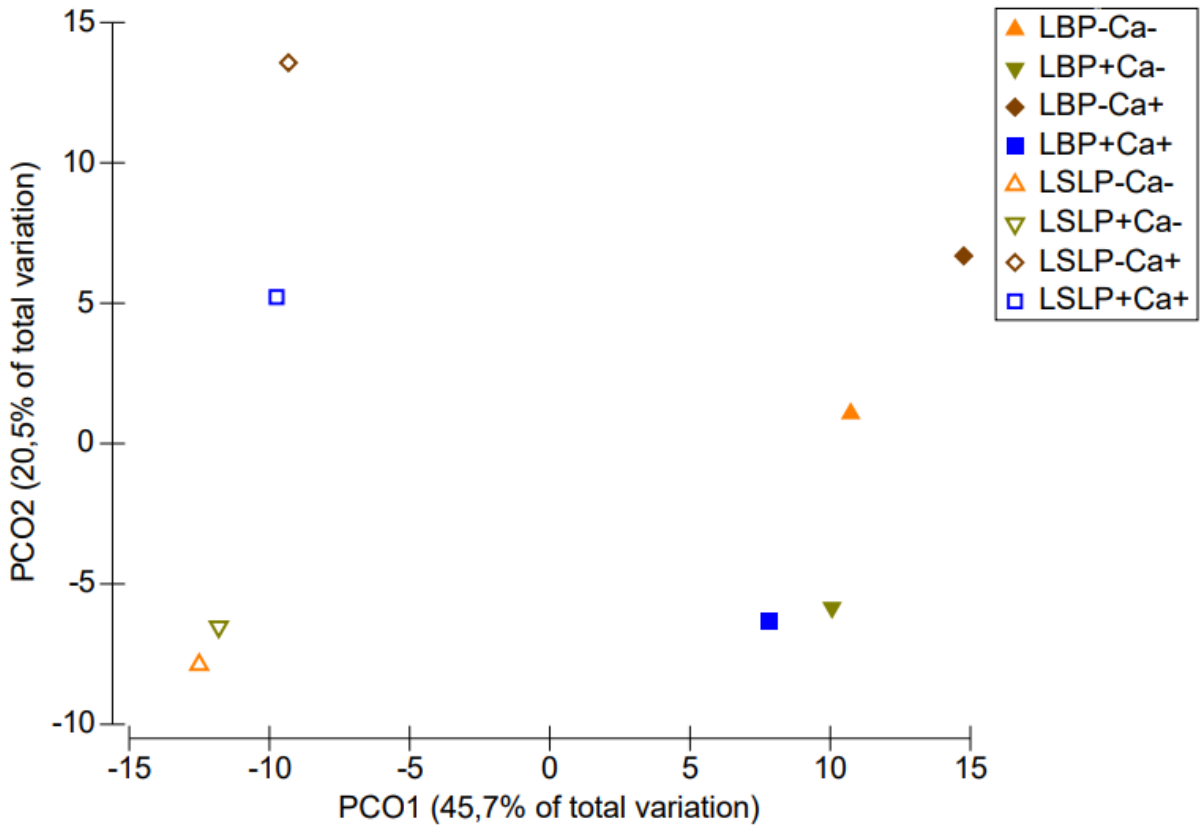
Suppl. figure 3.1. Distribution of ASV‘s in single GIT sections across all samples in both breeds. The number in parenthesis is the observed number of ASVs in each group.



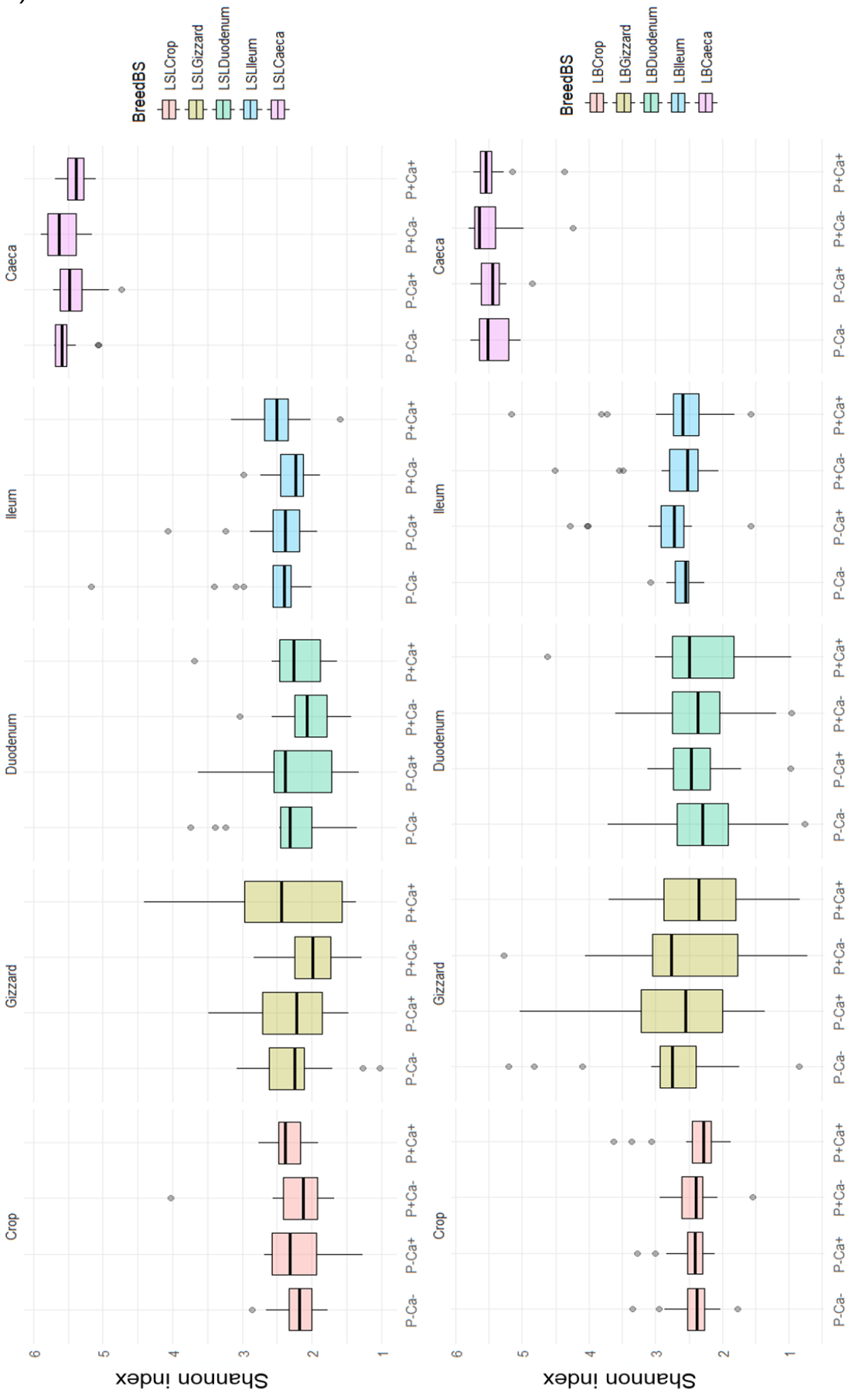
Suppl. figure 3.2. Barplot of the average relative abundance at phylum level (a) and genus level (b) separated by breed.



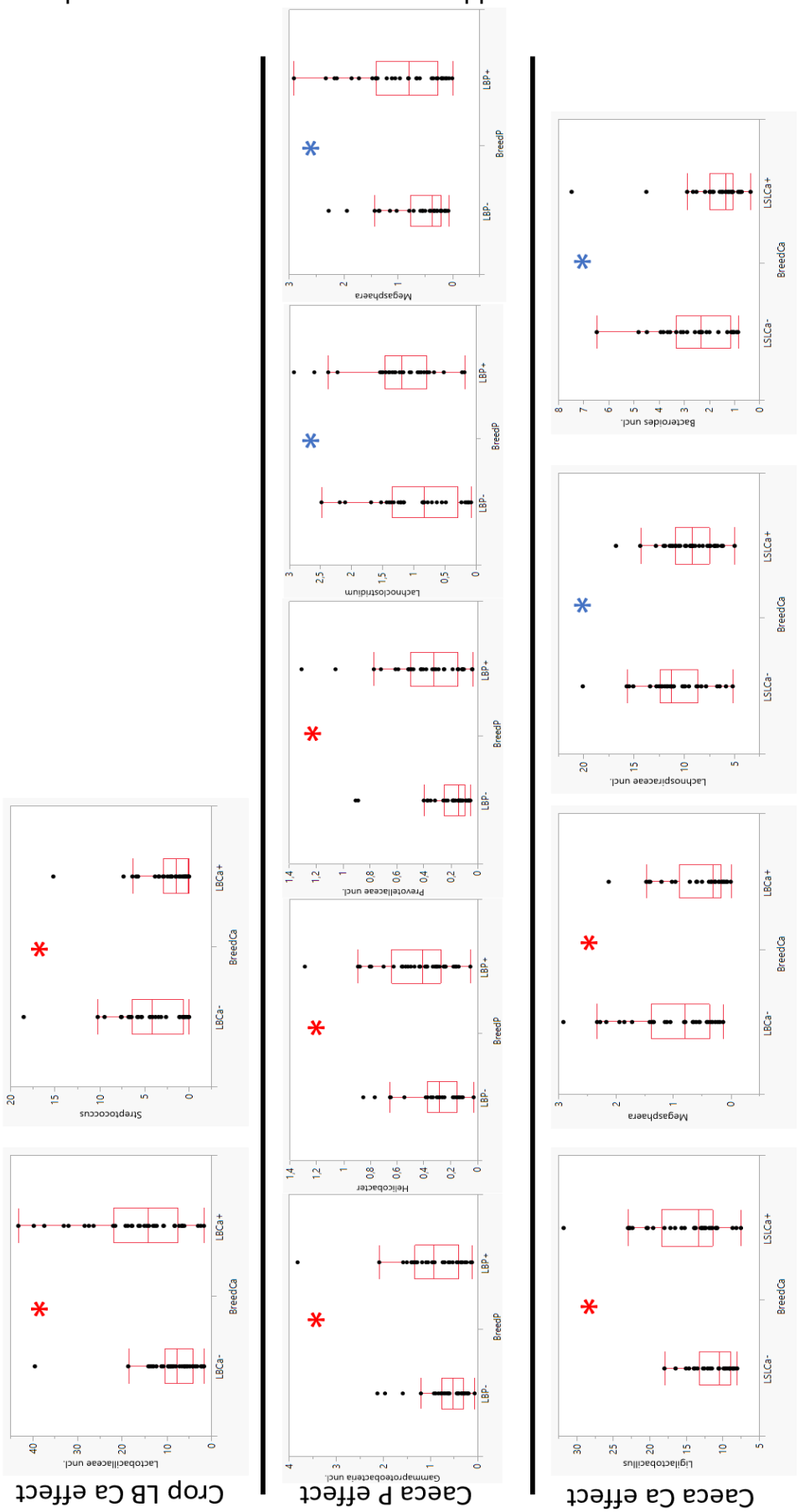
Suppl. figure 3.3. Centroids of the caecal ASV composition separated by Ca / P supplementation and breed.



Suppl. figure 3.4. Boxplot of Shannon diversity index separated by the breed, section (color) and Ca / P combination of the diet**.**



Suppl. fig. 3.5. Boxplot of the significant (red asterisk) and trending (blue asterisk) changes in crop and caeca related to Ca or P supplementation.



3.13.2 Supplementary Tables

S3.1 (excel file). Significant differences at genus level between breeds and GIT sections within the breeds; ONLY SIGNIFICANT RESULTS ARE SHOWN.

Uncl. Lachnospiraceae

Level	- Level	Score Mean Difference	Std Err Dif	Z	p-Value	Hodges-Lehmann	Lower CL	Upper CL	Difference
LSLGizzard	LSLCrop	32.05	6.98	4.59	0.0002	4.12	0.93	23.04	++
LSLDuodenun	LSLCaeca	-21.60	6.45	-3.35	0.0278	-4.52	-6.97	-0.41	---
LSLIleum	LBIleum	-24.10	6.98	-3.45	0.0197	-3.05	-6.98	-0.27	--
LSLIleum	LSLCaeca	-33.64	6.81	-4.94	<.0001	-4.91	-6.97	-2.32	---
LSLCrop	LBCrop	-46.02	7.03	-6.55	<.0001	-5.67	-9.03	-3.03	---
LSLCrop	LSLCaeca	-53.22	6.89	-7.72	<.0001	-6.70	-8.18	-5.08	----

Uncl. Lactobacillaceae

Level	- Level	Score Mean Difference	Std Err Dif	Z	p-Value	Hodges-Lehmann	Lower CL	Upper CL	Difference
LSLIleum	LBIleum	63.55	6.98	9.11	<.0001	17.65	13.54	21.98	+++++++++
LSLCrop	LSLCaeca	62.45	6.89	9.06	<.0001	22.71	18.41	27.43	++++++++++
LSLIleum	LSLCaeca	60.74	6.81	8.91	<.0001	14.71	10.53	18.69	+++++++
LSLCrop	LBCrop	54.13	7.03	7.71	<.0001	20.40	14.05	25.93	++++++++++
LSLGizzard	LBGizzard	45.65	6.74	6.78	<.0001	13.70	6.07	21.01	+++++++
LSLDuodenun	LBDuodenum	38.57	6.33	6.09	<.0001	11.91	4.94	19.29	++++++
LSLGizzard	LSLCaeca	30.54	6.76	4.52	0.0003	11.28	2.08	17.63	+++++
LSLDuodenun	LSLCaeca	27.97	6.45	4.34	0.0006	10.42	2.20	16.41	+++++
LSLIleum	LSLCrop	-26.01	7.03	-3.70	0.0081	-7.28	-13.39	-1.19	----
LBDuodenum	LBCrop	-29.70	6.73	-4.42	0.0004	-4.60	-7.89	-1.27	--
LBDuodenum	LBCaeca	-29.73	6.56	-4.53	0.0003	-4.12	-5.72	-1.44	--
LSLGizzard	LSLCrop	-31.09	6.98	-4.45	0.0004	-11.36	-19.19	-3.46	-----
LBIleum	LBCrop	-32.29	6.98	-4.63	0.0002	-4.16	-7.47	-1.41	--
LBGizzard	LBCrop	-33.15	6.79	-4.88	<.0001	-4.67	-8.09	-1.66	--
LBGizzard	LBCaeca	-33.82	6.63	-5.10	<.0001	-3.75	-5.38	-1.38	--
LSLDuodenun	LSLCrop	-34.52	6.72	-5.14	<.0001	-12.34	-19.95	-4.99	------
LBIleum	LBCaeca	-36.08	6.84	-5.27	<.0001	-3.26	-4.70	-1.58	--

Lactobacillus

Level	- Level	Score Mean Difference	Std Err Dif	Z	p-Value	Hodges-Lehmann	Lower CL	Upper CL	Difference
LSLIleum	LBIleum	55.74	6.98	7.99	<.0001	13.59	9.43	17.71	++++++
LSLIleum	LSLGizzard	36.44	6.90	5.28	<.0001	7.95	3.55	12.43	++++
LSLDuodenun	LSLCaeca	36.44	6.45	5.65	<.0001	11.66	5.29	19.44	+++++
LSLCrop	LBCrop	33.42	7.03	4.76	<.0001	7.41	2.71	12.10	+++
LSLDuodenun	LSLCrop	33.19	6.72	4.94	<.0001	11.74	4.29	19.70	+++++
LSLGizzard	LBGizzard	31.05	6.74	4.61	0.0002	6.43	2.28	10.57	+++
LSLIleum	LSLCaeca	30.83	6.81	4.52	0.0003	5.07	1.80	8.23	++
LSLDuodenun	LBDuodenum	26.21	6.33	4.14	0.0014	13.47	3.55	22.63	++++++
LSLIleum	LSLCrop	21.17	7.03	3.01	0.0776	4.23	-0.22	8.84	++
LSLIleum	LSLDuodenun	-20.76	6.63	-3.13	0.0549	-7.32	-15.41	0.15	---
LBIleum	LBDuodenum	-23.61	6.76	-3.49	0.0171	-6.98	-13.92	-0.66	---
LBGizzard	LBDuodenum	-25.79	6.53	-3.95	0.0032	-8.27	-14.71	-1.42	----
LBCrop	LBCaeca	-30.33	6.81	-4.45	0.0004	-6.14	-9.96	-1.91	---
LSLGizzard	LSLDuodenun	-40.18	6.57	-6.12	<.0001	-15.30	-23.47	-8.00	-------
LBIleum	LBCaeca	-46.29	6.84	-6.77	<.0001	-7.86	-10.53	-4.87	----
LBGizzard	LBCaeca	-48.84	6.63	-7.36	<.0001	-8.48	-11.37	-5.29	----

Ligilactobacillus

Level	- Level	Score Mean Difference	Std Err Dif	Z	p-Value	Hodges-Lehmann	Lower CL	Upper CL	Difference
LBIleum	LBDuodenum	58.51	6.76	8.66	<.0001	13.63	9.00	22.16	++++++++
LBIleum	LBGizzard	55.05	6.82	8.07	<.0001	13.03	8.12	20.93	+++++++
LSLIleum	LSLGizzard	40.33	6.90	5.84	<.0001	3.59	1.84	5.27	++
LBIleum	LBCrop	36.15	6.98	5.18	<.0001	7.83	2.97	15.22	++++
LSLIleum	LSLDuodenun	31.95	6.63	4.82	<.0001	2.85	0.99	4.54	++
LBIleum	LBCaeca	27.51	6.84	4.02	0.0023	5.49	1.15	13.84	+++
LSLDuodenun	LSLCrop	-25.41	6.72	-3.78	0.0061	-1.99	-3.94	-0.34	-
LSLGizzard	LSLCrop	-34.07	6.98	-4.88	<.0001	-2.75	-4.56	-1.04	--
LSLCrop	LBCrop	-34.37	7.03	-4.89	<.0001	-3.50	-5.98	-1.24	--
LBGizzard	LBCrop	-36.75	6.79	-5.41	<.0001	-4.99	-7.74	-2.26	---
LBDuodenum	LBCrop	-45.22	6.73	-6.72	<.0001	-5.81	-8.20	-3.35	---
LSLIleum	LSLCaeca	-45.23	6.81	-6.64	<.0001	-4.55	-6.40	-2.64	---
LBGizzard	LBCaeca	-48.14	6.63	-7.26	<.0001	-6.99	-9.37	-4.51	----
LSLCrop	LSLCaeca	-51.43	6.89	-7.46	<.0001	-5.30	-7.29	-3.45	---
LBDuodenum	LBCaeca	-55.04	6.56	-8.39	<.0001	-7.93	-9.85	-5.69	----
LSLDuodenun	LSLCaeca	-56.48	6.45	-8.76	<.0001	-7.27	-9.35	-5.48	----
LSLGizzard	LSLCaeca	-59.15	6.76	-8.75	<.0001	-8.03	-10.01	-6.22	----

S3.2 (excel file) Global test

PERMANOVA table of results. only statistically significant results are shown.

ALL						
Source	df	SS	MS	Pseudo-F	P(perm)	Unique perms
Breed	1	1.12E+05	1.12E+05	67.554	0.0001	9913
Bodyside	9	7.92E+05	88035	53.28	0.0001	9832
Calcium	1	3533.3	3533.3	2.1384	0.0334	9936
Phosphorus	1	4804.2	4804.2	2.9076	0.0071	9921
BreedxBodyside	9	80443	8938.1	5.4095	0.0001	9810
BreedxCalcium	1	4732.3	4732.3	2.8641	0.0071	9916
BreedxPhosphorus	1	2698.6	2698.6	1.6332	0.0942	9920
CalciumxPhosphorus	1	4087.1	4087.1	2.4736	0.0157	9911
BreedxCalciumxPhosphorus	1	4894.8	4894.8	2.9624	0.0076	9927
Res	598	9.88E+05	1652.3			
Total	677	2.09E+06				

CROP						
PERMANOVA table of results						
Source	df	SS	MS	Pseudo-F	P(perm)	Unique perms
Calcium	1	2476.5	2476.5	2.5901	0.052	999
Breed	1	42527	42527	44.477	0.001	999
CalciumxBreed	1	2173.4	2173.4	2.2731	0.082	999
Res	139	1.33E+05	956.14			
Total	146	1.85E+05				

GIZZARD						
PERMANOVA table of results						
Source	df	SS	MS	Pseudo-F	P(perm)	Unique perms
Breed	1	30075	30075	11.287	0.001	996
Res	127	3.38E+05	2664.5			
Total	134	3.84E+05				

DUODENUM						
PERMANOVA table of results						
Source	df	SS	MS	Pseudo-F	P(perm)	Unique perms
Breed	1	29185	29185	12.355	0.001	997
Res	111	2.62E+05	2362.3			
Total	118	3.08E+05				

ILEUM						
PERMANOVA table of results						
Source	df	SS	MS	Pseudo-F	P(perm)	Unique perms
Breed	1	62051	62051	39.646	0.001	998
Res	137	2.14E+05	1565.1			
Total	144	2.88E+05				

CAECA						
PERMANOVA table of results						
Source	df	SS	MS	Pseudo-F	P(perm)	Unique perms
Phosphor	1	3422.1	3422.1	2.0072	0.002	997
Calcium	1	4280.2	4280.2	2.5105	0.001	998
Breed	1	15601	15601	9.1507	0.001	997
PhosphorusxCalcium	1	2425.7	2425.7	1.4228	0.052	998
PhosphorusxBreed	1	2291.8	2291.8	1.3443	0.079	999
CalciumxBreed	1	4170.4	4170.4	2.4461	0.001	998
PhosphorusxCalciumxBreed	1	2687.8	2687.8	1.5765	0.022	999
Res	124	2.11E+05	1704.9			
Total	131	2.46E+05				

S3.3 (excel file) Anosim summary of supplementation effect (red), breed effect (green/blue) at ASV level / e.g. LB P+ caeca samples are significantly different from LB P- caeca samples.

Phosporus	LSL P+	LSL P-	LB P+	LB P-
LSL P+				
LSL P-				
LB P+	Cr,G,D,I,Cae			
LB P-		Cr,G,D,I,Cae	Cae	

Calcium	LSL Ca+	LSL Ca-	LB Ca+	LB Ca-
LSL Ca+				
LSL Ca-	Cae			
LB Ca+	Cr,G,D,I,Cae			
LB Ca-		Cr,G,D,I,Cae	Cr,Cae	

Crop	Pairwise Tests Groups	R Statistic	Significance Level %	Possible Permutations	Actual Permutations	Number >= Observed	p value	p.adj
	LBCropCa-, LBCropCa+	0.079	0.3	Very large	999	2	0.003	0.0036
	LBCropCa-, LSLCropCa-	0.581	0.1	Very large	999	0	0.001	0.003
	LBCropCa+, LSLCropCa+	0.257	0.1	Very large	999	0	0.001	0.002
	LBCropP+, LSLCropP+	0.378	0.1	Very large	999	0	0.001	0.003
	LBCropP-, LSLCropP-	0.395	0.1	Very large	999	0	0.001	0.002

Gizzard	Pairwise Tests Groups	R Statistic	Significance Level %	Possible Permutations	Actual Permutations	Number >= Observed	p value	p.adj
	LBGizzardP+, LSLGizzardP+	0.153	0.1	Very large	999	0	0.001	0.003
	LBGizzardP-, LSLGizzardP-	0.182	0.2	Very large	999	1	0.002	0.003
	LBGizzardCa-, LSLGizzardCa-	0.197	0.2	Very large	999	1	0.002	0.003
	LBGizzardCa+, LSLGizzardCa+	0.142	0.1	Very large	999	0	0.001	0.003

Duodenum	Pairwise Tests Groups	R Statistic	Significance Level %	Possible Permutations	Actual Permutations	Number >= Observed	p value	p.adj
	LBDuodenumP+, LSLDuodenumP+	0.268	0.1	Very large	999	0	0.001	0.003
	LBDuodenumP-, LSLDuodenumP-	0.125	0.1	Very large	999	0	0.001	0.002
	LBDuodenumCa-, LSLDuodenumCa-	0.254	0.1	Very large	999	0	0.001	0.003
	LBDuodenumCa+, LSLDuodenumCa+	0.144	0.2	Very large	999	1	0.002	0.003

Ileum	Pairwise Tests Groups	R Statistic	Significance Level %	Possible Permutations	Actual Permutations	Number >= Observed	p value	p.adj
	LBIleumP+, LSLIleumP+	0.436	0.1	Very large	999	0	0.001	0.003
	LBIleumP-, LSLIleumP-	0.535	0.1	Very large	999	0	0.001	0.002
	LBIleumCa-, LSLIleumCa-	0.498	0.1	Very large	999	0	0.001	0.003
	LBIleumCa+, LSLIleumCa+	0.492	0.1	Very large	999	0	0.001	0.002

Caeca	Pairwise Tests Groups	R Statistic	Significance Level %	Possible Permutations	Actual Permutations	Number >= Observed	p value	p.adj
	LBCaecaP+, LBCaecaP-	0.056	0.6	Very large	999	5	0.006	0.0072
	LBCaecaP+, LSLCaecaP+	0.243	0.1	Very large	999	0	0.001	0.003
	LBCaecaP-, LSLCaecaP-	0.344	0.1	Very large	999	0	0.001	0.002
	LBCaecaCa-, LSLCaecaCa-	0.339	0.1	Very large	999	0	0.001	0.003
	LBCaecaCa+, LSLCaecaCa+	0.327	0.1	Very large	999	0	0.001	0.002
	LSLCaecaCa+, LSLCaecaCa-	0.169	0.1	Very large	999	0	0.001	0.0012

S3.4 (excel file) PERMANOVA table of results: Functional prediction – Pathways ONLY SIGNIFICANT RESULTS ARE SHOWN.

Source		df	SS	MS	Pseudo-F	P(perm)	Unique perms
breed		1	7.0111	7.0111	3.3904	0.02	998
breedxPhosphorus		1	5.3792	5.3792	2.6012	0.05	999
Res		124	256.43	2.068			
Total		131	280.28				

ko02010	ABC transporters		Membrane transport		Environmental Information Processing					breed effect
Level	- Level	Score Mean Difference	Std Err Dif	Z	p-Value	Hodges-Lehman	Lower CL	Upper CL	Difference Plot	
LSL_P+Ca-	LB_P+Ca-	10.7917	3.5	3.08333	0.0428	0.00182	0.000092	0.0036202	+++++++	
ko00190	Oxidative phosphorylation		Energy metabolism		Metabolism					breed effect
Level	- Level	Score Mean Difference	Std Err Dif	Z	p-Value	Hodges-Lehman	Lower CL	Upper CL	Difference Plot	
LSLP-Ca-	LBP-Ca-	-10.355	3.281388	-3.1557	0.0343	-0.0002	-0.00039	-0.000015	-----	
Functions										
Steel-Dwass test										
K06607	myo-inositol catabolism protein IolS [EC:1,1,1,-]									Ca. Breed. P
Level	- Level	Score Mean Difference	Std Err Dif	Z	p-Value	Lehman	Lower CL	Upper CL	Difference Plot	
LSLP-Ca+	LSLP-Ca-	11.4921	3.342844	3.43781	0.0136	2.8E-05	2.83E-06	0.000078	++++	
LSLP-Ca-	LBP-Ca-	-10.355	3.281388	-3.1557	0.0343	-3E-05	-8.1E-05	-6.60E-07	----	
LSLP-Ca-	LSLP+Ca-	-11.2321	3.47011	-3.2368	0.0266	-5E-05	-9.5E-05	-3.31E-06	-------	
k22230	scyllo-inositol 2-dehydrogenase (NADP+) [EC:1,1,1,-]									Ca. Breed
Level	- Level	Score Mean Difference	Std Err Dif	Z	p-Value	Lehman	Lower CL	Upper CL	Difference Plot	
LSLP-Ca+	LSLP-Ca-	10.2222	3.342537	3.05822	0.0461	2.3E-05	-4.20E-07	0.000047	+++++	
LBP-Ca+	LBP-Ca-	-9.7738	3.243511	-3.0133	0.0526	-3E-05	-5.9E-05	0.0000001	-----	
LSLP-Ca-	LBP-Ca-	-14.3929	3.281388	-4.3862	0.0003	-4E-05	-6.8E-05	-0.000016	-------	
K01771	1-phosphatidylinositol phosphodiesterase [EC:4,6,1,13]									Breed effect
Level	- Level	Score Mean Difference	Std Err Dif	Z	p-Value	Lehman	Lower CL	Upper CL	Difference Plot	
LSLP-Ca+	LBP+Ca+	-10.369	3.661964	-2.8316	0.0873	#######	-0.00001	4.20E-07	----	
LSLP-Ca+	LBP-Ca+	-11.5919	3.309315	-3.5028	0.0109	#######	-1.8E-05	-1.66E-06	-------	
k015521	D-inositol-3-phosphate glycosyltransferase [EC:2,4,1,250]									Breed effect
Level	- Level	Score Mean Difference	Std Err Dif	Z	p-Value	Hodges-Lehman	Lower CL	Upper CL	Difference Plot	
LSLP-Ca-	LBP-Ca-	-9.83403	3.281388	-2.9969	0.0552	-2E-05	-3.7E-05	0.0000012	----	
k17209	inositol transport system permease protein									Breed effect
Level	- Level	Score Mean Difference	Std Err Dif	Z	p-Value	Hodges-Lehman	Lower CL	Upper CL	Difference Plot	
LSLP-Ca+	LBP-Ca+	-11.062	3.308982	-3.343	0.0188	#######	-1.8E-05	-1.02E-06	-------	
k17210	inositol transport system ATP-binding protein									Breed effect
Level	- Level	Score Mean Difference	Std Err Dif	Z	p-Value	Hodges-Lehman	Lower CL	Upper CL	Difference Plot	
LSLP-Ca+	LBP-Ca+	10.9295	3.307981	3.30398	0.0214	1.21E-06	5.00E-08	3.26E-06	++++	
k17215	inositol transport system ATP-binding protein									Breed effect
Level	- Level	Score Mean Difference	Std Err Dif	Z	p-Value	Hodges-Lehman	Lower CL	Upper CL	Difference Plot	
LSLP-Ca+	LBP-Ca+	-11.062	3.309315	-3.3427	0.0188	-3E-05	-5.4E-05	-2.46E-06	------	

S3.5 (excel file) Relative abundance of core microbiota separated by gastrointestinal section, strain and sample type Significant core bacteria changes between gastrointestinal sections and breed.

		CROP		GIZZARD		DUODENUM		ILEUM		CAECA		ava. abu.
		LSL	LB	LSL	LB	LSL	LB	LSL	LB	LSL	LB	across all samples [%]
ASV62	*uncl. Lactobacillus*	30.16	11.46	11.46	18.615	3.925	17.44	6.345	23	5.115	1.05	12.86
ASV63	Megamonas funiformis	13.05	16	16	4.665	3.885	6.235	6.565	8.615	5.82	0.215	8.11
ASV137	Ligilactus salivarius	6.6	10.8	10.8	2.7	4.2	3.5	4.1	4.8	5.5	0.4	5.34
ASV197	Lactobacillus helveticus	18.61	10.59	10.59	10.895	4.025	20.84	7.79	22.515	8.775	1.9	11.65
ASV561	uncl. Fuscatenibacter	2.8	2.1	2.1	0.9	0.5	1.1	0.8	1.4	0.6	0.1	1.24
	sum [%]	71.2	50.9	50.9	37.8	16.5	49.1	25.5	60.4	25.8	3.7	39.19

Significant core bacteria changes between gastrointestinal sections and breed

ASV62

Level	- Level	Score Mean Difference	Std Err Dif	Z	p-Value	Hodges-Lehmann	Lower CL	Upper CL	DifferencePlot
LSLCrop	LSLCaeca	67.8082	6.893981	9.8359	<.0001	29.8016	25.9902	34.5665	++++++++++++
LBCrop	LBCaeca	67.2431	6.813302	9.8694	<.0001	7.9867	5.7767	11.8364	+++
LSLIleum	LSLCaeca	67.1269	6.813302	9.8523	<.0001	21.5901	17.3271	25.5147	+++++++++
LBIleum	LBCaeca	66.0524	6.839979	9.6568	<.0001	3.8785	2.3436	5.2778	++
LSLIleum	LBIleum	63.6582	6.976316	9.1249	<.0001	17.6768	13.7394	22.2162	++++++++
LSLGizzard	LSLCaeca	61.5385	6.760636	9.1025	<.0001	17.5979	8.3545	22.9235	++++++++
LSLDuodenum	LSLCaeca	55.6966	6.44638	8.64	<.0001	17.1439	8.6838	22.9589	++++++++
LBDuodenum	LBCaeca	53.7869	6.560643	8.1984	<.0001	2.9317	1.4034	5.5139	+
LSLCrop	LBCrop	53.3692	7.025232	7.5968	<.0001	20.56	14.0045	26.0562	+++++++++
LBGizzard	LBCaeca	50.2777	6.633443	7.5794	<.0001	2.2975	1.0852	4.3249	+
LSLGizzard	LBGizzard	49.0599	6.737626	7.2815	<.0001	13.6187	6.3672	21.0071	++++++
LSLCaeca	LBCaeca	45.9545	6.658328	6.9018	<.0001	0.6222	0.3428	0.968	
LSLDuodenum	LBDuodenum	35.7101	6.330145	5.6413	<.0001	11.7793	4.6641	19.1122	+++++
LSLIleum	LSLCrop	-25.1125	7.025232	-3.5746	0.0129	-7.3113	-13.4394	-1.0655	---
LBDuodenum	LBCrop	-29.9429	6.726962	-4.4512	0.0004	-4.354	-7.8092	-1.23	--
LSLGizzard	LSLCrop	-32.4938	6.980301	-4.6551	0.0001	-12.171	-19.9728	-3.9989	-----
LBIleum	LBCrop	-33.4223	6.976316	-4.7908	<.0001	-4.1638	-7.4793	-1.4018	--
LSLDuodenum	LSLCrop	-34.2737	6.721111	-5.0994	<.0001	-12.5267	-20.4645	-5.1374	-----
LBGizzard	LBCrop	-39.7973	6.791201	-5.8601	<.0001	-5.3311	-8.6619	-2.3541	--

ASV63

Level	- Level	Score Mean Difference	Std Err Dif	Z	p-Value	Hodges-Lehmann	Lower CL	Upper CL	DifferencePlot
LBCrop	LBCaeca	68.9855	6.813302	10.1251	<.0001	14.6195	11.3923	18.2118	++++++++++
LSLCrop	LSLCaeca	68.8906	6.893981	9.9929	<.0001	11.3154	8.4244	15.4106	++++++++
LSLIleum	LSLCaeca	67.2431	6.813302	9.8694	<.0001	7.3982	4.569	10.4522	+++++
LBIleum	LBCaeca	66.8891	6.839979	9.7791	<.0001	4.9163	2.7527	6.3941	+++
LSLDuodenum	LSLCaeca	57.5112	6.44637	8.9215	<.0001	4.5442	2.88	7.6428	+++
LSLGizzard	LSLCaeca	57.0935	6.760636	8.445	<.0001	2.9677	1.1423	5.1055	++
LBDuodenum	LBCaeca	53.349	6.560605	8.1317	<.0001	4.6267	2.1085	5.5211	+++
LBGizzard	LBCaeca	46.3997	6.633443	6.9948	<.0001	1.744	0.5044	4.2108	+
LSLIleum	LSLGizzard	29.7101	6.90479	4.3028	0.0007	3.5733	0.8718	6.8496	++
LBIleum	LBGizzard	24.0809	6.818326	3.5318	0.0151	1.8346	0.1771	4.573	+
LSLIleum	LSLCrop	-22.1997	7.025232	-3.16	0.0506	-3.8763	-7.7109	0.0129	---
LSLDuodenum	LSLCrop	-34.0575	6.721111	-5.0672	<.0001	-6.0098	-9.8581	-2.428	----
LSLGizzard	LSLCrop	-47.27	6.980301	-6.7719	<.0001	-7.7415	-11.0842	-4.2815	-----
LBDuodenum	LBCrop	-47.8336	6.726928	-7.1108	<.0001	-9.4728	-13.2425	-6.0848	------
LBIleum	LBCrop	-54.6647	6.976316	-7.8358	<.0001	-9.4035	-12.9814	-6.112	------
LBGizzard	LBCrop	-58.4738	6.791201	-8.6102	<.0001	-11.2746	-14.8869	-8.5388	--------

ASV561

Level	- Level	Score Mean Difference	Std Err Dif	Z	p-Value	Hodges-Lehmann	Lower CL	Upper CL	DifferencePlot
LSLCrop	LSLCaeca	70.4858	6.893981	10.2242	<.0001	2.19119	1.78866	2.68606	++++++++++
LBCrop	LBCaeca	68.9855	6.813224	10.1252	<.0001	1.76887	1.31523	2.26483	+++++++++
LBIleum	LBCaeca	66.3121	6.839903	9.6949	<.0001	0.47405	0.34734	0.65393	++
LSLIleum	LSLCaeca	66.1976	6.813302	9.7159	<.0001	1.28201	1.11198	1.473	++++++
LSLIleum	LBIleum	54.0302	6.976316	7.7448	<.0001	0.83697	0.56635	1.0833	++++
LBDuodenum	LBCaeca	52.9736	6.560455	8.0747	<.0001	0.50687	0.22728	0.745	+++
LSLGizzard	LSLCaeca	49.1602	6.760628	7.2715	<.0001	0.63335	0.26117	1.00751	+++
LSLDuodenum	LSLCaeca	49.1085	6.44637	7.618	<.0001	1.01856	0.59152	1.34909	+++++
LBGizzard	LBCaeca	44.8118	6.633133	6.7557	<.0001	0.27062	0.08644	0.52938	+
LSLCaeca	LBCaeca	32.7424	6.658154	4.9176	<.0001	0.06439	0.02129	0.10442	
LSLIleum	LSLGizzard	30.0764	6.90479	4.3559	0.0006	0.61188	0.18748	0.98952	+++
LSLGizzard	LBGizzard	24.4335	6.737593	3.6264	0.0107	0.31311	0.03649	0.75717	++
LSLDuodenum	LBDuodenum	21.1129	6.330134	3.3353	0.0292	0.47364	0.01471	0.94742	++
LSLIleum	LSLCrop	-41.8269	7.025232	-5.9538	<.0001	-0.94786	-1.50541	-0.45622	-----
LSLDuodenum	LSLCrop	-43.1354	6.721111	-6.4179	<.0001	-1.29546	-1.96609	-0.69689	------
LBDuodenum	LBCrop	-51.2256	6.726962	-7.615	<.0001	-1.19962	-1.71226	-0.8465	------
LSLGizzard	LSLCrop	-54.9205	6.980301	-7.8679	<.0001	-1.53276	-2.13551	-1.00184	--------
LBGizzard	LBCrop	-61.8403	6.791193	-9.106	<.0001	-1.41218	-1.88521	-1.04684	-------
LBIleum	LBCrop	-63.9617	6.976316	-9.1684	<.0001	-1.2433	-1.69799	-0.87465	------

ASV137

Level	- Level	Score Mean Difference	Std Err Dif	Z	p-Value	Hodges-Lehmann	Lower CL	Upper CL	DifferencePlot
LSLCrop	LSLCaeca	70.4858	6.893981	10.2242	<.0001	5.72023	4.1892	7.0426	++++++
LBCrop	LBCaeca	68.9855	6.813302	10.1251	<.0001	8.70125	7.1287	11.2747	+++++++++
LSLIleum	LSLCaeca	68.9855	6.813302	10.1251	<.0001	4.28134	3.2007	5.2873	+++++
LBIleum	LBCaeca	65.9947	6.839979	9.6484	<.0001	4.35871	3.2947	5.4839	+++++
LSLDuodenum	LSLCaeca	55.86	6.44638	8.6653	<.0001	2.77287	1.9948	3.8163	+++
LBDuodenum	LBCaeca	51.2845	6.560643	7.817	<.0001	2.48058	1.5159	4.1893	+++
LSLGizzard	LSLCaeca	49.5576	6.760636	7.3303	<.0001	2.07764	0.9222	3.0667	++
LBGizzard	LBCaeca	42.6437	6.633443	6.4286	<.0001	2.28571	1.0966	4.5535	++
LSLIleum	LSLGizzard	34.6688	6.90479	5.021	<.0001	2.02669	0.7758	3.2679	++
LBIleum	LBGizzard	20.5909	6.818326	3.0199	0.076	1.74297	-0.0909	3.4865	++
LSLIleum	LSLDuodenum	19.6298	6.627765	2.9617	0.0893	1.23289	-0.0742	2.5979	+
LSLIleum	LSLCrop	-21.6008	7.025232	-3.0748	0.065	-1.384	-2.8787	0.0366	-
LSLDuodenum	LSLCrop	-36.1263	6.721111	-5.3751	<.0001	-2.59807	-4.283	-1.0952	---
LSLCrop	LBCrop	-37.0358	7.025232	-5.2718	<.0001	-3.57701	-5.9657	-1.5086	----
LBIleum	LBCrop	-44.2918	6.976316	-6.3489	<.0001	-4.45376	-6.8604	-2.3808	-----
LSLGizzard	LSLCrop	-47.7948	6.980301	-6.8471	<.0001	-3.37676	-4.9013	-1.9749	----
LBDuodenum	LBCrop	-49.1844	6.726962	-7.3115	<.0001	-5.93467	-8.2648	-3.7799	------
LBGizzard	LBCrop	-49.9552	6.791201	-7.3559	<.0001	-6.17667	-8.5919	-3.9353	-------

ASV197

Level	- Level	Score Mean Difference	Std Err Dif	Z	p-Value	Hodges-Lehmann	Lower CL	Upper CL	DifferencePlot
LSLCrop	LSLCaeca	67.2955	6.893981	9.7615	<.0001	16.9408	13.989	19.8249	++++++++++
LSLIleum	LSLCaeca	66.8074	6.813302	9.8054	<.0001	21.1764	18.2415	24.0946	++++++++++++
LBIleum	LBCaeca	65.4754	6.839979	9.5725	<.0001	7.6997	5.1753	9.4788	+++++
LBCrop	LBCaeca	61.798	6.813294	9.0702	<.0001	8.5818	4.4492	11.2758	+++++
LSLIleum	LBIleum	59.2166	6.976316	8.4882	<.0001	14.0921	10.2684	17.9391	++++++++
LSLDuodenum	LSLCaeca	58.7699	6.44638	9.1167	<.0001	19.0758	13.1218	23.4892	++++++++++
LBDuodenum	LBCaeca	50.8152	6.560643	7.7455	<.0001	5.9741	3.228	8.0657	++++
LSLGizzard	LSLCaeca	47.232	6.760636	6.9863	<.0001	8.4774	2.8374	12.8483	+++++
LSLCaeca	LBCaeca	45.7727	6.658328	6.8745	<.0001	1.2484	0.7718	1.6588	+
LSLIleum	LSLGizzard	45.0089	6.90479	6.5185	<.0001	11.6901	7.3053	16.8763	+++++++
LSLDuodenum	LBDuodenum	40.4243	6.330145	6.386	<.0001	13.3246	7.0384	18.9753	++++++++
LSLCrop	LBCrop	40.1664	7.025232	5.7174	<.0001	9.0513	4.5995	13.3489	+++++
LBIleum	LBGizzard	37.9536	6.818326	5.5664	<.0001	4.7688	1.9321	7.595	+++
LSLGizzard	LBGizzard	36.45	6.737626	5.4099	<.0001	5.7275	1.9546	11.5299	+++
LBGizzard	LBCaeca	32.9179	6.633434	4.9624	<.0001	1.6717	0.3413	3.374	+
LSLIleum	LSLCrop	19.8858	7.025232	2.8306	0.1261	3.7484	-0.4796	8.0833	++
LBIleum	LBDuodenum	10.8424	6.755466	1.605	0.8462	1.4454	-1.5779	4.643	+
LSLIleum	LSLDuodenum	6.9624	6.627765	1.0505	0.9891	1.894	-3.9734	7.7166	+
LSLDuodenum	LSLCrop	6.6077	6.721111	0.9831	0.9932	1.9372	-4.0331	7.8536	+
LBIleum	LBCrop	-5.2554	6.976316	-0.7533	0.9991	-0.8344	-4.6556	2.7454	
LBDuodenum	LBCrop	-13.0128	6.726962	-1.9344	0.6453	-2.0313	-6.282	1.3997	-
LBGizzard	LBDuodenum	-24.6594	6.533796	-3.7741	0.0062	-3.0678	-6.2389	-0.3884	--
LSLGizzard	LSLDuodenum	-31.941	6.566465	-4.8643	<.0001	-9.6481	-16.2082	-3.506	------
LSLGizzard	LSLCrop	-35.7252	6.980301	-5.118	<.0001	-8.1199	-12.8047	-3.3464	-----
LBGizzard	LBCrop	-37.2359	6.791193	-5.483	<.0001	-4.9453	-9.4563	-2.1304	---

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CHAPTER IV

Gut microbiota variations during the productive lifespan of two high-yielding laying hen breeds

4. Gut microbiota variations during the productive lifespan of two high-yielding laying hen breeds

4.1 Abstract

Gut microbiota affects nutrient digestion, pathogen inhibition, gut epithelium nourishment, endocrine activity, and interaction with the gut-associated immune system. In laying hens, previous studies focused their research on using feces, or specific gastrointestinal (GIT) sections and analyzed single production stages in the life of laying hens and the animals' response to particular conditions such as a change in the diet. This study aimed to characterize the active intestinal microbial community in two commercially laying hen breeds: Lohmann Brown-Classic (LB) and Lohmann LSL-Classic (LSL), during their complete productive lifespan. All birds were kept under the same diet, housing, and management conditions. Digesta samples of the crop, gizzard, duodenum, ileum, and caeca were collected at 10, 16, 24, 30, and 60 weeks of life to represent the production stages. RNA was extracted from 500 samples and analyzed by target amplicon sequencing. Phylogenetic analysis of the bacterial sequences was assessed using Mothur, followed by multivariate statistical analysis. A statistical significance was observed for the breed, GIT section, the production period and the combination of all factors ($p < 0.05$). Depending on the breed, the detected genera differed in the abundance level within GIT sections or production stages. The most significant shifts in the active microbiota of the laying hens were observed from the early life on (week 10) and with the transition into the laying period between weeks 16 and 24. Furthermore, deep analysis using metagenomic shotgun sequencing of weeks 16 and 24 revealed the functional shifts during this phase. Functional profiling showed differences between the breeds besides up- and downregulated functions with the onset of the laying phase. The taxonomical profile has been compared with the 16S rRNA gene amplicon sequencing results to indicate methodical differences. We conclude that the breed and production stage impact intestinal microbiota dynamics.

4.2 Introduction

Laying hens are challenged during their lifespan and the animals face physiological changes during this period [1]. Many molecular pathways influence the aging process, and the body's reaction, interaction and interplay of those vary additionally with the exposition to environmental factors [2–4]. The diet intake must change depending on the growing phase and the corresponding animal needs, especially minerals like phosphorus (P) or calcium (Ca), which play a major role in age-related changes in body growth and egg production. An imbalanced diet might affect animal health and productivity [5,6].

The post-hatch period is characterized by morphological and functional adaptations of the gastrointestinal tract (GIT) to utilize solid feed [7]. After week 15, the laying hens reproductive tract matures, which leads to the onset of egg-laying after this period [8,9]. Between weeks 16 and 24 of life, the laying hens pass from a period before the onset of egg production to approximately 88% of the maximum egg production [10,11]. Moreover, the diet changes from a grower to a pre-layer/ layer diet, which implies a higher Ca supplementation. The bird morphology also passes through different changes, such as an increase in the oviduct length, oviduct weight [12] and a higher allocation of the nutrient proportion to the reproductive development between week 19 and 23 compared to later stages (week 68) [13]. After week 30, the egg-laying peak will be achieved, the hens growth is terminated and the egg production decreases towards the 60 weeks of life [14].

Especially the development from week 16 to week 24 is of high importance as it is a challenge in laying hens' organism due to the start of egg production and the body's transition to lay eggs as previously described. The eggshell composes primarily of Ca; consequently, the Ca needs are increased two weeks before the beginning of the laying period [6], and the hens' higher Ca retention is a preparation for the Ca output in the form of the egg shells [4]. In addition, the P intake affects bone development, indirectly impacting egg production or shell quality [1]. Overall, Ca and P are primarily absorbed in the small intestine [3]. Therefore, reducing one or both minerals results in a reduced growth rate and lower bone mineralization [2,15].

Overall, the gut microbiome of laying hens evolves from day one after hatching and the microbiome has to adapt quickly as the organisms' priority shifts from growth to the egg-laying onset and the termination of body growth. While the bacteria found in early life are already similar to those in the matured hens, the relative abundance tends to

fluctuate a few weeks before stabilizing [5]. The GIT of hens develops from a slowly diverse habitat in the first week to higher bacterial diversity in later stages [5]. The recently published studies on laying hens microbiota mainly focused on specific life stages, caecal samples or epithelial tissues of the intestine [16–19]. Despite the importance of the caeca, the other GIT segments have unique physiological functions that are essential for the animal.

Therefore, two commercially used breeds Lohmann Brown-Classic (LB) and Lohmann LSL Classic (LSL) have been selected and compared across five production period stages that represent the whole productive lifespan [2]. The choice of the breeds relied on the fact that they are commonly used breeds for egg production in Europe. LB dominates in Austria and is used in worldwide markets [20]. Furthermore, they have medium egg size, high laying performance and can adapt to different housing situations. Moreover, the similar egg-laying performance and the differences in body weight, phytate degradation and bone metabolism allow for distinguishing the microbial variations between both commercially used breeds [21–24]. Therefore, we hypothesized that the active intestinal microbiota is age-dependent and investigate LB and LSL breeds across the five GIT sections crop, gizzard, duodenum, ileum and caeca over the whole productive lifespan. In addition, shotgun metagenomic analysis was used to gather further insights into the main functions and pathways between weeks 16 and 24.

4.3 Material and Methods

4.3.1 Ethical statement

The ethics committee of the Regierungspräsidium Tübingen approved the experimental design and management procedures by following the German welfare regulation (Project no., HOH50/17TE). The study was performed at the Agricultural Experiment Station of the University of Hohenheim, Germany.

4.3.2 Experimental design and sample collection

Sommerfeld et al (2021) provided a detailed description of the entire experimental setup [23]. In total, 50 Lohmann Brown-Classic (LB) and 50 Lohmann LSL-Classic (LSL) hens were used. All hens of both breeds were kept under the same conditions and were from the same hatch. Diets were based on corn and soybean meal to ensure minimum plant intrinsic phytase activity. The laying hens were fed the same diet, and

diet composition was adjusted to the requirements and level of feed intake in each production period (10, 16, 24, 30, and 60 weeks of life) [23].

All hens were hatched together on deep litter bedding until ten days before slaughter. Then, ten hens per strain were randomly chosen and kept individually in a randomized block design in metabolic units (1 m^3). During the sampling periods, the room temperature was set to 18-22°C.

Feed and tap water were provided for ad libitum consumption. Two hours before slaughtering, the feed was deprived, followed by 1h of ad libitum access to feed for gut fill standardization. The 20 hens on each sampling day were stunned by a gas mixture of 35% CO_2, 35% N_2, and 30% O_2 and immediately decapitated.

Samples were collected from the GIT sections crop (Cr), gizzard (G), duodenum (D), ileum (I) and caeca (Cae). The GIT sections were longitudinally opened, and the digesta was collected with a sterile spoon and stored in RNA later at −80°C until further analysis. RNA was extracted using Trizol (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions with a preliminary step of bead beating (30s, 5.5 m/s) in a FastPrep instrument (MP Biomedicals, Santa Ana, CA, USA). Extracted RNA per sample was treated with the DNase kit (Invitrogen), and cDNA synthesis using SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen). Library preparation was performed according to the Illumina protocol described by Roth et al. [25]. Briefly, the V1–2 regions of the 16S rRNA gene was amplified in a three-step PCR using PrimeSTAR® HS DNA Polymerase kit (TaKaRa, Beijing, China). The first two PCRs were prepared in a total volume of 25 µL using 1 µL of DNA template, 0.2 µM of primer, and 0.5 U Taq prime start HS DNA, and the third PCR was prepared in a total volume of 50 µL. An initial denaturation at 95°C for 3 min was followed by 10 cycles (pre and first PCR) or 20 cycles (third PCR) of denaturation at 98°C for 10 s, annealing at 55°C for 10 s, and an extension at 72°C for 45 s, and then a final extension of 72°C for 2 min. Libraries were pooled by index, standardized and purified using SequalPrep Normalization Kit (Invitrogen Inc., Carlsbad, CA, USA), and sequenced using 250 bp paired-end sequencing chemistry on an Illumina Novaseq 6000 platform. Due to the production stage effect on the microbiota of birds during weeks 16 and 24, DNA for shotgun metagenomics was extracted from crop, ileum and caeca samples, using FastDNA™ SPIN Kit for Soil (MP Biomedicals, Santa Ana, CA, USA, Base catalog number: 6560200) according to the manufacturer's instructions. Samples were sequenced using 150 bp paired-end sequencing on a NovaSeq6000.

4.3.3 Bioinformatics and Statistical Analysis

The bioinformatic analysis was performed with the tool Mothur v1.44.3 [26]. Raw reads (forward and reverse fastq file) were assembled with the function make.contigs. Reads with ambiguous bases, with homopolymers (> 8) and longer than 355 bp were removed. Sequences were aligned to the silva.seed v1.38.1 reference database (https://www.arb-silva.de/) [27], chimeras were identified using vsearch [28] and removed. Sequences were classified by using the silva reference and taxonomy set silva.seed v1.38.1 in combination with the Bayesian classifier. The amplicon sequencing variants (ASVs) of the output were filtered to fulfil a minimum of 50 reads across all samples resulting in a total of 6.795 ASVs. An average of 27.201±10.844 reads were obtained per sample, and out of 500 samples, nine were deleted due to low number of reads (< 5.000). The cut-off for bacterial taxonomy classification followed the recommendations of Yarza et al. (2014) [29]. Sample reads were standardized, and a sample-similarity matrix based on the Bray-Curtis similarity coefficient [30] was created using Primer6 [31]. PERMANOVA routine was used to study the significant differences and interactions between breeds, GIT sections and production stages [31]. For the visual hierarchical clustering and ordination of the community structures, a two-dimensional principal coordinate analysis (PCoA) was created, whereby the centroids representing the average plotting position of each breed and each section were ordinated. The differences in the microbial community structure between the different groups were identified using analysis of similarities (ANOSIM), pair-wise comparison tests [32] and the non-parametric comparison, using JMP®Pro (Version 16.1 SAS Institute Inc., Cary, NC, 1989–2021) by Dunn's All-Pairs Rank Comparison Test [33]. Groups of samples were considered significantly different if the p-value was lower than 0.05. The similarity percentage analysis (SIMPER) was used to calculate the similarity between and within the group combinations and to identify the ASVs contributing to the observed dissimilarities [32]. P-values of ANOSIM analysis were adjusted with the Benjamin-Hochberg method (FDR) [34]. The Shannon diversity index and richness were calculated using the phyloseq library in R v4.1.

Shotgun metagenomic raw reads were quality controlled, and the host genome (Chicken reference database GRCg6a (GCA_000002315.5)) was removed by the fully automated metagenomic pipeline SqueezeMeta v1.5.2 [35]. In total, 105 out of 120 samples remained with a host level below 80%. The assembly was done using Megahit [36], short contigs (< 200 bps) were removed, and contig statistics were done using

prinseq [37]. RNAs were predicted using Barrnap [38], 16S rRNA sequences were taxonomically classified using the RDP classifier [39] and the tRNA/tmRNA sequences were predicted using Aragorn [40]. ORFs were predicted using Prodigal [41], similarity searches for GenBank [42], for KEGG [43] were done using Diamond [44] and HMM homology searches were done by HMMER3 [45]. Read mapping against contigs was performed using Bowtie2 [46]. Kyoto Encyclopedia of Genes and Genomes (KEEG) Orthology (KO) databases were used to identify the functional categories at level 1 and 3. Further, the KO reads were filtered (keep all > 10) and the unknown KOs were removed. The results were finally visualized after the differential gene expression analysis via DESeq2 [47] in R v4.2.1.

4.4 Results

4.4.1 Experiment evaluation

A total of 6.795 ASVs were detected in the active microbiota across 491 samples and 88% of the ASVs were assigned to Firmicutes, 6% to Bacteroidetes, 2% to Proteobacteria, 1% to Actinobacteria, 1% to unclassified (uncl.) Bacteria and the rest are phyla with abundances lower than 1% (e.g. Deferribacteres 0.7%, Campilobacterota 0.35%). Further, the genera *Ligilactobacillus* (37%), *Lactobacillus* (18.8%), uncl. Lachnospiraceae (17.5%) and *Blautia* (2.2%) account for more than 75% of the total relative abundance of 167 genera across all samples.

PERMANOVA routine identified significant differences for the factors breed, production stage and GIT section ($p < 0.02$) as well as the interactions of breed x production stage, breed x GIT section, production stage x GIT section, and the triple interaction breed x GIT section x production stage ($p < 0.05$) (suppl. Table, S4.1). The factors production stage and breed are also significant within each GIT section ($p < 0.03$). The principal coordinate analysis (PCoA) shows different clusters depending on the GIT section and production stage for the centroids, respectively each unique sample (Figure 4.1, Suppl. fig. 4.1). Especially in the caeca, week 10 clusters apart from the other production stages, and the GIT microbiota composition varies the most towards week 16 and 24. Shannon diversity index showed statistical significance between caeca and all other GIT sections in both breeds ($p < 0.05$) (Suppl. fig. 4.2), and the overall average Shannon index is highest in caeca, followed by crop, ileum, gizzard and duodenum.

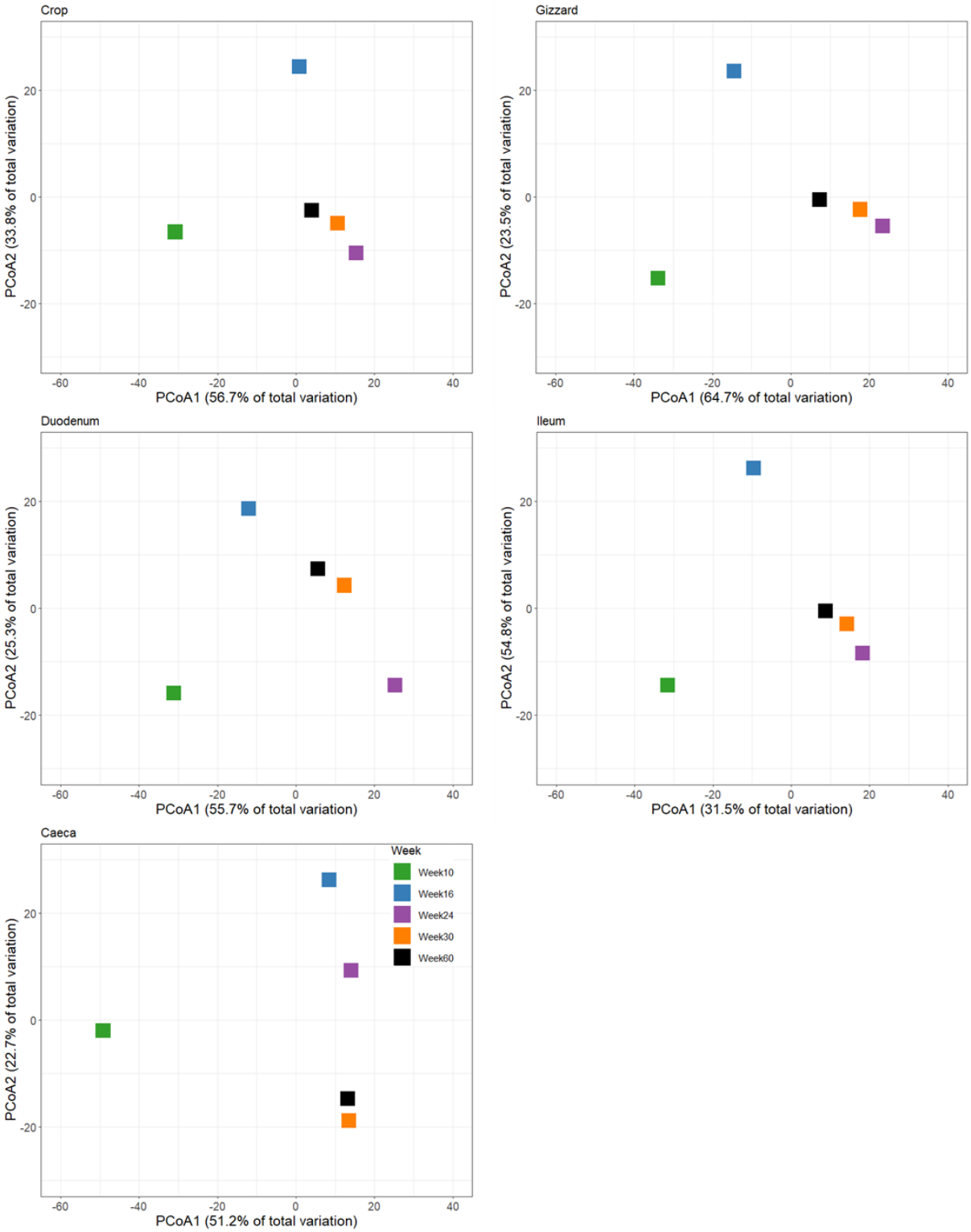


Figure 4.1: Centroids PcoA separated by section and production stage (week10-60) of sampling.

Regarding the production stage, the Shannon index differed depending on the GIT section x breed

combination (Figure 4.2). Significant differences between the production stages of the corresponding breed and GIT section combination were observed (suppl. Table, S4.2).

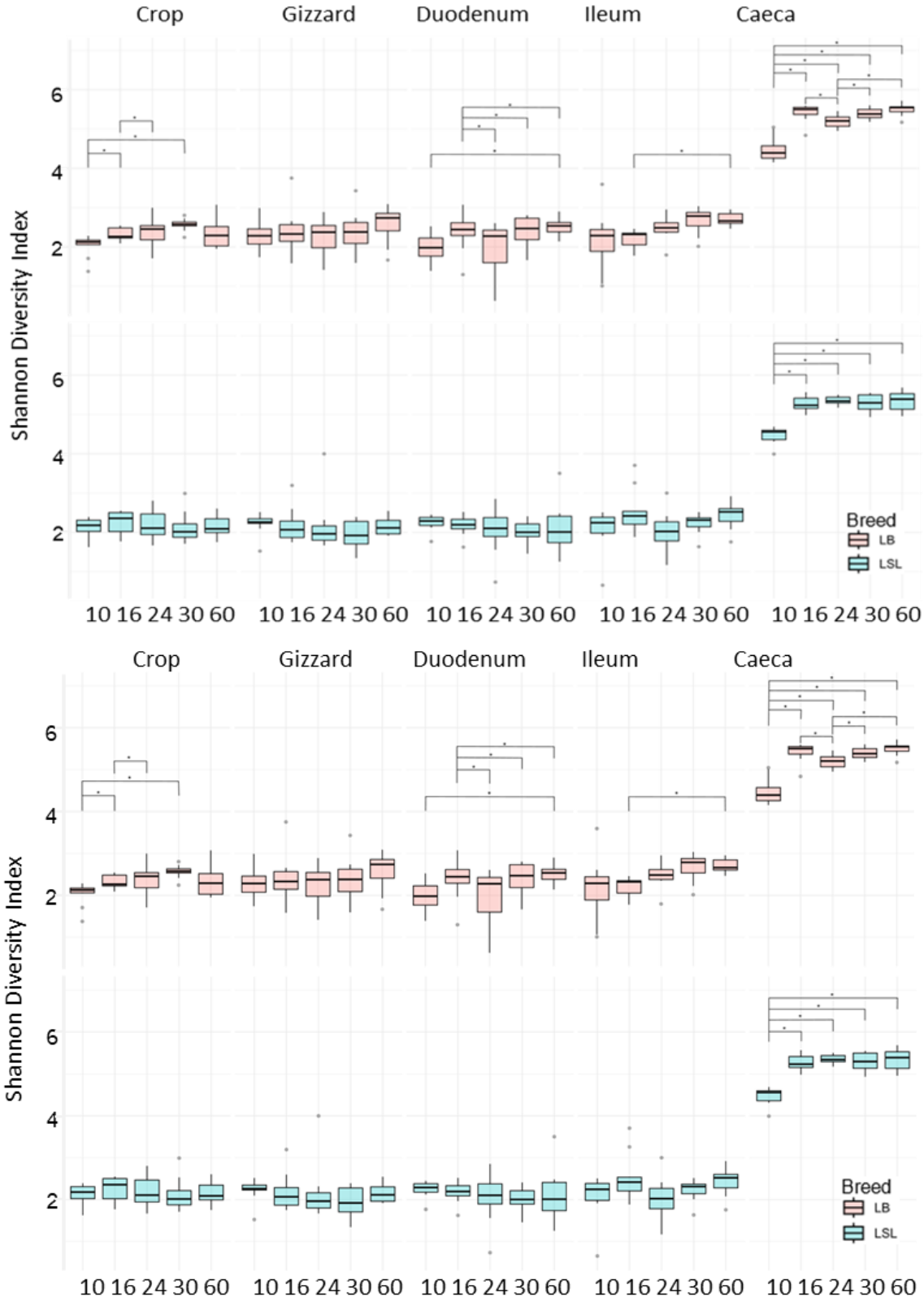


Figure 4.2: Boxplot of Shannon diversity index separated by breed (color), GIT section and production stage (* = p.adj. >0.05).

4.4.2 Genera comparison between production stages, breeds and GIT sections

The five investigated production stages across the laying hens' lifespan show differences in the average relative abundance [av.abu.] in the most abundant genera across all GIT sections and breeds (Figure 4.3), which account for more than 93% of the total relative abundance. *Ligilactobacillus* (LSL 44%; LB 30%) was the most abundant genus, followed by *Lactobacillus* (LSL 16%; LB 21%) and uncl. Lachnospiraceae (LSL 16%; LB 20%).

Ligilactobacillus was on average more present in the crop (LSL 62%; LB 46%), and the abundance decreased towards the caeca (LSL 15%; LB 14%). Regarding the production stages, *Ligilactobacillus* was less abundant in week 10 (LSL 32%; LB 18%), with an increase towards week 16 (LSL 41%; LB 30%) and week 24 (LSL 47%; LB 35%) and stabilized on the further weeks (LSL 50-51%; LB: 32-36%).

In contrast, *Lactobacillus* was in higher abundance in LB than LSL, except in the caeca. The highest levels were found in week 16 (LSL 30%; LB 32%) except for LSL caeca (week 60: 28%) and LB duodenum (week 24: 45%). The overall average abundance of *Lactobacillus* increased from week 10 (LSL 13%; LB 23%) to week 16 (LSL 30%; LB 32%), decreased in week 24 (LSL 15%; LB 16%) and week 30 (LSL 9%; LB 16%) and increased in LSL in week 60 to 15%, while the relative abundance was constant in LB (16%).

Uncl. Lachnospiraceae was less abundant in week 16 (LSL 5%; LB 8%) in all GIT sections except in LSL caeca (week 10: 4%). In total, the highest abundance of uncl. Lachnospiraceae was detected in the crop of 10 weeks old LB (46%). The average abundance increased from the lowest level in week 16 towards week 24 (LSL 14%; LB 19%). This change strongly depended on the GIT section and persisted in the caeca within the productive stages (LSL 5%; LB 6%).

Blautia, the fourth most abundant genus, was less abundant in week 10, where it was almost undetectable (LSL 0.06%; LB 0.04%) followed by week 16 (LSL & LB: 0.6%). The highest levels were found in week 24 for LSL (LSL 5%; LB 4%) and in week 30 for LB (LB 7%; LSL 2%). The highest production stage levels of *Blautia* per breed were observed in the ileum in week 24 (LSL 13%; LB 7%) and gizzard in week 30 (LSL: 4%; LB 18%). Significant abundance differences of the top genera between breed, GIT section and production stages are shown in the supplementary table S4.3.

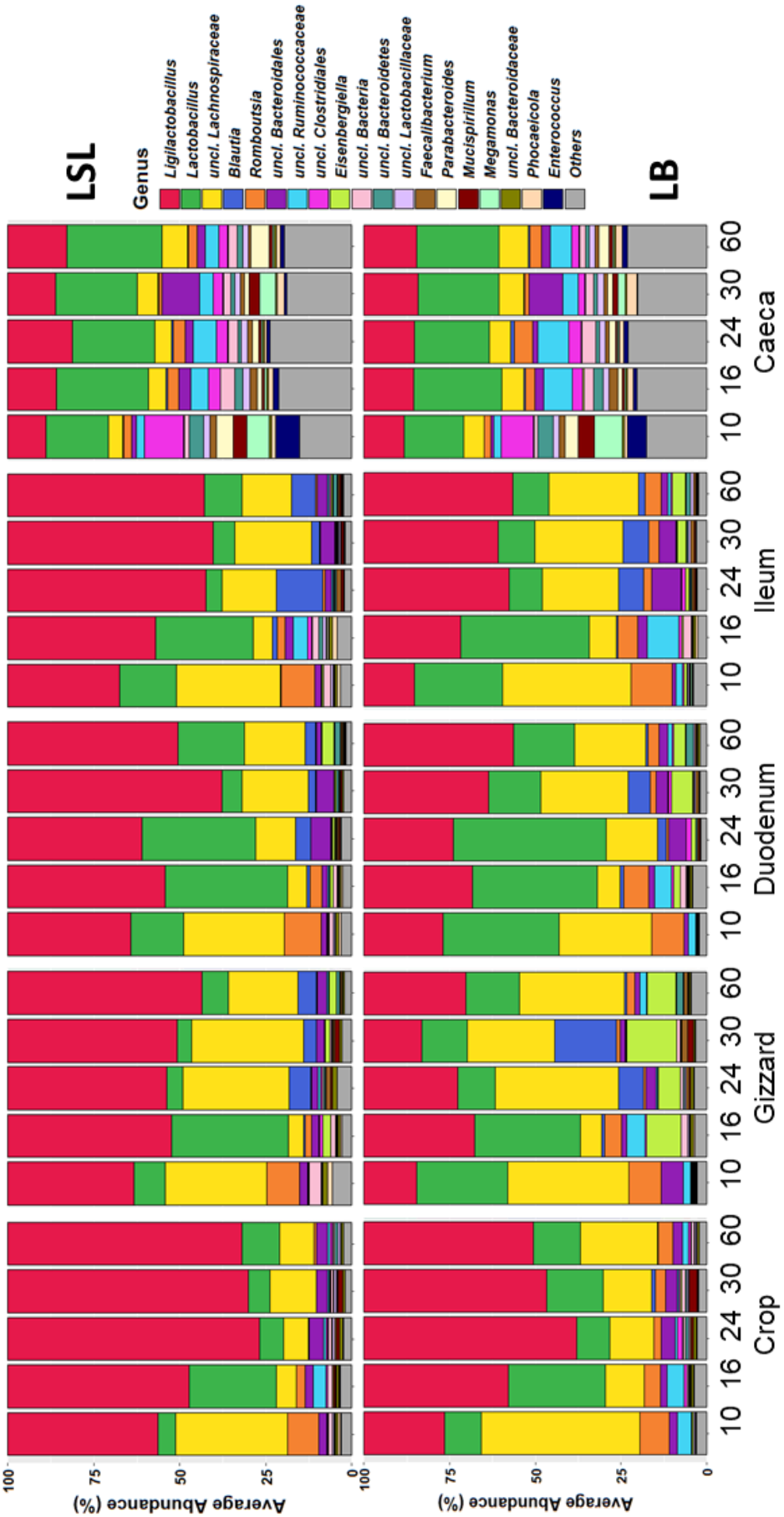


Figure 4.3: Top genera across the breeds separated by GIT section and production stages 10, 16, 24, 30 & 60.

3.3 From onset of egg production to peak egg production

Previous reports in this animal experiment [23,48–53] described distinctive shifts with the onset of the laying phase between week 16 and 24 as a major transition phase for the laying hens' organism with the start of sexual maternity, morphological changes in regards to the oviduct and the resulting egg production onset up to nearly the maximum of the egg production. For this reason, a particular focus will be further given to these two production stages.

The ANOSIM analysis reveals significant effects of production stage and breed in all GIT sections except for crop LB vs LSL (suppl. Table, S4.4). The week 24 clusters apart from week 16, and variations between each breed within each GIT section can be observed (Figure 4.4). The top fifteen ASVs (av.abu. > 1%) account for 65% (week

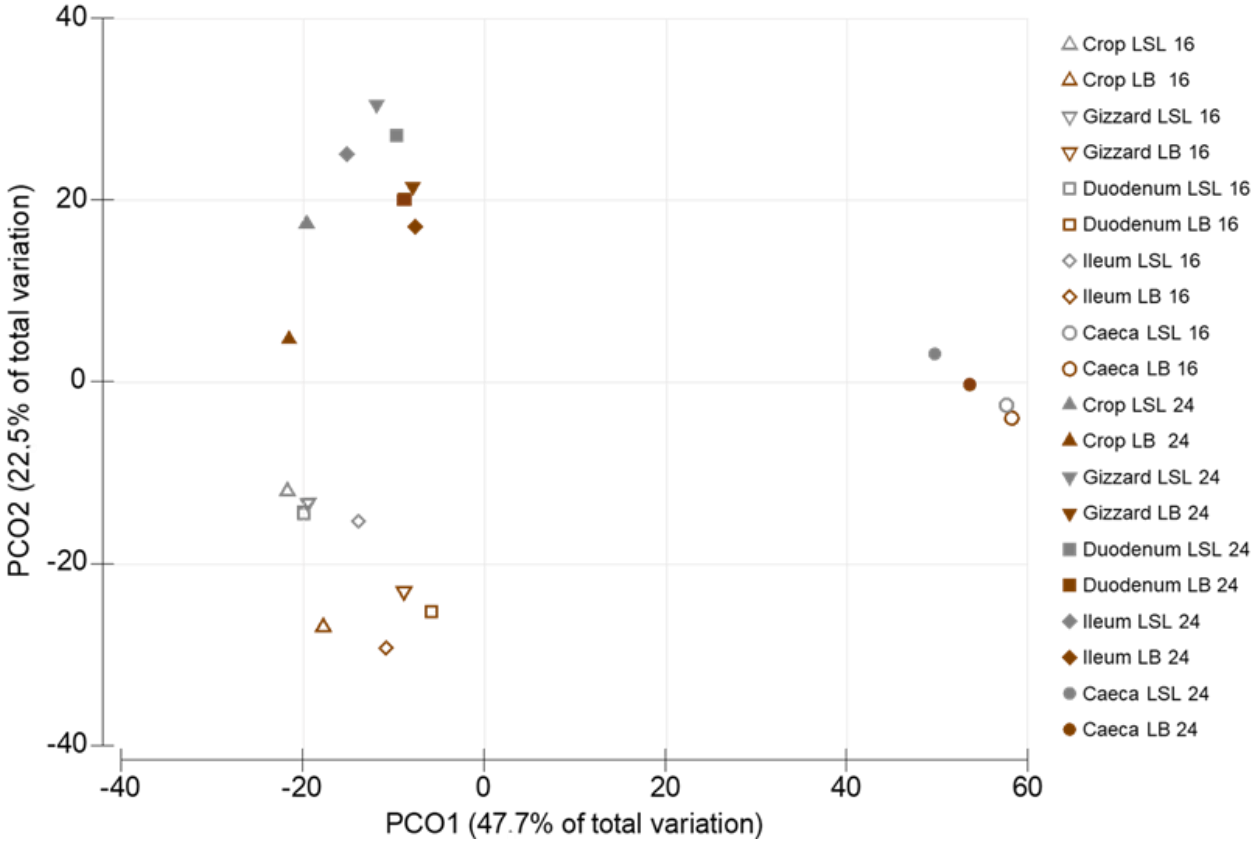


Figure 4.4: PCO plot of the Centroids depicted by GIT section, breed and week combination of the production stages week 16 and 24.

16) to 67% (week 24) of the active intestinal bacteria (suppl. fig.4.3 a, b). Further bacterial abundance differences between the production stages are described in suppl. table S4.5. Four ASVs showed a statistically significant production stage effect ($p < 0.05$) besides significant differences between GIT sections within breeds (uncl. Bacteroidaceae, uncl. Lachnospiraceae, *Clostridium sp*. and *Lactobacillus*

delbrueckii). Uncl. Bacteroidaceae significantly differed between week 16 and week 24 in ileum samples for both breeds and uncl. Lachnospiraceae in the gizzard of LSL. *Clostridium sp.* showed a significant production stage effect in crop and duodenum of LSL, whereas *Lactobacillus delbrueckii,* revealed significant differences in the duodenum of both breeds and ileum of LSL. Other ASVs belonging to the top fifteen were taxonomically assigned to *Lactobacillus gallinarum, Ligilactobacillus aviaries*, uncl. Oscillospiraceae, *Ligilactobacillus salivarius, Lactobacillus kitasatonis* and uncl. Eisenbergiella. This visualization and analysis on ASV-level support the previously shown significant differences in breed, GIT section and production stages and highlight the importance of the onset of the laying phase in laying hens.

A total of 105 samples of the crop, ileum and caeca of the two laying hen breeds were further analyzed by shotgun metagenomics. The taxonomical assignation of the metagenomic data using DNA differs in relative abundance levels from the 16s rRNA active microbiota output. Overall, 3.513 features were identified after filtering, with an additionally 7% unmapped and 10% completely unclassified remaining in the metagenomic data, which were removed from the ongoing analysis. The overall most abundant genera were *Lactobacillus* (av.abu 29%), *Limosilactobacillus* (av.abu 8%) and *Ligilactobacillus* (av.abu 7%), followed by *Mediterraneibacter* (av.abu 2%), *Faecalibacterium* (av.abu 2%), uncl. Bacteroidetes (av.abu. 1.3%) and *Blautia* (av.abu 1%) (suppl. fig. 4.4a). Even though the production stage had no significant effect on the relative abundance by analyzing the metagenomic taxonomy data, GIT section effects could be observed within the breeds (Suppl. table, S4.6). Further a similar clustering of the GIT sections and differences between the two production stages as in the 16s rRNA data was observed (suppl. fig. 4.4b). However, the active microbiota shows depending on the genus a greater variety in the microbial composition between breeds and production periods. In addition, the Shannon diversity index was significantly higher in the active microbiota (3.3 vs. 2.5 ($p < 0.05$)). Nevertheless, the most significant shift observed was in LSL of week 16 to week 24.

The metagenomic data was further subjected to the KEGG Orthology (KO) database and assigned to 11.489 KOs. The changes in KOs were more affected by the production stage than the breed. Nevertheless, a trend between the two breeds were observed in the crop and the ileum while in the caeca, the microbiota composition difference was significant in the PERMANOVA analysis ($p < 0.05$). Furthermore, the

production stage was affecting the microbiota composition significantly for both breeds and all GIT sections ($p < 0.05$).

The MA-plots picture the log2 fold changes attributed to the production stage variable, respectively, breed over the normalized counts for all samples (Figure 4.5 & 4.6). The

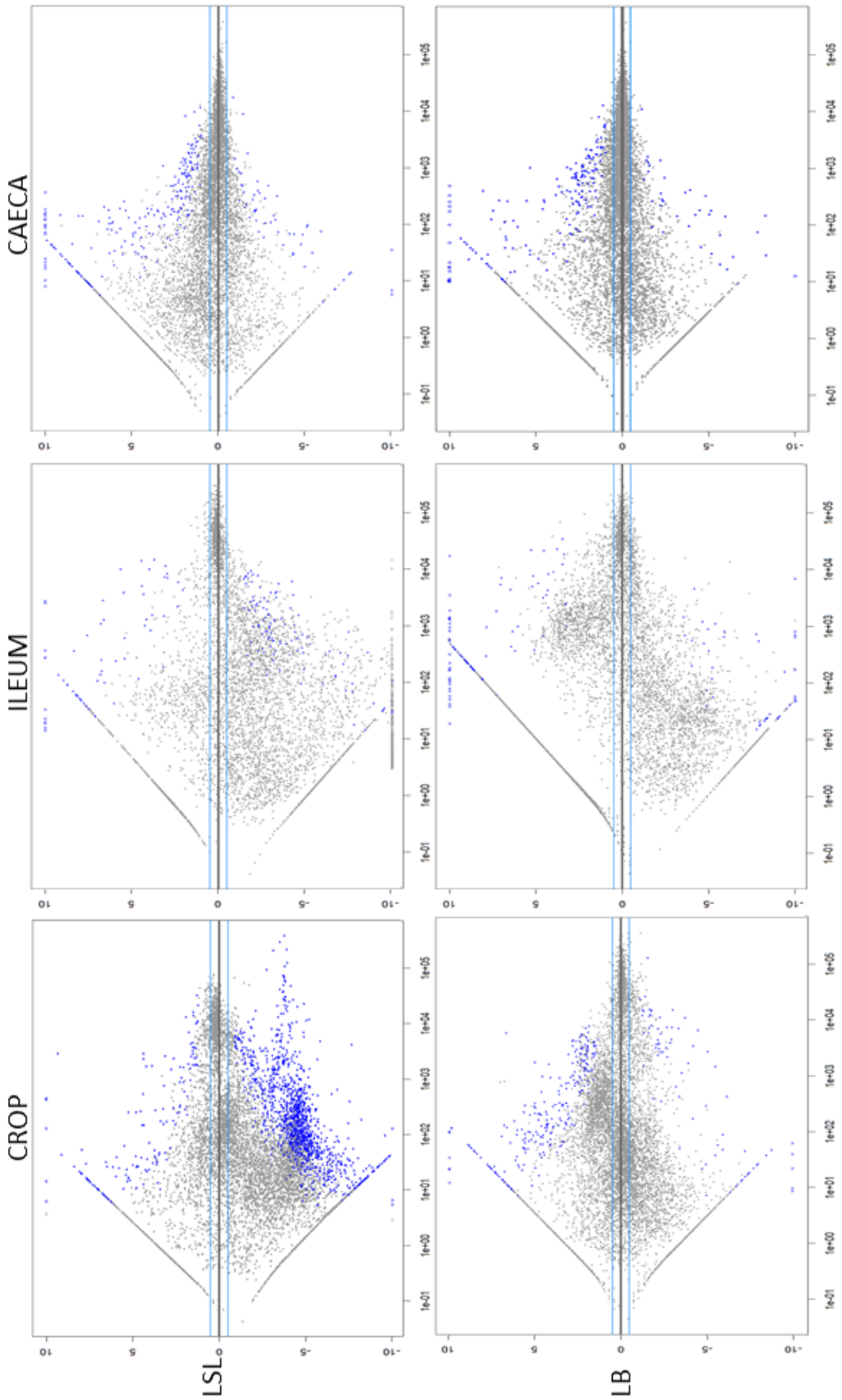


Figure 4.5: MA-plot of the significant by the production stage (week 24 vs 16) affected functions depicted by breed – GIT section combination (blue = p adj. < 0.05).

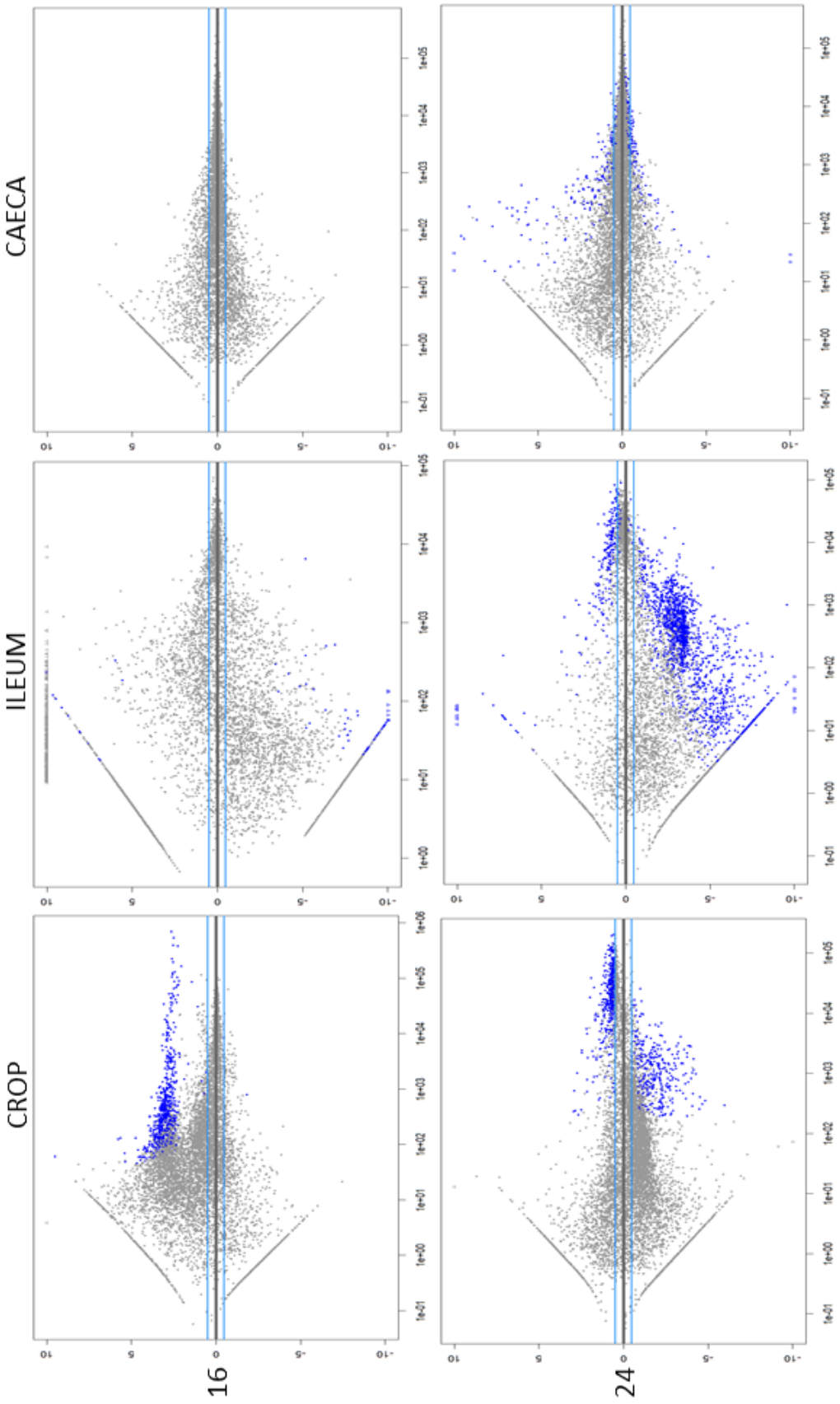


Figure 4.6: MA-plot of the significant by the breed (LSL vs LB) affected functions depicted by breed – production stage combination (blue = p adj. < 0.05).

noise associated with the log2 fold change from low-count genes was removed. The colored dots represent adjusted p-values lower than 0.05, and dots falling out of the window are plotted as open triangles up or down. Further, the log2 fold change indicates the up- or down-regulation of a specific function or pathway. Overall, an average downregulation of functions was observed for the production stage shift (Suppl table S4.7). Except for week 16 caeca, a breed effect for significantly different KOs in all GIT sections at both production stages was observed. The top down- and up-regulated significant KOs ($p < 0.05$) were selected based on the log2 fold change (> 2) in combination with the lfc shrinkage (> 0.5) to take also the functions with lower reads into account (Suppl. Fig. 4.5 & 4.6). The analysis showed that functional pathways change with the transition to the laying phase, and the organism may adapt to this shift. These differences were mainly functions and pathways which were up-regulated in week 16 and down-regulated in week 24 or vice-versa and related to protein, carbohydrate, co-factors and vitamins, lipid metabolism, digestive system and amino acid metabolism. Regarding the comparison between breeds at the same production stage, the functions and pathways variations are mainly on genetic information processing, signal transduction, membrane transport and metabolisms, indicating a high activity of the central energy metabolism. This shows an influence of the production stage and breed in the metabolic pathways of the laying hens. Further information about specific up- and down-regulated functions is described in the supplementary table 4.6.

Regarding the P assimilation, an important inositol-phosphate pathway was part of the top 25 up- and downregulated pathways by comparing the breed at each production stage and within each GIT section. This pathway was represented by an up-regulated K13024 (inositol-hexakisphosphate/diphosphoinositol-pentakisphosphate 1-kinase [EC:2.7.4.24]) in the crop of LSL compared to LB in week 16 and up-regulated K22231 – K22233 (3-dehydro-scyllo-inosose hydrolase [EC:3.7.1.-], scyllo-inosose 3-dehydrogenase [EC:1.1.1.-], 5-keto-L-gluconate epimerase [EC:5.1.-.-]) and K17237 (inositol-phosphate transport system substrate-binding protein) in LB caeca compared to LSL in week 24.

Nevertheless, the laying phase onset affects significantly the regulation in functions and pathways in laying hens with differences between breeds.

4.5 Discussion

4.5.1 Microbial differences between production stages with a focus on week 16 and 24

Longitudinal studies of chicken GIT microbiota revealed changes in microbial composition and diversity [54,55]. However, this knowledge is scarce in laying hens', especially along the entire GIT in laying hens. Therefore, understanding the intestinal microbiota composition throughout the laying hen's productive lifespan is essential.

In this investigation, the laying hens were kept under the same living conditions to minimize the effect of external factors. In the first productive stages, the growth of the animals decreases from a weekly increase of 50 - 80% per week until the growth rate terminates after the 24th week [18,23]. This is in line with our results with higher levels of body weight gain in LB. Such a rapid growth demands the efficient functioning of the whole GIT in terms of increased nutrient uptake and the development of the intestine. This energy demand is covered by the substrate butyrate, which is mainly produced in the animals' large intestine with positive effects on gut health, growth performance, gut development and control of pathogens [56]. Butyrate producers often belong to *Faecalibacterium, Roseburia* or *Eubacterium* [57–59]. Further members of the genus *Proteobacteria*, *Escherichia* and *Blautia* were recently investigated in a lifetime experiment from weeks 1 to 60 in the caeca of laying hens [18]. Even though we could only detect *Faecalibacterium* and *Blautia*, it can be assumed that *Roseburia* is included in the uncl. Lachnospiraceae group and *Eubacterium* in uncl. Clostridiales. Such lack of assignation might be due to the use of a different 16S region (V1-V2 vs. V3-V4), the OTU discrimination on 97% compared to the ASV at 100% or the used taxonomic database. All of these differences are known for effects on the microbial composition [60–62].

However, the cumulated abundance of these present genus is highest on week 10 of both breeds, except for gizzard and reduce by the aging progress in combination with the ongoing reduction in animal growth. This is in line with Videnska et al. 2014 [18]. Further, age-depending variations were investigated by the dominance of Bacteroidetes in mature chickens compared to the dominance of Firmicutes within the early life [63], which is in line with our study. Other studies performed in the same animal experiment in regards to the animal nutrition, physiology, functional anatomy, livestock population genomics and functional genome analysis showed an age effect as the distinctive shift observed in the microbial composition [23,48–53].

Besides the storage function, the crop is essential for starch digestion, the breakdown of sugar to lactic and other acids [64] with an actual pH level between 4.1 - 6.2 [65], while lactic acids cause a lower pH [66]. Due to this, higher levels of e.g. *Lactobacillus* spp. will increase the lactic acid production and cause a lower pH. In general, the bacterial colonization starts immediately after hatch, especially the encore of Lactobacillus strains, prebiotics and organic acids improves colonization (62), ensuring gut health and a balanced crop microbiota [67]. The dominance from week 24 on (> 60%) and the significant shifts of *Lactobacillus* and *Ligilactobacillus* by comparing week 16 and 24 support these statements. In addition, the crop proved to be the GIT section with the highest possible probiotic intake, supporting proliferation of the commensal bacteria *Lactobacillus* spp. and improving animal health through further proliferation of the butyrate-producing *Clostridium* spp. [68,69]. This could not be observed in this trial, where an increase in *Lactobacillus* had no direct effect on *Clostridium*. The dominance of Lachnospiraceae members in the crop in week 10 shifts towards a higher abundance of *Lactobacillus* and *Ligilactobacillus* after week 16 and stabilizes in the following weeks. These results support recent studies showing that the birds crop is dominated by Lactobacilli [25,70,71].

Starch digestion continues in the gizzard where the food components are crushed under a low pH (69) ranging commonly between 4 and 5 in layers due to high calcium carbonate content in the diet [72–75]. Although a pH around 3.5 has also been reported for laying hens [76]. The gizzard's bacterial composition consists of mainly L*actobacillus*, Clostridiaceae, *Enterococci*, small amounts of lactose-negative *Enterobacteria* and coliforms [77–79]. Especially the acidic milieu preserves bacteria like *Lactobacillus*. We found a dominance of *Lactobacillus* and *Ligilactobacillus* within the first two productive stages, and high levels of uncl. Lachnospiraceae in week 10, in contrast to the levels in the crop. On the other hand, an increase in *Lactobacillus* in the gizzard might be influenced by the abundance in the crop due to the reflux of the digesta [80]. Besides the Lactobacilli, and the Clostridiaceae, to which the in this study found *Blautia* belongs, none of the detected bacteria had higher abundance in our study. However, gastro juices pepsin and hydrochloric acid can inhibit fermentation activity which occurs with lower bacterial amounts [77] and certain Lachnospiraceae and *Blautia* can produce acetate while growing on carbohydrates [81]. This might explain the variation in recent publications. Additionally, acetate positively correlated with *Lactobacillus [82]*, which might be beneficial aspects to intestinal structure and

health. Further higher levels of *Lactobacillus* were reported to increase egg weight and egg size and to decrease cholesterol levels in egg yolk [83]. In contrast to the varying abundance levels, no breed effect on the egg weight was observed [23].

In the duodenum, the first part of the small intestine, hydrolytic acid is released by receiving digestive enzymes and bicarbonate from the pancreas and liver. [77]. So far, *Lactobacillus*, Clostridia and *Enterococcus* are known colonizers from day 3 of life [84,85]. Especially the widespread Lactobacilli colonize the small intestine relatively fast within the first week of life [86]. This can be partly proved by the domination of *Lactobacillus* and *Ligilactobacillus* during all production stages with an average level of 53%-76% and uncl. Lachnospiraceae (Clostridia) (6-29%). The genus *Romboutsia* increased significantly from week 10 to week 24, 30 and 60 for LSL, respectively week 24 for LB, which correlates negatively with the feed efficiency in hens [87]. However, it was reported that the increase in *Lactobacillus* causes a decrease in *Romboutsia* [88] which might increase the feed efficiency again. It can be expected, the reduction of *Romboutsia* and the competitive exclusion through *Lactobacillus* is due to a lower pH induced by *Lactobacillus*. Due to the appearance of *Blautia* in the preliminary GIT section, it can be postulated, that *Blautia* in the duodenum is present to irrigate free hydrogens of fermenting anaerobes [89]. *Eisenbergiella* was one of the core bacteria in chicken [90] and showed negative correlations with e.g. pyruvate metabolism [91]. This is not in line with the present study as myo-inositol, and InsP6 levels (both influenced by pyruvate) are higher in weeks 30 and 60 [23], and the abundance of *Eisenbergiella* was highest in week 30 and less abundant bevor which rejects the genus considering a core bacteria. The duodenum is the major GIT section regarding Ca and P uptake [92]. While both nutrients are needed for eggshell formation, no additional effect with the laying onset was observed, and the duodenal microbiota might be less affected by this transition.

The ileum is the site for nutrient absorption, and the overall composition affects digestion and nutrient uptake [93]. The ileal digesta is dominated by *Lactobacillus* (up to 70%), followed by Clostridiaceae*, Streptococcus*, and *Enterococcus [94]*. We could prove the high abundance of *Lactobacilli* with a 44-70% range. The other genera were less abundant or not detected. The uncl. Clostridiales abundance increased towards week 24 and stabilized. Bacteria within this group might belong to Clostridiaceae and, as previously reported, detected in higher concentrations [94,95]. For *Lactobacillus* in LSL, an effect was observed for the production stages, supporting the Lactobacillaceae

dominance with the egg-laying onset [86]. In our study, genera of the family Lactobacillaceae dominated the ileum before the laying phase onset. The bacterial abundance shifts might be due to changes in butyrate producers, like in the proximal GIT sections, which support immunity and inhibit pathogen attachment [96].

The caeca is strictly anerobic and often used for microbial research due to the higher bacterial diversity and the link with the immune system and metabolism improvement [97]. Recently the caeca was dominated by Lactobacillus and Ligilactobacillus being the most abundant genera [25]. The dominance of these genera is in line with our study, on the other hand, the assumption of a stabile microbial composition could be rejected, as the composition is still changing from week 24 towards week 60. However, the caeca can be considered a more stable GIT section compared to the others sections but still underlies changes along the productive stages. The indicated changes after day 40 in the hens' life showed that a stabilized microbiome composition needs longer to stabilize, which is not following a previous study [98]. Furthermore, the absence of chickens' parents and higher zoohygienic standards induce a slower caeca microbiota establishment [70]. However, bacteria of Lachnospiraceae dominated in later development stages [86], which is in line with the dominance of uncl. Clostridiales in combination with significant shifts between the production stages. In the caeca, essential amino acids are produced, and non-starch polysaccharides are digested. It has the highest diversity index, supported by its complexity in metabolism and functionality compared to other GIT sections [89,97].

Overall, the beginning of the laying period affects the microbial composition. Therefore, each GIT section has its own bacterial composition through development along the life span and the microbial composition stabilizes at a different stage of production.

4.5.2 Shotgun-Metagenomic results between week 16 and 24

Previous reports in this animal experiment [23,48–53] described distinct shifts from week 16 to 24, therefore, shotgun metagenomics was utilized to evaluate the effects of age on bacterial functions and pathways in the crop, ileum and caeca. To our knowledge, no such comprehensive analysis was performed before on laying hen breeds.

The end of the growing phase caused significant shifts in protein, carbohydrate, cofactors, vitamins, and lipid metabolism. It was confirmed that differences in body weight, feed intake and feed utilization are based on energy metabolites and metabolic pathways changes and additionally the immune system has to adapt with the onset of

the laying period [23,50–52]. The laying hen organism is still focused on growth in week 16. With the transition to the peak egg-laying stage, growth is not persisting as the most important metabolism aim, leading to breed differences in the digestive system, amino acid metabolism, genetic information processing, signal transduction, membrane transport and metabolism.

The inositol-related functions are of main importance due to the Ca and P related assimilation pathways. Lower levels of MI concentrations were found in week 24 [23] which is in line to the downregulation of the inositol phosphate metabolism: 5-keto-L-gluconate epimerase (iolO) (K22233) from week 16 to 24 (part of the fourth step of the MI degradation pathway [99]). Breed depending differences have been found for the functions K22231-22233 and the inositol-phosphate transport system substrate-binding protein (inoE) (K17237) (MI-1-phosphate specific ABC transporter [99]) and the inositol-hexakisphosphate/ diphosphoinositol-pentakisphosphate 1-kinase (K13024) (InsP6 metabolizing enzyme [100]). These downregulations could not be explained by correlating to the InsP6 or MI levels [23]. Even though these inositol related pathways belong to the top group that distinguish the breeds, they are less represented between the production stages and, due to this, this pathway may not affect the laying hens' transition to the laying phase onset between the timepoints as highly as assumed and due to the span of 8 weeks between the two production periods, the major increase might not be reported in the experimental data.

By investigating the taxonomical assignation of the metagenomes, no significant shift was detected for the GIT sections on genus level between the production stages. However, we could prove, the 16S rRNA gene sequencing of the active microbiota (RNA) detects a different genera distribution in comparison to the DNA based shotgun sequencing which is in line with Durazzi et al. [101]. In contrast, the PCoA plots provide a similar clustering of the sections and production stages. Therefore, it is necessary to combine -omics techniques and standardized sequencing methods to generate a wide knowledge about the active and total laying hens' microbiota.

4.6 Conclusion

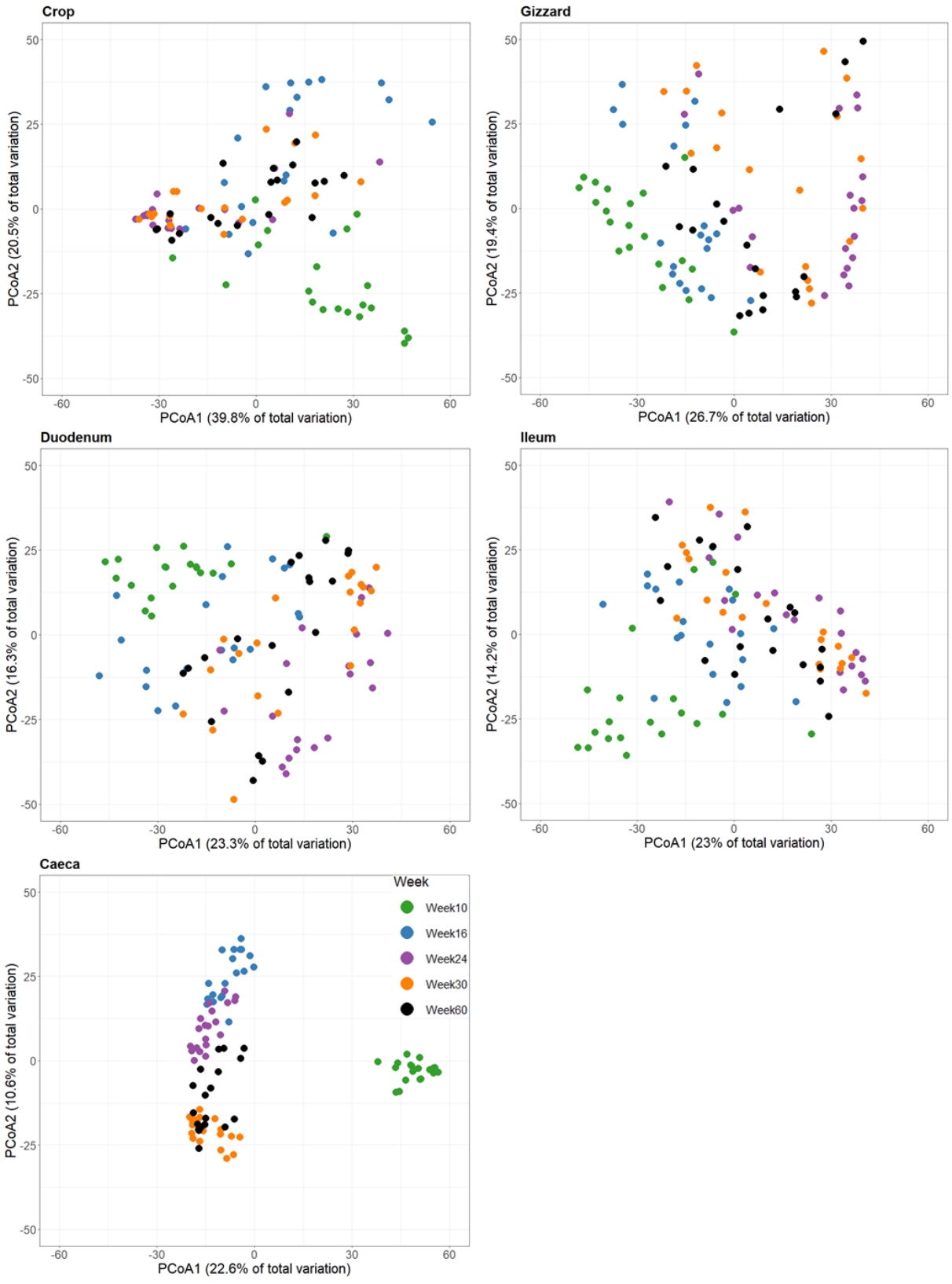
Even though the two laying hen breeds were offered the same diet and housed under similar conditions, the microbiota composition between the analyzed productive stages changes between the breed and GIT sections. The shift in the active microbiota community between weeks 16 and 24 supported the hypotheses of bacterial

fluctuations due to the starting of the laying period. However, it remains unclear if the changes in the feeding influenced the microbiota shifts or if the anatomical and physiological alterations affected the GIT microbiota. Furthermore, the shotgun metagenomic analysis revealed differences in regulating functions between the breeds and the two productive stages. Nevertheless, further studies on the intestinal microbiome functionality in connection to the host and the related differences between the observed production periods are necessary.

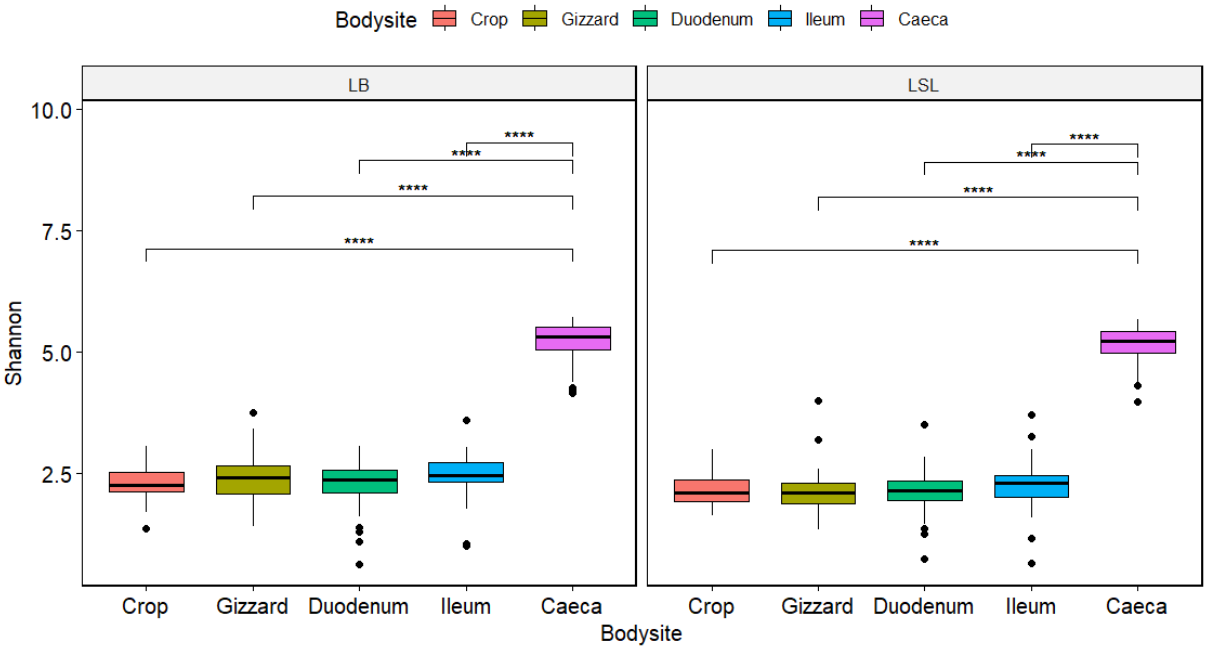
4.7 Supplementary Material

4.7.1 Supplementary Figures:

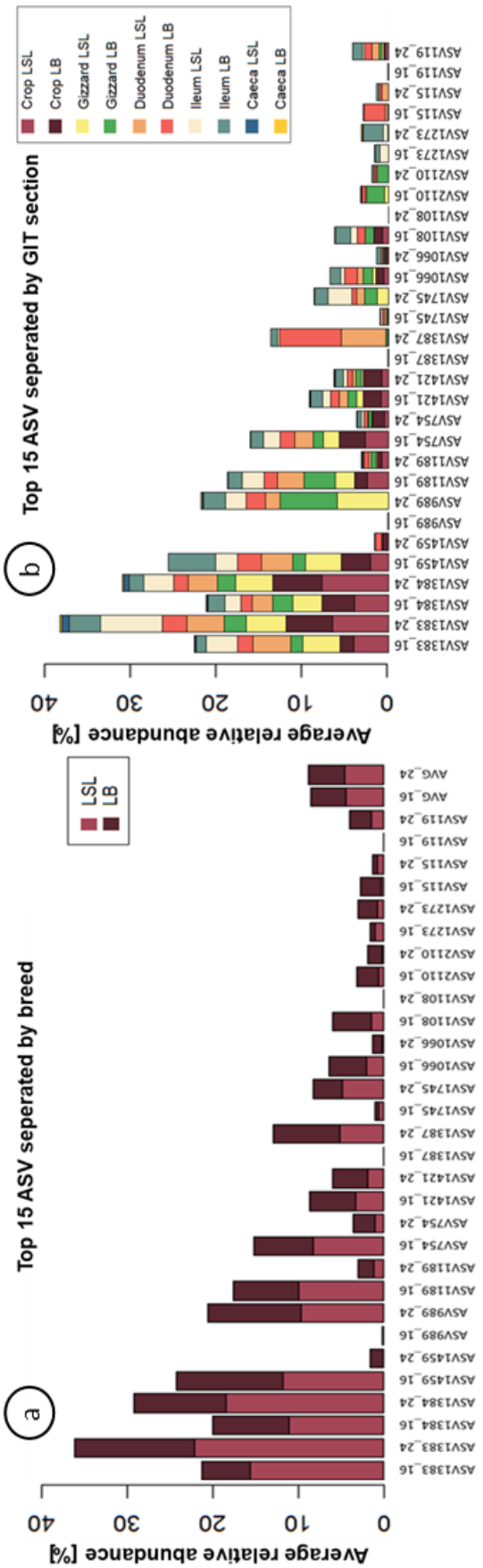
4.1 PcoA separated by section and production stage (week10-60) of sampling.



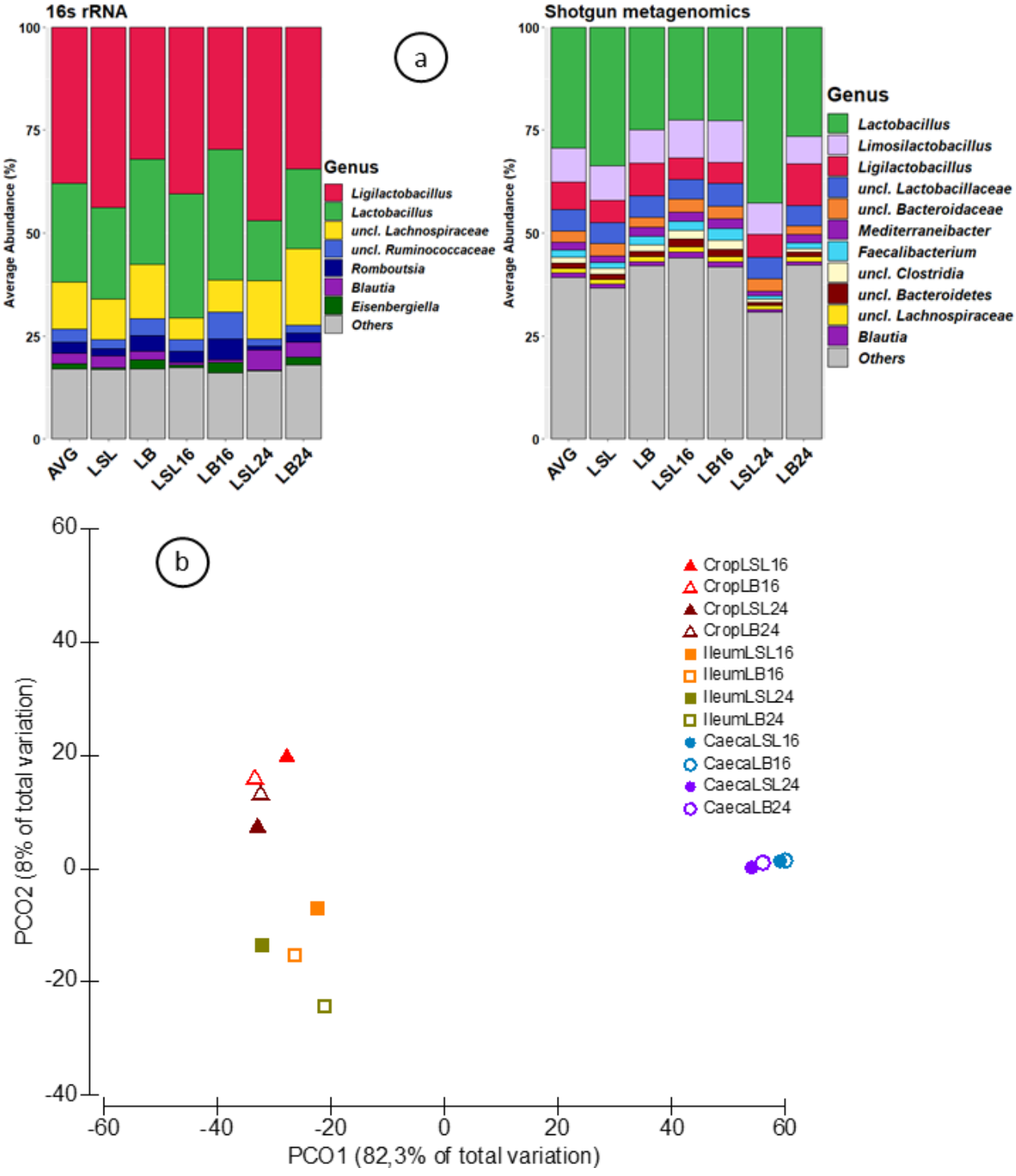
4.2 Boxplot of Shannon diversity index separated by breed and GIT section (color) (**** p = 0.001).



4.3 Barplots of the top 15 ASVs between week 16 and 24 depicted by breed (a) and GIT section x breed combination (b).

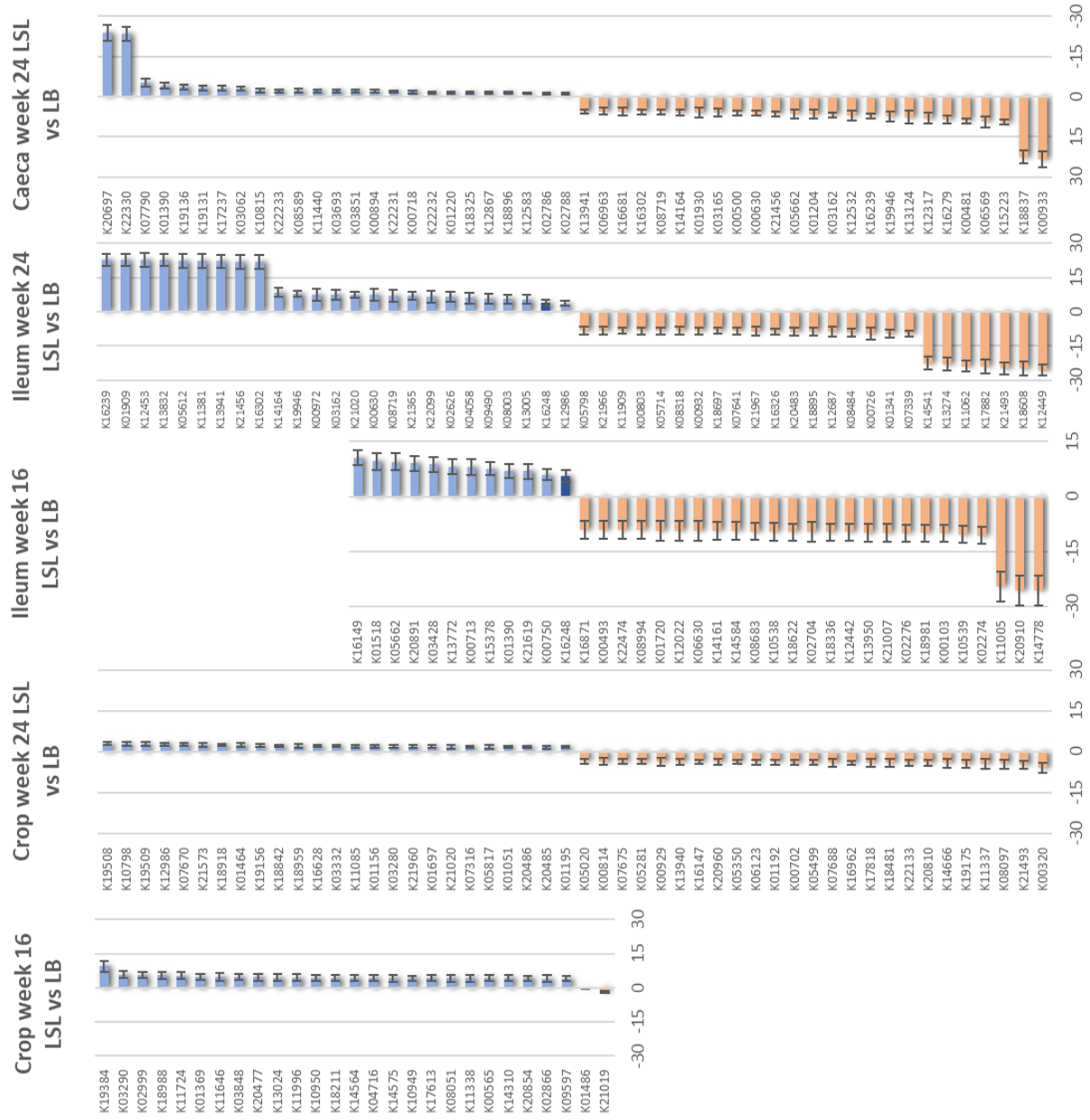


4.4 Av.abu. of the 16s rRNA and metagenomic taxonomic profile on genus level higher than 1% on average depicted by Breed and production stage (a), PcoA plot of the samples centroids separated by GIT section (crop), (ileum), (caeca), breed (LSL ; LB) and production stage (week16-24) comparison.



4.5 LefSe plots of the 25 top up and down regulated KOs. The dark blue and orange marked KOs are present in both breeds of the specific GIT section.

4.6 LefSe plots of the 25 top up and down regulated KOs. The dark blue marked KOs are present in the same GIT section for both breeds at the two production stages.



4.7.2 Supplementary Tables:

S4.1 (excel file) Global test. PERMANOVA table of results. only statistically significant results are shown.

S1. Global test

PERMANOVA table of results. only statistically significant results are shown.

Global test

PERMANOVA table of results						Unique
Source	df	SS	MS	Pseudo-F	P(perm)	perms
GIT section	4	3.94E+05	98466	67.915	0.001	998
timepoint	4	1.91E+05	47698	32.899	0.001	996
breed	1	49717	49717	34.291	0.001	997
GIT section x timepoint	16	1.72E+05	10760	7.4214	0.001	997
GIT section x breed	4	25025	6256	4.3151	0.001	999
breed x timepoint	4	25332	6333	4.368	0.001	999
GIT section x timepoint x breed	16	30200	1888	1.3019	0.02	994
Res	441	6.39E+05	1450			
Total	490	1.53E+06				

Crop

PERMANOVA table of results						Unique
Source	df	SS	MS	Pseudo-F	P(perm)	perms
breed	1	13523	13523	16.129	0.001	999
timepoint	4	45750	11437	13.641	0.001	999
breed x timepoint	4	7954.2	1989	2.3716	0.002	997
Res	88	73785	838.5			
Total	97	1.41E+05				

Gizzard

PERMANOVA table of results						Unique
Source	df	SS	MS	Pseudo-F	P(perm)	perms
breed	1	22079	22079	14.001	0.001	997
timepoint	4	68538	17135	10.865	0.001	998
breed x timepoint	4	9954.2	2489	1.578	0.03	999
Res	87	1.37E+05	1577			
Total	96	2.38E+05				

Duodenum

PERMANOVA table of results						Jnique
Source	df	SS	MS	Pseudo-F	P(perm)	perms
breed	1	12001	12001	7.3285	0.001	998
timepoint	4	68492	17123	10.457	0.001	998
breed x timepoint	4	11904	2976	1.8174	0.008	998
Res	88	1.44E+05	1638			
Total	97	2.36E+05				

Ileum

PERMANOVA table of results						Jnique
Source	df	SS	MS	Pseudo-F	P(perm)	perms
breed	1	20865	20865	12.676	0.001	996
timepoint	4	61762	15440	9.3802	0.001	997
breed x timepoint	4	10966	2742	1.6656	0.01	999
Res	89	1.47E+05	1646			
Total	98	2.40E+05				

Caeca

PERMANOVA table of results						Jnique
Source	df	SS	MS	Pseudo-F	P(perm)	perms
breed	1	6109.2	6109	3.9458	0.001	998
timepoint	4	1.18E+05	29516	19.064	0.001	998
breed x timepoint	4	14686	3672	2.3714	0.001	997
Res	89	1.38E+05	1548			
Total	98	2.77E+05				

S4.2. (excel file) Shannon diversity comparison depicted by breed, production stage, GIT section and the corresponding PERMANOVA table of results: only statistically significant results are shown.

Shannon	LSL	LB
Avg	2.764	2.929
Crop	2.171	2.319
Gizzard	2.129	2.388
Duodenum	2.109	2.264
Ileum	2.255	2.443
Caeca	5.141	5.196

LSL	10	16	24	30	60	AVG
Crop	2.141	2.264	2.172	2.109	2.166	2.1704
Gizzard	2.222	2.175	2.161	1.923	2.142	2.1246
Duodenum	2.244	2.168	2.029	2.02	2.082	2.1086
Ileum	2.083	2.508	2.026	2.227	2.454	2.2596
Caeca	4.472	5.277	5.351	5.289	5.338	5.1454
AVG	2.6324	2.8784	2.748	2.7136	2.8364	

LB	10	16	24	30	60	AVG
Crop	2.031	2.313	2.342	2.564	2.343	2.3186
Gizzard	2.29	2.425	2.262	2.392	2.577	2.3892
Duodenum	1.996	2.386	1.979	2.418	2.516	2.259
Ileum	2.166	2.2	2.478	2.666	2.706	2.4432
Caeca	4.482	5.413	5.192	5.389	5.502	5.1956
AVG	2.593	2.9474	2.851	3.0858	3.1288	

Level	- Level	Score Mean Difference	Std Err Dif	Z	p-Value	p adj	Hodges-Lehmann	Lower CL	Upper CL
CaecaLB30	CaecaLB10	9.9	2.645751	3.7419	0.0002	0.000795455	0.99293	0.66838	1.15733
CaecaLB60	CaecaLB10	9.9	2.645751	3.7419	0.0002	0.000795455	1.09896	0.74539	1.29944
CaecaLSL16	CaecaLSL10	9.9	2.645751	3.7419	0.0002	0.000795455	0.78343	0.57622	0.99159
CaecaLSL24	CaecaLSL10	9.9	2.645751	3.7419	0.0002	0.000795455	0.84267	0.70451	1.01858
CaecaLSL30	CaecaLSL10	9.9	2.645751	3.7419	0.0002	0.000795455	0.82826	0.56607	1.01128
IleumLB60	IleumLB16	9.9	2.645751	3.7419	0.0002	0.000795455	0.47817	0.26489	0.73782
CropLB30	CropLB10	9.7	2.645751	3.6663	0.0002	0.000795455	0.47549	0.33909	0.67388
CaecaLB16	CaecaLB10	9.5	2.645751	3.5907	0.0003	0.000881295	1.03826	0.56948	1.24405
CaecaLSL60	CaecaLSL10	9.39444	2.585573	3.6334	0.0003	0.000881295	0.8391	0.56414	1.09329
CaecaLB24	CaecaLB10	9.1	2.645751	3.4395	0.0006	0.00169746	0.79478	0.4486	0.97854
CaecaLB60	CaecaLB24	7.9	2.645751	2.9859	0.0028	0.00752193	0.32122	0.12293	0.48549
IleumLB30	IleumLB16	7.3	2.645751	2.7591	0.0058	0.01492647	0.49409	0.21155	0.7236
DuodenumLB60	DuodenumLB10	7.28333	2.585573	2.8169	0.0048	0.01253731	0.49998	0.15438	0.86526
IleumLB60	IleumLB10	7.1	2.645751	2.6836	0.0073	0.0185529	0.44745	0.16105	1.04751
IleumLB24	IleumLB16	6.7	2.645751	2.5324	0.0113	0.027685	0.21659	0.035	0.58639
CropLB30	CropLB16	6.65	2.585573	2.572	0.0101	0.02514736	0.2777	0.05824	0.44116
CropLB16	CropLB10	6.22778	2.585573	2.4087	0.016	0.03761996	0.19683	0.0256	0.46291
CaecaLB30	CaecaLB24	6.1	2.645751	2.3056	0.0211	0.04786574	0.19733	0.02571	0.35605
IleumLB60	CropLB60	6.1	2.645751	2.3056	0.0211	0.04786574	0.43691	0.07659	0.68773
IleumLSL24	IleumLB24	-6.1	2.645751	-2.306	0.0211	0.04786574	-0.48654	-0.81277	-0.06675
DuodenumLSL30	DuodenumLB30	-6.3	2.645751	-2.381	0.0173	0.04021347	-0.42348	-0.75451	-0.10743
CaecaLB24	CaecaLB16	-6.7	2.645751	-2.532	0.0113	0.027685	-0.26204	-0.4308	-0.05816
IleumLSL30	IleumLB30	-6.9	2.645751	-2.608	0.0091	0.02279652	-0.48619	-0.69246	-0.17822
CropLSL30	CropLB30	-7.3	2.645751	-2.759	0.0058	0.01492647	-0.54274	-0.77567	-0.21592
CropLSL60	CaecaLSL60	-8.88889	2.516611	-3.532	0.0004	0.001142191	-3.15878	-3.53339	-2.86512
DuodenumLSL60	CaecaLSL60	-8.88889	2.516611	-3.532	0.0004	0.001142191	-3.2915	-3.84409	-2.80832
GizzardLSL60	CaecaLSL60	-8.88889	2.516611	-3.532	0.0004	0.001142191	-3.18665	-3.47419	-2.91798
IleumLSL60	CaecaLSL60	-8.88889	2.516611	-3.532	0.0004	0.001142191	-2.85117	-3.22315	-2.50316
CropLB16	CaecaLB16	-9.39444	2.585573	-3.633	0.0003	0.000881295	-3.14266	-3.32412	-2.96182
DuodenumLB10	CaecaLB10	-9.39444	2.585573	-3.633	0.0003	0.000881295	-2.4782	-2.86384	-2.08627
GizzardLB16	CaecaLB16	-9.39444	2.585573	-3.633	0.0003	0.000881295	-3.0997	-3.36908	-2.75617
GizzardLSL30	CaecaLSL30	-9.39444	2.585573	-3.633	0.0003	0.000881295	-3.32304	-3.68506	-3.06903
CropLB10	CaecaLB10	-9.9	2.645751	-3.742	0.0002	0.000795455	-2.33838	-2.83265	-2.11068
CropLB24	CaecaLB24	-9.9	2.645751	-3.742	0.0002	0.000795455	-2.80618	-3.20288	-2.53239
CropLB30	CaecaLB30	-9.9	2.645751	-3.742	0.0002	0.000795455	-2.80533	-2.99119	-2.66147
CropLB60	CaecaLB60	-9.9	2.645751	-3.742	0.0002	0.000795455	-3.20274	-3.51549	-2.83695
CropLSL10	CaecaLSL10	-9.9	2.645751	-3.742	0.0002	0.000795455	-2.32053	-2.54648	-2.13387
CropLSL16	CaecaLSL16	-9.9	2.645751	-3.742	0.0002	0.000795455	-2.94771	-3.27199	-2.7367
CropLSL24	CaecaLSL24	-9.9	2.645751	-3.742	0.0002	0.000795455	-3.24039	-3.47045	-2.83337
CropLSL30	CaecaLSL30	-9.9	2.645751	-3.742	0.0002	0.000795455	-3.25177	-3.51804	-2.92651
DuodenumLB16	CaecaLB16	-9.9	2.645751	-3.742	0.0002	0.000795455	-3.04028	-3.25148	-2.61029
DuodenumLB24	CaecaLB24	-9.9	2.645751	-3.742	0.0002	0.000795455	-2.94374	-3.85462	-2.70677
DuodenumLB30	CaecaLB30	-9.9	2.645751	-3.742	0.0002	0.000795455	-2.91511	-3.24456	-2.64304
DuodenumLB60	CaecaLB60	-9.9	2.645751	-3.742	0.0002	0.000795455	-2.9816	-3.19272	-2.76617
DuodenumLSL10	CaecaLSL10	-9.9	2.645751	-3.742	0.0002	0.000795455	-2.23065	-2.41979	-2.05115
DuodenumLSL16	CaecaLSL16	-9.9	2.645751	-3.742	0.0002	0.000795455	-3.07209	-3.34415	-2.88246
DuodenumLSL24	CaecaLSL24	-9.9	2.645751	-3.742	0.0002	0.000795455	-3.2311	-3.58212	-2.95749
DuodenumLSL30	CaecaLSL30	-9.9	2.645751	-3.742	0.0002	0.000795455	-3.25495	-3.53514	-3.01312
GizzardLB10	CaecaLB10	-9.9	2.645751	-3.742	0.0002	0.000795455	-2.14376	-2.53388	-1.88721
GizzardLB24	CaecaLB24	-9.9	2.645751	-3.742	0.0002	0.000795455	-2.82251	-3.36218	-2.54376
GizzardLB30	CaecaLB30	-9.9	2.645751	-3.742	0.0002	0.000795455	-3.03957	-3.35022	-2.69281
GizzardLB60	CaecaLB60	-9.9	2.645751	-3.742	0.0002	0.000795455	-2.80603	-3.24151	-2.59553
GizzardLSL10	CaecaLSL10	-9.9	2.645751	-3.742	0.0002	0.000795455	-2.24876	-2.42192	-2.05096
GizzardLSL16	CaecaLSL16	-9.9	2.645751	-3.742	0.0002	0.000795455	-3.18107	-3.43821	-2.86171
GizzardLSL24	CaecaLSL24	-9.9	2.645751	-3.742	0.0002	0.000795455	-3.36835	-3.57226	-3.1384
IleumLB10	CaecaLB10	-9.9	2.645751	-3.742	0.0002	0.000795455	-2.17796	-2.90809	-1.82173
IleumLB16	CaecaLB16	-9.9	2.645751	-3.742	0.0002	0.000795455	-3.18529	-3.48132	-3.03162
IleumLB24	CaecaLB24	-9.9	2.645751	-3.742	0.0002	0.000795455	-2.69343	-2.91444	-2.46881
IleumLB30	CaecaLB30	-9.9	2.645751	-3.742	0.0002	0.000795455	-2.65127	-2.97128	-2.44249
IleumLB60	CaecaLB60	-9.9	2.645751	-3.742	0.0002	0.000795455	-2.82561	-2.96225	-2.61101
IleumLSL10	CaecaLSL10	-9.9	2.645751	-3.742	0.0002	0.000795455	-2.25153	-2.54275	-2.06031
IleumLSL16	CaecaLSL16	-9.9	2.645751	-3.742	0.0002	0.000795455	-2.90512	-3.2055	-2.53797
IleumLSL24	CaecaLSL24	-9.9	2.645751	-3.742	0.0002	0.000795455	-3.33797	-3.67101	-3.023
IleumLSL30	CaecaLSL30	-9.9	2.645751	-3.742	0.0002	0.000795455	-3.04568	-3.24839	-2.80995

S4.3. (excel file) Significant differences of top 20 genus between breeds, GIT sections and production stages. Red marked (production stage effect), green marked (production stage effect between weeks 16 and 24).

S4.4. (excel file) Anosim summary of breed effect (red), production stage effect (green) at ASV level.

ANOSIM
Global Test
Sample statistic (Global R): 0,653
Significance level of sample statistic: 0,1%
Number of permutations: 999 (Random sample from a large number)
Number of permuted statistics greater than or equal to Global R: 0

Pairwise Tests Groups	R Statistic	Significance Level %	Possible Permutations	Actual Permutations	Number >= Observed	P value	P adj.
Duodenum LB 16, Duodenum LB 24	0.622	0.1	92378	999	0	0.001	0.00160256
Duodenum LSL 16, Duodenum LSL 24	0.538	0.1	92378	999	0	0.001	0.00160256
Caeca LB 16, Caeca LSL 16	0.203	1.4	92378	999	13	0.014	0.0175
Caeca LB 16, Caeca LB 24	0.861	0.1	92378	999	0	0.001	0.00160256
Caeca LSL 16, Caeca LSL 24	0.876	0.1	92378	999	0	0.001	0.00160256
Gizzard LB 16, Gizzard LSL 16	0.203	2.5	92378	999	24	0.025	0.02920561
Gizzard LB 16, Gizzard LB 24	0.527	0.1	92378	999	0	0.001	0.00160256
Gizzard LSL 16, Gizzard LSL 24	0.684	0.1	92378	999	0	0.001	0.00160256
Ileum LB 16, Ileum LB 24	0.606	0.1	92378	999	0	0.001	0.00160256
Ileum LSL 16, Ileum LSL 24	0.53	0.1	92378	999	0	0.001	0.00160256
Crop LB 16, Crop LB 24	0.374	0.8	92378	999	7	0.008	0.01052632
Crop LSL 16, Crop LSL 24	0.612	0.1	92378	999	0	0.001	0.00160256
Caeca LB 24, Caeca LSL 24	0.628	0.1	92378	999	0	0.001	0.00160256
Ileum LB 24, Ileum LSL 24	0.186	2.3	92378	999	22	0.023	0.02764423

S4.5 (excel file) Relative abundance of top 15 microbiota (higher than 1%) separated by breed, GIT section and production stage; PERMANOVA table of results. only statistically significant results are shown (Red -> production stage effect).

TOP 15 ASV	[%]	LSL AVG	LB AVG	Crop LSL	Crop LB	Gizzard LSL	Gizzard LB	Duodenum LSL	Duodenum LB	Ileum LSL	Ileum LB	Caeca LSL	Caeca LB	AVG	
ASV1383	16	15.9	5.9	19.4	7.7	21.0	6.9	21.1	8.8	17.3	5.7	0.9	0.3	10.9	Ligilactobacillus aviarius
ASV1384	16	11.3	9.2	18.9	18.4	16.5	11.1	11.5	6.5	8.8	9.4	0.5	0.3	10.2	Ligilactobacillus salivarius
ASV1459	16	12.0	12.7	10.2	16.3	20.1	7.4	17.3	13.3	12.4	26.7	0.0	0.0	12.4	Lactobacillus kitasatonis
ASV989	16	0.0	0.1	0.0	0.0	0.0	0.0	0.0	0.3	0.0	0.0	0.0	0.0	0.0	Lachnospiraceae uncl.
ASV1189	16	10.1	7.9	12.1	7.0	10.8	17.3	15.0	7.4	12.5	7.7	0.1	0.2	9.0	Lactobacillus delbrueckii
ASV754	16	8.4	7.1	13.0	14.7	8.8	5.7	10.6	8.0	9.4	6.9	0.2	0.2	7.7	Ligilactobacillus aviarius
ASV1421	16	3.4	5.5	4.3	9.9	3.5	5.1	4.7	5.0	4.2	6.8	0.3	0.6	4.5	Clostridia uncl.
ASV1387	16	0.0	0.0	0.1	0.2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	Lactobacillus delbrueckii
ASV1745	16	0.5	0.5	0.0	0.0	0.4	0.9	0.9	1.2	1.3	0.3	0.0	0.0	0.5	Blautia uncl.
ASV1066	16	2.0	4.5	2.5	4.7	1.9	4.9	3.5	7.1	2.3	5.8	0.0	0.2	3.3	Clostridium sp.
ASV1108	16	1.4	4.6	3.4	4.9	0.0	4.8	0.0	4.3	3.7	8.7	0.0	0.3	3.0	Oscillospiraceae uncl.
ASV2110	16	0.7	2.5	0.0	0.0	2.2	10.1	0.9	1.9	0.1	0.4	0.0	0.0	1.6	Eisenbergiella uncl.
ASV1273	16	1.0	0.6	0.1	0.0	0.0	0.1	0.0	0.0	4.8	2.4	0.1	0.4	0.8	Ligilactobacillus salivarius
ASV115	16	0.3	2.5	0.0	0.0	0.0	0.4	1.6	11.8	0.0	0.3	0.0	0.1	1.4	Lactobacillus gallinarum
ASV119	16	0.0	0.1	0.0	0.0	0.0	0.0	0.1	0.0	0.0	0.2	0.0	0.0	0.0	Bacteroidales uncl.
AVG	16	4.5	4.2	5.6	5.6	5.7	5.0	5.8	5.1	5.1	5.4	0.2	0.2	4.4	
SUM	16	67.2	63.6	84.1	84.0	85.4	74.6	87.1	75.8	77.0	81.2	2.3	2.6	65.4	
ASV1383	24	22.7	14.2	31.4	25.9	22.5	12.4	20.7	14.1	34.6	17.4	4.2	1.2	18.4	Ligilactobacillus aviarius
ASV1384	24	18.9	11.0	37.4	27.5	20.9	10.0	16.4	8.6	16.8	8.0	2.9	0.8	14.9	Ligilactobacillus salivarius
ASV1459	24	0.0	1.6	0.0	3.5	0.0	0.4	0.0	3.5	0.0	0.4	0.0	0.0	0.8	Lactobacillus kitasatonis
ASV989	24	9.8	11.2	0.0	0.0	28.8	31.9	8.4	10.7	11.4	12.6	0.4	0.8	10.5	Lachnospiraceae uncl.
ASV1189	24	1.2	1.8	3.6	2.9	0.5	2.8	1.3	2.6	0.5	0.7	0.2	0.2	1.5	Lactobacillus delbrueckii
ASV754	24	1.0	2.6	2.2	6.7	0.6	1.8	0.4	2.1	1.6	2.4	0.1	0.3	1.8	Ligilactobacillus aviarius
ASV1421	24	1.9	4.3	3.9	10.1	1.2	3.1	1.7	3.2	2.2	4.3	0.3	0.6	3.1	Clostridia uncl.
ASV1387	24	5.3	7.9	0.0	0.0	0.3	1.3	25.1	34.7	1.1	3.5	0.0	0.0	6.6	Lactobacillus delbrueckii
ASV1745	24	4.9	3.5	0.0	0.0	6.3	7.2	4.5	2.6	13.4	7.3	0.2	0.3	4.2	Blautia uncl.
ASV1066	24	0.2	1.2	0.2	2.1	0.1	0.6	0.1	0.6	0.7	2.2	0.0	0.2	0.7	Clostridium sp.
ASV1108	24	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	Oscillospiraceae uncl.
ASV2110	24	0.2	1.7	0.0	0.0	0.0	6.4	0.6	1.2	0.1	0.9	0.0	0.0	0.9	Eisenbergiella uncl.
ASV1273	24	0.7	2.4	0.0	0.0	0.3	0.1	0.1	0.0	2.7	10.9	0.5	0.8	1.5	Ligilactobacillus salivarius
ASV115	24	0.8	0.6	0.0	0.0	0.0	0.0	3.6	1.4	0.2	1.4	0.0	0.0	0.7	Lactobacillus gallinarum
ASV119	24	1.5	2.5	1.8	1.0	0.9	1.6	4.0	4.3	0.8	5.8	0.0	0.0	2.0	Bacteroidales uncl.
AVG	24	4.6	4.4	5.4	5.3	5.5	5.3	5.8	6.0	5.7	5.2	0.6	0.3	4.5	
SUM	16	68.9	66.4	80.5	79.8	82.3	79.5	86.9	89.7	86.0	77.9	8.9	5.2	67.7	
ALL	AVG	4.5	4.3	5.5	5.5	5.6	5.1	5.8	5.5	5.4	5.3	0.4	0.3	4.4	

ASV1383

Level	- Level	Score Mean Difference	Std Err Dif	Z	p-Value
CropLSL24	CaecaLSL24	109,700	25,59369	4,28621	0.0035
CropLB24	CaecaLB24	106,700	25,59369	4,16900	0.0058
IleumLSL24	CaecaLSL24	105,300	25,59369	4,11430	0.0074
DuodenumLSL16	CaecaLSL16	94,700	25,59369	3,70013	0.0409

ASV1384

Level	- Level	Score Mean D	Std Err Dif	Z	p-Value
CropLB24	CaecaLB24	135,100	25,62616	5,27196	0.0001
CropLSL24	CaecaLSL24	132,600	25,62616	5,17440	0.0001
CropLSL16	CaecaLSL16	122,600	25,62616	4,78417	0.0003
CropLB16	CaecaLB16	114,517	26,32838	4,34955	0.0026
GizzardLSL16	CaecaLSL16	109,200	25,62616	4,26127	0.0039

ASV1459

Level	- Level	Score Mean D	Std Err Dif	Z	p-Value
IleumLB16	CaecaLB16	82,7000	21,17918	3,90478	0.0179

ASV989

Level	- Level	Score Mean D	Std Err Dif	Z	p-Value
GizzardLSL24	GizzardLSL16	89,700	23,57851	3,80431	0.027

ASV754

Level	- Level	Score Mean D	Std Err Dif	Z	p-Value
CropLB16	CaecaLB16	128,378	26,31484	4,87853	0.0002
CropLSL16	CaecaLSL16	112,900	25,61298	4,40792	0.002
DuodenumLSL16	CaecaLSL16	103,500	25,61298	4,04092	0.0101
GizzardLSL16	CaecaLSL16	97,100	25,61298	3,79105	0.0285

ASV1421

Level	- Level	Score Mean D	Std Err Dif	Z	p-Value
CropLB16	CaecaLB16	132,194	26,32838	5,02099	0.0001
CropLB24	CaecaLB24	128,900	25,62616	5,03002	0.0001
IleumLB16	CaecaLB16	121,200	25,62616	4,72954	0.0004
IleumLSL16	CaecaLSL16	112,100	25,62616	4,37444	0.0023
DuodenumLSL16	CaecaLSL16	110,600	25,62616	4,31590	0.003
CropLSL16	CaecaLSL16	106,400	25,62616	4,15201	0.0063
GizzardLB16	CaecaLB16	104,194	26,32838	3,95750	0.0144
GizzardLSL16	CaecaLSL16	93,900	25,62616	3,66422	0.0471

ASV1387

Level	- Level	Score Mean Difference	Std Err Dif	Z	p-Value
DuodenumLSL24	DuodenumLSL16	122,600	23,63834	5,18649	0.0001
DuodenumLB24	DuodenumLB16	117,100	23,63834	4,95382	0.0001
DuodenumLB24	CropLB24	114,100	23,63834	4,82690	0.0003
IleumLSL24	IleumLSL16	109,900	23,63834	4,64923	0.0006
DuodenumLSL24	CropLSL24	95,600	23,63834	4,04428	0.01

ASV1066

Level	- Level	Score Mean D	Std Err Dif	Z	p-Value
DuodenumLSL16	CaecaLSL16	98,200	25,61487	3,83371	0.024
IleumLB16	CaecaLB16	94,600	25,61487	3,69317	0.0421
CropLSL24	CropLSL16	-93,800	25,61487	-3,66194	0.0476
DuodenumLSL24	DuodenumLSL16	-98,800	25,61487	-3,85714	0.0218

ASV2110

Level	- Level	Score Mean D	Std Err Dif	Z	p-Value
GizzardLB24	CropLB24	123,800	25,02850	4,94636	0.0001
GizzardLB16	CropLB16	118,111	26,38236	4,47690	0.0014
DuodenumLB16	CropLB16	98,817	25,71434	3,84286	0.0231
GizzardLB16	CaecaLB16	94,794	25,71434	3,68644	0.0432
IleumLB24	CropLB24	92,100	25,02850	3,67980	0.0443
DuodenumLB24	CropLB24	92,000	25,02850	3,67581	0.045

ASV1273

Level	- Level	Score Mean D	Std Err Dif	Z	p-Value
IleumLSL16	GizzardLSL16	130,900	25,28496	5,17699	0.0001
IleumLSL16	DuodenumLSL16	127,500	25,28496	5,04252	0.0001
IleumLSL16	CropLSL16	120,200	25,28496	4,75381	0.0004
IleumLB24	CropLB24	114,000	25,28496	4,50861	0.0012
IleumLB16	CropLB16	112,550	25,97783	4,33254	0.0028
IleumLB24	DuodenumLB24	110,000	25,28496	4,35041	0.0026
IleumLB24	GizzardLB24	103,300	25,28496	4,08543	0.0084
CropLB24	CaecaLB24	-93,700	25,28496	-3,70576	0.04

ASV119

Level	- Level	Score Mean D	Std Err Dif	Z	p-Value
IleumLB24	IleumLB16	106,700	24,79908	4,30258	0.0032
IleumLB24	CaecaLB24	100,100	24,79908	4,03644	0.0103
CropLSL24	CaecaLSL24	98,000	24,79908	3,95176	0.0147
IleumLSL24	IleumLSL16	97,500	24,79908	3,93160	0.016
GizzardLB24	CaecaLB24	93,900	24,79908	3,78643	0.029
IleumLSL24	CaecaLSL24	91,500	24,79908	3,68965	0.0427

S4.6 (excel file) Significant different genera between breeds, GIT sections and production stages. (metagenomic data).

Lactobacillus

Level	- Level	Score Mean Difference	Std Err Dif	Z	p-Value
IleumLSL16	CaecaLSL16	65.925	18.01735	3.65897	0.0167
IleumLSL24	CaecaLSL24	57.9	13.61984	4.25115	0.0014
CropLSL24	CaecaLSL24	54	13.61984	3.9648	0.0048
CropLB16	CaecaLB16	52.2	13.61984	3.83264	0.0084
CropLB24	CaecaLB24	46.3	13.61984	3.39945	0.0446

Limosilactobacillus

Level	- Level	Score Mean	Std Err Dif	Z	p-Value
CropLB16	CaecaLB16	81.9	13.61984	6.01329	<.0001
CropLB24	CaecaLB24	63.4	13.61984	4.65497	0.0002
CropLSL16	CaecaLSL16	62.4	13.61984	4.58155	0.0003

Ligilactobacillus

Level	- Level	Score Mean	Std Err Dif	Z	p-Value
CropLB16	CaecaLB16	68.9	13.61984	5.0588	<.0001
IleumLSL16	CaecaLSL16	63.275	18.01735	3.51189	0.0294
CropLSL16	CaecaLSL16	59.3	13.61984	4.35394	0.0009
IleumLB24	CaecaLB24	54.6278	13.99305	3.90392	0.0062
IleumLSL24	CaecaLSL24	49.7	13.61984	3.64909	0.0174
IleumLSL24	CropLSL24	48.4	13.61984	3.55364	0.0251

Blautia

Level	- Level	Score Mean	Std Err Dif	Z	p-Value
IleumLSL24	CaecaLSL24	-47	13.2665	-3.54276	0.0261
IleumLB24	CaecaLB24	-54.0389	13.63003	-3.96469	0.0049
CropLB16	CaecaLB16	-56	13.2665	-4.22116	0.0016
CropLB24	CaecaLB24	-56.5	13.2665	-4.25885	0.0014
CropLSL16	CaecaLSL16	-58.2	13.2665	-4.38699	0.0008

Uncl. Bacteria

Level	- Level	Score Mean Difference	Std Err Dif	Z	p-Value
CropLSL16	CaecaLSL16	-49.1	13.61984	-3.60504	0.0206
IleumLSL24	CaecaLSL24	-56.1	13.61984	-4.11899	0.0025
CropLB16	CaecaLB16	-59.3	13.61984	-4.35394	0.0009

Mediterraneibacter

Level	- Level	Score Mean	Std Err Dif	Z	p-Value
IleumLSL24	CaecaLSL24	-52.2	13.47576	-3.87362	0.0071
CropLSL16	CaecaLSL16	-53.2	13.47576	-3.94783	0.0052
CropLB16	CaecaLB16	-53.6	13.47576	-3.97751	0.0046
IleumLB24	CaecaLB24	-55.2167	13.84503	-3.98819	0.0044
CropLB24	CaecaLB24	-66.9	13.47576	-4.96447	<.0001

Uncl. Firmicutes

Level	- Level	Score Mean	Std Err Dif	Z	p-Value
CropLB24	CaecaLB24	-49.7	13.61984	-3.64909	0.0174
IleumLSL16	CaecaLSL16	-63.175	18.01735	-3.50634	0.03
CropLB16	CaecaLB16	-75.4	13.61984	-5.53604	<.0001
CropLSL16	CaecaLSL16	-82	13.61984	-6.02063	<.0001

Faecalibacterium

Level	- Level	Score Mean	Std Err Dif	Z	p-Value
IleumLB24	CaecaLB24	-48.1722	13.90939	-3.46329	0.0352
IleumLSL24	CaecaLSL24	-51.05	13.53841	-3.77075	0.0107
CropLB24	CaecaLB24	-55.05	13.53841	-4.06621	0.0032
CropLSL16	CaecaLSL16	-57.2	13.53841	-4.22502	0.0016
CropLB16	CaecaLB16	-61.05	13.53841	-4.50939	0.0004

Uncl. Lactobacillaceae

Level	- Level	Score Mean Difference	Std Err Dif	Z	p-Value
CropLB16	CaecaLB16	76.6	13.61984	5.62415	<.0001
CropLB24	CaecaLB24	65.1	13.61984	4.77979	0.0001
CropLSL16	CaecaLSL16	57.9	13.61984	4.25115	0.0014
CropLSL24	CaecaLSL24	50.4	13.61984	3.70048	0.0142

Uncl. Eubacteriales

Level	- Level	Score Mean	Std Err Dif	Z	p-Value
CropLSL24	CaecaLSL24	-46.1	13.61984	-3.38477	0.047
IleumLSL24	CaecaLSL24	-50.2	13.61984	-3.6858	0.015
CropLB24	CaecaLB24	-55	13.61984	-4.03823	0.0036
CropLB16	CaecaLB16	-58.5	13.61984	-4.29521	0.0012
CropLSL16	CaecaLSL16	-68.5	13.61984	-5.02943	<.0001

uncl. Bacteroidaceae

Level	- Level	Score Mean	Std Err Dif	Z	p-Value
CropLSL16	CaecaLSL16	-49.2	13.51619	-3.64008	0.018
CropLSL24	CaecaLSL24	-50.7	13.51619	-3.75106	0.0116
CropLB16	CaecaLB16	-51.4	13.51619	-3.80285	0.0094
CropLB24	CaecaLB24	-57.25	13.51619	-4.23566	0.0015
IleumLB16	CaecaLB16	-69	23.41073	-2.94737	0.2115
IleumLSL24	CaecaLSL24	-70.05	13.51619	-5.18267	<.0001

Uncl. Bacteroidales

Level	- Level	Score Mean	Std Err Dif	Z	p-Value
CropLSL24	CaecaLSL24	-50	13.6186	-3.67145	0.0159
CropLB24	CaecaLB24	-52.1	13.6186	-3.82565	0.0086
CropLB16	CaecaLB16	-53.25	13.6186	-3.91009	0.0061
CropLSL16	CaecaLSL16	-60.65	13.6186	-4.45347	0.0006
IleumLSL24	CaecaLSL24	-63.9	13.6186	-4.69211	0.0002

Uncl. Bacteroides

Level	- Level	Score Mean Difference	Std Err Dif	Z	p-Value
IleumLSL24	CaecaLSL24	-47.1	13.61786	-3.45869	0.0358
CropLSL24	CaecaLSL24	-56.4	13.61786	-4.14162	0.0023
CropLB24	CaecaLB24	-60	13.61786	-4.40598	0.0007
CropLSL16	CaecaLSL16	-62.2	13.61786	-4.56753	0.0003
CropLB16	CaecaLB16	-64	13.61786	-4.69971	0.0002

Uncl. Clostridia

Level	- Level	Score Mean	Std Err Dif	Z	p-Value
CropLSL24	CaecaLSL24	-47.4	13.61984	-3.48022	0.0331
CropLSL16	CaecaLSL16	-49.9	13.61984	-3.66377	0.0164
CropLB16	CaecaLB16	-60.1	13.61984	-4.41268	0.0007
IleumLSL24	CaecaLSL24	-64.2	13.61984	-4.71371	0.0002

Uncl. Bacteroidetes

Level	- Level	Score Mean	Std Err Dif	Z	p-Value
IleumLB24	CaecaLB24	-49.15	13.77433	-3.56823	0.0237
IleumLSL24	CaecaLSL24	-52.1	13.40694	-3.88605	0.0067
CropLB16	CaecaLB16	-53.4	13.40694	-3.98301	0.0045
CropLB24	CaecaLB24	-56.3	13.40694	-4.19932	0.0018
CropLSL16	CaecaLSL16	-56.5	13.40694	-4.21423	0.0017

Uncl. Lachnospiraceae

Level	- Level	Score Mean	Std Err Dif	Z	p-Value
IleumLSL24	CaecaLSL24	-51.9	13.6198	-3.81063	0.0091
IleumLB24	CaecaLB24	-52.1722	13.99302	-3.72845	0.0127
CropLB16	CaecaLB16	-53.25	13.6198	-3.90975	0.0061
CropLB24	CaecaLB24	-59.75	13.6198	-4.38699	0.0008
CropLSL16	CaecaLSL16	-61.8	13.6198	-4.53751	0.0004

The following additional material can only be found in an appendix in electronic form/on CD-ROM:

S4.7 (excel file): Significant KOs separated in GIT sections subdivided by production stages and breed (yellow marked – Inositol phosphate metabolism related; red marked – consistent significant function across factor; blue marked - Log2foldchange < 2).

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CHAPTER V

DISCUSSION

5. General discussion

Poultry production worldwide increased within the last decades, and further growth is expected due to the increase in the human population [1,2]. The adequate feeding of the animals requires the availability of feed resources and essential elements like P or Ca. Especially a sustainable production system is needed to feed the animals according to their requirements and maintain performance, save resources, and reduce the excretion of undigested nutrients.

Recent studies in non-ruminants showed substantial InsP6 degradation from plant materials without phytase supplementation and low levels of P and Ca [3–5]. Nevertheless, global P resources stored in rock phosphate are limited and challenge future food production [6,7]. Depending on the animal breed and species, the requirement differs consequently. For example, laying hens and female quails need more Ca within the laying period for eggshell formation than male conspecifics. Therefore, a reduction in dietary P or non-phytate P in the feed without adverse effects on the health or productivity of the animals is necessary for future feeding strategies and might help to overcome the lack of nutrient availability. Especially the animal associated microbiota in the GIT combined with the diet plays a central role in this concerns [8,9]. Interactions between the microbes in the GIT and the intestinal wall occur due to the nutrients which modulates the microbiota itself [10]; consequently beneficial, commensal and pathogenic bacteria compete for nutrients and the attachment sites in the GIT. Understanding these relationships can ensure to maintain the animal's health and promote the productivity.

5.1 Research standardization and comparability

Comparability of microbiome research studies in animals is still impossible and requires standardization of methods. Furthermore, the variance in the used breeds is high worldwide, and even if two breeds of the same species are studied, the breed effect can superimpose differences between microbiota changes regarding the dietary treatments [11]. Especially in terms of comparability, a reference protocol and methodological standardization is needed within the study which is also linked to result and study reproducibility [12]. This is also true for animal facilities. An adequate standardization is needed due to environmental conditions to promote the comparability [13].

Each extraction method has its own advantages and disadvantages. For instance, even the extraction method changes the output, with significant abundance differences by investigating the bacterial taxa [14]. The in this project used TRIzol is well established to extraction and isolate nucleic acids, extra cellular vesicles and proteins from the same biological sample on various fields like human, plant, animals bacteria or viruses [15]. Due to this, further alterations between the microbiome and the genome, transcriptome and proteome can be compared on the same cell mass. This reagent separates molecules from one another based on the interaction of cellular components to phenol and guanidine [16]. However, TRIzol is labeled by the manufacturer MDS as acute oral, dermal and inhalation toxicity due to the vaporization so it should be handled with care. Additional, it can lead to skin corrosion and irritation and can cause serious health hazard from chemical burns, permanent scarring and is mutagenic [17]. Therefore, experiments should always be performed with lab coat, phenol resistant nitrile gloves under a hood. The TRIzol extraction has been widely used since its introduction in 1987 especially due to providing comparable DNA yields to other methods and up to 50% higher RNA yields [18,19]. Moreover, the short period of time necessary to extract molecules is a benefit of the extraction method (protein extraction in less than 4 hours [20]).

Microbiota studies can target DNA or RNA [21]. DNA analysis enables the investigation of a sample's overall bacterial composition and amount and provides a static view of organisms, includes the isolation recombinant DNA constructs (e.g. bacteriophages, plasmids) and the isolation of chromosomal or genomic DNA from prokaryotic or eukaryotic organisms [22]. RNA analysis, on the other hand, represents the active microbiota transcript by active genes and can give a closer understanding of cells or bacteria performing specialized tasks. Furthermore, RNA is an unstable molecule with a very short half-life after extraction [23] and underlies good laboratory technique and RNase-free conditions. Moreover, comparing the expressed (active) genes of different bacteria and their change over time or in response to varying stimuli is important to understand the state of the art, modulation, and influences in gut microbiota. DNA samples should be RNA-free and RNA samples should be DNA-free as the quality of scientific research is directly affected by contaminations [24]. Even if both are guaranteed, differences in the microbial composition between RNA and DNA can be observed ([25], Chapter IV).

The 16S rRNA gene has been the centerpiece of the sequence-based bacterial analysis for the last decades, encounter nine hypervariable regions (V1-V9) and is involved in the secondary structure of the small ribosomal subunit [26]. While the 16S hypervariable regions can vary significantly among bacteria, the 16S gene as a whole maintains greater length homogeneity than the 18S rRNA, the eukaryotic counterpart, which can further facilitate alignment [27] but strives to differentiate closely related species within the analysis [28]. In contrast to the human microbiome research that often used the V4 or V3-V5 regions [29], we used the V1-V2 region. This region has been shown to have the best performance, compared to others, in terms of two times higher numbers of species assignation of bacteria [30]. Therefore because of the persisting difficulty in selecting a common region for target amplicon sequencing, we decided to use the V1-V2 region to maintain knowledge along the poultry GIT and enable comparability across our research studies [11,31–33]. Moreover, it was reported, that the selected variable region affected the microbial analysis in the caeca in chicken [34]. Due to this, a standardized selection of specific regions within research topics can increase knowledge and improve linkage or networking between effects on the microbiome.

The bioinformatical analysis of target sequencing data can be performed on several pipelines. The most common ones are Mothur [35], QIIME [36], QIIME2 [37], DADA2 [38], USEARCH [39]. All of these pipelines follow the same procedure. Forward and reverse reads are assembled, and low-quality reads are filtered and deleted from the dataset besides low abundant ASVs (amplicon sequencing variants) / OTUs (operational taxonomic units) and chimeras [35,36]. A separation in clustered molecular OTUs or ASVs highlighting single-nucleotide differences can also be performed. Until recently, it was common to cluster sequences with more than 97% similarity over the whole length to multiple reference sequences and define them as OTU [40]. However, it is recommended to use ASVs as they show the exact biological sequences in the sample [41]. The OTUs or ASV have to be taxonomically assigned, and for this, different reference datasets are available (e.g. SILVA, RDP, NCBI or greengenes (GG)), which assign the domain, phylum, class, order, family, genus or species to the given sequence [42]. Many differences exist between the reference datasets as curation, the number of references, or additional features as mapping the taxonomy [42]. As a result, the output quality varies regarding the abundance levels,

especially on genus level and the diversity index used as well as between bioinformatical pipelines (Fig. 5.1).

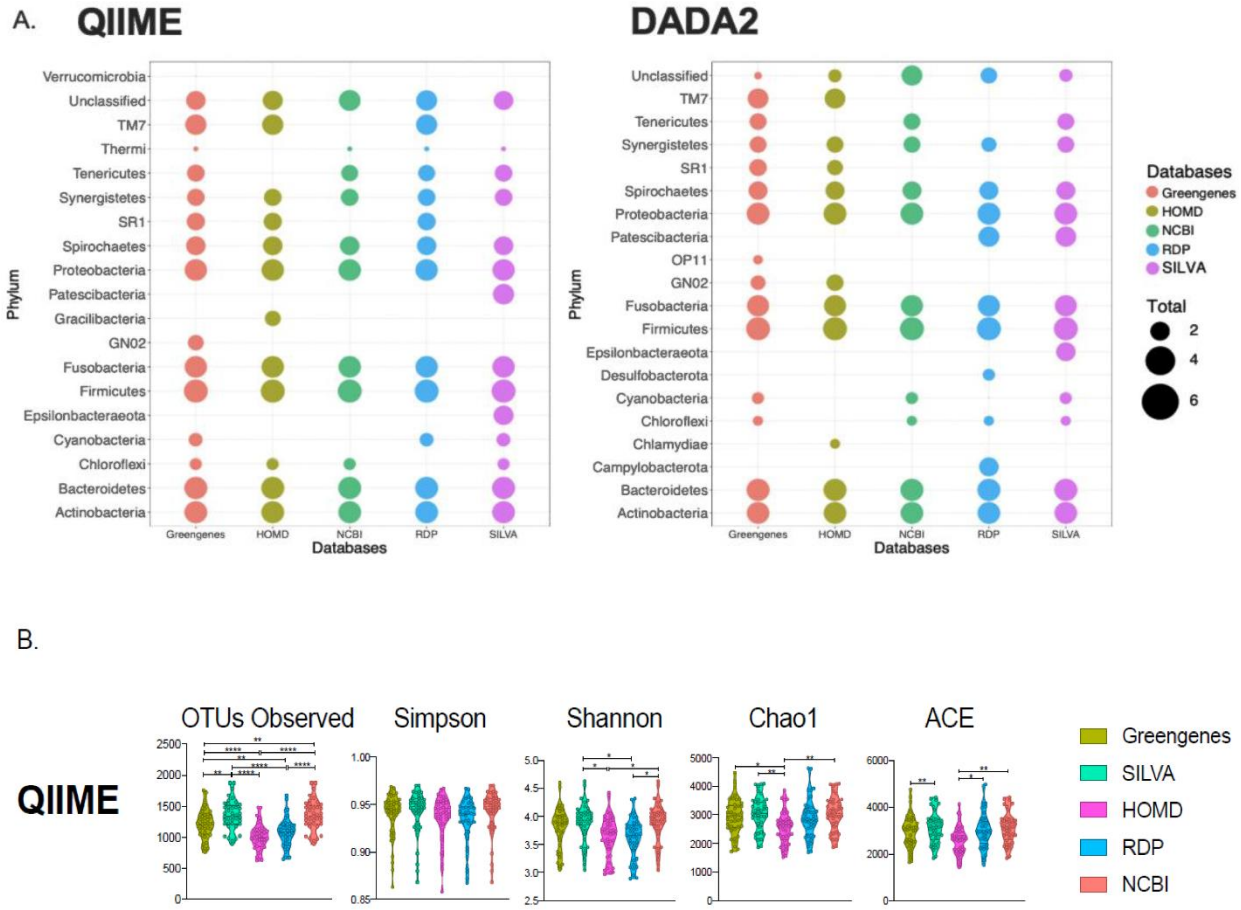

Figure 5.1: (A) Dotplot of phylum abundances from Quantitative Insights Into Microbial Ecology (QIIME) and the Divisive Amplicon Denoising Algorithm (DADA2) pipelines, comparing the five reference databases. Total abundances are log10 transformed. (B) α-diversity measurements for QIIME pipeline. p-values are assigned as ≤ 0.05 (*), < 0.002 (**), < 0.0002 (***), and < 0.0001 (****) [43].

Currently, no criteria are defined for data curation or the validation of annotations, which can affect the reproducibility and limits the comparability and it was reported, the choice of the database has a significantly higher impact on the taxonomy output than the used pipeline [43]. However, most of the given pipelines use SILVA or greengenes database on default. Greengenes was last renewed and actualized in 2013, and it has the lowest number of sequences compared to the others [42]. SILVA reference database is continuously improving with the latest release in August 2020, version 138.1 [44]. Moreover, the performance regarding taxonomy mapping is higher in SILVA than in RDP or greengenes. The ongoing approaches in the taxonomic assignation lead to an indeed wide range of changes in terms of classification. One of these changes was recently published by reclassifying the genus *Lactobacillus*, one of the

most common genera present in poultry GIT. The genus *Lactobacillus* was split into 25 genera, with 23 novel ones [45]. Due to the better annotation results and the recent classification changes, OTUs and ASVs in this study were aligned to the SILVA database within the two pipelines QIIME and Mothur, which we have chosen in this thesis as they produced both comparable richness and diversity results [46].

Recently, only single 'omic' approaches are commonly used within studies and often on specific GIT sections or treatments. Due to this, a limited comparability and inhibited understanding of the complete microbiome in animals is given. These concerns can be overcome by incorporating new and more approaches on the same biological samples in the future to overcome mis-understanding or -interpreting the results. Additionally, up-to-date databases can avoid mis-classification and improve the valid taxonomic assignation which is essential to understand the role of the microbes in the microbiome [47,48].

5.2 Quails microbiota and the effect of phosphorus and calcium utilization, feed intake, feed conversion, and body weight gain

The Japanese quail (*C. coturnix japonica*) were domesticated for over 800 years in Japan and used in egg and meat production in the Far East. They have been considered an animal model for poultry since 1959 [38]. Since then, were used to study genetics, overall growth development, animal nutrition, gut microbiota, physiology, and toxicology [49], but only in small cohorts of animals. Due to this, 760 ileum digesta samples derived from a large cohort from a previous study that used an F_2 design [50] were analyzed for microbiota characterization. By reducing mineral P and Ca from the diet, we proved that the ileal microbiota varies even though the animals were under the same diet and identical environmental conditions. Information regarding phosphorus utilization (PU), calcium utilization (CaU), feed intake (FI), feed conversion (FC), and body weight gain (BWG) was used to understand their influence on the microbiota structure of male and female quails.

A major problem of fowl microbiota studies is OTUs taxonomically annotated as "unclassified" for highly abundant bacteria [51,52]. This was also the case for the present study. The most abundant OTUs, contributing to more than 70% and belonging to the Japanese quail core microbiota community were uncl. *Lactobacillus*, uncl. *Clostridiaceae 1*, *Clostridium sensu stricto*, *Escherichia coli* and *Streptococcus alactolyticus*. Uncl. *Clostridiaceae1*, was positively correlated with PU, CaU, FI, and

BWG. Negative correlations were observed for *Clostridium sensu stricto* with FC, *Streptococcus alactolyticus* with BW, PU CaU and FI with *Escherichia coli* and uncl. *Lactobacillus* with FI. Even though Lactobacillus was positively correlated with egg production and feed conversion [53], only one negative correlation to FI was revealed in the present study. However, the presence is assumed to be beneficial for the carbohydrate transformation to lactic acid, pathogen adhesion inhibition, and a decreased pH in the ileum [54]. Furthermore, *Streptococcus alactolyticus*, a gram-positive lactic acid bacterium, is related to host well-being and is non-pathogenic. *S. alactolyticus* was first isolated from chicken feces and the pig intestine and is known to ferment glucose, fructose, and cellobiose [55]. Even though the relative abundance was low (3-16%), statistical differences between gender were detected for FC and BWG and within gender for all parameters (p-value < 0.1). Recent studies have shown that diet and host genotype influence *Streptococcus* species [56]. Still, no correlations of the abundance with gender, PU, CaU, or other performance traits have been found. It can be assumed that the high abundance of uncl. *Clostridiaceae* is due to the high proportion of corn [50], which favored the abundance of Clostridia in a recent avian study [57]. Despite the high abundance of *Clostridium sensu stricto* in the study, which is associated with pathogenesis and can be an indicator for an imbalanced microbiota [58], no effect was investigated on the animals and further no effects on the BWG due to higher levels of *Clostridium sensu stricto* were investigated as it was suggested by Apajalathi et al. [57].

Escherichia coli, as an enteropathogenic bacteria, can be a potential carrier for diseases in humans and animals [59]. However, it is also a common colonizer in the avian GIT with no profound effect on animal health. Despite the slight difference in the relative abundance of *E. coli* between high and low groups, statistical significance was observed between the high female and male groups for PU and CaU. Within the gender, PU, CaU and FC significantly differed in female groups and FC additionally in male groups. It can be assumed quails are predisposed to accommodate members of the family Enterobacteriaceae [60] in contrast to chicken surveys [54,61].

Even though birds were housed under identical conditions and were offered the same diet, gender had a substantial effect on the ileal microbial community in the Japanese quail. However, it remains unclear if the change in microbiota composition and function caused the differences in the performance parameters or if the microbiota composition followed the mechanisms that caused differences in PU and CaU. The comparability

to other studies is compromised, as recent studies have focused on a different age of the quails (4-8 weeks) [52,60,62], and knowledge regarding quails' microbiota during their lifespan is still scarce.

5.3 Laying hens and the influences on the GIT microbiota

The modern hen has been genetically selected for high productivity and efficiency [63]. Still, the recommended daily energy and nutrient intake has to be adjusted to the specific production objectives and environmental factors to maintain animal health and welfare [10]. Several factors that influence the poultry microbiome are the genetics [64], age, breed, GIT section, (Chapter IV), the fed diet [11], housing systems [65], health and feed additives (Figure 5.2), and they also interfere with animal production.

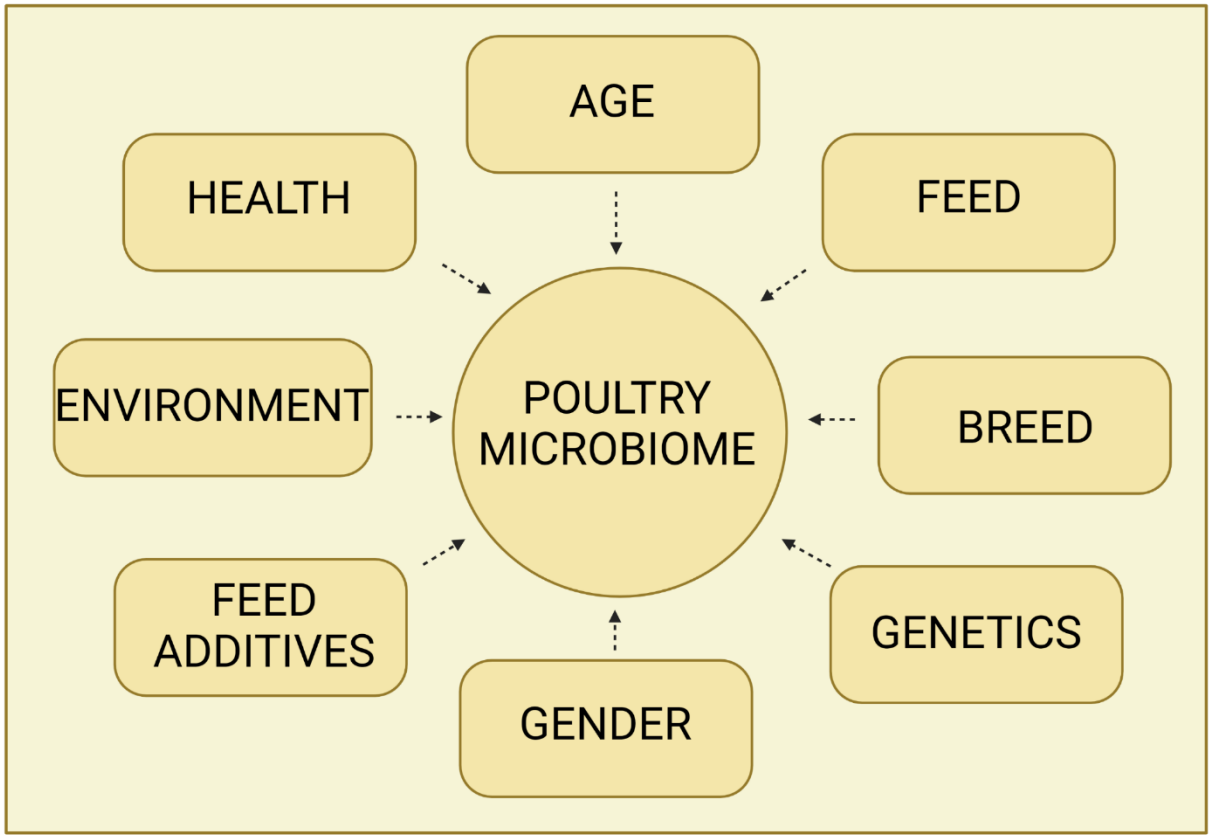

Figure 5.2: Endo- and exogenous factors affecting the poultry microbiome (created with BioRender.com).

The host-microbiota relationship can be commensal, symbiotic, or pathogenic, and the GIT microbiota varies depending on the fed diet and the exposed environment [66]. Furthermore, the gut microbiota is beneficial for immune system development and highly correlated to optimal animal health and productivity [67].

5.3.1 The dietary P and Ca effects on the microbiota of LB and LSL hens

P and Ca play an important role in animal metabolisms in poultry, especially in laying hens, mainly in bone development and eggshell formation [68,69]. A reduction of 20% compared to the recommended P and Ca levels in the diet was expected to significantly affect LB and LSL hens' metabolisms through changes in the microbial composition [11]. In contrast, no dietary influences on microbiota were revealed, and the most significant effect was driven by the two different laying hen breeds [11]. Such breed disparities have already been reported [70] and explained by different P absorption mechanisms [72]. In a companion study, higher inositol-5 and inositol-6 phosphate concentrations have been observed in the gizzard and caeca of LB [11,73]. Additionally, a decrease to 0.15% of available P was not affecting animal growth, productive performance and mRNA expression of P transporters in hens [74].

Even though the breed had a stronger impact than the diet on microbial dynamics [11], the bacterial groups detected revealed similar results of bacterial abundance levels between the breeds, which is in line with previous studies [75–77] and the highest diversity found in caeca of laying hens was also consistent with the literature [54]. Breed differences were found in the relative abundance of the shared microbial composition members on phylum and genus level [11]. Fewer Firmicutes and higher amounts of Bacteroidetes were present in LB [11]. It was reported, that fewer levels of Firmicutes correlate with a decrease in bacteria like *Peptostreptococcus* [71] which was not detected in our study [11]. Moreover, Bacteroidetes were significantly enriched in LB, which was associated with later laying stages and resulted in a decrease in Firmicutes [78]. Consequently, the LB might enter later laying phases earlier than LSL, resulting in microbial differences at the same timepoint [11].

Regarding P and Ca effect on the microbiota, *Ligilactobacillus, Megasphaera,* Lachnospiraceae, Bacteroides, *Helicobacter,* Prevotellaceae, *Lachnoclostridium, Streptococcus,* and Lactobacillaceae were affected by the fed diets [11]. Lachnospiraceae is a butyrate producer, which is crucial for the metabolism of epithelial tissue [79]. Due to this, the lower abundance and lower Ca levels in the diet might negatively impact gut health [11]. On the other hand, the relative abundance of *Megasphaera* decreased with higher levels of Ca in the diet, which might reduce the SCFA production in LSL since it is known to be part of the SCFA production in laying hens [11,80]. Moreover, the prevalence of *Ligilactobacillus* and members of the family Lactobacillaceae changed depending on Ca and P levels. They are common GIT

colonizers in laying hens and are usually associated with GIT health, productive performance, and immune system regulators [81,82].

Furthermore, the average abundance of genus *Streptococcus* members increased with higher Ca levels in LB [11]. This genus is associated with productive performance with a negative correlation to feed conversion ratio [80], which probably led to a reduced daily feed intake in a companion study [73]. Regarding the immune system, reduced P levels increased immune cell numbers and the mitogen-induced response of innate and adaptive immune cells [83]. In contrast, the abundance of the potential pathogen *Helicobacter* increased with higher levels of P in the diet, which could have indicated an effect on the immune system [84,85]. However, even if the relative abundance of the most discriminant ASVs varied by the breed or the fed diet, the assumed shift in functions by a P and Ca reduction was not observed, as for example, in a study with probiotic supplements compared to the standard diet [86].

The recent studies in layers revealed that members of Lactobacillaceae, Bacteroidaceae, Lachnospiraceae, Ruminococcaceae, Veillonellaceae, Prevotellaceae, Clostridiaceae, Rickenellaceae or Enterobacteriaceae were shared between animals and set up the core microbiome [87,88]. None of the studies combined the information of the whole GIT sections, targeted the active microbiome, or considered the necessary coverage to belong to the core microbiome to 50% [89] or up to 75% [87]. However, in a recent study, five bacteria could be detected as core microbiome (uncl. *Lactobacillus*, *Megamonas funiformis, Ligilactobacillus salivarius, Lactobacillus helveticus* and uncl. *Fusicatenibacter)*, present in 97% of the samples with a prevalence of more than 0.01% [11]. The genus *Lactobacillus* was recently proven to be part of the core microbiome in the ileum and caeca of laying hens [87,88] and is a common host-adapted lactic acid bacteria in the GIT [45]. A beneficial effect was reported on the egg-size and -weight [90]. In contrast, the LSL with higher abundances of *Lactobacillus* showed lighter egg weights [11,73]. The hydrogen consumer *M. funiformis* was previously found in the caeca of laying hens [90]. This characteristic bacterium in adult hens accounted additionally to the core microbiome in a recent study [89]. Higher abundances of this species in crop, ileum, duodenum and gizzard samples have never been found [11] and in contrast to Gan *et al.* [80], it almost disappeared in the caeca. The genus *Megamonas* was recently described as an important fermenter of glucose into acetate and propionate [91,92], including beneficial effects on the host health. It can be postulated that the major glucose

fermentation occurs in the upper GIT sections and might be replaced by other SCFA producers. *L. salivarius* was part of the core microbiome in laying hens [87] anis commonly isolated from birds' intestines or feces. This species is known to respond to food-borne pathogens due to antibacterial activity that affects the microbiome and the host immune system [93]. The higher abundance of *L. salivarius* in LSL [11] led to higher amounts of leukocytes, thrombocytes, monocytes, T cells, T helper cells and cytotoxic T than in LB [83], which might be a consequence of response to potential pathogens or breed-dependent reactions to the housing conditions [94]. In contrast, the early GIT colonizer *L. helveticus* [81] positively correlated to Ca absorption and bone metabolism *in vitro* [95]. The species was less abundant in the crop than duodenum and ileum, with the major changes observed between the investigated breeds [11]. This difference might result from a more intense immune response and increased blood components in the LSL [83]. *Uncl. Fusicatenibacter*, part of the family Lachnospiraceae was observed in the ileum and caeca of laying hens [77], permanently present from week 1 to week 40 [96] and is associated with GIT health [79]. This bacterial group was more abundant in the crop and might be involved in the initial feed digestion with *M. funiformis* [11]. Studying the active core microbiome can help to expand the knowledge of the role of bacteria within and across the microbiome and understand the functional importance of the core to the host. Due to these findings, it can be hypothesized that the recommended nutritional amounts, especially Ca and P, are higher than the laying hen’s organism needs. A further reduction can still ensure animal health, productivity on high levels, and fewer emissions of non-digested nutrients to the environment via feces.

5.3.2 The age effect on the microbiota

In addition to investigating dietary treatment effects on the animal microbiome, longitudinal studies of the chicken GIT revealed changes in microbial composition and diversity [97,98] (Figure 5.3).

There is still a lack of knowledge about the age effect in laying hens. This is especially true along the entire GIT in laying hens. However, age effects and a substantial shift were already observed in other studies in animal nutrition, physiology, functional anatomy, livestock population genomics and functional genome analysis [99–105]. Therefore, understanding the gut microbiota composition throughout the laying hen's productive life is essential.

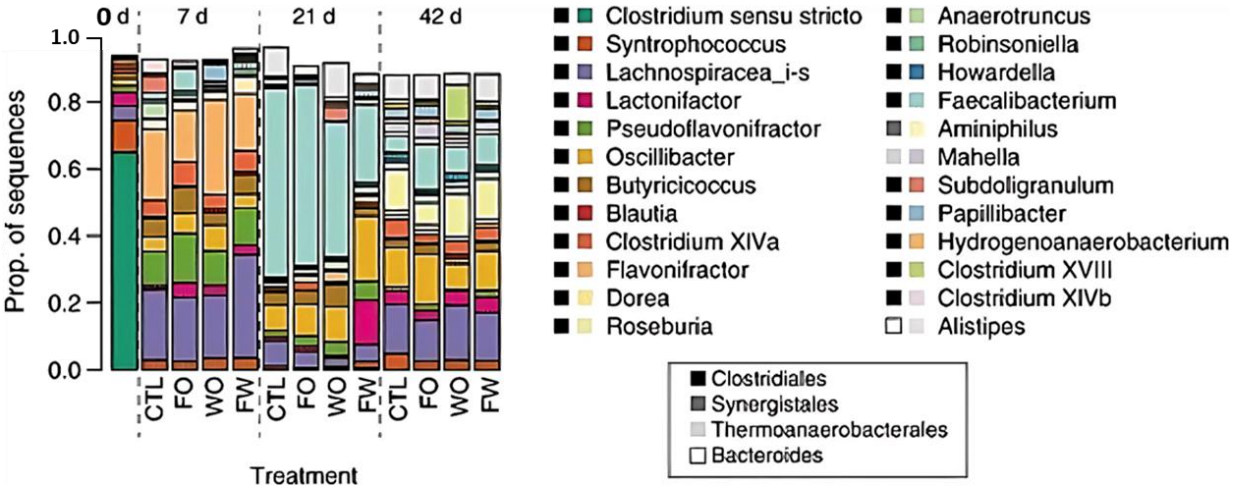

Figure 5.3: Relative abundance at the genus level for sequences by treatment and time with taxonomic classifications performed with the RDP classifier as described in the text. Only sequences with a total relative abundance greater than 5% are shown. For day-of-hatch birds and each subsequent time point (7d, 21d, and 42 d post-hatch), the relative proportions are shown for each treatment. Day-of-hatch birds were proportionally high in Clostridium but low quantitatively. Treatment designations are Ctl, control; FO, feed-only; WO, water-only; and FW, feed and water as described in the text [89].

Within the 10 to 16 weeks of life, the general growth rate increases from 50 to 80% per week in addition to the highest body weight gain and finally the growth rate terminates after the age of 24 weeks [105,106]. With this rapid growth comes the need for the efficient functioning of the entire GIT in terms of increased nutrient uptake and intestinal development. The substrate butyrate, mainly produced in the animal's large intestine, covers this energy requirement and positively affects intestinal health and development, growth performance, and pathogen control [107]. Members of the genera *Faecalibacterium, Roseburia,* and *Eubacterium* are often detected as butyrate producers [108–110]. Moreover, Proteobacteria, *Escherichia,* and *Blautia* have been shown to play a role in butyrate production in a laying hen experiment from week 1 to 60 [106].

Due to the reduced energy demand by the reduced growth rate, the cumulative abundance of these genera increases in the animals aging process (Chapter IV, [106]). Age effects on the microbial composition can be detected on the phylum level as the dominance of Firmicutes within the early life decreases, and the abundance of Bacteroidetes increases [88].

Overall, the variation of the microbial composition regarding age can be observed within each GIT section separately. With its storage function, the crop is the first site of

fermentation and is essential for starch digestion and the sugar breakdown to lactic and other acids [111]. Higher amounts of lactic acid produced by *Lactobacillus* spp. cause a lower pH, and the actual level in the crop varies from pH 4.1 to 6.2 [112,113]. The bacterial colonization in the crop starts immediately after hatch, and recent studies showed a dominance of lactobacilli in adult chickens [11,114,115]. By investigating the lifespan in separate, the microbial composition shifts from the dominance of Lachnospiraceae members to a higher abundance of Lactobacillus and Ligilactobacillus after week 16 and stabilizes in the following weeks (Chapter IV). Especially *Lactobacillus* strains, prebiotics and organic acids improve the prevention of pathogen colonization [112] to ensure intestinal health and an overall balanced crop microbiota [116]. This is supported by the dominance (> 60%) of *Lactobacillus* and *Ligilactobacillus* in the crop from week 24 on and the distinctive shift with the start of the laying period after week 16 (Chapter IV). In addition, it was hypothesized, that the crop has the highest possible probiotic intake, supporting the proliferation of commensal Lactobacillus spp. and improving animal health through a further increase of butyrate-producing Clostridium spp. [117,118].

The starch digestion continues in the gizzard, where the food is additionally crushed and, due to the calcium carbonate in the diet, has a pH ranging from 4 to 5 [118–122]. However, a pH of around 3.5 was also reported in laying hens [123]. The main bacteria in the gizzard comprise L*actobacillus*, Clostridiaceae, *Enterococci*, small amounts of lactose-negative *Enterobacteria,* and coliforms [127–129]. Due to the acetic milieu, bacteria like *Lactobacillus* are preserved. Regarding age-affected microbiota changes, the gizzard is dominated by *Lactobacillus* and *Ligilactobacillus* from week 10 to week 60 (Chapter IV). The beneficial effects on intestinal structure and health might be due to the positive correlation of acetate with *Lactobacillus* [130]. High levels of *Lactobacillus* also increase egg weight and size in combination with a decreased cholesterol level in the egg yolk [131]. In contrast to the age effect on each breed's microbial community and the corresponding breed differences, Sommerfeld et al (2020) reported no breed influence on the egg weight [105]. The high levels of *Lactobacillus* and *Ligilactobacillus* might also be due to the reflux of the digesta [132]. Besides the high Lactobacilli levels, higher *Blautia* (Clostridiaceae) levels could be detected with the laying phase onset (Chapter IV). In a study, lower bacterial amounts were detected with the presence of gastro juices pepsin, and also the hydrochloric acid can inhibit the fermentation activity [127], while certain Lachnospiraceae and *Blautia*

can produce acetate by growing on carbohydrates [133]. These influences might explain the variation in recent publications.

The small intestine receives digestive enzymes and bicarbonate from the pancreas and the liver [127]. Besides the widespread Lactobacilli, Clostridia and *Enterococcus* are the main colonizers from day 3 of life, dominating the first part of the small intestine, the duodenum [124–126]. This could partly be proved by the domination of *Lactobacillus* and *Ligilactobacillus* from week 10 to week 60 (av. abu. 53-76%) and the members of Lachnospiraceae (Clostridia) (6-29%) (Chapter IV). Further, age significantly affected the genus *Romboutsia* that decreased after week 10. Although, *Romboutsia* was reported for negative correlations with the feed efficiency in hens [134]. Lactobacillus increase with a lower abundance of *Romboutsia* which might increase the feed efficiency again [80,134]. The reduction of *Romboutsia* might be due to the competitive exclusion as an increasing abundance of Lactobacilli cause a lower pH in the GIT. After week 24, Blautia was detected in higher abundance, and it can irrigate free hydrogens of fermenting anaerobes and it is also present in preliminary GIT sections [135]. *Eisenbergiella* was negatively correlated to pyruvate metabolism [136]. Higher levels of *Eisenbergiella* were observed in week 30 compared to previous weeks (Chapter IV) which is contrary to the highest levels of in the pyruvate metabolism involved InsP6 and myo-inositol in week 30 and 60 [105]. This might neglect the negative influence of this bacteria and indicate positive influences on pyruvate. The duodenum is the section with the major Ca and P uptake within the GIT [137]. Even though both nutrients are involved and necessary for eggshell formation, no other effect with the transition to the egg-laying phase was observed in the microbiota (Chapter IV).

The overall microbial composition significantly affects the digestion and nutrient uptake in the ileum [138]. The ileal microbiome consists of mainly *Lactobacillus* (up to 70%), followed by Clostridiaceae *Streptococcus*, and *Enterococcus* [139]. The dominance of Lactobacillus was observed from weeks 10 to 60, with an abundance between 44 - 70% of the total microbial composition (Chapter IV). The age effect on the dominance of *Lactobacillus* supports the increase in the egg-laying onset [125]. The dominance of Lactobacillaceae members before the onset and the continuing growth in the proximal sections might also help the animal's immunity and inhibit pathogen attachment [140]. After an increase in the abundance of Clostridiales towards week 24, in later stages, the abundance stabilized at the same level (Chapter IV). These bacteria might belong

to the family Clostridiaceae which have been reported in higher levels in later stages [139,141].

The caecal bacterial community can be described as strictly anaerobic, linked to the immune system and metabolism improvement, which is why it is often used in microbial studies [142]. The across the complete GIT observed highest diversity index and its complexity in metabolism and functionality in comparison to other sections support the research focus on the caeca in poultry [135,142]. Within the caeca, essential amino acids are produced, and non-starch polysaccharides are digested. It is dominated by *Ligilactobacillus* and *Lactobacillus [11]*. Even though the caeca are often reported for their stable microbial composition during the animal's lifespan, this assumption could be rejected, as the composition is still changing from week 24 up to week 60 (Chapter IV). The stabilizing process in the caeca of hens takes more than the previously described 40 days [143]. Especially due to the absence of the chickens parents and nowadays higher zoohygienic standards, the caecal microbiota establishes slower [114]. Compared to other sections, the caeca can be considered more stable to endo- or endogen factors [143], but the microbial compositions still underlie a variance along the productive stages.

Each gastrointestinal section establishes its own bacterial composition and develops continuously along the productive lifespan. Further, a stabilizing plateau depends on the section itself and the specific time point. Even though bacterial changes emboss the growing phase in the pullet’s life, the egg-laying onset is a significant transition phase that occurs with bacterial microbiota variations.

Besides the microbiota analysis along the productive lifespan in laying hens, the distinctive shift from week 16 to week 24 has also been observed in many fields of animal science [99–105]. Due to this, and for the first time, a shotgun metagenomics approach was conducted to evaluate the age-affected bacterial functions and pathways of the sections crop, ileum, and caeca of two different laying hen breeds at two productive stages (Chapter IV). Especially the growing phase revealed significant effects on protein, carbohydrate, cofactors, vitamins and the lipid metabolism (Chapter IV). These differences align with differences in the overall hen body weight, feed intake, and - utilization which are based on energy metabolites and metabolic pathways in addition to immune regulatory mechanisms [101–103,105]. The hen’s organism is still focusing on growth at week 16. With the transition towards week 24, breed variations become more distinct in the digestive system, amino acid metabolism, genetic

information processing, signal transduction, membrane transport, and metabolism (Chapter IV). Especially inositol-related functions are important for Ca and P assimilation, and it can be assumed these functions are higher expressed within the laying period. However, lower myo-inositol concentrations were observed at week 24 [105], and the inositol phosphate metabolism: 5-keto-L-gluconate epimerase (iolO) (K22233), which is the fourth step of the MI degradation [144] was significantly downregulated towards week 24 (Chapter IV). In contrast to significant breed effects, the age did not affect significant differences in the inositol phosphate metabolism (Chapter IV). Significant differences were revealed in the inositol phosphate metabolism functions K22231-22233, the inositol-phosphate transport system substrate-binding protein (inoE) (K17237) (MI-1-phosphate specific ABC transporter [144]) and the inositol-hexakisphosphate/ diphosphoinositol-pentakisphosphate 1-kinase (K13024) (InsP6 metabolizing enzyme [145]). However, these significant breed-affected down-regulations did not align with the InsP6 or myo-inositol levels [105]. Moreover, the inositol-related functions were less represented in the dataset regarding age effects and might be less affected by the transition to the egg-laying phase than expected (Chapter IV). On the other hand, significant effects might not be reported due to the timespan of 8 weeks between the samplings (Chapter IV).

Even though two laying hen breeds were kept under similar conditions and diets, the microbiota composition varies between productive stages. The strong bacterial shift from week 16 to week 24 supports the hypothesis of bacterial fluctuations with the laying phase onset (Chapter IV). It remains unclear if the shift in the bacterial community is influenced by the change to a layer diet or if anatomical and physiological alterations affect the intestinal bacteria composition (Chapter IV).

5.4 Study limitations and future perspectives

The microbiome can be understood as an organ system [146]. There is still research needed to tackle the mechanisms that drive the microbiota changes and the influence of external factors, age, the host genome, and diseases on it. All these factors act synergistically and influence the microbial community. Therefore, research has to focus on understanding the whole system independently of focusing on specific organs or tissues of the animal.

The work presented in this thesis is part of a large-scale study including animal scientists from different areas. Such studies offer great potential to gain deeper

understandings of the microbiome-host interaction under various factors. Overall, the P-Fowl project is set up to concisely understand different mechanisms in the animals' organism and their reply to changing factors. By combining the knowledge gained new hypothesis can be formulated. Regarding the quail, it would be interesting to see how the animals respond to dietary changes in regards to Ca and P levels reduction below recommended concentrations and if the microbiota is still in line with the present work, especially due to microbiota variability according to the animal's genetics. The inclusion of P in the layer's diets can be further decreased to levels lower than 20% below the recommendation. Such a challenge to the animals might uncover adapted gut microbiota and guide the scientific community to re-think the current inclusion levels. Genetics also impacted the microbiota in quails, which should be further considered in the following experiments to evaluate the genetic predisposition regarding P and Ca utilization. Due to the strong effect on the microbiota between weeks 16 and 24, this period should be deeper analyzed weekly to investigate the ongoing changes towards the onset of the laying phase in combination with other omics approaches to identify the underlying functions and precise circumstances of the laying hen. However, the profound individual study effects by breed, age, diet or gender have been found to have strong impacts on the overall GIT microbiome for the animals.

Especially large datasets like the one of the laying hens challenge in terms of data handling (considering breeds, diets, GIT sections, sample types, or lifetime stages [87]) and enable gathering room for interpretation. Although individual variations in the dataset could be observed [32], on average high animal numbers, reduce the effect of individuals and reduce the model to the average present by the specific treatment (e.g. breed, diet, etc.) and increase the statistical power of significant effects [147,148].

Nevertheless, having more animals in experiments is more complicated due to strict ethical committees, which limits the possibility of larger cohorts. Regarding this, consideration must be given to reducing invasive sampling methods and finding solutions, such as fecal swabbing to correlate the fecal intestinal microbiota to performance data (e.g. Ca utilization), genetics, and other host-related parameters. Another option is to use *in vitro* systems to test hypotheses before applying them to an animal experiment [149–151].

Especially the microbiome, which is directly in touch with the diet and the basis for nutrient assimilation, can lead to a differentiation in animal performance in regards to e.g. feed intake, body weight gain, P and Ca utilization [32]. Therefore, targeting the

change-causing parameters on the microbiome can establish sustainable animal production in poultry and adjust the species-specific nutrition strategies. The results of this project provide deeper insights and knowledge into bacterial interactions while deepening our understanding of microbiota variations across the gastrointestinal tract and productive stages of layers and quails. Including new approaches will potentially bring new information to deeply investigate and interpret the given data to improve the animal’s health and performance.

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CHAPTER VI

SUMMARY

6. Summary

The microbiome's composition in the gastrointestinal tract (GIT) is subject to several changes and influences. In addition to breed, sex, or diet, age affects the GIT microbiome dynamics of laying hens and quails. From the first day, the microbiome develops and increases its bacterial load to thousands of species. Then, depending on the diet fed, the animal's microbiome and associated active bacteria vary and directly influence the animal's nutrient uptake and efficiency. Omics technologies give insights into changes in microbes in the GIT (crop, gizzard, duodenum, ileum, caeca). In addition, they can reveal how feed supplements such as calcium (Ca) or phosphorus (P) can affect host health and performance through alterations in the microbiome.

The Japanese quail has been an established animal model for nutritional and biological studies in poultry for the last 60 years. In particular, its short development time makes it a convenient model for microbiome research. However, compared to broiler microbiome research, the quail microbiome is still poorly understood. Animals of the breed *Coturnix japonica* were housed under the same conditions, fed a diet with P below recommendation, and the ileum microbiota characterized. Microbiota relations with gender and higher or lower predisposition of the birds for PU, CaU, FI, BWG, and FC were described (Chapter II). In addition, these performance parameters influenced the relative average abundance of bacteria like *Candidatus* Arthromitus, *Bacillus,* and *Leuconostoc*. Gender affects specific bacterial groups of the GIT, such as *Lactobacillus, Streptococcus, Escherichia,* and *Clostridium*, which differ in average abundance between male and female quails. Despite the comprehensive microbiota analysis, the interplay between animal genetics, diet, sex, and microbiome functionality is not yet understood.

The laying hen breeds Lohmann LSL-Classic and Lohmann Brown-Classic are used worldwide. Little is known about the interaction with microbiome composition, performance, dietary effects, and changes during the productive life that might help develop feeding strategies and microbiome responses on a large scale. Because of the importance of P and Ca in poultry diet, the research in Chapter III was conducted to challenge laying hens with reduced dietary P and Ca and describe the effect on GIT active microbiota. The breed was the primary driver of microbial differences. A core microbiome of active bacteria, present along the complete GIT, was revealed for the first time and consisted of five bacteria detected in 97% of all samples, including digesta and mucosa samples (uncl. *Lactobacillus*, *Megamonas funiformis*,

Ligilactobacillus salivarius, Lactobacillus helveticus, uncl. *Fuscatenibacter*). Furthermore, significant microbial differences between the GIT sections and between the breeds were described. Minor dietary effects of the P and Ca reduction on the microbiota showed that a further decrease in Ca and P supplementation might be possible without affecting the gut microbial composition and bird performance.

Furthermore, the microbiome of laying hens was characterized at five productive stages (weeks 10, 16, 24, 30, and 60) to analyze the age effect on the GIT microbiome (Chapter IV). Although the two breeds of laying hens were offered the same diet and housed under similar conditions, the active microbiota composition changed between the analyzed productive stages, the breed and the GIT sections. The major shift occurred between weeks 16 and 24 and supported the hypothesis of bacterial fluctuations due to the onset of the laying period. Those changes occurred mainly in the abundance of the genera *Lactobacillus* and *Ligilactobacillus*. However, it remains unclear whether the dietary changes, due to the development of the birds, influenced the microbiota shifts or if the anatomical and physiological modifications influenced the GIT microbiota. Furthermore, the shotgun metagenomic analysis revealed differences in regulatory functions and pathways between breeds, sections, and the two production stages. Different relative abundance levels of the microbial composition were observed between the RNA-based targeted sequencing and the DNA-based shotgun metagenomics.

In conclusion, the comprehensive characterization of the microbiota in the GIT of quails and two high-yielding breeds of laying hens contributes to a broader knowledge of the microbiome dynamics within the fowl GIT. Age and breed play a more important role than diet in influencing the dynamics of microbial composition in laying hens, and individual performance and sex in quails. Research characterizing the microbiome in poultry and its effect on diet and host genetics will help improve feeding and breeding strategies in the future and reduce excretion of nutrients into the environment while ensuring overall animal health.

CHAPTER VII

ZUSAMMENFASSUNG

7. Zusammenfassung

Die Zusammensetzung des Mikrobioms im Gastrointestinaltrakt (GIT) unterliegt verschiedenen Veränderungen und Einflüssen. Neben Rasse, Linie, Geschlecht oder Ernährung wirkt sich auch das Alter auf die Dynamik des GIT-Mikrobioms von Legehennen und Wachteln aus. Vom ersten Tag an entwickelt sich das Mikrobiom und erhöht seine bakterielle Besiedelung auf Tausende von Arten. Desweiteren variiert das Mikrobiom des Tieres und die damit verbundenen aktiven Bakterien je nach der gefütterten Nahrung und beeinflussen direkt die Nährstoffaufnahme und Effizienz des Tieres. Omics-Technologien geben Aufschluss über Veränderungen der Mikroben im GIT (Kropf, Muskelmagen, Zwölffingerdarm, Ileum, Blinddarm). Darüber hinaus können sie aufzeigen, wie sich Futterzusätze wie Kalzium (Ca) oder Phosphor (P) durch Veränderungen im Mikrobiom auf die Gesundheit und Leistung des Wirts auswirken können.

Die japanische Wachtel ist seit 60 Jahren ein etabliertes Modelltier für ernährungswissenschaftliche und biologische Studien an Geflügel. Vor allem ihre kurze Entwicklungszeit macht sie zu einem geeigneten Modell für die Mikrobiomforschung. Im Vergleich zur Mikrobiomforschung bei Masthähnchen ist das Mikrobiom der Wachtel jedoch noch wenig erforscht. Daher wurde die Microbiota des Ileums von Tieren der Rasse Coturnix japonica, welche unter identischen Bedingungen, einschließlich der Fütterung gehalten wurden, charakterisiert, wobei der Phosphorgehalt unter der allgemeinen Empfehlung lag. Es wurden Beziehungen zwischen der GIT Mikrobiota und dem Geschlecht sowie einer höheren oder niedrigeren Prädisposition der Tiere für Phosphorverwertung, Kalziumverwertung, Futteraufnahme, Körpergewichtszunahme und Futterverwertung beschrieben (Kapitel II). Darüber hinaus beeinflussten diese Leistungsparameter die relative durchschnittliche Abundanz von Bakterien wie *Candidatus* Arthromitus, Bacillus und Leuconostoc. Das Geschlecht wirkt sich auf bestimmte Bakteriengruppen des GIT aus, wie z. B. *Lactobacillus, Streptococcus, Escherichia* und *Clostridium*, die sich in ihrer durchschnittlichen Abundanz zwischen männlichen und weiblichen Wachteln unterscheiden. Trotz der umfassenden Mikrobiota-Analyse ist das Zusammenspiel zwischen Tiergenetik, Ernährung, Geschlecht und Mikrobiom-Funktionalität noch nicht verstanden.

Die Legehennenlinien Lohmann LSL-Classic und Lohmann Brown-Classic werden weltweit eingesetzt. Über die Wechselwirkung zwischen der Zusammensetzung des

Mikrobioms, der Leistung, den Auswirkungen der Ernährung und den Veränderungen während der produktiven Lebensabschnitte, die zur Entwicklung von Fütterungsstrategien und Reaktionen des Mikrobioms in großem Maßstab beitragen könnten, ist wenig bekannt. Aufgrund der Bedeutung von P und Ca in der Geflügelernährung wurden die Untersuchungen in Kapitel III durchgeführt, um Legehennen mit reduziertem P und Ca zu füttern und die Auswirkungen auf die aktive Mikrobiota im GIT zu beschreiben. Die Linie war der Hauptfaktor für die mikrobiellen Unterschiede. Ein Kernmikrobiom aktiver Bakterien, das entlang des gesamten GIT vorhanden ist, wurde zum ersten Mal aufgedeckt und bestand aus fünf Bakterien, die in 97% aller Proben, einschließlich Digesta- und Schleimhautproben, nachgewiesen wurden (uncl. Lactobacillus, Megamonas funiformis, Ligilactobacillus salivarius, Lactobacillus helveticus, uncl. Fuscatenibacter). Außerdem wurden signifikante mikrobielle Unterschiede zwischen den GIT-Abschnitten und zwischen den Linien beschrieben. Geringfügige diätetische Auswirkungen der P- und Ca-Reduzierung auf die Mikrobiota zeigten, dass eine weitere Verringerung der Ca- und P-Supplementierung möglich sein könnte, ohne die Zusammensetzung des Darmmikrobioms und die Leistung der Tiere zu beeinträchtigen.

Darüber hinaus wurde das Mikrobiom von Legehennen in fünf Produktivitätsstadien (10, 16, 24, 30 und 60 Wochen) charakterisiert, um den Alterseffekt auf das GIT-Mikrobiom zu analysieren (Kapitel IV). Obwohl die beiden Legehennenlinien das gleiche Futter erhielten und unter ähnlichen Bedingungen gehalten wurden, änderte sich die Zusammensetzung der aktiven Mikrobiota zwischen den untersuchten Produktionsstadien, der Linen und den GIT-Abschnitten. Die größte Verschiebung fand zwischen der 16. und 24. Woche statt und unterstützte die Hypothese der bakteriellen Fluktuationen aufgrund des Beginns der Legeperiode. Diese Veränderungen betrafen vor allem die Häufigkeit der Gattungen Lactobacillus und Ligilactobacillus. Es bleibt jedoch unklar, ob die Veränderungen in der Ernährung aufgrund der Entwicklung der Vögel die Verschiebungen in der Mikrobiota beeinflusst haben oder ob die anatomischen und physiologischen Veränderungen die GIT-Mikrobiota beeinflusst haben. Darüber hinaus ergab die Shotgun-Metagenomanalyse Unterschiede in den Regulationsfunktionen und -Metabolismuswegen zwischen den legehennenlinien, Sektionen und den beiden Produktionsstadien. Zwischen der gezielten Sequenzierung auf RNA-Basis und der Shotgun-Metagenomik auf DNA-

Basis wurden unterschiedliche relative Häufigkeiten der mikrobiellen Zusammensetzung festgestellt.

Zusammenfassend lässt sich sagen, dass die umfassende Charakterisierung der Mikrobiota im GIT von Wachteln und zwei Hochleistungslinien von Legehennen zu einem breiteren Wissen über die Dynamik des Mikrobioms im GIT von Geflügel beiträgt. Alter und Linie spielen eine wichtigere Rolle als die Ernährung, wenn es darum geht, die Dynamik der mikrobiellen Zusammensetzung bei Legehennen und die individuelle Leistung und das Geschlecht bei Wachteln zu beeinflussen. Die Forschung zur Charakterisierung des Mikrobioms bei Geflügel und seiner Auswirkungen auf Ernährung und Wirtsgenetik wird dazu beitragen, Fütterungs- und Zuchtstrategien in Zukunft zu optimieren und die Ausscheidung von Nährstoffen in die Umwelt zu verringern und gleichzeitig die Gesundheit der Tiere insgesamt zu gewährleisten.

CHAPTER VIII

APPENDIX

8. Appendix

8.1 Additional material of Chapter II and Chapter IV

The following additional material can only be found in an appendix in electronic form/on CD-ROM:

8.2 Curriculum vitae

Personal Data

Name:	Roth, Christoph Florian
Date of Birth:	20.04.1994
Place of Birth:	Augsburg, Germany
Nationality:	German

Education

Since 11/2019	Research for PhD, Institute of Animal Science, Department of Livestock Microbial Ecology, University of Hohenheim, Stuttgart, Germany
2018 - 2019	M.Sc. Agricultural science, University of Hohenheim, Stuttgart, Germany
2016 - 2018	B.Sc. Agricultural biology, University of Hohenheim, Stuttgart, Germany
2014 - 2018	B.Sc. Agricultural science, University of Hohenheim, Stuttgart, Germany
2012 – 2014	B.Sc. Automotive engineering, University of Stuttgart, Germany
2004 – 2012	Simpert-Kraemer Gymnasium, 86381 Krumbach

Internships

Since 09/2011	Agriculturally operational helper at Georg Mang, Bergweg 3, 86498 Bebenhausen - Maintenance / repair of agricultural laboratories - Troubleshooting and analysis of faults - Support in agriculture and livestock - Soil cultivation, sowing, fertilization, harvesting - Animal transport-qualification - Animal inspection, feeding and milking
05/2016 – 07/2016	Agriculturally operating experience Erich Maier, Breitenthal
01/09/2019 – 30/09/2019	Weißenhorner Milch Manufaktur (Molkery), Siemensstraße 5, 89264 Weißenhorn

Publications

D. Borda-Molina, **C. Roth**, A. Hérnandez-Arriaga, D. Rissi, S. Vollmar, M. Rodehutscord, J. Bennewitz, A. Camarinha-Silva. 2020. Effects on the Ileal Microbiota of Phosphorus and Calcium Utilization, Bird Performance, and Gender in Japanese Quail. DOI: 10.3390/ani10050885.

I. Sand, C. Gaio, W. Achilles, Y. Kranch, F. M. Lenz, W. Hartmann, D. Horlacher, M. Krause, **C. Roth**, P. Weyers, G. Manek, M. Däuber, K. Krön, J. O. Schroers, M. Funk, J. Grube. 2021, Milchziegenhaltung – Produktionsverfahren planen und kalkulieren; KTBL-Datensammlung, ISBN 978-3-945088-80-7

M. Arsalan Iqbal, H. Reyer , M. Oster , F. Hadlich, N. Trakooljul, A. Perdomo-Sabogal, S. Schmucker, V. Stefanski, **C. Roth**, A. Camarinha Silva, K. Huber, V. Sommerfeld, M. Rodehutscord, K. Wimmers, S. Ponsuksili. 2022. Multi-Omics Reveals Different Strategies in the Immune and Metabolic Systems of High-Yielding Strains of Laying Hens. DOI: 10.3389/fgene.2022.858232.

C. Roth, T. Sims, M. Rodehutscord, J. Seifert, A. Camarinha-Silva. 2022. The active core microbiota of two high-yielding laying hen breeds fed with different levels of calcium and phosphorus. DOI: 10.3389/fphys.2022.951350.

S. Ponsuksili, F. Hadlich, A. Perdomo-Sabogal, H. Reyer, M. Oster,N. Trakooljul, M. ArsalanIqbal, S. Schmucker, V. Stefanski, **C. Roth**, A. Camarinha-Silva, K. Huber, V. Sommerfeld, M. Rodehutscord, K. Wimmers. 2022. The dynamics of molecular, immune and physiological features of the host and the gut microbiome and their interactions before and after onset of laying in two hen strains. https://doi.org/10.1016/j.psj.2022.102256.

B. Rios-Galicia, J.S. Sáenz, T. Yergaliyev, **C. Roth**, A. Camarinha-Silva, J. Seifert. 2023. Novel taxonomic and functional diversity of bacteria from the upper digestive tract of chicken. https://doi.org/10.1101/2023.05.10.540237.

Place, Date Signature

8.2 Annex 3

Declaration in lieu of an oath on independent work
according to Sec. 18(3) sentence 5 of the University of Hohenheim's Doctoral Regulations for the Faculties of Agricultural Sciences, Natural Sciences, and Business, Economics and Social Sciences

1. The dissertation submitted on the topic:
   "Comprehensive characterization of microbiota in the gastrointestinal tract of quails and two high yielding laying hen breeds"
   is work done independently by me.

2. I only used the sources and aids listed and did not make use of any impermissible assistance from third parties. In particular, I marked all content taken word-for-word or paraphrased from other works.

3. I did not use the assistance of a commercial doctoral placement or advising agency.

4. I am aware of the importance of the declaration in lieu of oath and the criminal consequences of false or incomplete declarations in lieu of oath.

I confirm that the declaration above is correct. I declare in lieu of oath that I have declared only the truth to the best of my knowledge and have not omitted anything.

_____ _____

Place, Date Signature

Acknowledgement

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Ferner danke ich Herrn Prof. Dr. Jörn Bennewitz. Ohne dich Jörn, wäre ich nicht auf Amélia und dieses Promotionsthema aufmerksam geworden. Ich bedanke mich herzlichst für jeglichen Diskurs, sei er geschäftlicher oder privater Natur gewesen, während der zahlreichen Exkursionen, Staffelläufe oder währrend des Studiums, ua. bei der Betreuung meiner Bachelorarbeit.

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